Application of a Fluorescent Substrate / Inside-Out Transporter Vesicle Assay for Identifying Inhibitors of MRP Transport

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BD Biosciences

October 20, 2010

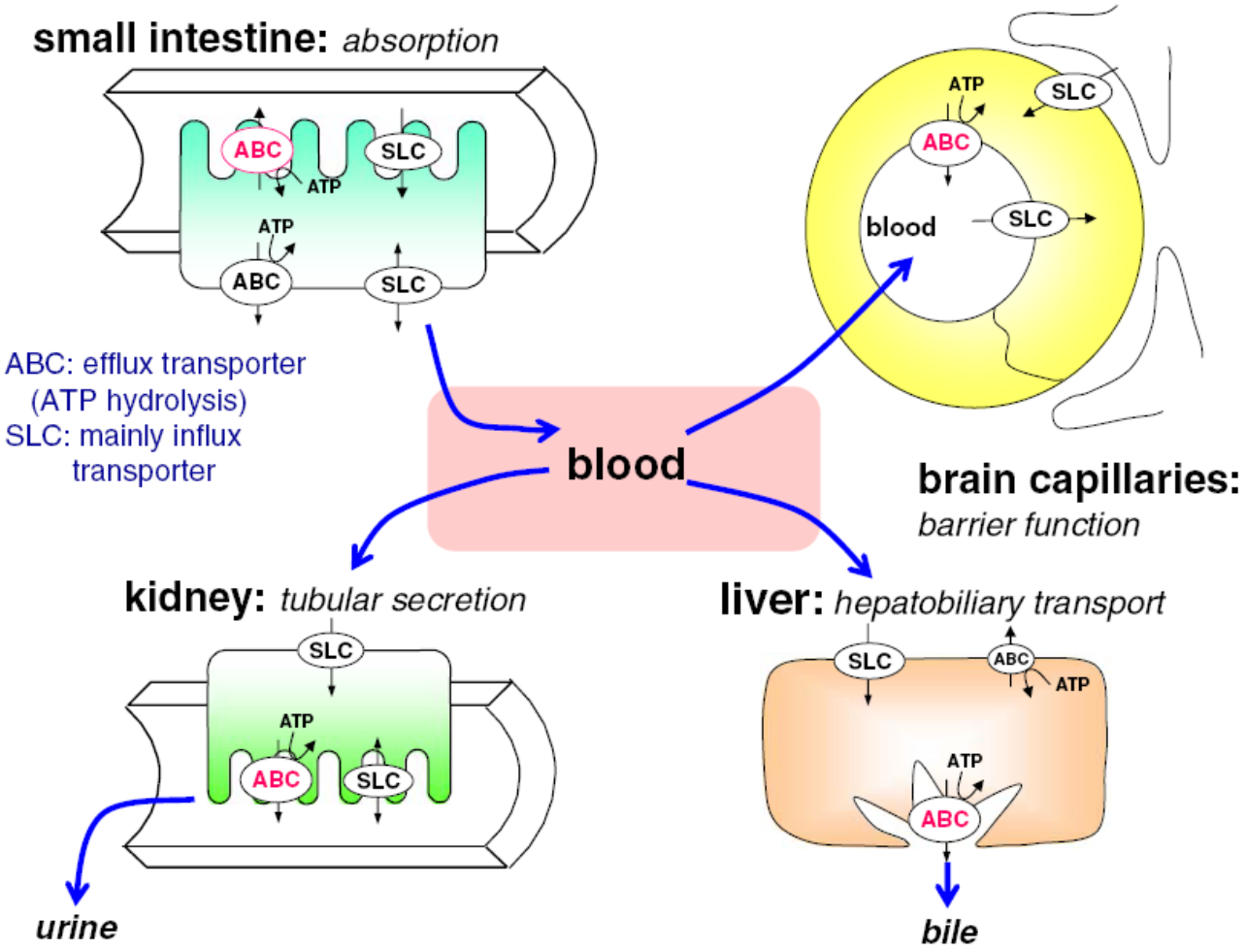
BD Gentest™ Transporter Seminar Series

- Today's seminar is the first in a series of transporter seminars that BD will present through 2010 and 2011.
- The upcoming titles and dates are as follows:
  - **Application of the colorimetric ATPase assay for accessing ABC transporter inhibition and stimulation**: **December 8, 2010**
  - **Application of inside-out vesicles for accessing ABC transporter inhibition and direct transport: February 9, 2011**
  - **Application of Transportocytes for identifying inhibitors and substrates of SLC transporters: April 7, 2011**
  - **Drug transport efflux and uptake assays using plated and suspension hepatocytes: May 5, 2011**

Today's Topics

- Overview of the important role of drug transporters in drug pharmacokinetics
- Overview of *in vitro* transporter models
- Application of a fluorescent substrate in characterizing transporter inhibitors using a vesicle assay
  - Advantages
  - Assay procedure
  - Application in characterizing drug interactions with MRP transporters

