Modeling of P-glycoprotein-involved epithelial drug transport in MDCK cells

SHINYA ITO,1 CINDY WOODLAND,2 BALÁZS SARKADI,2 GUIDO HOCKMANN,1 SCOTT E. WALKER,2 AND GIDEON KOREN1
1Division of Clinical Pharmacology and Toxicology, Departments of Pediatrics and Pharmacology, Hospital for Sick Children, Research Institute, and University of Toronto; 2Sunnybrook Health Science Center, Toronto, Ontario, Canada M5G 1X8; and 3The National Institute of Haematology, Blood Transfusion, and Immunology, H-1113 Budapest, Hungary

Ito, Shinya, Cindy Woodland, Balázs Sarkadi, Guido Hockmann, Scott E. Walker, and Gideon Koren. Modeling of P-glycoprotein-involved epithelial drug transport in MDCK cells. Am. J. Physiol. 277 (Renal Physiol. 46): F84–F96, 1999.—P-glycoprotein (P-gp) on the apical membranes of epithelial cells is known as a drug efflux pump. However, unclear is its integral quantitative role in the overall epithelial drug transfer, which also involves distinct diffusion processes in parallel and sequence. We used a simple three-compartment model to obtain kinetic parameters of each drug transfer mechanism, which can quantitatively describe the transport time courses of P-gp substrates, digoxin and vinblastine, across P-gp-expressing MDCK cell monolayers grown on permeable filters. Our results show that the model, which assumes a functionally single drug efflux pump in the apical membrane with diffusion across two membranes and intercellular junctions, is the least complex model with which to quantitatively reproduce the characteristics of the data. Interestingly, the model predicts that the MDCK apical membranes are less diffusion permeable than the basolateral membrane for both drugs and that the distribution volume of vinblastine is 10-fold higher than that of digoxin. Additional experiments verified these model predictions. The modeling approach is feasible to quantitatively describe overall kinetic picture of epithelial drug transport. Further model refinement is necessary to incorporate other modes of drug transport such as transcytosis. Also, whether P-gp solely accounts for the pump function in this model awaits more studies.

P-glycoprotein; multidrug resistance; diffusion; itraconazole

Drug translocation across epithelial cells consists of multiple independent processes such as active transport and diffusion, which are functionally connected to each other in parallel and sequence. Although this network of diffusion and transport appears simple, it is not easy to comprehend the relative contribution of each membrane process to overall cellular drug transport. A typical example is transepithelial transport of drugs via P-glycoprotein (8, 18), an ATP-dependent drug efflux pump (12) also known as multidrug resistance (MDR) protein because of its association with cellular resistance to various cytotoxic agents. Epithelia such as renal tubular cells express one of the isoforms of the protein (i.e., MDR1 in humans) on their apical membranes (32), and its drug transport function is unidirectional, extruding drugs out of the cells into the lumen. Preserving their polarizing characteristics, these cells grown on permeable filters enable extensive in vitro investigations of the P-glycoprotein-mediated transport (5, 15, 26, 31).

Studies in these polarized epithelia have shown that the expression of MDR1-type P-glycoprotein is associated with enhanced basal-to-apical net transfer of diverse classes of compounds such as vinca alkaloids (31), cyclosporin (25), and digoxin (3, 25, 31). Interpretation of these findings has been based on intuitive face validation of the conceptual model of P-glycoprotein as an apically located active drug efflux pump (Fig. 1; Refs. 5, 25, 31), which is assumed from the known localization of P-glycoprotein in the epithelial cells (32). However, to our knowledge, transport data of P-glycoprotein substrates have never been quantitatively analyzed according to this functional model. Although a steady-state linear kinetic model was previously applied to transport of vinblastine in MDCK cells (15), uncertainty of the parameter estimates was not explicitly determined. Hence, in intact epithelial cells, it is difficult to infer quantitative relations among pump-mediated drug transport, equilibrium accumulation, and passive diffusion across two distinct membrane domains. This knowledge is essential to understand biologic signiﬁcance of drug transporters and to gain insight into roles of other distinct drug transfer processes such as diffusion in disposition of xenobiotics in vivo.

In this report, we use mathematical modeling approach to quantitatively describe in vitro time course of net transepithelial transfer of P-glycoprotein substrates, digoxin and vinblastine, through P-glycoprotein-expressing MDCK renal tubular cells. Digoxin and vinblastine were used because their transport appears to be mediated mainly by P-glycoprotein (15, 31), if not entirely. In support of this notion, mice with a disruption of mdr1a gene encoding one of the drug-transporting P-glycoproteins showed substantial increase in digoxin and vinblastine plasma and tissue levels, indicating the major role of P-glycoprotein in eliminating these compounds in vivo (26, 27). The MDCK cell line was used because 1) P-glycoprotein-mediated drug transport has been extensively studied using this cell line; 2) its intercellular junction is very tight, rendering relatively high signal-to-noise ratio of the pump function; and 3) this distal renal tubular cell line, unlike the LCC-PK1 proximal renal tubular cell line, does not show transport signals of tetraethylammonium, a prototype substrate for the organic cation transport system (6), whose overlapping substrate specificity with P-glycoprotein (4) makes interpretation of experimental results difficult.

Our results show that transport of digoxin and vinblastine across the MDCK cell monolayers is ex-
plained quantitatively by a kinetic model, which assumes a functionally single unidirectional drug pump in the apical membrane and diffusion across both membranes. Interestingly, the model predicts that the MDCK apical membranes are less diffusion permeable than the basolateral membrane for both drugs and that the distribution volume of vinblastine is 10-fold higher than that of digoxin. Further experiments cross-validated the model by verifying these predictions.

MATERIALS AND METHODS

Materials

\[^{[3]}\text{H}\]digoxin (16.1 Ci/mmol) and \[^{[14]}\text{C}\]mannitol (55.1 mCi/mmole) were purchased from Du Pont Canada (Markham, Ontario, Canada). \[^{[3]}\text{H}\]vinblastine (11.2 Ci/mmole) was purchased from Amersham Canada (Oakville, Ontario). Fetal bovine serum was obtained from Gibco-BRL and was added to media (\(\alpha\)-MEM); Nunc TC inserts (25-mm external diameter) were from A/S Nunc. Vincristine, vinblastine, ketoconazole, and digoxin were purchased from Sigma. Itraconazole was donated by Janssen Pharmaceuticals. An anti-rabbit, horseradish peroxidase (HRP)-conjugated goat IgG was obtained from Boehringer. Calcein acetoxymethyl ester (calcein-AM) was obtained from Molecular Probes (Eugene, OR).

