An Overview of In Vitro Models for Studying Transporter Interactions

Pros, Cons and Practical Considerations

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In vitro Transporter Models

- Membrane Based Assays
  - ATPase assay
  - Vesicular transport assay

- Cell Based Assays
  - Uptake assays (suspended hepatocytes, transfected or transduced cell lines)
  - Bi-directional transport assay in polarized cell lines
  - Sandwich cultured hepatocytes
  - Dye efflux assay

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Membrane Vesicles

Origin

- Isolated primary cells (low expression level)
- Transduced insect cells: (insect background, high expression level)
  - baculoviral expression system
  - Sf9 (*Spodoptera frugiperda*)
  - Hi5 (*Trichopulsia ni*)
- Transduced mammalian cell line (high expression level, mammalian background)
- Selected mammalian cell line (mammalian background)
- Artificial membrane vesicles

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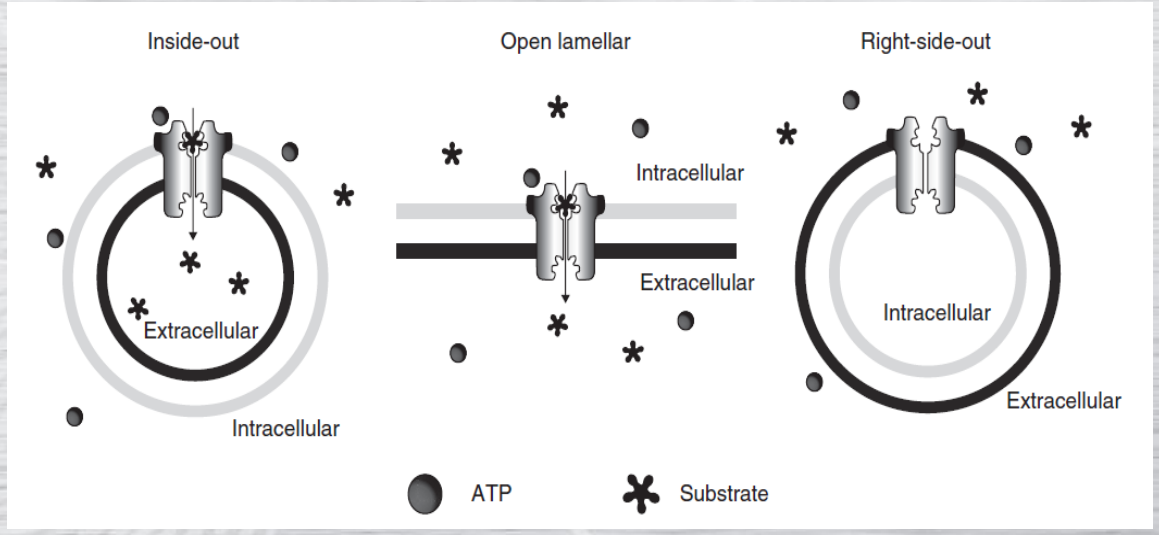
Membrane Vesicles

Method

- Original protocol Steck, *et al.* 1970
- Buffers with low ionic strength and divalent ions
- Homogenization methods: nitrogen cavitation, Potter-Elvehjem
- Ultracentrifuge steps
- Crude membrane fraction → stored at -80°C



Glavinas *et al* 2008

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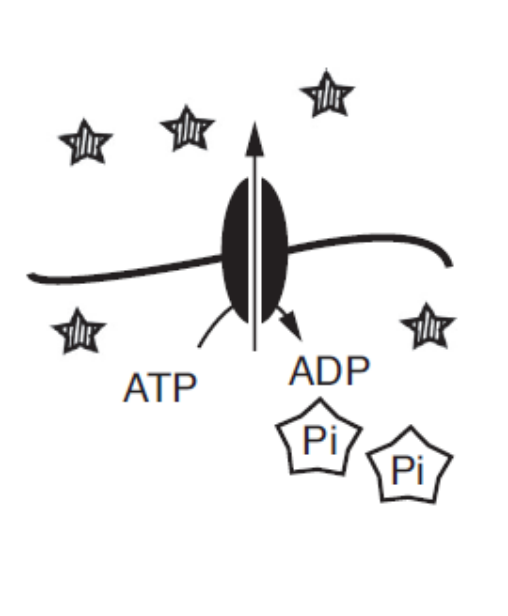
ATPase Model

- ✓ Suitable for ABC transporters only (MDR1, BCRP, MRPs)
- ✓ Inexpensive, non-radioactive
- ✓ Indication on the nature of interaction (esp. highly permeable drugs)



- × Indirect assay (not measuring actual transport)
- × Not accepted by regulatory agencies
- × Pi liberated at low concentration may not be detectable

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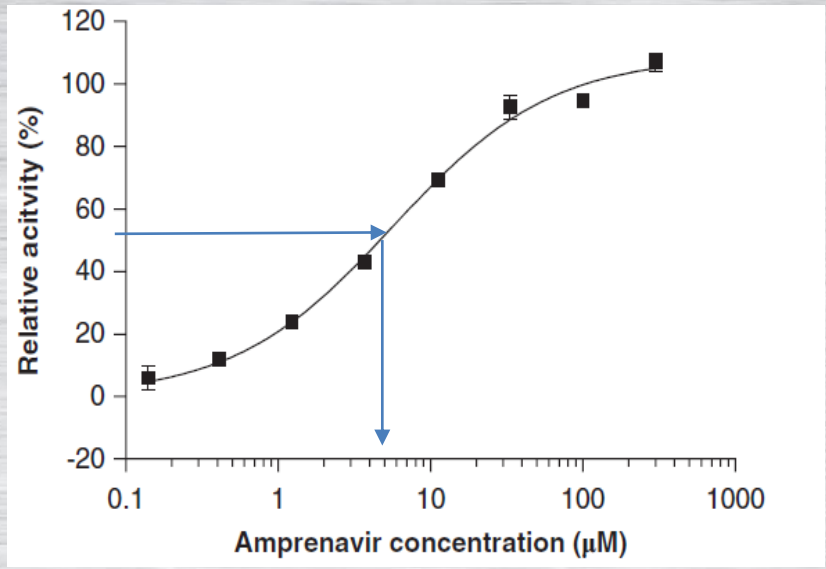
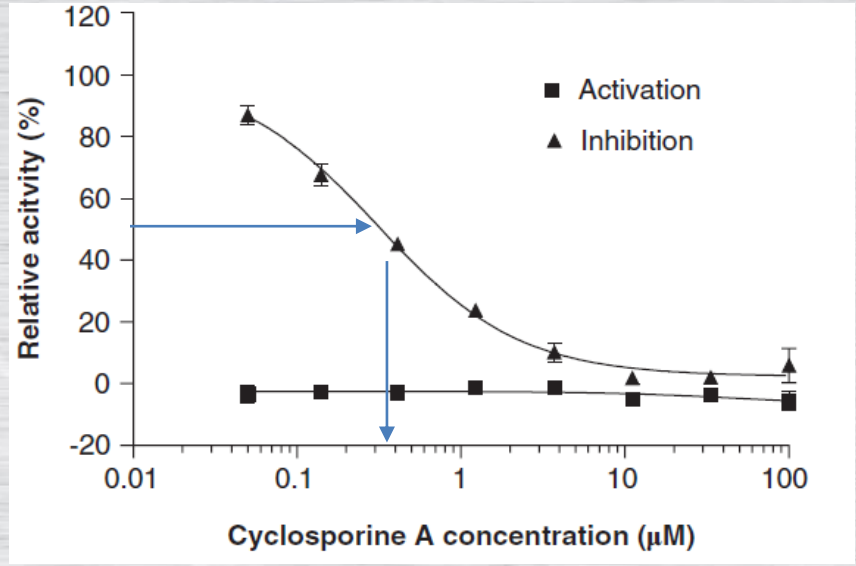
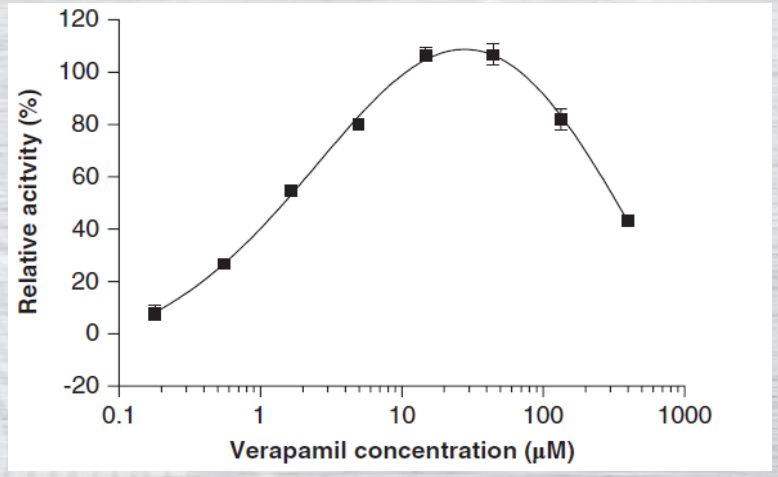
ATPase Model







<u>Outcome</u>

- EC50 and/or IC50

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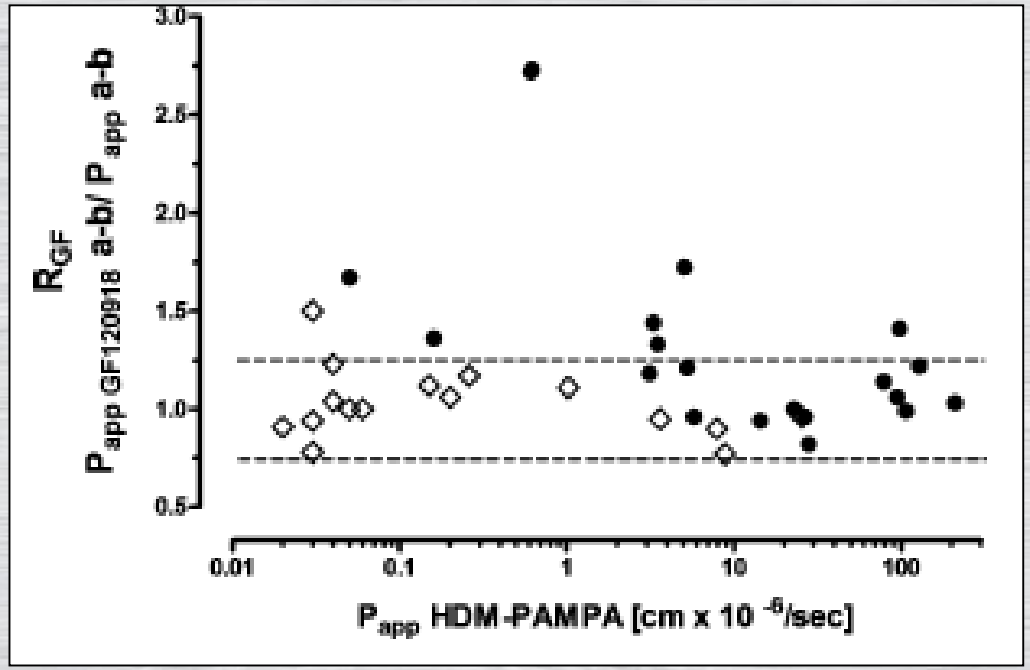
In Vitro P-gp Assays – Correlation Analysis

