Data-Based Mathematical Modeling of Vectorial Transport across Double-Transfected Polarized Cells

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ABSTRACT:
Vectorial transport of endogenous small molecules, toxins, and drugs across polarized epithelial cells contributes to their half-life in the organism and to detoxification. To study vectorial transport in a quantitative manner, an in vitro model was used that includes polarized MDCKII cells stably expressing the recombinant human uptake transporter OATP1B3 in their basolateral membrane and the recombinant ATP-driven efflux pump ABCC2 in their apical membrane. These double-transfected cells enabled mathematical modeling of the vectorial transport of the anionic prototype substance bromosulfophthalein (BSP) that has frequently been used to examine hepatobiliary transport. Time-dependent analyses of $^3$H-labeled BSP in the basolateral, intracellular, and apical compartments of cells cultured on filter membranes and efflux experiments in cells preloaded with BSP were performed. A mathematical model was fitted to the experimental data. Data-based modeling was optimized by including endogenous transport processes in addition to the recombinant transport proteins. The predominant contributions to the overall vectorial transport of BSP were mediated by OATP1B3 (44%) and ABCC2 (28%). Model comparison predicted a previously unrecognized endogenous basolateral efflux process as a negative contribution to total vectorial transport, amounting to 19%, which is in line with the detection of the basolateral efflux pump Abcc4 in MDCKII cells. Rate-determining steps in the vectorial transport were identified by calculating control coefficients. Data-based mathematical modeling of vectorial transport of BSP as a model substance resulted in a quantitative description of this process and its components. The same systems biology approach may be applied to other cellular systems and to different substances.

Vectorial transport of small molecules is an important process in polarized cells. Uptake across the basolateral membrane and efflux across the apical membrane domain of polarized cells contribute to the elimination and often detoxification of substances. In mammals, endogenous compounds and drugs are taken up from the blood into hepatocytes and subsequently effluxed mainly into bile.

Several factors affect vectorial transport of small molecules. First, unidirectional transport does not occur at a sufficient rate in the absence of the respective transport proteins in the basolateral and apical membranes, as evidenced by studies in hereditary mutants lacking certain transport proteins (Jansen et al., 2001; Keppler et al., 2001; Schinkel and Jonker, 2003) or in cell lines stably transfected with cDNAs encoding transport proteins (Cui et al., 2001; Sasaki et al., 2002; Hagenbuch and Meier, 2004). Second, it is affected in the intact organism by blood flow, intravascular binding to proteins, and intracellular metabolism.

To build a mathematical model of vectorial transport, we used a well defined cellular system developed previously (Cui et al., 2001). Polarized cells grown on filter membrane supports were stably transfected with cDNAs encoding the human uptake transporter for organic anions, OATP1B3 (König et al., 2000), and the human apical conjugate export pump ABC2, also known as multidrug resistance protein 2 (MRP2) (Büchler et al., 1996). Such double-transfected cells exhibit transporter-mediated substrate flux from the basolateral to the apical compartment and, for most compounds, very little intracellular metabolism (Cui et al., 2001; Keppler, 2005; Letschert et al., 2005). Bromosulfophthalein (BSP) is a substrate for both transport proteins, OATP1B3 (König et al., 2000) and ABC2 (Cui et al., 2001). Moreover, BSP is an established test compound for studies of hepatobiliary elimination in humans and animals (Wolkoff, 1994). Math-

ABBREVIATIONS: OATP1B3, human organic anion-transporting polypeptide, member 1B3; ABC2, human ATP-binding cassette transporter, subfamily C, member 2; MRP2, multidrug resistance protein 2; BSP, bromosulfophthalein; MDCKII, Madin-Darby canine kidney cells strain II; Abcc4, canine ATP-binding cassette transporter, subfamily C, member 4; ABC3, human ATP-binding cassette transporter, subfamily C, member 3; Endo$_{ap}$, apical endogenous efflux transporter; Endo$_{bl}$, basolateral endogenous efflux transporter; Endo$_{bl}$, basolateral endogenous uptake transporter.
emirical modeling was performed in this focused cellular system to
mediate the vectorial transport of BSP. The same modeling approach
can be applied to other polarized cellular systems and to a variety
of different substances, thus opening the perspective of quantitative and
predictive modeling and the understanding of vectorial transport sys-
tems.

Materials and Methods

Cell Culture and Cell Lines. MDCKII cells permanently expressing high
levels of recombinant human OATP1B3 or OATP1B3 together with ABCC2
were cultured as described previously (Cui et al., 2001; Fig. 1).