Cell culture. MDCK cells obtained from the American Type Culture Collection (Rockville, MD) were grown in \(\alpha\)-MEM containing 10% fetal bovine serum without antibiotics under an atmosphere of 95% air-5% CO\(_2\) at 37°C. They were subcultured twice weekly using 0.02% EDTA and 0.05% trypsin. NIH 3T3 cells overexpressing P-glycoprotein were kind gifts of Dr. M. M. Gottesman.

Immunoblotting and P-Glycoprotein Quantitation

Immunoblotting using a monoclonal antibody (C219; Ref. 18) against P-glycoprotein was performed as follows. Crude plasma membrane fractions were obtained from MDCK cells (23) and electrophoresed on 7.5% SDS-PAGE gels. Transfer to nitrocellulose was blocked overnight in 4% skim milk Tris-NaCl-Tween solution. The blocked nitrocellulose strips were incubated with C219 for 3 h at room temperature. Binding of C219 monodonal antibody was visualized employing the enhanced chemiluminescence detection procedure (ECL, Amersham).

We also used an anti-MDR1 P-glycoprotein polyclonal antibody (4077), which recognizes the NH\(_2\)-terminal half of MDR1 across different species including dogs (30), from which the MDCK cell line was derived. After washing twice in a protein-free (HPMI) medium, the cultured cells were dissolved and sonicated in a disaggregation buffer (1, 24). The second antibody (anti-rabbit HRP-conjugated goat IgG) was used in 20,000× dilution. HRP-dependent luminescence was detected in a similar manner to C219.

The amounts of P-glycoprotein were quantified by excising the respective bands recognized by the antibody 4077 from the polyvinylidene difluoride (PVDF) membrane and measuring their luminescence in a liquid scintillation counter (Beckman LS 6000, Single Photon Monitor mode). The standard was the known amounts of P-glycoprotein (MDR1) in the isolated Sf9 cell membranes (14).

Detection of multidrug resistant associated protein (MRP) with the R1 (rat) monoclonal anti-human MRP antibody, kindly provided by Dr. R. Schaper, was carried out as previously described (1). The second antibody (anti-rat, peroxidase-conjugated IgG was used in 1,000× dilution. HRP-dependent luminescence on the immunoblots (ECL, Amersham) was determined by autoradiography.

Calcein Accumulation Assay for P-Glycoprotein and MRP Function

Calcein-AM, a nonfluorescent hydrophobic compound, is converted to fluorescent calcein by cytoplasmic esterase after entering cells. Whereas P-glycoprotein (MDR1) appears to be a pump for calcein-AM, MRP seems to be a pump for both calcein-AM and its fluorescent free form, calcein, as well as its glutathione conjugate (13). Because of differences in their inhibitor specificity, it is possible to differentiate between the functions of the two proteins (13). Calcein accumulation was measured by incubating the cells in HPMI medium (2.5 × 10\(^5\) cells/ml) containing 0.25 µM calcein-AM as described elsewhere (15, 25). The multidrug resistance activity factor (MAF; Refs. 13 and 14) was calculated according to the equation: MAF = (\(F^*\) - F)/\(F^*\), where \(F^*\) and F designate the dye accumulation rate in the presence and absence, respectively, of an inhibitor of the respective multidrug transporter. Prostaglandin A\(_1\) at 10 µM was used for maximum inhibition of MRP (13), whereas 100 µM verapamil was used for maximum inhibition of both MRP and P-glycoprotein (14). MAF, an empirical index ranging from 0 to 1, is correlated with the expression levels of the transporters in a nonlinear fashion, which becomes asymptotic to unity at a high level of transporter expression (14).

Filter Preparation

MDCK cells in the incubation medium were seeded at a density of about 5 × 10\(^5\) cells/cm\(^2\) on the inorganic permeable membranes attached to 25-mm tissue culture inserts (0.2 µm pore size, 4.15-cm\(^2\) growth area; Nunc TC inserts). The inserts were placed in cluster plates, fed every 48 h, and incubated in the same conditions as described above. Experiments were conducted 10 days after seeding.

Transport Experiment

The incubation was started at 37°C immediately after the culture media of both compartments were replaced with...
preconditioned incubation media; only the media bathing one side of the monolayers (either basal or apical) contained 0.1 µM [3H]digoxin or 0.1 µM [3H]vinblastine. [14C]mannitol, 1.45 µM, was also added to the drug-containing media as a paracellular flux marker.

Two kinds of experiments were conducted: 24-h time course experiments, and 3-h time course experiments to examine side dependency of inhibitors. For 24-h incubation experiments, basal-to-apical and apical-to-basal transport was measured in the presence or absence of 5 µM itraconazole, a potent P-glycoprotein inhibitor (10), added to both basal and apical sides. When side dependency of inhibition was examined, basal-to-apical digoxin (0.1 µM) or vinblastine (0.1 µM) fluxes were monitored for 3 h in the presence or absence of inhibitors added to either basal or apical side of the monolayers.

Twenty-five microliters of the medium was sampled at given time intervals, and the radioactivity (dpm) was measured with scintillation counting. We discarded results from cell monolayers with [14C]mannitol fluxes greater than 2 SD of the mean mannitol flux in each experimental condition.

**Cell Viability Assay**

To ensure that overall cellular function is not disturbed even after 24-h exposures to drug, viability of the MDCK cells over prolonged periods of exposure to various concentrations of digoxin, vinblastine, and itraconazole was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bro-mide (MTT) assay (20). Briefly, MDCK cells were plated into 96-well plates in α-MEM with 10% fetal bovine serum and incubated at 37°C in 5% CO2. At 24- and 48-h incubation with various concentrations of digoxin, vinblastine, or itracona-zole, 20 µl of a 5 mg/ml MTT solution was added to wells and incubated at 37°C in 5% CO2 for 4 h. Supernatants were aspirated off, then 100 µl of acidified isopropanol was added and incubated in the dark at room temperature for 30 min. Plates were agitated for 2–3 min until a uniform color was achieved, then read on an ELISA reader at 490 nm. Percentage of cell survival was calculated by dividing the average value at each drug concentration by the average control value, multiplying by 100. This percentage was designated as the viability rate.

**Cellular Accumulation of Digoxin and Vinblastine**

The cellular accumulation of radiolabeled vinblastine from the basal side of the monolayers in the presence and absence of vincristine added to either the apical or the basal side was examined at 10, 30, and 60 min of incubation. Also, digoxin uptake from the basal side at 60 min was examined and compared to corresponding vinblastine uptake. The procedure has been described elsewhere (35). The uptake results were expressed as a ratio of the amount of drug in the cell lysates to that of the initial incubation medium.

