PROVEN GLOBAL CONTRACT RESEARCH EXPERTISE
FROM DISCOVERY THROUGH CLINICAL SUPPORT

Challenges & Solutions In Today's In Vitro Transporter Research Landscape

Joanna Barbara, Ph.D.
XenoTech
Vice President of Scientific Operations

Overview

- Introduction
  - Transporters in drug development
  - 2017 FDA in vitro drug-drug interaction (DDI) guidance transporter updates
- Case study 1: solute carrier transporter inhibition
- Case study 2: P-gp transport in Caco-2 cells
- Case study 3: permeability in Caco-2 cells
- Conclusions: lessons learned

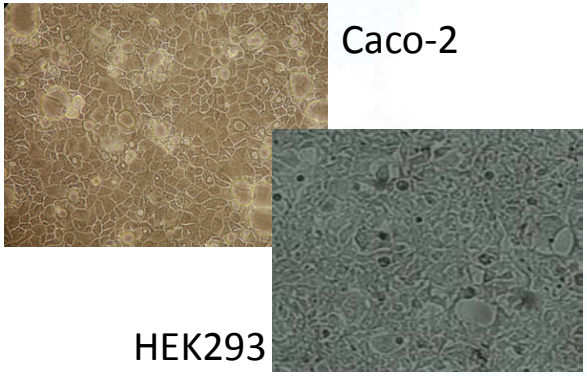
Transporter assays in drug development

- In vitro drug transporter assays performed throughout drug development to answer myriad questions (DDI focus)
  - Cell permeability studies
  - Inhibition / substrate potential
- Range from simple screens to kinetic assessments in complex assay formats

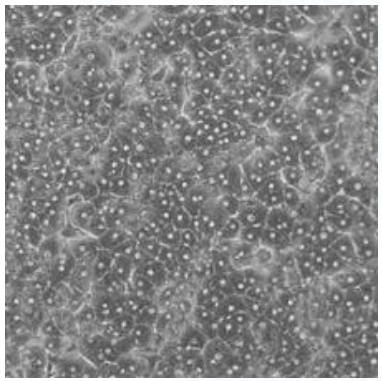
- When the compounds behave, things are straightforward
- When drugs misbehave, understanding the data and establishing a path forward can be challenging

Assay formats

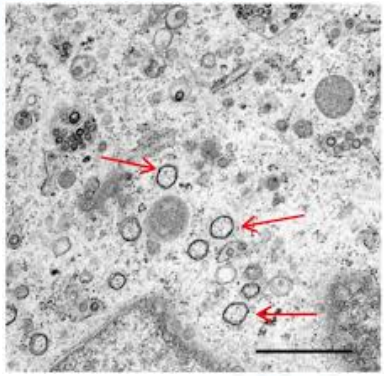
- Numerous human transporter proteins
- In vitro study designs based on availability of a test system to study the protein in question
- Generally



Polarized/transfected cell lines



Hepatocytes

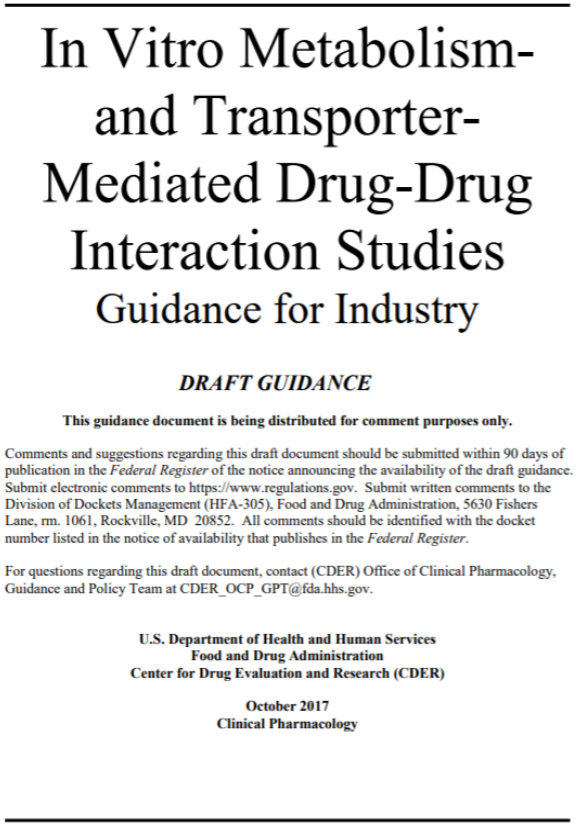


Membrane vesicles

- Each test system needs appropriate conditions and controls to be useful

Transporter assays in 2017 FDA DDI guidance

- Several changes related to transporter assays were made
- Some easily accommodated
  - e.g., 30 min preincubation for OATP assays
- Some were more challenging
  - e.g., 2 inhibitors for each transporter
- Some spoke to practical considerations and the ways data should be interpreted
  - What we are about to talk about…



In Vitro Metabolism- and Transporter-Mediated Drug-Drug Interaction Studies

Guidance for Industry

DRAFT GUIDANCE

This guidance document is being distributed for comment purposes only.

Comments and suggestions regarding this draft document should be submitted within 90 days of publication in the *Federal Register* of the notice announcing the availability of the draft guidance. Submit electronic comments to https://www.regulations.gov. Submit written comments to the Division of Dockets Management (HFA-305), Food and Drug Administration, 5630 Fishers Lane, rm. 1061, Rockville, MD 20852. All comments should be identified with the docket number listed in the notice of availability that publishes in the *Federal Register*.

