Toward a Unified Model of Passive Drug Permeation II: The Physiochemical Determinants of Unbound Tissue Distribution with Applications to the Design of Hepatoselective Glucokinase Activators

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Abstract

In this work, we leverage a mathematical model of the underlying physiochemical properties of tissues and physicochemical properties of molecules to support the development of hepatoselective glucokinase activators. Passive distribution is modeled via a Fick-Nernst-Planck approach, using in vitro experimental data to estimate the permeability of both ionized and neutral species. The model accounts for pH and electrochemical potential across cellular membranes, ionization according to Henderson-Hasselbalch, and passive permeation of the neutral species using Fick’s law, and passive permeation of the ionized species using the Nernst-Planck equation. The mathematical model of the physiochemical system allows derivation of a single set of parameters governing the distribution of drug molecules across multiple conditions both in vitro and in vivo. A case study using this approach in the development of hepatoselective glucokinase activators via organic anion-transporting polypeptide–mediated hepatic uptake and impaired passive distribution to the pancreas is described. The results for these molecules indicate the permeability penalty of the ionized form is offset by its relative abundance, leading to passive pancreatic exclusion according to the Nernst-Planck extension of Fickian passive permeation. Generally, this model serves as a useful construct for drug discovery scientists to understand subcellular exposure of acids or bases using specific physiochemical properties.

Introduction

The glucokinase enzyme, which facilitates the phosphorylation of glucose to glucose-6-phosphate, is a key regulator of glucose homeostasis through its actions in the liver, pancreas, gut, and brain. Of these tissues, the physiologic role of glucokinase in the liver and pancreas is most well understood. In hepatocytes, glucokinase determines the rate of hepatic glucose uptake and plays a key role in glycogen synthesis and the regulation of hepatic glucose production. In pancreatic β cells, glucokinase regulates glucose-stimulated insulin secretion, whereby insulin secretion in response to glucose is amplified. Because of these important physiologic roles, there has been considerable interest in the development of glucokinase activators for the treatment of type 2 diabetes mellitus. Much of this effort has followed from the identification of small molecule activators that reduce the S0.5 (e.g., Km of glucose) and increase the Vmax of glucokinase through interactions at an allosteric site (Grimsby et al., 2003). Although initially such glucokinase activators have demonstrated robust glucose lowering in early clinical trials, the incidence of mechanism-related hypoglycemia has posed severe challenges to the development of these agents (Matschinsky, 2013). This challenge has led to a variety of approaches to maintain efficacy while obviating hypoglycemic risk (Matschinsky, 2009, 2013; Pfefferkorn et al., 2012a,b). From a chemical design perspective, two such approaches have been described previously, namely: 1) the design of partial activators and 2) the design of activators that preferentially distribute to the liver. Efforts related to the latter approach have provided the first oral hepatoselective glucokinase activators, PF-049 [(5S)-6-(3-cyclopentyl-2-(4-(trifluoromethyl)-1H-imidazol-1-yl)-3-cyclopentylpropanamido) nicotinic acid] (Pfefferkorn et al., 2012b) and PF-051 (6-[(2S)-2-(4-(cyclobutanesulfonyl)-1H-imidazol-1-yl)-3-cyclopentylpropanamido] pyridine-3-carboxylic acid) (Stevens et al., 2013) (Fig. 1). Following continuous intravenous infusion in rats over 4 days, unbound liver concentrations of PF-049 and PF-051 are 5-fold greater than unbound plasma concentration (Stevens et al., 2013).

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In the same study, substantial exclusion from the pancreas and muscle was observed with unbound tissue-to-plasma ratios ($K_{pu}$) of $\leq 0.17$. The net effect of liver uptake and pancreatic exclusion provides liver-to-pancreas selectivity of greater than 60-fold (Fig. 2).

On a mechanistic level, the substantial hepatic uptake of this activator was achieved by selecting molecules with affinity for active hepatic uptake via organic anion-transporting polypeptide (OATP) 1B1 and OATP1B3. However, the mechanisms underpinning the observed distributional impairment to the pancreas and muscle are less obvious. The possibility of active efflux from both the pancreas and muscle is highly unlikely. In addition, although both compounds have relatively poor permeability at physiologic pH (transcellular permeability $\approx 1.5 \times 10^{-6}$ cm/s), this attribute alone cannot be used in a first-principles explanation of distributional impairment to a nonclearing organ at equilibrium according to Fick’s first law of diffusion. A perhaps more relevant characteristic of these compounds is their low $pK_a$ (~3.5), which indicates that the molecules will largely exist in the ionized form (~99.98%).Unlike neutral molecules, the diffusive flux of charged molecules is driven by both entropic factors (e.g., concentration gradient) and enthalpic factors via the electrical potential (e.g., membrane voltage). This membrane voltage, which is maintained by ion channels and transporters in virtually all eukaryotic cells, confers a relative negative voltage to the cell interior as well as the interior of various organelles. As such, this aspect of cell physiology could conceivably contribute to the observed pancreatic and muscle exclusion of carboxylic acids that exist almost exclusively in the anionic form at physiologic pH. Conversely, the electrophoretically driven concentrative uptake of cations has been extensively applied to the imaging of tumor cells, therapeutic targeting to tumor mitochondria (Modica-Napolitano and Singh, 2002; Hickey et al., 2008), and even the measurement of membrane potential (Hockings and Rogers, 1996).

In this work, we have characterized the pH-dependent permeability of PF-049 and PF-051 in a variety of standard in vitro cell systems.

**Fig. 1.** Chemical structure of PF-049 (A) and PF051 (B) is shown. Note the similar local structural motif around the carboxylic acid groups (shown in brackets).

