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Measuring Intrinsic Kinetic Constants for P-glycoprotein Mediated Quinidine Efflux Transport Using Intracellular Unbound Concentration in MDR1-MDCKII Cells

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Abstract

Evaluating the potential impact of p-glycoprotein (p-gp, MDR1) on drug absorption and disposition is an imperative task for preclinical stage drug development. In attempt to determine drug transport kinetic constants, cell-based *in vitro* system has been widely used. However, it was observed the apparent permeability (Papp) measured in cell monolayers cannot sufficiently describe the efflux activity of apically located P-gp activity, due to the dosing concentration not corresponding to the intracellular drug amount. Here, we demonstrate the measurement and the use of intracellular unbound concentration (Ccell,u) to describe intrinsic disposition kinetic parameters.

MDR1-MDCKII cell monolayers were tested using 96 well Transwell™ transport system after dosing B->A direction, 100 nM of [3H]-quinidine (5 Ci/mmol) incubated for 30 – 120 min. Also, dose-response curve was prepared after 90 min for concentration ranging 0.1 – 30 µM. After permeabilizing and extracting the cellular contents with 1:1 water:acentonitrile, the transported amount to receiving compartment and cellular concentrations were determined. Ccell,u was measured in the presence of high potassium buffer to eliminate the membrane potential-driven cellular accumulation. Based on the free drug hypothesis, which assumes the concentration of unbound drug measured from extracellular compartment equals Ccell,u, the intracellular fraction unbound (fu,cell) can be obtained by the ratio of the extracellular to the total cell concentration. Kinetic constants were plotted against dosing concentration (to result apparent parameter) vs. Ccell,u (intrinsic).

After 120 min incubation of 100 nM quinidine, pseudo steady-state was reached for transport rate, resulting decreased dosing concentration (0.046 µM), total cellular accumulation (~2.9 µM) and increased receiver concentration (~0.052 nM). If shorter incubation (30 min) was used, apparent permeability was over-predicted to 122%, which demonstrates the necessity of reaching steady-state for intracellular concentration. Quinidine fu,cell increased from 0.005 to 0.016 over total cellular concentration ranging 20 to 2000 µM (cell volume 1.6 µL/cm^2). When dosed 0.1 – 30 µM for 120 min, transport rates against dosing concentration showed a dose-dependent linear relationship. However, when transport rate of control cells (MDCK without MDR1) was subtracted, and MDR1 transport rate was plotted against Ccell,u, the intrinsic apical P-gp efflux constants were determined: Vmax (70 pmol/min/cm^2) and Km = 1.5 µM.

In conclusion, the intrinsic kinetic parameters acquired with intracellular unbound concentration suggests much higher activity (Vmax/Km ~47 µl/min/cm^2) of the Pgp mediated clearance compared to that of the apparent parameters (Vmax/Km ~5.6 µl/min/cm^2). In order to correctly measure kinetic constants for apically expressed P-gp, intracellular unbound concentration, time course study as well as dose response study should be carried out. Simulation result of transcellular disposition for quinidine using intrinsic kinetic parameters demonstrated more reliable prediction than the apparent values.

Apparent vs. Intrinsic Permeability on Efflux Transporter Activity

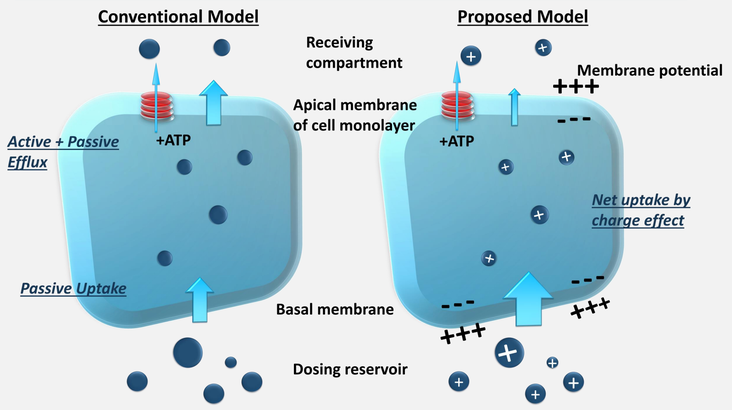
- The apparent permeability is what we measure in a standard bidirectional transport assay as transport rate across cell monolayer. It includes both flux due to the transporter activity and passive diffusion, normalized to the dosing conc.