For questions regarding this draft document, contact (CDER) Office of Clinical Pharmacology, Guidance and Policy Team at CDER_OCP_GPT@fda.hhs.gov.

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)

October 2017
Clinical Pharmacology

Factors for consideration

- FDA 2017 DDI guidance emphasized need for more rugged transporter study designs, considering
  - Stability in the test system
  - Non-specific binding to cells and experimental apparatus
  - Solubility limits
  - Effect of additive serum protein
  - Effect of prefiltration
  - Effect of cytotoxicity
  - Effect of other experimental steps

- Why?

Case 1: solute carrier (SLC) inhibition

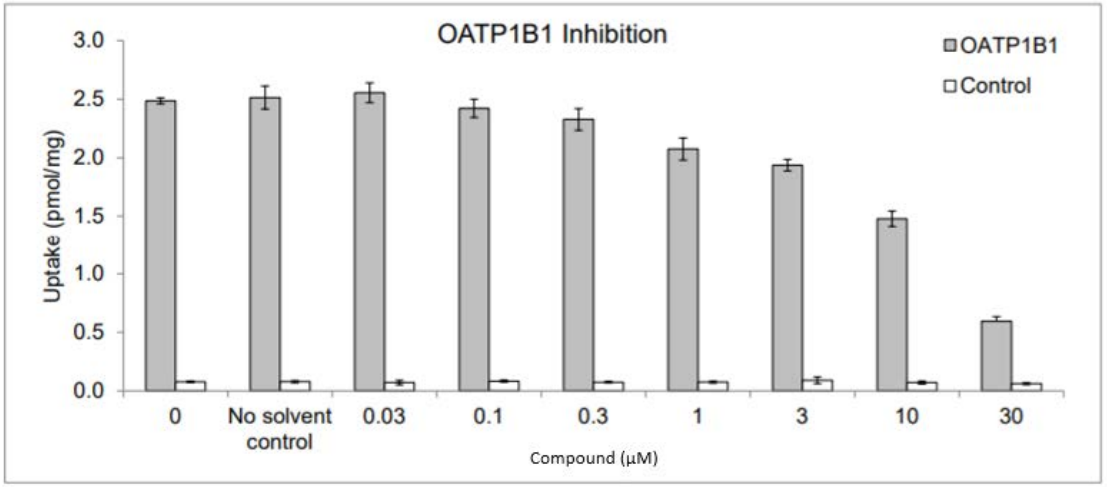
- Study scope: substrate and inhibition potential of compound for
  - OATP1B1
  - OATP1B3
  - OAT1
  - OAT3
  - OCT2
  - MATE1
  - MATE2-K

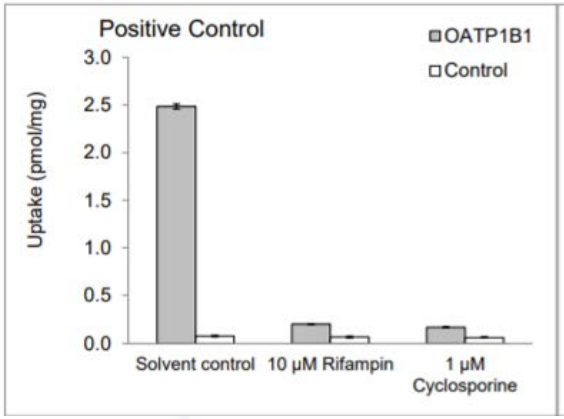
- Discussion will focus on inhibition for DDI potential

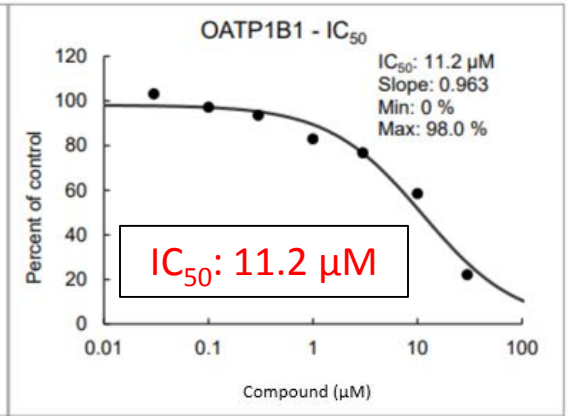
Case 1: SLC inhibition data – positive result