Von Richter *et al* 2008



Transporter effect in monolayer efflux assay

No transporter effect in monolayer efflux assay

- compounds that show ATPase activation

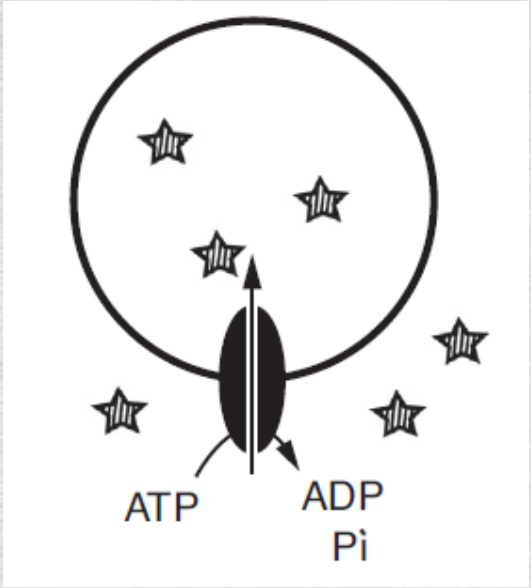
◊ compounds that show no ATPase activation

Polli *et al* 2001

Class	EAC Result	Number of Compounds	Mean P_{app} A → B	Median P_{app} A → B	Range of P_{app} A → B	Remarks
				nm/s		
IIA nontransported substrates	NYY	10	535	598	316–714	Very high P_{app} A → B 10/10 with P_{app} >300 nm/s

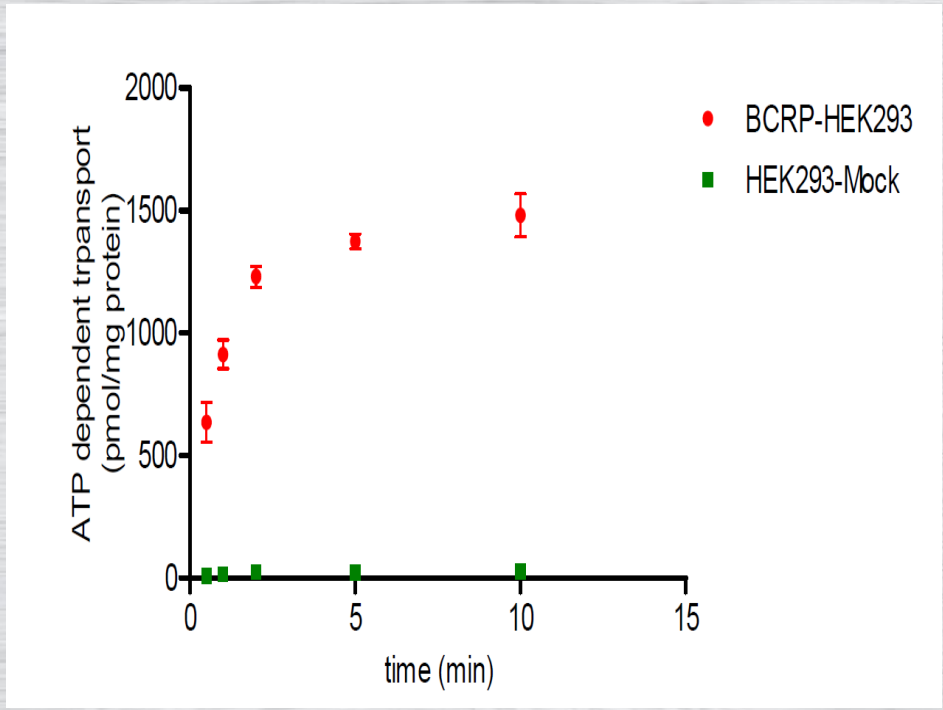
Vesicular Transport Assay

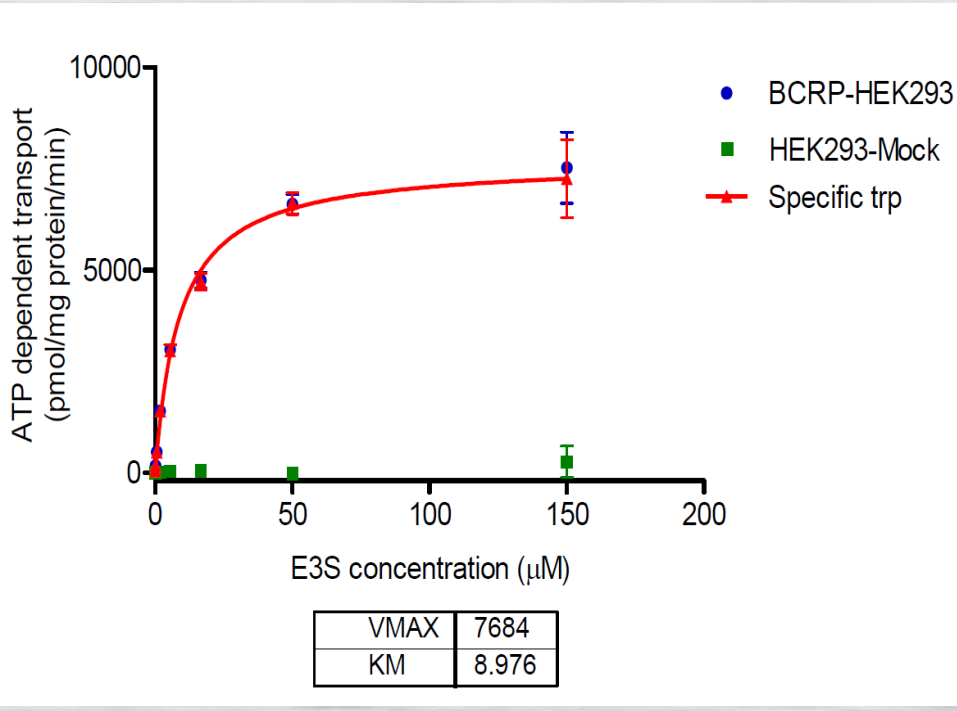
- ✓ Quick, simple, and high throughput
- ✓ Flexible readout (LSc, Fluo, LC/MS)
- ✓ Inhibition mode: permeability is not a factor
- ✓ Mammalian membrane vesicles available, providing more physiologically relevant system
- × Substrate testing only for low permeability compounds



Substrate Mode

Direct Measurement





VMAX	7684
KM	8.976

<u>Outcome</u>

Michaelis-Menten kinetics → K_m and V_{max} determination

$$V_0 = \frac{V_{max}[S]}{(K_M + [S])}$$

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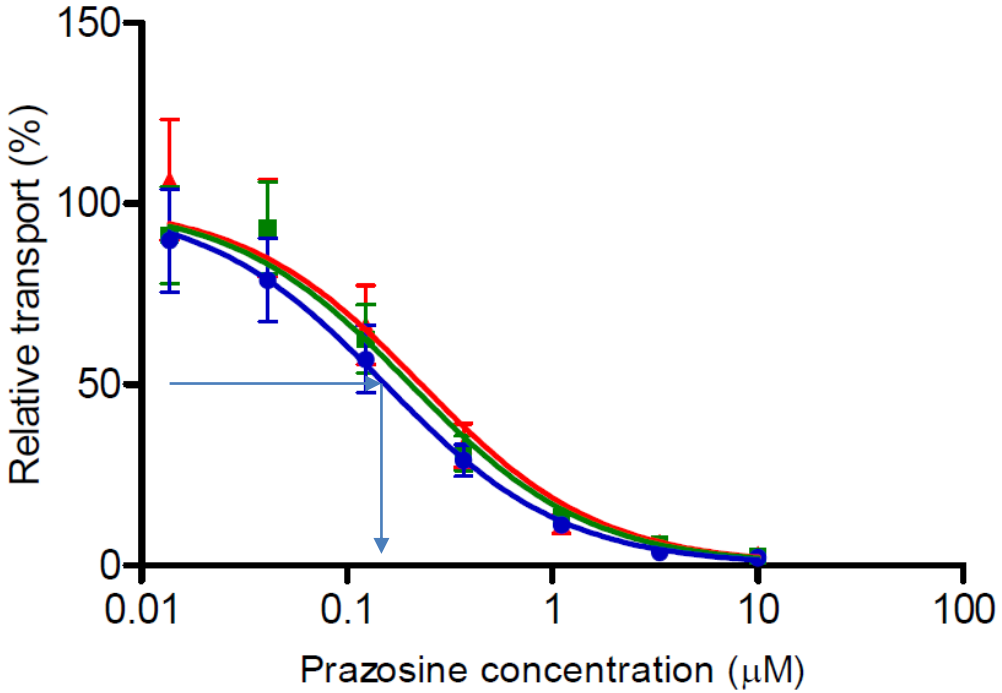
Inhibition Mode



	Exp1	Exp2	Exp3
IC50	0.2306	0.2045	0.1541

<u>Outcome</u>

- Probe substrate vs unknown TA
- IC_{50} (or K_i)
- Max inhibition

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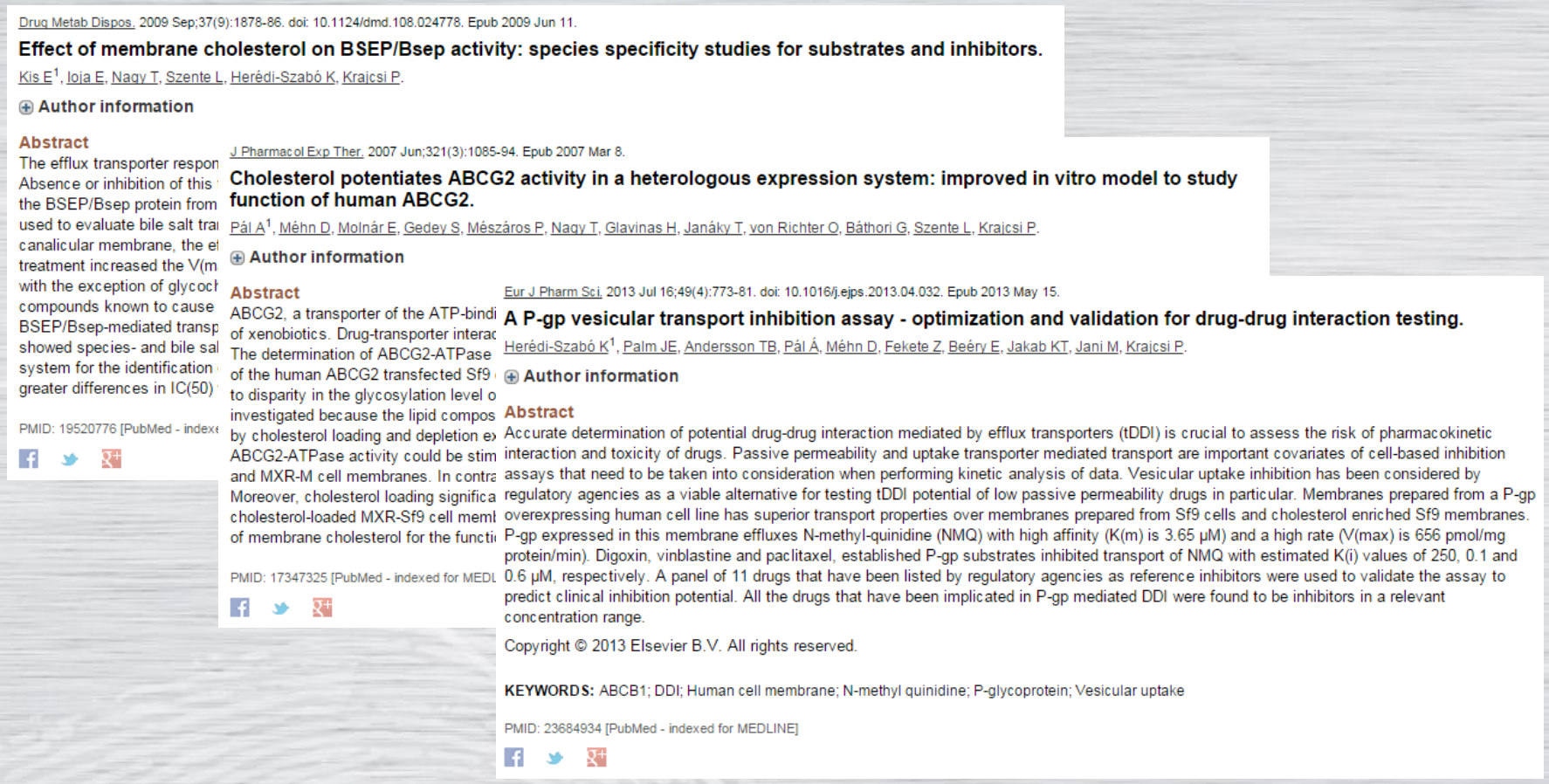
Insect or Mammalian Vesicles?