**Fig. 2.** Steady state unbound tissue-to-plasma exposure of PF-049 and PF-051 determined in rats post-4-day infusion. Error bars represent the S.D. in the in vivo unbound tissue/plasma ratios determined within rats (n = 3). Unbound exposures were calculated using mean unbound fractions. For PF-051, $f_{plasma}$, $f_{pancreas}$, $f_{liver}$, and $f_{muscle}$ were 0.38, 0.17, 0.17, and 0.41 (%CV $\leq 10$), respectively. For PF-049, $f_{plasma}$, $f_{pancreas}$, $f_{liver}$, and $f_{muscle}$ were 0.096, 0.11, 0.07, and 0.19 (%CV $< 16$), respectively.
using a mathematical model of cellular distribution similar to that previously reported for the prediction of tumor cell accumulation of lipophilic bases (Trapp and Horobin, 2005). The model accounts for ionization (according to Henderson-Hasselbalch), passive permeation of neutral molecules (according to Fick’s law), and passive permeation of ionized molecules across polarized cell membranes (according to the Nernst-Planck equation). The model also accounts for the physiologic values relevant to the cell systems employed in this work. The resulting analysis provides a first-principles explanation for the in vivo distributional impairment that is observed not only with regard to the pancreas, but also with regard to muscle.

Materials and Methods

Reagents. The hepatoselective glucokinase activators PF-049 (Pfefferkorn et al., 2012b) and PF-051 (Stevens et al., 2013) (Fig. 1) were used for all the experimental work listed below.

Experimental Evaluation of pH-Dependent Distribution in Wild-Type and OATP-Transfected Human Embryonic Kidney 293 Cells. Human embryonic kidney 293 (HEK-293), HEK-293/OATP1B1, and HEK-293/OATP1B3 cells were seeded at a density of 5.6 × 10^5 cells/well on 48-well poly-D-lysine-coated plates and cultured for 72 hours. For the uptake assay, the cells were washed three times with uptake buffer at 37°C (Hanks’ balanced salt solution with 20 mM HEPES, pH 7.4) and allowed to equilibrate to uptake buffer at either pH 6.0 or 7.4 for 15 minutes. After the equilibration period, buffer was discarded and the cells were incubated for 5 minutes with 200 μl uptake buffer at either pH 6.0 or 7.4 containing 1 μM compound on a heated plate shaker set to 37°C and 150 rpm. The assay was run at pH 6.0 and 7.4, allowing one to maintain cell viability while varying the ionization fraction of the compound in the media. Cellular uptake was terminated by quickly washing the cells four times on ice with 200 μl ice-cold uptake buffer. The cells were then lysed with 200 μl proprietary internal standard (mol. wt. 686) solution in methanol, and the lysate was injected onto a liquid chromatography tandem mass spectrometry (LC-MS/MS) system. The total cellular protein content was determined by using a Pierce bicinchoninic acid protein assay kit, according to the manufacturer’s specifications. Model equations for the intracellular uptake of the compound are described by eq. 5. The relevant physiologic parameters for HEK-293 cells are given in Table 1.

Bioanalysis. LC-MS/MS analysis was conducted on a Sciex Triple Quad 4000 mass spectrometer (turbospray ionization source) with a

<table>
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<th>Nucleus</th>
<th>Lysosome</th>
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Single mitochondrial surface area (SA): 1.19 × 10^{-7} cm^2
Single mitochondrial volume: 2.26 fl (used to estimate number of mitochondria)
Nuclear SA: (estimate from fractional volume and spherical symmetry)
Single lysosomal volume: 0.5 fl
Single lysosomal SA: (estimate from spherical symmetry)
Shimadzu LC-10 high-performance liquid chromatography system and Gilson 215 autosampler. The mass spectrometer was controlled by Analyst 1.4.2 software. The liquid chromatography method consisted of a step gradient with 25 μl samples loaded onto a 1.5 × 5 mm Shodex Ken GmbH (ODP 13 μm particle size column using 95% ammonium acetate buffer (2 mM), 2.5% methanol, and 2.5% acetonitrile. Samples were eluted with 10% ammonium acetate buffer (2 mM), 45% methanol, and 45% acetonitrile. The detection was in positive mode with monitored transitions: 397.1→261.1 (PF-049), 447.2→261.3 (PF-051), 472.3→436.2 (terfenadine, internal standard), and 687.0→320 (CP-628374, internal standard). Peak area counts of analyte compound and internal standard were integrated using DiscoveryQuant Analyze as an add-on to Analyst 1.4.2.

Mathematical Model of Drug Distribution into Wild-Type and OATP-Transfected HEK-293 Cells. A model similar to that published previously by Trapp and Horobin is used to characterize the distribution of PF-049 and PF-051 in wild-type (WT) and OATP-transfected HEK-293 cells (Trapp and Horobin, 2005; Trapp et al., 2008). Key components of the model are illustrated in Fig. 3. The physiologic parameters for HEK-293 cells are taken from the literature (www.sigmaaldrich.com/content/dam/sigmaaldrich/docs/Sigma/General_Information/1/r022.pdf; Søgaard et al., 2001) (Table 1). The associated mathematics are described below. Although the model is applied to acidic molecules, the derived equations are described both for monoprotic acids and bases to facilitate more generalized application.

