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

Piet Borst

Koen van de Wetering

The Netherlands Cancer Institute

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NKI-AVL

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

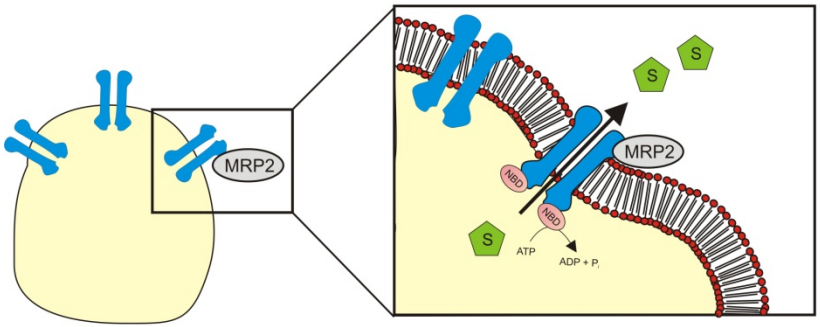
1) Vesicular uptake studies: inside-out vesicles containing the MRP of interest.

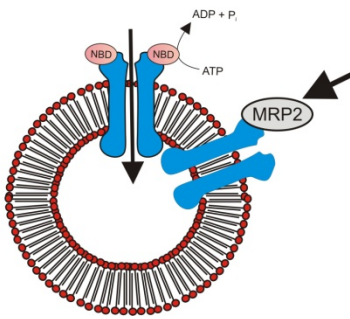
2) Cellular assays (efflux/transwell/cytotoxicity).

3) *In vivo* pharmacokinetics in MRP knockout mice.

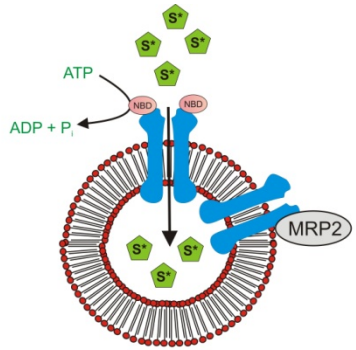
Vesicular uptake studies
how does it work?

Preparation of membrane vesicles





cell lysis
&
differential centrifugation



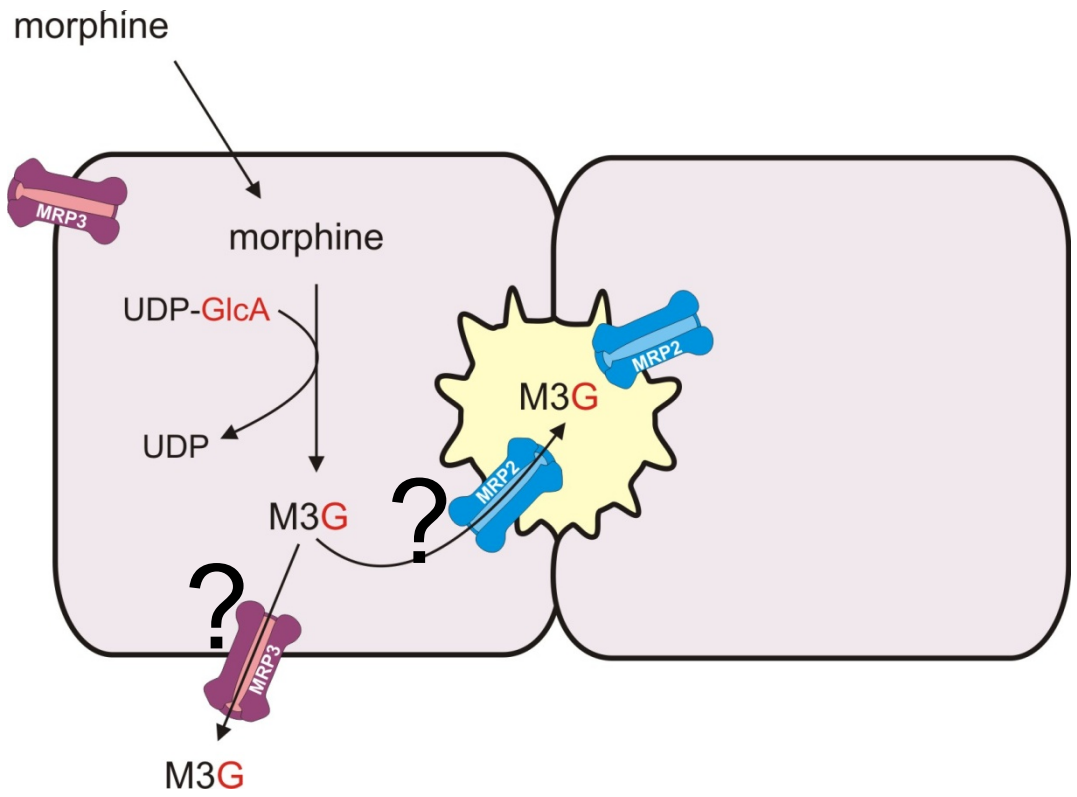
filter vesicles

wash filter

count filter

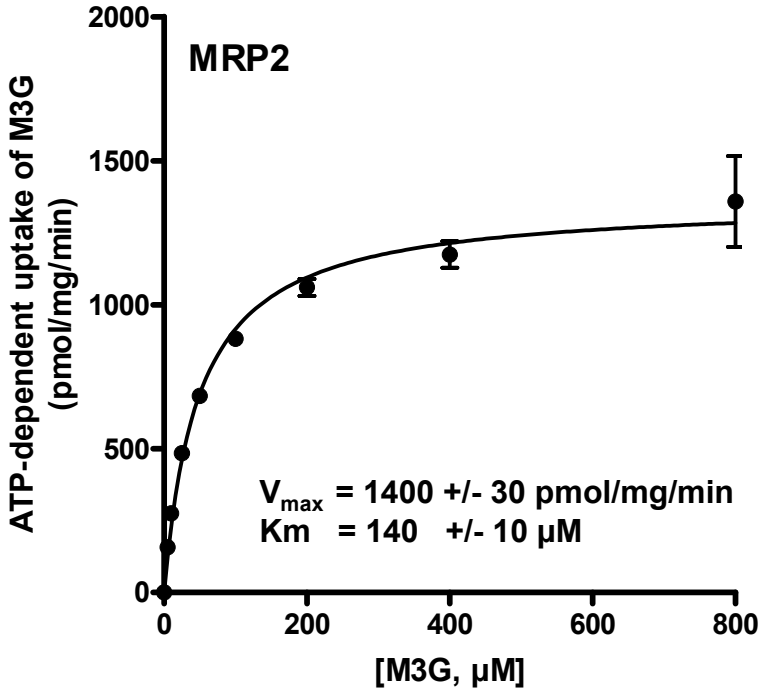
In vivo pharmacokinetics in Mrp knockout mice.