Drug Metab Dispos. 2009 Sep;37(9):1878-86. doi: 10.1124/dmd.108.024778. Epub 2009 Jun 11.

Effect of membrane cholesterol on BSEP/Bsep activity: species specificity studies for substrates and inhibitors.

Kis E[1], Ioja E, Nagy T, Szente L, Herédi-Szabó K, Krajcsi P.

Author information

Abstract

The efflux transporter respon
Absence or inhibition of this
the BSEP/Bsep protein from
used to evaluate bile salt tra
canalicular membrane, the et
treatment increased the V(m
with the exception of glycoch
compounds known to cause
BSEP/Bsep-mediated transp
showed species- and bile sal
system for the identification
greater differences in IC(50)

PMID: 19520776 [PubMed - index

J Pharmacol Exp Ther. 2007 Jun;321(3):1085-94. Epub 2007 Mar 8.

Cholesterol potentiates ABCG2 activity in a heterologous expression system: improved in vitro model to study function of human ABCG2.

Pál A[1], Méhn D, Molnár E, Gedey S, Mészáros P, Nagy T, Glavinas H, Janáky T, von Richter O, Báthori G, Szente L, Krajcsi P.

Author information

Abstract

ABCG2, a transporter of the ATP-bindi
of xenobiotics. Drug-transporter interac
The determination of ABCG2-ATPase
of the human ABCG2 transfected Sf9
to disparity in the glycosylation level o
investigated because the lipid compos
by cholesterol loading and depletion ex
ABCG2-ATPase activity could be stim
and MXR-M cell membranes. In contra
Moreover, cholesterol loading significa
cholesterol-loaded MXR-Sf9 cell memb
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PMID: 17347325 [PubMed - indexed for MEDL

Eur J Pharm Sci. 2013 Jul 16;49(4):773-81. doi: 10.1016/j.ejps.2013.04.032. Epub 2013 May 15.

A P-gp vesicular transport inhibition assay - optimization and validation for drug-drug interaction testing.

Herédi-Szabó K[1], Palm JE, Andersson TB, Pál Á, Méhn D, Fekete Z, Beéry E, Jakab KT, Jani M, Krajcsi P.

Author information

Abstract

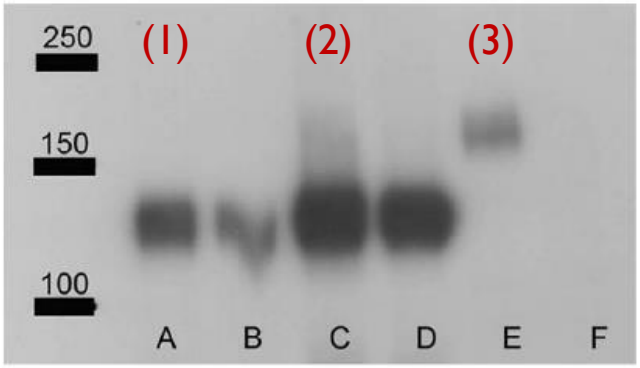
Accurate determination of potential drug-drug interaction mediated by efflux transporters (tDDI) is crucial to assess the risk of pharmacokinetic interaction and toxicity of drugs. Passive permeability and uptake transporter mediated transport are important covariates of cell-based inhibition assays that need to be taken into consideration when performing kinetic analysis of data. Vesicular uptake inhibition has been considered by regulatory agencies as a viable alternative for testing tDDI potential of low passive permeability drugs in particular. Membranes prepared from a P-gp overexpressing human cell line has superior transport properties over membranes prepared from Sf9 cells and cholesterol enriched Sf9 membranes. P-gp expressed in this membrane effluxes N-methyl-quinidine (NMQ) with high affinity (K(m) is 3.65 μM) and a high rate (V(max) is 656 pmol/mg protein/min). Digoxin, vinblastine and paclitaxel, established P-gp substrates inhibited transport of NMQ with estimated K(i) values of 250, 0.1 and 0.6 μM, respectively. A panel of 11 drugs that have been listed by regulatory agencies as reference inhibitors were used to validate the assay to predict clinical inhibition potential. All the drugs that have been implicated in P-gp mediated DDI were found to be inhibitors in a relevant concentration range.

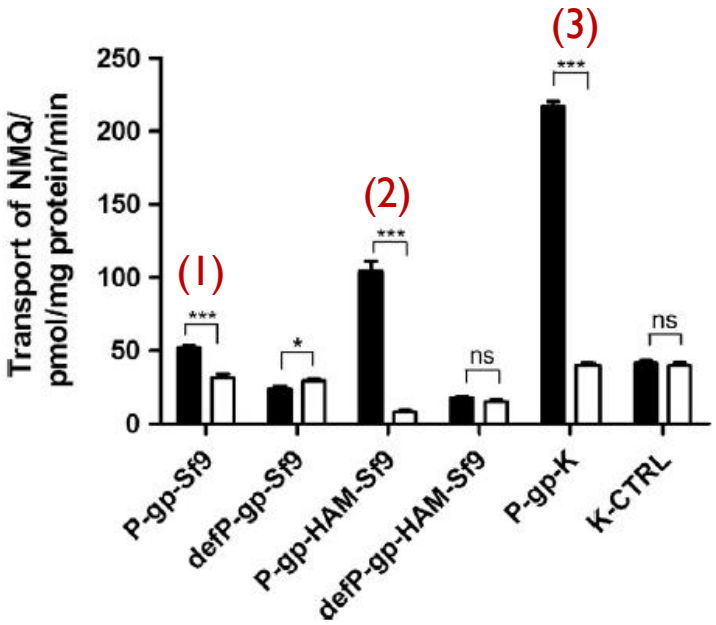
KEYWORDS: ABCB1; DDI; Human cell membrane; N-methyl quinidine; P-glycoprotein; Vesicular uptake

PMID: 23684934 [PubMed - indexed for MEDLINE]

- Solvo has shown the importance of cholesterol and/or improved transport activity in mammalian membranes for multiple efflux transporters- BSEP, BCRP, MDR1

Insect or Mammalian Vesicles?





Transporter **protein expression** is higher in insect cell membranes

-Note that proteins run at different sizes due to full post-translational modification (ie. full glycosylation) in mammalian cells

However, transporter **activity** is higher in mammalian cell membranes, even after loading the insect membranes with cholesterol

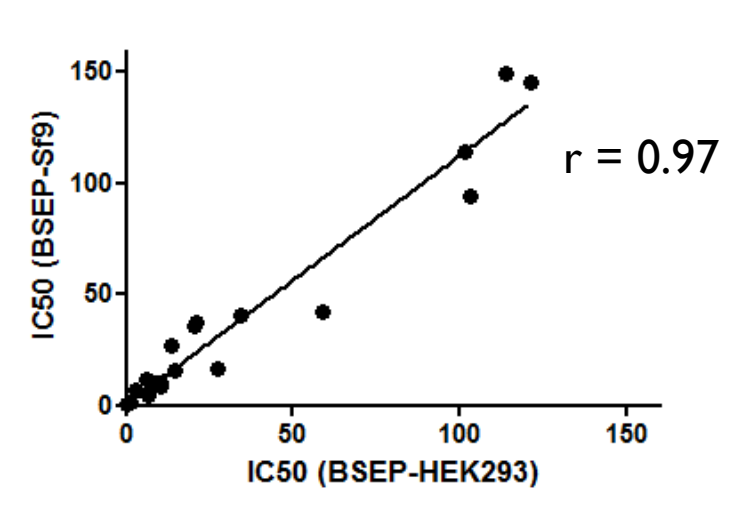
(1) MDR1 insect membrane

(2) Cholesterol-loaded MDR1 insect membrane

(3) MDR1 mammalian membrane

Herédi-Szabó *et al.* (2013) *Eur J Pharm Sci*

Why Use HEK293 Vesicles?

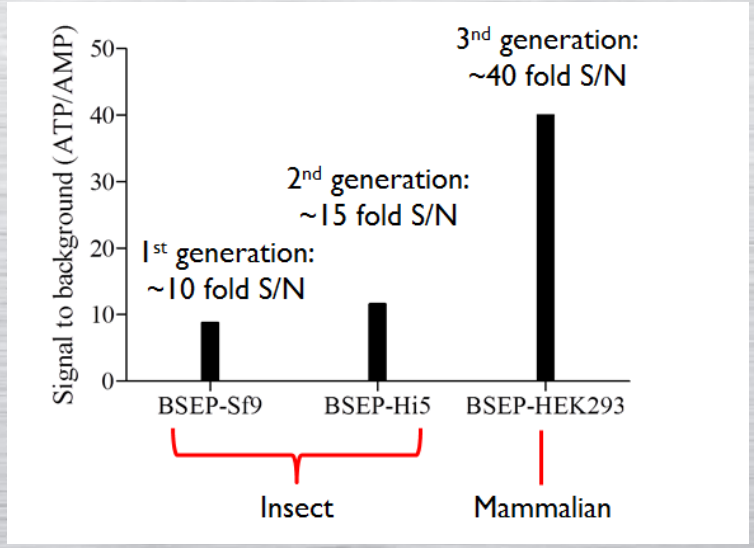


IC50 values correlate well between BSEP-Sf9 and BSEP-HEK293

Comparison study using 31 reference compounds show no difference in IC50 values using BSEP-Sf9 or BSEP-HEK293 vesicles

Superior assay performance

~40-fold dynamic range using BSEP-HEK293 vesicles, compared to ~10-fold using traditional Sf9 insect membranes



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Cell Lines

- **Primary cells**
  - Can be isolated from intact tissue

- **Cell lines without recombinant transporters**
  - Immortalized polarized cell lines (vectorial transport, Caco-2)
  - Chemically selected cell lines overexpressing certain transporter (dye efflux assays, K562-MDR)

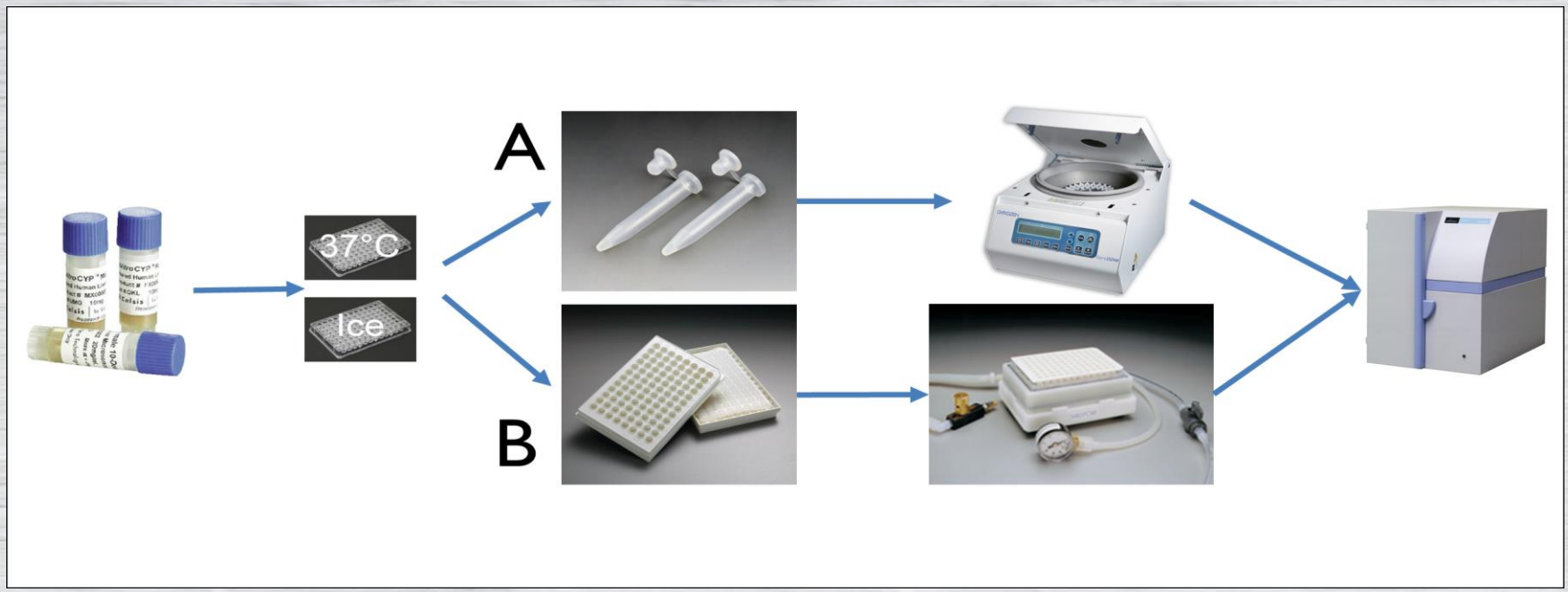
- **Transfected/transduced cell lines**
  - Stably or transiently expressed recombinant transporter in various cell lines
  - Single or double transfection/transduction (efflux and/or uptake transporters)
  - Cell lines used for transfection/transduction:

