Study design – practical considerations for *in vitro* transporter studies

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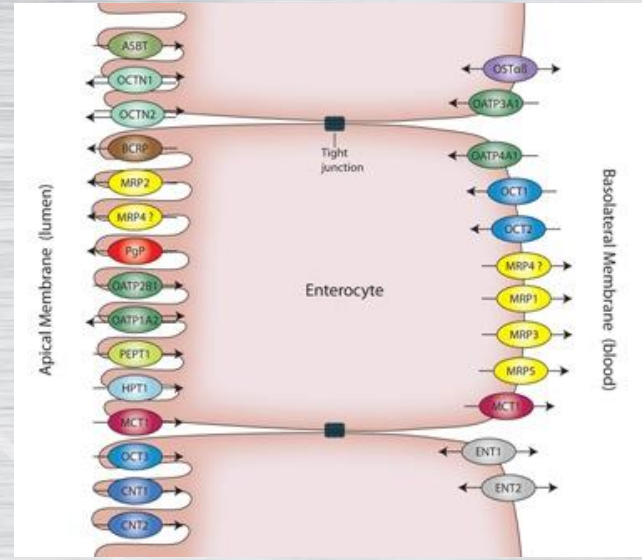
Regulatory compliance

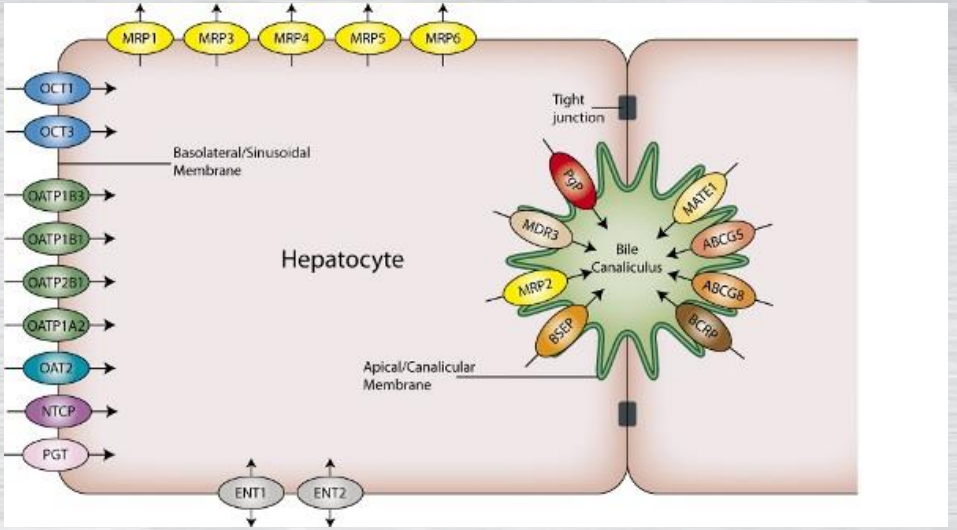
- Test system requirements
- Recommendations for substrate identification
- Recommendations for inhibitor DDI predictions