Immunofluorescence Microscopy. MDCKII cells were grown on ThinCert
membrane inserts (diameter, 6 mm; pore size, 0.4 µm; pore density, 1 ×
10³/cm²; Greiner Bio-One; Frickenhausen, Germany; Letschert et al., 2005) for
3 days at confluence and induced with 10 mM sodium butyrate for 24 h to
enhance the expression of recombinant proteins (Cui et al., 1999). Fixation
and permeabilization were performed as described previously (Cui et al., 2001).
OATP1B3 was detected by the antisera SKT (König et al., 2000), ABCC2
was detected by the antisera EAG (Cui et al., 1999), and canine Abcc4 was
detected by the purified antiserum SNG (Rius et al., 2003). Nuclei were stained
with propidium iodide. Confocal laser scanning microscopy was performed
with an LSM 510 META apparatus (Carl Zeiss, Jena, Germany).

Transport Studies. [3H]BSP (0.5 TBq/mmol) was obtained from Hartmann
Analytic (Braunschweig, Germany) (Cui et al., 2001). MDCKII cells were
grown on ThinCert membrane inserts (diameter, 24 mm; pore size, 0.4 µm; pore
density, 1 × 10³/cm²; Greiner Bio-One) for 3 days at confluence and
induced with 10 mM sodium butyrate for 24 h (Cui et al., 1999). The cells were
washed in prewarmed (37°C) transport buffer (142 mM NaCl, 5 mM KCl, 1
mM KH₂PO₄, 1.2 mM MgSO₄, 1.5 mM CaCl₂, 5 mM glucose, and 12.5 mM
HEPES, pH 7.3). The [3H]-labeled BSP was added to transport buffer and added
to the basolateral compartment (1.5 ml) at the concentration indicated.
After incubation at 37°C, radioactivity in the apical compartment (1.0
ml) was measured by sampling of aliquots from the apical compartment. Cells
were washed twice with ice-cold transport buffer containing 0.5% bovine
serum albumin and three times with ice-cold transport buffer. Intracellular
radioactivity was determined after lysing the cells with 0.2% sodium dodecyl
sulfate. For preloading studies, cells were washed after incubation at 37°C with
the labeled substrate as described above. Subsequently, cells were further
incubated at 37°C with transport buffer (1.0 ml in the basolateral and 1.0 ml in the
apical compartment) in the absence of labeled substrate, and radioactivity was
determined as described above.

The paracellular leakage was determined by the addition of 1 µM [3H]-insulin
(BIOTREND, Köln, Germany) to the basolateral compartment and measure-
ment of the radioactivity appearing in the apical compartment. The paracellular
leakage was less than 2% of the radioactivity added for all MDCKII cell clones
examined in this study.

Numerical Analysis. Ordinary differential equations were derived from the
model depicted in Fig. 2 by assuming Michaelis-Menten kinetics in the linear
regime. The kinetic behavior of the transporters OATP1B3 (König et al., 2000)
and ABCC2 (Cui et al., 1999) has been characterized, and Michaelis-Menten
constants were determined. The equations were integrated by ODESSA (Leis
and Kramer, 1988a,b). To ensure that we could identify all parameters while
performing the multieperiment fit, we used a penalized likelihood as cost-
function for parameter estimation. This likelihood includes prior knowledge of
the parameter distribution (see Supplemental Data). The resulting cost-function
was minimized using an optimization routine of the Gauss-Newton type
(Hanson and Haskell, 1982; Peifer and Timmer, 2007). Because measurement
errors show a linear dependence on the estimated mean value, we re-estimated
the standard deviations by applying a linear error model to minimize the
fluctuations in the estimated standard deviations (see Supplemental Data).
Results

The Cell System. MDCKII cells were grown in a polarized fashion
on filter membrane supports, and transport of BSP, a substrate for
both OATP1B3 and ABCC2, was studied in this system. The expres-
sion and sorting of the transport proteins were confirmed by immu-
nofluorescence and confocal laser scanning microscopy (Fig. 1). The
cell line represents an improved version of the double-transfectant
described previously (Cui et al., 2001).

MDCKII cells were also analyzed for the expression and localiza-
tion of endogenous transport proteins, as suggested by the mathemat-
ical modeling. Endogenous canine Abcc4 was localized to the baso-
lateral membrane of the MDCKII cells (Fig. 1E). Thus, this
endogenous basolateral efflux pump functions in addition to the
recombinant human transport proteins. ABCC4 has a broad substrate
specificity (Kruh et al., 2007) that includes BSP (data not shown). The
influxes, effluxes, and concentration pools are summarized in Fig. 2.