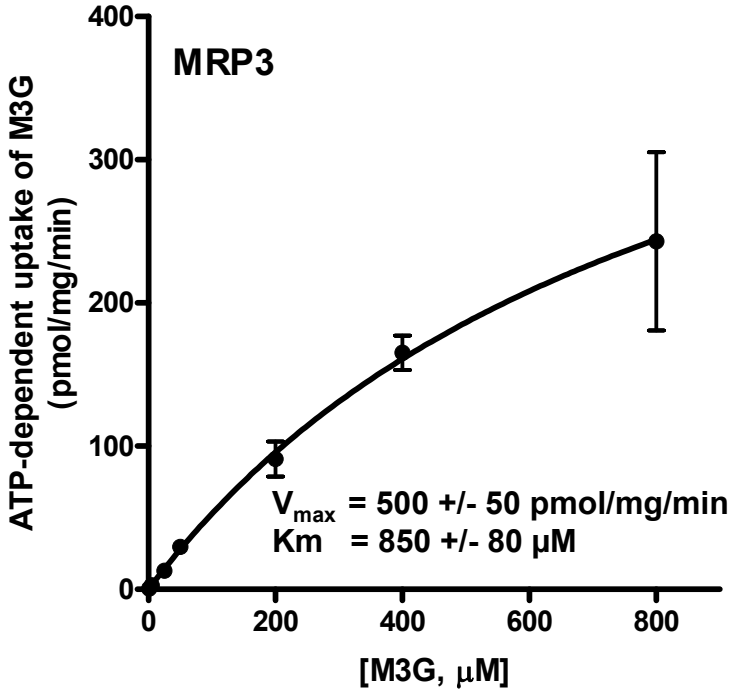
Example: disposition of morphine in *Mrp2*$^{-/-}$ and *Mrp3*$^{-/-}$ mice



Transport of morphine-3-glucuronide by MRP2 and MRP3in vesicular uptake experiments

inspired guesswork





Techniques used to study the MRPs

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

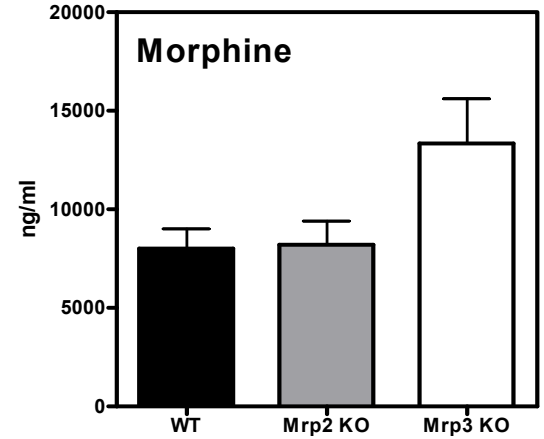
2) Cellular assays (efflux/transwell/cytotoxicity).

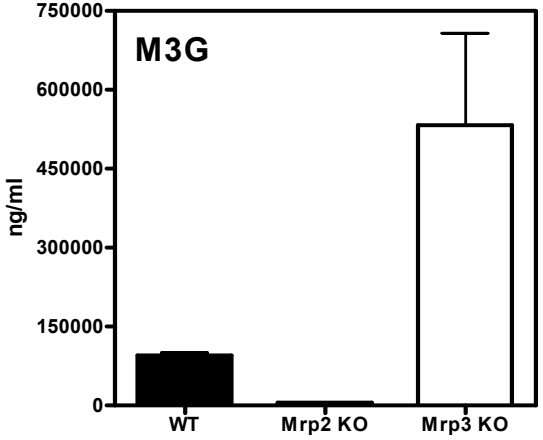
3) *In vivo* pharmacokinetics in MRP knockout mice.

Morphine and M3G levels in plasma and bile of *Mrp2*$^{(-/-)}$, *Mrp3*$^{(-/-)}$, and WT mice

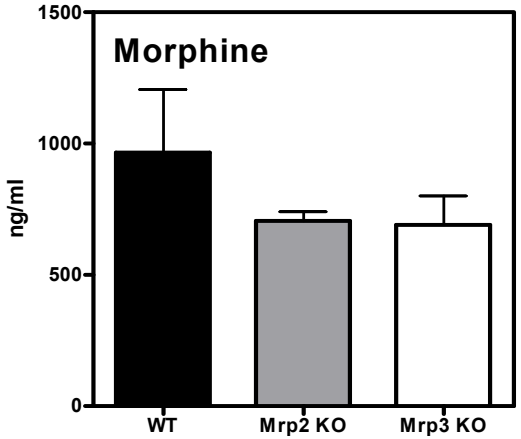
30 min after i.p. injection of morphine (15 mg/kg)

