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MEMBRANE TRANSPORTERS – first line of defence against xenobiotics

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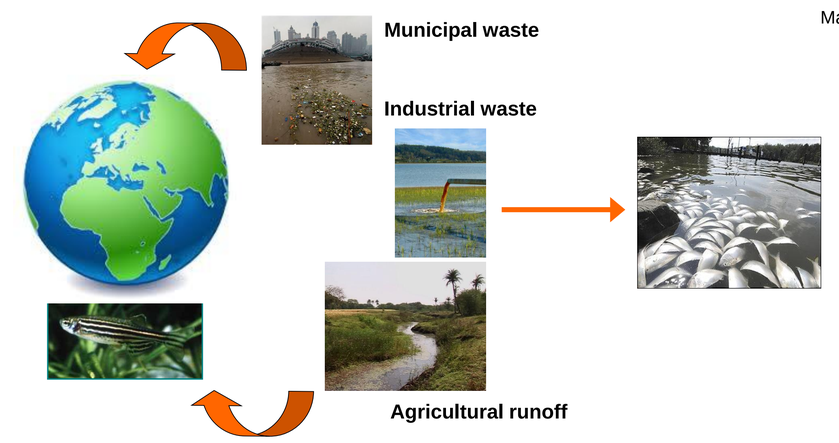
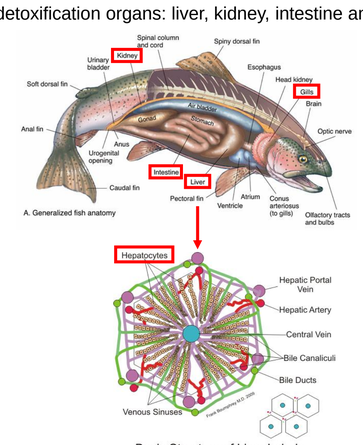
Introduction

1) Anthropogenic contamination in aquatic environment



2) Fish as model species

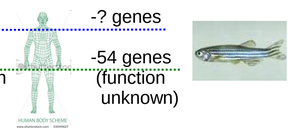
Main detoxification organs: liver, kidney, intestine and gills

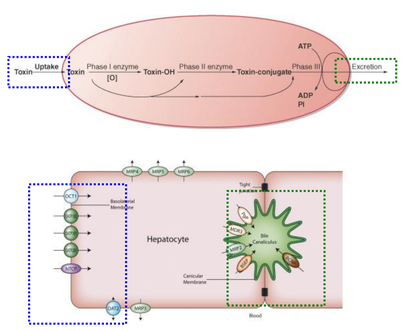


3) Role of membrane transporters in cellular ADME (*Absorption, Distribution, Metabolism, Excretion*)

Phase 0 in toxicology: Uptake transporters - **SLC21 and SLC22** families -28 genes -? genes

Phase III in toxicology: Efflux transporters - **ABC** superfamily (subfamilies Abcb, Abcc and Abcg involved in xenobiotic transport) -49 genes (mostly known function) -54 genes (function unknown)



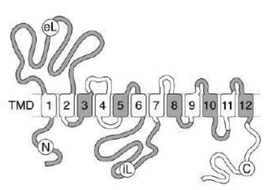


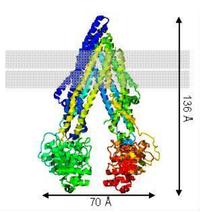
Uptake transporters *Efflux transporters*

Structure and transport mechanism

SLC21,22 - facilitated diffusion, anion exchangers or unknown mechanism

ABCs – ATP driven transport





How to study membrane transporters? From sequence to function!

Model species: zebrafish (*Danio rerio*)

- genome available
- ~ 77% of human ABC genes have zebrafish ortholog
- breeding in the lab: quick and easy
- excellent model for embryonic development + knock-down studies

1) Genome study

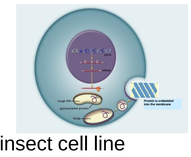
How to find target gene sequence in the genome? Blast search through genome databases (NCBI, ENSEMBL)

How to determine gene orthology relationships with other vertebrate genes of target gene families (SLC and ABC)? Multiple sequence alignment (muscle algorithm) + Phylogenetic analysis (maximum likelihood method)

2) Expression study

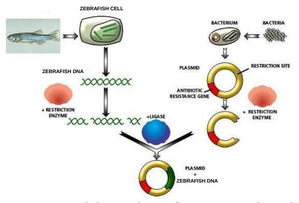
Is the target gene expressed on the mRNA level and what is its tissue expression pattern? Zebrafish dissection → RNA isolation from zebrafish tissues → Primer design → Real-Time PCR (SYBR Green dye)

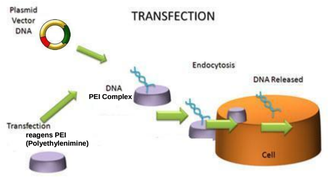
3) Functional study



How to 'catch' target gene from zebrafish and determine its function?