**Itraconazole Assay**

Itraconazole disposition in the MDCK cells was examined as follows. Itraconazole (5 µM) was added to both sides of the monolayers at time 0. After 48-h incubation under the usual culture conditions, apical and basal media were collected, and itraconazole was quantified with a modified HPLC method (for the original method, see Ref. 34). The inter- and intra-assay coefficients of variation were less than 5%. The quantification limit for itraconazole was set at 10 nM.

**Modeling of the Drug Transport**

We developed a two-stage method to account for differences of paracellular diffusion monitored by mannitol among the four different conditions: basal-to-apical and apical-to-basal transport of drug in the presence or absence of itraconazole. This method necessitates only one assumption: that mannitol flux is proportional, if not identical, to paracellular fluxes of digoxin and vinblastine. First, the paracellular flux coefficient was estimated (first-stage fitting), which was then incorporated into the disposition equations for the final fitting of the drug transfer data (second-stage fitting).

**Estimation of Paracellular Diffusion (first Stage).** The time courses of [14C]mannitol fluxes in each condition were fit to the following equations

\[
\frac{dA}{dt} = (B - A) \cdot \rho/2
\]

\[
\frac{dB}{dt} = (A - B) \cdot \rho/2
\]

in which A and B are concentrations of mannitol in the apical and basal compartment; \( \rho \) is a paracellular diffusion constant (ml/h) of mannitol through the cell monolayer; and the denominator is a volume of the apical and basal chamber (i.e., 2 ml). The estimated four different diffusion rates (\( \rho \)) for each of the four conditions were expressed as a ratio to the diffusion constant of mannitol in the basic condition, in which drug and mannitol were added to the basal side in the absence of itraconazole; the calculated ratio was designated as a relative paracellular diffusion constant (\( \rho' \)).

**Model Construction (second Stage).** Based on the criteria (Table 1), three main models were designed (Fig. 2). All three models are based on cellular localization and function of P-glycoprotein as a unidirectional drug efflux pump on apical membranes. Model A is based on the original model (15). Model B also assumes another drug efflux system on the basal side, as suggested from the localization of MRP, although MDCK does not show significant MRP function (see results). Model C is a two-compartment model assuming the renal tubular cells to be a volumeless single barrier (instead of a substantial compartment) between the apical and basolateral compartments; this was examined for its conceptual simplicity. As variants of the most plausible model (model A), we also tested the following models: 1) model A with separate diffusion and distribution volume parameters for itraconazole-added conditions; 2) model A with an influx unidirectional pump on the basal membrane; and 3) model A with a second intracellular compartment.

There exist endocytosis-transcytosis processes that may contribute to overall drug transfer from the basal to the apical side of epithelial cells (22). Also, drugs distributed into intracellular organelles are likely to be extruded to extracellular space via vesicular trafficking. In this study, these modes of drug transport were not taken into account, because experimental validation was difficult.

**Model A.** Model A is the physiologically defined model with a single active transport system in the apical membrane (Fig. 2A), which is based on the conceptual model of P-glycoprotein.

<table>
<thead>
<tr>
<th>Table 1. Criteria for model construction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Compartmental modeling is used.</td>
</tr>
<tr>
<td>2) Model parameters possess biological meanings.</td>
</tr>
<tr>
<td>3) Model has two extracellular compartments to represent the two culture chambers separated by the cell monolayer in the experimental system.</td>
</tr>
<tr>
<td>4) Model is consistent with existing data on the apical localization of P-glycoprotein in the MDCK cells.</td>
</tr>
</tbody>
</table>

Models are constructed on the basis of the physical and biological characteristics of the drug transport mechanisms.
as the apical pump in the renal tubular cells. The disposition
transport across the MDCK renal tubular cells.

Fig. 2. Compartment models of cellular digoxin and vinblastine
another drug efflux system in the basal membrane.
apical membrane and with three diffusion processes; this is based on
ment model with one unidirectional active transport system in the
overall diffusion parameter for the 2-compartment model; and k and
are the intrinsic clearance of drug via the apical pump and that of
the basal drug efflux pump, respectively.
as the apical pump in the renal tubular cells. The disposition
equations are described as follows

\[
\frac{dA}{dt} = \frac{[C \cdot (k \cdot \theta + \delta_1) + B \cdot p' \cdot \delta_3 - A \cdot (\delta_1 + p' \cdot \delta_3)]}{2}
\]
\[
\frac{dC}{dt} = A \cdot \delta_1 + B \cdot \delta_2 - C \cdot (k \cdot \theta + \delta_1 + \delta_2) / V
\]
\[
\frac{dB}{dt} = \frac{[C \cdot \delta_2 V + A \cdot p' \cdot \delta_3 - B \cdot (\delta_2 + p' \cdot \delta_3)]}{2}
\]
in which A and B are concentrations of the drug in the apical and
basal compartment; C denotes the amount of the drug in the
the cellular compartment; \(\delta_1\), \(\delta_2\), and \(\delta_3\) are diffusion parameters of drug expressed as volume per unit time through
apical membrane, basal membrane, and cellular junctions, respectively; \(p'\) is the relative paracellular diffusion constant of
each condition obtained by the first stage mannitol fitting
(see above); k is the intrinsic clearance of drug via the pump
in the absence of itraconazole; \(\theta\) is a fraction \((1 \geq \theta \geq 0)\) of
the remaining pump parameter (k) in the presence of itracona-
ze and V is the apparent cellular distribution volume of
drug. Itraconazole was assumed to inhibit the pump function
(k) only. The denominator of the first and third equations is 2
ml, which is the volume of the apical and basal compartments
in this experimental setting.

MODEL B. Model B is different from Model A in that it has
another drug efflux pump on the basal membranes (Fig. 2B).
Although there has been no experimental evidence suggest-
ing that the MDCK basal membrane expresses a carrier-
mediated drug efflux system for digoxin or vinblastine,
we studied this model to explore such a possibility. Behind this
attempt was the notion that MRP is expressed in basolat-
eral membranes of LLC-PK1, a proximal renal tubular cell line.
The general disposition equations are as follows

\[
\frac{dA}{dt} = \frac{[C \cdot (k \cdot \theta + \delta_1) V + B \cdot p' \cdot \delta_3 - A \cdot (\delta_1 + p' \cdot \delta_3)]}{2}
\]
\[
\frac{dC}{dt} = A \cdot \delta_1 + B \cdot \delta_2 - C \cdot (k \cdot \theta + \lambda + \delta_1 + \delta_2) / V
\]
\[
\frac{dB}{dt} = \frac{[C \cdot (\lambda + \delta_2) V + A \cdot p' \cdot \delta_3 - B \cdot (\delta_2 + p' \cdot \delta_3)]}{2}
\]

which are the same as model A except for the term involving \(\lambda\)
(an intrinsic clearance of the active transporter in the basal
membrane). Itraconazole was assumed to inhibit the apical
pump function (k) only.

