Influence of Passive Permeability on Apparent P-glycoprotein Kinetics

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Purpose. The objectives of this work were to evaluate the importance of moderate passive permeability on apparent P-glycoprotein (P-gp) kinetics, and demonstrate that inspection of basolateral to apical and apical to basolateral (BL-AP/AP-BL) permeability ratios may result in a compound being overlooked as a P-gp substrate and inhibitor of another drug's transport via P-gp inhibition.

Methods. The permeability ratios of nicardipine, vinblastine, cimetidine, and ranitidine were determined across Caco-2 monolayers that express P-gp, in the presence and absence of the specific P-gp inhibitor, GF120918. In addition, the permeability ratio of vinblastine was studied after pretreatment of Caco-2 monolayers with nicardipine, ranitidine, or cimetidine. Similar studies were repeated with hMDR1-MDCK monolayers.

Results. The permeability ratios for cimetidine and vinblastine were >2. The permeability ratios for nicardipine and ranitidine were close to unity, and were not affected by the addition of GF120918. Based solely on ratios, only compounds with moderate transcellular permeability (vinblastine and cimetidine) would be identified as P-gp substrates. Although the permeability ratios appeared to be unity for nicardipine and ranitidine, both compounds affected the permeability of vinblastine, and were identified as substrates and inhibitors of P-gp. Studies performed in hMDR1-MDCK cells confirmed these experimental results. Data were explained in the context of a kinetic model, where passive permeability and P-gp efflux contribute to overall drug transport.

Conclusions. Moderate passive permeability was necessary for P-gp to reduce the AP-BL drug permeability. Inspection of the permeability ratio after directional transport studies did not effectively identify P-gp substrates that affected the P-gp kinetics of vinblastine. Because of the role of passive permeability, drug interaction studies with known P-gp substrates, rather than directional permeability studies, are needed to elucidate a more complete understanding of P-gp kinetics.

KEY WORDS: P-glycoprotein; passive permeability.

INTRODUCTION

It has been suggested that P-glycoprotein (P-gp) effects are dependent on intracellular drug concentration, although the exact mechanism by which P-gp limits intestinal drug transport has not yet been elucidated (1–4). Therefore, the extent of passive membrane permeability may effect multidrug resistance and its modulation. Eytan et al. investigated the relationship between the transmembrane movement rate of several rhodamine dyes and the ability of these dyes to act as P-gp substrates (5). It was concluded that compounds with fast transmembrane movement overcame multidrug resistance (MDR) protein efflux compared to those with slow transmembrane movement. This suggests that a drug may appear to be uninfluenced by P-gp, by traversing the cell membrane faster than P-gp can efflux the drug (6).

Compounds are often identified as substrates of P-gp when directional permeability studies are performed in an MDR expressing cell monolayer. A ratio of basolateral to apical (BL-AP) to apical to basolateral (AP-BL) permeability >1 suggests that an efflux mechanism may play a role in the transport of the compound. As the results will show, this ratio method may only be effective when the compound exhibits moderate transcellular permeability.

The objectives of this work were to evaluate the importance of moderate passive permeability on apparent P-gp kinetics, and demonstrate that inspection of BL-AP/AP-BL permeability ratios may result in a compound being overlooked as a P-gp substrate and inhibitor of another drug's transport via P-gp inhibition.

MATERIALS AND METHODS

Materials

Caco-2 cells were obtained from ATCC (Rockville, MD). Transwell® inserts were purchased from Corning-Costar (Cambridge, MA). Radiolabeled ^{14}C-mannitol (specific activity of 51.5 mCi/mmole) was received from DuPont NEN (Boston, MA). ^{3}H-vinblastine sulfate (specific activity of 12.5 Ci/mmol) was received from Amersham Life Science (Piscataway, NJ). Nicardipine, cimetidine, and ranitidine were USP grade. hMDR1-MDCK cells overexpressing the human MDR1 gene were obtained from the Netherlands Cancer Institute (7). Hank's balanced salt solution (HBSS) and phosphate buffered saline (PBS) were purchased from Sigma Chemical Co. (St. Louis, MO). Cell culture media was obtained from GIBCO-BRL (Grand Island, NY). All organic solvents were HPLC grade. All other chemicals were reagent grade. GF120918 was a gift from Glaxo Wellcome, Inc.