Transporters Across Human Tissues

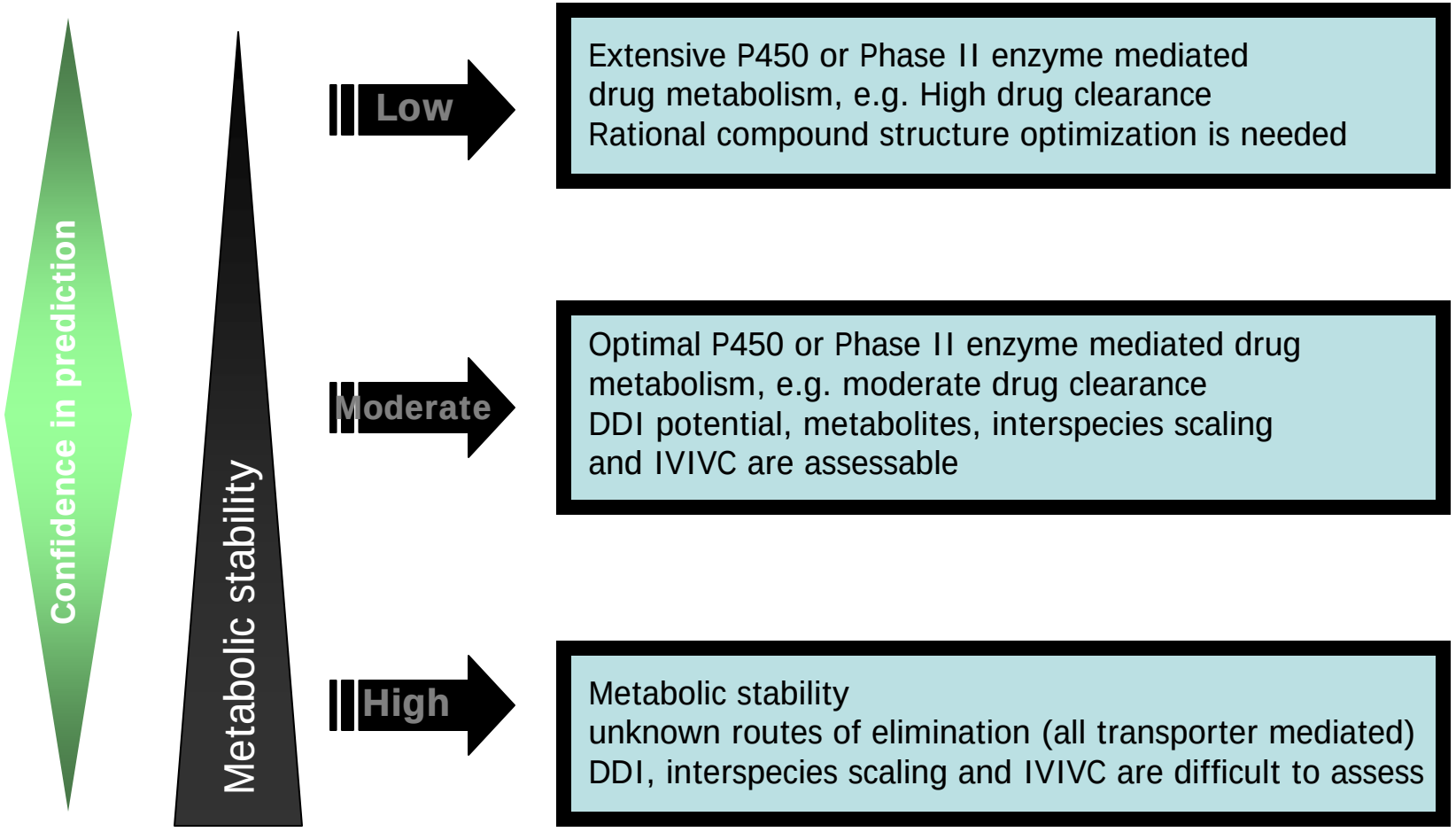


Kitamura S, et al. Naunyn Schmiedebergs Arch Pharmacol. **377(4-6)**:617-28 (2008).

Transporters in Drug Pharmacokinetics and Toxicity

- **Systemic exposure: Oral bioavailability and Organ Disposition**
  - Drug absorption in small intestine (BCRP, MDR1/P-gp) (Topotecan, sulphasalazine)
- **SLC transporter involved DDI**
  - OATP1B1: increase in statin AUC when co-administrated with cyclosporine
- **MDR1/P-gp involved DDI**
  - Digoxin clearance reduced when co-administrated with Quinidine, Ritonavir, and other P-gp inhibitors
- **Inhibition of MRP2/BSEP induced toxicity**
  - Cholestasis
  - Hyperbilirubinemia

Transporter-Mediated Drug Clearance – Challenge in Drug Discovery



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Selected Human Transporters

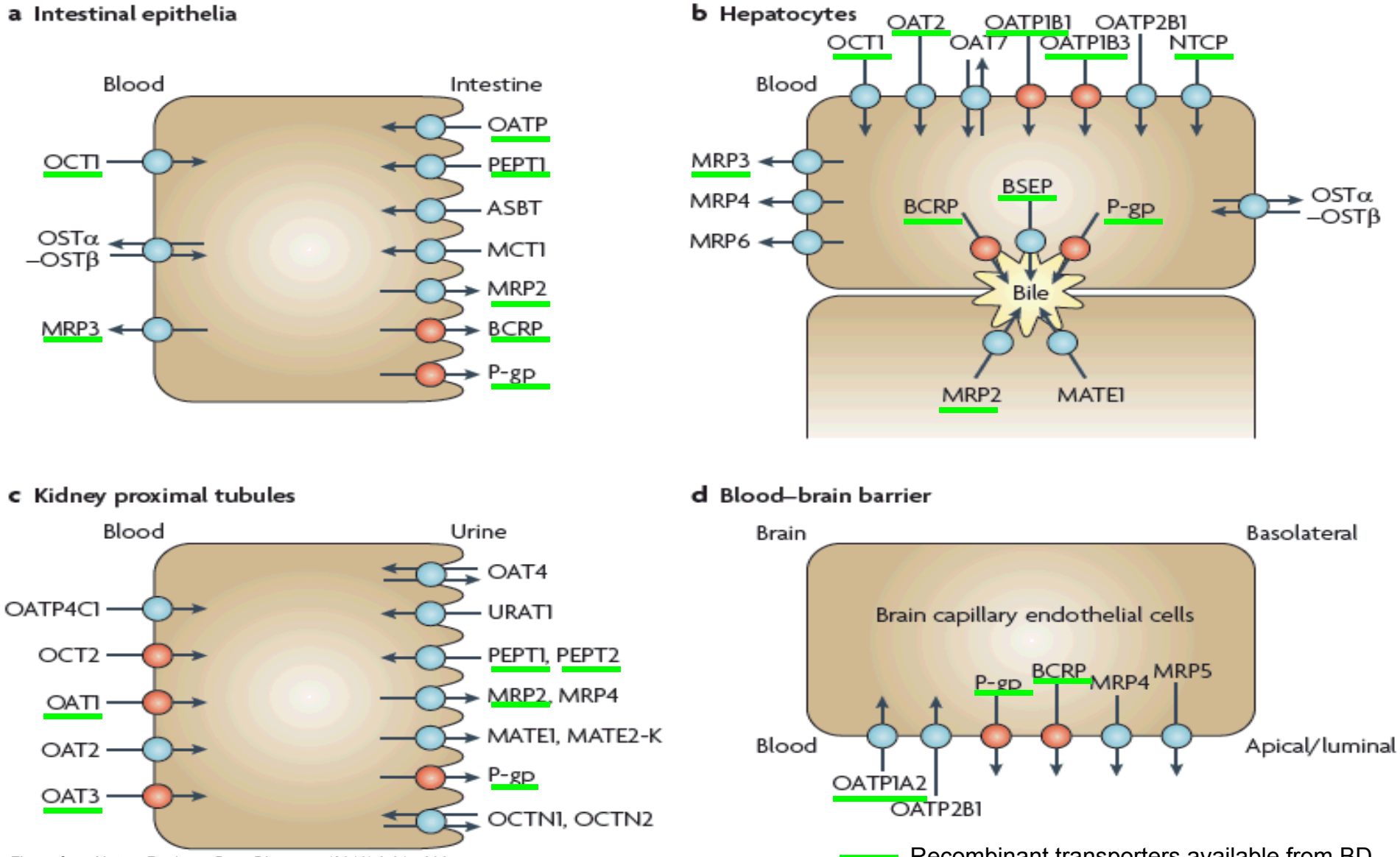


Figure from Nature Reviews: Drug Discovery (2010) 9:215-236

Recombinant transporters available from BD

Drug Transporters of Emerging Clinical Importance in the Absorption and Disposition of Drugs