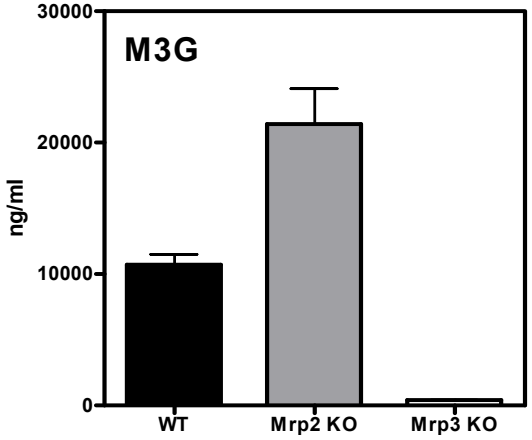
bile



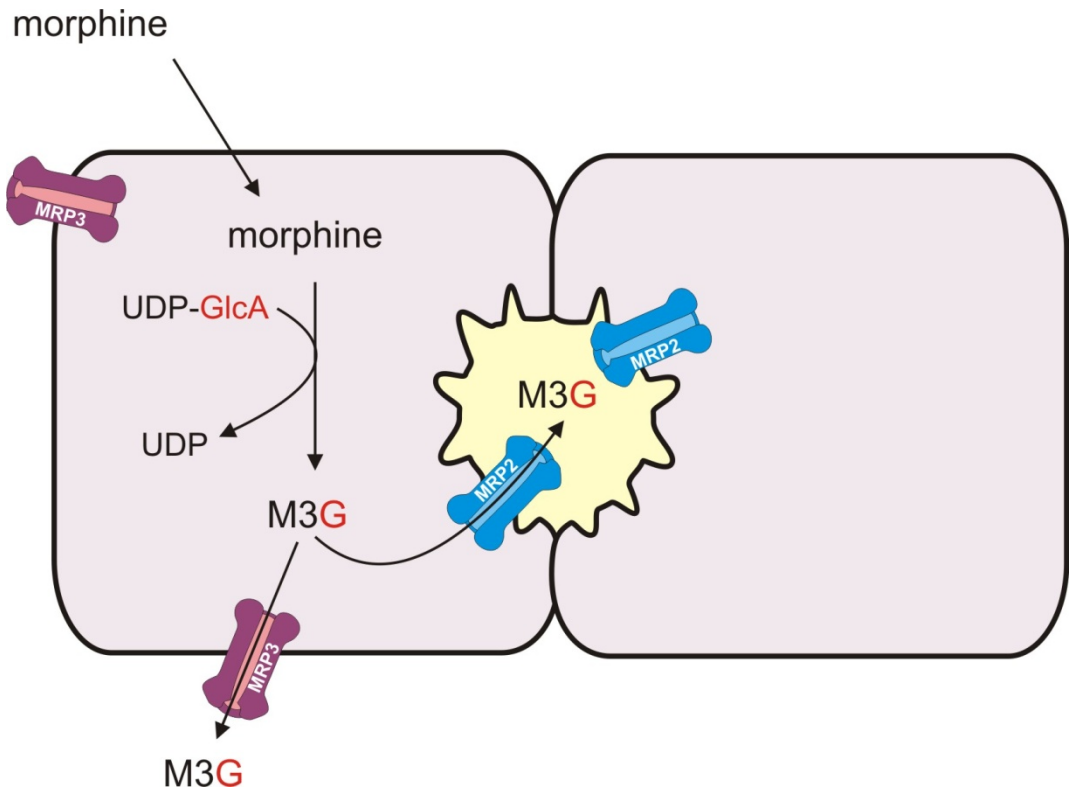


plasma





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

NKI-AVL
The Netherlands Cancer Institute
Antoni van Leeuwenhoek Hospital

Finding the function of MRPs

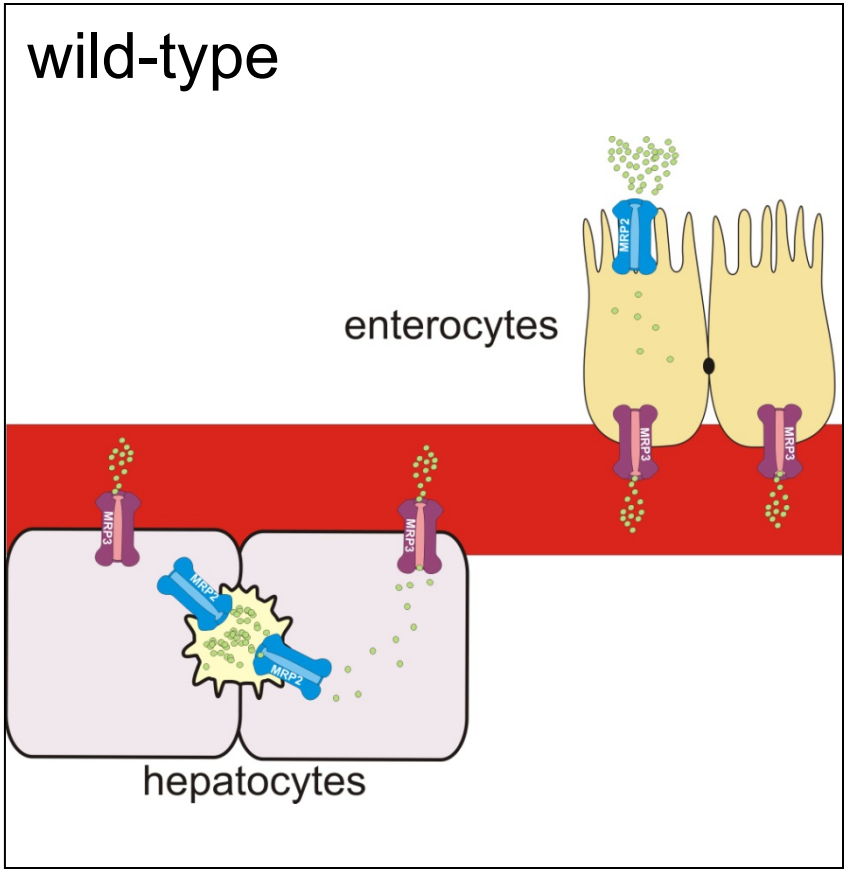
- Inspired guesswork and screening available organic anions for transport.
- Phenotype of KO mice, double KOs, triple KOs, etc. (and human counterparts).
- Systematic analysis of altered metabolites in body fluids of KO mice: **metabolomics.**

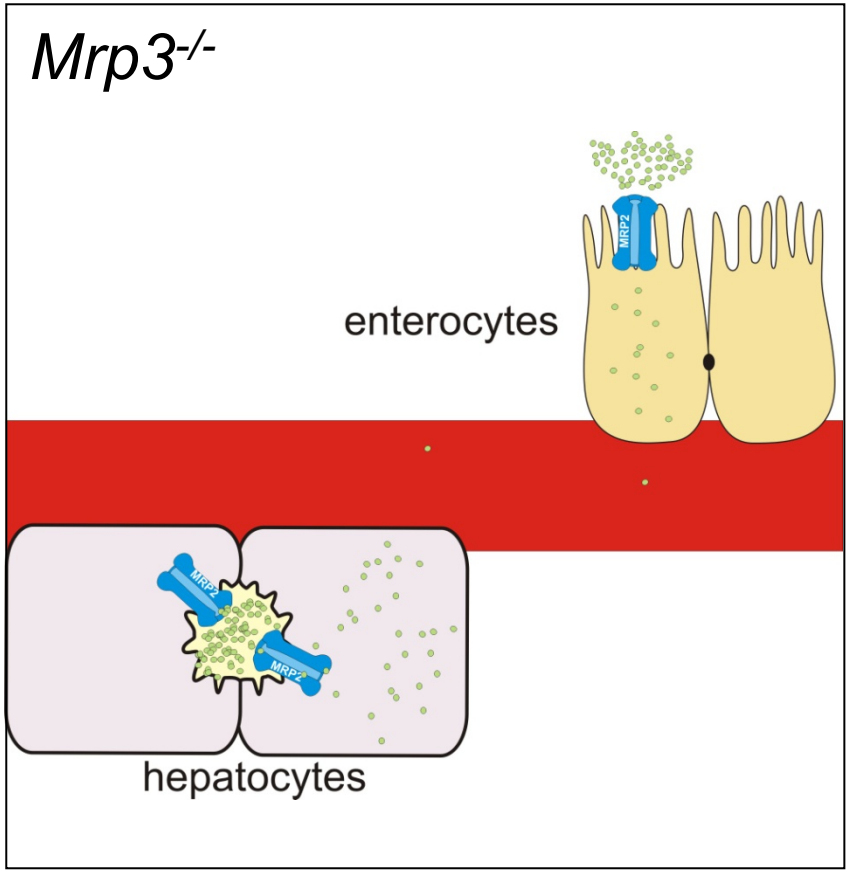
The exact physiological role of MRP3 is unclear.

