Bias in Estimation of Transporter Kinetic Parameters from Overexpression Systems: Interplay of Transporter Expression Level and Substrate Affinity

Anand Balakrishnan,¹ Naissan Hussainzada, Pablo Gonzalez, Marival Bermejo, Peter W. Swaan, and James E. Polli

Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, Maryland (A.B., N.H., P.G., P.W.S., J.E.P.); and Department of Pharmacy and Technology, University of Valencia, Valencia, Spain (M.B.)

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ABSTRACT

The objective was to investigate the interplay between transporter expression levels and substrate affinity in controlling the influence of aqueous boundary layer (ABL) resistance on transporter kinetics in an over-expression system. Taurocholate flux was measured across human apical sodium-dependent bile acid transporter (hASBT)-Madin-Darby canine kidney monolayers on different occasions and kinetic parameters estimated with and without considering ABL. In error-free simulation/regression studies, flux values were generated across a range of J\text{max}, K_i, and substrate concentrations. Similar evaluation was performed for transport inhibition studies. Additionally, simulation/regression studies were performed, incorporating 15% random error to estimate the probability of successfully estimating K_i. Across different occasions, experimental J\text{max} and K_i estimates for taurocholate were strongly associated (p < 0.001; \rho^2 = 0.82) when ABL was not considered. Simulation/regression results indicate that not considering ABL caused an overestimation of this association, such that K_i estimates were highly positively biased at high hASBT expression. In reanalyzing taurocholate flux data using the ABL-present model, K_i was relatively constant across occasions (~5 μM) and not associated with J\text{max} (p = 0.24; \rho^2 = 0.13). Simulations suggest that J\text{max} and K_i collectively determined ABL influence, which is most prominent under conditions of low monolayer resistance. Additionally, not considering ABL lead to negatively biased K_i estimates, especially at high J\text{max}. Error-inclusive simulation/regression studies indicated that the probability of successfully estimating K_i depended on the contribution of ABL resistance to flux; when flux became increasingly ABL-limited, probability of success decreased. Results indicate that ABL resistance can bias K_i and J\text{max} estimates from overexpression systems, where the extent of bias is determined by transporter expression level and substrate affinity.

Transfected cell models overexpressing specific transporters are a powerful tool to characterize substrate requirements of the transporter, including its ability to translocate drugs and prodrugs (Herrera-Ruiz et al., 2003; Tolle-Sander et al., 2004; Balakrishnan et al., 2005; Balakrishnan and Polli, 2006). Relative to native cells and in vivo systems, transfected cell models frequently have the advantage of characterizing a transporter without confounding variables, such as other simultaneously expressing transporters with overlapping substrate requirements. This benefit is achieved in part through high expression of the transporter of interest.

We recently developed a stably transfected cell model for the human apical sodium-dependent bile acid transporter (hASBT), using MDCK cells (Balakrishnan et al., 2005) that possesses several favorable properties, including high hASBT expression. This hASBT-MDCK model was further developed to yield kinetic estimates of substrates and/or inhibitors (e.g., J\text{max}, K_i, and K_i) that can be used for subsequent quantitative-structure activity relationship (QSAR) analysis. Thus, overexpression systems can be used suitably for functional characterization of transporters.

In general, transporter expression level is expected to vary between occasions (i.e., vary between passages or cell culture conditions) (Yu et al., 1997; Polli et al., 2001). As an example, Fig. 1 summarizes the kinetic analysis of hASBT-mediated

ABBREVIATIONS: hASBT, human apical sodium-dependent bile acid transporter; MDCK, Madin-Darby canine kidney; HBSS, Hanks’ balanced salt solution; QSAR, quantitative-structure activity relationship; ABL, aqueous boundary layer; UWL, unstirred water layer; HRP, horseradish peroxidase; OAT, organic anion transporter.
of expression levels. However, this variability is expected to be constant across occasions and independent of expression levels. As anticipated, flux across hASBT-MDCK monolayers varied severalfold across occasions. As anticipated, day-to-day variation in hASBT expression and exhibited strong, positive association with J\textsubscript{max} estimates. However, J\textsubscript{max} and K\textsubscript{t} were estimated using a transport model that does not consider contribution of aqueous boundary layer (ABL) resistance. The present manuscript considers ABL as a lumped parameter composed of the apical unstirred water layer (UWL), support filter, and basolateral UWL. These diffusional barriers are encountered in flux studies across a monolayer grown on support filters. These diffusion barriers are lumped together, since it is difficult to reliably delineate them experimentally. Previous theoretical and experimental reports considered the presence of a single UWL situated apical to the biological membrane (i.e., an apical UWL) and indicated that failure to consider the UWL can result in inaccurate K\textsubscript{t} estimates (Winne, 1973, 1977, 1978; Thomson and Dietschy, 1977, 1980; Thomson, 1979; Barry and Diamond, 1984; Sinko et al., 1996). These reports have indicated the potential for UWL effects on active transport. Based on our previous data and reports by others, we hypothesized that high transporter expression can lower monolayer resistance, resulting in ABL-limited transport of solutes, leading to biased kinetic estimates if ABL is not considered.

One distinction between the present study and previous reports is the use of an overexpression system, where high expression level of transporter was achieved. Previous reports have clearly demonstrated UWL effect on K\textsubscript{t} estimates, but it is not clear how variability in high transporter expression level impacts the quality of kinetic estimates, as observed in Fig. 1. Use of a monolayer flux assay also underpins a second difference between the present study and previous reports. Previous models assumed a single UWL followed by a biological membrane (Winne, 1973, 1977, 1978; Thomson and Dietschy, 1977, 1980; Thomson, 1979). These studies evaluated in vivo perfusion studies, where the UWL represents a diffusion barrier between the bulk and the membrane. Unfortunately, the diffusion barriers in a monolayer flux configuration are less determinant since they are difficult to reliably delineate experimentally. Previous models assumed a single UWL followed by a second difference between the present study and previous reports. Objective 2 was conducted through error-free simulations of both transport and inhibition studies. Objective 3 was carried out through simulations incorporating 15% random error.

Given the broad use of overexpression systems to characterize transporter kinetics and given transporter expression variability over time, these results have implications in the development of high expression cell culture assays, as well as QSAR models and drug discovery efforts that rely on accurate kinetic parameter estimates.

**Materials and Methods**

**Transport Studies: ABL-Absent Model.** Figure 2 illustrates two competing models to describe bile acid transport across an hASBT-MDCK monolayer. The models differ in the absence or presence of an ABL. In Fig. 2A, the ABL-absent model is illustrated and via the support filter. The present study puts forth a transport model that reflects the cell culture monolayer flux assay. This transport model was extended to identify an inhibition model, an area unexplored by previous studies.

This study evaluates the interplay of transporter expression levels and substrate affinity in determining the role of ABL resistance on transporter kinetics using hASBT as a model transport system. The objectives were to 1) evaluate the effect of J\textsubscript{max} on the contribution of ABL resistance to transporter kinetics, 2) identify global kinetic conditions when ABL needs to be explicitly considered to accurately estimate kinetic parameters, and 3) identify scenarios under which kinetic estimates are not reliable in spite of ABL consideration, due to ABL-dominated transport kinetics. Objective 1 was carried out through a combination of empirical laboratory studies as well as error-free simulation/regression studies. Objective 2 was conducted through error-free simulations of both transport and inhibition studies. Objective 3 was carried out through simulations incorporating 15% random error.