SLC transporters		ABC transporters	
Transporters/alias	Tissue Distribution	Transporters/alias	Organ/cells
OATP1B1/OATP-C, OATP2 (SLCO1B1)	Liver	MDR1/P-gp (ABCB1)	Intestine, Kidney, liver, brain
OATP1B3/OATP-8 (SLCO1B3)	Liver	BCRP/MXR (ABCG2)	Intestine, liver, kidney, brain, placenta, breast
OAT1 (SLC22A6)	Kidney, placenta	BSEP (ABCB11)	Liver
OAT3 (SLC22A8)	Kidney, liver, brain	MRP2/cMOAT (ABCC2)	Liver, kidney, intestine
OCT2 (SLC22A2)	Kidney, brain	MRP3 (ABCC3)	Liver, intestine
OATP1A2/OATP-A (SLCO1A2)	Brain	MRP4 (ABCC4)	Kidney, liver,
OATP2B1/OATP-B (SLCO2B1)	Liver	MDR3 (ABCB4)	Liver
OCT1 (SLC22A1)	Liver, intestine		
PEPT1 (SLC15A1)	Intestine, kidney		
PEPT2 (SLC15A2)	Kidney, lung		
MATE1 (SLC47A1)	Kidney, liver, skeletal muscle		
MATE2-K (SLC47A2)	Kidney		

Membrane Transporters in Drug Development, ITC, *Nature Review/Drug Discovery*, March (vol 9):215-236 (2010).

Overview of In Vitro Transporter Models

SLC transporters:

- Expressed in *Xenopus* oocyte system (Transportocytes)
  - OATP, OCT, OAT, and NTCP
  - Uptake assays for both radiolabeled and non-radiolabeled compounds
- SLC transporters overexpressed in mammalian cell lines
- Hepatocyte suspension assay using oil-filtration method

ABC transporters:

- Inside-out vesicles:
  - MRP, BCRP, and BSEP
  - Uptake assays for both radiolabeled and non-radiolabeled compounds
- Membranes:
  - P-gp, MRP, and BCRP
  - ATPase assay
- Polarized cell line expressing human P-gp, MRP2 or BCRP
- Sandwich cultured hepatocytes-ABC transporter on canalicular membrane

BD Gentest ABC Transporter Membranes / Vesicles

Membranes for ATPase Assay

- Human MDR1 (P-gp)
- Mouse Mdr1a/1b
- Rat Mdr1a/1b
- Cyno Mdr1
- Rhesus Mdr1
- Beagle Dog Mdr1

- Human MRP1
- Human MRP2
- Human MRP3
- Rat Mrp1
- Rat Mrp2

- Human BCRP (Arg482)
- Mouse Bcrp
- Rat Bcrp

Inside-out Vesicles for Uptake Assay

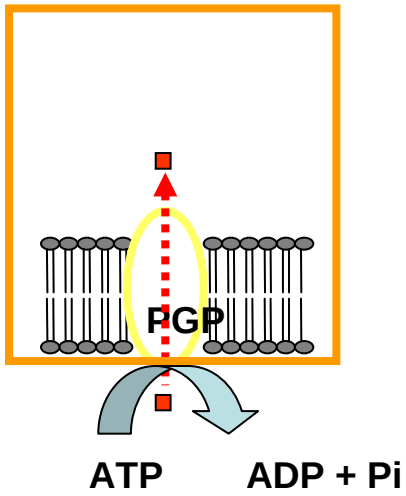
- Human MRP1
- Human MRP2
- Human MRP3
- Rat Mrp1
- Rat Mrp2

- Human BSEP
- Rat Bsep

- Human BCRP (Arg482)
- Rat Bcrp
- Mouse Bcrp

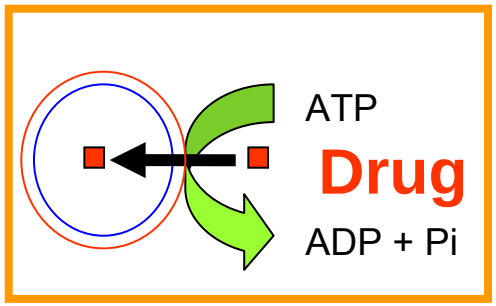
ABC Transporter Assays

ATPase Assay



- BD Gentest ABC transporter membrane
- Indirect Assay: measures the ability of a drug-stimulated ATP hydrolysis (substrates and inhibitors)
- Rapid, High-throughput
- Colorimetric assay (any compound)
- No extractions/separations/transfers

Vesicles Uptake Assay



- BD Gentest ABC transporter Vesicles
- Direct Assay: Measurement of transporter-mediated uptake of drugs into vesicles (Substrates and inhibitors)
- Can be high throughput: using 96-well plate and cell harvester / vacuum manifold
- Assays developed for both radiolabeled and non-radiolabeled compounds

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Transporter Assay Kits

- ATPase Kit (cat. no 459006)
  - One kit supports all BD ABC transporter membrane ATPase assays
  - 5 plates of 96-well plate assay
  - Probe substrates for P-gp, MRP1, MRP2, MRP3 and BCRP.
  - Colorimetric reagents in single use aliquot
  - 2 Year shelf-life when store at -20ºC
  - Reagents only
- MRP/BCRP Vesicle Kit (cat. no. 459010)
  - One kit supports all BD MRP and BCRP transporter vesicle assays
  - 200 assays
  - Probe substrate for MRP1, MRP2, MRP3 and BCRP
  - Fluorescent substrate (CDCF) for MRP2 and MRP3
  - 2 Year shelf-life
  - Reagents only
- BSEP Vesicle Kit (cat. no 459011)
  - Supports BSEP transporter vesicle assays
  - 200 assays
  - Probe substrate for BSEP
  - 2 Year shelf-life
  - Reagents only
- 10 X Wash Buffer for MRP/BCRP Vesicle Assay (cat. no 450600)
- 10 X Wash Buffer for BSEP Vesicle Assay (cat. no 450601)