- Inhibition of OATP1B1 observed

OATP1B1



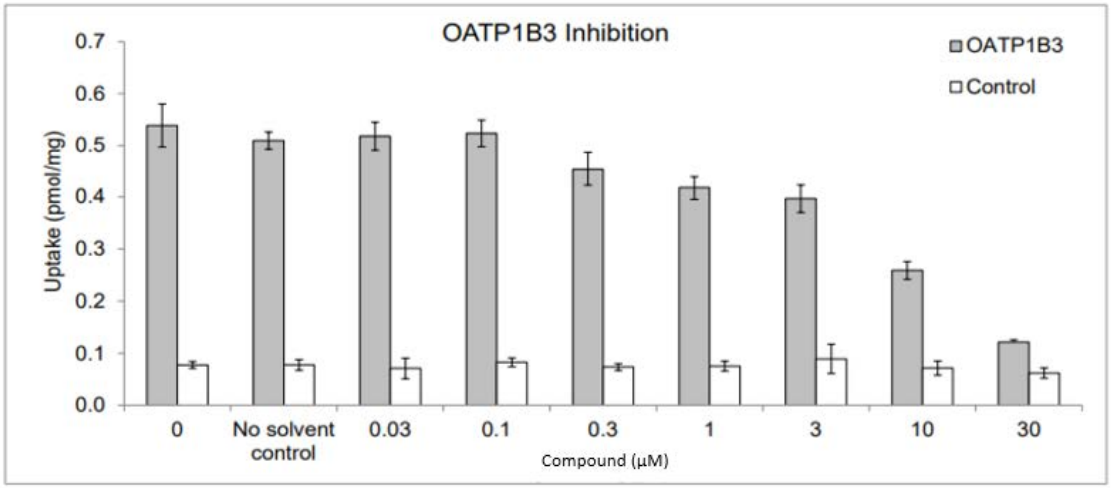


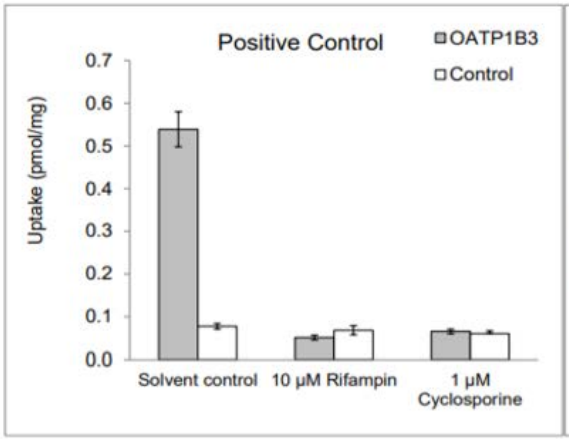


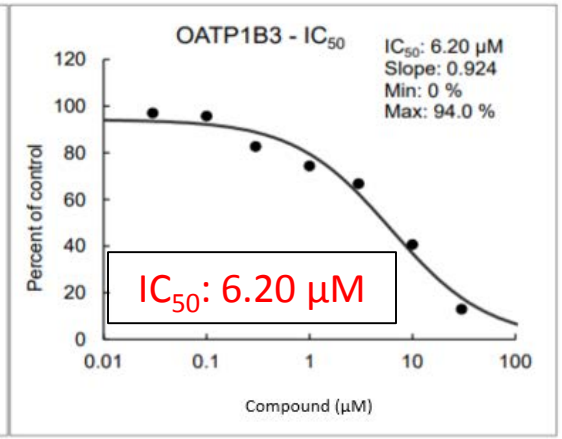
Case 1: SLC inhibition data – positive result