- *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

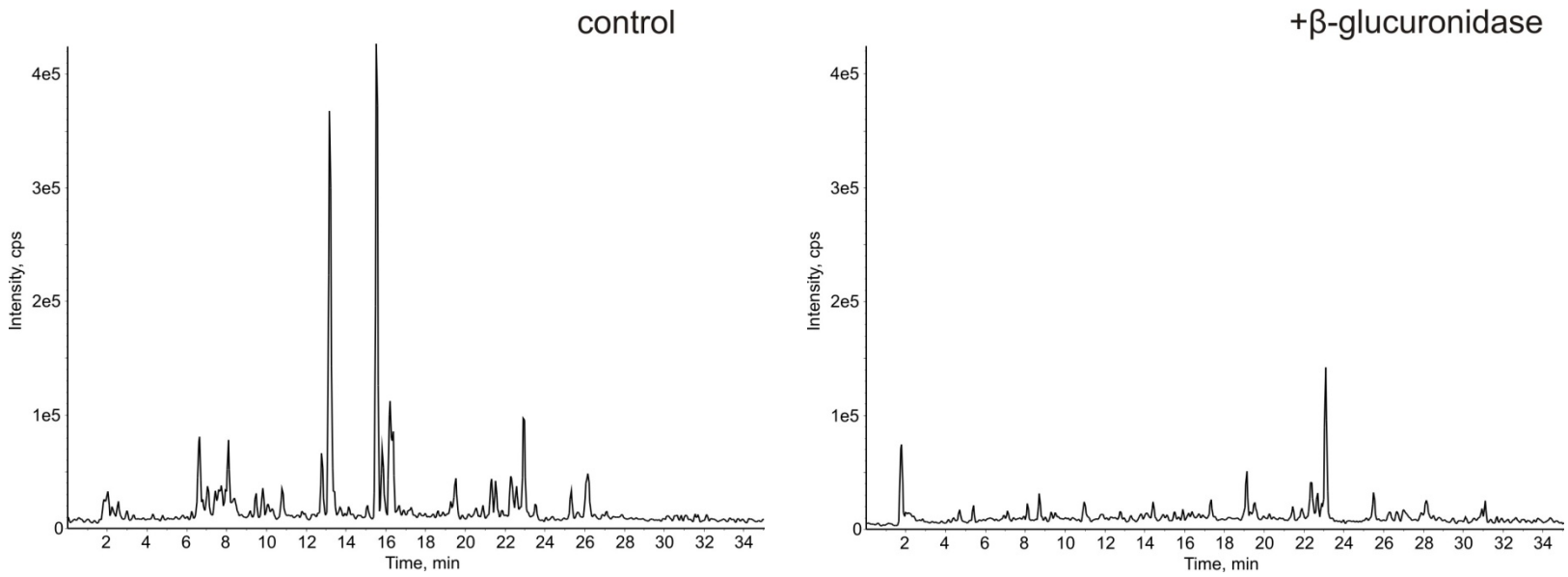




Rationale

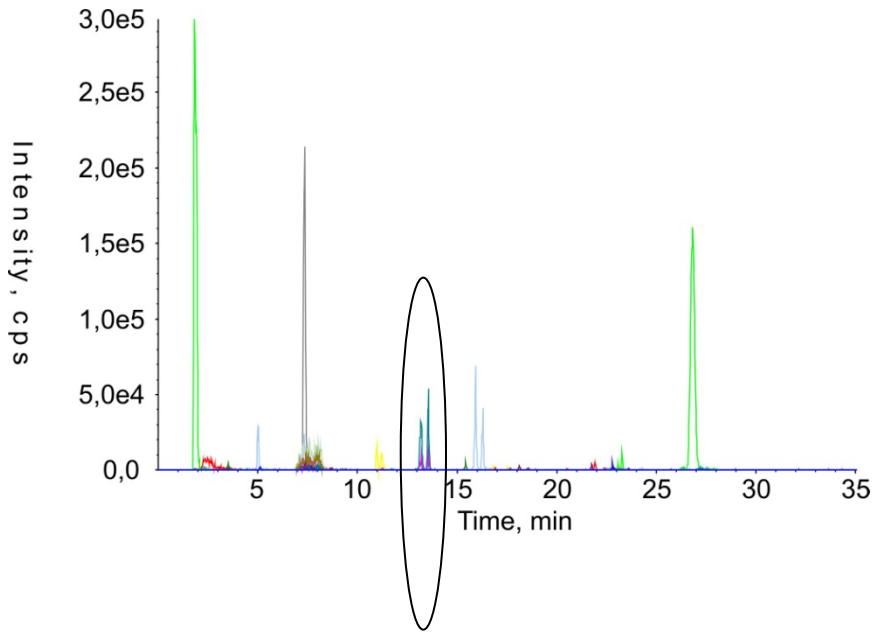
- Substrates of MRP3 should have a lower abundance in plasma (and urine) of mice that lack Mrp3.
- MRP3 has a preference for glucuronidated compounds
- During mass spectrometry, compounds containing a glucuronic acid moiety have a specific fragmentation pattern after collision-induced dissociation.

