Poster 447

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The Correlations between Transporter Protein Expression, Apparent Transcellular Permeability and Intrinsic Efflux Permeability: A Case Study on BCRP/ABCG2 Transporter

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ABSTRACT

Measuring a drug's apparent transcellular permeability (P_{app}) across polarized epithelial cell monolayers is widely used to quantify its active efflux mediated by ABC (ATP Binding Cassette) transporters such as P-gp and BCRP. Early experimental observations by others suggested that there was a non-linear correlation between efflux transporter expression and basolateral to apical (B>A) P_{app} [ref1]; subsequent analysis suggested that the calculated apical efflux activities might have a linear association with transporter expression [ref2]. Using BCRP transporter as a case study, this presentation will present sophisticated and definitive in vitro studies and in silico modeling to demonstrate the correlations between BCRP protein expression levels, the conventional B>A apparent permeability, and the "intrinsic" apical efflux permeability that should be solely dependent on transporter activities. In the studies, MDCK-II cell monolayers were transiently transfected with varying amounts of BCRP encoding plasmids to create cell models with a wide range of BCRP expression levels; basolateral transporter OATP1B1 was also co-expressed at a fixed level to facilitate basolateral uptake of polar substrates, such as Estradiol-17 beta Glucuronide (E17bG), in order to assess their apical efflux mediated by BCRP. Membrane BCRP proteins were quantified using LC/MS/MS method; Figure 4A showed an excellent linear correlation (R^2=0.96) between the BCRP protein levels and the amount of DNA used in transfections. Apparent B>A permeability of BCRP model substrates, E17bG and Genistein, were measured in the BCRP expressing MDCK-II monolayers. To assess the "true" apical efflux activities of BCRP, we used a novel method involving measuring substrate cellular concentrations (C_{cell}) at two time points when C_{cell} was at (quansi) steady state, and the net B>A substrate transport between the two time points. Figure 4B and 4C show the correlations between BCRP expression and the apparent B>A permeability ($P_{app,B>A}$), and the intrinsic BCRP mediated apical efflux permeability ($P_{eff,int}$) of E17bG. Clearly, $P_{app,B>A}$ exhibited a saturable association with, whereas $P_{eff,int}$ has a linear correlation (R^2=0.98). Follow-up mechanistic in silico modeling of BCRP mediated transcellular transport further confirmed our experimental observations and further suggested that the saturation in $P_{app, B>A}$ could be dependent on a number of factors such as basolateral transport and cellular unbound fraction. Our studies demonstrated, for the first time with definitive experimental data, the linear correlation between BCRP protein expression and the intrinsic BCRP efflux permeability, but not the commonly used apparent permeability. This results can be extremely useful in extrapolating in vitro efflux assay results and in building computational models for efflux transporters.

The BCRP-MDCK permeability assay is a valuable tool for the identification and characterization of BCRP substrates and inhibitors. The system avoids the complexities of multiple transporters by only focusing specifically on BCRP, and apparent permeability (P_{app}) is commonly used for quantitative measurements in most laboratories. However, it may be oversimplified with this measurement since it did not take into account with dynamic intracellular substrate concentrations, especially when cells contain both uptake and efflux transporters. To improve the accuracy, we proposed to use an intrinsic efflux permeability (P_{int}) performed with two time points and normalized with their intracellular concentrations. In our experimental designs, we transfected MDCK-D2 cells with OATP1B1 (20 ng/uL DNA) on the basal site, and BCRP (2-40 ng/uL) on the apical site. Estradiol-17 beta glucuronide was used as the substrate for both transporters and we then compared relationships between BCRP DNA amounts and P_{app}, P_{int}, and protein levels. Protein levels were accurately determined by LC-MS/MS. The results demonstrated that transfected DNA amounts are more linear with protein levels (R^2=0.96) and P_{int} (R^2=0.98), than that with P_{app}.

MATERIALS AND METHODS

- LC-MS/MS for BCRP protein quantification:
  Membrane proteins were extracted with PrteoExtract kit. After extraction, 40 µg membrane proteins were treated with dithiothreitol and iodoacetamide trypsin (1 µg) for reduction and alkylation, and digestion, respectively. After overnight digestion, an internal standard (BCRP stable isotope labeled peptide, SSLLDVLAAR^-$^{13}C_6{}^{15}N_4$) was added and API4000 mass spectrometer was used to determine BCRP protein levels.

