

Passive Influx and Ion Trapping Are More Relevant to the Cellular Accumulation of Highly Permeable Low-Molecular-Weight Acidic Drugs than Is Organic Anion Transporter 2

Dallas Bednarczyk

Novartis Institutes for BioMedical Research, Cambridge, Massachusetts

Received February 16, 2021; accepted May 17, 2021

ABSTRACT

Recently published work suggests that highly permeable low-molecular-weight (LMW) acidic drugs are transported by organic anion transporter 2 (OAT2). However, an asymmetric distribution of ionizable drugs in subcellular organelles where pH gradients are significant may occur in the presence of an inhibitor relative to its absence (e.g., lysosomal trapping). In the present study, OAT2-mediated transport of highly permeable LMW anions could not be demonstrated using OAT2 transfected cells, despite robust transport of the OAT2 substrate penciclovir. Moreover, a rifamycin SV (RifSV)-dependent reduction in the accumulation of highly permeable LMW anions previously observed in hepatocytes could be qualitatively reproduced using HepG2 cells and also in Madin-Darby canine kidney (MDCK) cells, which lack expression of OAT2. Neither HepG2 nor MDCK cells demonstrated meaningful penciclovir transport, nor was the cellular accumulation of the highly permeable LMW anions sensitive to competitive inhibition by the neutral OAT2 substrate penciclovir. Both cell lines, however, demonstrated sensitivity to the mitochondrial uncoupler *p*-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP) in a manner similar to RifSV. Furthermore, the transepithelial MDCK permeability

of the highly permeable LMW anions was measured in the absence and presence of RifSV and FCCP at concentrations that reduced the cellular accumulation of anions. Neither inhibitor, nor the OAT2 inhibitor ketoprofen, reduced the transepithelial flux of the anions as would be anticipated for transported substrate inhibition. The findings presented here are aligned with cellular accumulation of highly permeable LMW anions being significantly determined by ion trapping sensitive to mitochondrial uncoupling, rather than the result of OAT2-mediated transport.

SIGNIFICANCE STATEMENT

The manuscript illustrates that passive influx and ion trapping are more relevant to the cellular accumulation of highly permeable low-molecular-weight acidic drugs than is the previously proposed mechanism of OAT2-mediated transport. The outcome illustrated here highlights a rare, and perhaps previously not reported, observation of anionic drug trapping in a compartment sensitive to mitochondrial uncoupling (e.g., the mitochondrial matrix) that may be confused for transporter-mediated uptake.

Introduction

Organic anion transporter 2 (OAT2) is a transporter expressed in the liver and kidney, but also has reported mRNA distribution in brain, stomach, ileum, among other tissues (Shen et al., 2017). The transport mechanism (e.g., tertiary active transport, etc.) for OAT2 is currently ill-defined within the literature. Furthermore, the literature has implicated OAT2 in the disposition of drugs (Bi et al., 2019). Correspondingly, there is a desire to investigate OAT2 more thoroughly to determine its role in the disposition and clearance of drugs. Kimoto et al. (2018) investigated the role of OAT2 in the transport and disposition of highly permeable low-molecular-weight (LMW) acidic drugs, and concluded that the class of compounds were OAT2 substrates. However, an alternative explanation

for the apparent OAT2-mediated uptake may be supported by additional considerations.

OAT2-mediated uptake of highly permeable LMW acid drugs may appear to work in practice, but in theory, it should not work. The high passive permeability should minimize the impact of the transporter because of high capacity of the passive inward flux as well as outward leak that would act to equilibrate concentrative transport into the cell. Thus, an apparent discontinuity exists between high passive permeability and asymmetric transporter-mediated uptake into cells in the absence and presence of an inhibitor. Furthermore, it has been noted that cellular accumulation is not necessarily evidence that accumulating drugs are actively transported (Balaz, 2012). To investigate the apparent discontinuity, an analogous observation was considered from the literature. Literature reports observations of lysosomal accumulation of highly permeable bases, like propranolol, which collect in cells by means of pH gradient trapping that can be reduced by the presence of an inhibitor of lysosomal acidification like baflomycin (Bednarczyk and Sanghvi, 2020). Yet, propranolol is highly permeable across a cell monolayer or an artificial membrane system absent transporters (Wohnsland and

This work received no external funding.

No conflict of interest is declared by the author.

<https://doi.org/10.1124/dmd.121.000425>

ABBREVIATIONS: CI, confidence interval; FCCP, *p*-trifluoromethoxy carbonyl cyanide phenyl hydrazone; HBSS, Hanks' balanced salt solution; HEK, human embryonic kidney; LE, low efflux; MDCK, Madin-Darby canine kidney; LMW, low molecular weight; OAT2, organic anion transporter 2; P_{app} , apparent permeability; RifSV, rifamycin SV.

Faller, 2001; Bednarczyk and Sanghvi, 2020). Thus, the observation of asymmetric cellular accumulation of a highly permeable compound in the absence and presence of an inhibitor is not necessarily reflective of transporter-mediated uptake of the substrate, but may be due to a non-specific passive accumulation mechanism like lysosomal trapping and apparent inhibition, where the inhibitor is depleting the pH gradient. When considering a parallel mechanism to lysosomal trapping for highly permeable LMW acidic drugs, a compartment of alkaline pH is necessary. Such an alkaline compartment exists, the mitochondrial matrix, having a pH of ~8 (Llopis et al., 1998; Rossignol et al., 2004). Thus, the examination of a hypothesis considering the nonspecific accumulation of highly permeable acidic drugs in an alkaline compartment as a mechanism for cellular accumulation is possible.

The data presented by Kimoto et al. (2018) may be hypothesized to be a nonspecific effect rather than a transporter-mediated process. The authors reported that multiple highly permeable LMW acidic drugs demonstrated uptake ratios of greater than 1.5-fold in a heterologous expression system. They also leveraged cellular accumulation data from plated human hepatocytes in the absence or presence of a suprapharmacological concentration of rifamycin SV (RifSV) and observed reduced cellular accumulation of the acidic drugs in the presence of RifSV when compared with vehicle control. However, this observation was inconsistent for the drugs tested, where the cellular accumulation of diclofenac, pioglitazone, rosiglitazone, and tolcapone were increased in the presence of RifSV, but when a less specific 4°C condition was substituted as the RifSV condition for these compounds, a reduced cellular accumulation was observed. Moreover, the plated hepatocyte data suggests that molecules like piroxicam and warfarin are inherently poorly permeable in the presence of RifSV, which is inconsistent with parallel artificial membrane permeability assay data where high membrane permeability is observed for piroxicam and warfarin in the absence of transporters (Wohnsland and Faller, 2001). The observations may reflect another mechanism(s) other than OAT2-mediated transport and the RifSV effect in hepatocytes. Questions surrounding the observations merit further evaluation of the impact of OAT2-mediated transport on highly permeable LMW acidic drugs, and whether the RifSV effect is nonspecific.

Presented in this manuscript is data illustrating that the cellular accumulation of highly permeable LMW anionic drugs is not dependent on OAT2-mediated transport, but rather appears to be by a nonspecific mechanism that is sensitive to other acids, like RifSV at high concentrations, and to mitochondrial uncouplers such as *p*-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP). Furthermore, the cellular accumulation of highly permeable LMW anionic drugs appears to be insensitive to competitive inhibition by the neutral OAT2 substrate penciclovir.

Materials and Methods

Cellular Accumulation in HEK-Vector and HEK-OAT2 Cells

Chemicals and Reagents. Penciclovir and gliclazide were purchased from Tokyo Chemical Industry Co. (TCI America, Portland, OR), FCCP was purchased from Enzo (New York, NY), and piroxicam was purchased from Alfa Aesar (Haverhill, MA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or were acquired through the Novartis compound hub at >95% purity. Cell culture media, buffer solutions, and supplements were from Corning (Tewksbury, MA). Chromatography reagents were obtained from JT Baker (Radnor, PA).

Cell Lines and Culture Conditions. Human embryonic kidney (HEK) Flp-In cells were cultured at 37°C under a 5% CO₂ atmosphere at 95% relative humidity in DMEM containing 10% heat-inactivated fetal bovine serum (Corning Life Sciences, Acton, MA), penicillin-streptomycin (100 U/ml), 2 mM Ala-Gln, and nonessential amino acids. HEK-Flp-In cells containing OAT2 (Cheng et al., 2012) also included 100 µg/ml hygromycin in the continuous culture

media, but absent when plated for assay purposes. Cells were passaged twice weekly into an Omnitray (Nunc, Thermo Fisher Scientific, Rochester, NY) at a density of approximately 2,000,000 cells for continuous culture. For assay purposes, cells were seeded at a density of approximately 100,000 cells/well of a (poly)D-lysine-coated 96-well solid support plate (Greiner Bio-One North America Inc., Monroe, NC) in the absence of the selection agent and cultured for a period of two days.

HepG2 cells were cultured at 37°C under a 5% CO₂ atmosphere at 95% relative humidity in DMEM containing 10% heat-inactivated fetal bovine serum, penicillin-streptomycin (100 U/ml), and 2 mM Ala-Gln. Cells were passaged weekly into an Omnitray (Nunc, Thermo Fisher Scientific, Rochester, NY) at a density of approximately 500,000 cells for continuous culture. For assay purposes, cells were seeded at a density of approximately 75,000 cells/well of a 96-well solid support plate (Greiner Bio-One North America Inc., Monroe, NC) and cultured in the media noted above for a period of four days with media being changed on the third day of culture.