Neutral loss (176 Da) scan of wild type mouse plasma

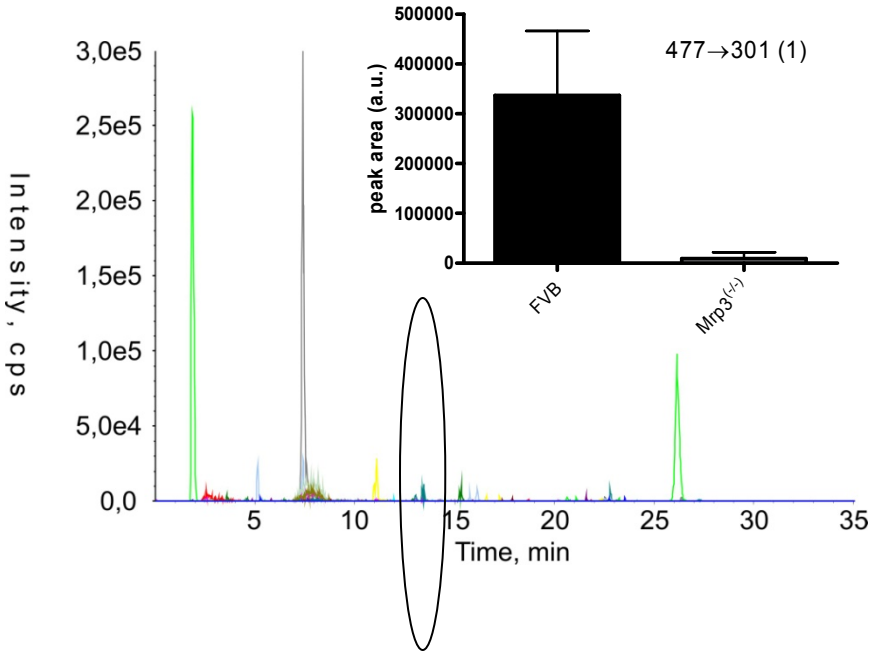


Detection of unknown glucuronides in mouse plasma

wild type mouse plasma

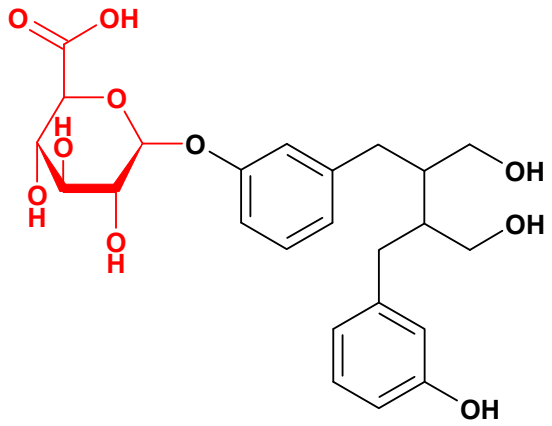


Mrp3$^{-/-}$ *mouse* plasma



Hypothesis peak m/z 477: Enterodiol-glucuronide (educated guess)

Enterodiol-glucuronide



Mw 478.3 (m/z = 477)

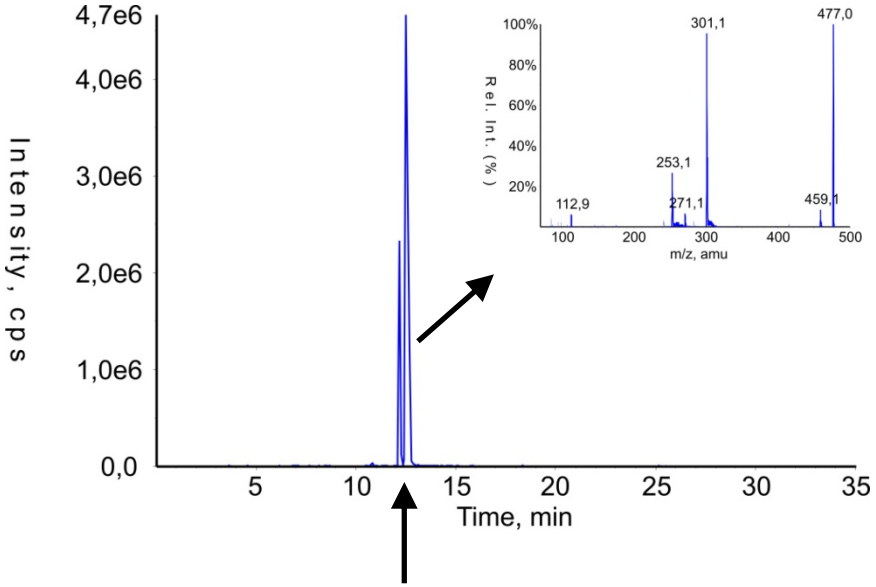
Enterodiol:

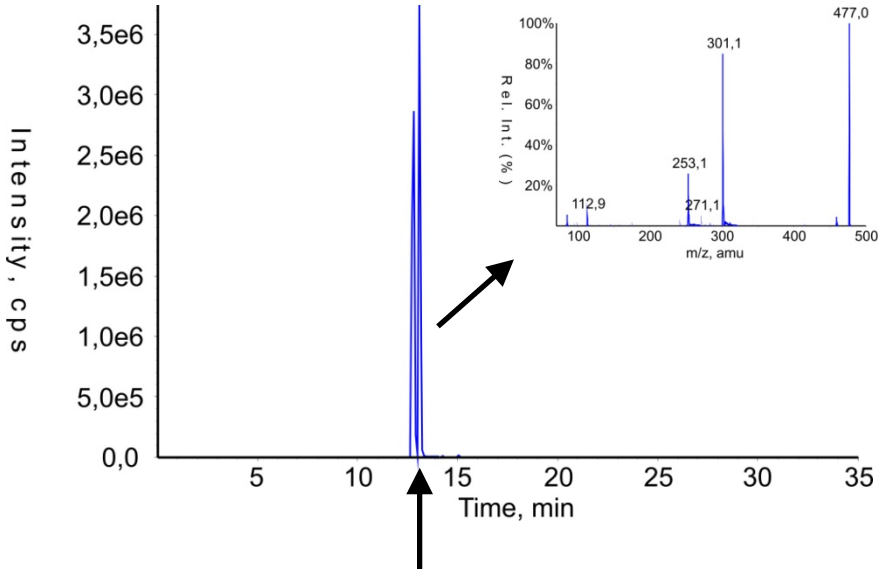
- Lignan
- Precursor present in many plants
- Formed in the gut by resident bacteria
- Known to be glucuronidated