The Henderson-Hasselbalch relationship is used to calculate the concentration of the ionized and neutral species based on total concentration, pH of the molecules, and compartmental pH. Equations 1 and 2 describe this calculation for acids and bases, respectively:

\[
[A^-] = C \frac{10^{pK_a}}{10^{pH} + 10^{pK_a}} \quad \text{and} \quad [HA] = C \frac{10^{pK_a}}{10^{pH} + 10^{pK_a}}
\]

where \(C\) is the total concentration of the drug molecule, \([A^-]\) and \([HA]\) are the concentrations of the ionized species of the acid and base, respectively. Flux across each membrane and equilibrium distributions are estimated using the previously described Fick’s Nernst-Planck framework for compounds that are highly ionized (i.e., compounds with \(pK_a\) significantly different from physiologic pH) (Ghosh et al., 2014). In this relationship, permeation of the ionized charge can contribute to the total flux even if the ionic permeability is (as expected) much smaller than the permeability of the neutral species. The flux equations across a single membrane corresponding to acids and bases are as follows:

\[
J_{acid} = P_n \cdot SA \cdot \left( [HA]_0 - [HA]_1 \right) - P_e \cdot SA \cdot \frac{z \Delta \phi F}{RT} \left( [A^-]_1 e^{-z\Delta \phi F / RT} - [A^-]_0 \right)
\]

\[
J_{base} = P_n \cdot SA \cdot \left( [B]_0 - [B]_1 \right) - P_e \cdot SA \cdot \frac{z \Delta \phi F}{RT} \left( [BH^+]_1 e^{z\Delta \phi F / RT} - [BH^+]_0 \right)
\]

where \([HA]_0\) and \([HA]_1\) \(([B]_0\) and \([B]_1\)) are the neutral concentrations of an acid (base) on either side of the membrane, \([A^-]_0\) \(([BH^+]_0)\) and \([A^-]_1\) \(([BH^+]_1)\) are the ionized concentrations of the acid (base) on either side of the membrane, \(z\) is the charge of drug molecule, \(F\) is Faraday’s constant, \(\Delta \phi\) is the electric potential across the membrane, \(RT\) is the product of the gas constant and absolute temperature, and \(SA\) is the relevant surface area. Finally, \(P_n\) and \(P_e\) are the permeability constants of the neutral and ionized species, respectively. Accounting for cell volumes and intracellular binding, eqs. 1 and 3 can be used to model the kinetics of unbound acidic drugs (e.g., PF-049 and PF-051):

\[
[BH^+] = C \frac{10^{pK_a}}{10^{pH} + 10^{pK_a}} \quad \text{and} \quad [B] = C \frac{10^{pK_a}}{10^{pH} + 10^{pK_a}}
\]
Correspondingly, eqs. 2 and 4 can be used to derive a similar expression for simple bases. The parameters $S_{\text{plasma}}$ and $S_{\text{organelle}}$ are the exposed surface area of the plasma membrane and estimated surface areas of the organelles, $fu$ is the unbound fraction of the drug within the cell, $C_{\text{plaque}}$ is the active uptake component of the OATP1B1, OATP1B3-transfected cells, and $V_{\text{buffer}}, V_{\text{cytosol}},$ and $V_{\text{organelle}}$ are the volume of the buffer, the cytosolic volume, and the organelle volumes, respectively. Estimated volumes of single organelles from the literature for HEK-293 are found in Table 1 and are used to estimate the organelle volumes and organelle volumes, respectively. The organelle volumes and organelle volumes, except for mitochondria for which an ellipsoidal symmetry assumption was used. Note that in this simplified picture, the $fu$ is taken to be organelle-independent and is instead just a general property of the cell systems. The organelle volumes and surface areas for HEK-293 cells are estimated from the literature and listed in Table 1. The uptake experiments in WT, OATP1B1-transfected cells, and OATP1B3-transfected cells were taken out to 5 minutes with samples measuring total intracellular concentration taken in triplicate at 0.5, 1, 1.5, 2, and 5 minutes at both pH 6.0 and 7.4. For each compound, all experimental data points are loaded into Mathematica (Wolfram Research, Champaign, IL; version 9.0.1, 2013) and fit simultaneously seeking a single unique set of parameters for $P, P_u, C_{\text{plaque}}, C_{\text{cytosol}}, C_{\text{organelle}}, V_{\text{cytosol}}$, and $V_{\text{organelle}}$ using the NonlinearModelFit procedure combined with the numerical global optimization algorithm NMinimize using the default settings.

**Experimental Determination of Transwell Permeability in Madin-Darby Canine Kidney–Low Expression Cells.** The cell-culturing conditions for the in-house low-efflux transporter Madin-Darby canine kidney–low expression (MDCK-LE) cell line (cells isolated from Madin-Darby canine kidney cells, selected for low endogenous efflux transporter expression levels) are discussed in more detail elsewhere (Di et al. 2011; Varma et al. 2011). Briefly, MDCK-LE cells were cultured at 37°C, 5% CO₂, 95% relative humidity in minimum essential medium that contained 10% fetal bovine serum, 1% nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% L-glutamine. Cells are passaged each week at about 90% confluency. Cells are then trypsinized and resuspended in complete media to obtain a cell suspension of 2.5 × 10⁶ cells/ml and then plated onto 96-well membrane inserts, with each insert receiving a volume of 75 μl. Plates were used on day 4 for transport studies. Cell inserts are washed with prewarmed (37°C) transport buffer before the experiment. Monodirectional transport studies were performed with 2 μM drug solution in transport buffer with 0.1% dimethylsulfoxide. Drug solution was added to the donor wells, and buffer was added to the receiver wells to initiate the transport assay. The plates were incubated at 37°C, and samples from both the donor and receiver were taken at 0 minute and 90 minutes for analysis. Permeability of Nadolol, a low permeability marker, was measured to assess the cell monolayer integrity. Transepithelial electrical resistance values were measured across the cell membranes prior to beginning the experimentation and at the last sample collection time point to assess the integrity of the MDCK-LE monolayers (transepithelial electrical resistance ≥250 Ω cm²). Permeability was determined at donor pH 5.5, 6.5, and 7.4, with receiver medium pH always 7.4.