    MDCK II, LLC-PK1, HEK293, CHO

Pros and Cons of Cell Lines

Type	Example	Transporters	Pros and Cons
Primary	Rat Brain Endothelial cells	- P-gp - BCRP	+ Phyisiologically relevant - Cells are leaky - Low signals (expression) - No specificity
Immortalized	Caco-2	- P-gp - BCRP	+ Phyisiologically relevant + Tight monolayers + Good signals - No specificity, inhibitors needed
Single Transfected/Transduced	MDCKII	- P-gp - BCRP	+ Tight monolayers + Good signals + Specific - Multiple cell lines - Background signals
Double Transfected/Transduced	MDCKII	Efflux + Uptake Multiple efflux Multiple uptake	Lack of specificity, many controls needed, but sometimes only option

Suspension Hepatocyte Uptake Assay



- ✓ Quick and easy assay
- ✓ Hepatocytes can be cryopreserved
- ✓ Good tools for identifying substrate of hepatic uptake transporters
- ✓ Assess the contribution of passive vs active processes

- × Individual transporter can not be determined
- × Not suitable for measuring canalicular efflux
- × Differences between donors (pooled donors)

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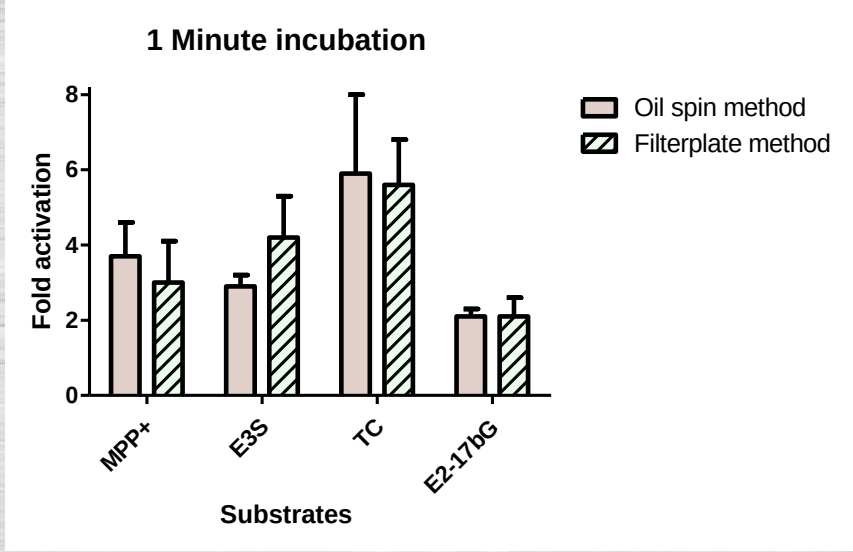
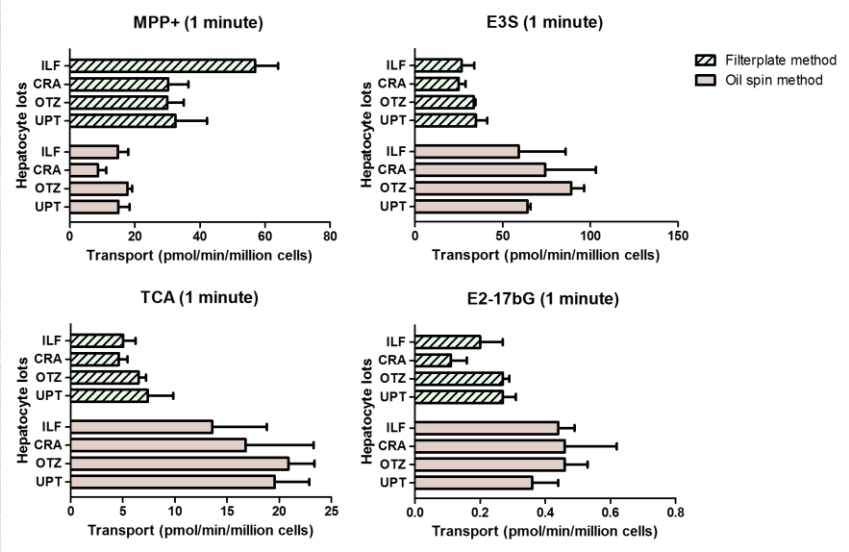
Suspension Hepatocyte Uptake Assay





Outcome:

- Uptake of TA (passive vs active)

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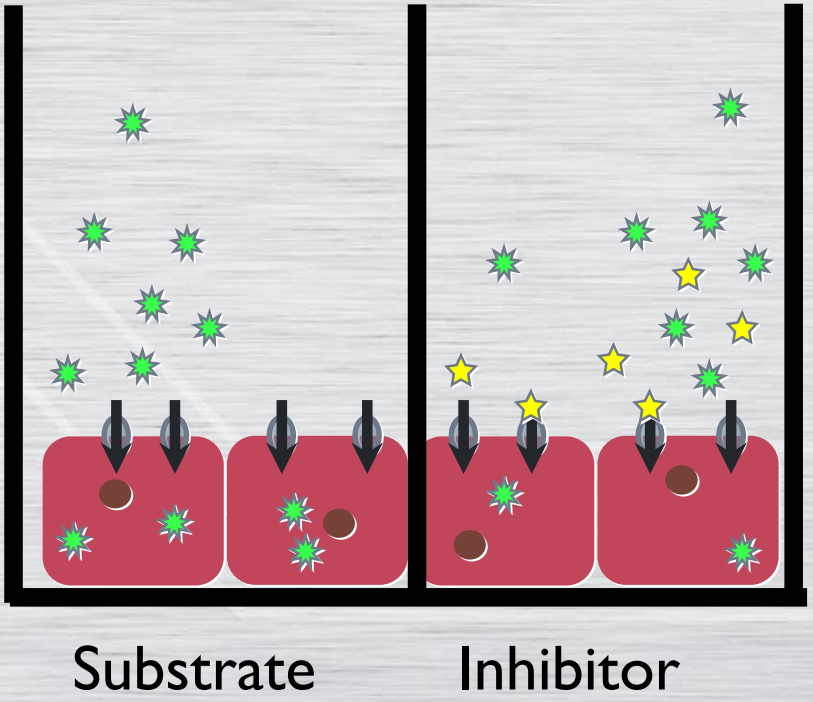
Transfected/Transduced Cell Lines Uptake Assays

- ✓ Quick and easy assay
- ✓ Flexible readout (LSc, Fluo,LC/MS)
- ✓ Inhibition mode: permeability is not a factor
- ✓ Various expression systems



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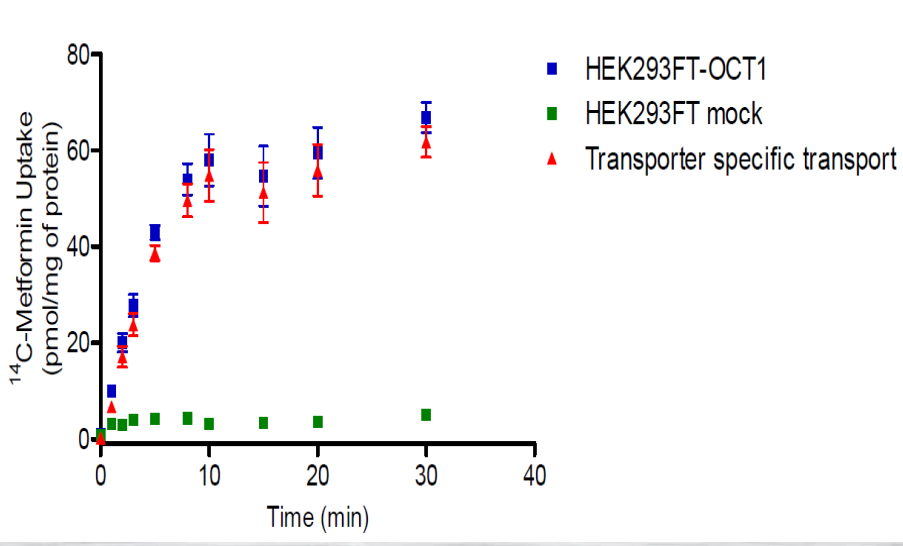
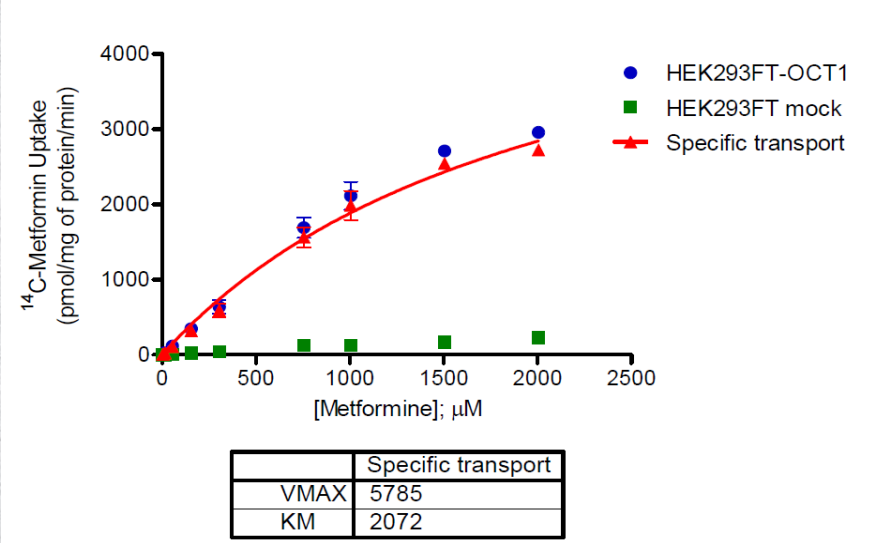
Substrate Mode





	Specific transport
VMAX	5785
KM	2072

- Fold accumulation over control cells (≥2) ± inhibitor

<u>Outcome</u>

- Substrate or not
- Kinetic parameters (linear phase of uptake with time, K_m and V_{max})