LC/MS chromatograms of MRM 477/301

Unknown glucuronide in screen

In vitro generated enterodiol-glucuronide



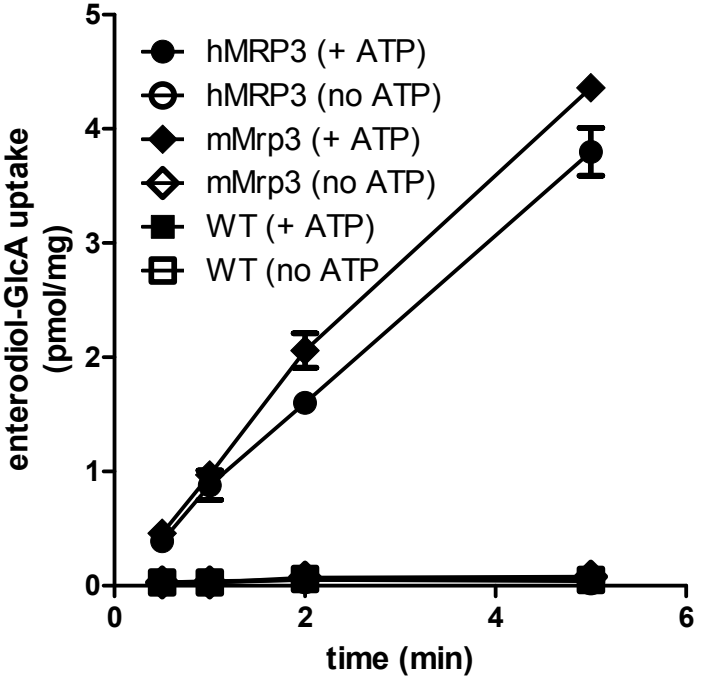


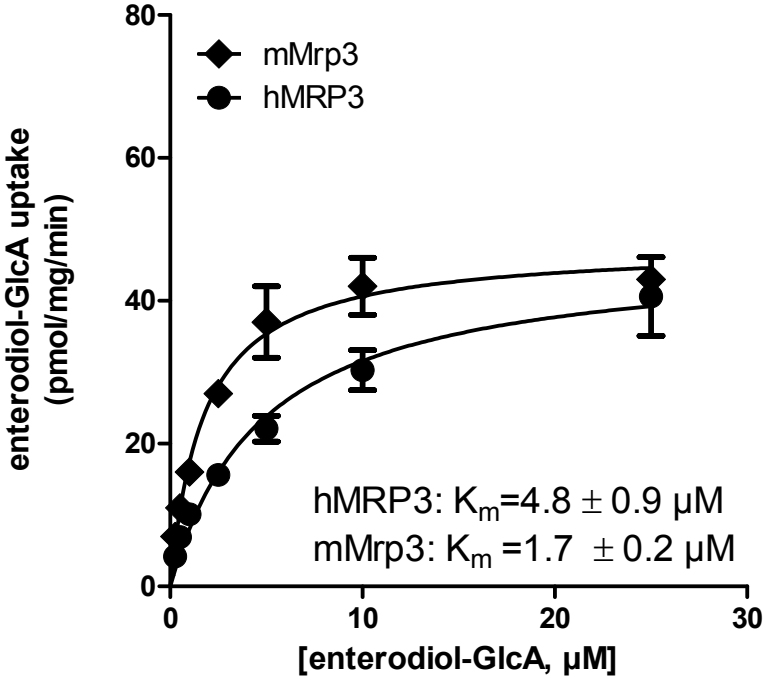
Unknown compound in screen is: **enterodiol-glucuronide**

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.

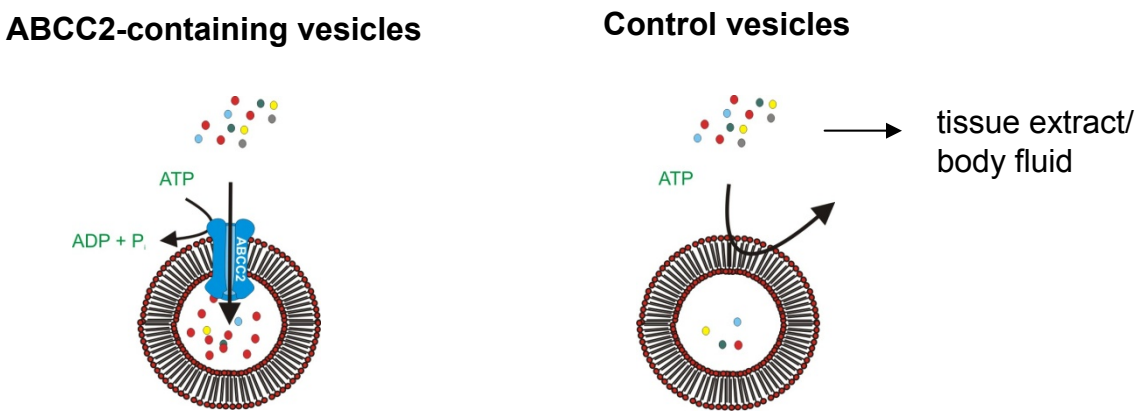
Confirmation of enterodiol-GlcA transport by MRP3/Mrp3 in vesicular transport experiments





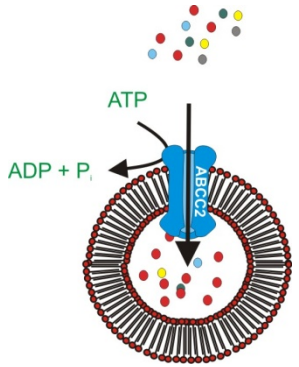
Vesicular transport assays

- 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.



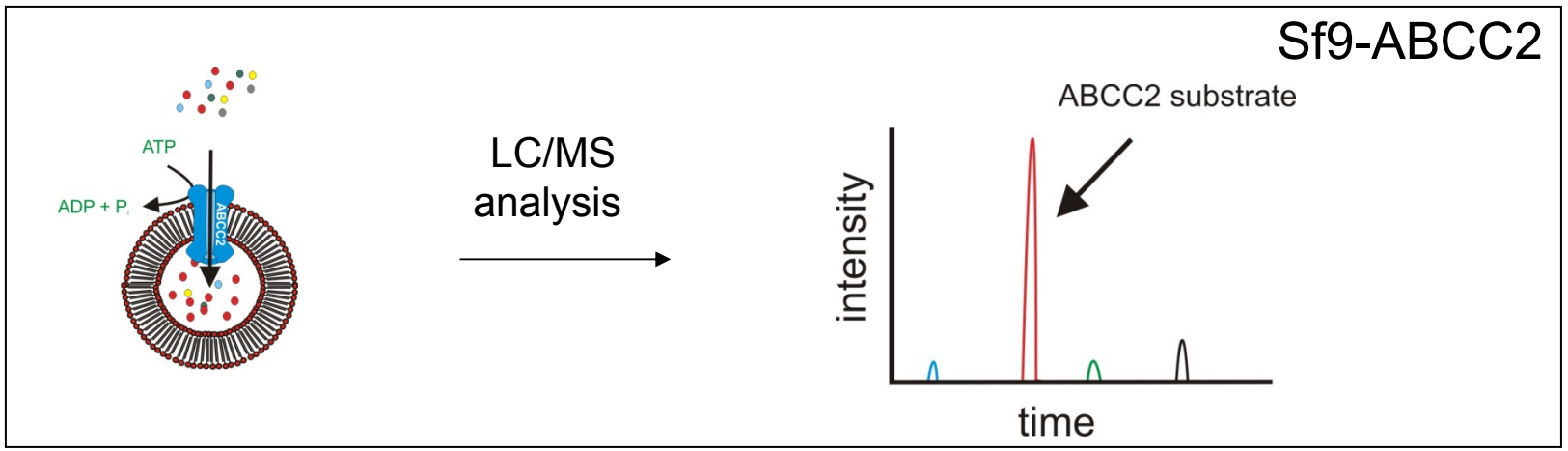
Transportomics: combination of vesicular transport assays and metabolomics