- OATP1B1/BCRP co-expressing MDCK-D2 cells:
  MDCK-D2 cells were transfected using in situ transfection technology, Opti-Expression, which allows consistent and effective transfection of polarized cell monolayers. Cells were transfected with plasmids encoding the OATP1B1 (20 ng/µL) with BCRP (2, 5, 10, 20, and 40 ng/µL) transporters. A plasmid encoding green fluorescent protein (GFP) as mock control.

- [^{3}H]-Estradiol-17 beta Glucuronide (E17bG), was used as test compound in current study, and [^{14}C]-Mannitol was used as a control substance for the monolayer integrity in all the assays. Intracellular retention, B→A flux of [^{3}H]-E17bG, and dosing solution were measured.

RESULTS

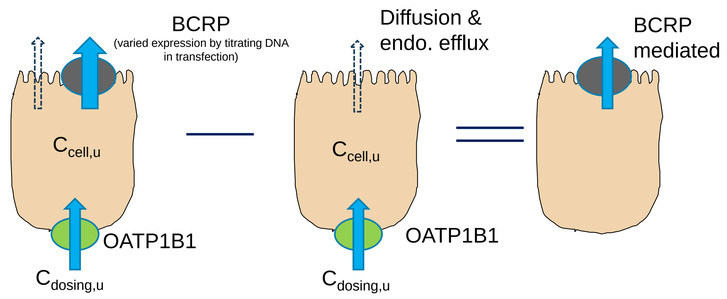

Figure 1. Experimental determination of BCRP mediated apical efflux in OATP1B1/BCRP co-expressing MDCK cells. The human BCRP mediated efflux was expressed in permeability difference between OATP1B1/BCRP and OATP1B1 cell models. Two permeability terms are defined:
Apparent transcellular permeability: $P_{app,B>A}=J_{apical}/C_{dosing,u}$;
Intrinsic apical efflux permeability: $P_{eff,int}=J_{apical}/C_{cell,u}$.

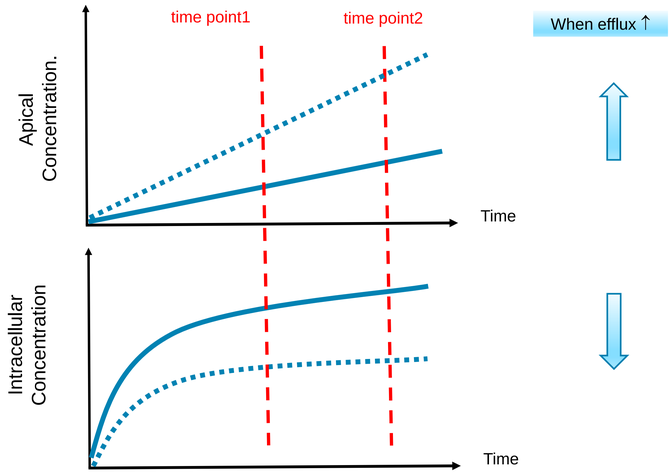

Figure 2. Schematic illustration of dynamic changes in apical and intracellular substrate concentrations when the substrate is dosed from basal side. BCRP expression level not only affects the flux into apical compartment, but also influences substrate intracellular concentration. Measuring intracellular unbound substrate concentration allows assessment of the "true" apical efflux mediated by BCRP. Because intracellular concentration can vary over a wide range, we used a two time-point experiment design to access the apical flux between two time points during which the intracellular concentration reaches quasi-steady state. In such case, apical efflux permeability can be precisely evaluated as $P_{eff,int}=\Delta J_{apical}/AVG(C_{cell,u.})$.

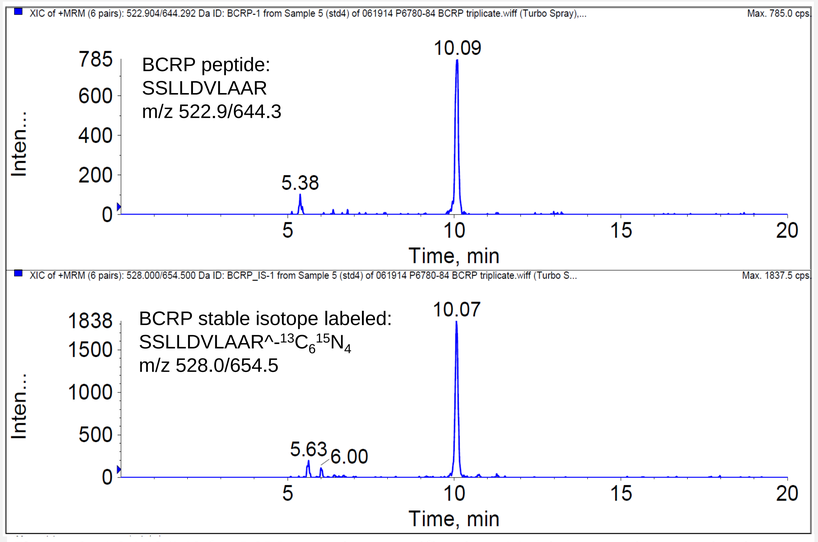

Figure 3. LC-MS/MS for BCRP protein quantification. Multiple reaction monitoring (MRM) chromatograms of the BCRP (SSLLDVLAAR) from the synthetic surrogate substrate peptide (top), and the stable isotope labeled peptide is served as the internal standard (bottom).