**Model-Based Predictions of Transcellular Permeability in MDCK-LE.** The underlying physicochemical transport and uptake processes involved in the MDCK-LE Transwell permeability measurement are shown in Fig. 4. A few key simplifying assumptions will be made to derive a tractable model in line with the experimental protocols described in the previous section. As described above, paracellular leakage of MDCK-LE cells was monitored using Nadolol. As such, permeation from the donor to the receiver compartment is expected to occur purely through transcellular passive permeation across the Transwell cellular layer. Additionally, the unstirred water layer effect for these compounds is negligible as the permeability of these compounds are well below the aqueous diffusion depletion limit (Ghosh et al., 2014).

Steady state flux conditions are expected to occur when the flux $J_{\text{in}}$ across the apical side of the Transwell layer equals the flux $J_{\text{out}}$ coming out of the basolateral side: $J_{\text{in}} = J_{\text{out}} = J$. Under such steady state flux conditions, uptake...
into organelles and nonspecific binding no longer contributes to the flux. Using eq. 3 to represent the flux across each membrane and taking advantage of the fact that the sign on the membrane potential $\Delta \phi$ across the donor-side plasma membrane is equal and opposite to the membrane potential $\Delta \phi$ across the receiver side, $J_{\text{in}}$ and $J_{\text{out}}$ can be derived as follows:

$$J_{\text{acid, in}} = P_{\text{n}} \cdot SA \cdot \left( [HA_D] - [HA_R] \right) - P \cdot SA \cdot \frac{\Delta \phi F}{RT} \left( [A^-] - [A^+] \right)$$

$$J_{\text{acid, out}} = P_{\text{n}} \cdot SA \cdot \left( [HA_R] - [HA_D] \right) - P \cdot SA \cdot \frac{\Delta \phi F}{RT} \left( [A^-] - [A^+] \right)$$

(6)

where the subscripts $D$, $i$, and $R$ represent the donor, intracellular, and receiver compartments, respectively. Substituting in our Henderson-Hasselbalch relationship (eq. 1) yields:

$$J_{\text{acid, in}} = P_{\text{n}} \cdot SA \cdot \left( \frac{10^{pK_a}}{10^pH_D + 10^{pK_a}} - \frac{10^{pK_a}}{10^pH_R + 10^{pK_a}} \right)$$

$$- P \cdot SA \cdot \frac{\Delta \phi F}{RT} \left( \frac{10^pH_D}{10^pH_R + 10^{pK_a}} \right)$$

(7)

$$J_{\text{acid, out}} = P_{\text{n}} \cdot SA \cdot \left( \frac{10^{pK_a}}{10^pH_R + 10^{pK_a}} - \frac{10^{pK_a}}{10^pH_D + 10^{pK_a}} \right)$$

$$- P \cdot SA \cdot \frac{\Delta \phi F}{RT} \left( \frac{10^pH_R}{10^pH_D + 10^{pK_a}} \right)$$

(8)

Under steady state conditions, $J_{\text{acid, in}} = J_{\text{acid, out}} = J$, yielding a system of two equations (eqs. 7 and 8) and two unknowns ($J_{\text{acid}}$ and the free intracellular drug concentration $C_D$). This allows one to solve for the flux $J_{\text{acid}}$ as a function of the two measurable quantities ($C_D$ and $C_R$):

$$J_{\text{acid}} = \frac{C_D \left( 10^{pH_D} + 10^{pK_a} \right) \left( \frac{\Delta \phi F}{RT} P \cdot SA \cdot 10^{pH_D} + \left( e^{\frac{\Delta \phi F}{RT}} - 1 \right) 10^{pK_a} P_{\text{n}} \cdot SA \right)}{2 \left( e^{\frac{\Delta \phi F}{RT}} - 1 \right) (10^{pH_D} + 10^{pK_a})(10^{pH_R} + 10^{pK_a})}$$

(9)

which under sink conditions ($C_R \to 0$) can be simplified to:

$$J_{\text{acid}} = \frac{C_D \left( \frac{\Delta \phi F}{RT} P \cdot SA \cdot 10^{pH_D} + \left( e^{\frac{\Delta \phi F}{RT}} - 1 \right) 10^{pK_a} P_{\text{n}} \cdot SA \right)}{2 \left( e^{\frac{\Delta \phi F}{RT}} - 1 \right) (10^{pH_D} + 10^{pK_a})} \cdot \frac{\left( \frac{\Delta \phi F}{RT} P \cdot 10^{pH_D} + \left( e^{\frac{\Delta \phi F}{RT}} - 1 \right) 10^{pK_a} P_{\text{n}} \cdot SA \right)}{P_{\text{app}}}$$

(10)

Using eqs. 2 and 4, a similar analysis for bases in the Transwell assay, yields the following expression for the flux:
which under sink conditions becomes:

\[
J_{\text{base}} = \frac{C_D \left( \frac{\Delta \delta F}{RT} \right) \left( \frac{\Delta \delta F}{RT} P_{\text{app}} \cdot \text{SA} \cdot 10^{K_{\text{p}}} \right)}{2 \left( \frac{\Delta \delta F}{RT} - 1 \right) \left( 10^{\Delta \delta F} D_f + 10^{K_{\text{p}}} \right)}
\]

where

\[
P_{\text{app}} = \frac{P_{\text{app, acid}}}{\text{RT}} - \frac{\Delta \delta F}{RT} \frac{P_{\text{app, acid}}}{\text{RT}} + \Delta \delta F \frac{P_{\text{app, acid}}}{\text{RT}}
\]

Under well stirred sink conditions for strong acids and bases, eqs. 10 and 12 provide an expression for the relative pH-dependent flux across the in vitro Transwell assay. In the current analysis, eq. 10 is used to predict the pH-dependent Transwell permeabilities of both PF-049 and PF-051 in MDCK-LE cells using the estimates for \( P_{\text{app}} \) and \( P \) obtained from modeling uptake kinetics in MDCK-LE cells and the physiologic parameters (Simmons, 1984; Ishikawa et al., 1998) listed for MDCK-LE cells in Table 1. This allows one to examine both the translatability of these transmembrane passive permeability constants across different cell types and the utility of the given mathematical framework.