- cloning of full length transcripts into expression vectors (pcDNA plasmid, pACHLT vector)
- transfection of recombinant plasmid into the host cells (human HEK293 cell line for SLCs, insect cell line Sf9 for ABCs: system optimization with specific transfection reagens (lipofectin, polyethylenimine)

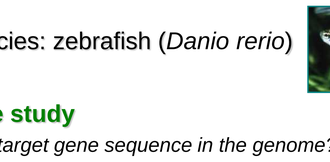




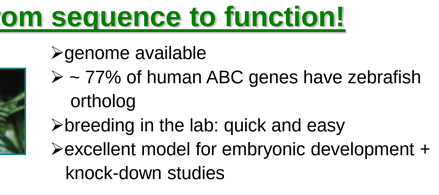
- development of functional assays for determining substrate and inhibitors of membrane transporters:

a) SLCs: uptake assay using transiently transfected HEK293 with radiolabeled chemicals → liquid scintillation counter + inhibition assay (coexposure of radiolabeled substrate ([H^3]S) and unknown compound)

b) ABCs: ATPase assay on isolated membrane vesicles from SF9 cells containing target ABC genes



SUBSTRATE INHIBITOR



Results and discussion:

1) Phylogenetic analysis

1A) UPTAKE TRANSPORTERS

- **SLC21 (Oatp)** (Fig. 1A)
  - **12** zebrafish genes identified and annotated
  - orthologs or co-orthologs: Oatp1c1, 2a1, 2b1, 3a1 and 3a2, 4a1, 5a1 and 5a2
  - **new** SLC21 subfamilies: 1d1, 1e1, 1f1 and 1f2
  - no orthologs of Oatp1a and Oatp1b subfamilies present in non-mammalian vertebrates

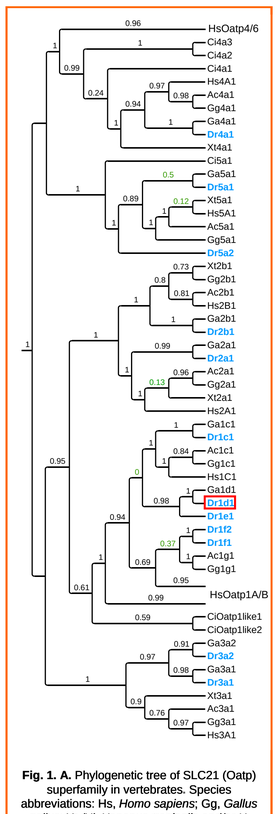


Fig. 1. A. Phylogenetic tree of SLC21 (Oatp) superfamily in vertebrates. Species abbreviations: Hs, *Homo sapiens*; Gg, *Gallus gallus*; Xt /*XI, Xenopus tropicalis and/or X. laevis; Dr, Danio rerio; Ga, Gastrosteus aculeatus.*

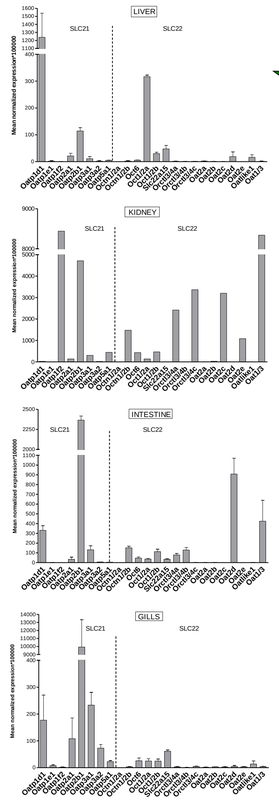


Fig. 2A. Expression pattern of SLC21 and SLC22 genes in tissues important for detoxification of adult female zebrafish. Data are expressed as a fold mean of 3 independent pools ±SE (normalized to the housekeeping gene EF1a).