MODEL C. Model C is a two-compartment model with
a single active transport system (Fig. 2C). This simple two-
compartment model assuming the renal tubular cells to be a
volumeless single barrier (instead of a substantial compart-
ment) between the apical and the basolateral compartments
was tested because it is a simpler approach providing an
easy-to-comprehend kinetic picture. The general disposition
equations are as follows

\[
\frac{dA}{dt} = \frac{[B \cdot (k \cdot \theta + p' \cdot \delta) - A \cdot p' \cdot \delta)]}{2}
\]
\[
\frac{dB}{dt} = \frac{[A \cdot p' \cdot \delta - B \cdot (k \cdot \theta + p' \cdot \delta)]}{2}
\]

where \(\delta\) is a hybrid diffusion constant of the three distinct
diffusion processes: apical membranes, basolateral mem-
branes, and intercellular junctions. Itraconazole was as-
umed to inhibit the pump function (k) only.

Fitting. We used MLAB (Civilized Software, Bethesda,
MD) in the least-squares fitting. This program uses the
Marquardt-Levenberg least-squares method for curve fitting,
which is characterized by its superior fitting efficiency even
when there are more than two model compartments with
relatively few data points. The fitting was performed with the
average data set of four filters for each of the four different
initial conditions; i.e., drug was added to either the basal or
the apical side in the presence and absence of itraconazole.
When the experimental results were fit to those models,
several initial estimates of the parameters were used to avoid
entrapment in local minima. The weighting factor employed
in the fitting was the reciprocal of the variance weight values
formed by the standard deviation estimates of each data
point. The constraints given to the fitting were the positivity
of all parameters and a range of fractional pump inhibition
by itraconazole (from 0 to 1). Itraconazole was assumed to
change the pump function only. In all models, first-order
movement of drug was assumed.

Simulation. To examine the influences of the apical pump
function changes on overall cellular drug disposition, time
courses of drug transport in the best-fit model (model A) were
simulated using the same modeling computer program
(MLAB) with the pump parameter ranging from 100% to 0%
of control.

Model diagnosis and selection of a best-fit model (Table 2).
To interpret the fitting results, two major questions need to be
answered: 1) How well does the model fit the data? and 2) Does this model fit the data better than an alternate model?

GOODNESS OF FIT (HOW WELL DOES THE MODEL FIT THE DATA?).
The assessment is done by a combination of multiple meth-
ods. The fitting with the least-squares method provides
residual sum of squares (RSS) as an indicator of the average
deviation of the fitting curve from the data. Here, two
different situations exist (19). First, if the error variances of
the experimental data are explicitly known and given, chi-
squared test can be used to statistically evaluate the overall
size of the RSS; specifically, compatibility of the size of RSS
with the given experimental errors is tested. Also, qualitative
assessment to identify apparent systematic misfitting or
outright fitting errors can be done by inspecting the graph of
the fitting curve superimposed on the data points and the
derived R-squared value. On the other hand, if the error
variance of data is unknown (such as in this study), chi-
squared test is meaningless. Instead, the qualitative assess-
ment is done.
In addition to these overall evaluations of the fitting, randomness of the residuals (i.e., random deviation of the fitting curve from the data) should be confirmed either qualitatively by patterning the residuals in a plot against time, or quantitatively by using the runs test (19). The runs test compares the number of runs of consecutive positive and negative residuals with that of runs expected from random distribution. Systematic or correlated errors identified by these methods may be the results of model error, experimental error, or failure of the weighted least-squares algorithm. In this study, both approaches were used.

**BEST-FIT MODEL (DOES THIS MODEL FIT THE DATA BETTER THAN AN ALTERNATE MODEL?)**. We selected a best-fit model as a minimal model, whose data fitting (i.e., residual sum of squares) is statistically indistinguishable from those of more complex models. In general, higher order, more complex models tend to fit data closer than do lower order, simpler models, giving smaller residual sum of squares. However, data fitting per se may simply reflect experimental errors rather than physiological truth. Taking these into account, we used a combination of analysis of variance (F test) and parsimony criteria such as Akaike's information criterion (AIC) and Schwartz criterion (SC) to select a best model (Table 2).

F test was used to examine statistical significance of difference in the best weighted residual sum of squares between given two models. When the F statistic becomes significant (i.e., $P < 0.05$), the model with a greater residual sum of squares was rejected in favor of the other. The least complex of the remaining models was chosen as the best model, and the selection was further confirmed using AIC and SC, which represent goodness of fit (i.e., lower weighted residual sum of squares) and simplicity of the model (i.e., fewer numbers of model parameters) (19): $\text{AIC} = n \cdot \ln (S) + 2r$; and $\text{SC} = n \cdot \ln (S) + r \cdot \ln (n)$. In these equations, $n$ denotes the number of data points; $S$ is the weighted residual sum of squares; and $r$ is the number of parameters estimated. The model with the lowest value of AIC and SC is considered as the simplest model that still provides fitting statistically as good as that of more complex models. In addition to the above statistical criteria, overall goodness of fit such as randomness of residuals (see GOODNESS OF FIT ..., above) and uncertainty of parameter estimates were also considered.

**Data Analysis**

The results are expressed as means ± SD unless otherwise stated. ANOVA and Student's t-test were used where appropriate. P values of less than 0.05 were considered statistically significant.

**RESULTS**

**Immunoblotting of P-Glycoprotein and MRP**

The levels of expression detected by the C219 monoclonal and the 4077 polyclonal antibodies were higher in MDCK cells than in wild-type LLC-PK1 cells (Fig. 3).

**Fig. 3. Immunoblotting of P-glycoprotein.** A: Western blotting by mouse monoclonal antibody (C219). Plasma membrane fractions from wild-type MDCK cells (lane 1) and the wild-type LLC-PK1 cells (lane 2) were loaded onto a 7.5% SDS-PAGE gel (100 µg protein/lane), transferred to nitrocellulose, and visualized using the enhanced chemiluminescence procedure (ECL, Amersham). B: Western blotting with rabbit polyclonal antibody (4077). After washing twice in a protein-free medium, the wild-type MDCK cells (lane 1), the wild-type LLC-PK1 cells (lane 2), and the P-glycoprotein-overexpressing NIH 3T3 cells (lane 3) were dissolved and sonicated in a disaggregation buffer. The second antibody (anti-rabbit HRP-conjugated goat IgG) was used in 20,000× dilution. ECL (Amersham) was used to visualize the protein.

