Evaluation of Efflux Transporter Function Using Caco-2 Transporter Knockout Cell Lines

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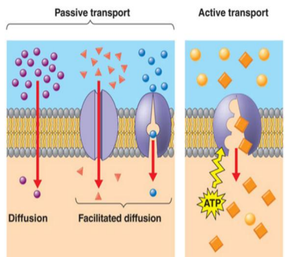
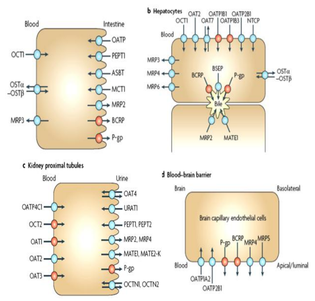
Introduction

- Drug transporters are membrane proteins that influence the ADME (Absorption, Distribution, Metabolism, Elimination) properties of drug molecules at the tissue level.
- Drug molecules can be substrates and/or inhibitors of these transporters and can contribute to clinically relevant dug-drug interactions (DDIs).
- Drug transporters are divided into two subfamilies:
  - **ATP-Binding Cassette (ABC) transporters -** Responsible for the efflux of a drug out of the cell.
    Ex. MDR1 (P-gp), BCRP, and MRP2
  - **Solute-Carrier (SLC) transporters** - Responsible for drug uptake.
    Ex. OATP, OAT and OCTs



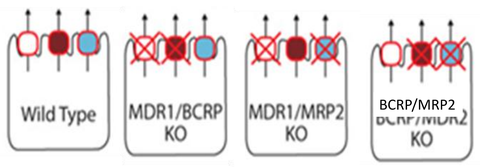
Drug Transporters Associated with ADME



Seven FDA essential transporters are highlighted in red circles. (*International Transporter Consortium* (ITC). 2010)

Overview

- *In vitro* cell models with intestinal epithelial cells like Caco-2 are a well-established method of determining drug permeability as well as substrate and inhibitor specificity of drugs for drug transporters.
- Caco-2 cells also express three important clinically relevant efflux transporters – MDR1 (P-gp), BCRP and MRP2.
- Caco-2 double knockout cells with single-transporter functionality were used in this study.



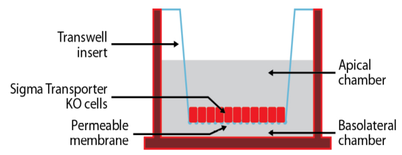
(Sigma Product Catalog, 2011)

Objectives

- Evaluate three different Caco-2 efflux transporter double knock out cell lines using various known efflux substrates.
- Use these KO cell lines as a tool to evaluate efflux transporter specificity for internal research compounds.

Materials and Methods

Cell Culture:
The Caco-2 and Caco-2 double knockout cell lines (Sigma-Aldrich) were seeded on 96-well Millipore®Millicell cell culture plates on a semi-permeable membrane support. Cells were fed every 3-4 days. The plates were used in assay between 21-26 days post-seeding.



Knockout Cells Evaluated:
MDR1/BCRP K.O. – MRP2 Functional
MDRP/MRP2 K.O. – BCRP Functional
BCRP/MRP1 K.O. – MDR1 Functional

Bidirectional Assay Procedure:
The assay was conducted using TECAN® automated liquid handling platform. In brief, all efflux control substrates were prepared at a final testing concentration of 1µM (Digoxin-2µM) in 10mM HBSS with HEPES assay buffer (pH 7.4). Aliquots of test solutions were dispensed in apical or basolateral compartments depending on the directionality of the assay. The plate was then incubated for 90min. Aliquots of samples were taken at time T0 and T90 from donor and T90 from acceptor compartments. Lucifer yellow permeability and trans-epithelial electrical resistance (TEER) were measured to confirm cell monolayer integrity.

Sample Analysis:
Sample analysis was performed using LC-MS/MS methods. Data processing was carried out using Gubbs Mass Spec Utilities Software (Gubbs, Inc. Alpharetta, GA).

Calculations:
Drug permeability in both directions was measured using the following equations:

Apparent Permeability

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

Efflux Ratio

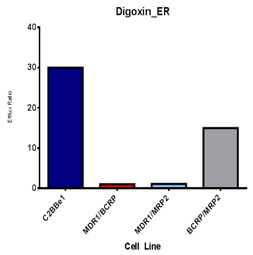
$$ER = \frac{P_{app}(B \rightarrow A)}{P_{app}(A \rightarrow B)}$$

P_{app} is expressed in units of 10^{-6} cm/s, A is the membrane growth area, C_0 is initial donor concentration, and dQ/dT is the slope of concentration vs. time.