- Inhibition of OATP1B3 observed

OATP1B3



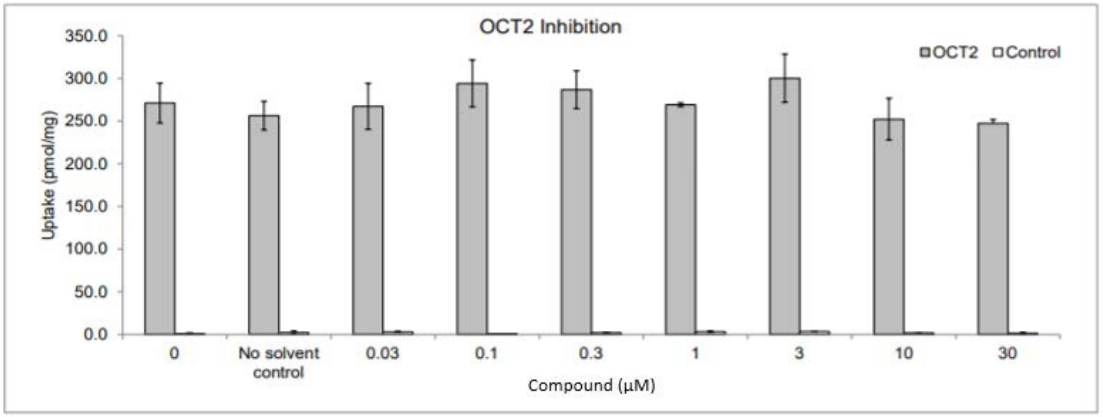


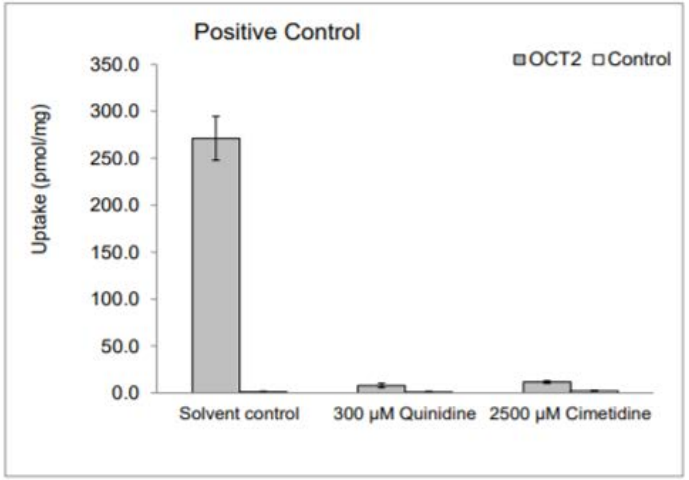


Case 1: SLC inhibition data – negative result

- No inhibition of OCT2

OCT2





Data interpretation: FDA basic model

- Starting point to evaluate need for clinical studies

Transporters	Equation	Cutoff
OATP1B1, OATP1B3	$1 + \frac{(f_{u,p} \times I_{in,max})}{IC_{50}}$	≥ 1.1
P-gp, BCRP	$\frac{I_{gut}}{IC_{50}}$	≥ 10
OAT1, OAT3, OCT2	$\frac{I_{max,u}}{IC_{50}}$	≥ 0.1
MATE1, MATE2-K	$\frac{I_{max,u}}{IC_{50}}$	≥ 0.02

Where

I_{max} = unbound plasma $C_{max.ss}$
F_a = fraction absorbed
F_g = intestinal availability
k_a = absorption rate constant
Q_h = hepatic blood flow
R_b = blood-to-plasma concentration ratio

$$I_{in,max} = I_{max} + \frac{(F_a F_g \times k_a \times Dose)}{Q_h / R_b}$$

If unknown, use $F_a F_g = 1$ and $k_a = 0.1$ min^{-1} as worst-case scenario
May have to assume $R_b = 1$ and $Q_h = 1.6$ L min^{-1}

Case 1: compound recovery data

- Recovery data for compound in HEK293 cells and apparatus
  - Compound incubated for 30 min in the presence and absence of cells

Sample	Theoretical concentration (µM)	Mean experimental concentration (µM)	CV (%)	Recovery (%)	Adsorption (%)
No cells	0.03	$(2.22 \pm 1.3) \times 10^{-2}$	4.7	74.0	26.0
	80	62.3 +/- 2.6	4.3	77.9	22.1
Control HEK cells	0.03	$(2.27 \pm 1.2) \times 10^{-2}$	4.5	75.5	24.5
	80	44.6 ± 9.6	22.3	55.8	44.2

Low recovery in the presence of cells

Case 1: Cutoff data for SLC transporter inhibition

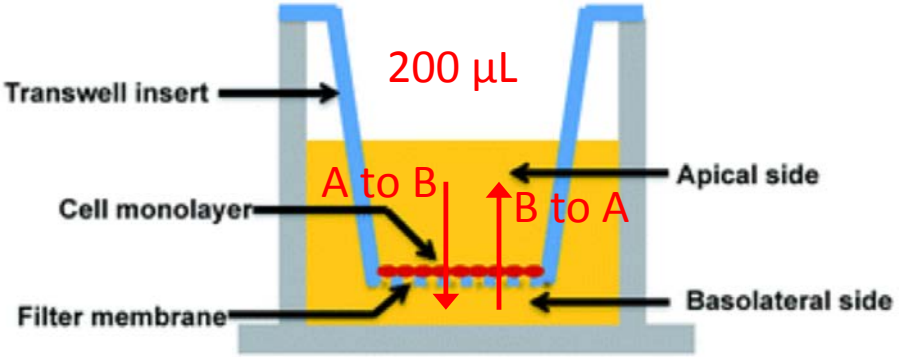
- Calculations performed using established IC_{50} values

Transporter	IC_{50} (µM)	Value	Cutoff	Inhibition potential
OATP1B1	11.2	1.0950	≥ 1.1	No
OATP1B3	**6.2**	**1.1715**	**≥ 1.1**	**Yes**
OAT1	35.6	0.0026	≥ 0.1	No
OAT3	11.2	0.0082	≥ 0.1	No
OCT2	>30	No inhibition	≥ 0.1	No
MATE1	22.3	0.0041	≥ 0.02	No
MATE2-K	>30	No inhibition	≥ 0.02	No

- But ~50% nonspecific binding, so theoretically IC_{50} values could be half the calculated values (at worst)
  - NSB-corrected IC_{50} values gave $R_{\mathbf{OATP1B1}}$ = 1.1899 **≥ 1.1**
  - OATP1B1 recommended for conservative scenario inhibition potential too

Case 2: P-gp substrate potential

- Study scope – P-gp transport in Caco-2 cells
- Assays performed in transwell format
- Polarized cell monolayer



- B to A: active transport
- A to B: passive permeability

Donor: side where drug is administered
Receiver: opposite side

Ye, Dawson and Lynch *Analyst*, 2015, 140: 83-97

Data processing: some math

- Permeability calculations based on Fick's first law:

$$J_{wall} = P_{wall} \times C$$

Where J_{wall} = flux P = permeability coefficient C = maximal intestinal concentration

- Normal transwell assay data processing:

$$P_{app} = \frac{V_R}{A \times C_{D0}} \times \frac{\Delta C_R}{\Delta t}$$

Where P_{app} = apparent permeability coefficient (cm/s) V_R = receiver volume (cm^3)
A = membrane surface area (cm^2) C_{D0} = donor concentration at time zero
$\Delta C_R / \Delta t$ = change in receiver concentration over time (s)

- Mass equation (for mass balance):

