Published in final edited form as:

J Pharm Sci. 2018 April ; 107(4): 1194–1203. doi:10.1016/j.xphs.2017.12.004.

Cellular Pharmacokinetic Model-based Analysis of Genistein, Glyceollin and MK-571 Effects on 5 (and 6)-Carboxy-2',7'-Dichlorofluorescein Disposition in Caco-2 Cells

Callie Drennen¹, Erin Gorse¹, and Robert E Stratford Jr.^{1,*}

¹Duquesne University School of Pharmacy, 600 Forbes Road, Pittsburgh, PA 15282

Abstract

Pharmacokinetic modeling was used to describe 5 (and 6)-Carboxy-2',7'-Dichlorofluorescein (CDF) disposition in Caco-2 cells following CDF or CDFDA (CDF diacetate) dosing. CDF transcellular flux was modeled by simple passive diffusion. CDFDA-dosing models were based on simultaneous fitting of CDF levels in apical, basolateral and intracellular compartments. Predicted CDF efflux was 50% higher across the apical versus the basolateral membrane. This difference was similar following apical and basolateral CDFDA dosing, despite intracellular levels being threefold higher following basolateral dosing, thus supporting non-saturable CDF efflux kinetics. A 3-compartment catenary model with intracellular CDFDA hydrolysis described CDF disposition. This model predicted that apical CDF efflux was not altered in the presence of MK-571, and that basolateral membrane clearance was enhanced to account for reduced intracellular CDF in the presence of this MRP inhibitor. Similar effects were predicted for glyceollin, while genistein exposure had no predicted effects on CDF efflux. These modulator effects are discussed in the context of model predicted intracellular CDF concentrations relative to reports of CDF affinity (measured by K_m) for MRP2 and MRP3. This model-based analysis confirms the complexity of efflux kinetics, and suggests that other transporters may have contributed to CDF efflux.

Introduction

Polyphenols derived from plants are a subset of phytochemicals that continue with high interest to be investigated for their health-promoting effects¹. Curcumin, genistein and resveratrol are three polyphenols that have received the most attention regarding their therapeutic potential in cancer, diabetes, and in cardiovascular and neurodegenerative diseases^{2–5}. Evaluation of their pharmacokinetic properties has revealed they have poor oral bioavailability due to efficient enterocyte extraction via glucuronide and sulfate conjugate formation, and transporter-mediated efflux of both untransformed compound and conjugate metabolites to the intestinal lumen⁶. The important role that intestinal transporters play in

*To whom correspondence should be addressed at stratfordr@duq.edu.

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determining the systemic availability of polyphenols has led to numerous studies to identify the transporters involved, and, through in vitro and in vivo work, has led to a fuller appreciation of the role transporters play not only in the absorption of these chemicals, but also in their downstream pharmacological effects and systemic elimination ⁷. Given that they are substrates for several transporters also important in the disposition of many pharmaceuticals, their potential to alter transporter-mediated drug disposition, especially given that they are available without a prescription, is high and merits increased attention on both research and regulatory fronts ⁸.

Glyceollin is a relatively new polyphenol to emerge with interest in its health-promoting and disease-sparing effects ⁹. Like genistein, it is produced from soy beans, but is referred to as a phytoalexin, being produced only under stressed conditions of plant growth. Preliminary studies conducted in rats demonstrated production of glucuronide and sulfate conjugates following oral administration ¹⁰. A sulfate conjugate was also identified upon exposure to Caco-2 cells ¹¹; thus, like other polyphenols, the possibility that conjugate metabolites would be exported to the lumen via BCRP and/or MRP2, much like genistein ¹² and resveratrol ¹³, is reasonable. Likewise, the potential that these conjugates could alter the efflux of another compound at the apical membrane was also evaluated by co-exposing Caco-2 cells to glyceollin and genistein ¹⁴; these studies demonstrated reduced genistein conjugate efflux in the presence of glyceollin. Companion studies conducted with substrates of BCRP (BODIPY-prazosin) ¹⁵ and MRP2 (CDF) ¹⁶ demonstrated that glyceollin reduced the transport of these substrates, suggesting that this polyphenol may inhibit BCRP and MRP2 function.

Increasingly, pharmacokinetic models describing drug and metabolite concentrations in whole cell in vitro models are recognized as useful tools to advance our understanding of the role that carrier-mediated transport plays in drug disposition, and in the assessment of drug safety and efficacy ^{17–19}. Their usefulness in predicting alteration of intracellular concentrations of victim drugs and their metabolites due to perpetrator drug alteration of transporter-mediated disposition has been demonstrated ^{20–21}. A recent application of modeling to decipher the various transporters involved in resveratrol disposition in HeLa cells demonstrated the usefulness of this approach to quantify MRP4-mediated contributions to overall glucuronide excretion ²². A surprising finding in the aforementioned studies with glyceollin in Caco-2 cells was a decrease in intracellular levels of CDF following exposure to MK-571, a well-recognized inhibitor of MRP2 and MRP3 ²³. Both transporters have been demonstrated to be responsible for CDF efflux, respectively, across the canalicular and serosal membranes of hepatocytes ^{16, 24–25}. Based on mRNA analysis, MRP2 and MRP3 were expressed in Caco-2 cells ^{26–27}; however, a recent proteomic analysis of Caco-2 cells grown on filters for two to four weeks, demonstrated high expression of MRP2, but no detectable expression of MRP3 ²⁸. Observation of increased retention of compounds in the presence of MK-571 has been taken to implicate that they are MRP substrates; such has been the case for Caco-2 ^{22, 27}. Thus, the purpose of the present study was to apply a retrospective modeling analysis of CDF transport data that had been obtained from several experiments conducted in Caco-2 cells grown on filters to determine if such an approach could provide insight regarding the effects of MK-571 (i.e., normalization of basolateral to apical dosing CDF flux ratios from approximately 3 to 0.8, but in the presence of reduced

intracellular CDF for both dosing conditions). Population models that described the time course of CDF amount transported over two hours in the receiver compartment, and terminal donor compartment and intracellular CDF amounts, following apical and basolateral CDFDA dosing were developed to quantify CDF efflux across both apical and basolateral membranes.

Materials and Methods

Conduct of in vitro transport evaluations

Details of the materials used in the conduct of the CDF and CDFDA transport evaluations in the absence versus presence of MK-571, glyceollin and genistein have been described^{14, 29}. Likewise, conduct of the transport studies has also been described in these same two publications, and will be summarized in abbreviated fashion herein.