**Quantum Mechanics-Based Free Energies of Solvation Calculation.**

Conformational analysis was performed using the OPLS-2005 force field (Jorgensen et al., 1996; Kaminski et al., 2001) with the generalized Born surface area framework (Still et al., 1990). Selected low energy conformations according to OPLS-2005/generализated born-surface area were then energy-minimized in water using the B3LYP exchange-correlation energy functional (Becke, 1993; Stephens et al., 1994), the 6-31G* basis set (Frisch et al., 1984), and the Poisson-Boltzmann solver (Tanner et al., 1994) to describe solvent effects. The most stable conformation according to this level of theory was submitted to single-point energy calculations in water at the B3LYP/6-311G**++ level of theory (Marten et al., 1996). All calculations were performed within the Jaguar program (Bochevarov et al., 2013).

**Experimental Determination of In Vivo Kpua.** In the in vivo distribution of PF-049 and PF-051 in male Wistar-Han rat plasma, pancreas, and muscle was determined by measuring unbound tissue concentrations, as described previously (Pfefferkorn et al., 2012a; Stevens et al., 2013). PF-049 was dosed at 0.13 mg/ml in PBS (pH 7.4), and PF-051 was dosed at 1.21 mg/ml in 12% (w/v) sulfobutyl ether β-cyclodextrin acidified with 0.1 N HCl. Both drugs were administered through a jugular vein catheter using a flow rate of 4 µl/min (total dose volume 23.04 ml) for 4 days. Plasma, pancreas, and quadriceps muscle were collected from animals under anesthesia and flash frozen for storage until analysis. Drug concentrations were determined by LC-MS/MS analysis of methanol extracts of tissue homogenates. Tissue protein binding was determined in untreated tissue homogenates using equilibrium dialysis. Measured in vivo Kpua are expressed as the ratio of unbound drug in tissue relative to unbound drug in plasma.

**Model-Based Predictions of In Vivo Tissue-to-Plasma Ratios.** Equilibrium calculations of Kpua can be derived from the appropriate steady state conditions. For acids, setting eq. 7 to 0 and solving for the ratio \( C_I/C_D = K_{\text{puu, acid}}/K_{\text{puu, plasma}} \) yields the following expression for the unbound plasma to cytosol ratio:

\[
K_{\text{puu, plasma, cytosol}} = \frac{\left( 10^{\Delta \delta F} + 10^{K_{\text{p}}} \right) \left( \frac{\Delta \delta F}{RT} P_{\text{app}} + \Delta \delta F \right) P_{\text{app}}^{\Delta \delta F}}{\left( 10^{\Delta \delta F} + 10^{K_{\text{p}}} \right) \left( \frac{\Delta \delta F}{RT} P_{\text{app}} + \Delta \delta F \right) P_{\text{app}}^{\Delta \delta F}}
\]

and correspondingly a similar relationship for bases can be derived yielding:

\[
K_{\text{puu, base, plasma, cytosol}} = \frac{\left( 10^{\Delta \delta F} + 10^{K_{\text{p}}} \right) \left( \frac{\Delta \delta F}{RT} P_{\text{app}} + \Delta \delta F \right) P_{\text{app}}^{\Delta \delta F}}{\left( 10^{\Delta \delta F} + 10^{K_{\text{p}}} \right) \left( \frac{\Delta \delta F}{RT} P_{\text{app}} + \Delta \delta F \right) P_{\text{app}}^{\Delta \delta F}}
\]

For organelles within a cell, it can be easily shown that the equilibrium partitioning must be multiplicative in nature:

\[
K_{\text{puu, plasma, organelle}} = K_{\text{puu, plasma, cytosol}} \times K_{\text{puu, cytosol, organelle}}
\]

Correspondingly, the unbound partitioning between the cytosol and any organelle is also described by eqs. 14 and 15, in which the donor compartment is now the cytosol and the receiver compartment is now the organelle in question. Note that the equilibrium partition coefficients Kpua are independent of organelle or cell volume. As with the purely prospective predictions of the MDCK-LE observed permeabilities, eq. 16 is also used purely prospectively to predict in vivo Kpua using the permeability parameters derived from the transfect the HEK-293 assay.

The relevant parameters (pH, volume, and fractional volume) for estimating the unbound partitioning within muscle (Guderley et al., 2006; Sawant et al., 2011), liver (Peters, 1984; Saito et al., 1992; Plettenberg et al., 2008), and the pancreas (Nishiyama and Petersen, 1974; Morgan et al., 1986; Taga et al., 1993) are provided in Table 1. The key approximation that will be made within this framework is that the transmembrane permeation is similar across various cell and organelle types and that \( f_u \) is a whole tissue parameter. As the membrane composition may differ across tissue types, this is clearly a simplifying approximation that should be tested more thoroughly.