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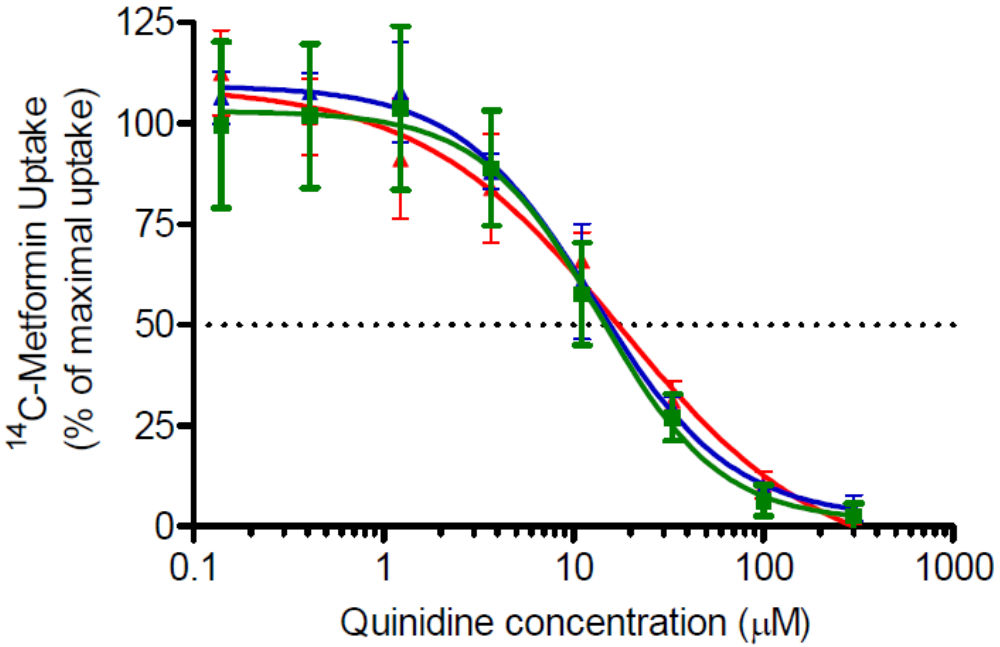
Inhibition Mode



	exp 1	exp 2	exp 3
IC50	17.62	13.05	13.85

- Probe substrate vs unknown TA

<u>Outcome</u>

- Max inhibition (solubility)
- IC_{50} (or K_i)

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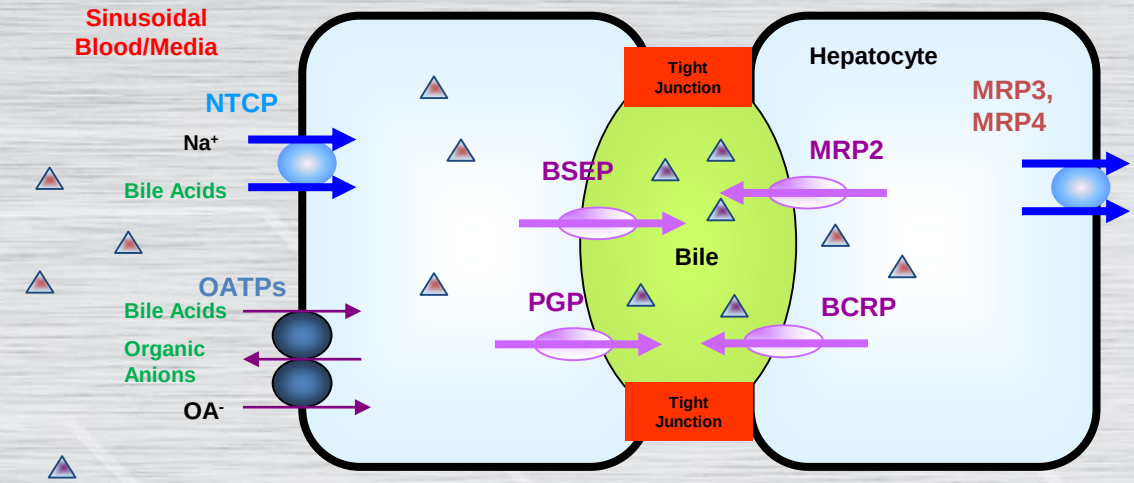
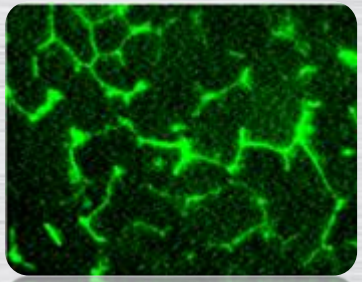
Sandwich Cultured Hepatocytes





Fluorescence microscopy image visualizing bile canaliculi using CDCF, a fluorescent probe accumulating in the bile canaliculi.

- ✓ Hepatobiliary disposition of drugs and metabolites
- ✓ Potential DDI
- ✓ Hepatic drug metabolism
- ✓ Direct toxicity measurement
- ✓ Basolateral uptake transporters are maintained
- ✓ Species differences (preclinical sp for human *in vivo* prediction)
- × Inter-donor differences
- × Matrigel (batch to batch variability, drugs must penetrate)

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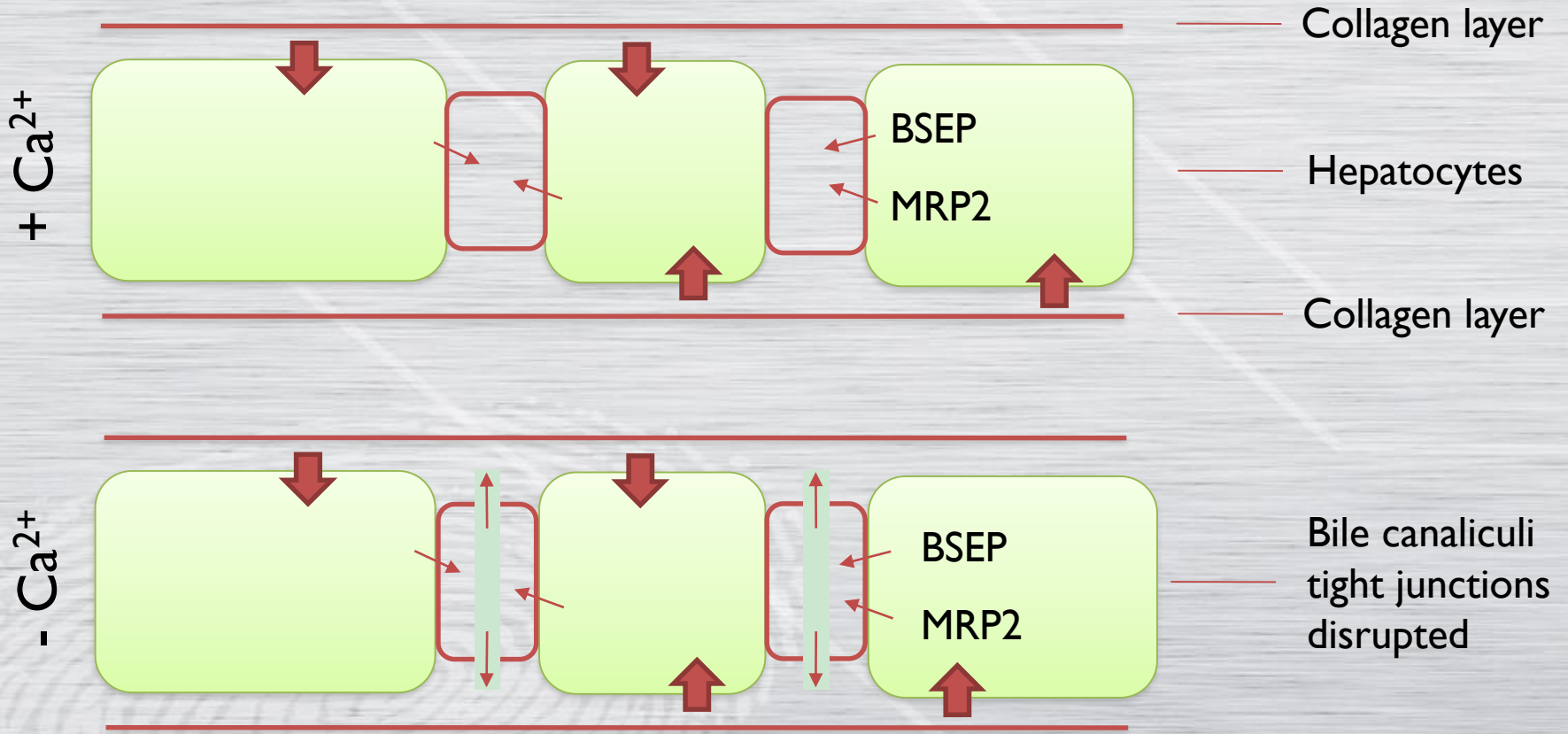
Sandwich Cultured Hepatocytes

B-Clear® Technology

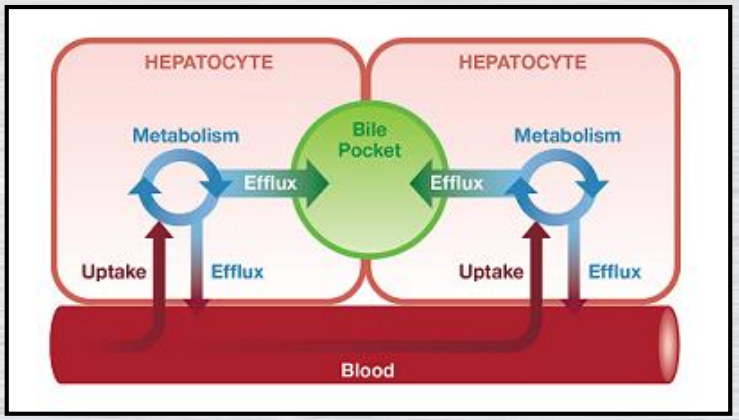
- Determine Biliary Efflux Rate by measuring in the presence and absence of Ca^{2+}
- Measure hepatic uptake

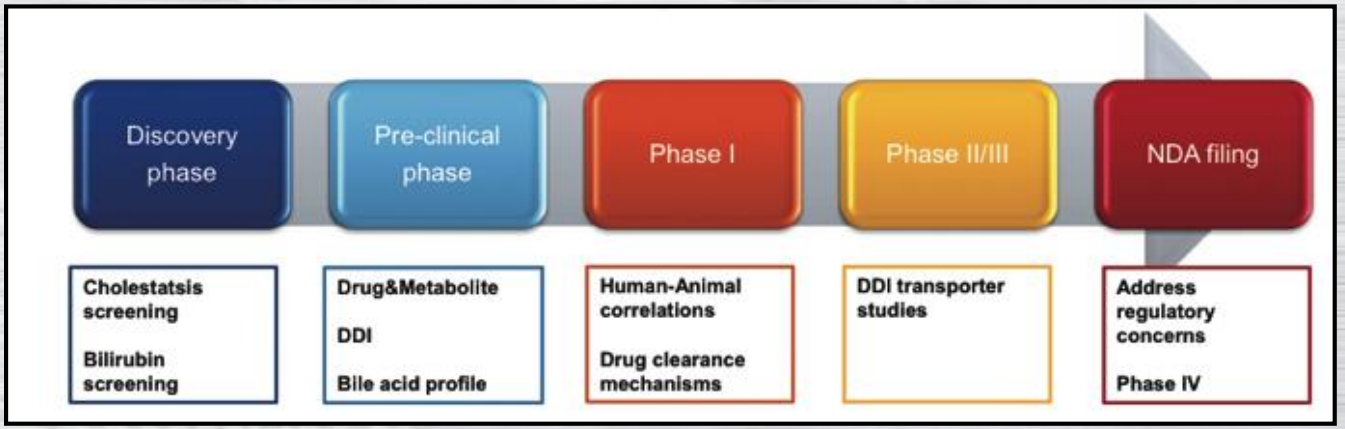


Sandwich Cultured Hepatocytes

B-Clear® Technology

- SOLVO partnering with BioIVT
- Hepatocytes
  - Human, rat, and mouse
- Provides info on all three clearance pathways
  - Efflux
  - Uptake
  - Metabolism