MDCK-LE cells are a Madin-Darby canine kidney (MDCK) low efflux (LE) cell line developed internally at Novartis (Dickson et al., 2019). MDCK-LE (MDCK hereafter) cells were cultured at 37°C under a 5% CO₂ atmosphere at 95% relative humidity in DMEM containing 10% heat-inactivated fetal bovine serum, penicillin-streptomycin (100 U/ml), and 2 mM Ala-Gln. Cells were passaged weekly into an Omnitray (Nunc; Thermo Fisher Scientific, Rochester, NY) at a density of approximately 500,000 cells for continuous culture. For assay purposes, cells were seeded at a density of approximately 75,000 cells/well of a 96-well solid support plate (Greiner Bio-One North America Inc., Monroe, NC), or 37,500 cells/well of a 96-well Transwell plate (Corning Life Sciences, Acton, MA), and cultured in the media noted above for a period of four days with media being changed on the third day of culture.

Cellular Accumulation Assay. The assay design was derived from Kimoto et al. (2018) to include a 10-minute preincubation with inhibitor and a 1-minute (HepG2 and MDCK) or 2-minute (HEK) uptake time point. Kimoto et al. (2018) used a 2-minute time point for HEK cellular accumulation in their work and a time range of 0.25–1 minute or 0.5–2 minutes for hepatocyte uptake investigation. Here, the 1-minute time point used for HepG2 and MDCK cellular accumulation captured a time point common to both time ranges (exceptions noted in respective figure legends). To initiate the assay, media was aspirated, and the cells were washed three times with Hanks' balanced salt solution (HBSS) containing 10 mM HEPES (pH 7.4). The cells were then equilibrated in the buffer solution for a period of 10 minutes at 37°C (included vehicle, 1 mM RifSV, 1 mM penciclovir, or 10 µM FCCP where indicated). At time = 0, the equilibration solution was aspirated, and the test compound (5 µM) in the presence of vehicle, 1 mM RifSV, 1 mM penciclovir, or 10 µM FCCP was added to each well. The test compound solution concentration of 5 µM was within the range of concentrations used by Kimoto et al. (2018), of 1 or 10 µM. After incubation with test compound for the indicated time period at 37°C, the assay solution was aspirated, and the cells were washed three times with HBSS containing 10 mM HEPES with an automated plate washer (BioTek, EL405, Winooski, VT). Subsequently, internal standard (glibenclamide in water:methanol, 50:50, v:v; or ganciclovir in water:methanol, 50:50, v:v, when penciclovir was the substrate) was added to each well and allowed to incubate for 10 minutes while shaking at 200 rpm. Standard curves were prepared in matrix. The 96-well plates were then centrifuged for 10 minutes at 4000g at 4°C. Samples from each well were then transferred to a 384-well plate. The 384-well plate was then centrifuged for 10 minutes at 4000g at 4°C. Samples were then subject to liquid chromatography/mass spectrometry analysis (below). Total protein determinations were made using a bicinchoninic acid assay kit with bovine serum albumin as the standard (Pierce Biotechnology, Rockford, IL).

Permeability Assay. The determination of the P_{app} and percent recovery was performed in the A→B direction as previously described (Dickson et al., 2019; Bednarczyk and Sanghvi, 2020). Compound test solutions were prepared in prewarmed HBSS with 10 mM HEPES (pH 7.4) containing vehicle or inhibitor; the final concentration was 10 µM for each permeability test compound. The solution plate was centrifuged for 2 minutes at 4000g prior to use in the assay to pellet any precipitate. To initiate the assay, media was aspirated, and the cells and basal chambers were washed three times with a buffer solution of HBSS containing 10 mM HEPES (pH 7.4). Buffer solution without test compound, but containing vehicle or inhibitor was added to the receiver chamber of the Transwell plate, and the compound test solution containing vehicle or inhibitor was

added to each respective donor well of the Transwell plate (Corning Life Sciences, Acton, MA). At time zero, sample of the donor solution was also sampled for further analysis. To this sample was added an internal standard solution (glibenclamide/ganciclovir in water:acetonitrile, 50:50, v:v). The assay was conducted for a period of 120 minutes at 37°C without shaking. At the time of assay termination, a sample was taken from each donor compartment. Additionally, a sample was taken from each acceptor compartment. To each of these samples was added the internal standard solution. The postassay transepithelial electrical resistance was measured using an EVOM² voltmeter (World Precision Instruments, Sarasota, FL). Concentration curves were prepared in the same matrix. The samples with internal standard were then centrifuged for 10 minutes at 4000g at 4°C. The samples and concentration curve samples were analyzed by liquid chromatography/mass spectrometry as noted below.

Mass Spectroscopy. For bioanalysis, the samples were loaded onto a Phenomenex XB-C18 column (100 × 2.1, 5 µm) or a Phenomenex PS C18 (50 × 2.1, 5 µm) by means of a CTC PAL autosampler (Zwingen, Switzerland). Penciclovir samples were loaded onto a Thermo Hypercarb column (20 × 2.1, 5 µm) because of poor retention on the above listed columns. Chromatography was performed at a flow rate of 0.9 mL/min using a biphasic gradient: 0.1% formic acid in water (A) and acetonitrile:water (99:1; v:v) + 0.1% formic acid (B). Chromatography was executed using the following gradient profile: 0–0.5 minutes, 5% B; 0.5–1.6 minutes, linear gradient to 100% B; 1.6–2.0 minutes, 100% B; 2.0–2.25 minutes, linear gradient to 5% B; and 2.25–2.90 minutes, 5% B. Mass spectroscopy was performed using an AB Sciex API5500 (Sciex, Framingham, MA) equipped with a turbo ion spray source. Compound concentration was calculated from the chromatographic peak area ratio of analyte to internal standard (glibenclamide or ganciclovir when penciclovir was the analyte) using Multiquant software V3.0 (Sciex, Framingham, MA).

Data Analysis. P_{app} values were determined using eq. 1,

$$P_{app} = (V_A / (S \times [D_0])) \times ([A_{120}] / t). \quad (1)$$

Percent recovery values were determined using eq. 2,

$$\% Recovery = 100 \times ([A_{120}] + [D_{120}]) / [D_0], \quad (2)$$

where V_A is the volume of the acceptor (mL), S is the surface area of the membrane, D_0 is the donor solution concentration at $t = 0$, D_{120} is the donor sample concentration at $t = 120$, A_{120} is the acceptor sample concentration at $t = 120$, and $t =$ time (seconds). P_{app} linearity over the 120-minute assay was assumed for the test compounds, because time linearity was observed for reference compounds demonstrating low and high permeability when characterizing the assay.

Cellular accumulation of the OAT2-expressing cells was compared with the corresponding control cells, where the OAT2-mediated transport was determined by subtraction of the substrate accumulation in cells expressing OAT2 from the substrate accumulation in cells expressing OAT2 (e.g., HEK-Flp-In-OAT2 – HEK-Flp-In-Vector = OAT2-mediated transport). S.D. of the OAT2-mediated transport was determined by means of propagation of errors where OAT2 and control cells have independent random error ($\delta z^2 = \delta x^2 + \delta y^2$). Similar analysis was performed for the HepG2 and MDCK data, where the inhibitor-sensitive cellular accumulation was determined by subtraction of the drug accumulation in presence of inhibitor from the drug accumulation in the absence of inhibitor (e.g., vehicle – RifSV = RifSV-sensitive cellular accumulation). S.D. of the inhibitor-sensitive cellular accumulation was determined by means of propagation of errors, where the vector and inhibitor conditions have independent random error ($\delta z^2 = \delta x^2 + \delta y^2$). The difference between the means, or net effect, was plotted as a mean and a 95% confidence interval (CI) (GraphPad Prism, San Diego, CA). Experiments where the 95% CI of the net effect (e.g., vehicle – RifSV) did not include zero, a P value was determined using a two-tailed unpaired t test (GraphPad Prism). In all cases where the 95% CI did not include zero, the P value was < 0.05 .

Results

Cellular Accumulation in HEK-Vector and HEK-OAT2 Cells.

The cellular accumulation of penciclovir, an established OAT2 substrate (Cheng et al., 2012; Hotchkiss et al., 2015; Mathialagan et al., 2018), and a series of highly permeable LMW organic anions previously classified as OAT2 substrates were investigated in HEK-Flp-In and HEK-Flp-In-OAT2 cells in the absence and presence of 1 mM RifSV (with