Caco-2 cells were seeded onto collagen-coated 0.4 μm -PTFE Transwell-Col[®] permeable supports (12 mm/12-well/1.12 cm^2) with 1.2×10^5 cells/mL and cultured for 18 to 24 days before use in a transport evaluation. On the day of a transport experiment, both sides of each Transwell insert were washed twice with 37°C pH 7.4 transport buffer and incubated on both sides for 30 minutes in a CO₂ incubator with either transport buffer containing only the cosolvent system used to prepare MK-571, glyceollin or genistein stocks (final concentration 0.1% dimethyl sulfoxide, 0.1% ethanol) for controls, or transport buffer with 10, 30 or 100 μM of one of the three agents. Following this pre-incubation, a 50 μM stock solution of CDF or CDFDA prepared in 1% dimethyl sulfoxide, 1% ethanol in transport buffer was added to achieve a final concentration of 5 μM CDF or CDFDA on either the apical side or basolateral side, and the 12-well plate placed on an orbital shaker (150 rpm) maintained at 37°C. The volume on the apical side was 0.5 mL, while on the basolateral side it was 1.5 mL. During an experiment, these volumes were held constant by taking a 0.4 mL sample for analysis of CDF concentration from the receiver side (basolateral compartment in an apical dosing experiment, and apical compartment in a basolateral dosing experiment) with immediate equivalent volume replacement. Receiver-side samples were taken every 30 minutes for 120 minutes (four samples). At the end of an incubation, a donor-side sample was taken. Also at 120 minutes, cells on monolayers were washed three times with ice-cold transport buffer (0.5 mL on apical side, and 1.5 mL on basolateral side), and then exposed on the apical side to 0.2 mL of methanol for 15 minutes at room temperature. Subsequently, the methanol was collected, centrifuged for 5 minutes at $2,000 \times g$ and saved for analysis of CDF concentration in Caco-2 cells at 120 minutes.

CDF concentrations in receiver, initial and final donor, and in cells were determined by comparison to CDF standards prepared by serial dilution in transport buffer over the range 7.8 to 1000 nM (separate standards were prepared for controls and a given agent (MK-571, glyceollin or genistein) at the final concentration of that agent used in a transport experiment). CDF measurements were made with a 96-well plate reader set to 485 nm/530 nm for excitation and emission wavelengths, respectively. Rate of CDF appearance ($d\text{CDF}/dt$, pmoles/min) into the receiver compartment was determined by linear regression analysis of the amount of CDF transported versus time between 30 and 120 minutes, thus zero-order, steady-state kinetics with no back diffusion of CDF were applied. In experiments

in which CDF was dosed, CDF clearance across the cells was determined using the following equation, where Cl refers to clearance (mL/min), dCDF/dt is the rate of CDF transport across the monolayer (pmol/min), and C_{donor} (pmoles/mL) is the average of the initial and final donor CDF concentrations.

$$Cl = \frac{dCDF/dt}{C_{donor}} \quad \text{Equation 1}$$

Modeling and simulation of CDF transport

There were five occasions in which CDF was dosed, and 16 occasions in which CDFDA was dosed. The data used to model CDF transport following CDF dosing have not been previously published. The data used to model CDF transport following CDFDA dosing have been published previously^{14, 29}. Each occasion consisted of 12 filters: 6 filters for apical dosing: 3 without (control) versus 3 with a defined concentration of an agent, and 6 filters for basolateral dosing: 3 without versus 3 with an agent. The range of Caco-2 passages used in these experiments was from 32 to 58.

Mass transport kinetic analyses were conducted using Phoenix[®] with NLME[™] 7.0 (Pharsight[®], Certara, L.P., Princeton, NJ, USA). A population approach was used to model CDF transport following either CDF or CDFDA dosing experiments in the AB and BA directions. Population parameter estimates (fixed effects) and between occasion and between filter variability within an occasion (random effects) were estimated using the method of quasi-random parametric expectation maximization (QRPEM), which is similar to SAEM (Stochastic Approximation EM) and IMP (Importance Sampling EM) methods in NONMEM[®]. There were 13 filters each for CDF dosing in the AB and BA directions (26 total filters). These were control filters only (MK-571, glyceollin and genistein were not evaluated when CDF was dosed). There were 48 filters each for apical and basolateral CDFDA dosing (96 total filters) in the absence of MK-571, glyceollin or genestein. For CDFDA dosing in the presence of 100 μ M of either MK-571, glyceollin, or genistein, there were 3 filters on three separate occasions for each agent for a total of 9 filters for apical CDFDA dosing and 9 for basolateral CDFDA dosing. On one occasion, 10 and 30 μ M of a given agent was also evaluated. In this case, there were 3 filters for each concentration and each CDFDA dosing direction. Modeling was conducted separately for each transport direction. In the case of CDF, combined AB and BA directional modeling was also conducted simultaneously since the transport model in this case was a two-compartment model (apical and basolateral compartments) with passive paracellular diffusion. CDF concentrations were not corrected for protein binding, which has been estimated to be between 15 – 20%¹⁶. A proportional error model was used to estimate residual unexplained variability between observed and predicted CDF amounts.

Mass transport of CDF and CDFDA following CDF and CDFDA dosing, respectively, were modeled as first-order kinetic processes. CDFDA transport was assumed to occur by passive diffusion, as has been shown to be the case in hepatocytes^{16, 24}. CDFDA hydrolysis followed by CDF efflux transport across apical and basolateral membranes following

CDFDA dosing were also modeled using first-order kinetics with back diffusion. Although receiver side samples were taken every 30 minutes with volume replacement, in the case of CDFDA dosing, simulations revealed that CDFDA back-diffusion was possible given that > 10% of the CDFDA dose could be transported to the receiver compartment during a two-hour incubation. Thus, back diffusion of CDFDA and CDF were incorporated into the model. In addition, a volume replacement component was incorporated into models in a similar manner to what has been described ³⁰.

The structure of the model used to describe CDFDA and CDF concentrations in apical, cell and basolateral compartments is shown in Figure 1. Differential equations used to model CDFDA and CDF amounts in the various compartments following CDF and CDFDA dosing are summarized in the Appendix. Apical, cell and basolateral compartment volumes were fixed to 0.5 mL, 0.0028 mL and 1.5 mL, respectively. The total cell compartment volume was based on a Caco-2 cell height of 25 μm ³¹ multiplied by the filter surface area (1.12 cm^2). Incorporation of CDFDA hydrolysis in transport buffer was not included in the models as this was experimentally found to be negligible, with a half-life > 100 hours, which translated to a clearance of 0.3×10^{-6} mL/min, at 37°C.