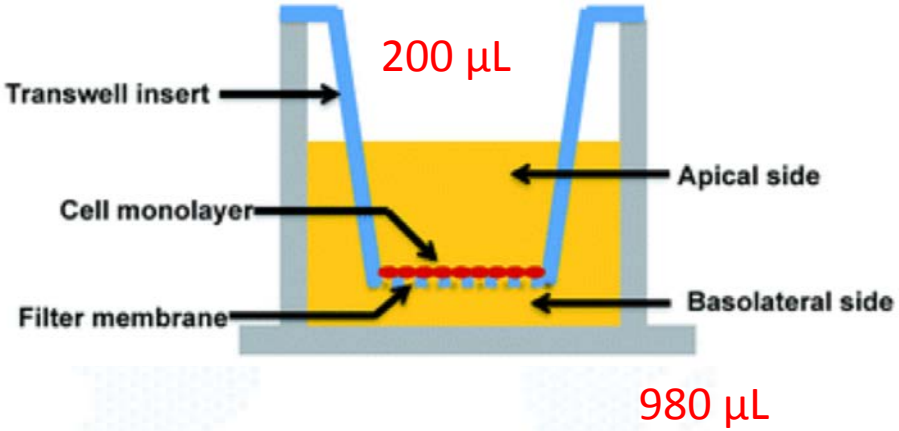
$$P_{app} = \frac{V_D}{A \times M_D} \times \frac{\Delta M_R}{\Delta t}$$

Where P_{app} = apparent permeability coefficient (cm/s) V_D = donor volume (cm^3)
A = membrane surface area (cm^2) M_D = donor amount (mol)
$\Delta M_R / \Delta t$ = change in receiver amount (mol) over time (s)

Youdim, Avdeef and Abbott *Drug Discovery Today,* 2003, 8 (21): 997 – 1003

Case 2: transwell substrate assay challenge

- Transwell assays can suffer from nonspecific binding problems
  - Different surface area: volume on A and B sides
  - Compound binds to the apparatus and also to the cell monolayer



- For substrate assays, need to be able to measure the compound to low levels

Ye, Dawson and Lynch *Analyst*, 2015, 140: 83-97

Case 2: recovery data in apparatus/control cells

- Recovery in the **absence** of cells

Theoretical concentration (µM)	Mean concentration stock (µM)	Deviation from nominal (%)	Mean concentration at 2 min (µM)	Recovery (%)	Mean concentration at 90 min (µM)	Recovery (%)
0.5	0.480	4.0	0.375	78.2	0.200	41.8
2.5	2.65	6.2	2.13	80.1	1.44	54.3
10	9.89	1.1	8.78	88.8	7.47	75.5
30	26.1	13.0	24.9	95.6	23.1	88.5

- Up to 58.2% lost through apparatus adsorption at the low concentrations

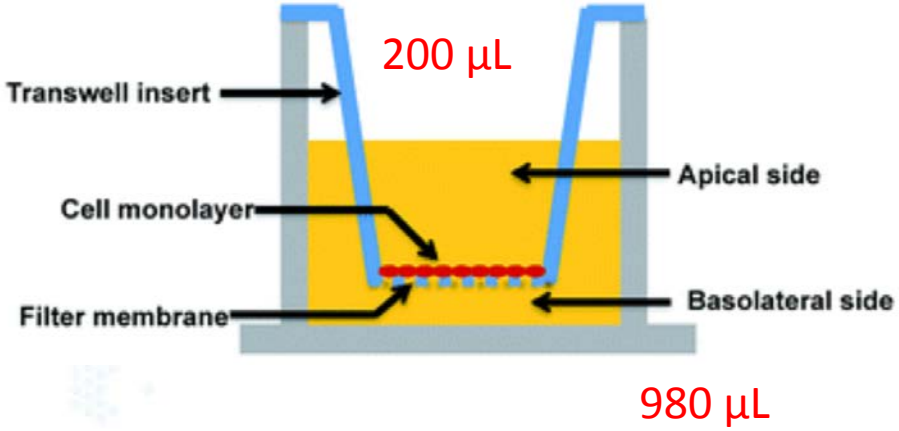
Case 2: recovery data in apparatus/control cells

- Recovery in the **presence** of control cells (90 min)

Theoretical concentration (µM)	Recovery apical to basal (%)	Recovery basal to apical (%)
1	41.2	74.8
3	55.5	69.7
10	54.0	76.6

All low recovery
A>B lowest

Can confuse conclusions



Case 2: Caco-2 P-gp substrate data

- P_{app} and efflux ratios

Theoretical concentration (µM)	Samples	P_{app} A > B ($1x10^{-6}$ cm s^{-1})	P_{app} B > A ($1x10^{-6}$ cm s^{-1})	Efflux ratio
0.3	No inhibitor	5.86	11.7	1.99
	Inhibitor	9.27	14.1	1.52
3	No inhibitor	8.54	16.2	1.90
	Inhibitor	13.3	18.3	1.46

- Per FDA DDI Guidance 2017: The following suggests that a drug is an in vitro P-gp substrate
  - A net flux ratio (or efflux ratio) of ≥2 in cells that express P-gp
  - A flux that is inhibited by at least one known inhibitor at a concentration at least 10x its K_i

Inconclusive??