Uptake and Efflux Transport in Polarized Cells. To acquire data,
several sets of transport experiments were performed. Vectorial transpor-
t of labeled BSP was measured over 60 min at high (10 µM; Fig. 3)
and low (10 nM; Fig. 4) concentrations. The intracellular content of
labeled BSP was significantly higher in MDCKII cells expressing

FIG. 1. Immunolocalization of recombinant human OATP1B3, ABCC2, and end-
genous Abcc4 in canine MDCKII cells. Cells were grown in a polarized fashion
on membrane inserts and analyzed by confocal laser scanning microscopy for the
localization of OATP1B3 (green in A and B), ABCC2 (red in C and D), and canine
Abcc4 (orange in E). Nuclei are stained in blue. A, C, and E are en face images at
the top of the cell monolayers; B and D are vertical sections through the cell
monolayers at positions indicated by the white lines in A and C. Scale bars, 10 µm.

FIG. 2. Cellular basis for the mathematical model of the vectorial transport in
polarized MDCKII cells. The cell system comprises three major compartments: the
basolateral compartment \([x_1]\), the intracellular compartment \([x_2]\), and the apical
compartment \([x_3]\). The extracellular binding sites \([x_4]\) represent the nonspecific
binding of the substrate to the filter membrane, and the intracellular binding sites
\([x_5]\) represent the binding of the substrate to intracellular proteins. Both extracellular
and intracellular binding sites have been experimentally verified. Six different
transport processes were identified: the basolateral uptake mediated by human
OATP1B3, the apical efflux mediated by human ABCC2, the basolateral endoge-

ous uptake \(\text{Endo}_{bl}\), and the basolateral and apical efflux processes
\(\text{Endo}_{ex-bl}\) and \(\text{Endo}_{ex-ap}\), respectively, and the paracellular leakage.
radioactivity inside the cells (intracellular content, \(x_e\)) and in the apical chamber (apical amount, \(x_a\)) was determined at the time points indicated. Data points with error bars represent mean values, given as nanomoles per milligram of cellular protein + S.D., from a triplicate determination reproduced independently at least once. The solid lines result from the mathematical modeling of the measured data.}

recombinant OATP1B3 than in the control MDCKII cells (Figs. 3 and 4, bottom), indicating that OATP1B3 is responsible for the uptake and intracellular accumulation of BSP. The release of BSP into the apical chamber was mainly detected in cells expressing recombinant OATP1B3 together with ABCC2 (Figs. 3 and 4, top), indicating that ABCC2 in the apical membrane efficiently mediates the efflux of BSP.

To assess the contributions of endogenous transport processes, the cells were preloaded (Fig. 5) by adding \(^3\)H-labeled BSP to the basolateral chamber for 30 min. After this preloading time, the amounts of radioactivity effluxed into the apical chamber and into the basolateral chamber (basolateral amount), as well as radioactivity accumulated inside the cells, were determined. BSP was strongly accumulated in cells expressing OATP1B3 and, to a lesser extent, in cells expressing OATP1B3 together with ABC2 (Figs. 5, middle). The MDCKII cells expressing recombinant OATP1B3 and ABC2 showed the highest efflux of BSP into the apical chamber (Fig. 5, top). However, the major BSP efflux into the basolateral chamber was observed in the cells that had reached the highest intracellular content (Fig. 5, bottom). This efflux is most probably mediated by the endogenous (canine) Abcc4 of the MDCKII cells (Fig. 1E).

**The Mathematical Model.** Our model incorporated the effects of several transport factors. First, as can be seen from measurements in MDCKII control cells (Figs. 3 and 4), there was a small but significant increase of BSP in the intracellular compartment and in the apical chamber. This observation suggests the existence of an endogenous basolateral uptake transporter (\(\text{Endo}_{\text{in},\text{mb}}\) in Fig. 2) as well as an apical endogenous efflux pump (\(\text{Endo}_{\text{ex},\text{ap}}\) in Fig. 2). Second, preloading experiments (Fig. 5) indicated the existence of a basolateral efflux pump (\(\text{Endo}_{\text{ex},\text{bl}}\) in Fig. 2). Third, based on \(^3\)Hjinulin transport experiments, we observed a low rate (less than 1% of total transport) of paracellular leakage (data not shown), which was also included in our model. Fourth, to determine the amount of \(^3\)H]BSP that bound to cytosolic proteins, we separated the cytosolic protein fraction by centrifugation at 20,000g and measured the amount of radioactivity in the resulting supernatant. We found that approximately 86% of the BSP bound to intracellular macromolecules. To account for this binding, we added intracellular binding sites to the model (\(x_b\) in Fig. 4). Finally, we incorporated the inulin transport experiments (Fig. 2). Third, based on \(^3\)Hjinulin transport experiments, we observed a low rate (less than 1% of total transport) of paracellular leakage (data not shown), which was also included in our model. Fourth, to determine the amount of \(^3\)H]BSP that bound to cytosolic proteins, we separated the cytosolic protein fraction by centrifugation at 20,000g and measured the amount of radioactivity in the resulting supernatant. We found that approximately 86% of the BSP bound to intracellular macromolecules. To account for this binding, we added intracellular binding sites to the model (\(x_b\) in Fig. 4). Finally, the intracellular content of BSP is approximately 7 times greater for the cells expressing recombinant OATP1B3 than for the control cells when determined at 60 min (Fig. 3). If transcellular diffusion played a significant role, the apical amount of BSP should be much greater for the OATP1B3-expressing cells than for the control cells. Thus, transcellular diffusion seems to be negligible and therefore was not factored into the model.