**Results**

**Characterization of pH-Dependent Distribution in WT and OATP-Transfected HEK-293 Cells.** PF-049 and its structural analog, PF-051, were analyzed to determine pH-dependent OATP1B1/OATP1B3 uptake, passive permeability, and fraction unbound within the cell. All relevant parameters were fit simultaneously using eq. 5 for all cell line (WT, OATP1B1, OATP1B3) data to derive a self-consistent single set of parameters. Kinetic data for PF-049 and PF-051 were reasonably well described by the proposed mathematical model for WT HEK-293 cells at both pH 6.0 and 7.4 (Figs. 5 and 6, open symbol/dashed lines). As expected, the estimated passive permeability of the ionized species (P) of each drug molecule is lower than the neutral species (P0). However, the ratio of these values is much larger for PF-049 (i.e., 357) than for PF-051 (i.e., 4.1). This is consistent with visual inspection of the kinetic data obtained in WT HEK cells, which indicate that cellular accumulation of PF-049 is more sensitive to pH than that of PF-051. The relatively larger P0 of
PF-049 manifests as a compound that is balanced between neutral-dominated and ionized permeability profiles in which cell uptake is more sensitive to the expected change to unionized fraction (Fig. 5, open symbols). In contrast, the relatively lower $P_{n}$ of PF-051 manifests as a completely ionized-dominated permeability profile in which cell uptake is insensitive to the expected change in unionized fraction (Fig. 6, open symbols). The potential physicochemical basis for this difference is addressed in the discussion.

Kinetic data for PF-049 and PF-051 were also well-described by the proposed mathematical model for OATP1B1- and OATP1B3-transfected HEK-293 cells at both pH 6.0 and 7.4 (Figs. 5 and 6, closed symbol/solid lines). As expected, accumulation is increased relative to WT HEK-293 cells. Data were best characterized by allowing for a pH-dependent active uptake rate. For example, consistent with what has been previously reported for OATP2B1-mediated uptake of statins (Varma et al., 2011), the estimated uptake rate of PF-051 was significantly larger at pH 6.0 relative to pH 7.4 (Table 2). A reverse trend was observed with respect to PF-049, although this did not reach statistical significance. The model-derived uptake rates indicate that PF-049 is transported more rapidly than PF-051 in 1B3- and 1B1-transfected HEK cells, respectively (Table 2). These results suggest that, unlike in rats (Fig. 2), PF-049 may have a human hepatoselectivity advantage over PF-051, although further investigation would be required to support this possibility.

**Prediction of pH-Dependent Transcellular Permeability in MDCK-LE Cells.** Both PF-049 and PF-051 demonstrated pH-dependent permeability in MDCK-LE cells (Table 3). As a test of the mathematical framework and translatability of passive permeabilities across cell lines, eq. 10 was used to predict these results, assuming the relevant physiologic parameters for MDCK-LE cells listed in Table 2 and values of $P_{n}$ and $P$ estimated from HEK-293 cells listed in Table 2. These predictions were generally consistent with the overall pH-dependent trend and the absolute values determined experimentally (Table 3, predicted versus observed). As observed with accumulation in WT HEK cells, the experimental and predicted $P_{obs}$ of PF-049 was more sensitive to pH than that for PF-051 (Table 3). This again can be attributed to the fact that PF-049 has a much larger ratio of $P_{n}$ and $P$. Given that both compounds have a $p_{K_{a}}$ of 3.5, the fraction unionized is low and proportionally related to the pH across the range of values examined (i.e., pH 7.4, 6.5, and 5.5, approximately 0.01, 0.1, and 1% unionized, respectively). As such, if overall MDCK permeation is predominately determined only by the neutral species, then the apparent permeability should be inversely proportional to pH. Likewise, the fraction ionized is high and relatively independent of pH across the range of values examined (i.e., pH 7.4, 6.5, and 5.5, approximately 99, 99.9, and 99.99% ionized, respectively). As such, if the overall MDCK permeation is predominately determined by the ionized species, then the apparent permeability should be independent of pH. The results listed in Table 3 are consistent with a contribution of both ionized and unionized fractions to the overall MDCK permeability (i.e., some sensitivity in $P_{app}$ short of a strict inverse proportionality). The relatively greater pH sensitivity of PF-049 (both observed and predicted) is attributed to the fact that it has a neutral permeability that is much greater than the ionized permeability (1868 versus 5.23 $\times\ 10^{-6}$ cm/s; Table 2). In contrast, PF-051 has a much lower neutral permeability relative to the ionized permeability (18.2 versus 4.43 $\times\ 10^{-6}$ cm/s; Table 2), a property that lends itself to being insensitive over the lower range of pHs studied.

**Prediction of In Vivo Unbound Tissue-to-Plasma Ratios in the Muscle and Pancreas.** Consistent with distributional impairment, the experimentally determined steady state $K_{puu}$ values of both PF-049 and PF-051 in rat muscle and pancreas were significantly lower than 1.

---

**Fig. 5.** Wild-type (dashed) and transfected 1B1 (A and B)/1B3 (C and D) uptake (solid) of PF-049 into HEK-293 cells at pH 7.4 (A and C) and 6.0 (B and D). Concentrations represent total cell-associated PF-049 (bound and free), as modeled.
This property of pancreatic exclusion contributes further to the pharmacological selectivity of these agents for liver glucokinase relative to glucokinase in the pancreas. As stated previously, these results cannot simply be explained by Fick’s law, as this would require $K_{puu}$ to equal unity at steady state. As an additional test of the proposed mathematical framework and the hypothesis that this exclusion is driven by membrane potential, eqs. 14 and 16 were used to predict $K_{puu}$, assuming the relevant physiologic pancreatic and muscle parameters listed in Table 1 and the values of $P$ and $P_n$ derived from HEK-293 cells listed in Table 2. As shown in Table 4, model predictions were generally consistent with experimental results for both compounds across both muscle and pancreas.

### Discussion


Results obtained by modeling the HEK-293 cell data indicate that the ionized species of PF-049 and PF-051 have similar permeabilities ($5.23 \times 10^{-6}$ and $4.43 \times 10^{-6}$ cm/s, respectively). Qualitatively, this is due to the similar structure around the carboxylic acid group that is expected to limit permeability in the ionized state (Fig. 1). The two compounds differ by substitution on the imidazole ring. PF-049 has a hydrophobic trifluoromethyl group, whereas PF-051 has a more hydrophilic cyclobutylsulfone group. For both compounds, the negative charge of the carboxylate group is expected to be a limiting factor for permeation with a modest contribution from the remaining substitutions.