The mean expression levels of P-glycoprotein quantified by the 4077 antibody were 0.22 µg/mg total cell protein for MDCK cells (~10% of the P-glycoprotein-overexpressing NIH 3T3 MDR1 cells: 2.25 µg/mg total cell protein). Total cell protein of MDCK was 212 µg/filter (4.15 cm²) on average. Taken together, MDCK cells were estimated to be expressing ~0.05 µg of P-glycoprotein per filter preparation, corresponding to ~0.3 pmol/filter (4.15 cm²).

The rat monoclonal antibody against human MRP failed to detect any significant antigens in the MDCK cells (data not shown). To circumvent the possible species differences of immunoreactivity of this antibody, we examined MRP function by the calcein accumulation assay (see below).

**Calcein Accumulation Assay**

Prostaglandin A₁, 10 µM, which behaves as an MRP inhibitor in this concentration range (14), caused only
METHODS). Viability rates of 99.6% at concentrations of 0.1 µM resulted in MDCK cell MTT Cell Viability Assay.

P-glycoprotein function. little MRP function, while they have relatively high verapamil). This suggests that MDCK cells possess little MRP function, while they have relatively high P-glycoprotein function.

Table 3. Mannitol paracellular diffusion

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Diffusion Parameter Estimates (µ), ×10^−3 ml/h</th>
<th>Standard Deviation of Estimates, ×10^−3 ml/h</th>
<th>Relative Diffusion Parameter (µ)</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Digoxin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−Itraconazole</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal to apical</td>
<td>2.05</td>
<td>0.299</td>
<td>1</td>
<td>0.998</td>
</tr>
<tr>
<td>Apical to basal</td>
<td>11.96</td>
<td>0.566</td>
<td>5.826</td>
<td>0.996</td>
</tr>
<tr>
<td>+Itraconazole</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal to apical</td>
<td>1.76</td>
<td>0.190</td>
<td>0.857</td>
<td>0.998</td>
</tr>
<tr>
<td>Apical to basal</td>
<td>27.49</td>
<td>2.180</td>
<td>13.39</td>
<td>0.995</td>
</tr>
<tr>
<td><strong>Vinblastine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−Itraconazole</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal to apical</td>
<td>5.06</td>
<td>0.456</td>
<td>1</td>
<td>0.999</td>
</tr>
<tr>
<td>Apical to basal</td>
<td>20.6</td>
<td>1.419</td>
<td>4.08</td>
<td>0.997</td>
</tr>
<tr>
<td>+Itraconazole</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal to apical</td>
<td>5.37</td>
<td>0.376</td>
<td>1.06</td>
<td>0.999</td>
</tr>
<tr>
<td>Apical to basal</td>
<td>17.87</td>
<td>1.613</td>
<td>3.53</td>
<td>0.997</td>
</tr>
</tbody>
</table>

Second-Stage Fitting Results

None of the fitting results of the three models was under active constraints at termination. For both drugs, models A and B provided significantly better fitting than a simpler model, model C (Table 4). There was no significant difference in the goodness of fit between model A and B (Table 4). However, model A showed the lowest values in the parsimony criteria of AIC and SC (Table 4). There was no systematic deviation of the model A fitting from the data points (see Fig. 4; and nonsignificant results of the runs test, data not shown). Therefore, model A is considered as the minimal model, whose fitting is statistically indistinguishable from those of a more complex model. Compared to digoxin, vinblastine fitting to model A showed relatively small RSS and more precise parameter estimates (CV < 40%).

Cross-validation of the Model Prediction

As shown in Table 5, the best-fit model (model A) predicted the following: 1) the apical membrane of the MDCK cell is less diffusion permeable than the basolateral membrane for both drugs; and 2) the distribution volume is 10-fold higher for vinblastine than that for digoxin, suggesting extensive cellular accumulation of vinblastine. These model predictions were experimentally cross-validated.

Second Stage Fitting Results

None of the fitting results of the three models was under active constraints at termination. For both drugs, models A and B provided significantly better fitting than a simpler model, model C (Table 4). There was no significant difference in the goodness of fit between model A and B (Table 4). However, model A showed the lowest values in the parsimony criteria of AIC and SC (Table 4). There was no systematic deviation of the model A fitting from the data points (see Fig. 4; and nonsignificant results of the runs test, data not shown). Therefore, model A is considered as the minimal model, whose fitting is statistically indistinguishable from those of a more complex model. Compared to digoxin, vinblastine fitting to model A showed relatively small RSS and more precise parameter estimates (CV < 40%).

Data fitting with the model A variants (see MATERIALS AND METHODS) did not converge to meaningful estimates and failed to provide better statistical results (not shown). These findings indicate that model A (the physiologically defined 3-compartment model with a functionally single active transport system in the apical membrane) appears to be the model of minimal complexity that can quantitatively reproduce the characteristics of our data set on digoxin and vinblastine transport in the MDCK cells (Fig. 4).

Cross-validation of the Model Prediction

As shown in Table 5, the best-fit model (model A) predicted the following: 1) the apical membrane of the MDCK cell is less diffusion permeable than the basolateral membrane for both drugs; and 2) the distribution volume is 10-fold higher for vinblastine than that for digoxin, suggesting extensive cellular accumulation of vinblastine. These model predictions were experimentally cross-validated.

Second Stage Fitting Results

None of the fitting results of the three models was under active constraints at termination. For both drugs, models A and B provided significantly better fitting than a simpler model, model C (Table 4). There was no significant difference in the goodness of fit between model A and B (Table 4). However, model A showed the lowest values in the parsimony criteria of AIC and SC (Table 4). There was no systematic deviation of the model A fitting from the data points (see Fig. 4; and nonsignificant results of the runs test, data not shown). Therefore, model A is considered as the minimal model, whose fitting is statistically indistinguishable from those of a more complex model. Compared to digoxin, vinblastine fitting to model A showed relatively small RSS and more precise parameter estimates (CV < 40%).

Data fitting with the model A variants (see MATERIALS AND METHODS) did not converge to meaningful estimates and failed to provide better statistical results (not shown). These findings indicate that model A (the physiologically defined 3-compartment model with a functionally single active transport system in the apical membrane) appears to be the model of minimal complexity that can quantitatively reproduce the characteristics of our data set on digoxin and vinblastine transport in the MDCK cells (Fig. 4).

