Finding physiological functions of drug transporters using KO mice, LC-MS and transportomics

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ABC transporters in Amsterdam

Ancient (start of MDR research in Amsterdam)
- Alexander van der Bliek

Old (start of KO’s of ABC transporters)
- Alfred Schinkel

Old (ABC transporters in trypanosomatids)
- Marc Ouellette (Pgp-A/MRP-A, the first MRP)
- Base J, a novel base in the DNA of trypanosomatids

Recent (drug resistance in mouse mammary cancer models)
- Sven Rottenberg
- Many others

Recent (LC-MS studies on KO mice; transportomics)
- Koen van de Wetering
- Robert Jansen
- Sunny Saphtu
MRPs-introduction

- 1990: Ouellette and Borst identify PgP-A (MRP-A) in Leishmania
- 1992: Susan Cole and Roger Deeley discover the Multidrug Resistance-associated Protein 1 (MRP1)
- 1997: Kool et al. show that MRP1 is part of a gene family in mammals; now 9 members of ABCC family.

- Most of these MRPs do not seem to be involved in MDR.
- All MRPs characterized thus far are multispecific organic anion transporters
Finding the function of MRPs

• Inspired guesswork and screening available organic anions for transport.

• Phenotype of KO mice, double KOs, triple KOs, etc. (and human counterparts).

• Systematic analysis of altered metabolites in KO mice.
Techniques used to study the MRPs

1) Vesicular uptake studies: inside-out vesicles containing the MRP of interest.

2) Cellular assays (efflux/transwell/cytotoxicity).

3) *In vivo* pharmacokinetics in MRP knockout mice.
Vesicular uptake studies

how does it work?

Preparation of membrane vesicles

filter vesicles

wash filter

count filter
*In vivo* pharmacokinetics in Mrp knockout mice.
Example: disposition of morphine in *Mrp2*<sup>-/-</sup> and *Mrp3*<sup>-/-</sup> mice
Transport of morphine-3-glucuronide by MRP2 and MRP3 in vesicular uptake experiments inspired guesswork

### MRP2

- **$V_{\text{max}}$**: 1400 ± 30 pmol/mg/min
- **$K_m$**: 140 ± 10 µM

### MRP3

- **$V_{\text{max}}$**: 500 ± 50 pmol/mg/min
- **$K_m$**: 850 ± 80 µM
Techniques used to study the MRPs

1) Vesicular uptake studies: inside-out vesicles containing the MRP of interest.

2) Cellular assays (efflux/transwell/cytotoxicity).

3) *In vivo* pharmacokinetics in MRP knockout mice.
Morphine and M3G levels in plasma and bile of Mrp2\(^{-/-}\), Mrp3\(^{-/-}\), and WT mice

30 min after i.p. injection of morphine (15 mg/kg)

**Bile**

**Plasma**

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**Morphine**

- WT: 5000 ng/ml
- Mrp2 KO: 5000 ng/ml
- Mrp3 KO: 15000 ng/ml

**M3G**

- WT: 50000 ng/ml
- Mrp2 KO: 150000 ng/ml
- Mrp3 KO: 600000 ng/ml

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**Morphine**

- WT: 1000 ng/ml
- Mrp2 KO: 5000 ng/ml
- Mrp3 KO: 15000 ng/ml

**M3G**

- WT: 5000 ng/ml
- Mrp2 KO: 20000 ng/ml
- Mrp3 KO: 450000 ng/ml
Conclusion: MRP2 and MRP3 are involved in the disposition of morphine
Disadvantages of inspired guesswork approach

- Only one substrate at the time can be studied.
- Experiments often involve use of radioactive compounds.
- Not available for all interesting compounds.
- After in vitro experiments in vivo tests are still needed to determine physiological relevance.
Characterization of the physiological roles of ABC efflux transporters by screening for their *in vivo* substrates using mass spectrometry

Koen van de Wetering
Finding the function of MRPs

- Inspired guesswork and screening available organic anions for transport.
- Phenotype of KO mice, double KOs, triple KOs, etc. (and human counterparts).
- Systematic analysis of altered metabolites in body fluids of KO mice: metabolomics.
The exact physiological role of MRP3 is unclear.