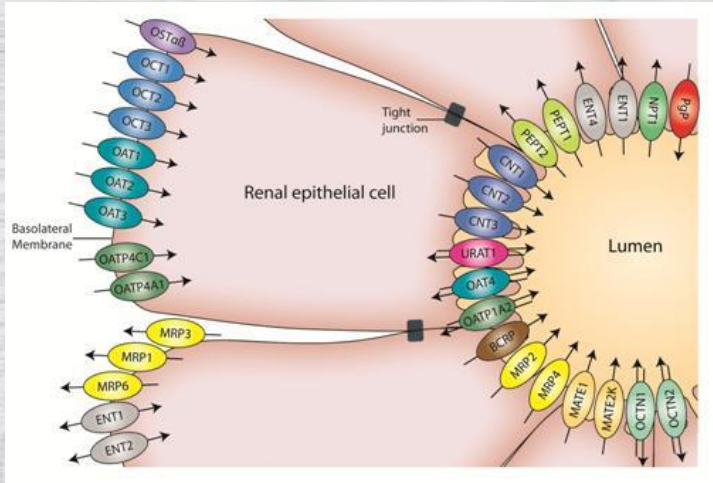
Other transporters / probes to consider

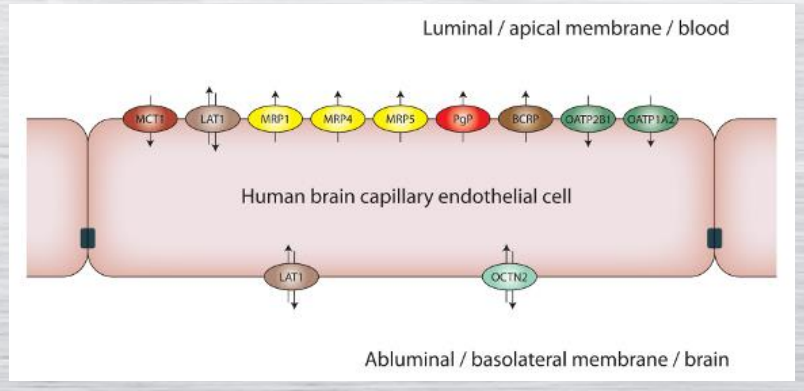
- Safety (BSEP) and screening considerations
- Biomarkers

Which transporters to study?









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FDA and EMA DDI Guidance on Transporters

		Inhibition studies		Substrate studies	
	Transporter	EMA	FDA	EMA	FDA
Efflux	P-gp (MDR1)	yes	yes	consider	yes
	BCRP	yes	yes	consider	yes
	BSEP	prefer	no mention	consider	no mention
	MRPs	no	no mention	consider	no mention
Uptake	OATP1B1	yes	yes	≥25% of elimination hepatic	≥25% of total clearance is hepatic or biliary
	OATP1B3	yes	yes		
	OCT1	consider	no mention	consider	no mention
	OAT1	yes	yes	consider	≥25% of total clearance is active renal
	OAT3	yes	yes		
	OCT2	yes	yes	consider	
	MATE1	consider	yes	consider	
	MATE2	consider	yes	consider	

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Requirements for the test systems

Transporter	In Vitro Systems
ABC Transporters	
BCRP, P-gp	Caco-2 cells, commercial or in-house membrane vesicles, knock-out/down cells, transfected cells (MDCK, LLC-PK_1, etc.)
Solute Carrier (SLC) Transporters	
OATPs	Hepatocytes, transfected cells (CHO, HEK293, MDCK, etc.)
OATs, OCTs	Transfected cells (CHO, HEK293, MDCK, etc.)
MATEs*	Commercial or in-house membrane vesicles, transfected cells (CHO, HEK293, MDCK)

- Recommended test system for each transporter (group)
- Vesicles are accepted but the compound's permeability should be considered
- Bidirectional permeability should be assessed, also on control cells
- For hepatic uptake transporters hepatocytes may also be used
- MATEs – direction of transport is pH dependent

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Requirements for the test systems

- Consistent and characterized transporter expression and function
- Positive and negative controls included in all assay
- Details of the assay should be available and reproducible
- As low as possible organic solvent content (≤ 1% v/v) and solvent control
- Characterize the effect of adding serum protein to the test system
- Transport studied should be conducted under linear transport rate conditions
- Acceptance criteria established (e.g. monolayer integrity, S/N, IC_{50} for inhibitor)
- Substrate can be readily measured with no interference from assay matrix

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Considerations for substrate testing

- The sponsor should evaluate multiple concentrations of the test drug to cover the range of clinically relevant concentrations.

- Typical study designs:
  - 4 concentrations, 1 time point
  - 2 concentrations, 2 time points
- Concentrations are selected based on measured or predicted human exposure:
  - BCRP, MDR1: gut exposure – oral dose/250 mL (solubility!)
  - OATPs: 10 x free hepatic inlet concentration

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

  - Renal transporters: 50 x unbound C_{max}
- Lowest concentration: LLOQ or specific activity

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Considerations for substrate testing

- Several factors may limit test drug concentrations in the in vitro assays, including aqueous solubility, nonspecific binding to the culture vessel, and cytotoxicity.

- The sponsor should evaluate the recovery (mass balance), stability, and/or nonspecific binding of the test drug.

- Aqueous solubility:
  - Routinely assessed using a microscope as the first step of the study
  - Concentration range modification
- Cytotoxicity:
  - Upon request – MTT assay
  - Most transporter assays are typically short (minutes)
  - Bidirectional permeability assay – 120 minutes – integrity test
- Non-specific binding
  - Plastic binding
    - Modeling assay conditions in the absence of cells/vesicles
  - Cellular association
  - Recovery assessment is part of standard design for bidirectional perm

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Considerations for substrate testing

- If the in vitro system expresses multiple transporters, the sponsor should conduct additional experiments to confirm the findings with two or more known potent inhibitors.