During model development, diffusional CDFDA clearance and hydrolysis were found to be highly correlated, consequently, CDFDA diffusional clearance was fixed in order to estimate CDFDA hydrolytic clearance in the apical and basolateral CDFDA dosing conditions. Thus, CDFDA transport was considered equivalent for both apical and basolateral dosing (passive diffusion) as already noted. The fixed value used for this clearance was determined through a parameter sensitivity analysis conducted in Phoenix using a naïve-pooled modeling approach. This application compared objective functions between models that differed in the value for CDFDA diffusional clearance. Clearance value estimates ranging in order of magnitude increments from 0.0001 to 100 mL/min were first evaluated, and then fine-tuned between 0.001 and 0.01 mL/min. For both dosing cases, diffusional clearances with the smallest objective function centered around 0.004 mL/min, so this was used in subsequent modelling work.

Changes in CDF efflux in the presence of MK-571, glyceollin or genistein were modeled as alterations to apical and/or basolateral membrane CDF efflux clearances according to Equation 2, where IC represents a specific concentration of an agent and β represents a linear adjustment factor estimated in the model. This equation was applied to both apical and basolateral membrane clearances, and either increases ($1 + \beta \times \text{IC}$) or reductions ($1 - \beta \times \text{IC}$) to efflux clearance were evaluated.

$$Cl_{cdf,membrane,IC} = Cl_{cdf,membrane,control} \times (1 \pm \beta \times IC) \quad \text{Equation 2}$$

In the case of modeling genistein effects on CDF transport, incorporation of this agent's observed stimulation of CDFDA hydrolysis ²⁹ was added in a similar manner as agent alterations to CDF efflux clearance by substituting hydrolytic clearance ($Cl_{hydrolysis}$) into

equation 2, and a positive effect on β to indicate genistein's observed enhancement. Models that did not include this stimulatory effect performed poorly.

The process for model development was to first perform a naïve-pooled analysis of the data, followed by incorporation of between occasion random effects, and finally evaluation of both between occasion and between filter random effects. At each stage, model goodness-of-fit was based on precision of the estimates for fixed and random effects, and visual inspection of observed versus predicted CDF amounts, and CWRES (conditional weighted residual) versus time and population predicted plots. These criteria were used to compare the different structural models incorporating the effects of MK-571, glyceollin or genistein (that is, stimulation or reduction of apical and/or basolateral CDF efflux).

Results

Analysis of CDF transport following exposure to CDF

Table 1 provides a summary of model-independent and model predicted CDF clearance across Caco-2 cells following exposure to 5 μ M CDF on five separate occasions. Based on application of Equation 1, model-independent clearance across the cell barrier was similar in the two directions ($p > 0.05$ by a two-tailed unpaired Student's *t*-test), and was smaller than lucifer yellow clearance ($8.7 \pm 0.24 \times 10^{-5}$ mL/min) determined in studies conducted contemporaneously and previously reported¹⁴. The results indicate that CDF transport is by passive diffusion through the paracellular pathway. Application of a population modeling approach based upon a simple two-compartment structure (apical and basolateral compartments) for a given transport direction provided similar clearance estimates to those measured by model-independent means. Simultaneous modeling of AB and BA directions resulted in a single clearance that was estimated with good precision. Goodness-of-fit graphical analyses of the combined model are summarized in Supplemental Figure 1 and provide further support that CDF transport was by passive diffusion following CDF application.

Analysis of CDF transport following exposure to CDFDA

Model-independent analysis—CDFDA was used to deliver CDF intracellularly to evaluate the effect of glyceollin and genistein on transporter-mediated efflux of CDF. Caco-2 cells have been shown to possess the carboxylesterase-1 (hCE-1) enzyme. hCE-1 is expressed in endoplasmic reticulum; thus, it is dispersed in the cytoplasm rather than expressed in the apical or basolateral membranes³². In support of a homogeneous distribution of CDFDA hydrolysis throughout the cell, fractional amounts of CDF in the three compartments were independent of transport direction. On average, there was 1.5 and 1.3% of CDF in the cell compartment at 120-minutes for apical vs. basolateral dosing, respectively. For the basolateral compartment, these values were 1.1 and 1.0%, respectively. The fraction of CDF present in the apical compartment was $4.0 \pm 2.33\%$ (mean $\pm$ standard deviation, $n = 16$ occasions) following apical dosing, which was not statistically different from $1.9 \pm 1.24\%$ in this compartment following basolateral dosing.

MK-571, a non-specific inhibitor of several MRPs^{23, 33} expressed on apical and basolateral membranes, was evaluated as a positive control to gauge glyceollin and genistein effects. Table 2 provides a summary of the effects of all three agents on CDF transcellular flux, and on the amount of CDF in the donor compartment and in cells at 120 minutes. In control experiments, CDF flux and cell content were approximately three times greater following basolateral dosing (mean ratios for flux and cell content were both 2.9). As reported previously¹⁴, CDF flux in the presence of glyceollin was significantly increased following apical dosing, and significantly decreased following basolateral dosing. These alterations were similar to those observed with MK-571. Importantly, these alterations could not be attributed to glyceollin or MK-571 alteration of CDFDA hydrolysis, as evidenced by no change in donor compartment amounts of CDF, and in vitro studies of Caco-2 cell homogenates demonstrating no effect on CDFDA hydrolysis of these two agents at 100 μM ¹⁴. In contrast, CDF flux was significantly enhanced following both apical and basolateral dosing in the presence of genistein, as also previously reported²⁹. For both dosing conditions, glyceollin and MK-571 reduced the amount of CDF in cells measured at the end of the two-hour transport experiment. In contrast, genistein increased cellular CDF following the two dosing conditions.