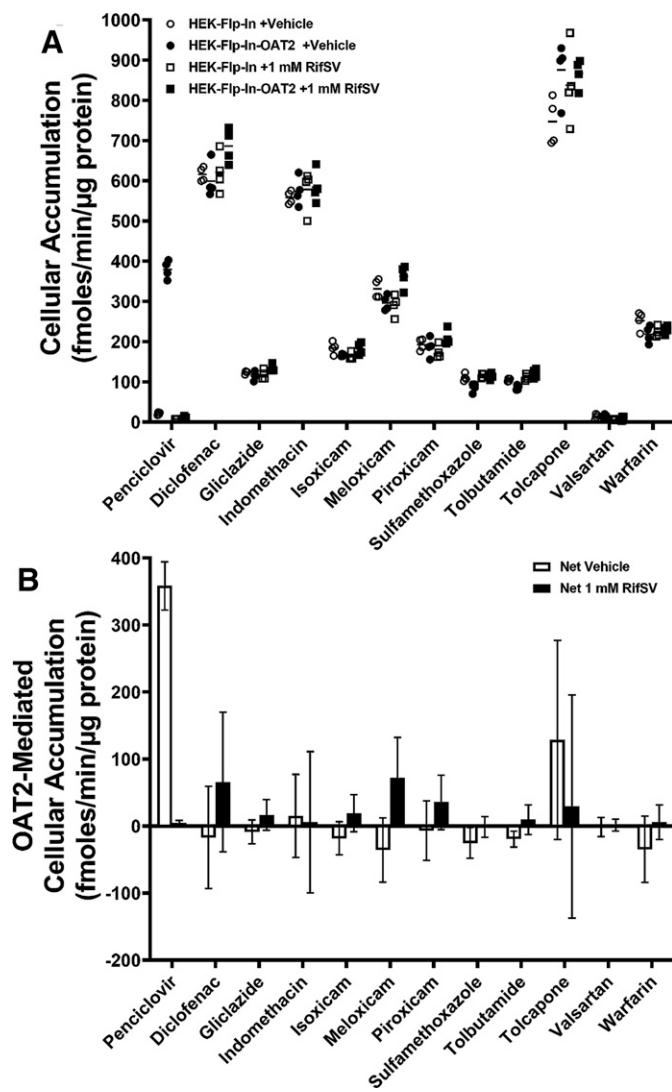


Fig. 1. (A) The 2-minute cellular accumulation of 5 µM compound in HEK-Flp-In or HEK-Flp-In-OAT2 cells in the presence of vehicle or 1 mM RifSV. Cells were preincubated with vehicle or 1 mM RifSV for a period of 10 minutes prior to initiation of the assay. HEK-Flp-In + vehicle (open circles), HEK-Flp-In-OAT2 + vehicle (filled circles), HEK-Flp-In + 1 mM RifSV (open squares), and HEK-Flp-In-OAT2 + 1 mM RifSV (filled squares). Represented is the mean (horizontal line) and four replicates per condition from a single experiment. (B) The OAT2-mediated cellular accumulation (HEK-Flp-In-OAT2 – HEK-Flp-In) in the presence of vehicle (white) or 1 mM RifSV (black). Error bars reflect the 95% CI for the respective compounds and conditions.

10-minute preincubation). Valsartan was included as a poorly permeable anionic-negative control. Penciclovir demonstrated robust OAT2-mediated transport, with an approximately 18-fold difference between the OAT2 and control conditions, with an OAT2-mediated accumulation of approximately 360 fmoles/min/µg protein that was reduced to 4 fmoles/min/µg protein in the presence of 1 mM RifSV (Fig. 1, A and B). By contrast, the HEK-Flp-In-OAT2 accumulation of the highly permeable LMW organic anions previously reported as OAT2 substrates demonstrated no meaningful difference from the control HEK-Flp-In condition in the absence of 1 mM RifSV, where no ratio (OAT2/Vector) exceeded 1.2, with most ranging from 0.9–1.1 (Fig. 1A). Additionally, the presence of 1 mM RifSV did not reduce the cellular accumulation relative to vehicle (Fig. 1, A and B). The lack of difference for the highly permeable LMW organic anions is more apparent in Fig. 1B, where the OAT2-mediated accumulation (OAT2 – Vector) values are

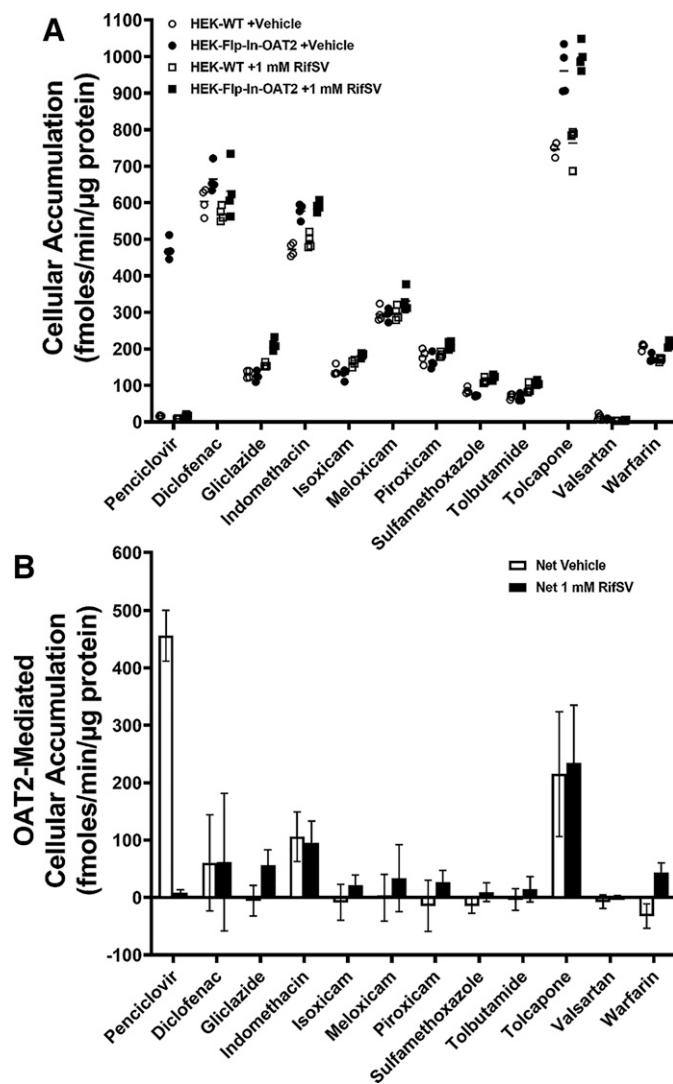


Fig. 2. (A) The 2-minute cellular accumulation of 5 μ M compound in HEK wild-type (WT) or HEK-Flp-In-OAT2 cells in the presence of vehicle or 1 mM RifSV. Cells were preincubated with vehicle or 1 mM RifSV for a period of 10 minutes prior to initiation of the assay. HEK-WT + vehicle (open circles), HEK-Flp-In-OAT2 + vehicle (filled circles), HEK-WT + 1 mM RifSV (open squares), and HEK-Flp-In-OAT2 + 1 mM RifSV (filled squares). Represented is the mean (horizontal line) and four replicates per condition from a single experiment. (B) The OAT2-mediated cellular accumulation (HEK-Flp-In-OAT2 – HEK-WT) in the presence of vehicle (white) or 1 mM RifSV (black). Error bars reflect the 95% CI for the respective compounds and conditions.

uniformly near zero, and the 95% CI ranges include zero. Only the positive control compound, penciclovir, stood out as an OAT2 substrate.

Cellular Accumulation in HEK Wild-Type and HEK-OAT2 Cells. When the OAT2-mediated uptake of highly permeable LMW acidic drugs of Kimoto et al. (2018) could not be reproduced in the HEK-Flp-In and HEK-Flp-In-OAT2 cells, a biologic difference was considered. Cheng et al. (2012) and Hotchkiss et al. (2015) used the HEK-Flp-In as a control for the HEK-Flp-In-OAT2 cells, whereas the Kimoto et al. (2018) publication used the HEK-Flp-In-OAT2 cells of Hotchkiss et al. (2015), but their control condition was wild-type HEK cells rather than the HEK-Flp-In. The HEK-Flp-In-OAT2 cell lines used in this investigation and those of Kimoto et al. (2018) originate from Dr. Ryan Pelis (Cheng et al., 2012; Hotchkiss et al., 2015). To address the possibility of a difference in cell types being responsible for the outcome, the previous investigation was repeated using wild-type HEK cells rather than HEK-Flp-In cells as the control. Again,

penciclovir demonstrated robust OAT2-mediated transport demonstrating a readily apparent difference between the OAT2 and control conditions, 29-fold and approximately 455 fmoles/min/ μ g protein OAT2-mediated accumulation that was reduced to 8 fmoles/min/ μ g protein in the presence of 1 mM RifSV (Fig. 2, A and B). Furthermore, the accumulation of the highly permeable LMW acidic drugs previously reported as OAT2 substrates demonstrated no meaningful difference from the control wild-type HEK cells condition in the absence of 1 mM RifSV, where no ratio (OAT2/Vector) exceeded 1.3 (Fig. 2, A and B). Additionally, the presence of 1 mM RifSV did not reduce the cellular accumulation of the highly permeable anions relative to vehicle (Fig. 2B). Indomethacin and tolcapone appeared to be exceptions, where a net positive difference was observed between the OAT2 cells and the wild-type HEK cells, however, the difference was insensitive to the inhibitor RifSV, indicating the difference was not due to an OAT2-mediated event (Fig. 2B; contrast with the OAT2 substrate penciclovir). The lack of difference for the highly permeable LMW organic anions is more apparent in Fig. 2B, where the OAT2-mediated transport (OAT2 – wild-type) values are uniformly near zero, and the 95% CI ranges include zero.