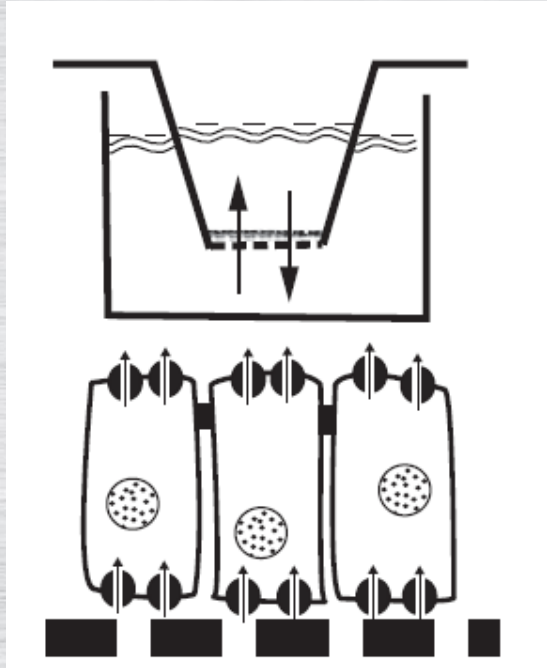




Webinar available online at- http://www.solvobiotech.com/webinars/understanding-hepatobiliary-disposition-the-importance-of-an-integrative-pl

Bi-Directional Transport Assay

- ✓ Single and double transfectant (MDCKII, LLC-PK1), or Caco-2
- ✓ Gold standard for modelling permeability
- ✓ Addresses active transport versus passive diffusion
- ✓ Various expression systems



- × Not suitable for low permeability compounds
- × Lab-to-lab variability in expression level

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Output

- P_{app} value (refers to permeability)
- Efflux Ratio (ER) = P_{app}B-A/P_{app}A-B or P_{app}B-A-P_{app}A-B
- Substrate: ER>2 and can be inhibited by a reference inhibitor
- Using a probe substrate inhibition potential (IC_{50}) can be calculated

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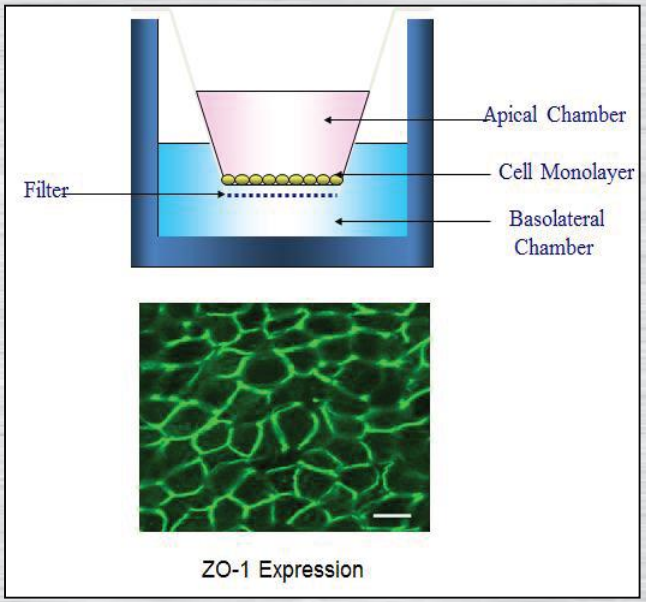
Proximal Tubule Cell Model

- Cells isolated from fresh, normal kidney, isolated <18 hours ex vivo
- Cells grown on Permeable filter HTS Transwell membranes to generate fully functional differentiated monolayers with TEER of ~140-180 Ω.cm²
- Reliable and regular supply of validated monolayers (1-2 kidneys/week)



Webinar available online at- http://www.solvobiotech.com/webinars/predictive-cross-species-proximal-tubule-cell-models-for-drug-development-a

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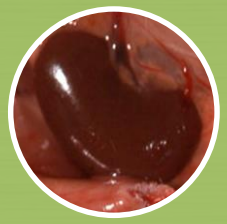
Brown CDA; Sayer R; Windass AS; Haslam IS; De Broe ME; D'Haese PC; Verhulst A (2008). Toxicol & Applied Pharmacol, 233: 428-438.
Huls M; Brown CDA; Windass AS; Sayer R; Van Den Heuvel JJMW; Heemskerk S; Russel FGM; Masereeuw R. (2008) Kid Int, 73: 220-225.
Verhulst A; Sayer R; De Broe ME; D'Haese PC; Brown CDA. (2008) Mol Pharmacol, 74:1084-1091

Renal Proximal Tubule Cell Monolayer

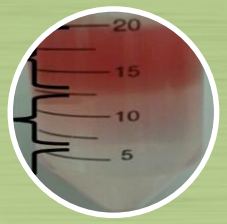


Cortex excised

Cortical slices taken from rat or human kidneys and minced.

Collagenase digestion

Minced tissue incubated with collagenase for 2 hours at 37 °C.



Density Separation

PTCs separated from heterogeneous cell populations using Percoll gradients.

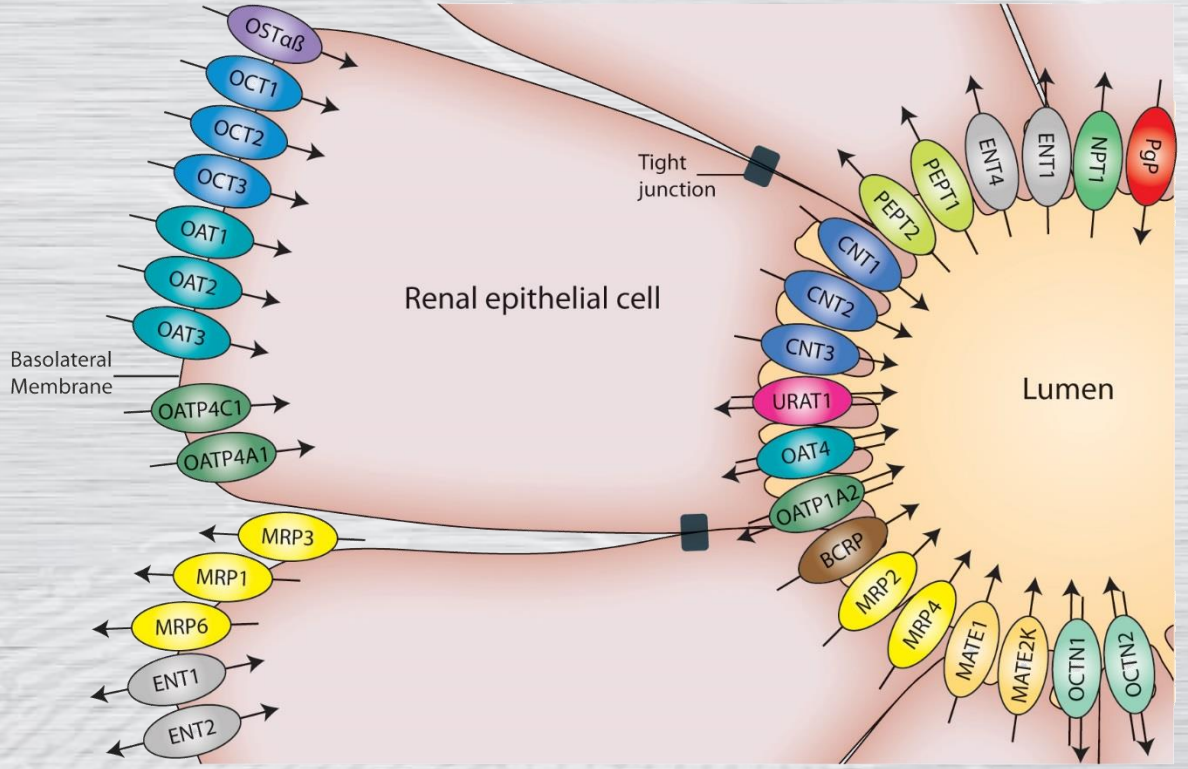
Cell culture

Isolated PTCs seeded on plastics and Transwell inserts and grown until confluent monolayer formation.

- ✓ Key renal transporters are expressed
- ✓ Potential nephrotoxicity
- ✓ Transporter mediated DDI
- ✓ Measuring physiological substrates (glucose, phosphate, urate)

- × Low S/N ratio compared to other system
- × Specific transporter can not be identified (lack of specific inhibitors)

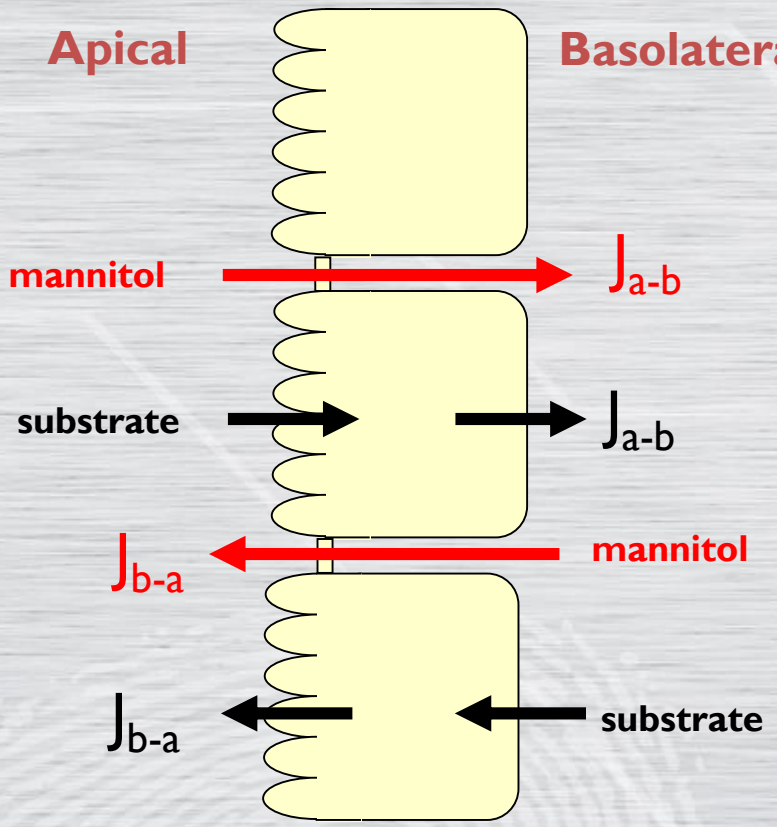
Summary of Drug Transporters in Human PTC Monolayers



R = qPCR P = protein F = Function

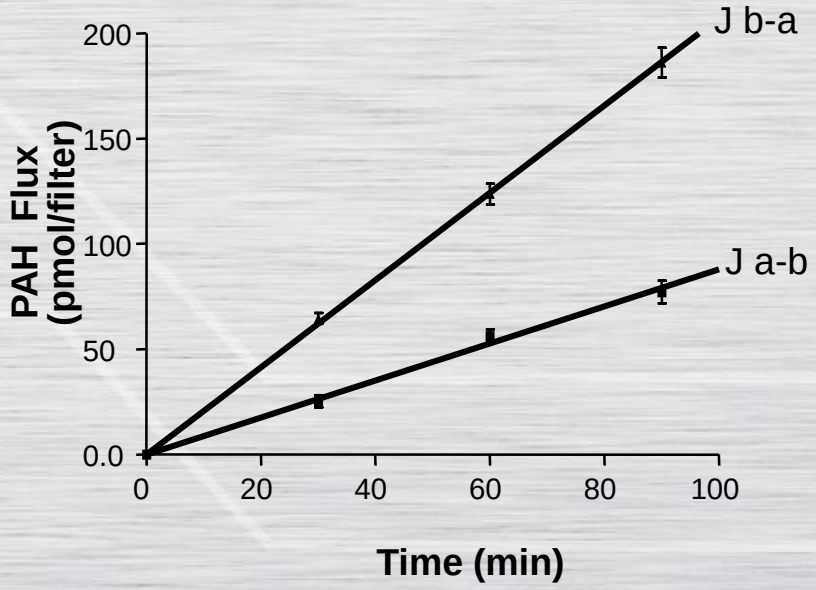
Transporter	Data
URAT1	R, F
GLUT9	R, F
OAT1	R, P, F
OAT3	R, P, F
OATP4C1	R, F
OCTN1	R
OCTN2	R, F
OCT1	R
OCT2	R, F
OCT3	R
MATE1	R, F
MATE2-K	R, F
MDR1	R, P, F
MRP2	R, F
MRP4	R, F
BCRP	R, P, F