Model-dependent analysis – Control experiments—A modeling and simulation approach was used to better understand CDF efflux from Caco-2 cells following its intracellular delivery via CDFDA dosing and hydrolysis. Figure 1 summarizes the 3-compartment model structure used. CDFDA and CDF predicted concentration time courses in the three compartments are shown in Figure 2. Simulations are shown for the 16 different occasions, thus reflecting mean predictions for each occasion for the three filters evaluated on a given occasion. This between-occasion variability was higher for CDF than CDFDA, reflecting the added variance associated with the CDF efflux across both apical and basolateral membranes. Following apical dosing, CDFDA concentrations in the donor and cell compartments declined in parallel and to approximately 50% of their initial values. Predicted CDFDA concentrations in the receiver compartment increased throughout the 120-minute incubation, and were eventually > 10% of donor concentrations, thus supporting incorporation of back diffusion into the model despite the use of a volume-replacement sampling approach. After basolateral dosing, CDFDA concentrations in donor and cell compartments also declined in parallel, but less so than observed following apical dosing. CDFDA concentration in the receiver compartment appeared constant from 30 to 120 minutes; these were approximately 10% of donor concentrations and > 10% of cell concentrations. Predicted CDF cell concentrations were approximately 3-fold higher following basolateral CDFDA dosing. CDF concentrations were approximately 5-fold larger than CDFDA cell concentrations following apical dosing, and 10-fold higher following basolateral dosing. CDF concentrations in apical and basolateral compartments increased throughout the 120 minutes and were roughly 2-orders of magnitude lower than CDF cell concentrations in the apical compartment, regardless of site of original CDFDA dosing, and approximately 2 to 3-orders of magnitude lower in the basolateral compartment than cell concentrations for either site of dosing.

Figure 3 demonstrates good agreement between observed and model predicted amounts of CDF in the three compartments for both CDFDA dosing conditions in control experiments. Observed results represent all 48 filters for a given dosing site (16 occasions x 3 filters/experiment), and demonstrate that, for all 3 compartments, between occasion variability was the major contributor to overall variability. Structural parameter estimates for the 3-compartment model under control conditions are summarized in Table 3, and represent analyses that account for between-occasion and within-occasion/between-filter variability. Various graphical summaries of model goodness-of-fit are summarized as Supplemental Figures 2 – 4. A non-parametric bootstrap analysis of 500 simulations with replacement for both transport directions is also summarized in Table 3, and demonstrated good agreement with the model-derived estimates, therefore indicating good accuracy of the final structural parameter estimates. Supplementary Figure 5 presents the results of a visual predictive check (VPC) following 1000 simulations, and shows good agreement between observed CDF amounts in the three compartments with corresponding predicted amounts.

Model-dependent analysis – Co-exposure of CDFDA with MK-571, glyceollin or genistein—Three key efflux transporters in the small intestine and the Caco-2 model that reside on the apical membrane are ABCB1, ABCC2 and ABCG2³⁴. Of the three, CDF is a substrate for only ABCC2^{35–36}. CDF is also a substrate for ABCC3¹⁶ and ABCC5. Model-based analysis of CDF concentrations in the three compartments supported a structure wherein CDF clearance across the apical membrane was not altered in the presence of MK-571, and clearance across the basolateral membrane increased with increasing concentrations of this pan-MRP inhibitor. Estimated enhancement of basolateral membrane clearance (given by the factor β) is summarized in Table 4. Based on Equation 2, predicted basolateral membrane clearance was approximately doubled in the presence of 100 μ M MK-571, regardless of CDFDA dosing location. Model-based analysis results were similar with glyceollin: a model structure with no effect on apical membrane clearance, and an enhancement in clearance across the basolateral membrane of a similar magnitude to that observed with MK-571 provided the best description of the data. In the case of genistein, incorporation of its stimulation of CDFDA hydrolysis that was observed in vitro²⁹ into the model was sufficient to describe the increase in the amounts of CDF in apical, cell and basolateral compartments, that is, models that incorporated stimulatory effects of genistein on apical and/or basolateral membrane clearance resulted in estimates of β with poor precision (CV > 50%), and did not alter visual fits of population or individual filter predicted versus observed CDF amounts in the three compartments. The model estimated β for stimulation of CDFDA hydrolysis is also shown in Table 4. Table 1 in Supporting Information provides a summary of all the estimated parameters for these three agents (MK-571, glyceollin and genistein). Estimates in the presence of these agents were consistent with those presented in Table 3.

Discussion

A modeling and simulation approach was applied to a data set derived from application of CDF and CDFDA to Caco-2 cells for the original purpose of evaluating the effects of glyceollin and genistein on MRP2 and MRP3 mediated transport of CDF in this in vitro

model of the small intestine. Initial work conducted by exposing cells directly to CDF resulted in CDF permeability that was lower than that measured for lucifer yellow and with no evidence of asymmetry in transport. These findings indicated that CDF was passively transported by paracellular diffusion, and, therefore, could not be used to deliver CDF intracellularly to evaluate glyceollin and genistein effects on transporter-mediated efflux. This result stands in contrast with liver perfusion and hepatocyte sandwich-culture approaches that identified Oatp-mediated uptake into rat hepatocytes¹⁶. Subsequently, CDFDA was used to deliver CDF intracellularly to evaluate potential alterations in efflux-mediated transport of CDF. Similar reliance on the high transcellular diffusional transport of the diester prodrug has been applied to understand the complementary role of biliary and sinusoidal membrane efflux of drugs and metabolites in the liver^{16, 24–25, 36–37}. CDFDA was also used in Caco-2 cells to investigate the effects of Vitamin D on MRP2 function³⁸, the effects of phytochemicals in a Caco-2-based efflux inhibition assay^{39–40}, and advocated as a screen for MRP2 interacting compounds in Caco-2 cells⁴¹.

A population modeling approach was used to provide insight regarding a consistent 3-fold asymmetry in CDF transcellular flux observed from several paired apical and basolateral CDFDA dosing occasions (Table 2). The relatively large between-versus within-occasion variability observed has been a consistent observation with the Caco-2 model⁴². For the three estimated parameters, between-occasion variance accounted for > 95% of total variance in estimated $Cl_{hydrolysis}$ and $Cl_{cdf,bl}$, and half of the variance for $Cl_{cdf,ap}$ following apical dosing, and between 80 to 90% of total variance for the three parameters following basolateral dosing. Measurement of CDF in cells at 120 minutes demonstrated a 3-fold higher concentration following basolateral dosing relative to apical dosing (Table 2). For both dosing cases, simulations demonstrated that CDF intracellular concentrations were constant between 30 and 120 minutes (Figure 2), which is attributed to rapid CDFDA diffusion and hydrolysis followed by rate-limiting CDF efflux across both membranes. Thus, the 3-fold larger CDF cell concentration following basolateral dosing was a consequence of the 3-fold larger dose of CDFDA needed to achieve the same CDFDA starting concentration (given the 3-fold larger volume of this compartment). One interpretation of the 3-fold higher flux with basolateral dosing is simply that transcellular clearance of CDF was equivalent for the two dosing sites, as concluded from Equation 1: rate = $Cl \times C$, with C referring to intracellular CDF concentration, and transcellular clearance (Cl_{AB} or Cl_{BA} , for apical and basolateral dosing, respectively) being a composite of the three estimated parameters according to Equations 3a and 3b below, for a given dosing site.