  P_{app} = Flux/C_{dosing}
  Flux is what we actually measure *in vitro*

- The intrinsic permeability of the apical membrane, $P_{int,a}$, is exclusively attributable to the outward transport driven by intracellular unbound concentration instead of dosing concentration. Difference between the $P_{int,a}$ in the presence and absence of MDR1 can be attributed to MDR1 efflux permeability, $P_{int,efflux}$.

  P_{int} = Flux/$C_{cell,u}$
  $P_{int,\ active\ efflux}$ = $P_{int,a,\ w\ MDR1}$ – $P_{int,a,\ no\ MRD1}$
  $C_{cell,u}$ is experimentally evaluated *in vitro*

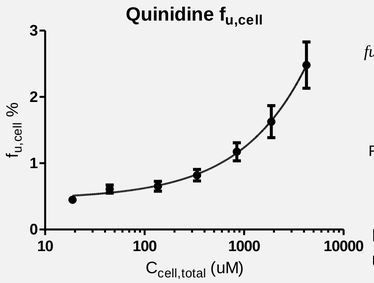
Mechanistic Understanding of Transcellular Transport



- B>A transcellular transport equals to cross-apical membrane transport. This is affected by the apical components (e.g. apical membrane potential and receiver concentration) and the intracellular unbound concentration.
- Basal components (e.g. dosing concentration) may indirectly affect transcellular transport through affecting intracellular concentration.
- Although concentration gradient may drive the net flux from B>A direction, ionized molecules entering (basal membrane) and leaving the cell (apical membrane) will have different intrinsic permeability (P_{int}) as electric potential gradient in two membranes are opposite.
- In order to measure the active efflux ($P_{int,\ active\ efflux}$) of the multi-drug resistant transporter P-gp, the intracellular unbound drug concentration should be determined.

Methods

1. MDR1-MDCK cells were cultured in PCF-membrane Transwell™ inserts (0.4 µm in pore size) for three days to reach confluency.
2. Dose [3H]-Quinidine to basal dosing reservoir (300 µl) in the presence of paracellular diffusion marker [14C]-mannitol, and measure the apical receiving compartment (150 µl) radio activity with various dosing concentration (for kinetics) and time course.
3. At the end of the assay, wash cells with PBS, lysis, then extract/measure radio activity associated with cells for intracellular concentration.
4. Using mass balance to quantify mass transport and concentration change over time, apical flux were adjusted by the paracellular transport and normalized to intracellular concentration per individual wells. MDCK cells without MDR1 served as background ($Flux_{\ no\ MDR1}$) to factor out the non-MDR1 component.
5. Cellular concentration (Ccell,total) was adjusted for unbound fraction measured separately measured in separate experiments.
6. For forward prediction using the mechanistic modeling calculator, we built the Opti-Sim simulator using Fick-Nernst-Planck equation with series of differential equation with mass balance.



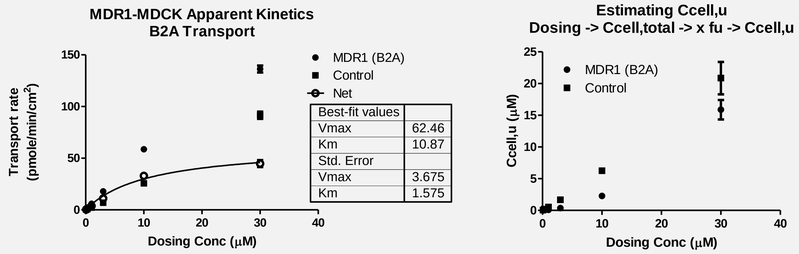
$$fu = 1 - \frac{1 - 0.0046}{1 + \left(\frac{[X]}{1.4 \times 10^6}\right)^{0.67}}$$

R^2: 0.9519

For details of the unbound fraction (fu) measurements, please refer to the poster, #448.

