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A NOVEL HIGH-THROUGHPUT METHOD FOR MEASURING INTRACELLULAR UNBOUND FRACTION

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ABSTRACT

The binding/acting sites of virtually all ABC (ATP Binding Cassette) transporters, major drug metabolizing enzymes, and nearly half of drug targets are intracellularly located. Therefore, being able to determine drug intracellular unbound concentration ($C_{cell,u}$) is of paramount importance for evaluating transporter-mediated active efflux, metabolism and pharmacodynamic responses of xenobiotics. One of the major challenges in determining $C_{cell,u}$ is due to the difficulty in measuring fraction unbound in cells ($f_{u,cell}$). Although a couple of methods (e.g. microdialysis-based techniques[1]) have been proposed, they require laborious and time consuming efforts, thus makes it unsuitable for assessing a large number of compounds. Here, we introduce a simple *in situ* high-throughput method for empirically estimating $f_{u,cell}$.

Assuming the steady state $C_{cell,u}$ will come to an equilibrium with the extracellular free drug concentration in medium, $C_{medium,u}$, we eliminated the cell membrane potential ($\Delta\Psi_{pm}$ = -40 mV, for MDCK plasma membrane) by applying high potassium "intracellular medium". Matching Cl^-, K^+, Na^+ and H^+ concentrations on the either side of the plasma membrane, the electric membrane potential became zero. MDCK-II cells were grown into polarized monolayer on 96-well Transwell™ inserts, and incubated with radioisotope labeled drugs applied to basal compartment, up to 3 hours. For low permeability hydrophilic compound metformin, MDCK-II cells were transiently transfected with basolaterally localized facilitative transporter OCT2 to decrease the time to reach steady-state. Apical compartment was kept dry to prevent drug efflux.

Total drug accumulation in cells were measured at the end of assay, and the unbound cytosolic concentration was determined from extracellular concentration at each time point. $f_{u,cell}$ was then calculated by dividing fitted steady-state C_{cell}. The mass balance was held greater than 90% after incorporating previously measured cell volume (1.6 µl/cm^2) and cytosol fraction (0.9). As a result, we determined $f_{u,cell}$ for various anionic, cationic and neutral compounds with varying logP values. For example, Atorvastatin $f_{u,cell}$ was determined as 3.6%, which is in agreement with 3 – 5 % that were previously measured with microdialysis method[2]. For quinidine, $f_{u,cell}$ exponentially increased from 0.5% to 2.5% (R^2=0.95) when dosed at concentrations ranging from 20 µM to 4000 µM. Surprisingly, low plasma-protein-binding hydrophilic metformin appeared to bind (or being sequested) substantially to intracellular components, $f_{u,cell}$ ranging from 50% to 70%.

In conclusion, elimination of membrane potential and Na^+ and H^+ gradient may provide a high-throughput method to measure $f_{u,cell}$, which can be further implemented for mechanistic modeling of cellular disposition processes. Transfection of facilitative transporter allows us to utilize the same principle without affecting the equilibrium level of $C_{cell,u}/C_{medium,u}$.

BACKGROUND

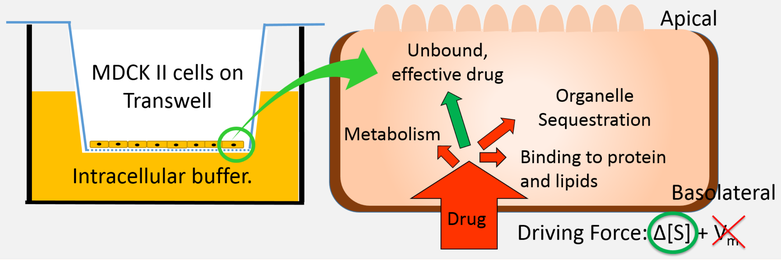
- Free drug hypothesis assumes ratio of the free drug concentration across the cell membrane (Kp,uu) to be 1 at steady state. However, due to existing membrane potential, positively charged molecules have Kp,uu greater than 1, while negatively charged molecules have less than 1.
- Independent from chemical gradient, membrane potential forces charged molecules down the gradient. Membrane potential is generated due to disparity of ion species distribution across the plasma membrane, demonstrated by Goldman-Hodgkin-Katz equation. V_m, electric potential; R, gas constant; T, absolute temperature; F, Faraday's constant; P, permeability of the ion.