Methods

Caco-2 Cell Culture

Caco-2 cells were plated on Transwell inserts using a previously reported 4-day growth protocol (8). Permeability studies were conducted in HBSS containing 10 mM HEPES buffer at 37°C and 50 oscillations/min. AP-BL and BL-AP permeability of 4 nM ^{3}H-vinblastine was determined to assess the presence of efflux transporters in this model. ^{3}H-vinblastine sulfate was quantified using a scintillation counter.

Effect of Passive Permeation on Apparent P-gp Kinetics

Permeabilities of nicardipine, ranitidine, and cimetidine were each determined in the AP-BL and BL-AP directions at donor concentrations of 10 μM. Nicardipine was quantified by HPLC with UV detection (237 nm) using a Phenomenex C8 column, 150 × 4.6 mm (Torrance, CA). Mobile phase consisted of 25 mM potassium dihydrogenphosphate, acetoni-

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Table 1. Directional Permeabilities Across Caco-2 Monolayers With and Without Inhibitor

Compound	Permeability (cm/sec) × 10^6 AP-BL	Permeability (cm/sec) × 10^6 BL-AP	Ratio of BL-AP/AP-BL permeability
10 μM Nicardipine	19.8 (± 0.7)	24.1 (± 0.3)	1.22 (± 0.05)
with GF120918	20.2 (± 0.7)	19.8 (± 0.8)	0.98 (± 0.05)
4 nM Vinblastine	3.83 (± 0.18)	12.9 (± 0.4)	3.37 (± 0.19)
with GF120918	6.15 (± 0.45)	5.91 (± 0.95)	1.04 (± 0.17)
10 μM Cimetidine	1.71 (± 0.08)	3.54 (± 0.04)	2.07 (± 0.10)
with GF120918	3.37 (± 0.07)	3.74 (± 0.11)	1.11 (± 0.04)
10 μM Ranitidine	1.23 (± 0.06)	1.59 (± 0.08)	1.29 (± 0.09)
with GF120918	2.21 (± 0.18)	2.22 (± 0.19)	1.01 (± 0.12)

Values are $n = 3$, except Vinblastine ($n = 8$). SEM on ratio values was calculated using the delta method.

trile, and methanol at a ratio of 49:45:6, respectively. Flow rate was 1.5 ml/min. Ranitidine and cimetidine analysis was performed using liquid chromatography–tandem mass spectrometry (LC-MSMS). Chromatography was performed on a Phenomenex Luna C18, 50 × 2 mm (i.d.), 3 μm particle size column at 40°C. Mobile phase consisted of two solvents: (A) 13 mM ammonium acetate pH 6.8 with 2% (v/v) methanol, and (B) methanol. The profile was 0–1.5 min 1% B; 1.5–4 min linear gradient to 95% B; 4–4.5 min hold at 95% B, 4.5–4.6 min to 1% B; 4.6–6.5 min hold at 1% B. Flow rate was 0.6 ml/min. Mass spectrometry was performed on a PE-Sciex (Foster City, CA) API3000 mass spectrometer equipped with a heated nebulizer (HN) source for atmospheric pressure chemical ionization (APCI) in the positive ion mode. Detection by tandem mass spectrometry was based on precursor ion transitions to the strongest intensity product ions. Instrumental conditions were optimized to afford best sensitivity.

All samples for LC-MSMS analysis were diluted to yield a final concentration of 25% (v/v) acetonitrile (9). Dose and donor solutions were diluted 5-fold in transport medium prior to adding acetonitrile to bring their concentrations into the working analytical range.

Drug Interaction Studies

The AP-BL and BL-AP permeabilities of vinblastine, nicardipine, ranitidine, and cimetidine were also determined after a 30-min pretreatment with 500 nM GF120918, a potent inhibitor of P-gp. Transport studies were conducted as stated above with 500 nM GF120918 in both the apical and basolateral compartments throughout the time course of the experiment. The purpose of these studies was to note any changes in directional permeability values after the inhibition of P-gp in the Caco-2 cells.

The AP-BL and BL-AP permeabilities of vinblastine were assessed in Caco-2 monolayers after a 30-min pretreatment with either 60 μM nicardipine, 3 mM ranitidine, or 3 mM cimetidine, to test the effect of these agents on P-gp kinetics. These concentrations were chosen based on the extent of passive permeability of these compounds. For ranitidine and cimetidine, the donor concentration was increased to facilitate drug concentration in the cytosol, where P-gp inhibition is assumed to occur (1,10). Transport studies were conducted as stated above. Pretreatment concentrations of each inhibitor were present in both the apical and basolateral compartments throughout the time course of the experiment.