NEW Products

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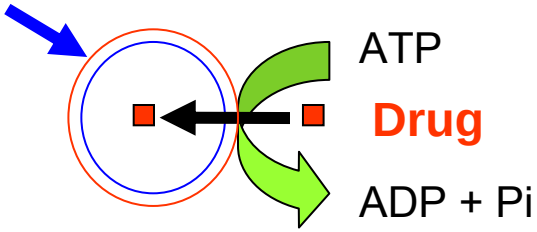
Vesicle Uptake Assays

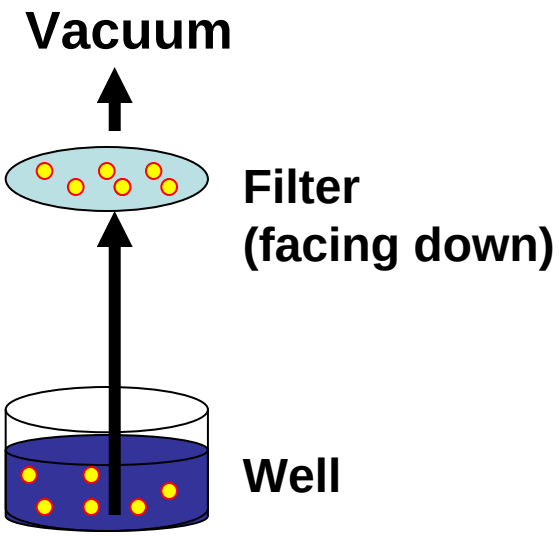
Using Radiolabeled Compounds in Vesicle Uptake Assays

- **Equipment**
  - Vacuum manifold/cell harvester and scintillation counter (if 96-well glass fiber filter plate is used, MicroBeta scintillation counter, Perkin Elmer is needed)
- **Advantages**
  - Extraction step in sample preparation is not required
  - Recovery is not an issue
  - Fast
- **Disadvantages**
  - Radiolabeled compounds are not always available
  - Waste removal and radiation license requirements associated with radiolabeled compound
  - Expensive

Vesicle Uptake Assay Using Cell Harvester – Radiolabeled Compound

Inside-out Vesicles

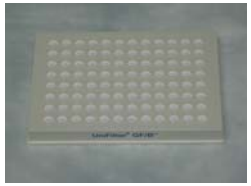




Equipment and Materials

Cell Harvester

Filter Plate



Microplate Scintillation Counter

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Using a Fluorescent Substrate in Vesicle Uptake Assays

- Equipment: Vacuum manifold and spectrophotometer
- Compared with the physiologically relevant, high-affinity substrate, LTC_4, CDCF is:
  - More robust MRP2 substrate, with similar kinetics as LTC_4
  - Optimal for high throughput screening of compound interactions with MRP2
- No need for radiolabeled compound
- Requires a simple elution step in sample preparation
- Less expensive, more convenient, higher S/N ratio compared to using radio-labeled compound

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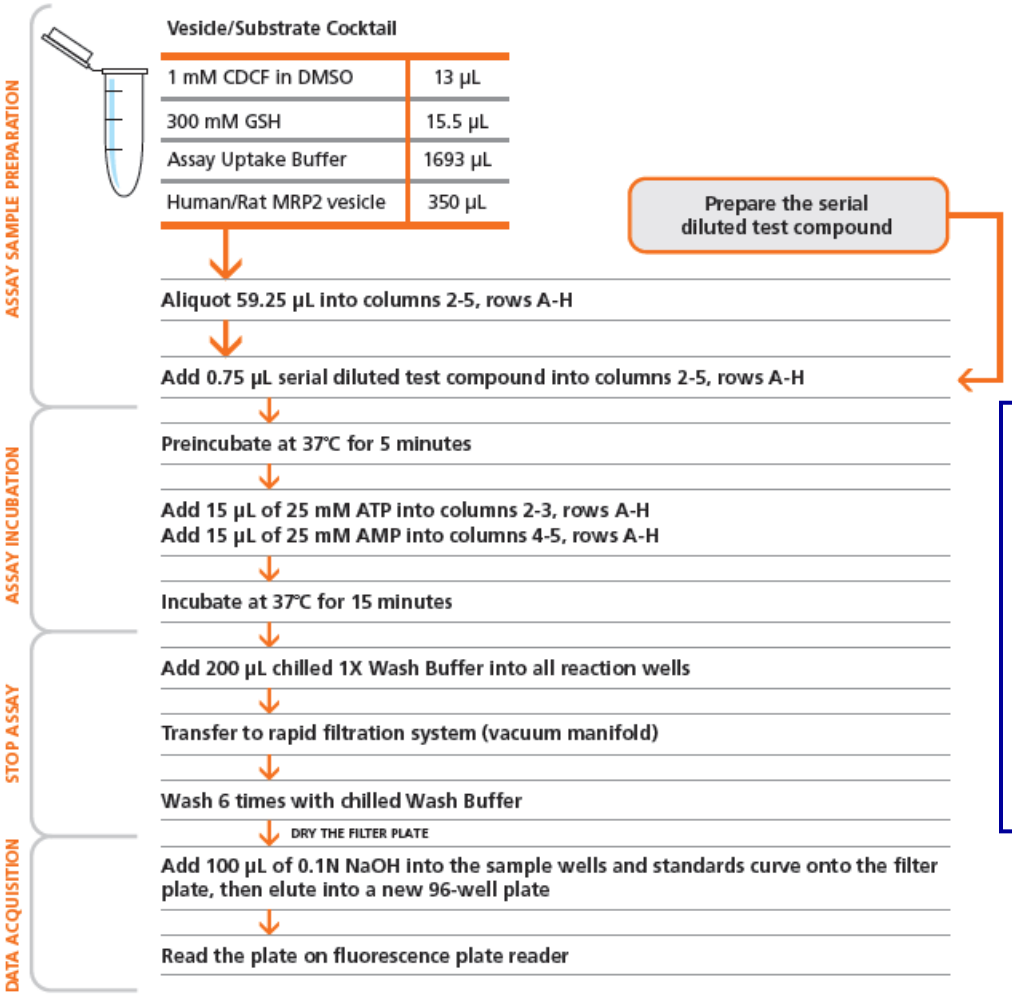
Vesicle Uptake Assay Using Vacuum Manifold – Fluorescent Compound