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**Materials and Methods**

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![Fig. 2. ABL-absent model and ABL-present model. A and B, two competing models describing bile acid transport across an hASBT-MDCK monolayer are considered and are denoted the ABL-absent model and the ABL-present model. C, ABL-present model for uptake studies. In A, the ABL-absent model is illustrated and only considers monolayer resistance to limit flux, resulting in eq. 1; the resistance due to the support filter and unstirred water layers(s) are considered negligible. Mechanisms of bile acid permeation across the monolayer are active hASBT transport and passive permeability. In B, the ABL-present model is illustrated. In eq. 7, the support filter, the apical unstirred water layer, and the basolateral unstirred water layer are collectively denoted as the ABL. In uptake studies (C), the term ABL includes only the apical unstirred water layer. The permeability across the ABL is P\textsubscript{ABL}, such that this term represents the permeability across barriers other than the cell monolayer.](image-url)
only considers monolayer resistance to limit flux. Mechanisms of bile acid permeation across the monolayer are active hASBT transport and passive permeability. hASBT is located in the apical membrane and is the dominant mechanism for bile acid absorption (Dawson et al., 2003; Balakrishnan et al., 2005).

From Fig. 2A, the ABL-absent model is as follows:

\[ J = \frac{J_{\text{max}} \times S}{K_t + S} + P_p \times S \]  

(1)

where \( J \) is bile acid flux when ABL is absent, \( J_{\text{max}} \) and \( K_t \) are the Michaelis-Menten constants for hASBT-mediated transport, \( S \) is bile acid substrate concentration, and \( P_p \) is the passive permeability coefficient. \( J_{\text{max}} \) and \( K_t \) are sometimes denoted \( V_{\text{max}} \) and \( K_m \), respectively, in the transporter literature.

**Transport Studies: ABL-Present Model.** The ABL-present model is illustrated in Fig. 2B. The term ABL includes the apical, basolateral, and basolateral UWL. ABL is considered to be a single resistivity \( R_{\text{ABL}} \) that represents the permeability of taurocholate across blank filter in the absence of cells.

From Fig. 2B, the apparent resistance to flux is as follows:

\[ R_{\text{app}} = R_{\text{ABL}} + R_{\text{mono}} \]  

(2)

where \( R_{\text{app}} \) is the apparent resistance, \( R_{\text{ABL}} \) is the resistance due to the ABL, and \( R_{\text{mono}} \) is the resistance due to the monolayer itself (i.e., monolayer resistance). Since permeability is the inverse of resistance,

\[ \frac{1}{P_{\text{app}}} = \frac{1}{P_{\text{ABL}}} + \frac{1}{P_{\text{mono}}} \]  

(3)

where \( P_{\text{app}} \) is the apparent permeability, \( P_{\text{ABL}} \) is the ABL permeability, and \( P_{\text{mono}} \) is the monolayer permeability. From the contributions of hASBT-mediated transport and parallel passive bile acid permeability to monolayer permeability,

\[ P_{\text{mono}} = \frac{J_{\text{max}}}{K_t + S} + P_p \]  

(4)

Substituting eq. 4 into eq. 3,

\[ \frac{1}{P_{\text{app}}} = \frac{1}{P_{\text{ABL}}} + \frac{1}{\left( \frac{J_{\text{max}}}{K_t + S} + P_p \right)} \]  

(5)

\[ P_{\text{app}} = \frac{P_{\text{ABL}} \times \left( \frac{J_{\text{max}}}{K_t + S} + P_p \right)}{P_{\text{ABL}} + \frac{J_{\text{max}}}{K_t + S} + P_p} \]  

(6)

Since \( J_{\text{ABL}} = P_{\text{app}} \times S \),

\[ J_{\text{ABL}} = \frac{P_{\text{ABL}} \times \left( \frac{J_{\text{max}}}{K_t + S} + P_p \right) \times S}{P_{\text{ABL}} + \frac{J_{\text{max}}}{K_t + S} + P_p} \]  

(7)

where \( J_{\text{ABL}} \) is bile acid flux when ABL is present. Equation 7 is denoted the ABL-present model.

**Comparison of the ABL-Present Model to the Uptake Model of Winne.** Equation 7 differs from the uptake model of Winne (1977), which can be written as follows:

\[ J_{\text{UWL}} = P_{\text{UWL}} \left( S + \frac{q}{2} \frac{K_t}{q} + \frac{J_{\text{max}}}{P_{\text{UWL}}} - S \right) \]  

\[ - \frac{q^2}{4} \left( \frac{K_t}{q} + \frac{J_{\text{max}}}{P_{\text{UWL}}} - S \right) + qK_t \]  

(8)

where \( J_{\text{UWL}} \) is the flux when a single UWL is present before the membrane, \( P_{\text{UWL}} \) is the permeability of the UWL, and \( q = P_{\text{UWL}}/P_p + P_{\text{UWL}} \cdot J_{\text{max}}, K_t, P_p, \) and \( S \) represent the same parameters in both eqs. 7 and 8. Equation 8 simplifies the original Winne equation by equating the area of the UWL to the area of the membrane as well as by substituting \( P_{\text{UWL}} \) for the ratio of the diffusivity coefficient to the UWL thickness.

Underpinning the difference between the ABL-present model and the Winne uptake model is the number and arrangement of diffusion barriers, relative to the biological membrane. In the uptake model of Winne, only an apical UWL is present. Winne et al. applied eq. 8 to assess the role of the UWL in the absorption of phenylalanine (Winne et al., 1979) using the perfused rat jejunal loop. Justification of Winne’s model would seem to be the presence of an UWL within the loop lumen, with no other significant diffusion barriers, except the jejunal loop membrane. Strength of the Winne uptake model is its differentiation between the bulk donor solution concentration (\( S \)) and the concentration at the UWL-membrane interface, particularly compared against the ABL-present model.

Winne’s model cannot be applied to transport studies across a cell monolayer since it fails to consider diffusional resistances other than the apical UWL. Complicating this scenario is the arrangement of a diffusion barrier on the luminal side of the monolayer (i.e., apical UWL) and two diffusion barriers on the other side (i.e., support filter and basolateral UWL). A further limitation is an insufficient understanding of these diffusion barriers since the permeability of each barrier is difficult to measure in a reliable manner. The approach in the present manuscript was to characterize these three barriers as a single resistivity \( R_{\text{ABL}} \) per eq. 2 (i.e., a single permeability \( P_{\text{ABL}} \) per eq. 3). This single barrier is denoted the ABL. \( P_{\text{ABL}} \) was estimated by measuring the permeability of taurocholate across blank filter in the absence of cells.

**Measure of Monolayer Resistance.** Equations 1 and 7 (i.e., the ABL-absent and ABL-present models) each allow for a nonlinear, Michaelis-Menten-type component as well as linear, passive transport component. The two models differ with respect to the contribution of the ABL on flux. In Fig. 2A, ABL is absent, such that the only rate-limiting barrier in eq. 1 is the monolayer. In Fig. 2B, ABL is present, reflecting that ABL contributes as a barrier to flux in eq. 7. Qualitatively, a high ABL resistance relative to monolayer resistance results in flux limited by ABL. In addition, high monolayer resistance relative to ABL results in flux limited by monolayer, as in Fig. 2A.