Assuming Michaelis-Menten kinetics in the linear regime, we derived the ordinary differential equations (Table 1) from the model shown in Fig. 2. This procedure is in accordance with similar modeling approaches (Liu and Pang, 2006; Turncliff et al., 2006). The rates of BSP uptake into cells may vary somewhat between different sets of experiments depending on the expression level of OATP1B3. Accordingly, we fitted our model to the data from all of the experiments, depending on the expression level of OATP1B3. This procedure is in accordance with similar modeling approaches (Liu and Pang, 2006; Turncliff et al., 2006). The rates of BSP uptake into cells may vary somewhat between different sets of experiments depending on the expression level of OATP1B3.
Mathematical modeling of vectorial transport controls the half-life of many endogenous and xenobiotic substances in the mammalian or-

Discussion

Vascular transport across polarized cells controls the half-life of many endogenous and xenobiotic substances in the mammalian or-

all parameters for the multieperiment fit (see Supplemental Data). Results of the fits are shown in Figs. 3 through 5.

We found it necessary to include the endogenous transporters in the model. We compared the complete model (Fig. 2) to models lacking selected endogenous transport processes. Each of the smaller models yielded a significantly worse fit of the data than the complete model (see Supplemental Data).

Rate-Determining Steps in Vectorial Transport. Based on our model (Fig. 2), we calculated the contribution of each transport process to the total amount of BSP transported into the apical chamber. The largest amount of BSP was transported by the recombinant uptake transporter OATP1B3, followed by the recombinant apical efflux pump ABC2, the endogenous efflux pump Endo\textsubscript{ex-bl} (probably Abcc4), and the endogenous uptake transporter Endo\textsubscript{in-bl} (Fig. 6A). The endogenous apical efflux Endo\textsubscript{ex-ap} and paracellular transport contributed the smallest fractions to the total transported amount. OATP1B3 accounted for approximately 44% of the overall transport, followed by ABCC2 with 28% and the endogenous efflux pump Endo\textsubscript{ex-bl} with approximately 19% (Fig. 6B). The endogenous transporters Endo\textsubscript{in-bl} and Endo\textsubscript{ex-ap} and paracellular BSP transport together account for less than 10% of the total transport. Since the endogenous efflux pump Endo\textsubscript{ex-bl} (probably Abcc4) transports BSP back into the basolateral chamber, its contribution to the total amount of BSP transported into the apical chamber is actually negative.

Control coefficients have frequently been used in metabolic control analysis to quantify the contribution of a given enzyme to the steady-state flux of metabolites (Schuster and Heinrich, 1992; Conradie et al., 2006). In this analysis, the fractional change of the steady-state flux of a metabolite $v_j$ is related to the fractional change of each transport process $J_p$, defined as $C_{jv} = J/vJdv/v$. For our system, we calculated the relationship of the fractional change of the total flux into the apical chamber, $J = dx_5/dt$, to the fractional change of each transport process in our model (Fig. 7). Since the rate for each transport process in our model is linear and has the form $v_j = p_jx_k$, the normalized control coefficients read:

$$C_{jv} = \frac{p_j}{v_j} \frac{\partial J}{\partial x_k}$$

where $p_j$ is the parameter controlling transport process $j$. The resulting control coefficients for the double-transfected and MDCKII control cells are depicted in Fig. 7.

For the double-transfected cells, the control coefficients of OATP1B3 and ABC2 were the dominant transport processes and were rate-determining in our system. This agrees with the fact that each of the single-transfected cells (i.e., OATB1B3 cells or ABC2 cells; Cui et al., 2001) exhibit significantly less total vectorial transport, whereas OAT1B3 and ABC2 were the dominant transport processes and the rate-determining step in vectorial transport clearly depends upon the expression level of the respective transport proteins.