$$Cl_{AB} = \frac{Cl_{hydrolysis} \times Cl_{cdf,bl}}{Cl_{cdf,ap} \times Cl_{cdf,bl}} \quad \text{Equation 3a (apical dosing)}$$

$$Cl_{BA} = \frac{Cl_{hydrolysis} \times Cl_{cdf,ap}}{Cl_{cdf,ap} \times Cl_{cdf,bl}} \quad \text{Equation 3b (basolateral dosing)}$$

These equations are based on those previously defined and used by others to account for multiple transport steps and transport pathways associated with transcellular transport^{43–44}. As in the Sun and Pang case, the equations are simplified by assuming 1) absence of, or equivalent binding/partitioning of CDF to apical, basolateral and intracellular proteins/membranes, and 2) linear (constant) clearance for the three terms. The first assumption is reasonable based on pK_a values of 3.6 and 4.9 for the two carboxylic acid groups⁴¹, indicating it is a dianion at pH 7.4 with expected poor membrane diffusion, and low protein binding in plasma¹⁶. Thus, in order for the two transcellular clearances to be equivalent, $Cl_{hydrolysis}$ would need to be equivalent for the two cases; as well, clearance across both membranes would also need to be equivalent, $Cl_{cdf,ap} = Cl_{cdf,bl}$. However, application of a 3-compartment catenary model consistently revealed an approximately 1.5-fold larger apical membrane clearance following both apical and basolateral dosing (Table 3). Importantly, membrane clearance estimates for a given membrane were similar for the two dosing conditions, which, given the three-fold higher intracellular concentration for basolateral dosing, supports the assumption that carrier-mediated CDF transport across both membranes was linear. While estimates of CDFDA hydrolysis clearance were different for the two dosing conditions (this parameter was 1.8-fold larger for basolateral dosing, Table 3), the two estimates are considered similar. This conclusion is based on the high correlation of this parameter with CDFDA diffusional clearance, the latter which was assumed to be equivalent across both membranes, and was not estimated in order to yield a hydrolytic clearance estimate with good precision. The assumption of equivalent CDFDA diffusional clearance can be questioned given the facile diffusion of CDFDA, which could exacerbate possible apical versus basolateral sided differences in unstirred water layer thickness, and reports of different surface areas for the two membranes⁴⁵. Importantly, fixing hydrolytic clearance for both dosing conditions, using the average of the two estimates shown in Table 3, resulted in inferior apical and basolateral dosing models based on all goodness-of-fit criteria, including that apical and basolateral membrane clearances were poorly estimated and physiologically untenable ($< 1 \times 10^{-10}$ in some cases). In summary, these observations support that apical membrane clearance of CDF was larger than basolateral membrane clearance. Application of Equations 3a–b with the 3-compartment model estimates, resulted in a ratio of basolateral to apical dosing transcellular CDF clearance of 2.9, which agrees with the empirical flux ratio estimate (Table 2).

The transport kinetics of the diester prodrug, adefovir dipivoxil, its monoester metabolite and adefovir in Caco-2 cells were modeled using 3- and 4-compartment catenary model structures³⁰. A 4-compartment model with hydrolysis occurring in the apical membrane as well as intracellularly yielded the smallest residual sum of squares. This model was inspired because of a marked asymmetry in total metabolite production, with nearly 2.7-fold higher average production of metabolites (expressed as % of dose) following apical administration of the prodrug, which the authors suggested was due to preferential apical membrane hydrolysis. For the following reasons, application of a 4-compartment model to describe CDF efflux following CDFDA dosing was not considered reasonable. Total CDF production was on average $6.7 \pm 3.10\%$ and $4.3 \pm 1.21\%$ (mean $\pm$ standard deviation) of the CDFDA dose following apical and basolateral dosing, respectively, which were not statistically different ($p > 0.05$). These CDF productions were much smaller than observed for adefovir

dipivoxil (93% and 34% total metabolite production for apical and basolateral dosing, respectively). Furthermore, the hCE-1 form of human carboxylesterase is expressed in Caco-2³². The enzyme is located in the lumen of the endoplasmic reticulum⁴⁶, thus it is considered an intracellular enzyme. A 3-compartment model is consistent with this subcellular distribution. CDFDA is hydrolyzed in the liver; presumably hCE-1 is involved since this is the more abundant enzyme, and it prefers substrates with large acyl groups and small alcohols, versus the opposite for hCE-2⁴⁶. In contrast, adefovir dipivoxil appears to be a better substrate for hCE-2 (bulky alcohol and small acyl leaving group), and, while hCE-2 has been reported to be poorly expressed in Caco-2³², this may have been sufficient to result in preferential hydrolysis in the apical membrane, consistent with a 4-compartment model. Finally, with regard to the present work, there is no a priori reason to consider that binding/partitioning of either CDFDA or CDF would be different between apical, basolateral and intracellular membranes.

The effects of MK-571 exposure on transcellular flux of CDF (increased following apical dosing and decreased following basolateral dosing) were consistent with preferential inhibition of MRP2. As mentioned, CDFDA has been used to deliver CDF intracellularly to Caco-2 in transcellular⁴¹, and efflux inhibition^{39–40} assays, and to evaluate the functional effects of Vitamin D exposure on presumably MRP2 function in Caco-2³⁸. A surprising finding in the present studies was a reduction in intracellular CDF in the presence of this pan-MRP inhibitor^{23, 33}. Given that CDF is a substrate for several of the MRPs that MK-571 inhibits (MRP2 and MRP3¹⁶, and MRP-5³³), expectation was that intracellular CDF would have increased as a result of inhibition of both apical and basolateral membrane MRP-mediated efflux. This expectation is supported by two studies which showed that MK-571 inhibited MRP2 mediated CDF transport in membrane vesicles prepared from transfected Sf9 cells, with a $K_i < 10 \mu\text{M}$ ^{47–48}. However, pharmacokinetic modeling indicated that MK-571 did not alter apical membrane clearance of CDF, and increased basolateral membrane clearance up to 2-fold in the presence of 100 μM MK-571, the highest concentration evaluated.