Cellular Accumulation in HepG2 Cells in the Absence and Presence of Inhibitors. Kimoto et al. (2018) investigated the cellular accumulation of highly permeable LMW organic anions in the absence and presence of 1 mM RifSV using plated human hepatocytes. A parallel investigation was pursued here using the human hepatic derived HepG2 cell line to determine whether the effect of 1 mM RifSV on the cellular accumulation of highly permeable LMW organic anions was unique to human hepatocytes. The experimental protocol was similar to that previously reported, where the HepG2 cells were preincubated with vehicle or 1 mM RifSV for 10 minutes with subsequent coincubation in a 1-minute uptake experiment. The observed data were qualitatively similar to that observed in the plated hepatocytes of Kimoto et al. (2018), where the highly permeable LMW organic anions gliclazide, isoxicam, meloxicam, piroxicam, tolbutamide, and warfarin demonstrated reduced HepG2 cellular accumulation in the presence of 1 mM RifSV yielding ratios (vehicle/RifSV) of 2.3–7.3-fold, and positive RifSV-sensitive drug accumulation (Fig. 3, A and B). By contrast, the much less permeable organic anion, valsartan, demonstrated little change in the cellular accumulation in the presence of RifSV (Fig. 3B). Despite the apparent uptake of the highly permeable anionic drugs, the established OAT2 substrate penciclovir demonstrated very little accumulation in the HepG2 cells, 22 fmoles/min/ μ g protein, reflecting little or no endogenous OAT2 activity in the cell line. In the presence of 1 mM RifSV, the cellular accumulation value fell below the limit of quantification (<9.9 fmoles/min/ μ g protein). Diclofenac and tolcapone demonstrated little RifSV sensitivity, similar to Kimoto et al. (2018), where a 4°C condition was necessary to produce inhibition of hepatocyte uptake of these two molecules.

To explore the specificity of the RifSV effect, an equivalent experiment was conducted using the neutral OAT2 substrate penciclovir as an inhibitor at a concentration of 1 mM, similar to RifSV. The reported K_m and IC_{50} values for penciclovir in OAT2 assays are 284 and 78 μ M, respectively (Cheng et al., 2012; Hotchkiss et al., 2015). In contrast to RifSV, the OAT2 substrate penciclovir was not able to meaningfully reduce the cellular accumulation of any of the highly permeable LMW organic anions, where no reduction of >15% was observed (Fig. 4, A and B). Penciclovir was not used as an inhibitor of penciclovir accumulation, because unlabeled compound was used, and analysis was by mass spectroscopy. The net effect of penciclovir on the HepG2 cellular accumulation of the highly permeable LMW organic anions can be further observed in Fig. 4B, where the penciclovir-sensitive cellular

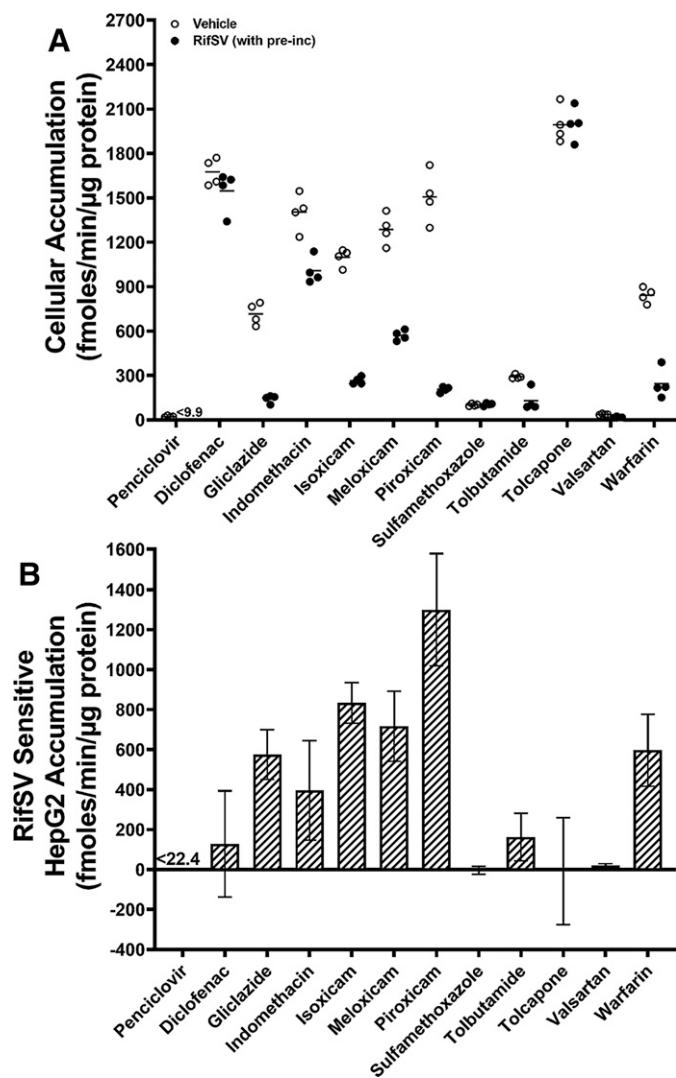


Fig. 3. (A) The 1-minute cellular accumulation of 5 µM compound in HepG2 cells in the presence of vehicle or 1 mM RifSV. Cells were preincubated (pre-inc) with vehicle or 1 mM RifSV for a period of 10 minutes prior to initiation of the assay. HepG2 + vehicle (open circles) and HepG2 + 1 mM RifSV (filled circles). Represented is the mean (horizontal line) and four replicates per condition from a single experiment. The penciclovir accumulation in the presence of RifSV fell below the limit of quantification (<9.9 fmol/min/µg protein) (B) The RifSV-sensitive HepG2 cellular accumulation (vehicle – RifSV). Error bars reflect the 95% CI for the respective compounds and conditions. Where the 95% CI of the RifSV-sensitive cellular accumulation did not include zero, a *P* value was determined using a two-tailed unpaired *t* test. In all cases where the 95% CI did not include zero, the *P* value was <0.05.

accumulation (vehicle – penciclovir condition) values were uniformly near zero, and the 95% CI ranges frequently included zero.

When the neutral OAT2 substrate penciclovir failed to reduce the cellular accumulation of the highly permeable LMW organic anions in a manner similar to RifSV by means of a presumed OAT2-mediated mechanism, a nonspecific mechanism of ion trapping was considered. To investigate the potential of a nonspecific mechanism to influence the cellular accumulation of highly permeable LMW organic anions, 10 µM FCCP, an acid and mitochondrial uncoupler, was used in place of RifSV. Illustrated in Fig. 5, A and B, inhibition with FCCP yielded a result qualitatively similar to RifSV (Fig. 3, A and B), where the highly permeable LMW organic anions, gliclazide, indomethacin, isoxicam, meloxicam, piroxicam, tolbutamide, tolcapone, and warfarin achieved FCCP-sensitive accumulation (vehicle – FCCP) meaningfully greater

than zero, where the 95% CI did not include the value zero (Fig. 5B). Vehicle/FCCP ratios for the same compound ranged from 1.6–10.6. No meaningful net effect was observed for penciclovir or valsartan accumulation in the MDCK cells in the absence and presence of 10 µM FCCP.

Cellular Accumulation in MDCK Cells in the Absence and Presence of Inhibitors. Because of the specificity concerns raised in the experiments using HepG2 cells, a cell line shown not to express OAT2, MDCK (Hotchkiss et al., 2015), was leveraged for further investigation. To investigate whether the effect of preincubation with 1 mM RifSV on the cellular accumulation of highly permeable LMW acidic drugs was specific to human hepatocytes or more generally liver-derived cells, the cellular accumulation was tested in MDCK cells. The experimental protocol was the same as that described for HepG2 cells above. The data were qualitatively similar to that observed for the

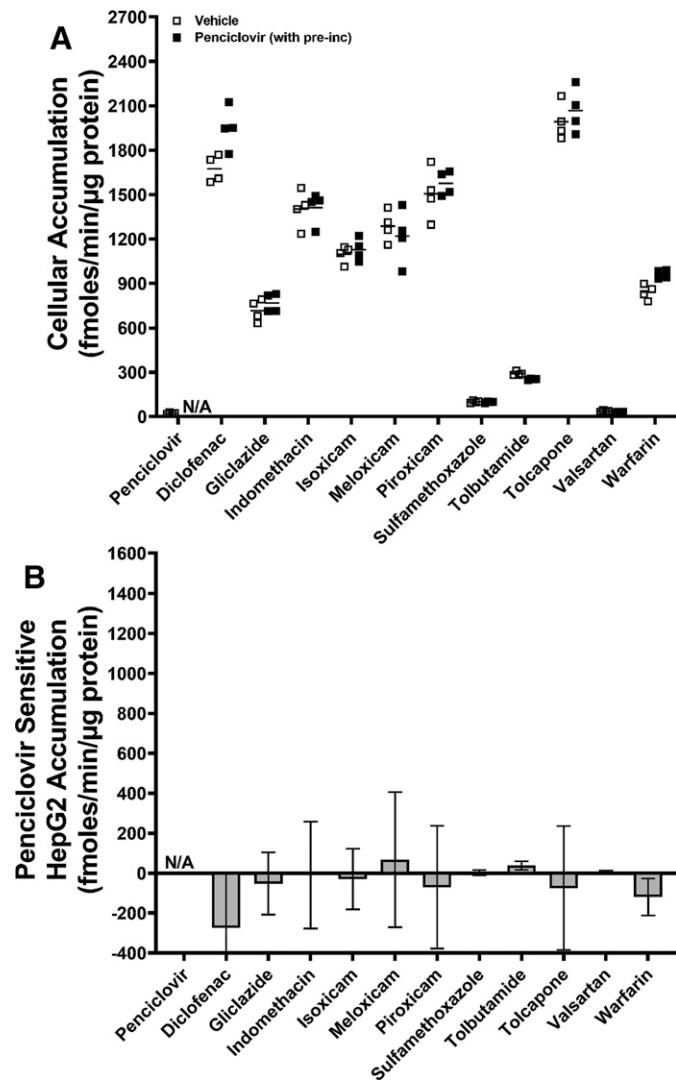


Fig. 4. (A) The 1-minute cellular accumulation of 5 µM compound in HepG2 cells in the presence of vehicle or 1 mM penciclovir. Cells were preincubated (pre-inc) with vehicle or 1 mM penciclovir for a period of 10 minutes prior to initiation of the assay. HepG2 + vehicle (open squares) and HepG2 + 1 mM penciclovir (filled squares). Represented is the mean (horizontal line) and four replicates per condition from a single experiment. Penciclovir was not used as an inhibitor of penciclovir transport. (B) The penciclovir-sensitive HepG2 cellular accumulation (vehicle – penciclovir). Error bars reflect the 95% CI for the respective compounds and conditions. Where the 95% CI of the penciclovir-sensitive cellular accumulation did not include zero, a *P* value was determined using a two-tailed unpaired *t* test. In all cases where the 95% CI did not include zero, the *P* value was <0.05.