Measurement of Trans-epithelial Fluxes



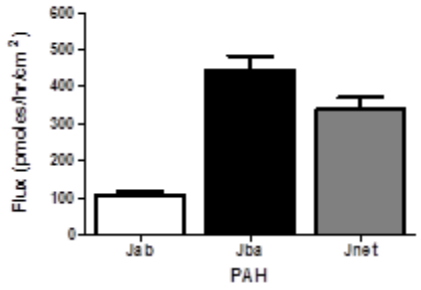
Transcellular Flux

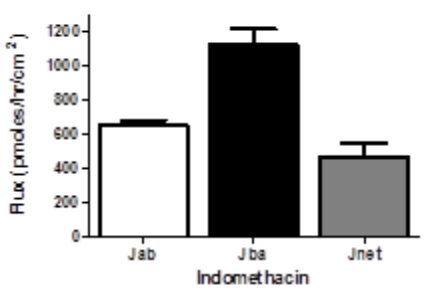
Paracellular Flux

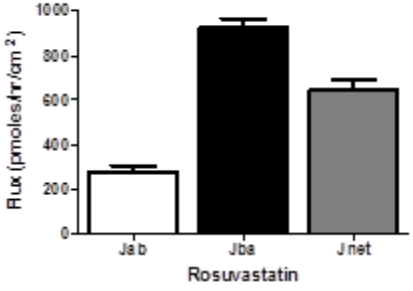


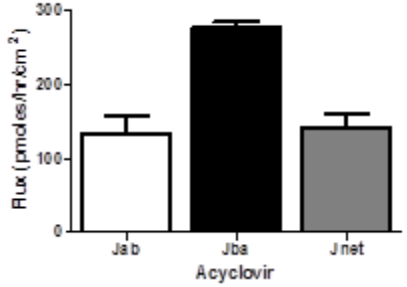
Brown et al Toxicol Appl Pharm 233 2008

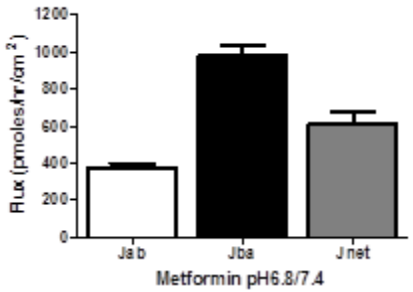
Handling a range of substrates...

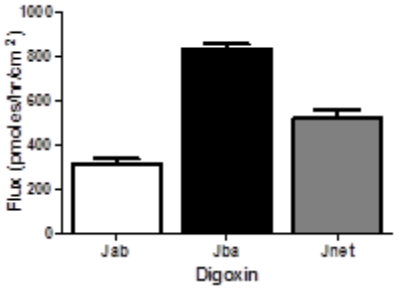


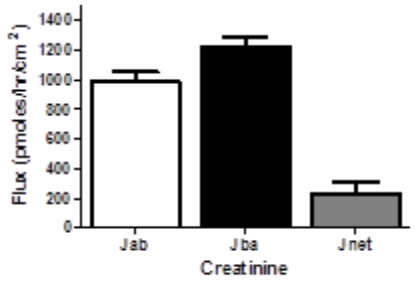


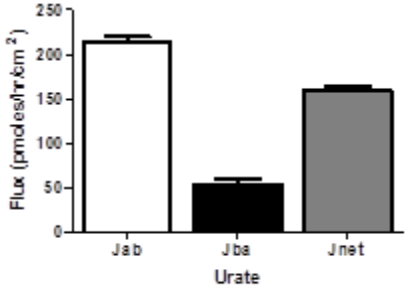


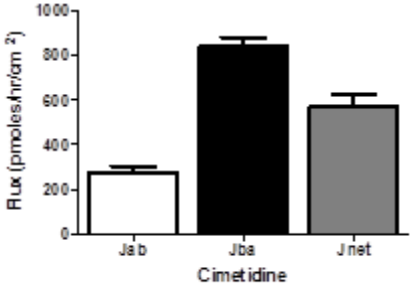












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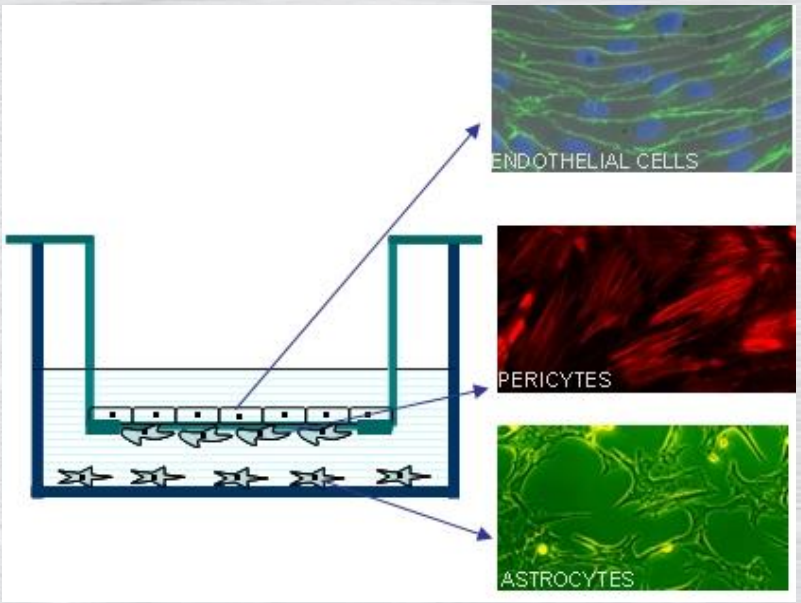
Proximal Tubule Cell Model

- Flux measurement, including determination of paracellular contribution to total flux.
- Net transport measurements.
- Measurement of intracellular drug and metabolite concentrations.
- Mechanistic understanding of the role and importance of individual transporters to the renal clearance pathway.
- Identification of potential transporter-mediated drug-drug interactions.
- Investigation of nephrotoxic potential using a panel of early damage biomarkers.

In Vitro Methods - Brain

- Brain capillary endothelial cells
  - Triple co-culture



✓ Physiologically close to *in vivo* BBB phenotype

× Can be variable, depending on the isolation (inter-batch differences)
× Low TEER values
× Species differences

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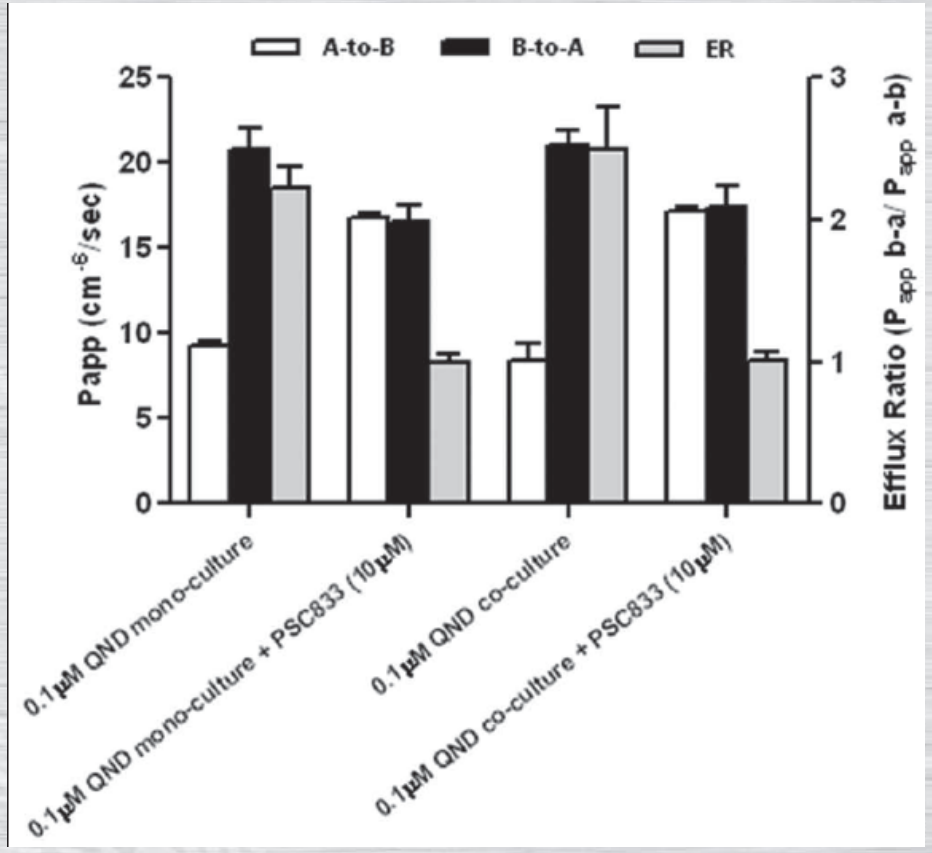
Primary Brain Endothelial Cell Culture



Outcome:

- Brain penetration (low permeability or transporter interaction)

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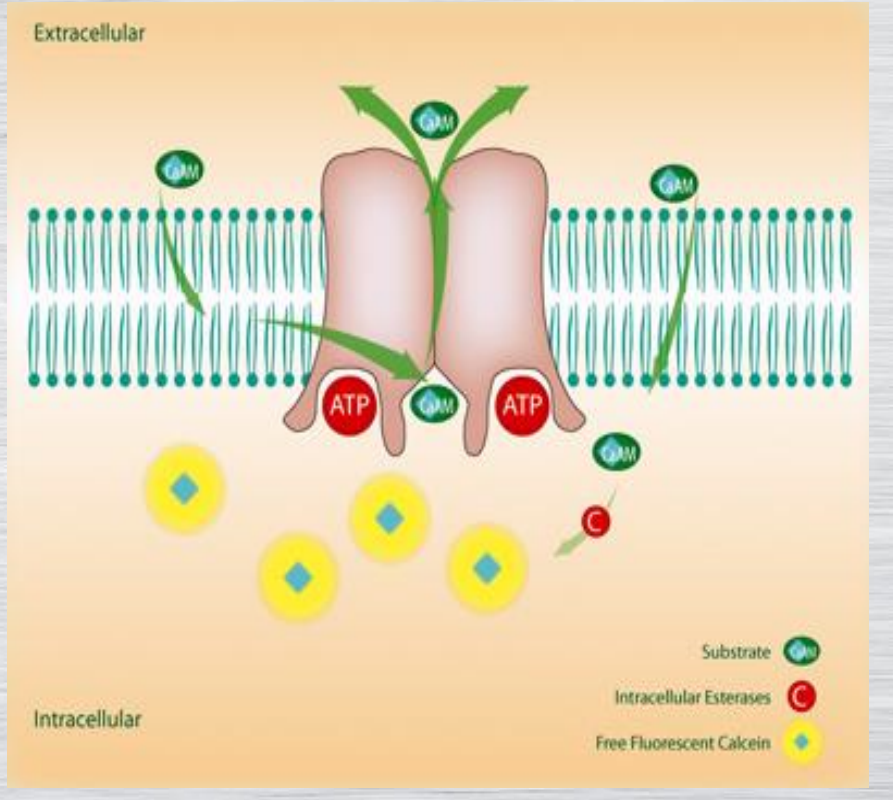
Cellular Dye Efflux Assay

- ✓ Quick and easy assay
- ✓ Fluorescent readout

- × Does not indicate the nature of the interaction
- × Not accepted by the regulatory agencies