Cross-validation of the Model Prediction

As shown in Table 5, the best-fit model (model A) predicted the following: 1) the apical membrane of the MDCK cell is less diffusion permeable than the basolateral membrane for both drugs; and 2) the distribution volume is 10-fold higher for vinblastine than that for digoxin, suggesting extensive cellular accumulation of vinblastine. These model predictions were experimentally cross-validated.

Diffusion across apical membranes is slower than across basal membranes. This prediction is compatible with the characteristics of barrier epithelia (37). If the lipid bilayer of the apical membrane is relatively impermeable, then access to the cytoplasm by diffusion from
the extracellular milieu should be slower through the apical membrane than through the basal membrane. Given that the drug entry port of P-glycoprotein exists in its cytoplasmic domain or in the lipid phase of the apical membrane, substrates are expected to engage in the transport more readily through the basal than through the apical membranes, because the relative diffusion impermeability of barrier epithelia is due to the characteristics of the outer leaflet of the apical membranes (21).

To cross-validate this model prediction, we examined the following compounds as inhibitors of the vinblastine and digoxin transport: digoxin, vinblastine, vincristine, verapamil, itraconazole, and ketoconazole. The reported P-glycoprotein substrates such as digoxin, verapamil, vinblastine, and vincristine inhibited digoxin and vinblastine basal-to-apical transport when they were added to the basal side; when added to the apical side, the inhibition was less remarkable (Fig. 5).

Despite the inhibition of the vinblastine basal-to-apical transport by basally added vincristine (Fig. 5A), cellular uptake of vinblastine was significantly increased (Fig. 6A). In contrast, apically added vincristine showed little effect on the vinblastine uptake as well as on the transport. These findings indicate that the inhibition of the basal-to-apical transport of vinblastine by the basally added P-glycoprotein substrates is due to blockage of vinblastine extrusion at the apical membranes. We previously showed the similar phenomenon for digoxin (17, 35). Taken together, these findings are compatible with our model prediction that apical membranes of MDCK cells are an efficient diffusion barrier relative to basal membranes for P-glycoprotein substrates.

In contrast to the P-glycoprotein substrates, itraconazole exhibited strong and almost instantaneous inhibition from the apical side (Fig. 5, C and H). When the cell monolayers were incubated with 5 µM itraconazole added to both sides at time 0, no significant accumulation of itraconazole in the apical side was observed after 48 h of incubation; the apical-to-basal concentration ratio was 0.97 ± 0.13 (n = 6). Its major metabolite, hydroxyitraconazole, was undetectable both in media and cell lysates. These findings suggest either that
Table 4. Second-stage fitting statistics for models of cellular drug transport

<table>
<thead>
<tr>
<th>Model</th>
<th>Degrees of Freedom, Number of Parameters</th>
<th>r²</th>
<th>Residual Sum of Squares</th>
<th>AIC</th>
<th>SC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digoxin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>58 (6)</td>
<td>0.9951</td>
<td>309.1</td>
<td>379.0</td>
<td>391.9</td>
</tr>
<tr>
<td>B</td>
<td>57 (7)</td>
<td>0.9951</td>
<td>311.6</td>
<td>381.5</td>
<td>396.6</td>
</tr>
<tr>
<td>C</td>
<td>61 (3)</td>
<td>0.9946</td>
<td>385.8</td>
<td>387.2</td>
<td>393.6</td>
</tr>
<tr>
<td>F test</td>
<td>F statistic</td>
<td>p value</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A vs. B</td>
<td>F₁ = 1.0</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A vs. C</td>
<td>F₃ = 4.80</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B vs. C</td>
<td>F₄,₅ = 3.39</td>
<td>&lt;0.025</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vinblastine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>58 (6)</td>
<td>0.9971</td>
<td>120.3</td>
<td>318.6</td>
<td>331.5</td>
</tr>
<tr>
<td>B</td>
<td>57 (7)</td>
<td>0.9972</td>
<td>119.2</td>
<td>320.0</td>
<td>335.1</td>
</tr>
<tr>
<td>C</td>
<td>61 (3)</td>
<td>0.9735</td>
<td>604.5</td>
<td>415.9</td>
<td>422.4</td>
</tr>
<tr>
<td>F test</td>
<td>F statistic</td>
<td>p value</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A vs. B</td>
<td>F₁ = 1.0</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A vs. C</td>
<td>F₃ = 77.8</td>
<td>&lt;0.005</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B vs. C</td>
<td>F₄,₅ = 58.0</td>
<td>&lt;0.005</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistical goodness of fit is assessed by Akaike's Information Criterion (AIC) and Schwartz criterion (SC), which combine the number of estimated parameters, the number of data points, and the best weighted sum of squares. The smaller the AIC and SC values, the better statistically. NS, not significant.

DISCUSSION

This study provides quantitative evidence that digoxin and vinblastine transport in the wild-type MDCK cells expressing P-glycoprotein can be explained by a three-compartment model, which assumes a functionally single itraconazole-inhibitable unidirectional transport mechanism in the apical membrane. Moreover, the model gives an overall kinetic picture of epithelial drug transport at a cellular level, elucidating functional contributions of each membrane drug translocation process. Interestingly, it predicts low diffusion permeability of the apical membranes for vinblastine and digoxin and significant kinetic differences between the two drugs. We were able to experimentally cross-validate these model predictions. This modeling approach is feasible to gain insight into kinetics of cellular drug handling.

Although modeling approach is one of the important analytical methods, there are limitations (Table 6). A