- Reaction
- **Stop reaction by transferring to vacuum manifold**
- **Wash on vacuum manifold**
- **Elute into a new 96-well plate**
- **Fluorescence reader**

This approach can be used for non-radiolabeled compounds, subject to LC-MS/MS measurements

Overview of Assay Procedure – Example of IC_{50} Study using CDCF as MRP2 Substrate



SERIAL DILUTION OF TEST COMPOUND

		Comp1 w ATP (µM)		Comp1 w AMP (µM)		Comp2 w ATP (µM)		Comp2 w AMP (µM)		STD Curve (CDCF) (nM)		
	1	2	3	4	5	6	7	8	9	10	11	12
A	–	200	200	200	200	200	200	200	200	200	200	–
B	–	100	100	100	100	100	100	100	100	100	100	–
C	–	50	50	50	50	50	50	50	50	50	50	–
D	–	25	25	25	25	25	25	25	25	0	0	–
E	–	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	–	–	–
F	–	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	–	–	–
G	–	3.125	3.125	3.125	3.125	3.125	3.125	3.125	3.125	–	–	–
H	–	0	0	0	0	0	0	0	0	–	–	–

Uptake activity with ATP

Uptake activity with AMP

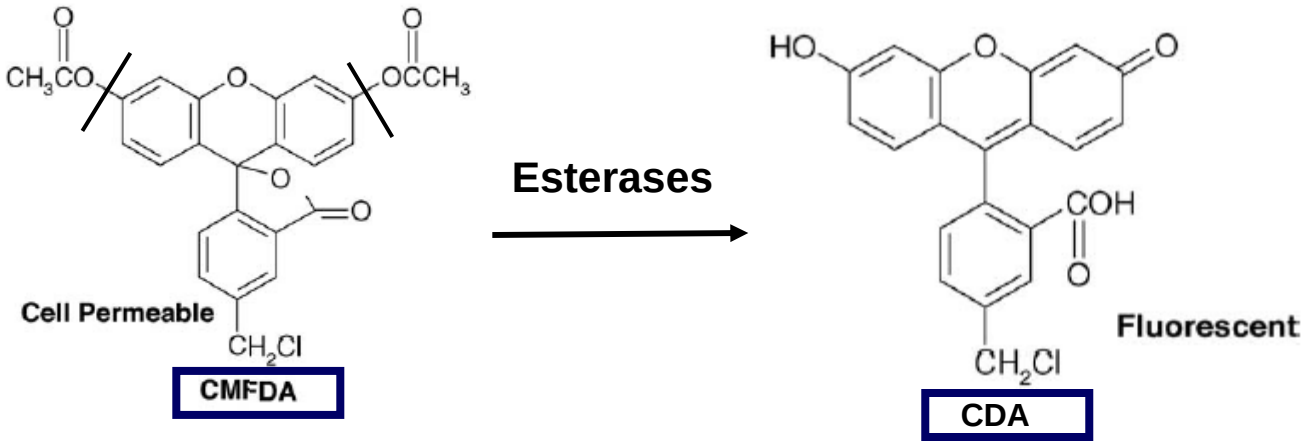
ATP-dependent uptake activity = Uptake activity with ATP (pmol/min/mg) - Uptake activity with AMP (pmol/min/mg)

Signal to Noise Ratio= Uptake activity with ATP (pmol/min/mg) / Uptake activity with AMP (pmol/min/mg)

IC_{50} Calculation:

$$IC_{50} = \frac{(50\% - \text{Low \% Inhibition})}{(\text{High \% inhibition} - \text{Low \% Inhibition})} \times (\text{High Con.} - \text{Low Con.}) + \text{Low Con.}$$

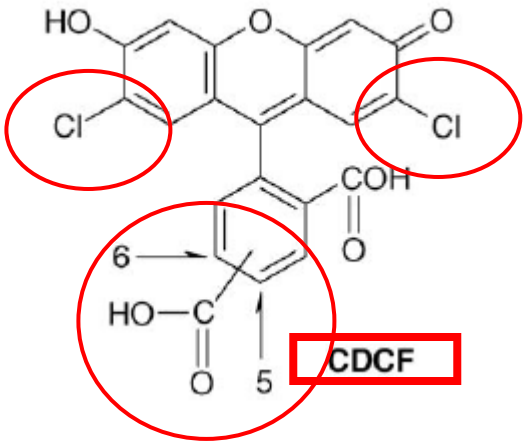
Structure of 5(6)-Carboxy-2,'7'-Dichlorofluorescein (CDCF)