An Efflux Ratio ≥ 2 indicates that the compound is an **efflux substrate.**

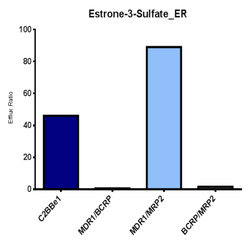
Summary of Results

Summary of Results for Digoxin



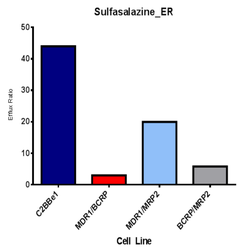
Analyte	Papp (AtoB) (x10^-6 cm/sec)	SD	Papp (BtoA) (x10^-6 cm/sec)	SD	Efflux Ratio	n	Type of cells
Digoxin	0.32	0.22	10	1.5	30	8	C2bbe1
	1.4	0.21	1.5	0.21	1.0	5	MDR1/BCRP K.O.
	1.5	0.52	1.6	0.56	1.1	5	MDR1/MRP2 K.O.
	0.64	0.12	9.7	2.7	15	5	BCRP/MRP2 K.O.

Summary of Results for Estrone-3-Sulfate



Analyte	Papp (AtoB) (x10^-6 cm/sec)	SD	Papp (BtoA) (x10^-6 cm/sec)	SD	Efflux Ratio	n	Type of cells
Estrone-3-Sulfate	0.35	0.29	16	3.4	46	5	C2bbe1
	1.0	0.51	0.62	0.22	0.59	2	MDR1/BCRP K.O.
	0.27	0.16	24	9.7	89	3	MDR1/MRP2 K.O.
	1.0	0.52	1.7	0.092	1.6	2	BCRP/MRP2 K.O.

Summary of Results for Sulfasalazine



Analyte	Papp (AtoB) (x10^-6 cm/sec)	SD	Papp (BtoA) (x10^-6 cm/sec)	SD	Efflux Ratio	n	Type of cells
Sulfasalazine*	0.36	0.22	16	2.3	44	5	C2bbe1
	0.75	0.035	4.4	0.15	3.0	3	MDR1/BCRP K.O.
	0.61	0.46	12	0	20	3	MDR1/MRP2 K.O.
	0.73	0.92	1.6	0.43	5.8	3	BCRP/MRP2 K.O.

*Sulfasalazine, with reference to literature, is said to be an MRP2 substrate. However, our data indicates that this compound is in fact a BCRP substrate

Efflux Ratio Comparisons for Additional Compounds Across the KO Cell Lines

Analyte	Wild Type	MDR1/BCRP K.O.	MDR1/MRP2 K.O.	BCRP/MRP2 K.O.	Transporter Specificity
Digoxin	30	1.0	1.1	15	P-gp
Estrone-3-Sulfate	46	0.59	89	1.6	BCRP
Sulfasalazine*	44	3	20	5.8	BCRP (MRP2)
Fexofenadine	10.0	1.3	1.3	8.0	P-gp
Nitrofurantoin	16	1.7	11	1.1	BCRP
Erythromycin	35	0.66	0.7	9	P-gp
Cladribine	19	1.4	4.9	2.5	BCRP
Cimetidine	17	2.5	38	1.6	BCRP

Efflux data for selected Small Molecule Inhibitors – Set 1

Efflux Ratio Data	C2BBe1 (WT)	MDR1/BCRP KO	MDR1/MRP2 KO	BCRP/MRP2 KO
Compound 1	93	1.6	13	14
Compound 2	78	1.1	20	11
Compound 3	160	1.1	25	27
Compound 4	140	N/A	31	7

Conclusion: Research compounds are substrates of MDR1 and BCRP efflux transporters.

Efflux data for selected Small Molecule Inhibitors – Set2

Efflux Ratio Data	C2BBe1 (WT)	MDR1/BCRP KO	MDR1/MRP2 KO	BCRP/MRP2 KO
Compound A	4	1.8	1.4	2.9
Compound B	520	120	7.2	43
Compound C	16	1.5	16	1.7

Conclusion: Research compounds are substrates of multiple efflux transporters with variable affinity.

Observations

- Three Caco-2 efflux transporter knockout cell lines were successfully evaluated for efflux transporter function.
- The assay accurately identified the substrates for efflux transporters that have been previously reported in the literature (as indicated in the table).

MDR1	BCRP	MRP2
Digoxin	Estrone-3-Sulfate	Sulfasalazine*
Fexofenadine	Nitrofurantoin	
Erythromycin	Cladribine	
Ranitidine	Furosemide	
	Cimetidine	

- Preliminary assay results for research compounds suggest multiple efflux transporters associated with compound efflux.

Conclusions

- Successfully evaluated and validated the Caco-2 efflux transporter knock out cell lines using different substrates.
- The Caco-2 knock out cell lines are important tool to identify specific substrate-transporter interactions thereby predicting possibility of important DDI's.
- Furthermore, these cell lines can be used to generate kinetic data (K_m and V_{max}) and inhibition data (IC_{50}) of research compounds.

References

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