Data processing binding correction

- Mass equation (for mass balance):

$$P_{app} = \frac{V_D}{A \times M_D} \times \frac{\Delta M_R}{\Delta t}$$

Where P_{app} = apparent permeability coefficient (cm/s)
A = membrane surface area (cm^2)
$\Delta M_R / \Delta t$ = change in receiver amount (mol) over time (s)
V_D = donor volume (cm^3)
M_D = donor amount (mol)

- Correction equation:

$$P_{app} = \frac{V_D}{A \times (M_D - M_{cells})} \times \frac{\Delta M_R}{\Delta t}$$

M_{cells} = amount of material in monolayer (mol)
$\Delta M_R / \Delta t$ = change in receiver amount (mol) over time (s)
M_D = donor amount (mol)

$$M_{cells} = M_{D0} - \left(M_{Dt} + \sum M_R \right)$$

Where M_{D0} = donor amount (mol) at time zero
M_{Dt} = donor amount (mol) at time *t*
ΣM_R = sum of receiver amount (mol) at all time points

Youdim, Avdeef and Abbott *Drug Discovery Today,* 2003, 8 (21): 997 – 1003

Case 2: Caco-2(P-gp) substrate data

- P_{app} and efflux ratios calculated accounting for monolayer material

Theoretical concentration (µM)	Samples	P_{app} A > B ($1x10^{-6}$ cm s^{-1})	P_{app} B > A ($1x10^{-6}$ cm s^{-1})	Efflux ratio
0.3	No inhibitor	15.8	16.6	1.05
	Inhibitor	21.6	22.6	1.05
3	No inhibitor	19.1	28.7	1.50
	Inhibitor	31.4	30.1	0.959

- Not a human P-gp substrate
  - Efflux ratio does not approximate 2 when M_{cells} is taken into account

Conclusive!

Case 3: Caco-2 permeability

- Study: Caco-2 permeability screen in transwell format
- Screen
  - 1 compound concentration; ± inhibitor (valspodar)
  - 1 time point
  - Quick LC-MS/MS method development
  - P_{app}, efflux ratio and recovery measurement
  - High and low permeability controls

Case 3: Caco-2 permeability screen

- Screening so minimal information; no structure

Sample	P_{app} A > B ($1x10^{-6}$ cm s^{-1})	P_{app} B > A ($1x10^{-6}$ cm s^{-1})	Efflux ratio	Recovery A > B (%)	Recovery B > A (%)
10 µM Compound	0	0	No data	150	387
10 µM Compound + inhibitor	0	0	No data	161	182
10 µM Digoxin	1.86	68.0	36.6	105	94.3
10 µM Mannitol	0.831	0.720	0.867	80.9	76.3
10 µM Caffeine	19.9	21.5	1.08	75.9	81.8

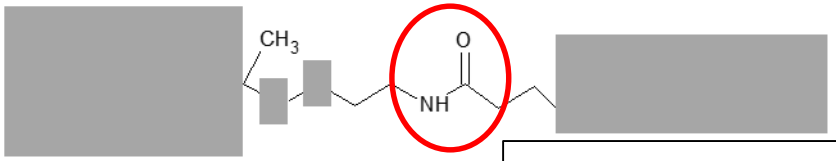
Digoxin – P-gp positive control
Mannitol – low permeability control
Caffeine – high permeability control

No P_{app} data – nothing in receiver samples - and impossibly high recovery

- Repeated assay and obtained same results
- Nonspecific binding again? Unlikely because HIGH recovery

Case 3: troubleshooting

- Screening: no supporting data available – requested structure

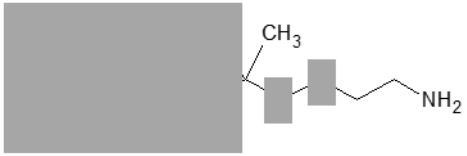


Compound

Monoisotopic Mass = 595.326618 Da

Amides can hydrolyze enzymatically and non-enzymatically in Caco-2 cells

2 Products proposed



Amide hydrolysis product A

Monoisotopic Mass = 285.096952 Da

Ionizable by electrospray (LC-MS/MS)