### Table 1

Ordinary differential equations deduced from the model by assuming linearized Michaelis-Menten kinetics

Here, $x_1$ is the basolateral amount and $x_2$ is BSP bound nonspecifically to the filter membranes. Unbound intracellular BSP is denoted by $x_3$, whereas $x_4$ is BSP bound to intracellular-binding proteins and $x_5$ is the apical amount of BSP. The rate constants $p_{1j}$ and $p_{5j}$ for OATP1B3 and ABC2, as well as $p_{2j}$ and $p_{3j}$ for Endo\textsubscript{ex-bl} and Endo\textsubscript{ex-ap}, are proportional to the respective concentrations of the transporters. $V_{bl}$ and $V_{ap}$ denote the volume of the basolateral and the apical chamber, respectively.

**Differential Equations**

1. $\frac{dx_1}{dt} = -p_{1j}x_1 - p_{3j}x_3 + p_{4j}x_4 + p_{5j}(x_6 - x_5) / V_{bl}$
2. $\frac{dx_2}{dt} = p_{1j}x_1 - p_{2j}x_2$
3. $\frac{dx_3}{dt} = p_{1j}x_1 - p_{2j}x_2 + p_{3j}x_3 - p_{3j}x_3 - p_{4j}x_2 + p_{5j}(x_6 - x_5)$
4. $\frac{dx_4}{dt} = p_{1j}x_1 - p_{2j}x_2 - p_{3j}x_3 + p_{5j}(x_6 - x_5)$
5. $\frac{dx_5}{dt} = -p_{2j}x_2 + p_{3j}x_3 + p_{7j}(x_1 / V_{ap} - x_4 / V_{ap})$
BSP in the basolateral compartment, inside the cells, and in the apical edge on the vectorial transport of endogenous and xenobiotic substances. Accordingly, predictions can be made for the time course of transport and for the relative contribution of single transport steps.

In conclusion, the polarized cell system for studies on vectorial transport (Cui et al., 2001; Sasaki et al., 2002) was successfully used for data-based mathematical modeling and resulted in the quantification of individual transport steps in a complex system. This quantitative modeling greatly expands the mostly qualitative previous knowledge on the vectorial transport of endogenous and xenobiotic substances. Accordingly, predictions can be made for the time course of transport and for the relative contribution of single transport steps.
to the overall transport. The calculation of control coefficients enabled the identification of rate-determining single steps in overall vectorial transport. Moreover, the modeling approach in this study has been useful for the identification of previously unexpected partial processes, such as the quantitatively important basolateral efflux (Endo\textsubscript{AP}, Mrp). This additional transport process is well explained by the detection of endogenous Abcc4 in the MDCKII cells. The inclusion of this process was necessary to obtain excellent agreement between the experimental data and the mathematical model. Thus, data-based quantitative mathematical modeling led to new qualitative as well as quantitative insight into the biological system. In this study, we focused on BSP as a well known model substance for the analysis of hepatobiliary elimination. However, this modeling approach may be applied to other substances, e.g., cholecystokinin octapeptide CCK-8, the vectorial transport of which has been characterized recently (Letschert et al., 2005), and other polarized cellular systems, such as quadruple-transfected MDCKII cells (Koppow et al., 2005).

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References


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1 Mathematical Model

The transport rate $v$ of a substrate $x$ mediated by a transporter is in general described by Michaelis-Menten kinetics (Stein, 1990), i.e. $v = \frac{v_{max} x}{K_m + x}$. Since the substrate concentrations in our experiments are smaller, respectively in the order of the Michaelis-Menten constants of OATP1B3 and ABCC2 (Letschert et al., 2004; Cui et al., 2001), we linearized the Michaelis-Menten equation resulting in $v \approx \frac{v_{max} x}{K_m} = px$. Thus, the translation of the model depicted in Fig. 2 into ordinary differential equations yields:

$$\frac{dx_1(t)}{dt} = -p_1x_1(t) - p_3x_1(t) + p_4x_3(t) - p_6x_1(t)(p_8 - x_2(t)) + p_7x_2(t) - p_{12}\left(\frac{x_1(t)}{V_{bl}} - \frac{x_5(t)}{V_{ap}}\right)$$

(1)

$$\frac{dx_2(t)}{dt} = p_6x_1(t)(p_8 - x_2(t)) - p_7x_2(t)$$

(2)

$$\frac{dx_3(t)}{dt} = p_1x_1(t) - p_2x_3(t) + p_3x_1(t) - p_4x_3(t) - p_5x_3(t) - p_9x_3(t)(p_{11} - x_4(t)) + p_{10}x_4(t)$$

(3)

$$\frac{dx_4(t)}{dt} = p_9x_3(t)(p_{11} - x_4(t)) - p_{10}x_4(t)$$

(4)

$$\frac{dx_5(t)}{dt} = p_2x_3(t) + p_5x_3(t) + p_{12}\left(\frac{x_1(t)}{V_{bl}} - \frac{x_5(t)}{V_{ap}}\right)$$