The ratio of flux from the ABL-present model to the flux from the ABL-absent model (\( J_{\text{ABL}}/J \)) is used to assess the impact of ABL on flux. Of note, \( J_{\text{ABL}}/J \) is also numerically equivalent to the fraction of total flux resistance that is attributed to the monolayer:

\[ F_{\text{mono}} = \frac{J_{\text{ABL}}}{J} \]  

(9)

where \( F_{\text{mono}} \) is the fraction of total flux resistance that is due to the monolayer. Appendix 1 derives eq. 9. Monolayer resistance excludes components of the ABL such as the support filter and apical and basolateral UWLs. From eq. 9, \( F_{\text{mono}}/J = 1 \) indicates that flux is limited by monolayer resistance only (i.e., \( F_{\text{mono}} = 1 \)). Meanwhile, \( J_{\text{ABL}}/J = 0.1 \) indicates that the monolayer contributes to only 10% of
the total resistance (i.e., $P_{\text{mono}} = 0.1$), with the ABL contributing the remaining 90%.

Transport Inhibition Studies. For competitive inhibition studies of taurocholate transport by bile acids, the following transport inhibition model describes taurocholate transport when ABL is absent:

$$ J = \frac{J_{\text{max}} \times S}{K_{t} (1 + I/K_{\text{p}}) + S} + P_{p} \times S $$

(10)

where $I$ is the concentration of inhibitor (i.e., inhibitory bile acid) and $K_{t}$ is inhibition constant. $S$ is substrate concentration (i.e., taurocholate concentration); $J_{\text{max}}$, $K_{t}$, and $P_{p}$ characterize substrate transport parameters. Equation 10 is denoted the ABL-absent inhibition model.

When the ABL is included, the competitive inhibition model becomes:

$$ J_{\text{ABL}} = \frac{P_{\text{ABL}} \times \left( \frac{J_{\text{max}}}{K_{t} (1 + I/K_{\text{p}}) + S} + P_{p} \right) \times S}{P_{\text{ABL}} + \left( \frac{J_{\text{max}}}{K_{t} (1 + I/K_{\text{p}}) + S} + P_{p} \right)} $$

(11)

where $P_{\text{ABL}}$ is ABL permeability. Equation 11 is denoted the ABL-present inhibition model. This model is derived in Appendix 2.

Taurocholate Transport across hASBT Monolayers. Taurocholate was used as a model substrate for hASBT. On 12 occasions, apical-to-basal taurocholate flux was measured across an hASBT-MDCK monolayer model, as described previously (Balakrishnan et al., 2005). Briefly, hASBT-MDCK cells were seeded at a density of 0.75 million cells/cm² on support filters without monolayers, as discussed below. Nonlinear regression to eqs. 1 and 7 was performed using Pearson correlation coefficient using SPSS 12.0 (SPSS Inc., Chicago, IL). Correlation among parameter estimates was evaluated using Pearson correlation coefficient using SPSS 12.0 (SPSS Inc.).

Flux data from each occasion were fitted to the ABL-absent model (i.e., eq. 1) as well as the ABL-present model (i.e., eq. 7), providing estimates for $J_{\text{max}}$, $K_{t}$, and $P_{p}$ for each of the two model scenarios. In eq. 7, $P_{\text{ABL}}$ was assigned, based upon diazepam uptake data, as discussed below. Nonlinear regression to eqs. 1 and 7 was performed using SigmaPlot 2000 (SPSS Inc.). Correlation among parameter estimates was evaluated using Pearson correlation coefficient using SPSS 12.0 (SPSS Inc.).

Taurocholate Transport across Support Filter without hASBT-MDCK Monolayer to Estimate $P_{\text{ABL}}$ for Transport Studies. It is difficult to directly measure the net effect of the aqueous boundary layer at the apical interface, the aqueous boundary layer at the basolateral interface, and the plastic filter (i.e., $P_{\text{ABL}}$) when cell monolayer is present. When an estimate of $P_{\text{ABL}}$ is sought, a common approach to measure $P_{\text{ABL}}$ is to use blank filter (Imanidis et al., 1996; Yu and Sinko, 1997). Hence, taurocholate transport across blank support filters (i.e., polyester Transwell without cell monolayer) was performed as described above. The underlying assumption in this approach is that the ABL in the ABL-present model in Fig. 2B is the same whether a monolayer is present or not present. Flux studies were performed using HBSS with 10 mM HEPES, pH 6.8, at 37°C and 50 rpm. At 5 min, sample was collected from the receiver compartment and allowed for sink conditions to be maintained. Taurocholate permeability across blank support filters was $6.61 \pm 6.0 \times 10^{-6}$ cm/s, which was taken to be equal to $P_{\text{ABL}}$. This value was in agreement with that of (Buur and Mork, 1992).

$P_{\text{ABL}}$ served as a lumped parameter in eqs. 7 and 11 to characterize ABL, since it is difficult to reliably measure the permeability of the apical unstirred water layer, the support filter, and the basolateral unstirred water layer individually. It should be noted that Yu and Sinko (1997) and Adson et al. (1994) have attempted to uncouple some of these barrier components, although active transport was not considered. Adson et al. (1994) calculated a value for the permeability of the support filter, based upon a porous membrane model and parameters provided by the manufacturer, an approach that may be unreliable (Yu and Sinko, 1997). Yu and Sinko (1997) used an empirical model to simultaneously estimate support filter permeability and the UWLs; the approach required “labor-intensive” flux studies across blank filters, where hydrodynamics was varied (Yu and Sinko, 1997).

Diazepam Uptake into hASBT-MDCK Monolayer to Estimate $P_{\text{ABL}}$ for Uptake Studies. Uptake of radiolabeled diazepam into hASBT-MDCK cells was assessed to estimate $P_{\text{ABL}}$ for uptake studies. By virtue of its high permeability across bilayers, diazepam is a commonly used marker for measurement of UWL resistance. After 30 s, uptake was stopped by freezing the cells instantaneously over dry ice in alcohol. This process rigidized cell membrane and aimed to minimize back diffusion. Cells were washed twice with ice-cold buffer and lysed using 1% Triton in 1 N NaOH. Diazepam in cell lysate was quantified using a scintillation counter. Diazepam permeability was $150 \pm 3 \times 10^{-6}$ cm/s, which was taken to be equal to $P_{\text{ABL}}$ for uptake studies. This $P_{\text{ABL}}$ value from the uptake configuration is greater than $P_{\text{ABL}}$ from the transport configuration, presumably reflecting that only the apical UWL is present in case of uptake configuration, whereas basolateral UWL and filter support are additional diffusion barriers in the transport configuration.