In all transport experiments, ^{14}C-mannitol was monitored to ensure monolayer integrity. All mannitol permeability values were less then 2.6×10^{-6} cm/sec.

hMDR1-MDCK

hMDR1-MDCK cells were passaged as described (11). For transport studies, cells were plated on 0.4 μm Transwell polycarbonate inserts at a seeding density of 664,000 cells/cm^2 (0.33 cm^2 per insert). Similar studies were performed using hMDR1-MDCK cells as previously described. Analyses of cimetidine, ranitidine, nicardipine, and vinblastine samples from the hMDR1-MDCK studies were determined by LC-MSMS using cassette analysis (9).

RESULTS

Table 1 summarizes the AP-BL and BL-AP permeabilities of nicardipine, vinblastine, cimetidine, and ranitidine in 4-day Caco-2 monolayers with and without the P-gp inhibitor, GF120918. The ratios of BL-AP/AP-BL permeabilities are also shown. Based solely on inspection of the BL-AP/AP-BL permeability ratio, only compounds with moderate permeability (vinblastine and cimetidine) would be identified as possible P-gp substrates. The BL-AP/AP-BL permeability ratios for nicardipine, a highly permeable compound, and ranitidine, a lowly permeable compound, were close to unity, and were not affected by the addition of GF120918. Based solely on this experiment, these compounds may not be expected to be P-gp substrates.

Table 2 summarizes the AP-BL and BL-AP permeabilities of vinblastine, as well as corresponding BL-AP/AP-BL ratios, after pretreatment of Caco-2 monolayers with nicardipine, ranitidine, or cimetidine. These compounds all affected the P-gp kinetics of vinblastine. Based on Table 1, where cimetidine's BL-AP/AP-BL permeability ratio was >2,

Table 2. Drug Interaction Studies: Vinblastine Permeability Across Caco-2 Monolayers

Compound	Permeability (cm/sec) × 10^6 AP-BL	Permeability (cm/sec) × 10^6 BL-AP	Ratio of BL-AP/AP-BL permeability
Vinblastine with 60 μM Nicardipine	7.83 (± 0.33)	6.01 (± 0.34)	0.77 (± 0.05)
Vinblastine with 3 mM Cimetidine	6.70 (± 0.78)	10.0 (± 1.64)	1.49 (± 0.30)
Vinblastine with 3 mM Ranitidine	5.54 (± 0.41)	9.43 (± 0.35)	1.70 (± 0.14)

Values are $n = 3$, except Vinblastine with Ranitidine ($n = 6$). SEM on ratio values was calculated using the delta method. Baseline BL-AP/AP-BL ratio for vinblastine is 3.37 (±0.19) from Table 1.

only cimetidine would have been predicted to have an effect on vinblastine. Even though their BL-AP/AP-BL permeability ratios were approximately 1, nicardipine and ranitidine each increased the permeability of vinblastine, and thus were identified as substrates of P-gp.

Table 3 summarizes the results of studies performed in hMDR1-MDCK cells. These results confirm those obtained using Caco-2 monolayers. For vinblastine alone, the overexpression of P-gp drives the AP-BL concentration to below the limit of quantitation (LOQ) of the LC-MSMS method (LOQ = 10 nM).

DISCUSSION

Effect of Passive Permeation on Apparent P-gp Kinetics

From directional transport studies, only compounds with moderate permeability (vinblastine and cimetidine) were identified as possible P-gp substrates. However, from drug interaction studies, nicardipine, ranitidine, and cimetidine affected the P-gp kinetics of vinblastine. Nifedipine results are consistent with the observation of Eytan et al., who concluded that compounds with fast transmembrane movement overcame MDR efflux compared to those with slower transmembrane movement. Results obtained using a mechanistic permeation model also suggest that compounds with high affinity to P-gp and high passive membrane permeability will overcome P-gp-mediated efflux (12). This model found that compounds with lower passive permeability have a better chance of being affected by P-gp, even with lower affinity to P-gp. Similarly, a pharmacokinetic model for estimating fraction dose absorbed after oral administration showed a greater fraction dose absorbed following P-gp inhibition, when the intracellular diffusion constant of the modeled drug was small (13). Here, the highly permeable compound nicardipine also supports this conclusion that high passive permeability can dominate P-gp effects. In addition, nicardipine stimulates P-gp ATPase activity (14). It should be noted that an alternative explanation is that nicardipine (and ranitidine) only interacts with P-gp to inhibit the transport of other drugs, but is not translocated.