(5)

Here, $x_1$ is the amount of BSP unbound in the basolateral chamber, $x_2$ is BSP bound unspecifically to the filter membrane, $x_3$ is unbound intracellular BSP, $x_4$ is intracellular BSP bound to intracellular proteins, and $x_5$ is the amount of BSP in the apical chamber. The rate constants for OATP1B3 and ABCC2 are $p_1$ and $p_2$ respectively; $p_3$, $p_4$, and $p_5$ are the rate constants of the endogenous basolateral uptake transporter Endo$_{in-bl}$, the
endogenous basolateral efflux pump $Endo_{ex-bl}$, and the endogenous apical efflux pump $Endo_{ex-ap}$, respectively. The parameters $p_6$ and $p_7$ are the association and dissociation constants for unspecific binding to the filter membrane, the total capacity of which is denoted by $p_8$. The association and dissociation constants for the unspecific binding to intracellular proteins are $p_9$ and $p_{10}$ respectively, whereas $p_{11}$ denotes the total amount of intracellular binding proteins. The diffusion parameter of the paracellular transport is represented by $p_{12}$. $V_{bl}$ and $V_{ap}$ are the volumes of the basolateral and apical chamber, respectively.

It is not possible to observe all five components separately. Only the total intracellular content, $x_3 + x_4$, and the apical amount $x_5$ can be determined. For the preloading experiments, the basolateral amount $x_1$ was also determined. Thus, the observation equations for a given set of parameters $\vec{p} = (p_1, p_2, \ldots, p_{12})$ read:

\[
\begin{align*}
y_1(t, \vec{p}) &= x_3(t, \vec{p}) + x_4(t, \vec{p}) \\
y_2(t, \vec{p}) &= x_5(t, \vec{p}) \\
y_3(t, \vec{p}) &= x_1(t, \vec{p})
\end{align*}
\]

### 1.1 Reduction of Parameter Space

Experiments showed that $86 \pm 1\%$ of the intracellular BSP is bound to intracellular proteins, independent of the total intracellular content of BSP under our conditions. Thus, for the steady state, the proportion of bound intracellular BSP to total intracellular BSP, $x_4/(x_3 + x_4)$, has to be equal to 0.86.

Solving the steady state of Eqn. 4 for $x_4/(x_3 + x_4)$ yields:

\[
\frac{x_4}{x_3 + x_4} = \left(\frac{p_{10}}{p_9} \frac{1}{p_{11} - x_4} + 1\right)^{-1} \equiv 86\%
\]

Since, as stated above, this identity is independent of the total intracellular content of BSP, the inequality $p_{11} \gg x_4$ has to hold. Thus, we get:

\[
\frac{p_{10}}{p_9} = \left(\frac{1}{0.86} - 1\right) (p_{11} - x_4) \approx \frac{0.14}{0.86} p_{11} \\
\Rightarrow p_{10} = \frac{0.14}{0.86} p_{11} p_9
\]
With $p_{11} \gg x_4$ and setting $k = p_9 p_{11}$, the model Eqs. 3 and 4 can be rewritten:

$$\frac{dx_3}{dt} = p_1 x_1 - p_2 x_3 + p_3 x_1 - p_4 x_3 - p_5 x_3 + \frac{0.14}{0.86} k x_4$$

$$\frac{dx_4}{dt} = k x_3 - \frac{0.14}{0.86} k x_4,$$

leading to a reduction of the number of parameters from 12 to 10.

## 2 Penalized Likelihood Estimator

As described in **Methods**, experiments have been accomplished with the following experimental setups: Experiments with a concentration of BSP in the basolateral chamber of 10 nM and 10 µM, as well as preloading experiments. Each of these experiments was performed for control MDCKII cells, cells expressing OATP1B3, and cells expressing OATP1B3 and ABCC2 (Figs. 3-5). To describe all data sets with the model (Fig. 2), the parameters of the model had to be fitted to the data simultaneously by a multi-experiment analysis. The advantage of such an analysis is that non-identifiabilities of parameters can be resolved. For example, when the model was fitted solely to the data of an experiment with cells expressing OATP1B3, the amount of BSP transported either by OATP1B3 or by the endogenous transporter $Endo_{m-ld}$ could not be distinguished. Thus, only the sum of the parameters $p_1$ and $p_3$ could be determined, but not their individual values.

Since in the experiments performed with control cells there is only one basolateral uptake process, it is possible to determine the parameter $p_3$ in these experiments. By multi-experiment analysis, this knowledge about the value of $p_3$ from the control experiments is used to eliminate the non-identifiabilities in the other experiments.