Simulations for Objective 1: Impact of Varying $J_{\text{max}}$ on ABL Contribution. The ABL-present model for transport (i.e., eq. 7) was used to simulate flux data. No error was incorporated into these simulations. Values for $K_{t}$, $P_{p}$, and $P_{\text{ABL}}$ were 5 μM, 0.5 $\times 10^{-6}$ cm/s, and 70 $\times 10^{-6}$ cm/s, respectively. These values were selected based upon experimental measurements of $K_{t}$, $P_{p}$, and $P_{\text{ABL}}$. Values for substrate concentration and $J_{\text{max}}$ ranged from 0.5 to 1000 μM and 0.00001 to 0.01 nmol/cm²/s, respectively.
based upon observed $J_{\text{max}}$ values. Error-free simulations were systematically performed for all combinations of parameter values. Error-free flux data were similarly simulated using the ABL-absent model (eq. 1).

Simulated $J_{\text{ABL}}$ data were subsequently regressed on to the ABL-absent model to yield estimates of $K_i$, $J_{\text{max}}$, and $P_e$. This approach quantified bias in $K_i$, $J_{\text{max}}$, and $P_e$ parameter estimates when ABL was ignored.

Simulations for Objective 2: Identification of Global Kinetic Conditions That Require ABL Consideration. Simulations were performed to identify global kinetic conditions when ABL needs to be explicitly considered to accurately estimate kinetic parameters (e.g., $K_i$, $J_{\text{max}}$ or $K_p$). Simulations were performed for transport and transport inhibition studies.

Transport data were simulated under both ABL-present and ABL-absent scenarios. $P_e$ and $P_{\text{ABL}}$ were fixed to be $0.5 \times 10^{-6}$ and $70 \times 10^{-6}$ cm/s, respectively. Values for $K_i$ and $J_{\text{max}}$ ranged from 0.1 to 10,000 $\mu$m and 0.00003 to 0.01 nmol/cm²/s, respectively. Substrate concentration was fixed to one-tenth the $K_e$ value for each simulation. The impact of ABL was assessed by the ratio $J_{\text{ABL}/J}$. $J_{\text{ABL}/J}$ greater than 0.9 indicated that ABL contribution is not significant. $J_{\text{ABL}/J}$ less than 0.9 indicated that ABL is significant, such that ABL needs to be considered to accurately estimate kinetic parameters.

Inhibition data were simulated using the ABL-absent and ABL-present models in a similar manner (i.e., eqs. 10 and 11). Substrate concentration, $K_i$, and $K_p$, values were 2.5, 5, and 50 $\mu$m, respectively. Inhibitor concentration ranged from 0.1 to 1000 $\mu$m. $J_{\text{max}}$ ranged from 0.00001 to 0.01 nmol/cm²/s. Simulated $J_{\text{ABL}}$ was subsequently regressed on the ABL-absent inhibition model to yield estimates of $K_i$, $K_p$, $J_{\text{max}}$, and $P_e$ were assigned to the values used in generating the simulated data. This approach quantified bias in $K_i$, $K_p$, parameter estimates when ABL was ignored.

Simulations for Objective 3: Identification of Global Kinetic Conditions When $K_e$ Estimates Are Unreliable as a Result of ABL Contribution. Equation 7 was used with $P_e = 0.5 \times 10^{-6}$ cm/s and $P_{\text{ABL}} = 70 \times 10^{-6}$ cm/s to generate initial error-free flux data. Fifteen scenarios were systematically evaluated for five levels of $K_e$ (1, 3, 5, 10, and 25 $\mu$m) at each of three levels of $J_{\text{max}}$ (0.0001, 0.0003, and 0.001 nmol/cm²/s). Substrate concentrations were 1/20-, 1/10-, 1/5-, 1/2-, 1-, 2-, 5-, 10-, and 20-fold of true $K_e$ value. For the scenario $K_i = 1 \mu$m and $J_{\text{max}} = 0.001$ nmol/cm²/s, substrate concentrations 40, 80, and 160 $\mu$m were also included. Flux data for every substrate concentration were simulated in triplicate for each occasion, mimicking triplicate design of in vitro laboratory studies. Random error reflecting percent coefficient of variation = 15% was incorporated into simulated flux data. For each of these 15 scenarios, simulations were performed on 100 occasions. Data from each occasion were regressed onto eq. 7 to estimate $K_i$, $J_{\text{max}}$, and $P_e$, with emphasis on $K_e$. For each simulation scenario, $K_e$ estimates from each of the 100 occasions was used to calculate the probability of calculating meaningful $K_e$ estimates (i.e., statistically differed from zero based upon 95% confidence interval). Nonlinear regression to eq. 7 was performed using WinNonlin Professional version 4.1 (Pharsight, Mountain View, CA), which was kindly donated by Pharsight.

Cell Surface Biotinylation and Immunoblotting. Figure 1 provides motivation for the present study, presuming that variation in $J_{\text{max}}$ was ascribed to variation in hASBt expression. The rank order agreement between cell surface hASBt expression level and $J_{\text{max}}$ was assessed though parallel studies involving Western blots and taurocholate transport studies.

hASBt-MDCK monolayers were grown as described above (Balakrishnan et al., 2005) and treated with varying levels of sodium butyrate to modulate hASBt expression level. hASBt-MDCK monolayers were treated with either 10 mM, 5 mM, or no sodium butyrate to yield monolayers corresponding to three levels of hASBt expression (i.e., high, intermediate, and low). Additionally, untransfected MDCK monolayers were also treated with 10 mM sodium butyrate as negative control.

Cell surface expression of hASBt was determined by treating cells with the membrane-impermeant biotinylation reagent sulfo-succinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate (Pierce Chemical, Rockford, IL) for 30 min at room temperature (Visieris et al., 2003). After several washes in phosphate-buffered saline with 0.1 mM CaCl₂ and 1 mM MgCl₂, cells were disrupted in lysis buffer (25 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM CaCl₂, 1% Triton X-100, and 0.5% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)) for 30 min at 4°C. Biotinylated proteins were recovered by overnight incubation with 100 $\mu$l of streptavidin-agarose beads per sample (Pierce Chemical Co.) at 4°C using end-over-end rotation. The following day, the beads were washed once with lysis buffer, once in high-salt lysis buffer (same as lysis buffer except with 500 mM NaCl and 0.1% Triton X-100), and finally twice with 50 mM Tris, pH 7.5. Biotinylated proteins were eluted using 100 $\mu$l of SDS-polyacrylamide gel electrophoresis Laemmli buffer, pH 6.8 (Sigma-Aldrich), for 10 min at 85°C. For each sample, 20 $\mu$l was applied to a 12.5% SDS-polyacrylamide gel electrophoresis gel (Bio-Rad, Hercules, CA) and transferred to a polyvinylidene difluoride membrane for immunoblotting. Since hASBt was constructed with a V5 epitope (Balakrishnan et al., 2005), hASBt was detected with the anti-V5/horse-radish peroxidase-conjugated antibody (1:1000 dilution; Invitrogen, Carlsbad, CA) directed to the V5 epitope located at the C terminus. Bands were detected by chemiluminescence using ECL Plus Western Blotting Detection system (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Blots were then striped via ECL Detection System protocols and reprobed for the presence of the constitutively expressed plasma membrane marker α-integrin, using anti-integrin α2/VLA-2α antibody, followed by donkey anti-mouse HRP-conjugated secondary antibody. α-integrin represents a positive control for cell surface biotinylation of plasma membrane proteins. To confirm cell membrane integrity during biotinylation, the same membrane was also probed for the absence of calnexin, a 90-kDa housekeeping protein present in whole-cell lysate preparations, using anti-calnexin antibody and donkey anti-rabbit HRP-conjugated secondary antibody. Calnexin was absent from biotinylated samples (data not shown), ensuring that only cell surface proteins were biotin-labeled and immunoblotted.