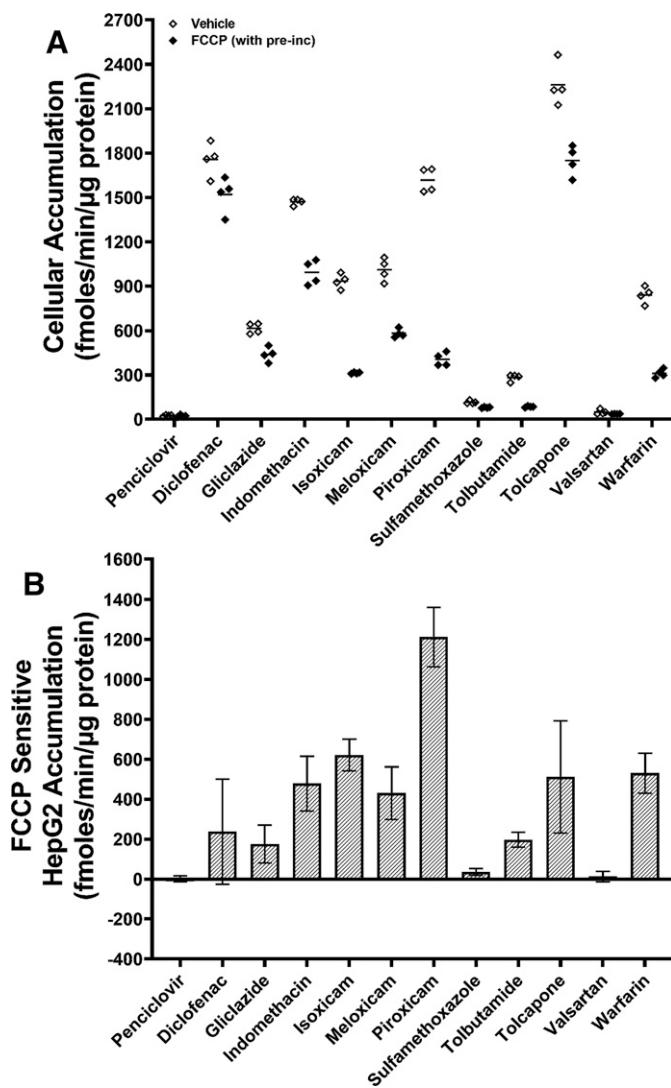


Fig. 5. (A) The 1-minute cellular accumulation of 5 µM compound in HepG2 cells in the presence of vehicle or 10 µM FCCP. Cells were preincubated (pre-inc) with vehicle or 10 µM FCCP for a period of 10 minutes prior to initiation of the assay. HepG2 + vehicle (open diamonds) and HepG2 + 10 µM FCCP (filled diamonds). Represented is the mean (horizontal line) and four replicates per condition from a single experiment. (B) The FCCP-sensitive HepG2 cellular accumulation (vehicle – FCCP). Error bars reflect the 95% CI for the respective compounds and conditions. Where the 95% CI of the FCCP-sensitive cellular accumulation did not include zero, a *P* value was determined using a two-tailed unpaired *t* test. In all cases where the 95% CI did not include zero, the *P* value was < 0.05.

HepG2 cells, where highly permeable LMW organic anions diclofenac, gliclazide, indomethacin, isoxicam, meloxicam, piroxicam, tolbutamide, tolcapone, and warfarin demonstrated a reduced MDCK cellular accumulation in the presence of 1 mM RifSV by 42%–92% (vehicle/RifSV ratios 1.7–13.0) (Fig. 6, A and B versus 3A and 3B). By contrast, the much less permeable compound, valsartan, demonstrated no appreciable shift in the cellular accumulation in the presence of RifSV, nor did the established OAT2 substrate penciclovir (Fig. 6B). Similar to the HepG2 cells, the MDCK cells appeared to transport the highly permeable anionic drugs in the absence of transport of the OAT2 substrate penciclovir.

The OAT2 substrate penciclovir was also used in the MDCK cells as an inhibitor. Similar to what was observed in the HepG2 cells, the OAT2 substrate penciclovir was not able to meaningfully reduce the

cellular accumulation of any of the highly permeable LMW organic anions in MDCK cells, where no reduction of >27% was observed with most vehicle/penciclovir ratios ranging between 0.8 and 1.2 (Fig. 7, A and B). The net effect of penciclovir on the MDCK cellular accumulation of the highly permeable LMW organic anions can be observed in Fig. 7B, where the penciclovir-sensitive (vehicle – penciclovir condition) values were frequently near zero, and the 95% CI ranges included zero in the majority of cases.

The mitochondrial uncoupler, FCCP, was also tested in the MDCK cells. Illustrated in Fig. 8, A and B, FCCP yielded a result qualitatively similar to that of RifSV in MDCK cells (Fig. 6, A and B) and HepG2 cells (Fig. 5, A and B). The highly permeable LMW organic anions, such as diclofenac, gliclazide, isoxicam, meloxicam, piroxicam, tolbutamide, tolcapone, and warfarin demonstrated meaningful cellular

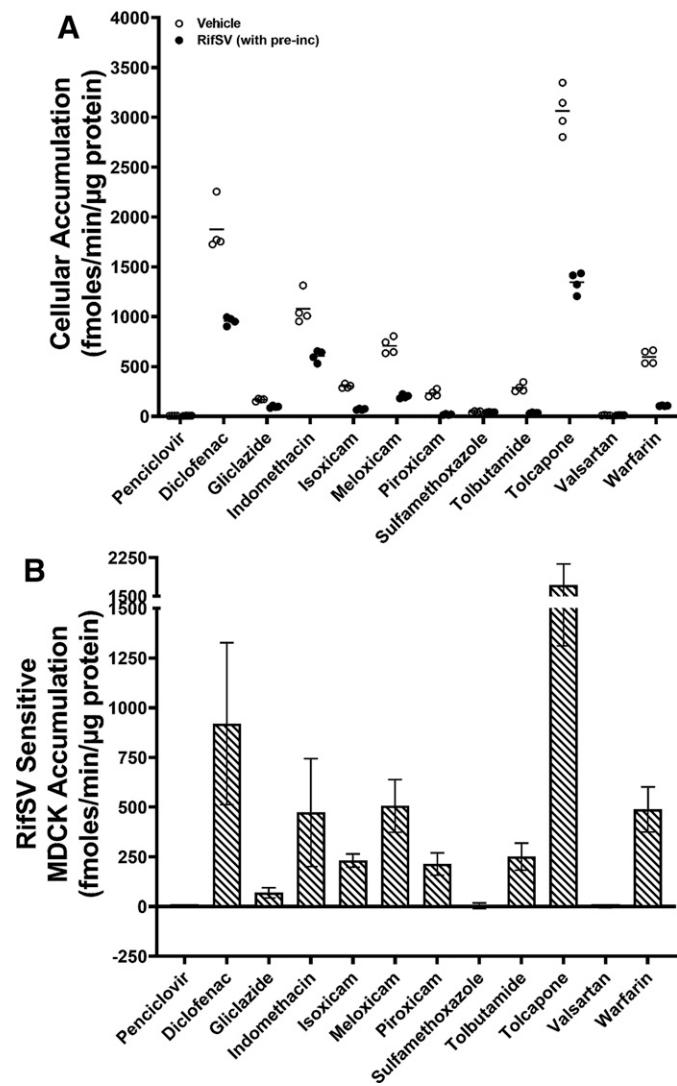


Fig. 6. (A) The 1-minute cellular accumulation of 5 µM compound in MDCK cells in the presence of vehicle or 1 mM RifSV. A 2-minute cellular accumulation was used for penciclovir and valsartan to achieve quantifiable levels of accumulation. Cells were preincubated (pre-inc) with vehicle or 1 mM RifSV for a period of 10 minutes prior to initiation of the assay. MDCK + vehicle (open circles) and MDCK + 1 mM RifSV (filled circles). Represented is the mean (horizontal line) and four replicates per condition from a single experiment. (B) The RifSV-sensitive MDCK cellular accumulation (vehicle – RifSV). Error bars reflect the 95% CI for the respective compounds and conditions. Where the 95% CI of the RifSV-sensitive cellular accumulation did not include zero, a *P* value was determined using a two-tailed unpaired *t* test. In all cases where the 95% CI did not include zero, the *P* value was < 0.05.