In addition to high permeability being able to mask a P-gp effect, low permeability may mask a P-gp effect as well. For the lowly permeable drug ranitidine, P-gp may be less effective in limiting transport, because presumably the intracellular ranitidine concentration is low. Ranitidine has been described to be predominantly transported via the paracellular route. With a low intracellular concentration, a drug that is an intrinsic P-gp substrate may not manifest BL-AP/AP-BL ratios above 1.0, because essentially small drug amounts are effluxed.

The role of passive permeability in apparent P-gp kinetics is presented here. However, an alternate explanation for these observations is a low affinity of the compound for P-gp (i.e., poor affinity; high K_m). In practice, it may be difficult to separate the influences of passive permeability and intrinsic drug affinity on P-gp kinetics.

Table 3. Directional Permeabilities Across hMDR1-MDCK Monolayers With and Without Inhibitor

Compound	Permeability (cm/sec) × 10^6 AP-BL	Permeability (cm/sec) × 10^6 BL-AP	Ratio of BL-AP/AP-BL permeability
10 μM Nicardipine	45.6 (± 2.2)	51.9 (± 3.0)	1.14 (± 0.09)
with GF120918	51.6 (± 1.2)	62.5 (± 0.8)	1.21 (± 0.03)
10 μM Vinblastine	<0.2	36.9 (± 1.9)	>18
with GF120918	1.47 (± 0.12)	2.88 (± 0.22)	1.96 (± 0.22)
10 μM Cimetidine	0.97 (± 0.32)	2.40 (± 0.18)	2.47 (± 0.84)
with GF120918	0.90 (± 0.13)	0.69 (± 0.10)	0.76 (± 0.15)
10 μM Ranitidine	0.95 (± 0.13)	1.47 (± 0.19)	1.55 (± 0.29)
with GF120918	0.52 (± 0.09)	0.57 (± 0.05)	1.11 (± 0.22)

Values are $n = 3$ in hMDR1-MDCK monolayers after 60 min. SEM on ratio values was calculated using the delta method.

Kinetic Model

Results obtained from this work were subjected to theoretical analysis, using a kinetic model of a monolayer. In Fig. 1, drug can permeate via the passive transcellular and passive paracellular routes, as well as be effluxed via P-gp. Sink conditions are assumed for passive permeability (i.e., no passive back diffusion). Net apical to basolateral flux ($J_{AP\text{-}BL}$) can be described by:

$$J_{AP-BL} = (P_{trans} + P_{para})C_d - \frac{V_{\max}C_{cyto}}{K_m + C_{cyto}} \quad (1)$$

where P_{trans} is passive transcellular permeability, P_{para} is passive paracellular permeability, C_d is the apical donor concentration, V_{max} is the maximal drug efflux, C_{cyto} is the unbound cytoplasmic drug concentration, and K_m is the intracellular drug concentration at half V_{max} and inversely reflects intrinsic drug affinity for P-gp. Protein-bound drug does not contribute to C_{cyto} (15).

C_{cyto} is assumed here to be the more relevant drug concentration (1). This model, in combination with the data obtained in the laboratory, can be used to explain the relative

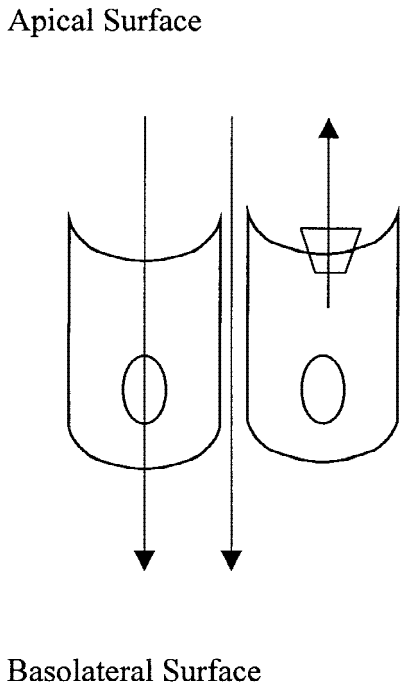

Fig. 1. Schematic representation of the passive and active components to efflux by P-glycoprotein.

importance of moderate passive intestinal permeability for P-gp effects. It should be noted Eq. (1) (and Fig. 1) is not inconsistent with the conclusions of Ito et al., who indicate that the apical membrane was less passive permeable than the basolateral membrane (16). In such a case, P_{trans} in Eq. (1) would essentially be equal to the apical membrane passive permeability.