Densitometry measurements of hASBt expression level were not performed, since densitometry unfortunately requires high protein expression and α-integrin was not sufficiently high for densitometric measurement. Rather, post hoc analysis of band density using pixel intensity was assessed using NIH Image software (version 1.6.3; (http://rsb.info.nih.gov/nih-image/index.html) for each sodium butyrate treatment level (as well as control untransfected MDCK monolayers). Using a standard rectangular box in NIH Image, pixel intensity of hASBt was measured and normalized against pixel intensity of α-integrin; pixel intensity ratio of hASBt versus α-integrin from each treatment was corrected for background control via subtraction of pixel intensity ratio from untransfected MDCK monolayers and interpreted as hASBt expression level (relative to integrin).

Taurocholate flux in the presence and absence of sodium was measured as described above. $J_{\text{max}}$ was estimated and compared with Western blot analysis. Results are shown in Appendix 3.

Results

Simulations for Objective 1: Impact of Varying $J_{\text{max}}$ on ABL Contribution. Taurocholate permeability across blank support filters was 66.1 ± 6.0 $\times 10^{-6}$ cm/s. Hence, $P_{\text{ABL}}$ was fixed at $70 \times 10^{-6}$ cm/s in all simulations.

Figure 3 is a contour plot of the ratio of $J_{\text{ABL}}$ versus $J$, as a function of $J_{\text{max}}$ and substrate concentration. $J_{\text{ABL}}/J$ greater than 0.9 indicates that ABL contribution was not significant. $J_{\text{ABL}}/J$ less than 0.9 indicates that ABL contribution is significant. At a very low $J_{\text{max}}$ of 0.00003 nmol/
cm²/s, J_{ABL} / J always exceeded 0.9, regardless of substrate concentration. For higher \( J_{\text{max}} \) scenarios, J_{ABL} / J dropped below 0.9 at low substrate concentrations, but generally returned to about unity at high substrate concentration. At very high \( J_{\text{max}} \) of 0.01 nmol/cm²/s, flux from the ABL-present model was 20-fold lower than flux from the ABL-absent model at low substrate concentration (i.e., \( J_{ABL} / J \) about 0.05). These simulations indicate that ABL limits flux significantly at high \( J_{\text{max}} \) and low substrate concentration. These simulations follow the results observed in earlier studies (Thomson and Dietschy, 1980). There was practical identity between fitted \( J_{\text{max}} \) and true \( J_{\text{max}} \) values, indicating that the use of the ABL-absent model did not bias \( J_{\text{max}} \) estimates. ABL only impacted \( K_{t} \) estimates and not \( J_{\text{max}} \). Interesting, the ABL-absent and ABL-present models provided equally good fits to simulated data, based on Akaike information criterion and \( R^2 \) (data not shown).

In the light of above-mentioned observations, Fig. 5 reevaluates experimental taurocholate data. The ABL-present model was used to obtain \( K_{t} \) and \( J_{\text{max}} \) estimates, whose values are plotted in Fig. 5. \( J_{\text{max}} \) ranged between 0.0001 to 0.001 nmol/s/cm². \( K_{t} \) ranged from about 1 to 12 \( \mu \)M with an average \( K_{t} \) estimate of approximately 5 \( \mu \)M. Compared with regression results from the ABL-absent model (i.e., Fig. 1), estimates of \( K_{t} \) were about 2- to 3-fold lower from the ABL-

---

**Fig. 3.** Contour plot showing the effect of ABL on flux for varying \( J_{\text{max}} \).

**Fig. 4.** Effect of not considering ABL resistance on \( K_{t} \) estimates. Simulations were performed using eq. 7 (i.e., ABL-present model) over a range of seven \( J_{\text{max}} \) values, using a \( K_{i} \) of 5 \( \mu \)M. This “true \( K_{i} \)” is illustrated with a broken line. Filled circles indicate \( K_{t} \) estimates from fitting simulated data to eq. 1 (i.e., ABL-absent model). Application of the ABL-absent model generally provided \( K_{t} \) estimates larger than 5 \( \mu \)M. \( K_{t} \) estimates at and below \( J_{\text{max}} \) of 0.00003 nmol/cm²/s were biased by 0 to 5%. For \( J_{\text{max}} \) of 0.0003 nmol/cm²/s, the \( K_{t} \) estimate was about 10 \( \mu \)M (i.e., 2-fold higher than true \( K_{t} \)). These results are consistent with experimental taurocholate data where larger \( K_{t} \) estimates were calculated at high \( J_{\text{max}} \) using the ABL-absent model (i.e., Fig. 1).

**Fig. 5.** Plot of taurocholate \( K_{t} \) estimates versus taurocholate \( J_{\text{max}} \) estimates across different days, using ABL-present model. In contrast to Fig. 1 where the ABL-absent model was used, there was no dependence of \( K_{t} \) on \( J_{\text{max}} \) when the ABL-present model was used. \( K_{t} \) was practically the same (about 8 \( \mu \)M) across different days, as expected. \( K_{t} \) estimates from the ABL-present model were about 3-fold lower than \( K_{t} \) estimates from the ABL-absent model, consistent with the simulations in Fig. 4.
present model. $J_{\text{max}}$ estimates were the same from each model from both models, as expected from above simulation results. Additionally, $P_s$ estimates were the same from both models (data not shown). In contrast to Fig. 1, there was no association between $K_i$ estimates and $J_{\text{max}}$ estimates in Fig. 5 ($p = 0.24; \rho^2 = 0.13$). This lack of association indicates that correcting for ABL influence lead to a more accurate and less biased estimate of $K_i$.

UWL effects in taurocholate uptake data were also corrected in an analogous fashion (see Appendix 4). It should be noted that the $K_i$ estimates for uptake and transport studies were similar (both $\sim 5 \mu M$), indicating that the configuration of the assay (i.e., uptake versus transport) did not affect $K_i$ estimate. This result supports the apical membrane serving as the rate-limiting barrier within the monolayer in transport studies. Hence, a basolateral transporter such as Osto-Ostβ is not contributing as the rate-limiting barrier. Osto-Ostβ is a heteromeric transporter that has been recently identified to translocate bile acids in the enterocyte basolateral membrane (Ballatori et al., 2005; Dawson et al., 2005).

Simulations for Objective 2: Identification of Global Kinetic Conditions That Require ABL Consideration.

Since high $J_{\text{max}}$ can lead to ABL-limited transport and subsequent positive bias in $K_i$ estimate, a second objective was to identify global kinetic conditions when ABL needs to be explicitly considered to accurately estimate kinetic parameters. This assessment was carried out via simulations of the ABL-absent and ABL-present models, using parameter values that reflect the range of observed parameters. The impact of ABL was assessed by $J_{\text{ABL}}/J$; $J_{\text{ABL}}/J$ less than 0.9 indicated that ABL is significant.