- 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



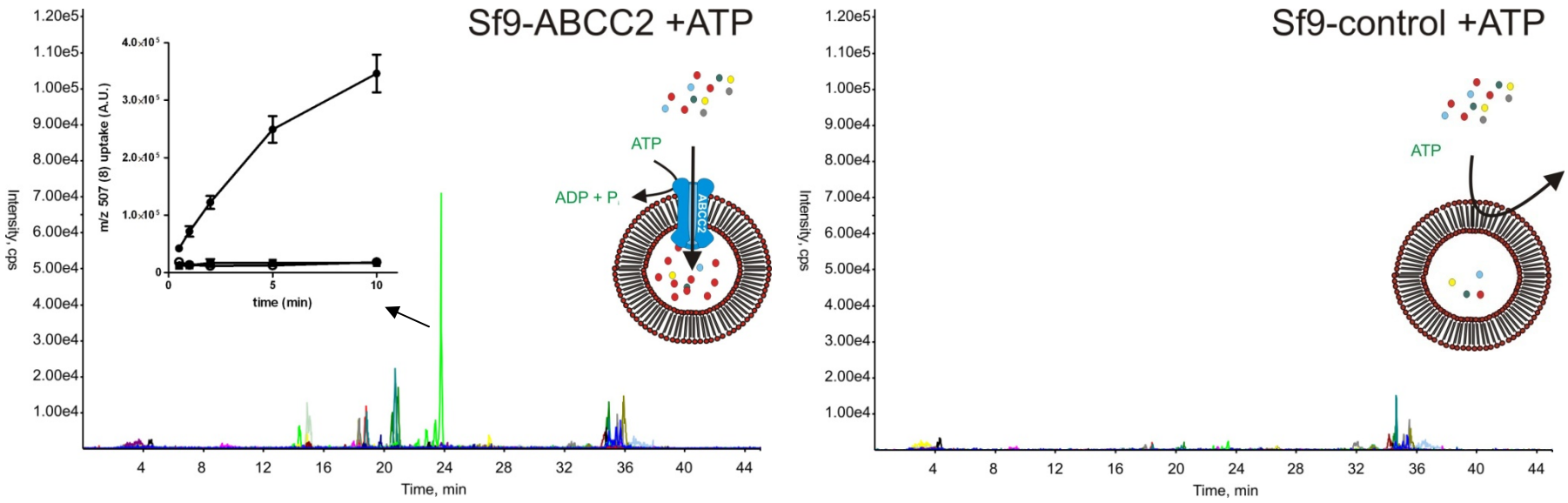
Transportomics: example ABCC2

- 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

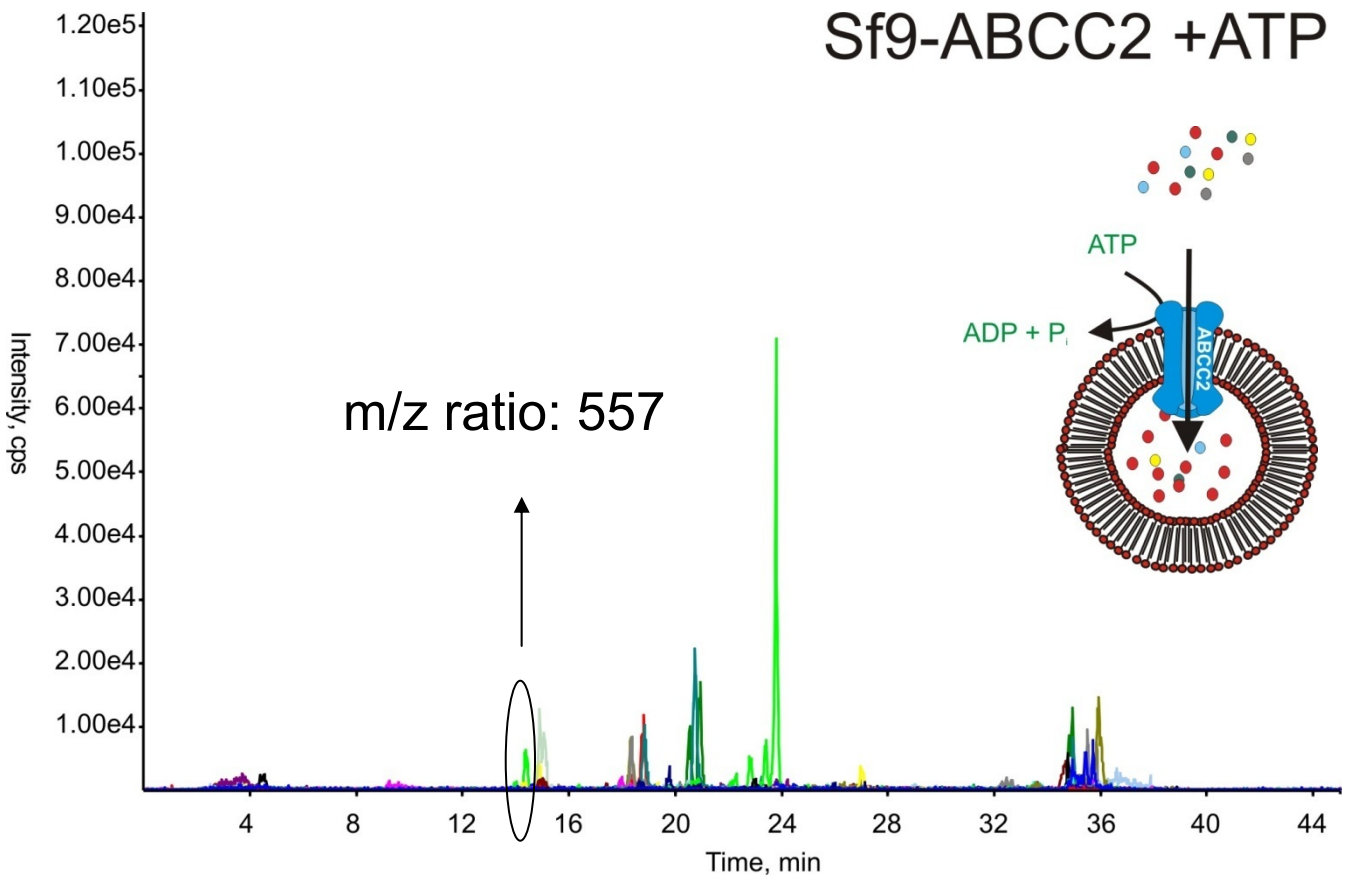
ABCC2-mediated transport of glucuronides from urine

Transport of glucuronides from mouse urine

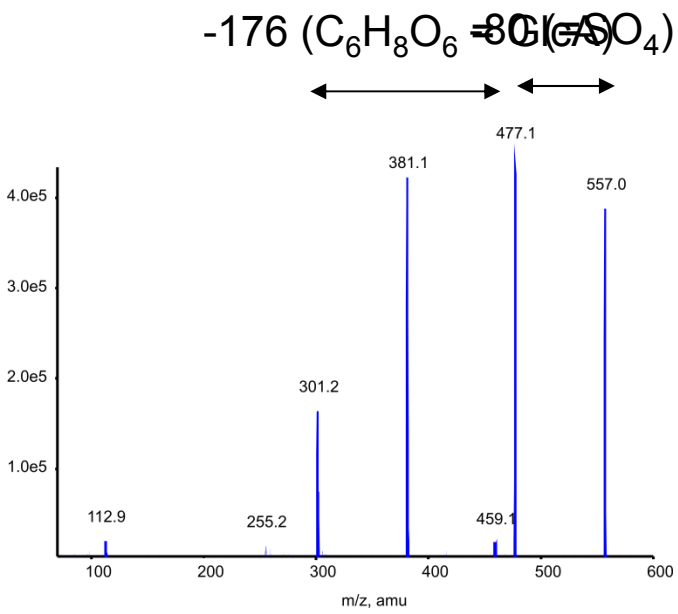


Detection: targeted metabolomics (compounds conjugated to glucuronic acid)