Results

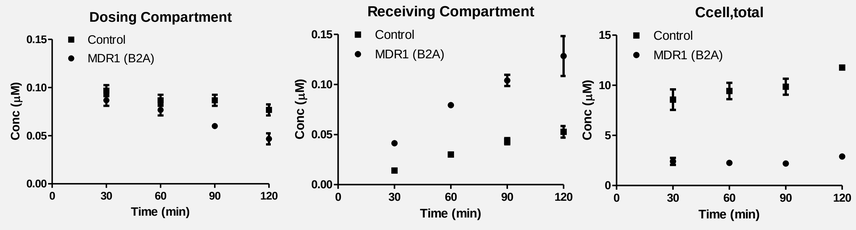
1. Transcellular Quinidine Transport: Apparent vs. Intrinsic Kinetics



	MDR1 (B2A)	Control
Best-fit values		
Pd	4.093	4.299
Vmax	70.07	~ 0.07633
Km	1.490	~ 4.856e+016
Std. Error		
Pd	0.2261	0.03999
Vmax	4.799	~
Km	0.1990	~

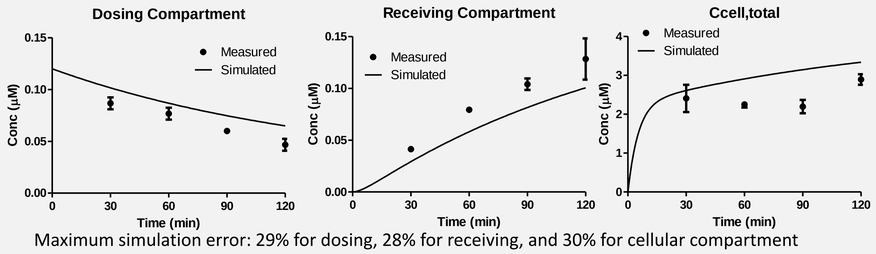
$$V = P_d \times C_{cell,\ u} + \frac{V_{max} \times C_{cell\ u}}{K_m + C_{cell,\ u}}$$

2. Time Course, 100 nM Quinidine



Forward Prediction Using Opti-Sim

- Paracellular transport of [3H]-Quinidine was estimated by the paracellular diffusion marker [14C]-mannitol. Paracellular transport rate was separately incorporate to increase the accuracy for transcellular transport.
- Based on the experimental values cell membrane potential was approximated to -28 mV.
- Passive permeability for quinidine was estimated from basal uptake permeability, approximating 61 x10^{-6} cm/s. Experimentally 59 x10^{-6} cm/s was measured without the membrane potential (use of intracellular buffer).
- Other simulation condition matched the B>A MDCK-MDR1 transcellular experiment settings.



Maximum simulation error: 29% for dosing, 28% for receiving, and 30% for cellular compartment

Conclusion

- Highly protein bound drug quinidine has about 0.5% fu,cell, which may be increased as cellular accumulation continues.
- Intrinsic p-gp activity (clearance) was approximately 47 µl/min/cm^2, that is about 8.4x greater than the apparent value of 5.6 µl/min/cm^2.
- Utilizing the unbound intracellular concentration in transcellular transport assay better explains high efficiency of Pgp in quinidine efflux, demonstrating the importance of intrinsic permeability (P_{int}) over Papp.
- Preliminary simulation using intrinsic membrane passive permeability, intrinsic efflux kinetic parameters, and series of differential equations predicted cellular disposition processes within 30% accuracy.