With respect to the prediction that MK-571 had no effect on apical membrane CDF efflux, two possibilities are suggested. Transport kinetics of CDF across the canalicular membrane of rat hepatocytes have been demonstrated to be biphasic: there was a high affinity state with an estimated K_m of 3 and 9 μM in Sprague-Dawley and Wistar rats, respectively, and a low affinity state with a K_m of 192 and 69 μM , respectively, in the two strains²⁴. Like the rat hepatocyte findings, others have demonstrated multiple binding sites in MRP2, concluding that either both sites can transport, or that there is an allosteric site^{47, 49–51}. Simulated CDF concentrations we observed averaged 10 and 24 μM following apical and basolateral dosing, respectively. Based on the intracellular CDF concentrations observed in the present studies, it is possible that the low affinity/high capacity form of MRP2 was primarily responsible for CDF efflux across the apical membrane; furthermore, this form may have different inhibition kinetics than the high affinity/low capacity form. Another possibility is that CDF efflux was by another transporter expressed on the apical membrane of Caco-2 cells, as has been suggested for canalicular membrane transport²⁴.

Observed stimulation of CDF efflux across the basolateral membrane in the presence of MK-571 could also be due to allosteric interactions between MK-571 and CDF transport via either or both MRP3 and MRP5. Allosteric interactions regarding MRP3 transport kinetics have been reported⁵². Thus, while MK-571 inhibition of MRPs expressed on the basolateral membrane has been observed²³, including specifically for calcein efflux in Caco-2 cells²⁷, systematic evaluation of its effects on CDF transport have not been examined. Other basolateral membrane transporters could also have participated in CDF efflux to compensate for MRP-mediated/MK-571 inhibited apical and basolateral transport. MRP3 compensates for MRP2 loss of function in perfused livers of Abcc2 (–/–) knockout mice³⁶, and presumably either or both MRP3 and MRP5 compensate in perfused liver from TR[–] (MRP2 genetically deficient) rats^{16, 24}. Alternatively, expression of OSTαβ has been reported in Caco-2^{28, 53}. This basolateral membrane transporter recognizes bile acids and other organic anions, including sulfate conjugate metabolites^{54–55}. CDF may be a substrate for this transporter.

Glyceollin effects on CDF apical and basolateral transport were similar to MK-571 effects. This phytoalexin has been shown to form glucuronide and sulfate conjugates following administration to rats¹⁰, and upon exposure to Caco-2 cells¹¹. As is the case with resveratrol, these conjugate metabolites could be substrates for apical and basolateral membrane transporters. Resveratrol and its conjugate metabolites inhibited MRP2-mediated efflux of bromosulphothalein⁵⁶, and MRP3-mediated transport of estradiol-17-β-glucuronide⁵⁷. Influence of natural products on drug disposition is a developing science⁸, and their effects on efflux transport are inherently complex⁵⁸. Additional studies will be needed to determine the effects of glyceollin and its conjugates on specific apical and basolateral transporters to gain insight regarding its effects on CDF disposition in Caco-2 cells.

Genistein is also conjugated in the intestine and in Caco-2 cells^{59–60}, and these conjugates were shown to be transported across both membranes¹². Genistein has been shown to inhibit MRP2 in TR[–] rats⁶¹. However, model structures that included stimulation of apical and basolateral CDF efflux to account for the observed increase in apical and basolateral compartment CDF in the presence of genistein were not superior to a model that predicted enhancement was a result of genistein's stimulation of CDFDA hydrolysis. Interestingly, probenecid was shown to increase CDFDA hydrolysis in rat hepatocytes¹⁶, and this effect was also taken into account to examine its inhibition of CDF efflux across the serosal membrane.

In summary, a pharmacokinetic model describing CDF disposition in Caco-2 cells has been developed. The model accounts for paracellular diffusion of CDF, intracellular hydrolysis of CDFDA following its transcellular diffusion to deliver CDF to the cytoplasm, and CDF efflux across both apical and basolateral membranes. The model predicts asymmetric efflux across the two membranes, with an approximate 1.5-fold larger apical membrane clearance regardless of apical or basolateral dosing of CDFDA. The model described MK-571 and glyceollin effects that increased basolateral delivery of CDF following apical dosing and reduced apical delivery following basolateral dosing as a stimulation of basolateral membrane CDF efflux with no alteration of transport clearance across the apical membrane.

By predicting intracellular CDF concentrations, the model provides some insight regarding transporter kinetics across the two membranes that help to rationalize the effects of modulators. At the least, the model provides a guide to the design of future experiments that would seek to clarify these observations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

A portion of this work was funded by Grant Number 2G12MD007595 from the National Institutes on Minority Health and Health Disparities (NIMHD), National Institutes of Health (NIH), Department of Health and Human Services (DHHS). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of NIMHD or NIH. The authors have no competing financial interests to declare.

Abbreviations

ABCB1	ATP Binding Cassette Sub-family B, member 1)
ABCC2	ATP Binding Cassette Sub-family C, member 2
ABCC3	ATP Binding Cassette Sub-family C, member 3
ABCG2	ATP Binding Cassette Sub-family G, member 2
AB	apical-to-basolateral
BA	basolateral-to-apical
BCRP	Breast Cancer Resistance Protein
CDF	5 (and 6)-Carboxy-2',7'-Dichlorofluorescein
CDFDA	CDF diacetate
Cl	clearance
MRP	multidrug resistance-associated protein
OSTαβ	heteromeric organic solute transporter

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Appendix

Model equations for CDF bidirectional transport, 3-compartment model

$$\begin{aligned} \text{deriv}(A1\text{apcdf}) &= -(\text{Cl}\text{dcd}\text{f} * \text{Capcd}\text{f}) + (\text{Cl}\text{dcd}\text{f} * \text{Cbldc}\text{f}) \\ \text{deriv}(A1\text{blcdf}) &= (\text{Cl}\text{dcd}\text{f} * \text{Capcd}\text{f}) - (\text{Cl}\text{dcd}\text{f} * \text{Cbldc}\text{f}) \\ \text{Capcd}\text{f} &= A1\text{apcdf} / V_{\text{ap}} \\ \text{Cbldc}\text{f} &= A1\text{blcdf} / V_{\text{bl}}, \end{aligned}$$

The term $\text{deriv}(A1\text{apcdf})$ is the rate of change of CDF in the apical compartment (expressed as pmoles/minute), and $\text{deriv}(A1\text{blcdf})$ is the rate of change of CDF in the basolateral compartment. V refers to volume (mL) in the apical (ap) or basolateral (bl) compartment. $\text{Cl}\text{dcd}\text{f}$ refers to transcellular CDF clearance by passive diffusion from (negative sign) or into (positive sign) a compartment. A1 is the amount of CDF dosed, which was 2500 pmoles for the AB direction of transport, and 7500 pmoles for the BA direction of transport, as calculated from an initial CDF concentration of 5 μM in either direction.