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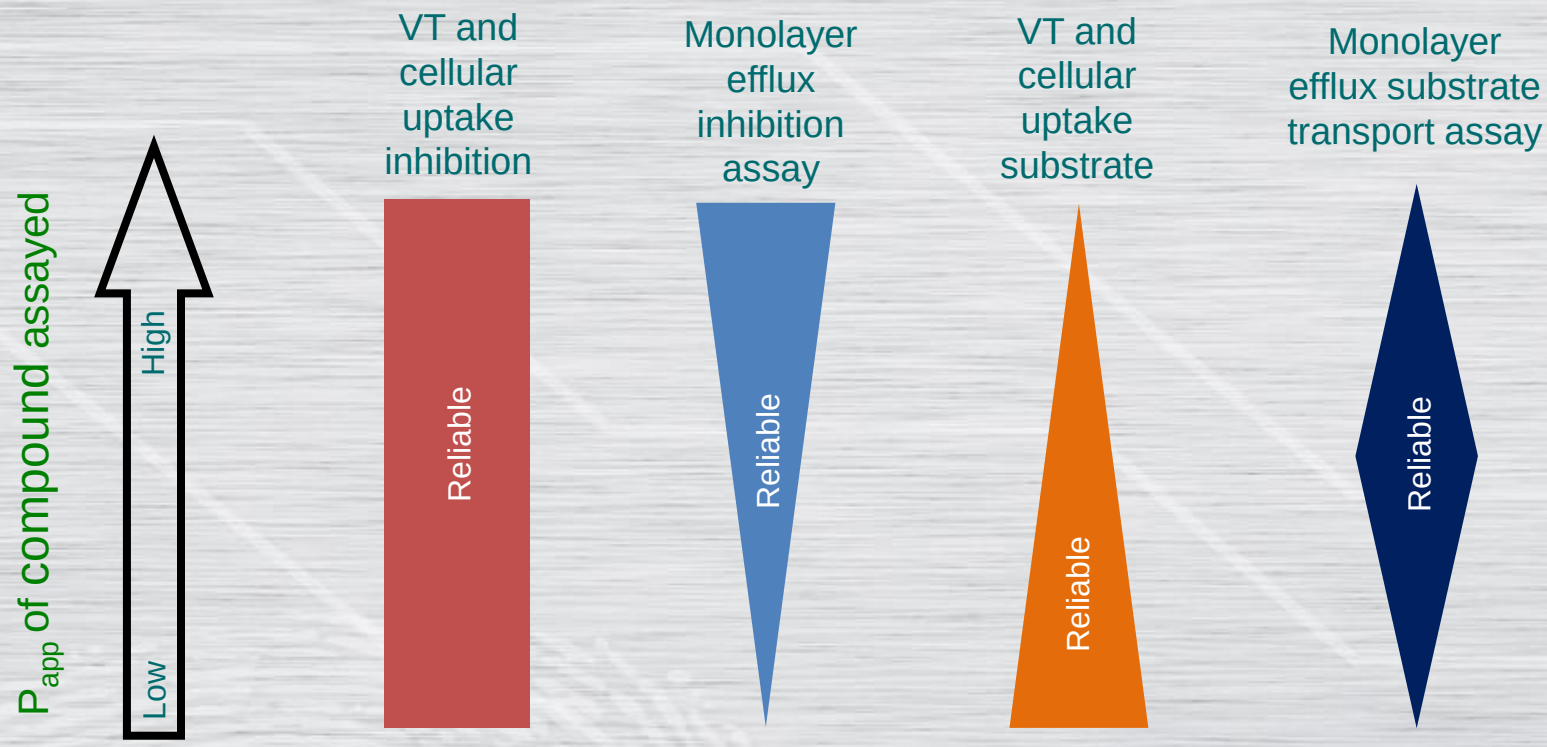
Recommendations for Choosing the Optimal Assay

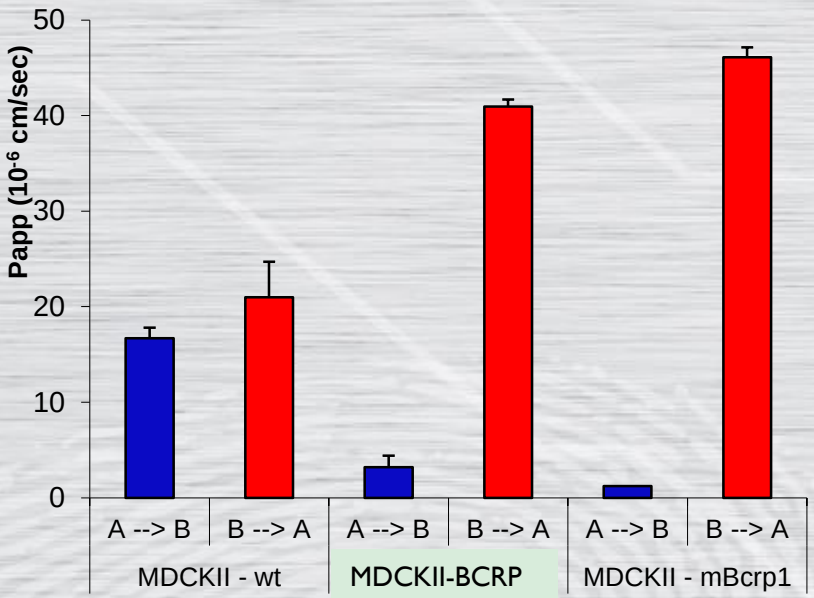


Application of different types of assays for Low-High permeability compounds

Importance of Permeability to Model Choice

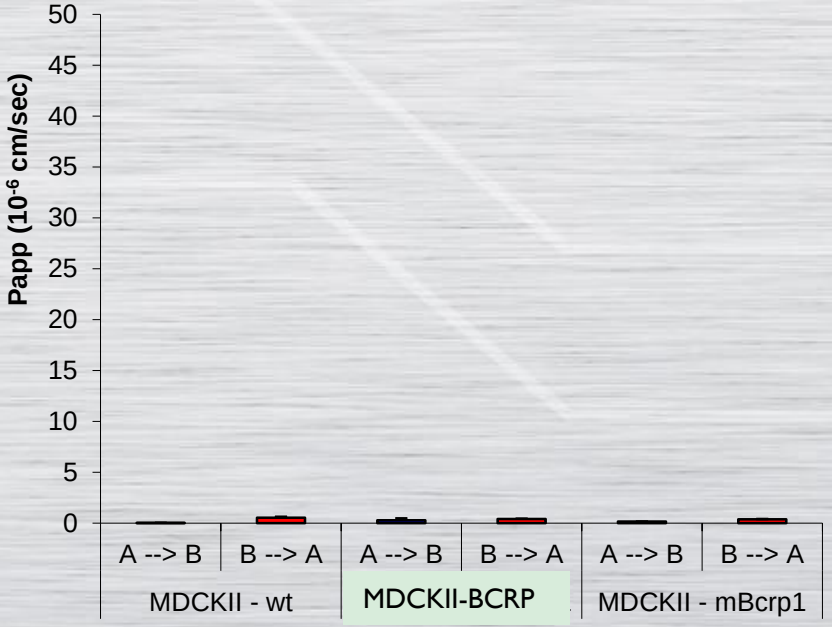
(medium permeability)

Dantrolene (10µM)



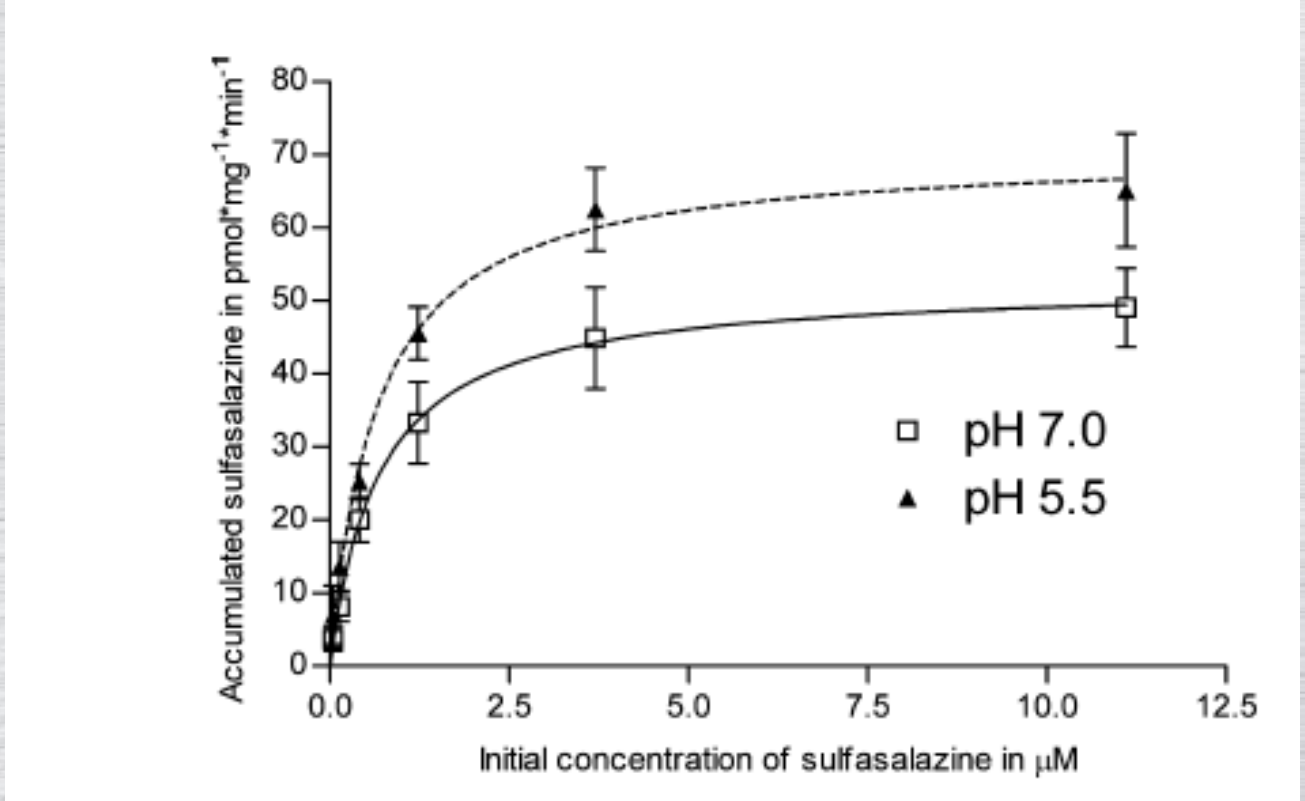
(low permeability)

Sulfasalazine (100µM)



Importance of Permeability to Model Choice - cont

Sulfasalazine (low permeability)



Jani et al (2009) Biol Pharm Bull

Summary Table

	ATPase	VT	Uptake	Dye efflux	Monolayer
Strengths	non-radioactive, inexpensive	measure trp kinetics, flexibile readout	Measure trp kinetics, flexibile readout	quick and easy	polarized cells, barrier-like (tight junctions)
Weaknesses	indirect assay, not accepted by the regulatory agencies	*optimally low passive permeability cmpds can be detected	*optimally low passive permeability cmpd s can be detected	doesn't indicate the nature of interaction, not accepted by the regulatory agencies	permeability of cpds, lengthy cell culturing for Caco-2
Opportunities	predict the substrate nature of highly permeable drugs	screen trp - mediated DDI	various expression systems	prediction of drug resistance for oncology drugs	distinguish passively diffused cpds from effluxed substrates, various expression systems
Threats	Pi liberated at low conc. is not detectable	detection of transport of high perm. cpds	detection of transport of high perm. cpds	May miss substrate due to permeability (low)	May miss substrate due to permeability (high or low)

* substrate

Thank you for your Attention!

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