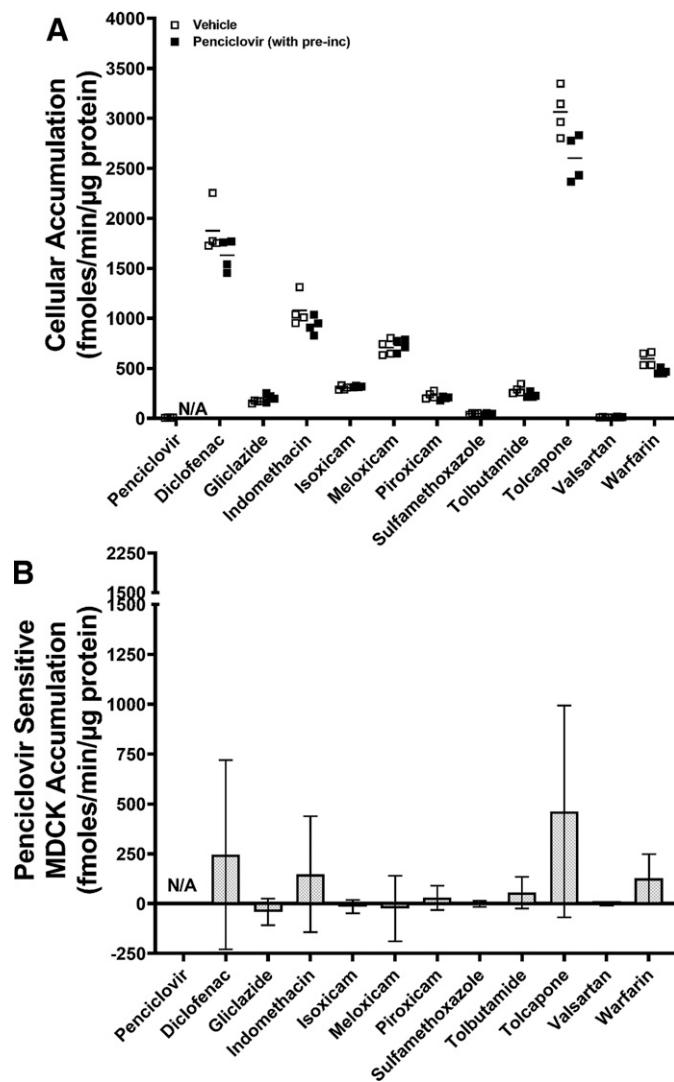


Fig. 7. (A) The 1-minute cellular accumulation of 5 μ M compound in MDCK cells in the presence of vehicle or 1 mM penciclovir. A 2-minute cellular accumulation was used for penciclovir and valsartan to achieve quantifiable levels of accumulation. Cells were preincubated (pre-inc) with vehicle or 1 mM penciclovir for a period of 10 minutes prior to initiation of the assay. MDCK + vehicle (open squares) and MDCK + 1 mM penciclovir (filled squares). Represented is the mean (horizontal line) and four replicates per condition from a single experiment. Penciclovir was not used as an inhibitor of penciclovir transport. (B) The penciclovir-sensitive MDCK cellular accumulation (vehicle – penciclovir). Error bars reflect the 95% CI for the respective compounds and conditions. The 95% CI of the penciclovir-sensitive cellular accumulation included zero in all cases.

accumulation shifts between the absence and presence of FCCP (Fig. 8B). No meaningful net effect was observed for neutral molecule penciclovir in the MDCK cells in the absence and presence of 10 μ M FCCP.

Permeability in MDCK Monolayers in the Absence and Presence of Inhibitors The MDCK cells are unique in that they are capable of forming monolayers and have utility for transepithelial permeability measurement. The observation of a RifSV-sensitive accumulation of highly permeable organic anions in the MDCK cells raised the question, are the highly permeable acidic drugs highly permeable because they are transported across a monolayer? To better distinguish the impact of RifSV on permeability or nonspecific cellular accumulation, a subset of compounds, penciclovir, piroxicam, tolbutamide, and warfarin, were used to investigate the apparent permeability in the absence or

presence of RifSV, ketoprofen, and FCCP. Ketoprofen, a highly permeable LMW acid, has been suggested as a relatively selective inhibitor of OAT2 (Mathialagan et al., 2018). The substrates were selected based on either a large observed net effect in the presence of RifSV and FCCP or low cellular accumulation in the presence of inhibitor (e.g., approaching that of penciclovir, Figs. 6 and 8). Table 1 illustrates that the transepithelial permeability of piroxicam, tolbutamide, and warfarin were not substantially reduced by any of the inhibitory conditions in the monolayer assay. The presence of RifSV, ketoprofen, or FCCP did not appear to diminish the intrinsic permeability of highly permeable LMW acidic drugs across a monolayer with observed reductions <10%–20% and S.D. values often overlapping with that of the vehicle control. Concentrations of ketoprofen and RifSV were limited to 100 μ M for the analysis of

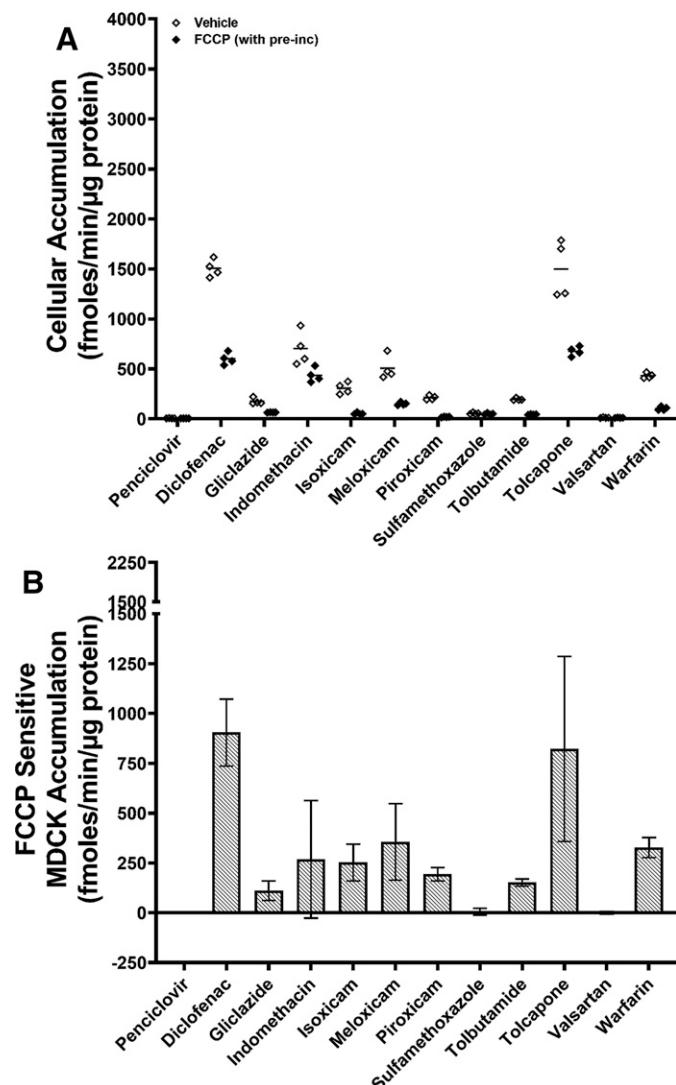


Fig. 8. (A) The 1-minute cellular accumulation of 5 μ M compound in MDCK cells in the presence of vehicle or 10 μ M FCCP. A 2-minute cellular accumulation was used for penciclovir and valsartan to achieve quantifiable levels of accumulation. Cells were preincubated (pre-inc) with vehicle or 10 μ M FCCP for a period of 10 minutes prior to initiation of the assay. MDCK + vehicle (open diamonds) and MDCK + 10 μ M FCCP (filled diamonds). Represented is the mean (horizontal line) and four replicates per condition from a single experiment. (B) The FCCP-sensitive MDCK cellular accumulation (vehicle – FCCP). Error bars reflect the 95% CI for the respective compounds and conditions. Where the 95% CI of the FCCP-sensitive cellular accumulation did not include zero, a P value was determined using a two-tailed unpaired t test. In all cases where the 95% CI did not include zero, the P value was < 0.05 .

TABLE 1

Compound apparent permeability (P_{app}) across MDCK monolayer in the absence and presence of inhibitor. Represented is the mean of three replicates per condition from a single experiment. Recovery reflects the percent recovery in the assay for the respective compounds.

| Compound | Inhibitor | P_{app} | | Recovery | |
|-------------|------------------------|----------------------------|------|----------|------|
| | | Mean $\times 10^6$ cm/s | S.D. | Mean % | S.D. |
| Penciclovir | Vehicle (1% DMSO) | 0.71 | 0.36 | 100 | 10.4 |
| Penciclovir | 100 μ M ketoprofen | 0.43 | 0.08 | 97 | 5.4 |
| Penciclovir | 10 μ M FCCP | 0.70 | 0.31 | 96 | 4.8 |
| Penciclovir | 100 μ M RifSV | 0.55 | 0.36 | 96 | 4.1 |
| Piroxicam | Vehicle (1% DMSO) | 19.1 | 5.2 | 100 | 18.7 |
| Piroxicam | 100 μ M ketoprofen | 18.4 | 0.7 | 88 | 2.7 |
| Piroxicam | 10 μ M FCCP | 16.1 | 2.6 | 89 | 14.7 |
| Piroxicam | 100 μ M RifSV | 16.2 | 0.8 | 87 | 1.5 |
| Tolbutamide | Vehicle (1% DMSO) | 14.7 | 0.3 | 84 | 0.8 |
| Tolbutamide | 100 μ M ketoprofen | 17.4 | 1.3 | 93 | 3.1 |
| Tolbutamide | 10 μ M FCCP | 16.2 | 1.8 | 90 | 6.3 |
| Tolbutamide | 100 μ M RifSV | 16.4 | 1.9 | 91 | 3.9 |
| Warfarin | Vehicle (1% DMSO) | 20.9 | 2.4 | 95 | 4.8 |
| Warfarin | 100 μ M ketoprofen | 18.7 | 2.1 | 91 | 4.6 |
| Warfarin | 10 μ M FCCP | 17.6 | 1.9 | 89 | 5.0 |
| Warfarin | 100 μ M RifSV | 20.8 | 2.2 | 101 | 3.2 |

inhibition, because higher concentrations, 300 μ M (ketoprofen and RifSV) and 1000 μ M (RifSV), resulted in compromised monolayer integrity in an independent experiment.