### 2.1 Global and Local Parameters

In multi-experiment analysis, one has to distinguish between global and local parameters. Global parameters are those that have the same value for each experiment, whereas local parameters can differ from one experiment to another. Global parameters are parameters
such as chemical rate constants. In our model, the parameters $p_6$, $p_7$, $p_9$, and $p_{10}$ are rate constants and are thus set to global parameters. Also, the total number of binding sites at the filter membrane, $p_8$, as well as the total number of intracellular binding sites, $p_{11}$, were assumed to be the same for all experiments and were thus treated as global parameters.

The amount and localization of the transport proteins varies depending on the time in culture, the induction of transporter expression and additional variance in the biological system. Thus, parameters that depend on protein concentrations, such as $v_{\text{max}}$ values, can attain different values in different experiments. In our model, parameters $p_1 - p_5$ are proportional to the number of transporters per cell and were treated as local parameters.

Inulin experiments measuring the paracellular leakage also showed fluctuations in the amount of transported BSP. Therefore, we treated the diffusion parameter $p_{12}$ as a local parameter as well.

The drawback of setting parameters to local ones is that for these parameters the advantages of the multi-experiment fit are lost. By definition, local parameters can vary independently and thus do not use the information provided by the other experiments. Again, this can lead to non-identifiabilities of parameters.

To resolve this issue, we introduced constrained local parameters (i.e., local parameters whose variations are bound to a predefined region around the mean of this parameter) over all experiments. We add constraints on the local parameters by introducing a penalized likelihood (Good and Gaskins, 1971).

### 2.2 Penalized Likelihood

To impose constraints on the variability of local parameters, we added a penalty term to the likelihood function that was to be maximized. In terms of Bayesian statistics, this penalty term contains prior knowledge of the distribution of the local parameters. Assuming Gaussian distributed local parameters, this penalty term reads:

$$
\rho(p_{jl}) = \frac{1}{\sqrt{2\pi\sigma_l}} \exp \left( -\frac{(p_{jl} - \bar{p}_l)^2}{2\sigma_l^2} \right),
$$

where $p_{jl}$ is the value of local parameter $l$ in experiment $j$, and $\bar{p}_l$ is the mean over all experiments. The standard deviation $\sigma_l$ determines the size of the interval around the
mean value $\bar{p}_l$ that is accessible to the local parameters. For very large values of $\sigma_l$, (i.e., $\sigma_l \rightarrow \infty$), the penalty term is zero, and the local parameters $p_l$ undergo no constraints. On the other hand, for $\sigma_l \rightarrow 0$, the smallest deviation of a local parameter $p_{jl}$ from the mean value $\bar{p}_l$ leads to an infinite value of the penalty term, forcing all local parameters $p_{jl}$ to attain the same value $\bar{p}_l$ (i.e., setting these local parameters to a global parameter).

If 66% of the local parameters $p_{jl}$ are forced to be within an interval around the mean value $\bar{p}_l$, where the upper border of the interval is $n_l$-times the lower border, we get $\sigma_l = \frac{n_l-1}{n_l+1}\bar{p}_l$. For our model, we chose $n_l = 3$ for OATP1B3 and ABCC2, and $n_l = 1.5$ for the endogenous transporters as well as for the paracellular transport.

Let $\sigma_{ijk}$ be the weight of the observed data, and $y_{ijk}$ and $y_k(t_{ij})$ be the estimated value at time $t_{ij}$ given the parameters $\bar{p}$. Then the penalized log-likelihood reads:

$$L(p) = \sum_{i,j,k} \left( \frac{y_{ijk} - y_k(t_{ij}, p))}{\sigma_{ijk}^2} \right)^2 + \sum_{j,l} \left( \frac{p_{jl} - \bar{p}_l}{\frac{n_l-1}{n_l+1}\bar{p}_l} \right)^2$$

Here, the first term is the usual least-square functional, with the sum running over data points $i$, all experiments $j$, and observables $k$. The second term is the logarithm of the penalty term with the sum running over all local parameters $l$ and all experiments $j$.

## 3 Error Model

As described in Methods, the estimated mean values and standard deviations of the experiment presented in Figs. 3-5 were determined by triplicate measurements. Triplicate measurements were performed to give an estimate about the confidence interval of the estimated mean value. These confidence intervals are also subject to statistical fluctuations and thus cannot be determined with certainty. For Gaussian distributed random numbers, the 95% confidence interval of the estimated variances is (Honerkamp, 1994):

$$\sigma^2 \pm 1.96\sqrt{\frac{2}{N-1}\sigma^2}$$

For triplicate measurements (i.e., for $N = 3$), these intervals are very large, resulting in strong fluctuations in the estimated standard deviations (Supplement Fig. 8).
The plot of the estimated standard deviations versus the estimated mean values suggests a linear dependency (see Supplem Fig. 8). Using this linear relationship, we can re-estimate the standard deviations by a linear fit for each experiment. Thus, not only the three replicate measurements are used for to estimate standard deviation, but all measurements of one experiment. This leads to more reliable estimates of the standard deviations.