Contour plots showing the theoretical reduction in apparent AP-BL permeability as a function of relative cytoplasmic concentration (*y*-axis) and apical solution concentration (*x*-axis) are shown in Fig. 2. The relative cytoplasmic concentration is the ratio of C_{cyto} vs. C_d. In each plot, the intrinsic drug K_m for P-gp was assumed to be 10 μM. V_{max} was assumed to be 1×10^{-6} μmol/cm^2/sec (17). P_{para} was assumed to be 5×10^{-7}cm/sec. To elucidate the contribution of passive permeability to the relative reduction in AP-BL permeability, P_{trans} was assigned values of 5×10^{-5} cm/sec, 5×10^{-6} cm/sec, and 0 cm/sec, for Figs. 2A, 2B, and 2C, respectively.

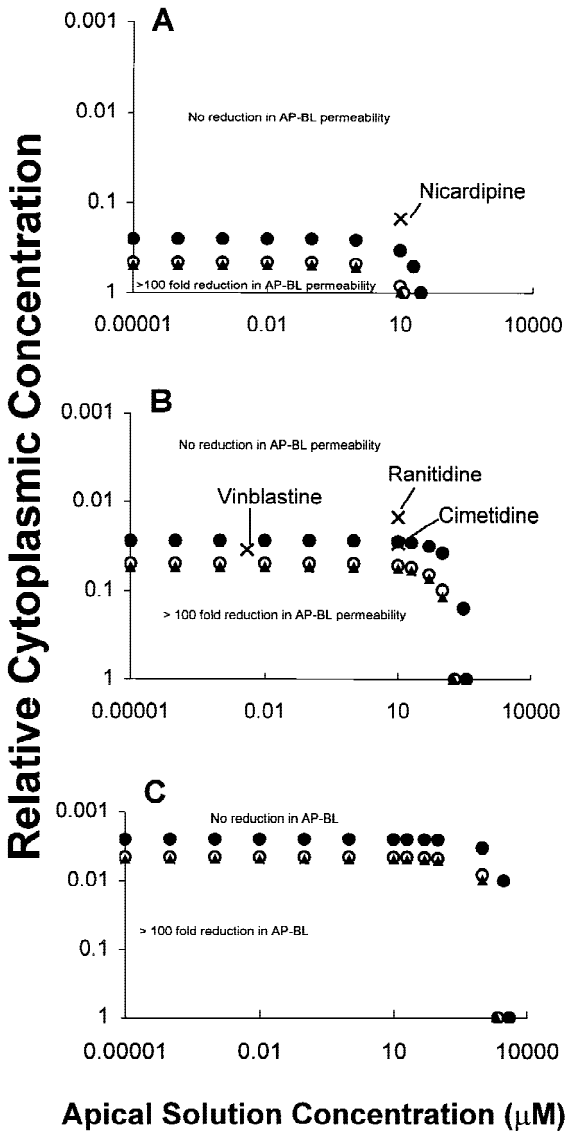

Fig. 2. Contour plots of the relative reduction in apparent AP-BL permeability (● = 2-fold; ○ = 10-fold; ▲ = 100-fold) due to active efflux by P-glycoprotein. Panels A, B, and C denote high, moderate, and extremely low passive transcellular permeability.

In each of these scenarios, P-gp would not reduce the AP-BL permeability when the relative cytoplasmic concentration is very low (e.g., $C_{cyto}/C_d = 0.001$). Assuming the source of P-gp efflux of drug is the cytosol, sufficient drug needs be present in the cytosol. On the other hand, high relative cytoplasmic concentration (bottom range of Figs 2A, 2B, and 2C) generally results in over a 100-fold reduction in permeability due to P-gp.

Under various conditions, a 2-fold, 10-fold, or 100-fold reduction in permeability can occur. For moderately permeable compounds (Fig. 2B), these P-gp effects occur between relative cytoplasmic concentration levels of 0.01 to 0.1. However, apical solution concentrations exceeding K_m (>10 μM) require higher relative cytoplasmic concentrations, due to P-gp saturation.