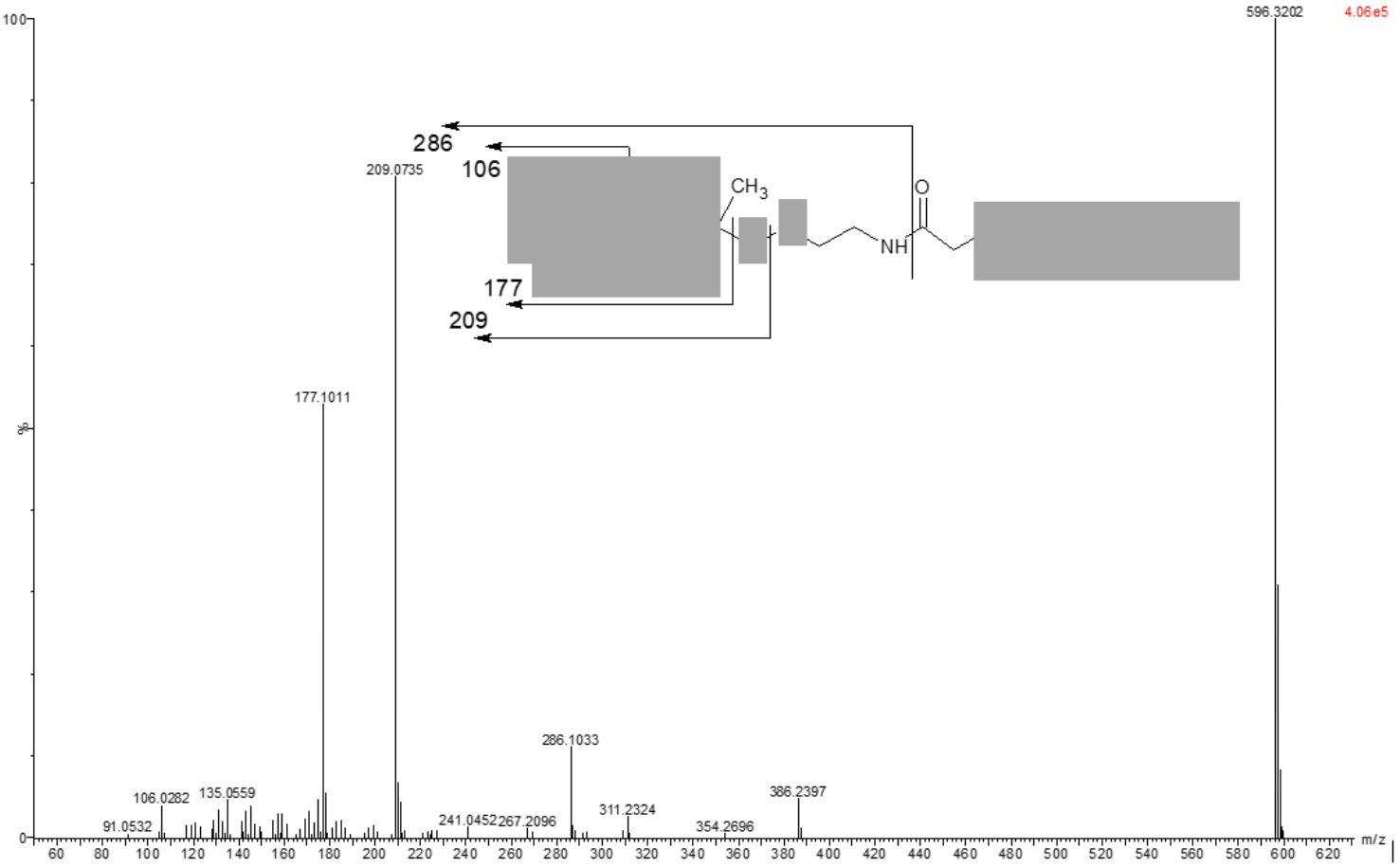
Amide hydrolysis product B

Monoisotopic Mass = 328.24023

Weakly ionizable by electrospray (LC-MS/MS)

Case 3: exploring hydrolysis hypothesis

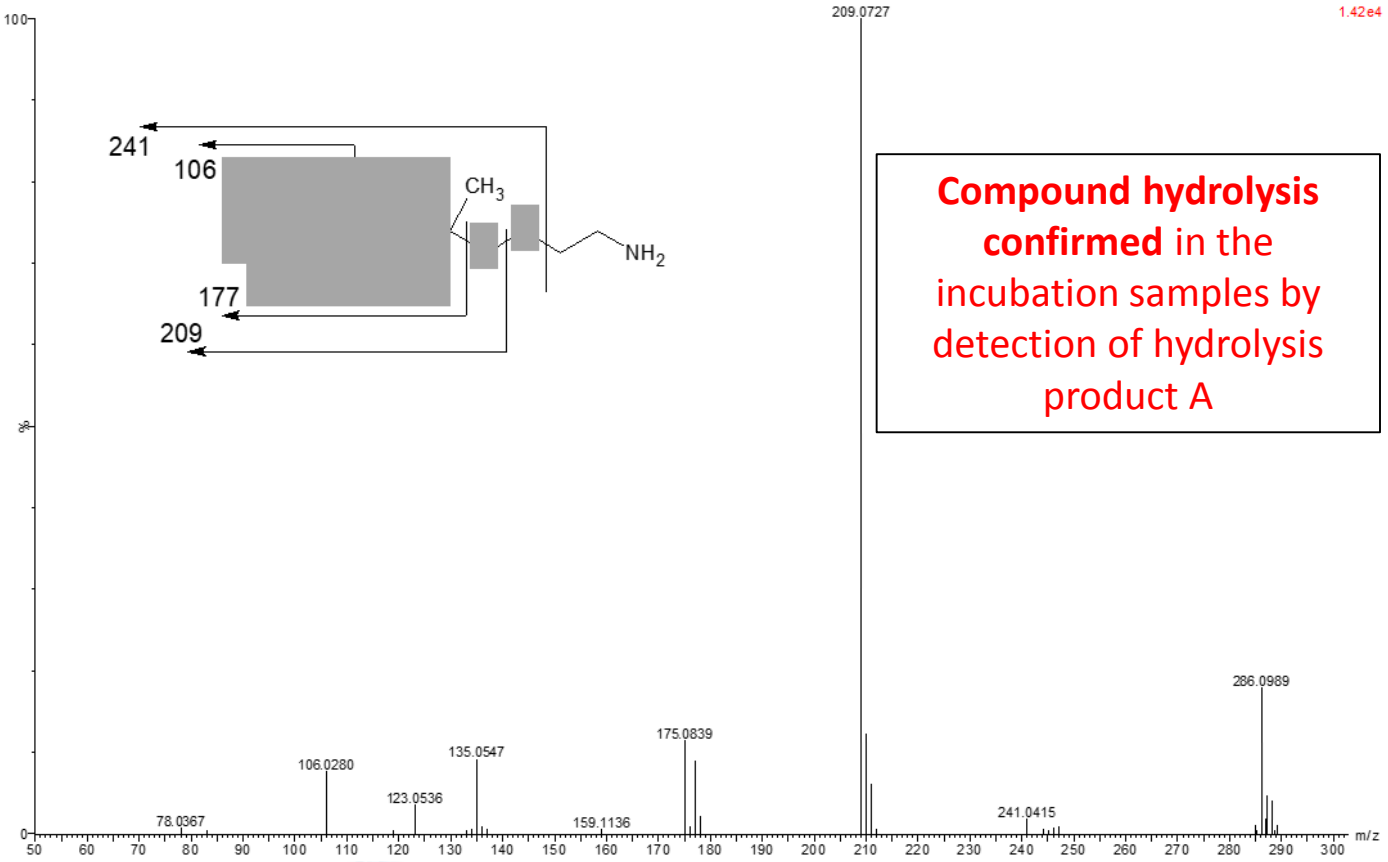
- High-resolution product ion spectrum for compound standard