$$V_m = \frac{RT}{F}\ln\left(\frac{P_K[K]_o + P_{Na}[Na]_o + P_{Cl}[Cl]_i}{P_K[K]_i + P_{Na}[Na]_i + P_{Cl}[Cl]_o}\right)$$

Our Hypothesis :

1. Eliminating membrane potential will result in true Kp,uu, as long as the equilibrium is reached.
2. For poorly permeable drugs, addition of facilitative transporter will shorten the time to reach the equilibrium, leaving cellular level unaffected.

Figure 1. The main driving for drug getting into cell is concentration gradient (Δ[S]).



METHODS

1. MDCK-II cells were grown on a polarized monolayer in 96-well permeable membrane insert plate (PCF-0.4um), for three days after seeding 60,000 cells/cm^2. Standard cell culture procedure was performed.
2. When measuring low permeability hydrophilic compound metformin, cells were transiently transfected with basolateral facilitative transporter OCT2 using *in situ* transfection technology, Opti-Expression™ . Assay was performed 48 hours after transfection, and compared to mock-transfected.
3. Radiolabeled compounds were dissolved in high K^+, low Na^+ intracellular buffer. Apical side of the cell layer was dry during incubation. After incubating 15, 30, 120 and 180 minutes with basal dosing (300 µl), drug concentration in the extracellular and intracellular compartments were measured by scintillation counter. Cell volume 1.6 µl/cm^2 and cytosol fraction 0.9 were determined previously using [3H]-H_2O.
4. After confirming the cell concentration reached an equilibrium,

$$f_{u,cell} = \frac{C_{cell,u}\ at\ eq.}{C_{cell,total}\ at\ eq.} = \frac{C_{medium,u}\ at\ eq.}{C_{cell,total}\ at\ eq.}$$

RESULTS

Table 1. Physicochemical Property and Estimated $f_{u,cell}$

Compound	Charge status at pH 7.4	Transporter[a]	$f_{u,cell}$ (%)	LogP[b]	Literature reported $f_{u,cell}$ (%)
Atorvastatin	-1	NA	3.68	5.39	3-5[2]
Bromosulfophthalein	-2	NA	1.97	2.34	
Cimetidine	0	NA	65.92	-0.11	
Creatinine	0	NA	73.8	0.22	
		OCT2	79.98		
Desacetyl-rifampicin	+1	NA	26.95	2.33	
Gemcitabine	0	NA	3.09	-1.47	
Metformin	+1	NA	73.38	-0.92	
		OCT2	60.81		
MPP+	+1	NA	22.75	-1.54	
Quinidine	+1	NA	0.65	2.51	
Rifampicin	-1	NA	8.03	2.77	
Ritonavir	+1	NA	1.51	5.22	1.6 [1]
Trimethoprim	2	NA	20.40	1.28	

[a]Cells were transiently transfected with basolateral facilitative transporter.
[b]Predicted using ChemAxon Physico-chemical property predictor (ChemAxon, Hungary)

Figure 2. Correlation between LogP and Log$f_{cell,u}$

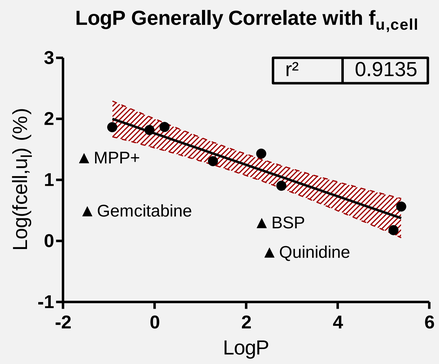
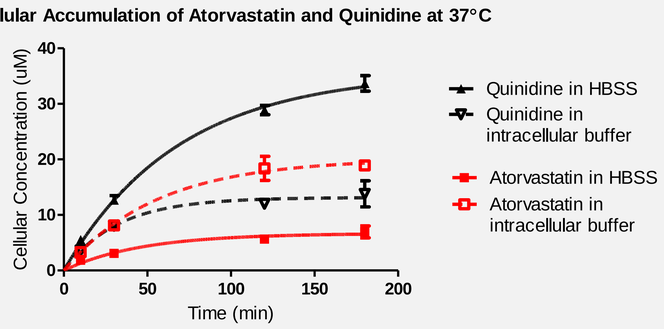


Figure 3. Cell membrane potential ($\Delta\Psi_{pm}$) effect on cellular accumulation for a cation, quinidine, and an anion, atorvastatin.