For highly permeable drugs (Fig. 2A), a 2-fold reduction in permeability is difficult to attain, because very high relative cytoplasmic concentrations need be achieved. For extremely low permeability drugs (Fig. 2C), P-gp can impact permeability over a theoretically wide range of conditions (10 pM to 100 μM), when relative cytoplasmic concentrations are greater than 0.003. However, it may be questionable whether extremely low permeable drugs can provide this level of intracellular drug concentration.

Data Interpretation Within the Kinetic Model

Results from the directional permeability studies of nicardipine, vinblastine, cimetidine, and ranitidine through Caco-2 monolayers are plotted on the theoretical plots (Fig. 2). The relative reduction in AP-BL permeability was taken to be the ratio of BL-AP/AP-BL permeability. For nicardipine, directional permeability studies indicated no reduction in AP-BL permeability at the donor concentration of 10 μM used in the study. This observation was in qualitative agreement with the theoretical plot shown in Fig. 2A. For a compound with rapid transmembrane movement, C_{cyto} would need to reach 35% of C_d in order to observe a reduction in AP-BL permeability. Such high C_{cyto} values are presumably not attained even in steady state transport studies, and may explain why nicardipine did not demonstrate apparent P-gp efflux, when studied alone. When plotted in Fig. 2A, with an apical solution concentration of 10 μM and an assumed relative cytoplasmic concentration of 0.15 (or less), nicardipine's AP-BL permeability would be predicted to be unchanged, as observed experimentally.

Reduction in AP-BL permeability was observed for both vinblastine and cimetidine. These observations can be interpreted in the context of Fig. 2B, and its underlying model (i.e., moderate Ptrains = 5×10^{-6} cm/sec). Vinblastine and cimetidine both exhibited moderate permeabilities (Table 1). Because the relative reduction in AP-BL permeability for vinblastine and cimetidine were 3-fold and 2-fold, respectively, Fig. 2B suggests $C_{cyto}/C_d \approx 0.03$. These cytoplasmic drug concentrations may be realistic. Further studies are warranted. Notable is that $C_d = 10$ μM for both nicardipine and cimetidine transport studies; however, a 2-fold relative reduction in AP-BL permeability was seen for cimetidine, but not for nicardipine. As plotted in Fig. 2A and 2B, the only assumed difference is nicardipine's high transcellular permeability and cimetidine's moderate transcellular permeability.

Also plotted in Fig. 2B is an estimate of ranitidine's dis-

position under P-gp influence. Experimentally, the BL-AP/AP-BL permeability ratio for ranitidine was 1.29. Using this model (i.e., $P_{trans} = 5 \times 10^{-6}$ cm/sec) $C_{cyto}/C_d \leq 0.015$. Hence, in Fig. 2B, the relative cytoplasmic concentration of ranitidine is assumed to be 2-fold less than that of cimetidine. This assumption may be reasonable, given that P_{trans} for ranitidine and cimetidine were 1.59×10^{-6} and 3.54×10^{-6} cm/sec, respectively (i.e., 2-fold different).

These theoretical contour plots are in agreement with the experimental data, and emphasize the combined roles of passive permeability, apical donor concentration, and intracellular free drug concentration on apparent P-gp kinetics. However, measurement of intracellular free drug concentration for each compound is needed for further model assessment.

Moderate passive permeability is required for the manifestation of P-gp effects (vinblastine and cimetidine). Such compounds are sufficiently able to enter the cell, where P-gp efflux can effectively compete against passive permeability. Compounds such as nicardipine, with rapid transmembrane movement, may pass through the cell faster than P-gp can remove it. Similarly, compounds such as ranitidine, whose passive permeability is low, may never reach the intracellular concentrations needed to be effluxed by P-gp. Simple inspection of the ratio of BL-AP/AP-BL permeability after directional transport studies is a limiting means to identify compounds that may affect P-gp kinetics. Drug interaction studies with known P-gp substrates are needed to identify whether a compound is a P-gp substrate.