Figure 6 plots $J_{\text{ABL}}/J$ as a function of $K_i$ at varying $J_{\text{max}}$ and illustrates the interplay of $J_{\text{max}}$ and $K_i$ in determining ABL impact. In Fig. 6, at low $J_{\text{max}}$ and high $K_i$ (i.e., poor substrate for transport), ABL is not significant, as observed by $J_{\text{ABL}}/J$ greater than 0.9 (i.e., $F_{\text{mono}} > 0.9$). Overall flux is controlled by monolayer resistance and not by ABL. Meanwhile, for large $J_{\text{max}}$ and/or low $K_i$, $J_{\text{ABL}}/J$ is low, which reflects a significant role of ABL. In the case of both high $J_{\text{max}}$ and low $K_i$, $J_{\text{ABL}}/J$ (i.e., $F_{\text{mono}}$) is less than 0.1, reflecting ABL accounts for over 90% of total resistance to transport. Figure 6 is consistent with observed taurocholate data plotted in Figs. 1 and 5, where ABL was modestly important for low $J_{\text{max}}$ and very important at high $J_{\text{max}}$. Similar dependence on $J_{\text{max}}$ is evident in Fig. 6.

These results comparing $J_{\text{ABL}}$ and $J$ indicate that ABL needs to be explicitly considered for all potent substrates (i.e., $K_i$ less than or equal to 10 $\mu M$), even in low expression systems (i.e., $J_{\text{max}} > 0.00003 \text{ nmol/cm}^2/\text{s}$). Additionally, at $J_{\text{max}}$ greater than 0.001 nmol/cm$^2$/s, as observed for hASBT-MDCK, ABL needs to be considered even when $K_i$ is as large as 100 $\mu M$. Given the high hASBT expression from the current hASBT-MDCK model, the simulations anticipate the need to employ the ABL-present model for all native bile acids. Interestingly, these simulations suggest lowered hASBT expression (i.e., lower $J_{\text{max}}$) as an approach to minimize ABL influence on transport. Future laboratory studies will employ hASBT-MDCK monolayers with reduced hASBT expression, especially for potent substrates, to minimize ABL influence.

Simulation studies were also performed for transport inhibition studies to identify global kinetic conditions that require ABL consideration for accurate $K_i$ estimation. Previous studies have not examined diffusion barrier effects on $K_i$ estimation. Figure 7 plots $J_{\text{ABL}}/J$ as a function of inhibitor concentration and $J_{\text{max}}$. At low $J_{\text{max}}$, $J_{\text{ABL}}/J$ was greater than 0.9, indicating lack of ABL effect. However, $J_{\text{ABL}}/J$ decreased with increasing $J_{\text{max}}$ and reached 0.4 and lower at $J_{\text{max}} = 0.01 \text{ nmol/cm}^2/\text{s}$, indicating significant ABL effect under these conditions. These observations are similar to the results from the corresponding transport simulations (Fig. 3). The consequence of low $J_{\text{ABL}}/J$ (observed in Fig. 7) on $K_i$ estimates is shown in Fig. 8. Figure 8 plots the true and fitted estimates for $K_i$ as a function of $J_{\text{max}}$. The true $K_i$ (i.e., the $K_i$ value employed in simulations) was 50 $\mu M$. For $J_{\text{max}}$ of 0.0001 nmol/cm$^2$/s, $K_i$ estimate was $\sim 27 \mu M$ (i.e., $\sim 2$-fold lower than true $K_i$). For progressively higher $J_{\text{max}}$, $K_i$ estimates exhibited very high negative bias ($\sim 10$-fold lower). These results indicate that failure to consider the contribution of ABL leads to erroneously low $K_i$ estimates, particularly at high $J_{\text{max}}$. Figure 9 further details the interplay of
random error. This evaluation aimed to identify conditions under which kinetic parameters cannot be precisely estimated due to the combination of ABL-controlled transport and modest experimental variability.

Table 2 indicates the probability of successfully estimating \( K_i \) estimates under different scenarios of \( J_{\text{max}} \) and \( J_{\text{max}} \) using error-inclusive simulated flux data. Estimation of \( K_i \) was deemed successful if the 95% confidence interval for the \( K_i \) estimate did not include zero. For each of the fifteen scenarios, Table 2 also denotes the percentage of total resistance that is attributed to ABL. As expected from objective 2 results, the probability of successful \( K_i \) estimation was low when \( J_{\text{max}} \) was high and true \( K_i \) was less than 10 \( \mu M \). For example, the probability was only 10% when true \( K_i = 1 \mu M \) and \( J_{\text{max}} = 0.001 \text{nmol/cm}^2/\text{s} \). In general, the probability of success increased for progressively lower \( J_{\text{max}} \) and progressively higher \( K_i \). For example, when true \( K_i = 5 \mu M \) and \( J_{\text{max}} = 0.0001 \text{nmol/cm}^2/\text{s} \), the probability was 91%. This trend is attributed to progressively lower contribution of ABL to overall control of flux. To a first approximation, the probability of failing to estimate \( K_i \) is equal to the percentage of resistance due to ABL. \( J_{\text{max}} \) was successfully estimated in almost all

<table>
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<th>TABLE 1</th>
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<tr>
<td>ABL influence on taurocholate inhibition kinetics</td>
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<td>Taurocholate ( K_i ) values were calculated using both the ABL-present inhibition model and the ABL-absent inhibition model. As expected from simulation results (Figs. 8 and 9), ( K_i ) values from ABL-absent inhibition model was always lower than the ( K_i ) estimates from ABL-present inhibition model. Values are the average of three observations ± S.E.M.</td>
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<th>TABLE 2</th>
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<tr>
<td>Probability of obtaining statistically significant ( K_i ) estimate under different scenarios of ( J_{\text{max}} ) and ( J_{\text{max}} )</td>
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<td>( K_i ) was estimated on 100 occasions for each scenario using simulated data that incorporated 15% random error. The probability of successful ( K_i ) estimation was the percentage of occasions when the 95% confidence interval for ( K_i ) did not include zero. Tabulated in parentheses is the percentage of total resistance due to ABL (percentage of ABL resistance). When ( J_{\text{max}} ) was high, probability of successful ( K_i ) estimation was low, especially when true ( K_i ) was less than 10 ( \mu M ). At progressively lower ( J_{\text{max}} ), the chance of successful ( K_i ) estimation improved. For most of the scenarios evaluated, the probability of successful ( K_i ) estimation depended on the contribution of ABL resistance to flux (i.e., percent ABL resistance). As flux became increasingly ABL-limited, chances of success decreased.</td>
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<th>( K_i )</th>
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<td>( \mu M )</td>
<td>( \text{Probability of Successful } K_i \text{ Estimation} )</td>
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occasions, as expected based on previous studies (Thomson and Dietschy, 1980) and results from objective 2 in the present study. A result not anticipated from error-free simulation is the modest decrease in successful $K_t$ estimation in Table 2 at high $K_t$. At $K_t$ of 25 µM, the above-mentioned trend was reversed, such that probability of success drops for progressively lower $J_{\text{max}}$. At high $K_t$ scenarios, the passive flux component contributes significantly to overall flux, especially when $J_{\text{max}}$ is low, leading to modest decrease in successful $K_t$ estimation.

