Poster
M1038

Comparison of Function and Relative Transporter Protein Concentrations in Caco-2 Cells with Single and Double Knockouts of the ABCB1, ABCG2, and ABCC2 Genes

Emile G. Plise, Jonathan Cheong, and Laurent Salphati
Drug Metabolism and Pharmacokinetics, Genentech, Inc. South San Francisco, CA

Genentech
A Member of the Roche Group

ABSTRACT

PURPOSE:
Compare the MDR1, BCRP, and MRP2 transporter function and their relative protein expression levels in Caco-2 wild-type versus single and double knockout cells.

METHODS:
Caco-2 wild-type (wt) cells and cells with single (KO) and/or double (DKO) knockouts for the ABCB1 (MDR1), ABCG2 (BCRP), and ABCC2 (MRP2) genes were purchased from Sigma-Aldrich (St. Louis, MO) in pre-cultured 24-well plates. Cells were dosed with 2.5 µM of prototypical probe substrates [digoxin (MDR1), topotecan (MDR1/BCRP) and vinblastine (MDR1/MRP2)] in HBSS containing 100 µM lucifer yellow. Samples were taken at 45, 90, and 135 minutes and were analyzed on an AB Sciex Qtrap 5500. A ProteoExtract kit was used to extract plasma membrane proteins from cells with the same passage. The proteins were reduced (DTT), alkylated (iodoacytamide) and digested with trypsin (24 h at 37°C). Signature peptide fragments and heavy peptides were used for calibration standards and internal standards, respectively. Peptide samples were analyzed on an Agilent 6460A MS in MRM mode.

RESULTS:
The endogenous transporter expression levels of MDR1, BCRP, and MRP2 in the Caco-2 wt cells were 0.48, 0.91, and 0.83 fmol/µg of plasma membrane protein, respectively. All three substrates exhibited B to A / A to B ratios >14 in the wild-type cells. In the MDR1 KO cells the ratios for the substrates markedly decreased [digoxin (1.3), topotecan (3.8), and vinblastine (2.9)] with ratios >1 suggesting BCRP (topotecan) or MRP2 (vinblastine) involvement. The transporter protein levels for all deleted genes were BLOQ and it appears the knockouts did not cause compensatory changes in the endogenous expression of the transporters examined.

CONCLUSIONS:
The endogenous levels of MDR1, BCRP, and MRP2 in the Caco-2 wt cells are sufficient to transport substrates. The function, protein levels, and the specific gene deletion of these transporters have been confirmed in both the KO and DKO cells. These Caco-2 cell lines could prove to be a valuable tool to study the transport of compounds without the use of non-specific or multiple MDR1, BCRP, and MRP2 inhibitors.

INTRODUCTION

Various members of the ABC transporter family (MDR1, BCRP, and MRP2) play an important role in absorption and distribution of xenobiotics. Caco-2 cells are a widely used model to assess passive permeability and MDR1, BCRP, and MRP2 mediated active efflux of compounds. The expression of multiple transporters in Caco-2 makes it more difficult to clearly determine which transporter is involved. The lack of inhibitor specificity for some transporters can further convolute assay results.

Zinc finger nuclease technology was used in Caco-2 cells to knockout the genes encoding the MDR1, BCRP, and MRP2 transporters. In this study we examined pre-plated Caco-2 cells for transporter function, quantitatively characterized the endogenous transporter protein expression, verified the single and double gene knockout on a protein level, and looked for compensatory changes in the endogenous transporter expression in KO cells.

MATERIALS and METHODS

Figure 1
Cell Culture for Assay Ready 24-well Plates

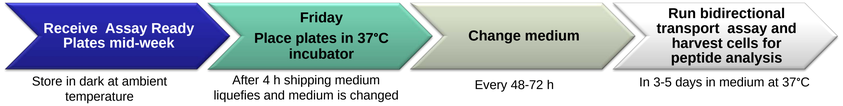


Figure 2
Peptide Preparation and Quantification of Transporters by LC-MS/MS

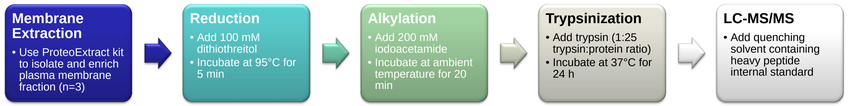


Table 1
Signature Peptides, Heavy Labeled Internal Standard Peptides and MRM Fragmentation