To quantify the physiochemical basis for why the neutral permeabilities for PF-049 and PF-051 are so different from each other, in contrast to the ionized permeabilities, the free energies of solvation were calculated using the self-consistent reaction field approach. The free energies of solvation are used as a simplified surrogate to estimate the free energies associated with stripping the water molecules upon insertion in the membrane. The negatively charged PF-049 and PF-051 were calculated to have hydration-free energies of $-279.56$ and $-286.56$ kcal/mol, respectively. The absolute numbers are so negative that the $27$ kcal/mol difference between the hydration-free energies becomes irrelevant; they both display equally low permeabilities for the ionized state. In the neutral state, PF-049 permeabilities ($5.23 \times 10^{-6}$ and $4.43 \times 10^{-6}$ cm/s, respectively). Qualitatively, this is due to the similar structure around the carboxylic acid group that is expected to limit permeability in the ionized state (Fig. 1). The two compounds differ by substitution on the imidazole ring. PF-049 has a hydrophobic trifluoromethyl group, whereas PF-051 has a more hydrophilic cyclobutylsulfone group. For both compounds, the negative charge of the carboxylate group is expected to be a limiting factor for permeation with a modest contribution from the remaining substitutions.

To quantify the physiochemical basis for why the neutral permeabilities for PF-049 and PF-051 are so different from each other, in contrast to the ionized permeabilities, the free energies of solvation were calculated using the self-consistent reaction field approach. The free energies of solvation are used as a simplified surrogate to estimate the free energies associated with stripping the water molecules upon insertion in the membrane. The negatively charged PF-049 and PF-051 were calculated to have hydration-free energies of $-79.56$ and $-86.56$ kcal/mol, respectively. The absolute numbers are so negative that the $7$ kcal/mol difference between the hydration-free energies becomes irrelevant; they both display equally low permeabilities for the ionized state. In the neutral state, PF-049

### Table 2

Model-derived parameters for passive permeability, fraction unbound, and active uptake determined in human embryonic kidney 293 cells

<table>
<thead>
<tr>
<th></th>
<th>PF-049</th>
<th>PF-051</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P$</td>
<td>5.23 (4.06..6.78)</td>
<td>4.43 (3.4..5.8)</td>
</tr>
<tr>
<td>$P_n$</td>
<td>1868 (1637..2132)</td>
<td>18.2 (13.9..23.8)</td>
</tr>
<tr>
<td>$f_{hc,ci}$</td>
<td>0.2 (0.15..0.237)</td>
<td>0.67 (0.63..0.7)</td>
</tr>
<tr>
<td>$C_{uptake\ 1b1\ (6.0)}$</td>
<td>2.0 (1.7..2.4)</td>
<td>0.77 (0.72..0.81)</td>
</tr>
<tr>
<td>$C_{uptake\ 1b1\ (7.4)}$</td>
<td>2.6 (2.3..3.0)</td>
<td>0.38 (0.34..0.41)</td>
</tr>
<tr>
<td>$C_{uptake\ 1b3\ (6.0)}$</td>
<td>0.59 (0.4..0.88)</td>
<td>0.38 (0.34..0.43)</td>
</tr>
<tr>
<td>$C_{uptake\ 1b3\ (7.4)}$</td>
<td>1.1 (0.85..1.35)</td>
<td>0.25 (0.22..0.28)</td>
</tr>
</tbody>
</table>

### Table 3

Predicted Transwell Madin-Darby canine kidney–low expression permeabilities at multiple pH

<table>
<thead>
<tr>
<th></th>
<th>PF-049</th>
<th>PF-051</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>Predicted</td>
<td>Experimental</td>
</tr>
<tr>
<td>5.5</td>
<td>10.18</td>
<td>7</td>
</tr>
<tr>
<td>6.5</td>
<td>1.87</td>
<td>2.4</td>
</tr>
<tr>
<td>7.4</td>
<td>1.06</td>
<td>1.4</td>
</tr>
</tbody>
</table>
and PF-051 have less pronounced hydration-free energies, $-23.60$ and $-31.19$ kcal/mol, respectively, again approximately $-7.0$ kcal/mol, favoring the hydration of the cyclobutylsulfone group. For a neutral molecule, the $-7.0$ kcal/mol extra hydration solvation-free energy is expected to play a more significant role. We hypothesize that it is this relative hydration penalty that leads to the much lower permeability for the neutral state of PF-051, yet similar permeability for the ionized fractions.

**Forward Predictions—MDCK-LE Transwell Prediction.** For both compounds, predictions employing the permeabilities derived from the HEK-293 cell data are in agreement with that observed in the MDCK-LE Transwell system across three different pHs. The transmembrane potential can serve to favor the hydration of the cyclobutylsulfone group. For a neutral molecule, the $-7.0$ kcal/mol extra hydration solvation-free energy is expected to play a more significant role. We hypothesize that it is this relative hydration penalty that leads to the much lower permeability for the neutral state of PF-051, yet similar permeability for the ionized fractions.