Discussion

Kinetics of hASBT-Mediated Taurocholate Flux Using ABL-Absent Model. hASBT is under investigation as a target to increase the oral absorption of drug candidates through a prodrug approach (Balakrishnan and Polli, 2006). An hASBT-MDCK monolayer model was developed previously (Balakrishnan et al., 2005). Specificity of hASBT-mediated transport of taurocholate was based upon a number of observations (e.g., hASBT-transfected versus mock-transfected studies, no-sodium versus sodium-containing studies, apical-to-basolateral versus basolateral-to-apical studies), lack of taurocholate inhibition by DIDS or probenecid, confocal imaging of hASBT in the hASBT-MDCK model, and similar $K_v$ estimates in monolayer transport and uptake studies). It should be noted that, although the hASBT-MDCK is used to elucidate the substrate requirements of hASBT, bile acids are substrates for the recently identified Ostα-Ostβ heteromeric transporter in the enterocyte basolateral membrane (Ballatori et al., 2005; Dawson et al., 2005). However, as noted above, results here do not reflect Ostα-Ostβ kinetic activity, but reflect hASBT kinetic activity, since $K_v$ estimates for uptake and transport studies were similar. Additionally, taurocholate transport was unaffected by the Ostα-Ostβ inhibitor bromosulphaelin (Seward et al., 2003; Balakrishnan et al., 2006).

Taurocholate flux across hASBT-MDCK monolayers are routinely performed. Motivation for the present study was the observed positive association between taurocholate $J_{\text{max}}$ and $K_v$ estimates, where the ABL-absent model was used. The ABL-absent model is the model typically used in analyzing flux data from cell culture transporter assay systems. $K_v$ and $J_{\text{max}}$ estimates exhibited significant variation across occasions. $K_v$ ranged from 2 to 27 µM. $J_{\text{max}}$ ranged between 0.0001 and 0.001 nmol/cm²/s. In Fig. 1, there was a strong linear association between $K_v$ and $J_{\text{max}}$ estimates ($r^2 = 0.82; p < 0.001$). Variability in $J_{\text{max}}$ can be ascribed to variability in hASBT expression (see Appendix 3). However, variability in $K_v$, particularly $K_v$ variation that is associated with $J_{\text{max}}$, was difficult to explain. This association yielded the following hypothesis: high transporter expression can lower monolayer resistance, resulting in ABL-limited transport of solutes, leading to biased kinetic estimates if ABL is not considered. Three objectives were pursued in challenging this hypothesis.

Simulations for Objective 1: Impact of Varying $J_{\text{max}}$ on ABL Contribution. The first objective was to evaluate the effect of $J_{\text{max}}$ on the contribution of ABL resistance to transporter kinetics. Objective 1 was carried out through a combination of empirical laboratory studies, as well as error-free simulation/regression studies. Results indicate that failure to consider ABL can lead to erroneously large $K_v$ estimates at high $J_{\text{max}}$. Results were consistent with experimental taurocholate data in Fig. 1, where high $K_v$ was associated with high $J_{\text{max}}$. Simulation results suggest that, in Fig. 1, $K_v$ estimates were over-estimated due to low monolayer resistance (i.e., low $F_{\text{mono}}$), particularly on occasions when $J_{\text{max}}$ was high.

Figure 5 reevaluates experimental taurocholate data. The ABL-present model was employed to obtain $K_v$ and $J_{\text{max}}$ estimates, whose values are plotted in Fig. 5. Compared with regression results from the ABL-absent model (i.e., Fig. 1), estimates of $K_v$ were about 2- to 3-fold lower from the ABL-present model. In contrast to Fig. 1, there was no association between $K_v$ estimates and $J_{\text{max}}$ estimates in Fig. 5 ($p = 0.24$; $r^2 = 0.13$). This lack of association indicates that correcting for ABL influence lead to a more accurate and less biased estimate of $K_v$. It should be noted that variability in $K_v$ was not completely eliminated. Contributions to the remaining unexplained variation are variation in flux measurements and parameter estimation error.

Simulations for Objective 2: Identification of Global Kinetic Conditions That Require ABL Consideration. The second objective was to identify global kinetic conditions when ABL needs to be explicitly considered to accurately estimate kinetic parameters. Objective 2 was conducted through error-free simulations of both transport and inhibition studies. Results indicate that ABL can generally modulate hASBT kinetics, particularly for all potent substrates, even in low expression systems. Additionally, at $J_{\text{max}}$ greater than 0.001 nmol/cm²/s, as observed for hASBT-MDCK, ABL needs to be considered even when $K_v$ is as large as 100 µM. Interestingly, results suggest lower hASBT expression as an approach to minimize ABL influence on transport. Simulation studies were also performed for transport inhibition studies, which revealed that failure to consider the contribution of ABL leads to erroneously low $K_v$ estimates, particularly at high $J_{\text{max}}$.

However, it should also be noted that ABL effect depends on substrate $K_v$ and $J_{\text{max}}$ in the particular assay system, and therefore ABL is not always significant. For example, ABL influence is not significant for a low-affinity transporter such as hPEPT1. Herrera-Ruiz et al. (2003) developed a stably transfected hPEPT1-MDCK cell line with varying expression levels. $K_v$ of glycyrl sarcosine was 400 µM. From Fig. 6 here, it can be inferred that ABL influence would not be significant for this substrate until very high $J_{\text{max}}$ (i.e., until over 0.001 nmol/cm²/s) is attained. Lack of effect was evident experimentally from Herrera-Ruiz et al. (2003) since glycyrl sarcosine $K_v$ estimate did not vary with varying $J_{\text{max}}$. A second example illustrates a modest ABL effect in an organic anion transporter (OAT3) study (Zhang et al., 2004). Uptake of estrone sulfate into rabbit OAT3-Chinese hamster ovary exhibited $K_v$ and $J_{\text{max}}$ of 4 µM and 0.000413 nmol/cm²/s, respectively, indicating high affinity of estrone sulfate for OAT3. From Figs. 4 and 6 here, low $K_v$ of estrone sulfate would seem to be overestimated by 2-fold.

Simulations for Objective 3: Identification of Global Kinetic Conditions When $K_v$ Estimates Are Unreliable as a Result of ABL Contribution. The third objective was to identify scenarios under which kinetic estimates are not reliable in spite of ABL consideration, due to ABL dominated
transport kinetics. Objective 3 was carried out through simulations incorporating 15% random error, to estimate the probability of successfully estimating \( K_i \). In general, the probability of success increased for progressively lower \( J_{\text{max}} \) and progressively higher \( K_i \). To a first approximation, the probability of failing to estimate \( K_i \) is equal to the percentage of resistance due to ABL.

These results have implication for the functional characterization and development of QSAR models of transporters. QSAR models rely on kinetic parameters (e.g., \( J_{\text{max}}, K_t \), and \( K_i \)) to relate structural features of substrate/inhibitor to activity. Data sets for QSAR analysis typically make up kinetic estimates collected across different occasions, including over years, and across laboratories. Since transport expression levels vary across occasions, results here imply the quality of kinetic parameter estimates can vary with occasion, if ABL effects are ignored. Biased kinetic parameters underestimate the affinity of potent substrates and overestimate the inhibition potency of modest and poor inhibitors, leading to error in QSAR models.