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REFERENCES

1. G. A. Altenberg, C. G. Vanoye, J. K. Horton, and L. Reuss. Unidirectional fluxes of rhodamine 123 in multidrug-resistant cells: evidence against direct drug extrusion from the plasma membrane. *Proc. Natl. Acad. Sci. U.S.A.* **91**:4654–4657 (1994).
2. M. Ramachandra, S. V. Ambudkar, D. Chen, C. A. Hrycyna, S. Dey, M. M. Gottesman, and I. Pastan. Human P-glycoprotein exhibits reduced affinity for substrates during a catalytic transition state. *Biochemistry* **37**:5010–5019 (1998).
3. A. B. Shapiro and V. Ling. Positively cooperative sites for drug transport by P-glycoprotein with distinct drug specificities. *Eur. J. Biochem.* **250**:130–137 (1997).
4. W. D. Stein, C. Cardarelli, I. Pastan, and M. M. Gottesman. Kinetic evidence suggesting that the multidrug transporter differentially handles influx and efflux of its substrates. *Mol. Pharmacol.* **45**:763–772 (1994).
5. G. D. Eytan, R. Regev, G. Oren, C. D. Hurwitz, and Y. G. Assaraf. Efficiency of P-glycoprotein-mediated exclusion of rhodamine dyes from multidrug-resistant cells is determined by their passive transmembrane movement rate. *Eur. J. Biochem.* **248**: 104–112 (1997).
6. G. D. Eytan, R. Regev, G. Oren, and Y. G. Assaraf. The role of passive transbilayer drug movement in multidrug resistance and its modulation. *J. Biol. Chem*. **271**:12897–12902 (1996).
7. R. Evers, N. H. Cnubben, J. Wijnholds, L. van Deemter, P. J. van Bladeren, and P. Borst. Transport of glutathione prostaglandin A conjugates by the multidrug resistance protein 1. *FEBS Lett.* **419**: 112–116 (1997).
8. K. A. Lentz, J. Hayashi, L. J. Lucisano, and J. E. Polli. Development of a more rapid, reduced serum culture system for Caco-2 monolayers and application to the Biopharmaceutics Classification System. *Int. J. Pharm.* **200**:41–51 (2000).
9. J. W. Polli, J. E. Humphreys, S. A. Wring, T. C. Burnette, K. D. Read, A. Hersey, D. Butina, L. Bertolotti, P. Pugnaghi, and C. S. Serabjit-Singh. Comparison of MDCK and bovine brain endothelial cells (BBECs) as a blood-brain barrier screen in early drug development. In M. Balls, A. VanZeller, and M. Halder (eds.), *Progress in the Reduction, Refinement and Replacement of Animal Experimentation,* Elsevier Science, New York, 2000, pp. 271–289.
10. N. F. Ho, P. S. Burton, R. A. Conradi, and C. L. Barsuhn. A biophysical model of passive and polarized active transport processes in Caco-2 cells: approaches to uncoupling apical and basolateral membrane events in the intact cell. *J. Pharm. Sci.* **84**: 21–27 (1995).
11. J. D. Irvine, L. Takahashi, K. Lockhart, J. Cheong, J. W. Tolan, H. E. Selick, and J. R. Grove. MDCK (Madin-Darby canine kidney) cells: A tool for membrane permeability screening. *J. Pharm. Sci.* **88**:28–33 (1999).
12. S. Doppenschmitt, H. Spahn-Langguth, C. G. Regardh, and P. Langguth. Role of P-glycoprotein-mediated secretion in absorptive drug permeability: An approach using passive membrane permeability and affinity to P-glycoprotein. *J. Pharm. Sci*. **88**: 1067–1072 (1999).
13. K. Ito, H. Kusuhara, and Y. Sugiyama. Effects of intestinal CYP3A4 and P-glycoprotein on oral drug absorption–theoretical approach. *Pharm. Res*. **16**:225–231 (1999).
14. R. L. Shepard, M. A. Winter, S. C. Hsaio, H. L. Pearce, W. T. Beck, and A. H. Dantzig. Effect of modulators on the ATPase activity and vanadate nucleotide trapping of human P-glycoprotein. *Biochem. Pharmacol*. **56**:719–727 (1998).
15. G. A. Sawada, L. R. Williams, B. S. Lutzke, and T. J. Raub. Novel, highly lipophilic antioxidants readily diffuse across the blood-brain barrier and access intracellular sites. *J. Pharm. Exp. Ther.* **288**:1327–1333 (1999).
16. S. Ito, C. Woodland, B. Sarkadi, G. Hockmann, S. Walker, and G. Koren. Modeling of P-glycoprotein-involved epithelial drug transport in MDCK cells. *Am. J. Physiol.* **277**:F84–F96 (1999).
17. J. Hunter, B. H. Hirst, and N. L. Simmons. Drug absorption limited by P-glycoprotein-mediated secretory drug transport in human intestinal epithelial Caco-2 cell layers. *Pharm. Res.* **10**: 743–749 (1993).