Transporter	Peptide	Parent ion (*m/z* 2)	Product ions (*m/z* 1) 1	2	3
MDR1	NTTGALTTR	467.8	618.3	719.4	820.5
	NTTGALTT**R**	472.8	628.3	729.5	830.5
BCRP	SSLLDVLAAR	522.8	757.5	644.4	529.3
	SSLLDVLAA**R**	527.8	767.4	654.4	539.3
MRP2	LTIIPQDPILFSGSLR	886.1	1330.9	989.5	664.9
	LTIIPQDPILFSGSL**R**	889.5	1337.5	996.6	668.5

Heavy labeled peptides **(blue)** have the same sequence as the light except R was replaced with [$^{13}C_6{}^{15}N_4$]**R**

Bidirectional Transport Experiments

Cells were equilibrated in HBSS for 30 minutes prior to the addition of compound and/or inhibitors (Table 2). All substrates were tested in quadruplicate with lucifer yellow (LY) in the donor chamber. LY permeability was used to assess monolayer integrity throughout the experiment and was read on a Molecular Devices M5 Plate reader (excitation 485 nM, excitation 535 nM). The apparent permeability (Papp, cm/s) was calculated for all compounds using equation 1. The B to A / A to B efflux ratio (equation 2) was calculated using the mean Papp values in both directions. Wells were not used if the LY Papp was ≥ 1.0 x 10^{-6} cm/s.

Table 2
Transporter Inhibitors used in C2BBe1 wt Cells

Transporter	Inhibitor	Conc. (uM)
MDR1	Zosuquidar (ZSQ)	3.0
BCRP	Ko143	1.0
MRP2	MK571	50

Equation 1
Apparent Permeability

$$Papp = \frac{dQ}{dt} \times \frac{1}{C_0} \times \frac{1}{A}$$

where A = the surface area of the insert (0.3 cm^2), C_0 is the initial concentration in the donor well, and *dQ/dt* is the rate.

Equation 2
B to A / A to B Efflux Ratio using Mean Papp Values

$$Efflux\ Ratio = \frac{Mean\ B\ to\ A\ Papp}{Mean\ A\ to\ B\ Papp}$$

RESULTS

Figure 3
Quantitative Transporter Expression of MDR1, BCRP, and MRP2 in C2BBe1 and Knockout Cells

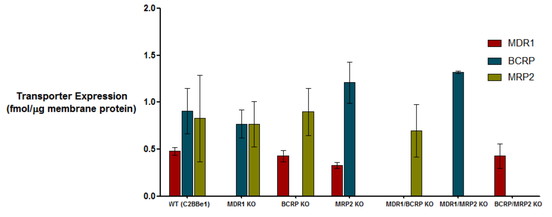
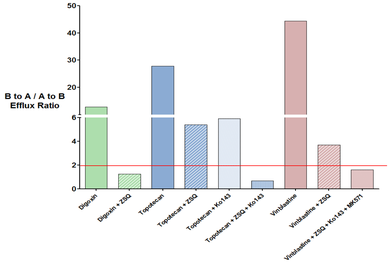
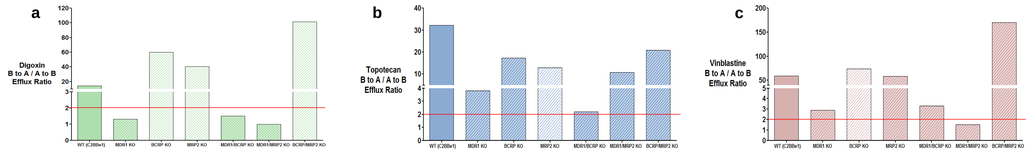


Figure 4
Inhibition of MDR1, BCRP, and MRP2 Efflux in C2BBe1 Cells



Figures 4a, b, and c
Efflux Ratios of Digoxin (a), Topotecan (b), and Vinblastine (c) in the C2BBe1 wt and Knockout Cell Lines



CONCLUSIONS

- The endogenous levels of MDR1, BCRP, and MRP2 in the Caco-2 C2BBe1 wt cells are sufficient to transport substrates.
- All three transporters in the C2BBe1 cell line could be inhibited with standard concentrations of inhibitors.
- The function, protein levels, and the specific gene deletion of these transporters have been confirmed in both the KO and DKO cells.
- These Caco-2 cell lines could prove to be a valuable tool to study the transport of compounds without the use of non-specific or multiple MDR1, BCRP, and MRP2 inhibitors.

Acknowledgements

We would like to thank Michael D. Mitchell from Sigma-Aldrich for providing the Caco-2 cells for this project. We would also like to graciously thank Bhagwat Prasad and Jashvant D. Unadkat from the University of Washington's Research Affiliates Program on Transporters (UWRAPT) for the quantifying transporter peptides.