Fig. 4A

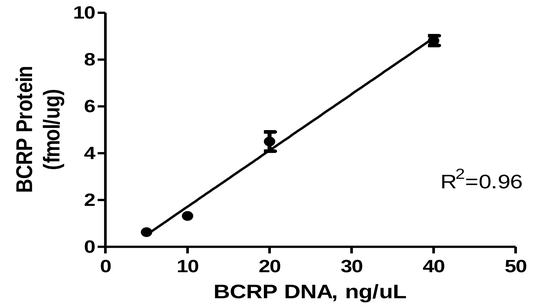

Fig. 4B

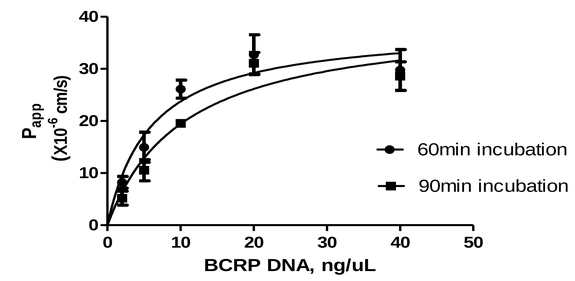

Fig. 4C

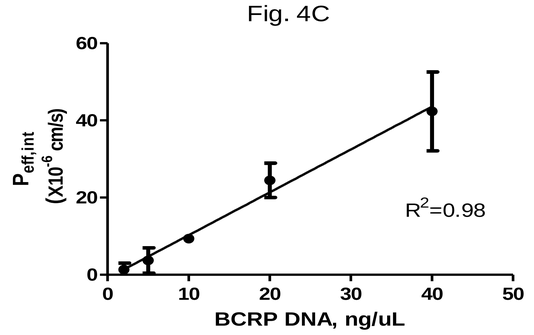

Figure 4. When titrated BCRP DNA in transfection of MDCK cells, the protein levels were determined by LC-MS/MS (4A), apparent transcellular permeability (Papp) of E17bG was calculated with the equation : $P_{app,B>A}=J_{apical}/C_{dosing,u}$ (4B), and intrinsic efflux permeability ($P_{eff,int,\ 60\rightarrow90min}$) of that was calculated with the eauation: $J_{apical}/C_{cell,u}$(4C).

CONCLUSIONS

1 With Opti-Expression transfection technology, protein expression levels are exceptionally linear with transfected DNA amounts (Fig. 4A). With this model, it largely reduces variation between cell types, and different membrane environment when studied in drug transport studies.

2. The relationship between apparent permeability P_{app} and BCRP expression is not linear, which is similar to the observation on P-gp (Ref. 1). In our studies, P_{app} reached maximum saturation at both time points (60 and 90 mins)(Fig. 4B) under high BCRP expression. This is because P_{app} is affected by the transport across both basal and apical membranes, and when apical efflux is high due to increased BCRP expression, the basolateral transport is likely becomes the rate-limiting step (Ref. 2).

3. In contrast, intrinsic apical efflux permeability $P_{eff,int,}$ evaluated with our two-time point transcellular transport method described in this poster, exhibited linear correlation with protein expression (Fig. 4C). Because $P_{eff,int}$ is a direct measure of active efflux activity, independent of basal transport and intracellular binding processes, the use of $P_{eff,int}$ instead of P_{app} in evaluating the kinetics and inhibition of ABC transporters should be considered.

REFERENCE

1. Shirasaka Y, Sakane T, Yamashita S. 2008. Effect of P- Glycoprotein Expression Levels on the Concentration-Dependent Permeability of Drugs to the Cell Membrane. J Pharm Sci. 97(1):553-65.

2. Tachibana T, Kitamura S, Kato M, Mitsui T, Shirasaka Y, Yamashita S, Sugiyama Y. 2010. Model Analysis of the Concentration-Dependent Permeability of P-gp Substrates. Pharm Res. 27(3):442-6.