Discussion

The cellular accumulation of highly permeable LMW acidic drugs was suggested to be mediated by OAT2 (Kimoto et al., 2018). The OAT2-mediated transport of highly permeable anionic drugs was primarily derived from observations of asymmetric accumulation between HEK-Flp-In-OAT2 cells and wild-type HEK cells and the use of a non-specific inhibitor, RifSV, with plated hepatocytes at suprapharmacological concentrations. In the current investigation, an asymmetric accumulation of highly permeable LMW acid drugs between OAT2-expressing cells and two separate HEK control cell lines could not be demonstrated in the absence or presence of RifSV despite robust transport and inhibition of the OAT2 substrate penciclovir (Figs. 1 and 2). Additionally, the present study illustrates that the RifSV effect observed in plated hepatocytes can be qualitatively reproduced in the human HepG2 cell line and in MDCK cells (Figs. 3 and 6) which lack expression of OAT2 as well as functional uptake of OAT2 substrates (Lepist et al., 2014; Hotchkiss et al., 2015). Moreover, the HepG2 and MDCK cells demonstrated no meaningful RifSV-sensitive penciclovir transport (Figs. 5 and 7). Furthermore, neither RifSV or FCCP, nor ketoprofen, reduced the transepithelial flux of the acidic drugs as would be anticipated for transported compounds in the presence of an inhibitor (Table 1).

The findings presented are aligned with the cellular accumulation of highly permeable organic anions being significantly determined by ion trapping in mitochondria rather than OAT2-mediated transport. Ion trapping of nondrug organic anions in mitochondria was previously observed (Rashid and Horobin, 1991). The process of organic anion trapping in mitochondria is analogous to the ion trap effect that underlies weak base accumulation in lysosomes, but the compound charge is opposite, and the mitochondrial matrix is alkaline (pH ~8) (Llopis et al., 1998; Rossigno et al., 2004; Trapp and Horobin, 2005; Balaz, 2012). The ion trapping effect of organic acids in mitochondria has been modeled, where the results illustrated that ion trapping yields a positive shift in total cellular accumulation (Trapp and Horobin, 2005; Scott et al., 2017). The physicochemical properties of

mitochondriotropics, molecules showing greater propensity for mitochondrial accumulation, have been considered in the literature (Trapp and Horobin, 2005; Horobin et al., 2007; Scott et al., 2017). The properties of nonspecific accumulation in mitochondria are expectedly broad. However, for weak acids, the general properties identified are log P values of 0–5, pK_a values distributed between 4 and 8 with peak accumulation at pK_a ~6, and sufficient permeability to access the mitochondrial matrix. The molecules examined here were highly permeable, so mitochondrial access would not be anticipated to be an issue. Similarly, the log P values of the molecules are moderate reflecting the low molecular weights (250–350) and high permeability values. Extensive membrane partitioning might be anticipated for very lipophilic molecules and poor permeability/lack of mitochondrial penetration for very hydrophilic molecules. The pK_a values also fall within the anticipated range of 4–8, where meloxicam (pK_a = 4.1) and diclofenac (4.2) represented molecules with the lowest pK_a values, and warfarin (5.9) and piroxicam (6.3) represented molecules with the highest pK_a values. The apparent mitochondrial accumulation was not extensively correlated with pK_a, so other factors like polar surface area or charge delocalization (e.g., carboxylic acid versus acyl sulfonamide) may also play a role.

Mitochondrial uncouplers like FCCP collapse the pH gradient across the inner mitochondrial membrane, correspondingly collapsing the driving force for organic acid accumulation in the mitochondrial matrix (Llopis et al., 1998). This mechanism can be applied to two permeable organic acids, where the application of one permeable acid would limit the mitochondrial matrix accumulation of another acid, appearing as transport inhibition. This is analogous to what has been observed for bases in lysosomes and is the foundation of lysosomal trapping assays, where a basic test compound is used to inhibit the accumulation of a probe base (Kazmi et al., 2013; Burger et al., 2015). The apparent saturability of the process can be explained similarly, where increasing concentrations of a highly permeable LMW acidic drug would decrease the mitochondrial matrix pH diminishing the driving force for accumulation and thus appearing to saturate the process. Again, an analogous process occurs for lysosomal trapping (Burger et al., 2015). Consistent with this, nonsteroidal anti-inflammatory drugs as a class demonstrate mitochondrial uncoupling activity that would diminish the driving force with increased accumulation of the nonsteroidal anti-inflammatory drug as either an inhibitor or substrate (Mahmud et al., 1996; Moreno-Sánchez et al., 1999; Nadanaciva et al., 2013). Furthermore, a structural RifSV analog that is also a monovalent acid, 3-formyl rifamycin SV, has demonstrated uncoupling in liver mitochondria (Inouye et al., 1977). Thus, there is a very probable mechanism where permeable anionic drugs passively diffuse into the mitochondrial matrix and are subject to ion trapping that can be inhibited with other anionic drugs, or more generally compounds that produce mitochondrial uncoupling through pH gradient disruption.

The mitochondrial ion trapping mechanism is supported by the observations presented here, where FCCP and RifSV reduced cellular accumulation of the highly permeable anionic drugs in HepG2 and MDCK cells. MDCK cells which do not show OAT2 expression or function (Lepist et al., 2014; Hotchkiss et al., 2015) demonstrated FCCP and RifSV sensitivity to the accumulation of the highly permeable anionic drugs, but the drug accumulation was insensitive to the presence of the neutral OAT2 substrate penciclovir (Figs. 6–8). The MDCK permeability assay offered an orthogonal test system to investigate the apparent discontinuity, where the anionic drugs are highly permeable in an MDCK monolayer assay but appear to require a transporter for cellular accumulation. If the high monolayer permeability was due to transport, flux across the monolayer should have been subject to inhibition by the same molecules that limited cellular accumulation, FCCP and RifSV, or the reported OAT2 inhibitor ketoprofen (Mathialagan et al., 2018).

However, the presence of FCCP, RifSV, or ketoprofen did not limit flux of the highly permeable acids across the MDCK monolayer; suggesting a transporter independent mechanism of permeability (Table 1). By contrast, penciclovir demonstrated poor permeability in the absence or presence of the inhibitors. Although it could be argued that a 1000- μ M concentration of RifSV was necessary to yield an inhibitory effect, FCCP did not substantially reduce the MDCK permeability of any of the compounds despite showing a similar response to 1000 μ M RifSV when assessed for cellular accumulation (Figs. 6 and 8). The inability to inhibit the transepithelial flux of the anionic drugs combined with the ability to inhibit the cellular accumulation of the same compounds grown on solid supports suggests a mechanism where high passive permeability facilitates entry into alkaline compartment followed by trapping of the ionized substituent; compounds capable of reducing the pH gradient can limit trapping. However, because the cellular entry and exit of the compounds is not transporter dependent, the same inhibitors cannot limit the passive flux of the highly permeable compounds across a cell monolayer.

The cellular accumulation data of the HepG2 and MDCK cells were qualitatively similar to that of Kimoto et al. (2018), but the OAT2 transfectant data could not be reproduced here. The experimental set up was quite similar, so uncertainties may arise because of small apparent differences in procedure. One notable difference in cell culture between previous work and this work was their supplementation of 1% pyruvate in the cell culture media when seeding for transport. The pyruvate supplementation results in media pyruvate concentrations >90-fold what is typically found in DMEM. It has been shown that the transient addition of lactate and pyruvate decrease mitochondrial matrix pH (Llopis et al., 1998). Furthermore, the energy substrate used in culture, of which pyruvate is one, has been shown to impact mitochondrial function, such as steady-state pH (Rossignol et al., 2004). It is unknown whether the pyruvate supplementation affects mitochondria or results in a differential compensatory mechanism in the HEK-Flp-In-OAT2 transfectants versus the HEK wild-type cells used in the previous work, but it may represent an opportunity for investigation. Culture conditions or cell type may also play a role in the lack of an RifSV effect observed here in the HEK cells. The passive accumulation of the highly permeable organic anions in the absence of inhibitor in HepG2 cells was frequently greater than that of the HEK cells used here. However, in the presence of RifSV or FCCP, the HepG2 total cellular accumulation of the highly permeable anionic drugs approached HEK cells, which were not impacted by the inhibitors. This further suggests that the effect is not OAT2-mediated accumulation, but is nonspecific. It perhaps reflects that the conditions used in the current study for the HEK cell culture result in a reduced pH gradient or a smaller alkaline compartment (e.g., fewer mitochondria) within the HEK cells relative to the HepG2 cells.