- *Mrp3*^-/- mice do not have an overt phenotype.
- We therefore want to set up a screen to test for alterations in (endogenous) glucuronidated compounds in plasma/urine.
Metabolomics
example: MRP3

wild-type

Mrp3⁻/⁻
Rationale

- Substrates of MRP3 should have a lower abundance in plasma (and urine) of mice that lack Mrp3.
- MRP3 has a preference for glucuronidated compounds
- During mass spectrometry, compounds containing a glucuronic acid moiety have a specific fragmentation pattern after collision-induced dissociation.
Neutral loss (176 Da) scan of wild type mouse plasma.
Detection of unknown glucuronides in mouse plasma

wild type mouse plasma

Mrp3\(^{-/-}\) mouse plasma

- Peak area (a.u.)
- Time, min
- Intensity, cps
Hypothesis peak m/z 477: **Enterodiol-glucuronide**
(educated guess)

Enterodiol-glucuronide

![Chemical structure of Enterodiol-glucuronide]

Enterodiol:
- Lignan
- Precursor present in many plants
- Formed in the gut by resident bacteria
- Known to be glucuronidated

Mw 478.3 (m/z = 477)
LC/MS chromatograms of MRM 477/301

Unknown glucuronide in screen

In vitro generated enterodiol-glucuronide

Unknown compound in screen is: enterodiol-glucuronide
Confirmation that identified compounds are substrate of MRP3

- Are lower levels due to absence of Mrp3 or to secondary effect(s)? Exclude false positive results

- Upregulation of other transporters and/or metabolizing enzymes in Mrp3−/− mice.

- Use in vitro assays to confirm that identified compounds are transported by MRP3.

- Check whether both mouse/human MRP3 transport identified substrate.
Confirmation of enterodiol-GlcA transport by MRP3/Mrp3 in vesicular transport experiments

- **hMRP3** (+ ATP)
- **hMRP3** (no ATP)
- **mMrp3** (+ ATP)
- **mMrp3** (no ATP)
- **WT** (+ ATP)
- **WT** (no ATP)

**Graphs:**
- **X-axis:** time (min)
- **Y-axis:** enterodiol-GlcA uptake (pmol/mg)

**Km Values:**
- hMRP3: $K_m = 4.8 \pm 0.9 \mu M$
- mMrp3: $K_m = 1.7 \pm 0.2 \mu M$
Vesicular transport assays

• Substrates of ABC transporters are present in many different organs/body fluids.
• Can the vesicular transport system be used to screen for substrates in these organs/body fluids?
• Need (unbiased) method to detect substrates taken up into the vesicles.
Transportomics: combination of vesicular transport assays and metabolomics

- Metabolomics aims at making (unbiased) profiles of small molecular compounds in biological samples

- Metabolomics, techniques:
  - LC or GC coupled to Mass Spectrometry (sensitive).
  - NMR (unbiased, but low sensitivity).

- LC/MS-based metabolomics flavors:
  - Targeted: (some) a priori knowledge needed.
  - Untargeted: no a priori knowledge needed.
Vesicular transport assays screen for substrates in biological samples.

Sf9-ABCC2

LC/MS analysis

intensity

time

ABCC2 substrate

Sf9-control

LC/MS analysis

intensity

time
Transportomics: example ABCC2

• also known as Multidrug Resistance Protein 2 (MRP2)
• Present in liver, kidney and gut.
• Involved in excretion of xenobiotics and metabolic waste products
• Absence of functional ABCC2 results in the Dubin-Johnson syndrome: increased circulating levels of bilirubin-glucuronide
Vesicular transport and metabolomics
ABCC2-mediated transport of glucuronides from urine

Transport of glucuronides from mouse urine

Detection: targeted metabolomics (compounds conjugated to glucuronic acid)
Identification of unknown glucurononides

m/z ratio: 557

Sf9-ABCC2 + ATP

m/z ratio: 557
Identification of unknown compound with m/z 557

-176 (C₆H₈O₆ - GlcA) = GlcA

**Unknown compound contains:**
1) Sulphate moiety
2) Glucuronic acid moiety

**Guess:** sulpho-enterodiol-glucuronide

**Mw 558 (m/z = 557)**

- Enterolignan
- Precursor present in food
- Plant-derived compound
- Known to be extensively glucuronidated/sulphated
Identification of compound with a m/z ratio of 557

Unknown compound in screen is: sulpho-enterodiol-glucuronide
Advantages of “Transportomics”

• Transport of several compounds can be studied in one experiment.
• Compounds do not need to be identified in order to study transport.
• Unanticipated substrates can be found (untargeted metabolomics).
• Less experimental animals needed to find physiological substrates.
• Can be used to find physiological substrates if knockout mice are not available (ABCC11 & ABCC12).
Disadvantages of “Transportomics”

• Less suitable for finding hydrophobic substrates.

• Less sensitive than liquid scintillation counting.

• Potential of (competitive) inhibition by other compounds present in body fluid (plasma?)

• Not possible to determine transport kinetics

• Long analysis time per sample.
Outlook

- Use Transportomics to study other members of the ABCC subfamily.
- Use untargeted metabolomics to detect substrates transported into the vesicles.
- Use of tissue extracts (liver?).
- Focus on **ABCC6**.
  - Absence of ABCC6 results in Pseudoxanthoma elasticum (PXE).
  - Ectopic calcification (soft tissues)
  - Due to absence of ABCC6 in the liver. Substrate transported from the liver into the circulation unknown.
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Some papers on ABC-transporters from the Borst lab