RESULTS (con't)

Figure 4. $f_{u,cell}$ and $C_{cell,total}$ for Quinidine was not linear.

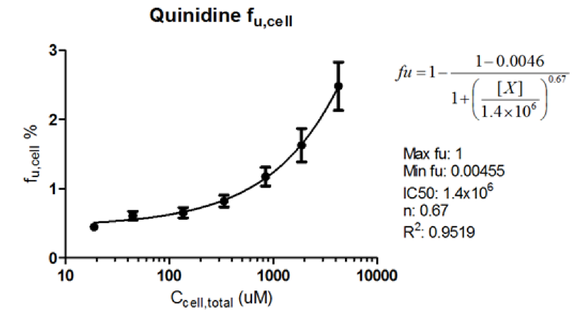
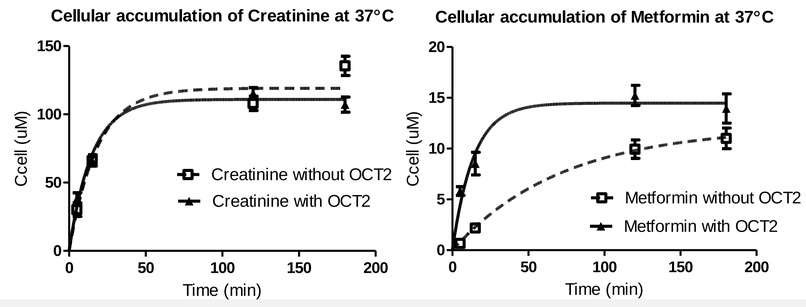


Figure 5. Basolateral facilitative transporter does not affect level intracellular equilibrium for creatinine. For poorly permeable metformin, OCT2 facilitated the cellular accumulation without affecting the level of equilibrium.



CONCLUSION

- Comparing to previously reported values using microdialysis methods, current *in situ* measurement can successfully estimate $f_{u,cell}$ (Table 1).
- Correlating $f_{u,cell}$ to logP suggests that main mechanism for cellular sequestration or binding is through lipophilic partitioning (Fig.2).
- Experimental controls for metabolism (gemcitabine), endogenous transporter substrates (BSP, MPP^+ and quinidine) indicated that substrates for the enzyme and transporters that are expressed in MDCK cells are not suitable current set up.
- Due to negative electric potential across the plasmamembrane, cationic quinidine permeability is accelerated, while anionic atorvastin is decelerated. Upon dosing with intracellular buffer that eliminates the membrane potential, quinidine equilibrates at lower concentration, while atorvastatin equilibrate at higher concentration. Both drug reached the equilibrium point within 2 hours of incubation (Fig.3).
- Fig. 3 results demonstrate that the free drug hypothesis is not valid under normal conditions where disparity of ion species created different membrane potential according to Goldman-Hodgkin-Katz equation. In return, Kp,uu is subject to change according to membrane potential.
- For cationic quinidine, $f_{u,cell}$ exponentially increased as the dosing concentration increased. This suggests, the binding/sequestration sites in cell may be saturable (Fig.4).
- Facilitative transporter, by definition, cannot concentrate in the absence of membrane potential as they dependent only on the concentration gradient. However, by adding OCT2, membrane permeability increases allowing faster equilibration, without affecting the level of plateau (Fig.5).

REFERENCES

[1]. Mateus A, et. al., Rapid Measurement of Intracellular Unbound Drug Concentrations, Mol. Pharmaceutics 2013, 10, 2467−2478

[2]. Cindy Xia, Using In Silico and In Vitro Methods to Understand the Clinical Impact of Efflux Transporter Polymorphisms, Applied Pharmaceutical Analysis, Sept 2013, Boston