Identification of unknown glucuronides



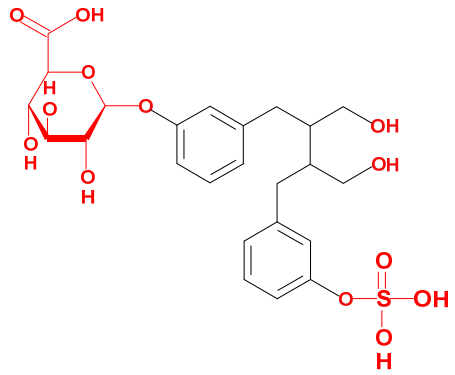
Identification of unknown compound with m/z 557



Unknown compound contains:

1) Sulphate moiety
2) Glucuronic acid moiety

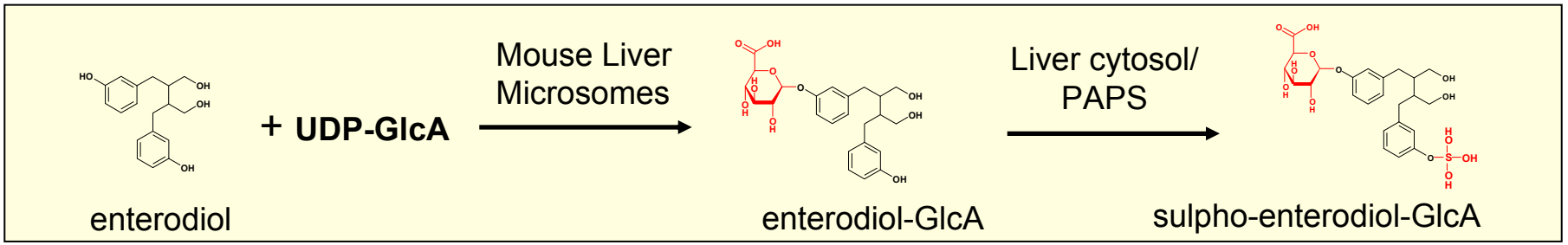
guess: sulpho-enterodiol-glucuronide



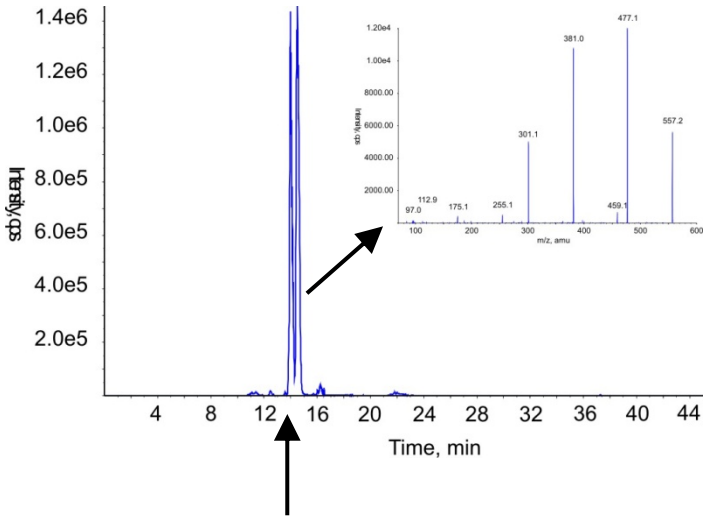
Mw 558 (m/z = 557)

- Enterolignan
- Precursor present in food
- Plant-derived compound
- Known to be extensively glucuronidated/sulphated

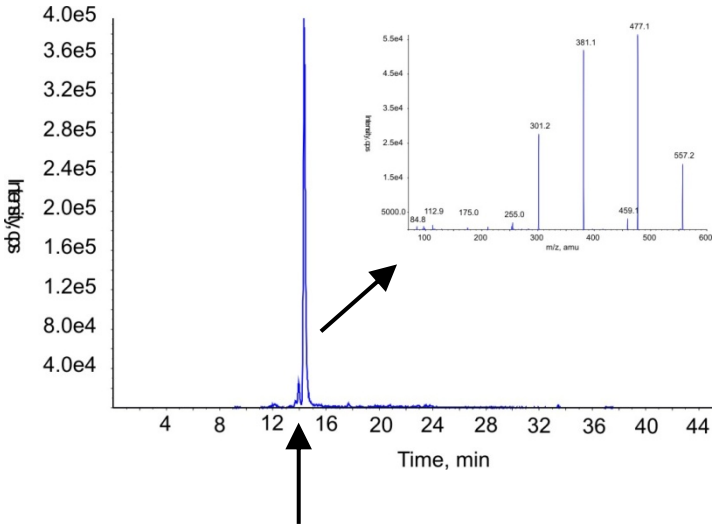
Identification of compound with a m/z ratio of 557



Unknown glucuronide in screen with m/z 557



In vitro generated sulpho-enterodiol-glucuronide



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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Old (start of KO's of ABC transporters)
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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

Some papers on ABC-transporters from the Borst lab

- Van de Wetering,K., Feddema,W., Helms,J.B., Brouwers,J.F., and Borst,P. (2009). Targeted metabolomics identifies glucuronides of dietary phytoestrogens as a major class of MRP3 substrates in vivo. Gastroenterol. *137*, 1725-1735.
- Krumpochova,P., Sapthu,S., Brouwers,J.F., De Haas,M., de Vos,R., Borst,P., and Van de Wetering,K. (2012). Transportomics: screening for substrates of ABC transporters in body fluids using vesicular transport assays. FASEB J *26*, 738-747.
- Van de Wetering,J.K. and Sapthu,S. (2012). ABCG2 functions as a general phytoestrogen-sulfate transporter in vivo. FASEB *26*, 4014-4024.
- Van de Wetering,K., Zelcer,N., Kuil,A., Feddema,W., Hillebrand,M., Vlaming,M.L., Schinkel,A.H., Beijnen,J.H., and Borst,P. (2007). Multidrug resistance proteins 2 and 3 provide alternative routes for hepatic excretion of morphine-glucuronides.

- De Wolf,C., Jansen,R., Yamaguchi,H., De Haas,M., Van de Wetering,K., Wijnholds,J., Beijnen,J., and Borst,P. (2008). Contribution of the drug transporter ABCG2 (breast cancer resistance protein) to resistance against anticancer nucleosides. Mol Cancer Ther. *7*, 3092-3102. Mol Pharmacol *72*, 387-394.
- Pajic,M., Iyer,J.K., Kersbergen,A., Van der Burg,E., Nygren,A.O., Jonkers,J., Borst,P., and Rottenberg,S. (2009). Moderate increase in Mdr1a/1b expression causes in vivo resistance to doxorubicin in a mouse model for hereditary breast cancer. Cancer Res. *69*, 6396-6404.