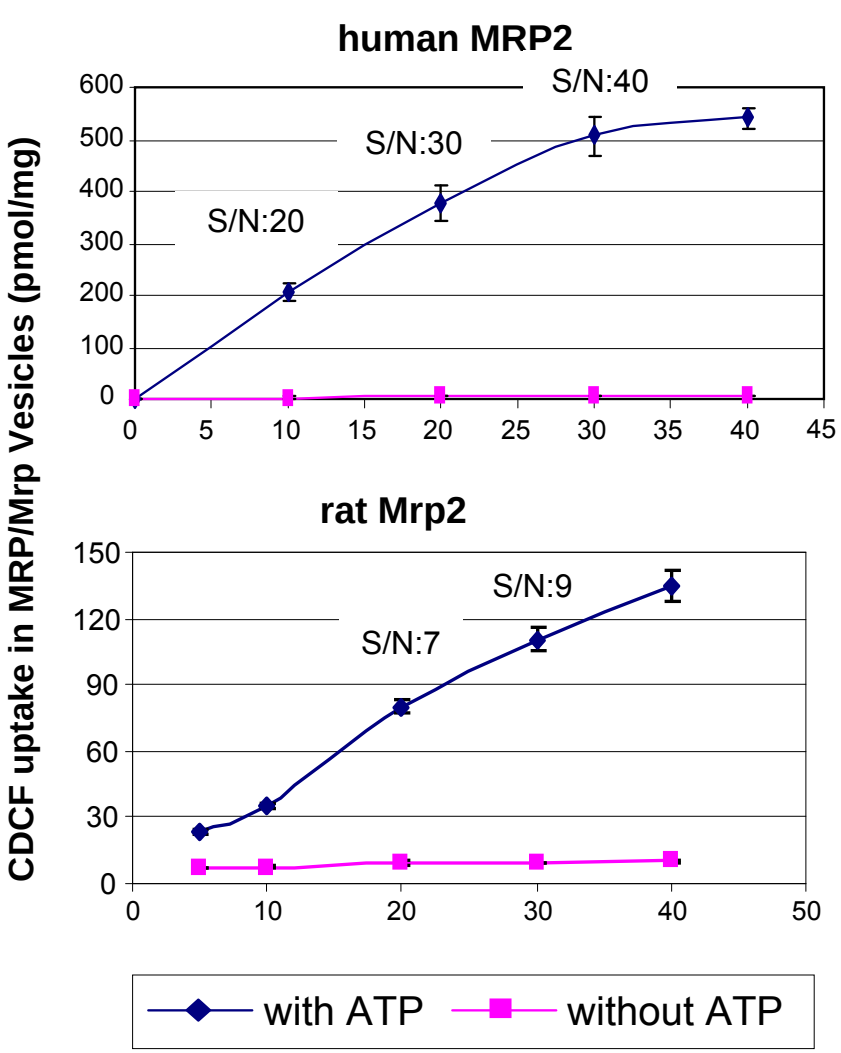
Fluorescent

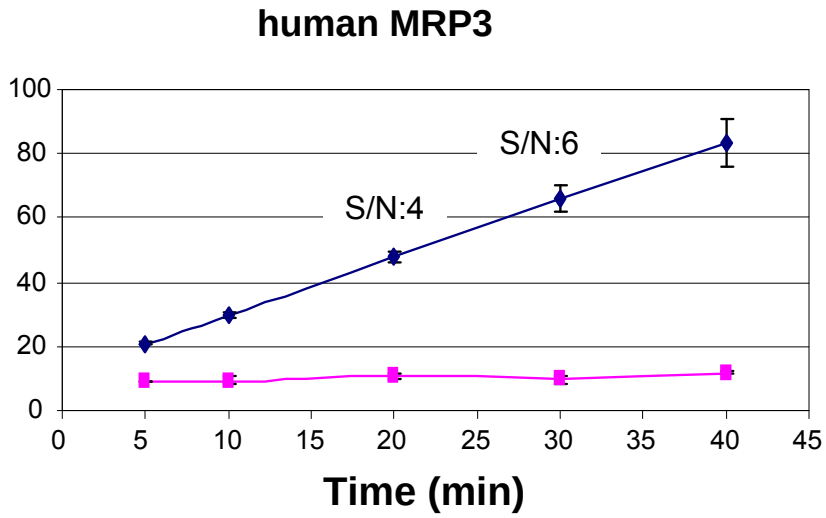
Ex: 485 nm

Em: 538 nm



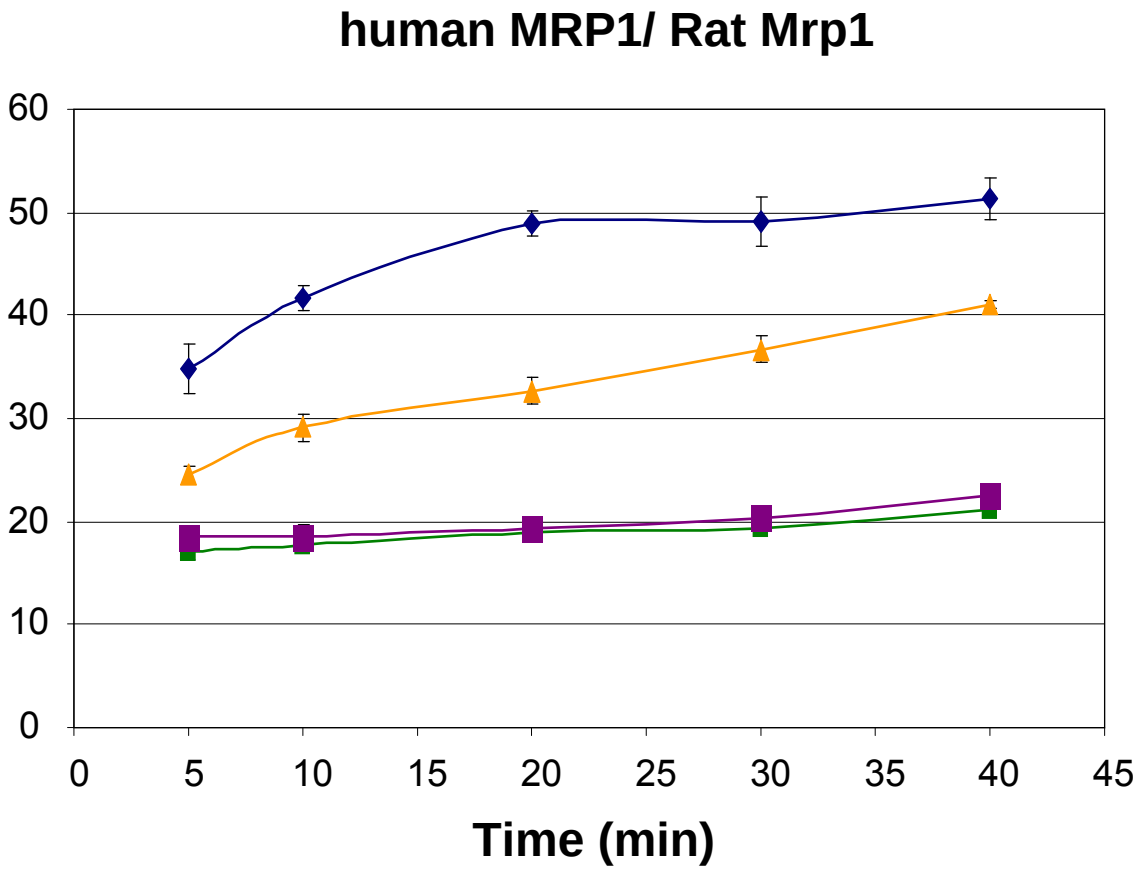
Time-Dependent Uptake of CDCF in MRP2/MRP3 Vesicle





ATP-dependent uptake of CDCF is linear for at least 30 minutes for hMRP2/rMrp2, hMRP3 membrane vesicles.