- Caco-2 assay:
  - MDR1: valspodar (PSC833) and verapamil
  - BCRP: Ko143 and novobiocin

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Considerations for inhibition testing

- Test-drug concentrations should generally be as high as possible to maximize the inhibition effect. However, the drug concentration should not exceed the drug's solubility limits or cause deleterious effects (e.g., cytotoxicity) in the cells.

- Step 1: choosing concentrations based on PK parameters if available (if not 100 or 300 µM)
- Step 2: solubility assessment in assay buffers (modify cc range if necessary)
- Step 3: cytotoxicity measurement (upon request; modify cc range if necessary)
- Step 4: transporter inhibition assay

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Considerations for inhibition testing

- Number of concentrations: two cc pre-screen for all transporters; follow up with 7 ccs if > 50% inhibition observed (IC_{50} or K_i determination)
- Top assay concentration should be chosen based on PK parameters
- Calculated IC_{50} values are compared with the relevant PK parameters for DDI prediction
- The probe substrate concentration should be at or below the K_m

Prediction of clinical DDI based on in vitro data

Transporters	FDA	EMA
Intestinal Efflux (MDR1 and BCRP)	$[I]_2/IC_{50} < 10$	$K_i\ (IC_{50}) \geq 0.1 \times [I]_2$
Hepatic Uptake (OATP1B1*, OATP1B3* and OCT1)	$R < 1.1$	$K_i\ (IC_{50}) \geq 25 \times [I]_{u,inlet,max}$ (following oral dosing) $K_i\ (IC_{50}) \geq 50\times$ unbound C_{max} (following IV dosing)
Hepatic Efflux (MDR1, BCRP, BSEP and MATE1)	NA	$K_i\ (IC_{50}) \geq 50\times$ unbound C_{max}
Renal Uptake (OAT1, OAT3 and OCT2)	Unbound $C_{max}/IC_{50} < 0.1$	$K_i\ (IC_{50}) \geq 50\times$ unbound C_{max}
Renal Efflux (MDR1, BCRP, MATE1 and MATE2-K)	Unbound $C_{max}/IC_{50} < 0.02$ for MATE1 and MATE2-K	$K_i\ (IC_{50}) \geq 50\times$ unbound C_{max}

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Considerations for inhibition testing

- The sponsor should consider a pre-incubation step with the test drug (for a minimum of 30 minutes) for OATP1B1 and OATP1B3 inhibition to evaluate whether TDI could result in a lower IC_{50} of the test drug. For example, recent data show that cyclosporine and its metabolite AM1 are time-dependent OATP1B inhibitors (Amundsen, Christensen et al. 2010; Gertz, Cartwright et al. 2013; Izumi, Nozaki et al. 2015).
- The sponsor could use positive and negative controls to calibrate their internal in vitro systems to generate cutoff values to inform potential future clinical DDI studies.

- A 30-minute preincubation with the inhibitor is included in all SLC transporter inhibition assays
- More information on time-dependent inhibition and assay calibration:
  - Péter Tátrai: Validating and optimizing in vitro assays for improved DDI prediction – assay calibration and time-dependent inhibition
  - Day 2 13:10

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Considerations for inhibition testing

- Inhibition can be substrate dependent; therefore, the sponsor should determine the inhibition constant of the test drug with a probe substrate that may also be used in later clinical studies. Alternatively, the sponsor may use a probe substrate that usually generates a lower IC_{50} for known inhibitors to avoid underestimating the interaction potential of the investigational drug.

Transporter	Test system	Probe substrate
MDR1	Bidirectional permeability assay	**Digoxin**
BCRP	Bidirectional permeability assay	Prazosin, E3S, **teriflunomide**, **chlorothiazide**
MDR1	Vesicular transport assay	NMQ
BCRP	Vesicular transport assay	E3S, **sulfasalazine**, **rosuvastatin**
MATE1, MATE2-K, OCT2 and OCT1	Uptake transporter assay	**Metformin**, TEA
OAT1	Uptake transporter assay	**tenofovir**
OAT3	Uptake transporter assay	E3S, **methotrexate**
OATP1B1	Uptake transporter assay	$E_2$17βG, **olmesartan**, **statins***
OATP1B3	Uptake transporter assay	CCK-8, **statins***