- Identical spectrum obtained for donor samples

Case 3: exploring hydrolysis hypothesis

- Product ion spectrum for product A in donor sample



Compound hydrolysis confirmed in the incubation samples by detection of hydrolysis product A

- Product A also detected in receiver samples

Case 3: back to the math

- Normal transwell assay data processing:

$$P_{app} = \frac{V_R}{A \times C_{D0}} \times \frac{\Delta C_R}{\Delta t}$$

Where P_{app} = apparent permeability coefficient (cm/s) V_R = receiver volume (cm^3)
A = membrane surface area (cm^2) C_{D0} = donor concentration at time zero
$\Delta C_R / \Delta t$ = change in receiver concentration over time (s)

- Dimensional analysis: all units cancel except cm s^{-1}
- So?
  - This is a relative assay and the units of concentration don't matter as long as they are consistent
  - Can calculate P_{app} using area ratio of the hydrolysis product without a reference standard

Case 3: corrected data

- Reinjected sample batch with longer LC method and monitored hydrolysis product A as well as compound

Sample	P_{app} A > B ($1x10^{-6}$ cm s^{-1})	P_{app} B > A ($1x10^{-6}$ cm s^{-1})	Efflux ratio	Recovery A > B (%)	Recovery B > A (%)
10 µM Compound	0	0	No data	79.9	101
10 µM Compound + inhibitor	0	0	No data	78.8	76.1
Amide hydrolysis product A	26.4	62.5	2.37	NA	NA
Amide hydrolysis product A + inhibitor	24.7	8.33	0.337	NA	NA

Amide hydrolysis product A values calculated using peak area ratio data

Sensible recovery
Useable P_{app} values

- Confirmed permeability results for parent compound
- Evaluated permeability characteristics of product A

Case 3: conclusion

- Confirmed active transport of hydrolysis product A by targeted metabolite monitoring
  - Efflux ratio > 2
  - Reduced in the presence of the P-gp inhibitor valspodar
- Confirmed instability of parent compound in incubation

- Conclusion: compound rapidly hydrolyzed to product A which then required active transport for efflux across the cells

Conclusion: lessons learned

- Critical factors highlighted in the 2017 FDA DDI guidance can have a big impact on interpreting transporter data
  - Solubility
  - Cytotoxicity
  - Nonspecific binding
  - Stability in the test system
- Remember practical factors of the assays and assumptions when troubleshooting
- Can reach useful conclusions in challenging situations

- Give partners the compound structure to access their knowledge and get the best results!

Acknowledgments

- Dr. Brian Ogilvie
- Andrea Wolff
- Rebekah Snyder
- Lois Haupt
- Andrea Rhoades
- Chase McCoy

End – Questions?

Joanna Barbara
VP – Scientific Operations
jbarbara@xenotechllc.com

www.xenotech.com

Featuring publications, posters, webinars and other useful resources.

Webinar Topic Request Form:

www.xenotech.com/scientific-resources/upcoming-webinars

XenoTech's Services & Products:

In Vitro ADMET PK & DDI
- Drug Transport
- Drug Metabolism
- Enzyme Inhibition & Induction
- Protein Binding
- Metabolite Identification
- ADME Screening
- Toxicology

In Vivo ADME/PK & Distribution
- QWBA
- Microautoradiography
- Excretion / Mass Balance
- Tissue Distribution
- Blood / Plasma & Lymphatic Partition Rate

Bioanalytical
Pharmacology
- In Vitro Ligand Binding & Radioreceptor Assays
- Immunoassays

Chemical Synthesis
- Radiolabeled Synthesis
- Metabolite Synthesis
- Peptide Synthesis

Consulting...

Cellular Products
- Hepatocytes (Cryo/Fresh, Genotyped...)
- Non-Parenchymal Cells (Kupffer Cells)

Subcellular Fractions
- Liver Microsomes
- S9 Fractions
- Cytosol
- Homogenate
- Lysosomes & Tritosomes
- Mitochondria
- Extrahepatic Fractions

Custom Products
- Various Species, Tissues & Preparations

Research Biobank
- Normal & Diseased Tissue Samples

Recombinant Enzymes

Substrates & Metabolites

Metabolite Production Kits

JCRB Cell Lines...