Model equations for CDF transport following CDFDA dosing (application) to the apical compartment, 3-compartment model

$$\begin{aligned} \text{deriv}(A1\text{cdfda}) &= -(\text{Cl}\text{dcd}\text{fda} * \text{Capcd}\text{fda}) + (\text{Cl}\text{dcd}\text{fda} * \text{Ccelcd}\text{fda}) \\ \text{deriv}(A\text{blcdfda}) &= (\text{Cl}\text{dcd}\text{fda} * \text{Ccelcd}\text{fda}) - (\text{Cl}\text{dcd}\text{fda} * \text{Cbldc}\text{fda}) \\ \text{deriv}(A\text{celcd}\text{fda}) &= -(\text{Ccelcd}\text{fda} * \text{Cl}\text{cd}\text{fda}\text{hydrolysis}) + (\text{Cl}\text{dcd}\text{fda} * \text{Capcd}\text{fda}) - 2 * (\text{Cl}\text{dcd}\text{fda} * \text{Ccelcd}\text{fda}) + (\text{Cl}\text{dcd}\text{fda} * \text{Cbldc}\text{fda}) \\ \text{deriv}(A\text{celcd}\text{f}) &= (\text{Ccelcd}\text{fda} * \text{Cl}\text{cd}\text{fda}\text{hydrolysis}) - (\text{Cl}\text{outapcd}\text{f} * \text{Ccelcd}\text{f}) - (\text{Cl}\text{outblcd}\text{f} * \text{Ccelcd}\text{f}) \\ \text{deriv}(A\text{blcdf}) &= (\text{Cl}\text{outblcd}\text{f} * \text{Ccelcd}\text{f}) + (\text{Cl}\text{dcd}\text{f} * \text{Capcd}\text{f}) - (\text{Cl}\text{dcd}\text{f} * \text{Cbldc}\text{f}) \\ \text{deriv}(A\text{apcdf}) &= (\text{Cl}\text{outapcd}\text{f} * \text{Ccelcd}\text{f}) - (\text{Cl}\text{dcd}\text{f} * \text{Capcd}\text{f}) + (\text{Cl}\text{dcd}\text{f} * \text{Cbldc}\text{f}) \\ \text{Cbldc}\text{fda} &= A\text{blcdfda} / V_{\text{bl}} \\ \text{Capcd}\text{fda} &= A1\text{cdfda} / V_{\text{ap}} \\ \text{Ccelcd}\text{fda} &= A\text{celcd}\text{fda} / V_{\text{cell}} \\ \text{Ccelcd}\text{f} &= A\text{celcd}\text{f} / V_{\text{cell}} \\ \text{Capcd}\text{f} &= A\text{apcdf} / V_{\text{ap}} \\ \text{Cbldc}\text{f} &= A\text{blcdf} / V_{\text{bl}} \end{aligned}$$

Amount (A), concentration (C), clearance (Cl) are as defined for CDF with appropriate subscripts. Clcdcfda refers to CDFDA transport by passive diffusion. Clcdcfdahydrolysis refers to CDFDA hydrolysis. Cloutapcdf and Cloutblcdf refer efflux clearance of CDF from the cell to the apical and basolateral compartments, respectively.

Model equations for CDF transport following CDFDA dosing (application) to the basolateral compartment, 3-compartment model

$$\begin{aligned} \text{deriv}(A1\text{cdcfda}) &= -(Cl\text{cdcfda} * C\text{blcdcfda}) + (Cl\text{cdcfda} * C\text{celcdcfda}) \\ \text{deriv}(A\text{apcdcfda}) &= (Cl\text{cdcfda} * C\text{celcdcfda}) - (Cl\text{cdcfda} * C\text{apcdcfda}) \\ \text{deriv}(A\text{celcdcfda}) &= -(C\text{celcdcfda} * Cl\text{cdcfdahydrolysis}) + (Cl\text{cdcfda} * C\text{blcdcfda}) - 2 * (Cl\text{cdcfda} * C\text{celcdcfda}) + (Cl\text{cdcfda} * C\text{apcdcfda}) \\ \text{deriv}(A\text{celcdcf}) &= (C\text{celcdcfda} * Cl\text{cdcfdahydrolysis}) - (Cl\text{outapcdf} * C\text{celcdcf}) - (Cl\text{outblcdf} * C\text{celcdcf}) \\ \text{deriv}(A\text{blcdf}) &= (Cl\text{outblcdf} * C\text{celcdcf}) - (Cl\text{dcdf} * C\text{blcdf}) + (Cl\text{dcdf} * C\text{apcdf}) \\ \text{deriv}(A\text{apcdf}) &= (Cl\text{outapcdf} * C\text{celcdcf}) + (Cl\text{dcdf} * C\text{blcdf}) - (Cl\text{dcdf} * C\text{blcdf}) \end{aligned}$$

Terms are as described for the CDFDA dosing 3-compartment model with apical compartment dosing.

Figure 1.

CDFDA/CDF 3-compartment transport model following CDFDA dosing. The cell on the left represents transport in the absence of MK-571, glyceollin or genistein; whereas, the cell on the right depicts the stimulatory effects of MK-571 and glyceollin on CDF efflux across the basolateral membrane, and genistein stimulation of CDFDA hydrolysis.

Figure 2.

Model predicted concentrations in the various compartments following CDFDA dosing. CDFDA concentrations following apical dosing (A) and basolateral dosing (C), and CDF concentrations following apical dosing (B) and basolateral dosing (D). The different colors used refer to apical compartment (red), cell compartment (blue) and basolateral compartment (green). The different lines for each compartment represent the mean predicted concentration for a dosing occasion.

Figure 3.

Observed (symbols) and model predicted (lines) CDF amounts in control experiments following CDFDA dosing. A–C: apical compartment dosing; D–F: basolateral compartment dosing. A and D: apical compartment; B and E: cell compartment; C and F: basolateral compartment. The different lines in each figure represent the mean predicted CDF amount for a dosing occasion, and each symbol represents an individual filter, of which there were three for each occasion.

Model independent and model predicted CDF clearance (mL/min) across Caco-2 cells following apical (AB transport direction) or basolateral (BA transport direction) exposure to 5 μ M CDF.