Table 5. Digoxin and vinblastine kinetic parameters derived from the models

<table>
<thead>
<tr>
<th>Drug</th>
<th>Parameters</th>
<th>Estimates</th>
<th>Standard Deviation</th>
<th>Unit</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digoxin</td>
<td>k</td>
<td>0.207</td>
<td>0.249</td>
<td>ml/h</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>δ₁</td>
<td>0.00246</td>
<td>0.0076</td>
<td>ml/h</td>
<td>309</td>
</tr>
<tr>
<td></td>
<td>δ₂</td>
<td>0.0925</td>
<td>0.0452</td>
<td>ml/h</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>δ₃</td>
<td>0.00148</td>
<td>0.00053</td>
<td>ml/h</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>θ</td>
<td>0.030</td>
<td>0.029</td>
<td>ml/h</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>0.0653</td>
<td>0.0184</td>
<td>ml</td>
<td>28</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>k</td>
<td>0.300</td>
<td>0.0619</td>
<td>ml/h</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>δ₁</td>
<td>0.0182</td>
<td>0.002</td>
<td>ml/h</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>δ₂</td>
<td>0.216</td>
<td>0.0289</td>
<td>ml/h</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>δ₃</td>
<td>0.00097</td>
<td>0.000385</td>
<td>ml/h</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>θ</td>
<td>0.0762</td>
<td>0.0108</td>
<td>ml/h</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>0.705</td>
<td>0.0863</td>
<td>ml</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>kbasal</td>
<td>0.0013</td>
<td>0.00092</td>
<td>ml/h</td>
<td>71</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>k</td>
<td>0.349</td>
<td>0.0982</td>
<td>ml/h</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>δ₁</td>
<td>0.0195</td>
<td>0.0026</td>
<td>ml/h</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>δ₂</td>
<td>0.222</td>
<td>0.0320</td>
<td>ml/h</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>δ₃</td>
<td>0.00072</td>
<td>0.00055</td>
<td>ml/h</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>θ</td>
<td>0.0652</td>
<td>0.0162</td>
<td>ml/h</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>0.725</td>
<td>0.0900</td>
<td>ml</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>kbasal</td>
<td>0.0558</td>
<td>0.0806</td>
<td>ml/h</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>Model C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digoxin</td>
<td>k</td>
<td>0.0604</td>
<td>0.0448</td>
<td>ml/h</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>δ</td>
<td>0.00238</td>
<td>0.0033</td>
<td>ml/h</td>
<td>139</td>
</tr>
<tr>
<td></td>
<td>θ</td>
<td>0.0517</td>
<td>0.0518</td>
<td>ml/h</td>
<td>100</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>k</td>
<td>0.350</td>
<td>0.113</td>
<td>ml/h</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>δ</td>
<td>0.0195</td>
<td>0.0029</td>
<td>ml/h</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>θ</td>
<td>0.0651</td>
<td>0.0172</td>
<td>ml/h</td>
<td>26</td>
</tr>
</tbody>
</table>

CV, coefficient of variation; k, intrinsic clearance of the pump; δ₁, diffusion parameters of apical membranes, basal membranes, and intercellular junctions, respectively; θ, remaining fraction of the pump in the presence of itraconazole; V, apparent distribution volume of drug; and kbasal, intrinsic clearance of the basally located drug efflux pump (e.g., multidrug resistant associated protein, MRP).
model is an abstract of a real system, simplifying an intricated network of events into a manageable format within limits of time and space resolution of the experimental data. As a result, a model can be used for simulation of the system behavior and prediction of the consequences. However, each system parameter in the model may not correspond to a single physical or molecular entity, because different molecular mecha-
nisms may be viewed kinetically as a single functional unit. Hence, the unidirectional transport mechanism on the apical membranes in our models may not be represented by P-glycoprotein alone, although our model describes the experimental data reasonably well.

MRP, another recently identified membrane drug transporter, is expressed on the basal membrane of LLC-PK1 renal tubular cells (both the wild-type and the human MRP gene transfected line) conferring the transport activity toward the basal side of the cells. MRP translocates both uncharged hydrophobic compounds and anionic substances such as glutathione conjugates of drug across the basal membranes. However, using a calcein accumulation assay, we were able to show that MRP is not a major contributor in our experimental system with MDCK cells.

Fig. 6. Uptake of drug by the MDCK cells. A: vinblastine uptake. Uptake of 0.1 µM [3H]vinblastine into MDCK cells from basal side was examined in presence and absence of 5 µM vincristine added to either the basal [VCR(B); open squares and solid lines] or apical side [VCR(A); open triangles and dashed lines] of the cell monolayers grown on permeable filters. After 10, 30, and 60 min of incubation at 37°C, culture inserts were rinsed and solubilized with 0.01% Triton X-100. Radioactivity of cell lysate was counted. While the basal-to-apical fluxes of vinblastine are decreased in presence of vincristine in basal side (as seen in Fig. 5), uptake is significantly increased. *P < 0.05, VCR(B) vs. control. **P < 0.01, VCR(B) vs. control, and VCR(B) vs. VCR(A). Uptake is unchanged in apical presence of vincristine [VCR(A)] compared with control. Results are expressed as means ± SD (n = 3–6) of cell-to-medium vinblastine ratio (%), i.e., a ratio between vinblastine recovered from the entire cell monolayer and that initially added to basal medium. B: digoxin and vinblastine uptake. Uptake of 0.1 µM [3H]vinblastine and 0.1 µM [3H]digoxin from basal side of MDCK cells was measured at 60-min incubation. Vinblastine uptake was nearly 10-fold higher than that of digoxin (n = 4, P < 0.001). Results are means ± SD of the cell-to-medium vinblastine ratio (%).

Although a comprehensive model remains to be tested, our model provided several interesting predictions. It has been widely accepted that the pump function of P-glycoprotein in epithelial tissues is proportional to the basal-to-apical fluxes and inversely proportional to the basal-to-apical fluxes of its substrates (Fig. 1; Refs. 5, 15, 27, 31). In contrast to this conventional notion, the rate of apical-to-basal vinblastine transport up to 4 h in our study did not show significant symmetrical increase (i.e., symmetrical to the decrease in the basal-to-apical direction) in the presence of the pump inhibitor. Despite the apparent violation of the criteria, the vinblastine data are well explained by the pump model. Although the digoxin apical-to-basal flux increased from the early period of incubation when P-glycoprotein is inhibited, the nearly 10-fold increase of concomitant paracellular diffusion (i.e., monitored by mannitol) made the interpretation difficult (Table 1). Our simulation further indicates that increase in apical-to-basal digoxin and vinblastine fluxes for the first several hours (Fig. 7) necessitates increase in diffusion across plasma membranes and/or intercellular junctions. These findings exemplify pitfalls of intuitive interpretation of transport kinetics without quantitative verification. The conventional, intuitive experimental criteria may not necessarily hold true. Interpreta-

![Diagram](http://aprenal.physiology.org/)
tion of transport data based on a multicompartment model should be supported by a quantitative modeling approach. Moreover, this has a significant implication when one interprets data regarding drug penetration across epithelial tissues from the apical to basal side such as seen in blood-brain barrier (30) and intestinal cells. If modification of P-glycoprotein function is associated with changes in initial apical (luminal)-to-basal drug translocation in these tissues, then concurrent alterations of diffusion may also have to be considered.

Asymmetry of apical and basal membrane diffusion was also predicted by the model. Barrier epithelia such as urinary bladder and collecting tubule are known to have apical membranes with very low permeability (9, 37), due to lower fluidity of the exofacial leaflet of the apical membranes (21). Our model prediction in MDCK cells, which are of distal tubular and collecting duct origin, is compatible with this functional difference in permeability between the apical and the basolateral membranes of barrier epithelia. However, it is unknown whether a difference in simple diffusion through plasma membranes accounts for this model prediction or whether differential expressions of carrier-mediated systems for facilitated diffusion such as aquaporins (i.e., pore-forming water channels) are also involved.