* In progress

Suggested clinical probes by the FDA

- MDR1: **digoxin**, dabigatran etexilate, fexofenadine
- BCRP: **rosuvastatin**
- OATPs: pitavastatin, pravastatin and rosuvastatin
- OCT2 and MATEs: **metformin**
- OAT1: adefovir and ganciclovir
- OAT3: benzylpenicillin

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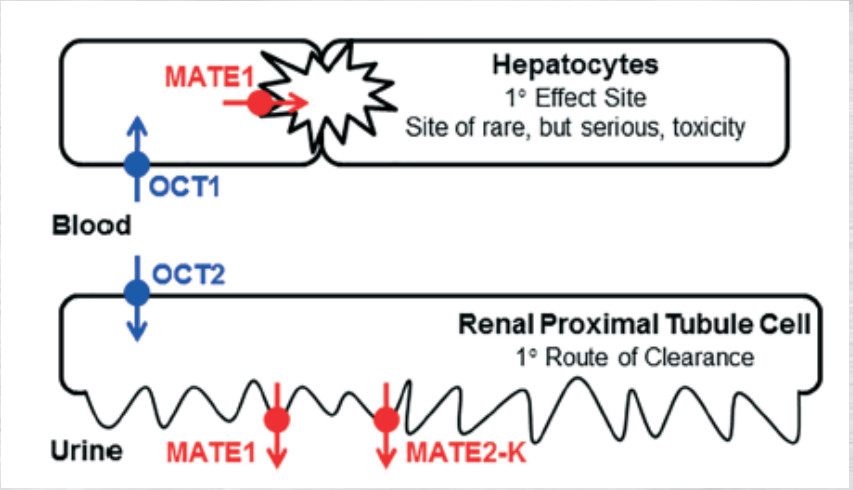
OCT1

- Polymorphic transporter
- Uptake of metformin into hepatocyte – key determinant for pharmacological effect
- Not an indicator for metformin PK (OCT2)
- Other compounds were suggested as clinical probes
  - **Sumatriptan**
  - Fenoterol
  - Ondansentron



Zamek-Gliszczynski, CPT 2018, Matthaei, CPT 2016

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Regulatory compliance

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Other transporters / probes to consider

- Safety (BSEP) and screening considerations
- Biomarkers

Discovery – screening considerations for special targets

- Simple test system
- Easy read-out
- Relevant probe substrate (e.g. co-medication)
- Possibility for automation

- Solvo solutions:
  - Large vesicle batches
  - Cell bank – cell line licensing
  - Validated clinically relevant probes

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Discovery – safety considerations (BSEP)

- Ranking cpds based on BSEP IC_{50}, 25 µM cutoff
- 79% of cpds with BSEP IC_{50} < 25 µM associated with DILI
- C_{ss} / BSEP IC_{50} > 0.1 gave 95% correlation with DILI incidence
- Recommendations:
  - BSEP VT screen for potent inhibitors
  - Confirmatory transporter assays (MRP2, MRP3 and MRP4) may be helpful

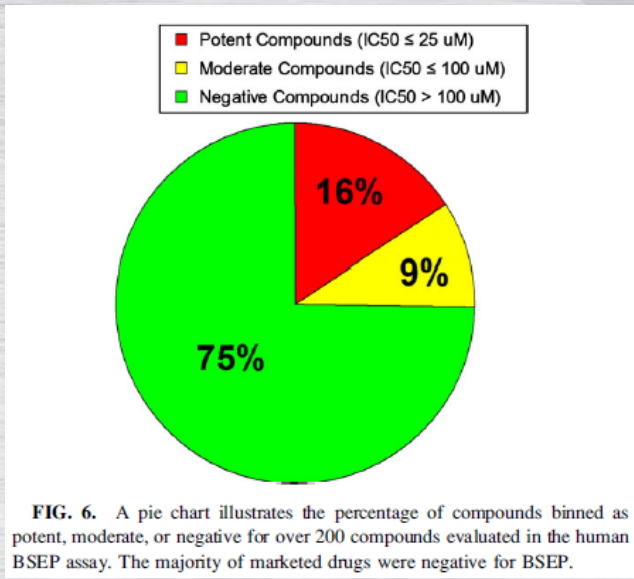

FIG. 6. A pie chart illustrates the percentage of compounds binned as potent, moderate, or negative for over 200 compounds evaluated in the human BSEP assay. The majority of marketed drugs were negative for BSEP.