Table 1

Model Independent Analysis		Model Based Analysis			
AB	BA	Estimate	AB	BA	AB/BA Combined
Mean (CV,%)	Mean (CV,%)	Population (RSE,%)	1.3×10^{-5} (19.0)	2.0×10^{-5} (10.9)	1.6×10^{-5} (11.7)
1.6×10^{-5} (30.3)	2.1×10^{-5} (3.3)	% BOFV (RSE,%)	45.1 (27.0)	18.0 (17.4)	35.7 (21.3)
		% RUV (RSE,%)	16.4 (20.0); 14.1 (16.5)	23.4 (33.8); 7.1 (12.6)	22.0 (29.2); 12.6 (15.4)

Values from model independent experiments are the mean of 5 separate occasions in both transport directions from n = 2 to 3 filters per occasion for a total of 13 filters for each transport direction. Abbreviations: CV is coefficient of variation obtained from model independent analysis, RSE is the relative standard error of the estimate of the model-based parameter estimate; BOFV is between occasion and filter variability expressed as %; RUV is residual unexplained variability for apical (top) and basolateral (bottom) compartment CDF concentrations, respectively, expressed as %.

Table 2

Measured transcellular CDF flux across Caco-2 monolayers, and CDF amounts in donor compartment and cells at 120 minutes following initial exposure to 5 μ M CDFDA (Control), and with either 100 μ M MK-571, glyceollin or genistein.

Condition	AB directional flux (pmol/min)		BA directional flux (pmol/min)		AB Donor (apical) CDF at 120 minutes (pmoles/well)		BA Donor (basolateral) CDF at 120 minutes (pmoles/well)		AB Intracellular CDF at 120 minutes (pmoles/well)		BA Intracellular CDF at 120 minutes (pmoles/well)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Control	0.28	0.004	0.81	0.018	101	14.5	143	23.2	29	2.2	83	6
MK-571	0.51	0.019	0.39	0.012	115	25.7	249	59.2	15	1.7	26	3.6
Glyceollin	0.63	0.041	0.53	0.041	95	2.8	187	9.9	15	1.3	30	9.4
Genistein	1.25	0.064	2.68	0.052	301	22.8	427	51.0	85	16.4	134	3.1

Flux was calculated from linear regression of the amount of CDF transported versus time from 30 to 120 minutes (zero-order and steady-state kinetic assumption). SEM refers to standard error of the mean. For each transport direction, there were 48 filters in control experiments and 9 filters for each agent. For a given transport direction, flux and cell content were significantly different from control in the presence of all three agents ($p < 0.05$) according to a 1-way ANOVA followed by Tukey's test for multiple comparisons. For a given transport direction, donor amounts in the presence of either MK-571 or glyceollin were not significantly different from control; whereas, donor amounts were different for genistein.

Table 3

Model predicted parameters describing CDFDA transport and hydrolysis, and CDF efflux transport across apical and basolateral membranes following apical or basolateral exposure to 5 μ M CDFDA.

Parameter	Parameter Estimate (% RSE)		Bootstrap Analysis	
	Apical Dosing	Basolateral Dosing	Apical Dosing	Basolateral Dosing
Cl_{cdfda}	0.004 (fixed)	0.004 (fixed)	----	----
$Cl_{hydrolysis}$	8.2×10^{-4} (<0.1)	1.5×10^{-3} (0.1)	1.0×10^{-2} ($0.9 - 1.2 \times 10^{-2}$)	1.6×10^{-3} ($1.2 - 1.9 \times 10^{-3}$)
$Cl_{cdf,ap}$	8.4×10^{-5} (<0.1)	8.5×10^{-5} (0.2)	2.2×10^{-5} ($1.7 - 2.6 \times 10^{-5}$)	8.3×10^{-5} ($7.1 - 10.8 \times 10^{-5}$)
$Cl_{cdf,bl}$	5.6×10^{-5} (0.1)	5.2×10^{-5} (0.2)	1.3×10^{-5} ($1.2 - 1.4 \times 10^{-5}$)	5.1×10^{-5} ($3.8 - 6.7 \times 10^{-5}$)
BOFV _{hydrolysis}	11.0 (20.6)	34.0 (20.4)	7.3 (19.2)	31.4 (33.5)
BOFV _{Cl_{cdf,ap}}	12.2 (20.5)	13.6 (20.6)	2.9 (79.2)	14.5 (8.6)
BOFV _{Cl_{cdf,bl}}	11.2 (21.4)	24.0 (20.4)	7.5 (21.9)	24.4 (41.7)
RUV _{bl} (proportional)	30.2 (13.7)	0.1 (160.0)	0.74 (0.67 – 0.94)	0.06 (0 – 0.21)
RUV _{cell} (proportional)	0.2 (101.1)	0.02 (161.6)	2.0 (0.02 – 2.4)	7.3 (0 – 270)
RUV _{ap} (proportional)	0.01 (99.6)	10.3 (1.5)	7.1 (5.9 – 8.4)	9.4 (3.9 – 18.5)

Results are presented as the population parameter estimates (% RSE is the percent relative standard error of the population estimate). Clearance parameters have units of mL/min. Cl_{cdfda} , diffusional clearance of CDFDA; $Cl_{hydrolysis}$ intracellular hydrolysis of CDFDA; $Cl_{cdf,ap}$, efflux clearance of CDF across the apical membrane; $Cl_{cdf,bl}$, efflux clearance of CDF across the basolateral membrane; BOFV, between occasion and filter variability; RUV, residual unexplained variability. BOFV and RUV results are expressed in percent. Apical, cell and basolateral compartment volumes were fixed to 0.5 mL, 0.0028 mL and 1.5 mL, respectively. Bootstrap analysis parameter estimates are expressed as median (5% and 95% boundaries of the 90% confidence interval), except for BOFV estimates, which are expressed as median (%RSE).

Table 4

Model-derived estimates of β -adjustment factor for enhancement of basolateral membrane clearance by MK-571 and glyceollin, and CDFDA hydrolysis clearance by genistein.

Agent	Apical Dosing Parameter Estimates (% RSE)		Basolateral Dosing Parameter Estimates (% RSE)	
	$Cl_{\text{hydrolysis}}$	$Cl_{\text{cdf,bl}}$	$Cl_{\text{hydrolysis}}$	$Cl_{\text{cdf,bl}}$
MK-571	----	1.7×10^{-2} (<0.1)	----	2.4×10^{-2} (0.4)
Glyceollin	----	2.2×10^{-2} (<0.1)	----	1.8×10^{-2} (2.9)
Genistein	6.9×10^{-3} (<0.1)	----	2.8×10^{-2} (0.6)	----