In some cases, in the HepG2 and MDCK cells, a lack of apparent mitochondrial accumulation was observed. Ion trapping requires a pH difference between cytoplasm and the mitochondrial matrix. The absolute pH of the respective compartments or the pH gradient between compartments may not be uniform between cell types; the membrane potential may differ as well. These differences may account for some of the dissimilarities in the cellular accumulation between various cell types. It may also influence the cellular accumulation response to RifSV and FCCP between cell lines as well. Diclofenac and tolcapone accumulation in the HepG2 cells showed little response to RifSV in this investigation. Perhaps the compartmental pH values or the corresponding gradient are less optimal for diclofenac and tolcapone accumulation and reduction by RifSV in HepG2 cells. Alternatively, Kimoto et al. (2018) observed a stimulation of diclofenac and tolcapone accumulation in hepatocytes in the presence of RifSV, but a

reduction at 4°C. Perhaps here the stimulatory mechanism of RifSV was marginal in HepG2 cells and offset by a reduction in mitochondrial trapping resulting in little net effect. Poor accessibility to the compartment may also play a role. For instance, sulfamethoxazole demonstrated very little accumulation in the HepG2 and MDCK cells at the 1-minute time point and may have insufficient exposure to the mitochondrial matrix to yield a robust response to RifSV or FCCP.

The possibility of an alternative cellular accumulation mechanism may exist. Lysosomal trapping is unlikely because of the low compartment pH that would not support the ionization and trapping of acids. Nonspecific binding to protein is another potential mechanism that cannot be readily dismissed. Protein binding of acidic drugs is well established, and RifSV and FCCP may be able to displace the binding of the highly permeable organic anions within cells. The Kimoto et al. (2018) work in hepatocytes would not appear to support nonspecific binding, where the authors observed a temperature-sensitive decrease in cellular accumulation for diclofenac, pioglitazone, rosiglitazone, and tolcapone. The nonspecific binding would be anticipated to be less sensitive to temperature than a compartmentalization mechanism. Nonspecific accumulation due to membrane potential represents another alternative mechanism, but the presence of FCCP causes a depolarization of the membrane that would favor anion accumulation rather than appear to inhibit it.

Recent reports of highly permeable LMW acidic drugs have concentrated on OAT2-mediated transport while not providing sufficient attention to compound physicochemical properties, subcellular pH gradients, and inhibitors of poor specificity (Bi et al., 2018a,b, 2019; Kimoto et al., 2018). The work presented here suggests that the cellular accumulation of highly permeable anionic drugs is not OAT2 dependent, but rather subject to ion trapping that is sensitive to mitochondrial uncoupling. The apparent inhibition is not primarily involved with transmembrane transport of the compound of interest, but rather secondary to its passive flux and subsequent ion trapping. In conclusion, no observable support for the OAT2-mediated transport of highly permeable anionic drugs could be identified in the current work. The cellular accumulation of highly permeable anionic drugs appeared to be passive and dependent on ion trapping in an alkaline compartment consistent with the mitochondrial matrix.

Acknowledgments

The author would like to thank Dr. Stephen Wright at the University of Arizona for his critical review of the manuscript.

Authorship Contributions

Participated in research design: Bednarczyk

Conducted experiments: Bednarczyk

Performed data analysis: Bednarczyk

Wrote or contributed to the writing of the manuscript: Bednarczyk

References

- Balaz S (2012) Does transbilayer diffusion have a role in membrane transport of drugs? *Drug Discov Today* 17:1079–1087.
- Bednarczyk D and Sanghvi MV (2020) The impact of assay recovery on the apparent permeability, a function of lysosomal trapping. *Xenobiotica* 50:753–760.
- Bi YA, Costales C, Mathialagan S, West M, Eatemadpour S, Lazzaro S, Tylaska L, Scialis R, Zhang H, Umland J, et al. (2019) Quantitative contribution of six major transporters to the hepatic uptake of drugs: “SLC-phenotyping” using primary human hepatocytes. *J Pharmacol Exp Ther* 370:72–83.
- Bi YA, Lin J, Mathialagan S, Tylaska L, Callegari E, Rodrigues AD, and Varma MVS (2018a) Role of hepatic organic anion transporter 2 in the pharmacokinetics of R- and S-warfarin: in vitro studies and mechanistic evaluation. *Mol Pharm* 15:1284–1295.
- Bi YA, Mathialagan S, Tylaska L, Fu M, Keefer J, Vildhede A, Costales C, Rodrigues AD, and Varma MVS (2018b) Organic anion transporter 2 mediates hepatic uptake of tolbutamide, a CYP2C9 probe drug. *J Pharmacol Exp Ther* 364:390–398.

- Burger H, den Dekker AT, Segeletz S, Boersma AW, de Brujin P, Debiec-Rychter M, Taguchi T, Sleijfer S, Sparreboom A, Mathijssen RH, et al. (2015) Lysosomal sequestration determines intracellular imatinib levels. *Mol Pharmacol* **88**:477–487.
- Cheng Y, Vapurcuyan A, Shahidullah M, Aleksunes LM, and Pelis RM (2012) Expression of organic anion transporter 2 in the human kidney and its potential role in the tubular secretion of guanine-containing antiviral drugs. *Drug Metab Dispos* **40**:617–624.
- Dickson CJ, Hornak V, Bednarczyk D, and Duca JS (2019) Using membrane partitioning simulations to predict permeability of forty-nine drug-like molecules. *J Chem Inf Model* **59**:236–244.
- Horobin RW, Trapp S, and Weissig V (2007) Mitochondriotropics: a review of their mode of action, and their applications for drug and DNA delivery to mammalian mitochondria. *J Control Release* **121**:125–136.
- Hotchkiss AG, Barrigan L, and Pelis RM (2015) Organic anion transporter 2 transcript variant 1 shows broad ligand selectivity when expressed in multiple cell lines. *Front Pharmacol* **6**:216.
- Inouye B, Uchinomi Y, Wachi T, and Utsumi K (1977) Effect of rifampicin derivatives on the ion compartmentation of biological membranes. *J Antibiot (Tokyo)* **30**:494–499.
- Kazmi F, Hensley T, Pope C, Funk RS, Loewen GJ, Buckley DB, and Parkinson A (2013) Lysosomal sequestration (trapping) of lipophilic amine (cationic amphiphilic) drugs in immortalized human hepatocytes (Fa2N-4 cells). *Drug Metab Dispos* **41**:897–905.
- Kimoto E, Mathialagan S, Tylaska L, Niosi M, Lin J, Carlo AA, Tess DA, and Varma MVS (2018) Organic anion transporter 2-mediated hepatic uptake contributes to the clearance of high-permeability-low-molecular-weight acid and zwitterion drugs: evaluation using 25 drugs. *J Pharmacol Exp Ther* **367**:322–334.
- Lepist EI, Zhang X, Hao J, Huang J, Kosaka A, Birkus G, Murray BP, Bannister R, Cihlar T, Huang Y, et al. (2014) Contribution of the organic anion transporter OAT2 to the renal active tubular secretion of creatinine and mechanism for serum creatinine elevations caused by cobicistat. *Kidney Int* **86**:350–357.
- Llopis J, McCaffery JM, Miyawaki A, Farquhar MG, and Tsien RY (1998) Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. *Proc Natl Acad Sci USA* **95**:6803–6808.
- Mahmud T, Rafi SS, Scott DL, Wrigglesworth JM, and Bjarnason I (1996) Nonsteroidal anti-inflammatory drugs and uncoupling of mitochondrial oxidative phosphorylation. *Arthritis Rheum* **39**:1998–2003.
- Mathialagan S, Costales C, Tylaska L, Kimoto E, Vildhede A, Johnson J, Johnson N, Sarashina T, Hashizume K, Isringhausen CD, et al. (2018) In vitro studies with two human organic anion transporters: OAT2 and OAT7. *Xenobiotica* **48**:1037–1049.
- Moreno-Sánchez R, Bravo C, Vásquez C, Ayala G, Silveira LH, and Martínez-Lavín M (1999) Inhibition and uncoupling of oxidative phosphorylation by nonsteroidal anti-inflammatory drugs: study in mitochondria, submitochondrial particles, cells, and whole heart. *Biochem Pharmacol* **57**:743–752.
- Nadanaciva S, Aleo MD, Strock CJ, Stedman DB, Wang H, and Will Y (2013) Toxicity assessments of nonsteroidal anti-inflammatory drugs in isolated mitochondria, rat hepatocytes, and zebrafish show good concordance across chemical classes. *Toxicol Appl Pharmacol* **272**:272–280.
- Rashid F and Horobin RW (1991) Accumulation of fluorescent non-cationic probes in mitochondria of cultured cells: observations, a proposed mechanism, and some implications. *J Microsc* **163**:233–241.
- Rossignol R, Gilkerson R, Aggeler R, Yamagata K, Remington SJ, and Capaldi RA (2004) Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells. *Cancer Res* **64**:985–993.
- Scott DO, Ghosh A, Di L, and Maurer TS (2017) Passive drug permeation through membranes and cellular distribution. *Pharmacol Res* **117**:94–102.
- Shen H, Lai Y, and Rodrigues AD (2017) Organic anion transporter 2: an enigmatic human solute carrier. *Drug Metab Dispos* **45**:228–236.
- Trapp S and Horobin RW (2005) A predictive model for the selective accumulation of chemicals in tumor cells. *Eur Biophys J* **34**:959–966.
- Wohnsland F and Faller B (2001) High-throughput permeability pH profile and high-throughput alkane/water log P with artificial membranes. *J Med Chem* **44**:923–930.

Address correspondence to: Dr. Dallas Bednarczyk, Novartis Institutes for BioMedical Research, 250 Massachusetts Ave., Cambridge, MA 02139. E-mail: dallas.bednarczyk@novartis.com