Although the molecular mechanism underlying these diffusion processes and its asymmetry is unclear, the prediction was cross-validated by our results showing that verapamil, vincristine, and vinblastine inhibit basal-to-apical digoxin transport more from the basal side of the monolayers; similarly, verapamil, vincris-

Fig. 7. Simulation of digoxin and vinblastine transport-time profiles. Based on model A and the estimated parameters (Table 5), digoxin (A and B) and vinblastine (C and D) transport was simulated for different levels of the pump function (solid lines, 100%; dashed lines, 50%, 25%, 10%, and 0%); y-axis represents drug concentrations expressed as % of the initial concentrations. A and C: changes of digoxin (A) and vinblastine (C) concentration in apical side for 2 h, when drugs are added to basal side at t ime 0. Insets of A and C: concentration changes in the basal and the apical sides over 50 h, when added to basal side at time 0. B and D: concentration changes of digoxin (B) and vinblastine (D) in basal side for 2 h, when added to apical side at time 0. Insets of B and D: concentration changes in basal side over 50 h, when added to apical side at time 0. Note that insets of B and D do not show the changes of concentration in apical side to provide a detailed view on basal side concentration changes. Note also that basal concentration changes for the initial 2 h in the condition shown in B and D are relatively insensitive to pump function changes.

Table 6. Advantages and limitations of modeling approach in cellular drug transport research

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimation of system parameters bearing possible biological significance</td>
<td>No guarantee of link between the parameters and the molecular entities</td>
</tr>
<tr>
<td>Determination of statistical uncertainty of the estimates</td>
<td>Ignoring functionally minor systems for simplicity</td>
</tr>
<tr>
<td>Quantitative prediction of overall system behavior</td>
<td>Dependence of model complexity on resolution of measurement</td>
</tr>
</tbody>
</table>


5). Octanol/water partition coefficients of these compounds, if the substrate entering the MDCK cells across the apical membrane is pumped out by P-glycoprotein before reaching cytoplasm, then it must be assumed that P-glycoprotein has two or more independent drug translocation processes: one for substrates presented to the pump through the outer leaflet of the apical membrane from extracellular side, and another for substrates accessing to the pump intracellularly. Otherwise, it is inexplicable that apically added excess amounts of these drugs conspicuously lack the inhibitory effects on the pump function (Fig. 5).

In contrast to the P-glycoprotein substrates, itraconazole almost instantly inhibits P-glycoprotein more from the apical than from the basal side. Ketoconazole, another azole antifungal and a P-glycoprotein inhibitor (29), shows no such differential inhibitory effects (Fig. 5). Octanol/water partition coefficients of these compounds are as follows in a rank order of lipophilicity: vinblastine, >1.6 \times 10^{-4}; ketoconazole, >2,000; vincristine, >150; and digoxin, ~50. Although the coefficient of itraconazole has not been reported, it is more lipophilic than ketoconazole. Clearly, hydrophobicity per se does not completely explain the side-dependent inhibitory effects of these substances on P-glycoprotein. Itraconazole and ketoconazole may interact with P-glycoprotein in a manner different from other typical P-glycoprotein substrates, probably by also interacting with the extracellular domain of P-glycoprotein. In fact, we were unable to detect a signal of itraconazole active transport; i.e., accumulation of itraconazole on the apical side against a concentration gradient during the 48-h incubation.

Certain assumptions made in this modeling analysis warrant discussion. First, metabolism of digoxin and vinblastine was assumed to be negligible. This assumption seems valid, because digoxin is not extensively metabolized in vivo. Vinblastine undergoes metabolism through cytochrome P-450 3A (CYP3A) enzymes, while other groups of CYP isozymes do not seem to mediate its metabolism (38). Although kidney expresses CYP3A, its expression level is less than 1% of the hepatic level (11), and its significant contribution to overall disposition of vinblastine is unlikely. Taken together, metabolism of these probe compounds seems small enough to be ignored.

Second, itraconazole was assumed to inhibit the pump function only. We do not know whether itraconazole directly affects the pump or perturbs membrane fluidity, thereby indirectly inhibiting the pump. This may further change passive diffusion of probe compounds across the membranes. Third, the intracellular distribution volume of drug was assumed not to be affected by itraconazole, although intracellular binding of drug may be altered by the inhibitors (33). We considered these possibilities, and assigned separate diffusion and volume parameters to the model for the data obtained in the presence of itraconazole. However, the volume and diffusion parameters did not differ in the presence or absence of itraconazole by more than 10%, and this separate parameter assignment did not improve the overall fitting (data not shown). This suggests that these potential effects are insignificant, although not completely ruled out. Therefore, we opted for simplicity and fitting efficiency to assume no change in the diffusion and distribution volume of digoxin and vinblastine by itraconazole.

Third, we assumed the first-order transport kinetics for P-glycoprotein. In this study, digoxin and vinblastine concentrations of greater than 0.1 \mu M were not used in the 24-h time course experiments, because of the possible damaging effects on the cells after prolonged exposures (see MTT assay results). Because K_m values of P-glycoprotein-mediated transport of digoxin and vinblastine are probably in the range of 1–10 \mu M (15, 16), our assumption of first-order kinetics appears valid. Given that the modeling approach is feasible, in situ determination of K_m and V_max may be possible, if the method is further refined.

In summary, using the model for P-glycoprotein-mediated epithelial drug transport, we were able to estimate kinetic parameters of digoxin and vinblastine transport across P-glycoprotein-expressing MDCK cells. Our simple model provides a kinetic framework with which to understand epithelial drug transport at a cellular level, predicting substantial difference in diffusion between the apical and the basal membranes. The modeling approach, however, is presently limited by resolution of experimental measurement. Detailed data on intracellular drug distribution and transcellular movement are needed, which will lead to designing more comprehensive models.

S. Ito is a recipient of a PMAC/Medical Research Council of Canada Career Award for Health Sciences. This work is supported by the Medical Research Council of Canada Grants MT-8544 and MT-13747.

Portions of this work were presented at the Annual Meeting of the American Society for Pharmacology and Experimental Therapeutics (San Diego, CA) in March 1997 and at the Annual Meeting of the American Society for Clinical Pharmacology and Therapeutics (New Orleans, LA) in March 1998.

Address for reprint requests and other correspondence: S. Ito, Div. of Clinical Pharmacology and Toxicology, Hospital for Sick Children, 555 University Ave., Toronto, Ontario, Canada M5G 1X8.

Received 27 October 1998; accepted in final form 24 March 1999.

REFERENCES