TABLE 2
Number of Compounds With Evidence of Liver Injury/Total Number of Compounds Fitting Column and Row Criteria (%)

Transporter Assay	C_{ss}/IC_{50} Ratio < 0.01	C_{ss}/IC_{50} Ratio < 0.1	C_{ss}/IC_{50} Ratio ≥ 0.1
BSEP	18/44 (41%)	34/70 (49%)	36/38 (95%)
MRP2	6/9 (67%)	9/13 (69%)	1/1 (100%)
MRP3	7/11 (64%)	17/23 (74%)	5/6 (83%)
MRP4	10/23 (53%)	26/39 (67%)	14/17 (82%)

Notes. The closer exposure values in humans approach *in vitro* potency values in the transporter assays, the stronger the association with liver injury. Conversely, as the exposure values fall further below the *in vitro* potency values, the weaker the association with liver injury.

Morgan, Toxicol Sci 2010 and 2013

Discovery – safety considerations (BSEP)

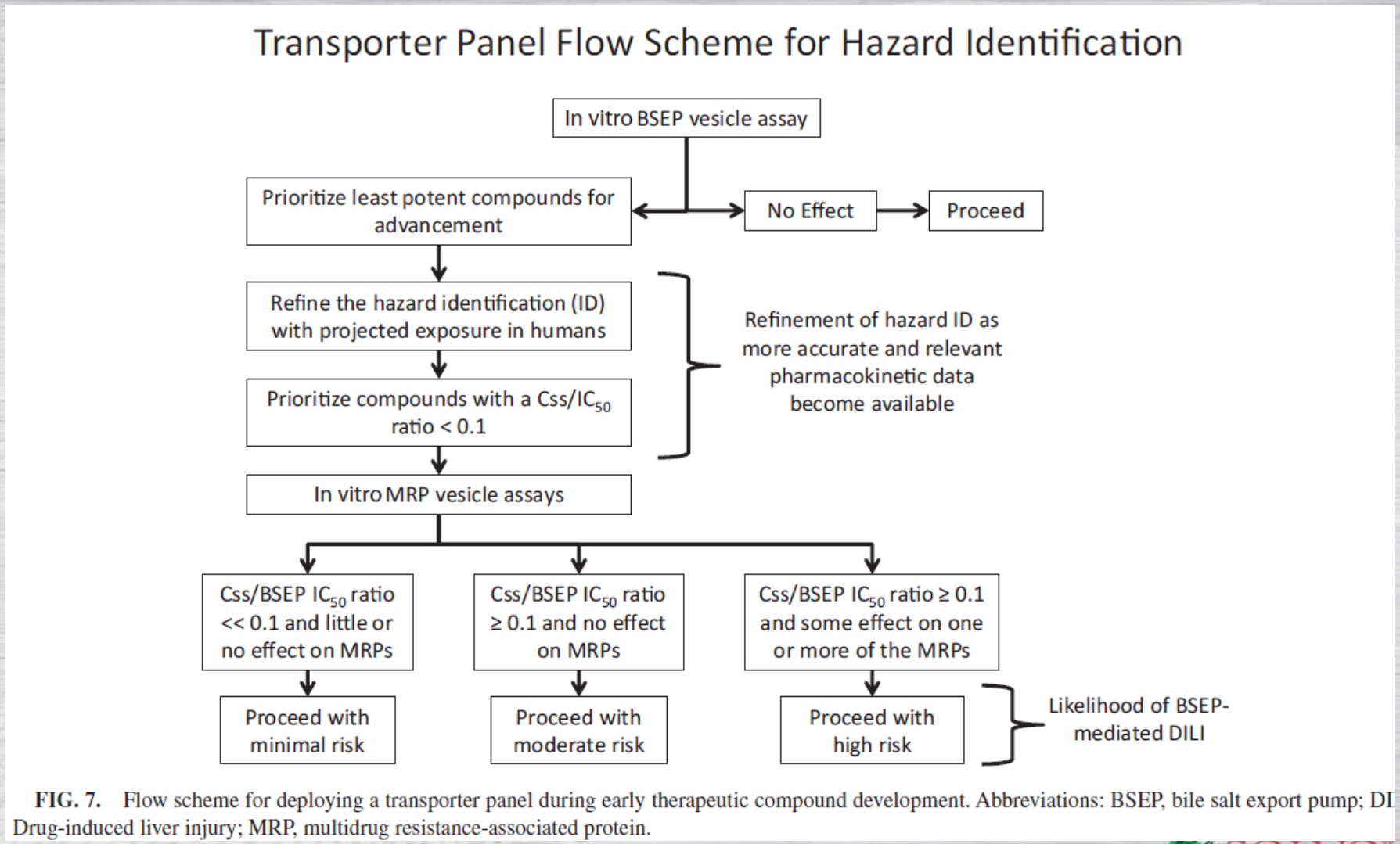

FIG. 7. Flow scheme for deploying a transporter panel during early therapeutic compound development. Abbreviations: BSEP, bile salt export pump; DI
Drug-induced liver injury; MRP, multidrug resistance-associated protein.

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Morgan, Toxicol Sci 2013

Discovery – screening considerations

- Target/indication specific screening
  - MDR1 CNS drugs
  - OATP1B1, OATP1B3 vs statin
  - ASBT (hypercholesterolaemia)
  - URAT1 (uricosuric drugs – inhibit uric acid re-absorption)
  - SGLT1 (antidiabetic)
  - SGLT2 (antidiabetic)
    - Recent warning on FDA's website regarding risk of SGLT2 inhibitors for development of ketoacidosis and urinary tract infections