In summary, our combined data indicate that the ABL resistance layer can have significant impact on carrier-mediated solute transport in overexpression systems. This effect was always observed for hASBT-MDCK, but it was most prominent on occasions when \( J_{\text{max}} \) was high. Failure to consider ABL lead to positively biased estimates of \( K_t \) and negatively biased estimates of \( K_i \). The extent of bias is determined collectively by expression level and substrate affinity, such that ABL effect is most prominent for conditions that lead to lowered monolayer resistance (i.e., high \( J_{\text{max}} \) and low \( K_i \)). Results provide three possibilities: 1) monolayer resistance is sufficiently high (i.e., low \( J_{\text{max}} \) and high \( K_i \)) such that ABL can be ignored and kinetic parameters can be estimated with accuracy and precision; 2) monolayer resistance is sufficiently high but ABL cannot be ignored for the accurate and precise estimation of kinetic parameters; and 3) monolayer resistance for substrate is low (high \( J_{\text{max}} \) and low \( K_i \)) such that flux is highly ABL-controlled, and even consideration of ABL does not allow for accurate and precise estimation of kinetic parameters.

**APPENDIX 1**

The objective of Appendix 1 is to derive eq. 9. For the ABL-present model:

\[
R_{\text{app}} = R_{\text{ABL}} + R_{\text{mono}}
\]  

(12)

Hence, in comparing the relative resistances in the ABL-absent model and the ABL-present model,

\[
F_{\text{mono}} = \frac{R_{\text{mono}}}{R_{\text{mono}} + R_{\text{ABL}}}
\]

where \( F_{\text{mono}} \) is the fraction of total flux resistance that is due to the monolayer.

\[
F_{\text{mono}} = \frac{1}{P_{\text{app}}} - \frac{1}{P_{\text{ABL}}}
\]

(13)

\[
F_{\text{mono}} = \frac{1}{P_{\text{app}}} \times \frac{1}{P_{\text{ABL}}}
\]

(14)

\[
F_{\text{mono}} = \frac{S/J}{S/J_{\text{ABL}}}
\]

(15)

**APPENDIX 2**

The objective of Appendix 2 is to derive the ABL-present inhibition model (i.e., eq. 11).

Substrate flux in the presence of an inhibitor when ABL is absent is described by

\[
J = \frac{J_{\text{max}} \times S}{K_t(1 + I/K_i) + S} + P_p \times S
\]

(17)

where \( I \) is the concentration of inhibitor (i.e., inhibitory bile acid), and \( K_t \) is inhibitory constant. \( S \) is substrate concentration (i.e., taurocholate concentration); \( J_{\text{max}}, K_t, \) and \( P_p \) characterize substrate transport parameters.

In the ABL-present model, since permeability is the inverse of resistance,

\[
\frac{1}{P_{\text{app}}} = \frac{1}{P_{\text{ABL}}} + \frac{1}{P_{\text{mono}}}
\]

(18)

where \( P_{\text{app}} \) is the apparent permeability, \( P_{\text{ABL}} \) is the ABL permeability, and \( P_{\text{mono}} \) is the monolayer permeability.

From eq. 17, monolayer permeability in the presence of inhibitor is

\[
P_{\text{mono}} = \frac{J_{\text{max}}}{K_t(1 + I/K_i) + S} + P_p
\]

(19)

Substituting eq. 19 into eq. 18,

\[
\frac{1}{P_{\text{app}}} = \frac{1}{P_{\text{ABL}}} + \frac{1}{P_{\text{mono}}} = \frac{1}{P_{\text{ABL}}} + \frac{1}{\frac{P_{\text{ABL}} \times J_{\text{max}}}{K_t(1 + I/K_i) + S} + P_p}
\]

(20)

\[
P_{\text{app}} = \frac{P_{\text{ABL}} \times J_{\text{max}}}{K_t(1 + I/K_i) + S} + P_p
\]

(21)

since \( J_{\text{ABL}} = P_{\text{app}} \times S, \)

\[
J_{\text{ABL}} = \frac{P_{\text{ABL}} \times \left(\frac{J_{\text{max}}}{K_t(1 + I/K_i) + S} + P_p\right) \times S}{P_{\text{ABL}} + \frac{J_{\text{max}}}{K_t(1 + I/K_i) + S} + P_p}
\]

(22)

**APPENDIX 3**

The objectives of Appendix 3 are to 1) provide support for the presumption that, in Fig. 1, variation in \( J_{\text{max}} \) can be ascribed to variation in hASBT expression; and 2) provide further support that ABL influence is modulated by \( J_{\text{max}} \). Figure 1 inspired the hypothesis that high transporter expression can lower monolayer resistance, resulting in ABL-limited transport of solutes, leading to biased kinetic estimates if ABL is not considered.

Figure 10 shows Western blot analysis of hASBT expressed at cell surface (Fig. 10A) as well as correlation between measure \( J_{\text{max}} \) and hASBT expression level (relative to inte-
were 19.6 ± 2.4, 15.3 ± 1.72, and 7.47 ± 0.50 μM for lanes A, B, and C, respectively. When the ABL-present model was applied to this same data, this association was abolished; \( K_t \) values were 3.24 ± 1.01, 3.73 ± 0.70, 5.77 ± 0.40 μM for lanes A, B, and C, respectively. Result here from hASBT-MDCK monolayers subjected to differing levels of induction by sodium butyrate provide further support that ABL influence is modulated by \( J_{max} \).

**APPENDIX 4**

The objective of Appendix 4 is to evaluate the ability of the ABL-present model to accurately estimate kinetic parameter estimates from uptake studies (i.e., \( J_{max} \) and \( K_t \)), relative to the Winne uptake model. Taurocholate uptake data from five occasions were regressed onto eqs. 7 and 8 using SigmaPlot 2000 (SPSS Inc.). Kinetic parameters were compared via the paired \( t \) test.

This comparison is motivated since Winne’s uptake model is tailored for uptake configuration. Winne’s uptake model would seem not be applicable to transport studies, by virtue of its high identifiability as an uptake model. Favorable performance of the ABL-present model in assessing uptake data, compared with the Winne’s uptake model, would support the ABL-present model to serve as a single form to analyze both transport and uptake data that require ABL consideration.

For each occasion, \( J_{max} \) and \( K_t \) values from the ABL-present model were statistically indistinguishable from \( J_{max} \) and \( K_t \) values from Winne’s uptake model (\( p > 0.05 \)). Additionally, the ABL-present model effectively eliminated the bias in \( K_t \) that otherwise results from ABL. As was observed from transport studies in Fig. 1, there was a positive, linear association between \( K_t \) and \( J_{max} \), when the ABL-absent model was applied to uptake data (\( r^2 = 0.79 \)). Application of the ABL-present model markedly attenuated the association (\( r^2 = 0.08 \)), as did the Winne uptake model.

Favorable performance of the ABL-present model in assessing uptake data supports the ABL-present model to serve as a single form to analyze both transport and uptake data that require ABL consideration.

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Address correspondence to: Dr. James E. Polli, Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, 20 Penn St., HSF2 Rm 623, Baltimore, MD 21201. E-mail: jpolli@rx.umaryland.edu