4 Model Selection

To justify the inclusion of the additional transport processes into our model, we compare the fit of the complete model with the fits of smaller models. Hereby, the smaller models are lacking either one of the endogenous transporters Endo_{in-bl}, Endo_{ex-bl} and Endo_{ex-ap}, as well as the paracellular transport. Supplement Fig. 9 shows the resulting fits of the smaller models in comparison with the complete model. Hereby, for simplicity only the components with the most significant deviation of the fit of the smaller models from the experimental data are displayed.

Also, the Akaike Information Criterion (AIC) (Akaike, 1973) supports the choice of the complete model as the best model, see Supplement Table 2.

5 Fitting Results

The cumulative distributions of the local parameters resulting from the optimization of the penalized likelihood are displayed in Supplement Fig. 10, and the values for the global parameters are given in Supplement Table 3.
References

Akaike H (1973) Information theory and an extension of the maximum likelihood principle. 

*Mol Pharmacol* 60:934–943.


Supplement Figure 8: The estimated standard deviations plotted against the estimated mean values. The statistical fluctuations are large, since the standard deviations were estimated from only three measurements.
Supplement Figure 9: Comparison of the fits of the smaller models with the fit of the complete model. (A) Experiments with 10 nM and 10 µM BSP showed an intracellular accumulation for control cells also. The model lacking the endogenous basolateral uptake transporter cannot explain this accumulation in the control cells. Thus, the model cannot describe the observed data. (B) For preloading experiments, the model without the endogeneous basolateral efflux transporter can explain the increase of BSP in the basolateral chamber to only partially. Specifically, it can by the amount that was previously bound non-specifically to the filter membrane during the preloading procedure. For any additional accumulation of BSP in the basolateral chamber, BSP has to be transported back from the intracellular into the basolateral compartment. (C) The increase of the apical amount of BSP for the preloading experiments cannot sufficiently be explained by the model lacking the endogenous apical efflux pump. (D) When the paracellular transport is missing in the model, the apical amount of BSP at early time points of the experiment is significantly smaller, and at later time points significantly larger than the experimental data. In the beginning, there is no BSP in the intracellular compartment, and thus nothing can be exported into the apical chamber. On the other hand, at later time points, when sufficient amounts of BSP have accumulated in the cells, the apical efflux pump leads to a larger increase of BSP in the apical chamber than experimentally determined.
Supplement Figure 10: Cumulative distribution of the local parameters. Each closed circle represents the value of a local parameter in one experiment. Altogether, there were nine experiments: BSP concentrations of 10 µM, 10 nM, and preloading, each for control, single-transfected (OATP1B3) and double-transfected (OATP1B3-ABCC2) cells. For the parameters $p_1$ and $p_2$, only six, respectively three values were determined since the control cells do neither express OATP1B3 nor ABCC2, and the single-transfected cells do not express ABCC2.
Supplement Table 2: Akaike Information Criteria (AIC) for each model. The AIC enables the quantitative comparison of different models, favoring the model with the smallest AIC.

<table>
<thead>
<tr>
<th></th>
<th>Complete Model</th>
<th>w/o Endo$_{in-bl}$</th>
<th>w/o Endo$_{ex-bl}$</th>
<th>w/o Endo$_{ex-op}$</th>
<th>w/o paracell.</th>
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<td>AIC</td>
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<td>3353</td>
<td>-566</td>
<td>-784</td>
<td>-818</td>
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</table>
Supplement Table 3: The values of the global parameters and their standard deviations of the resulting fit. Parameters $p_8$ and $p_{10}$ were fixed since they were not identifiable. The parameters for the volumes $V_{bl}$ and $V_{ap}$ were known from the experimental setup. The basolateral volume $V_{bl}$ was 1.0 ml for the preloading experiments and 1.5 ml for all other experiments.

<table>
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<th>Parameter</th>
<th>Unit</th>
<th>Mean</th>
<th>Std</th>
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<tbody>
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<td>$p_6$</td>
<td>mg/(min·nmol)</td>
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<tr>
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<td>min$^{-1}$</td>
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<td>0.002</td>
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<tr>
<td>$p_9$</td>
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<td>0.01</td>
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<tr>
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<td>min$^{-1}$</td>
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<td>1.6</td>
</tr>
<tr>
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<tr>
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<td>-</td>
</tr>
<tr>
<td>$V_{ap}$</td>
<td>ml</td>
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