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Biomarkers for transporters

- = endogenous probes (substrates) for DDI assessment
- Specificity
  - Selective substrate of a given transporter
  - Not a biomarker for a disease (bilirubin, bile acids, creatinine?)
  - Ideally reflects instant response vs delayed (compensatory) effects
- Predictive and translational
  - Correlate with extent of transporter inhibition
  - Reflect site of inhibition (gut, liver, kidney)
- Accessible
  - Blood or urine sampling
  - Can be monitored e.g. Phase I dose finding trials
- Reproducible
  - Rapid, accurate and reproducible detection (LC-MS/MS)
- Cost effective

Biomarker candidates for transporters

- Suggested liver (OATP1B) biomarkers:
  - Bilirubin (conjugated and unconjugated) – not selective enough, can be related to disease state
  - Coproporphyrins
  - DHEAS – might not be sensitive enough, more data needed
  - Conjugated and unconjugated BAs – not selective enough
  - Fatty acid dicarboxylates – from GWAS is an OATP1B1 substrate
- Kidney biomarkers (OCT2/MATEs)
  - Thiamine – TDI might be important only at high doses (reabsorption is dominant)
  - NMN
  - Tryptophan
  - Creatinine – controversial, besides TDI also a marker for AKI; no good correlation with metformin vs cimetidine

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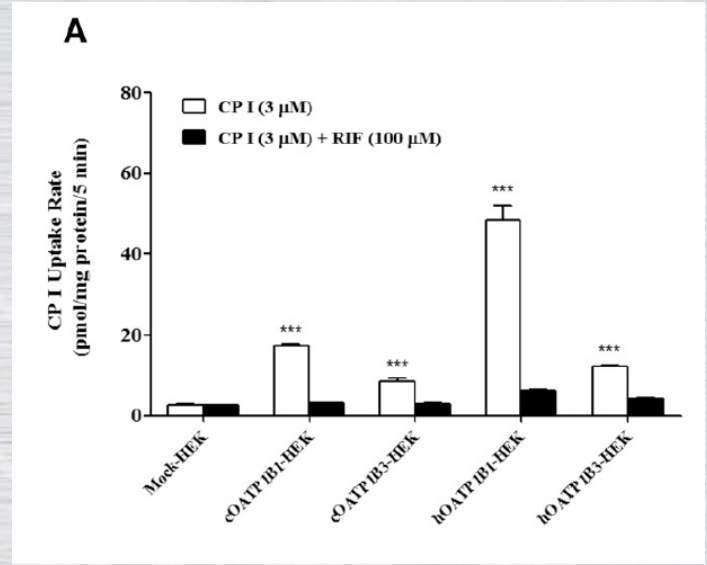
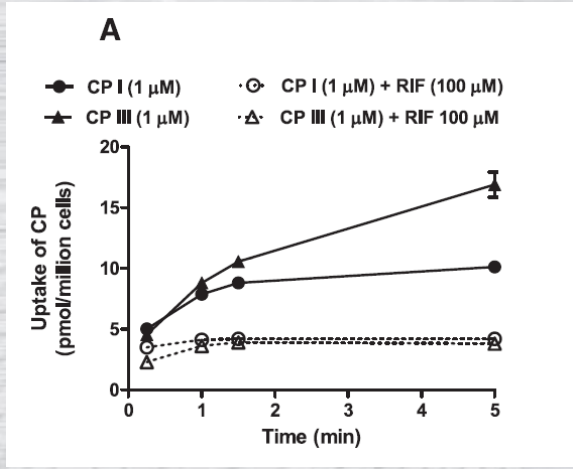
Chu et al J Pharm Sci 2017