<table>
<thead>
<tr>
<th></th>
<th>$K_{puu}$ (Observed)</th>
<th>$K_{puu}$ (Predicted)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF-049</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.25</td>
<td>0.24</td>
</tr>
<tr>
<td>Muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bulk</td>
<td>0.1</td>
<td>ND</td>
</tr>
<tr>
<td>Slow-twitch oxidative</td>
<td>ND</td>
<td>0.089</td>
</tr>
<tr>
<td>Fast-twitch oxidative</td>
<td>ND</td>
<td>0.084</td>
</tr>
<tr>
<td>Fast-twitch oxidative</td>
<td>ND</td>
<td>0.055</td>
</tr>
<tr>
<td>PF-051</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.10</td>
<td>0.22</td>
</tr>
<tr>
<td>Muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bulk</td>
<td>0.17</td>
<td>ND</td>
</tr>
<tr>
<td>Slow-twitch oxidative</td>
<td>ND</td>
<td>0.074</td>
</tr>
<tr>
<td>Fast-twitch oxidative</td>
<td>ND</td>
<td>0.068</td>
</tr>
<tr>
<td>Fast-twitch oxidative</td>
<td>ND</td>
<td>0.041</td>
</tr>
</tbody>
</table>

ND, not determined.

$K_{puu}$ was not predicted for bulk muscle as relative fractions of different muscle types were unknown.

$K_{puu}$ was experimentally determined only as bulk muscle.

An acid will follow a Nernst-Planck distribution only when the product of $P$ and $[A^-]$ exceeds that of the product of $P_n$ and $[HA]$. Both PF-049 and PF-051 have $K_{puu}$ values of 3.5. The 6300-fold ratio of ionized to neutral species at physiologic pH is expected to completely offset the observed $P_n$ to $P$ ratios for PF-049 and PF-051 (i.e., 357 and 4, respectively). In such cases, the enthalpic contribution from the membrane potential (eqs. 14 and 15) will govern the equilibrium partitioning of the drug and lead to a degree of intracellular exclusion. Conversely, if the product of $P_n[HA]$ exceeds that of $P[A^-]$, these equations collapse back to the classic pH partitioning equations, the underlying mechanism that governs, for instance, cationic accumulation of drugs within lysosomes (Kauffmann and Krise, 2007; Fridén et al., 2011). In this sense, the derived equilibrium equations for steady state partitioning ($K_{puu}$) (eqs. 14 and 15) should be considered as simply an organelle-specific generalization of classic pH partitioning behavior.

Fick’s law and classic pH partitioning hold for weak acids and weak bases with a characteristic localized charge in the ionized form that renders the ionized species impermeable to a hydrophobic barrier.

**Fig. 7.** Predicted apparent permeability of a hypothetical acid (red) and base (blue) with intrinsic permeabilities $P = 5.23 \times 10^{-5}$ cm/s and $P_n = 1868 \times 10^{-5}$ cm/s as a function of $pK_a$ across a MDCK-LE Transwell system at physiologic pH (7.4). The observed Transwell permeability is lower for acids in comparison with bases with the same physiochemical properties ($P$, $P_n$).
Fig. 8. Predicted steady state pancreatic $K_{pu}$ for the acidic glucokinase agonists PF-049 and PF-51 in pancreatic cells. The plot represents the relationship to $P$ and $P_{n}$ for an acid with a $pK_{a}$ of 3.5.

However, in cases in which charge delocalization yields a lipophilic acid (or base) or the $pK_{a}$ of the drug is such that the abundance of the charged species is many orders of magnitude higher than its neutral counterpart, the enthalpic contribution of membrane potential must be considered. Using these basic physiochemical properties, we are able to design glucokinase agonists with a greater therapeutic index of the compound than simply taking a pure transporter-driven hepatoselective uptake approach (Fig. 2). This approach not only maximizes the known therapeutic index, minimizing hypoglycemic risk due to insulin stimulation, but also has the advantage of minimizing other off-target risk by minimizing exposure to nontarget tissues.

As has been shown with our glucokinase agonists, unbound tissue/organelle accumulation or exclusion is driven by a mixture of the physiochemical properties of the drug ($P$, $P_{n}$, $pK_{a}$) and the physiologic properties of the cell and its relevant organelles (membrane potential $\phi$, $pH$). In Fig. 8, the predicted $K_{pu}$ in the pancreas for an acidic compound with a fixed $pK_{a}$ of 3.5 as a function of the neutral permeability $P$ and the ionized permeability $P_{n}$ is shown in this figure. PF-049 and PF-051 are shown in red and blue, respectively, illustrating how, for the case of the two clinical candidates, the compounds have a balance of properties that provide for pancreatic exclusion at the target site. It is also clear that there is a large optimum (i.e., low $K_{pu}$) space for molecules with $pK_{a}$ values in this range. We note that this relationship is only valid for molecules with a $pK_{a}$ of 3.5 and that the optimum space becomes increasingly restricted at higher $pK_{a}$ values (data not shown).

Conclusions
We have shown that a single set of physiochemical properties of a compound ($P$, $P_{n}$, $pK_{a}$) can be used to derive a self-consistent parameterization of time-dependent uptake measurement as well as Transwell permeability measurements. Furthermore, it has been shown that, in combination with a few key physiologic parameters, the same set of parameters can be used to derive the equilibrium-unbound $K_{pu}$ in noncleaving organs through a nontransporter-mediated efflux or uptake mechanism. Analytical expressions for the equilibrium $K_{pu}$ for tissues and organelles of interest have been derived. We have shown that physiochemical properties of our glucokinase agonist compounds govern exposure in certain organelles and tissues, providing an optimal therapeutic index by minimizing exposure in nonhepatic tissues.

Authorship Contributions
Participated in research design: Ghosh, Scott, Maurer, Guimaeraes, Tu, Scialis.
Conducted experiments: Rotter, Scialis.
Contributed new reagents or analytic tools: Varma, Feng, Scialis.
Performed data analysis: Ghosh, Guimaeraes, Tu.
Wrote or contributed to the writing of the manuscript: Ghosh, Scott, Maurer, Guimaeraes, Tu, Varma, Litchfield.

References


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