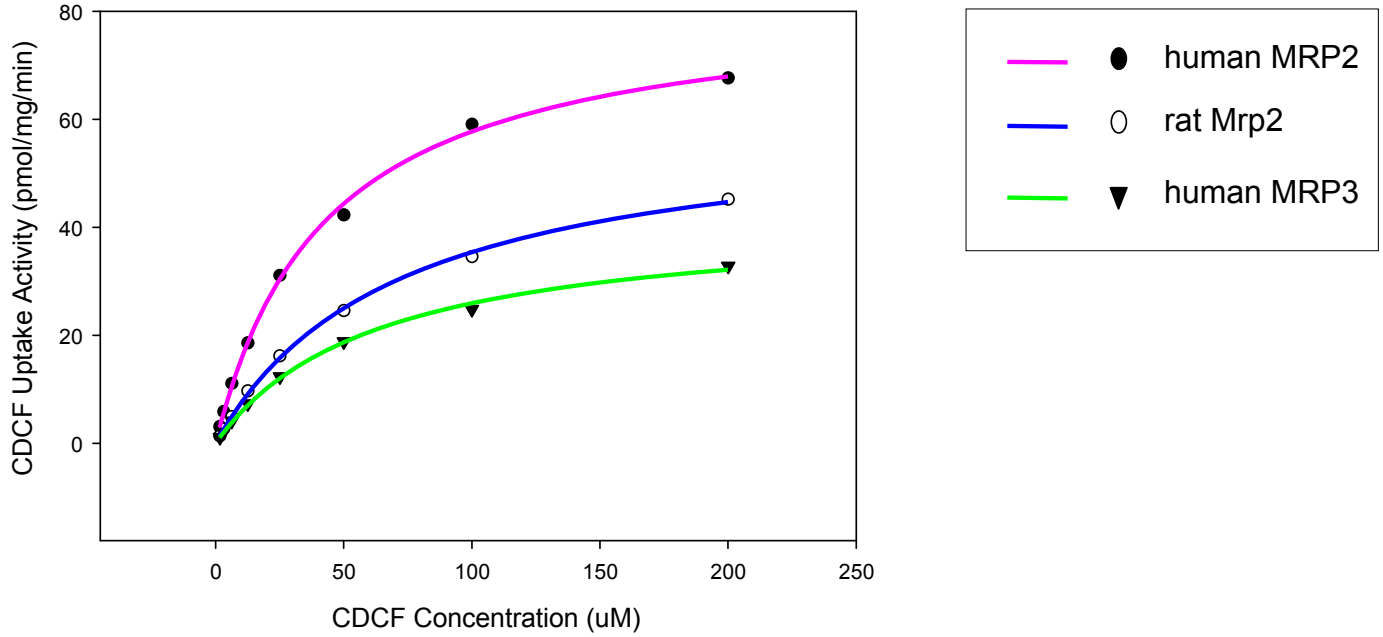
CDCF is not Transported by hMRP1/rMrp1



No time-dependent increase was observed in ATP-dependent uptake of CDCF in hMRP1/rMrp1 membrane vesicles.