Specificity of CP-I and CP-III transport

- Substrates of human and cyno OATP1B1 and OATP1B3
- Not substrates for OAT1/2/3/4,OCT2, MATE1 or MATE2-K
- Transfected cells and hepatocytes





Shen et al JPET 2016

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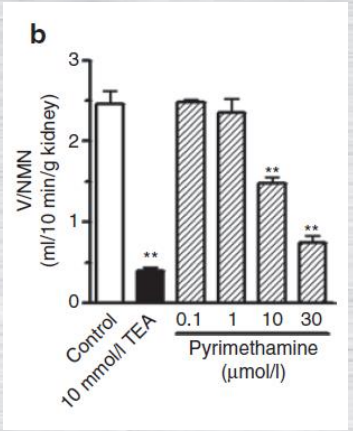
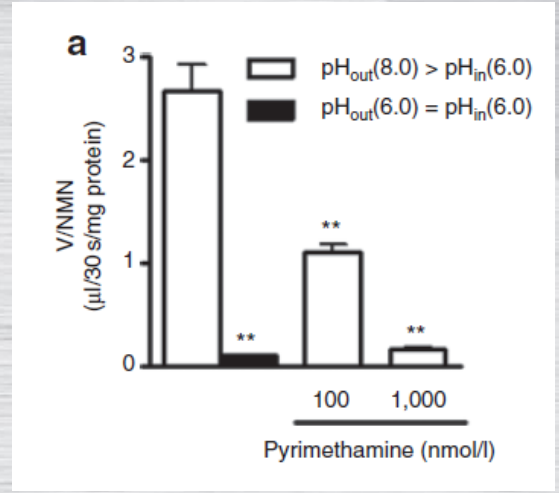
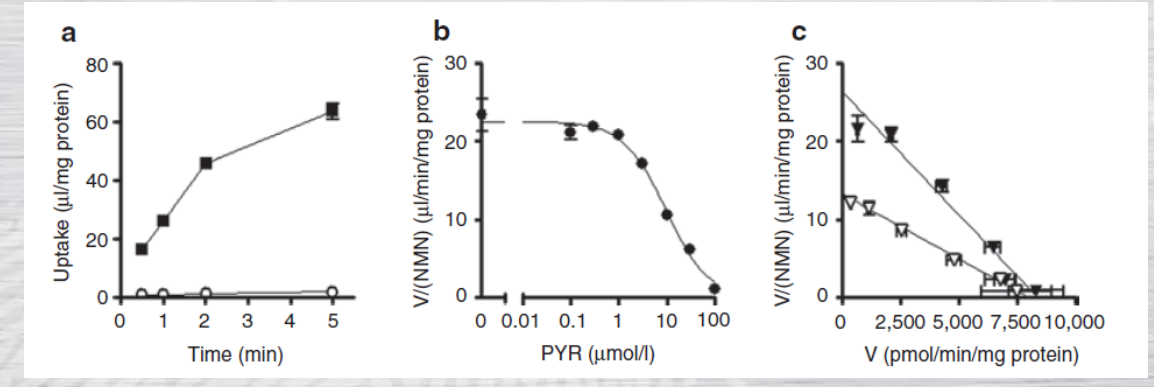
Specificity of NMN transport

- N-methyl nicotinamide
- Substrate for OCT2, MATE1, MATE2-K
- Can be inhibited by PYR in vitro in a competitive manner
- Transfected cells and mouse kidney slices and human BBMVs







Ito et al CPT 2012

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Take home messages

- Regulatory compliance is important
- New details are provided for better in vitro study design
  - Physchem properties
  - Validated assay systems
  - Concentration range based on PK parameters
- In vitro results can be used for clinical DDI predictions
  - Importance of probe substrate selection
  - Improved cutoff values
  - In-house assay calibration is recommended
- There are other transporters beyond the guidance documents!

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Thank you for your attention!

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