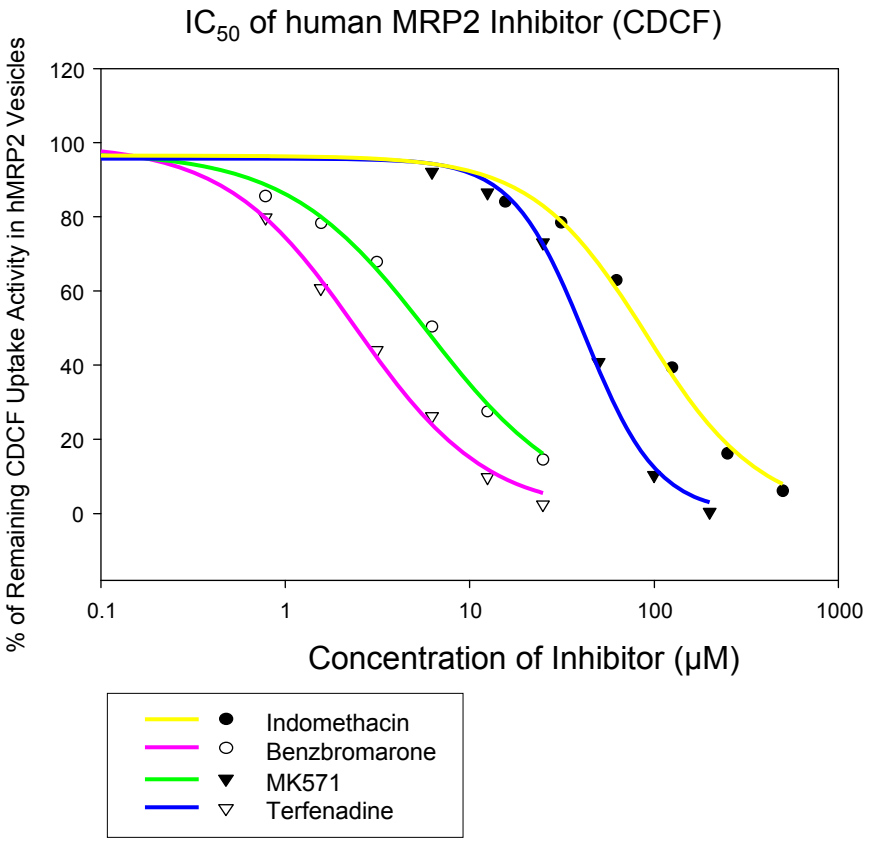
Kinetic Study of CDCF in Human MRP

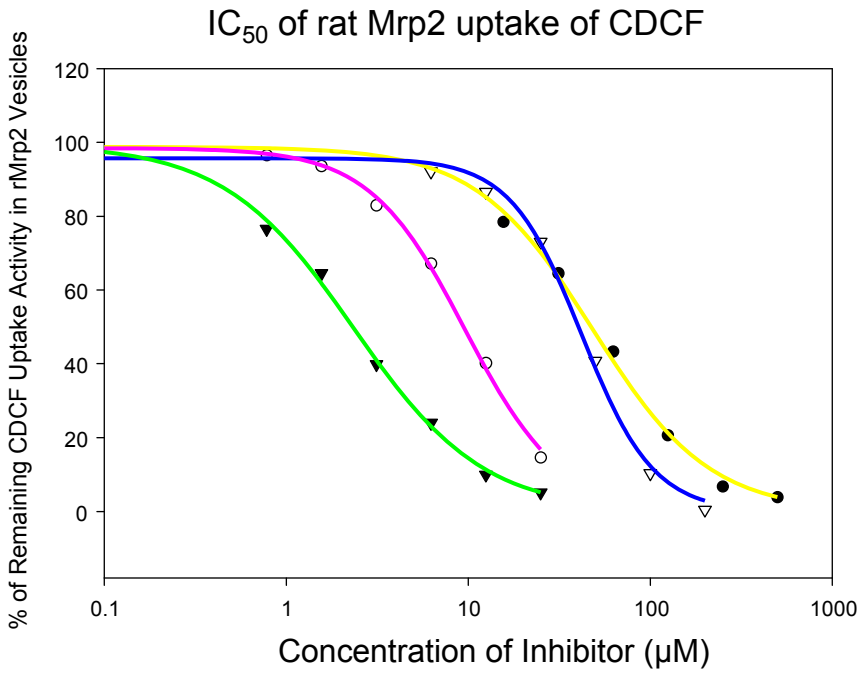
Km of CDCF in MRP2 and MRP3



Vesicles	K_m values (μM)		V_{max} (pmol/mg/min)		**Average K_m**	**Average V_{max}**
hMRP2	23.4	22.5	186	245	22.95	215.5
hMRP3	49.3	63	29.2	42.3	56.15	35.75
rMRP2	57.5	71.1	54	60.6	64.3	57.3

MRP2/Mrp2 IC_{50} Assay Using CDCF as a Substrate





Comparison of Inhibitory Effects of MRP2 Modulators on CDCF Transport in Human MRP2 and rat Mrp2

Human MRP2

Test compounds	IC_{50} (μM)		Average IC_{50} (μM)
	Day 1	Day 2	
MK571	5.9	6.4	6.2
Terfenadine	48.6	42.9	45.8
Benzbromarone	2.6	3.9	3.3
Indomethacin	84.6	96.9	90.8

Rat MRP2

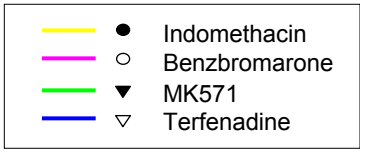
Test compounds	IC_{50} (μM)		Average IC_{50} (μM)
	Day 1	Day 2	
MK571	2.5	2.3	2.4
Terfenadine	42.9	49.7	46.3
Benzbromarone	10.2	13.8	12
Indomethacin	52.7	54.9	53.8

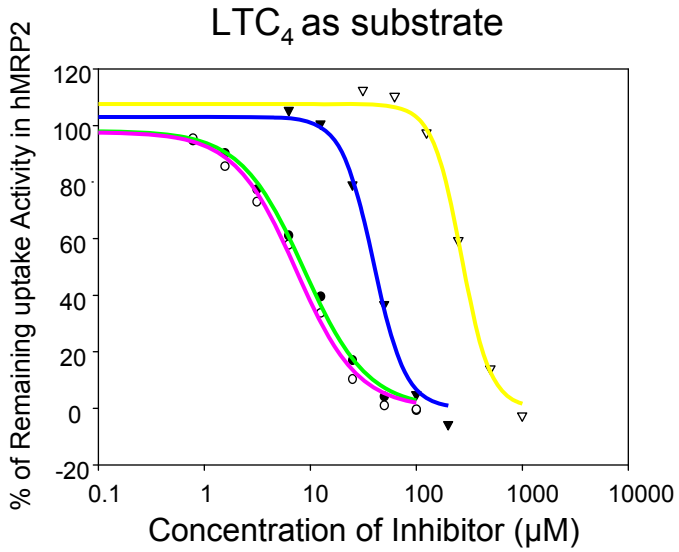
- Four test MRP2 modulators can inhibit ATP-dependent CDCF transport in both human and rat MRP2/Mrp2 vesicles.
- The rank order of the inhibitory potency of four test modulators is slightly different between human and rat.
- Benzbromarone and MK571 are more potent inhibitors than Indomethacin and Terfenadine for both human and rat MRP2/Mrp2.

CDCF is an Alternative to LTC_4 as an MRP2 Substrate

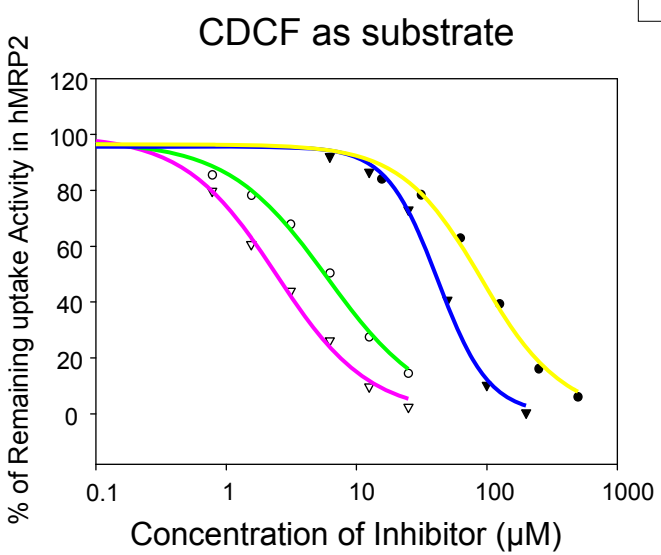
Like LTC_4 (Leukotriene C_4), CDCF is transported by MRP2/Mrp2 in time-dependent, concentration dependent manner following Michaelis-Menten Kinetics.

Comparison of IC_{50} values of human MRP2 modulators.





Test compounds	IC_{50} (μM)
Benzbromarone	7.8
MK571	9.2
Terfenadine	41.6
Indomethacin	281.5



Test compounds	IC_{50} (μM)
Benzbromarone	3.3
MK571	6.2
Terfenadine	45.8
Indomethacin	90.8

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Summary

- Awareness is increasing on the importance of drug transporters in drug pharmacokinetics, safety and efficacy profiles.
- Vesicle uptake assays are direct assays to determine transporter substrates or inhibitors.
- CDCF is a more robust and convenient tool to characterize drug interactions with MRP transporters vs radiolabeled substrates.

Summary of BD Biosciences In Vitro Transporter Models

- **ABC Transporters**
  - Inside-out vesicles
    - Uptake and inhibitory assays
    - MRP/BCRP vesicle kit (**459010**); BSEP vesicle kit (**459011**)
    - 10X wash buffer for MRP/BCRP vesicle assay (**450600**)
    - 10X wash buffer for BSEP vesicle assay (**450601)**
  - Membranes
    - ATPase assay
    - ATPase assay kit (**459006**)
- **SLC Transporters**
  - Expressed in *Xenopus* oocyte system (transportocytes)
    - Uptake and inhibitory assays
  - Hepatocyte suspension assay using oil-filtration method
    - Uptake and inhibitory assays

Questions?

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