- **SLC22 (Oatp)** (data not shown)
  - **17** zebrafish genes identified and annotated
  - orthologs or co-orthologs: Oct1/2 a i b, Oct6, Octn1/2 a i b, Oat1/3, Oat2a- e, Slc22a15, Slc22a17, Orctl3/4a-c
  - no orthologs of human OAT4-6 in other vertebrates including zebrafish

2) Expression study

- Main detoxification tissues: liver, kidney, intestine and gills
- Dominantly expressed SLC and ABC transporters are shown in Figs 2A and 2B, respectively

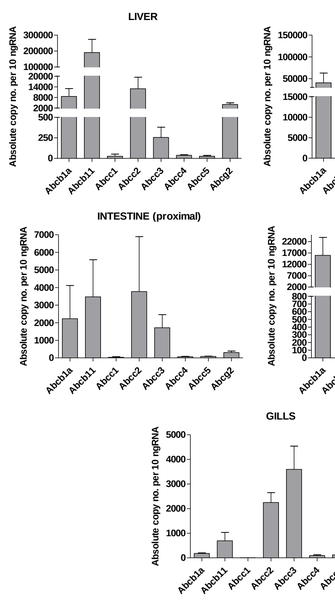


Fig. 2B. Expression pattern of ABC genes in tissues important for detoxification of adult female rainbow trout (*Oncorhynchus mykiss*). Data are expressed as a fold mean of 3 independent pools ±SE (normalized to the housekeeping gene EF1a).

1B) EFFLUX TRANSPORTERS

- **ABCB subfamily** (Fig. 1B); ABCC and ABCG phylogenies not shown
  - **ABCB: 12** zebrafish genes identified and annotated, clear orthology relationships
  - **additional genes** in comparison to mammals: B1b, B3b, B11b

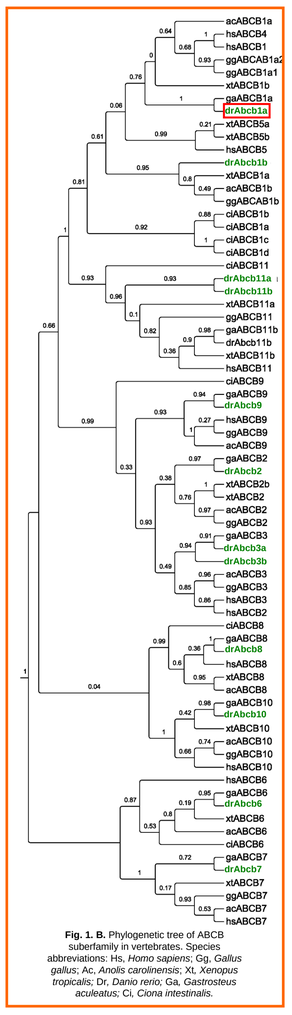


Fig. 1. B. Phylogenetic tree of ABCB subfamily in vertebrates. Species abbreviations: Hs, *Homo sapiens*; Gg, *Gallus gallus*; Ac, *Anolis carolinensis*; Xt, *Xenopus tropicalis*; Dr, *Danio rerio*; Ga, *Gastrosteus aculeatus*; Ci, *Ciona intestinalis.*

3) Functional characerization of Oatp1d1 and Abcb1a and their (eco)toxicological relevance

- Oatp1d1 is a high affinity [H^3]estrone-3 sulfate transporter (Fig. 3) → [H3]E3S can be used for the inhibition assay on the optimized system

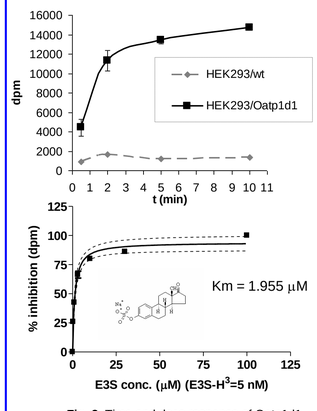


Fig. 3. Time and dose response of Oatp1d1 mediated estrone-3 sulfate (E3S) uptake.

- Oatp1d1/HEK293 inhibition assay showed similarities in interaction with model substrates and inhibitors of human OATP1 proteins (Fig 4.): Oatp1d1 is different then Oatp1C1, but similar to 1A2, 1B1 and 1B3.

Fig. 4. Model substrates and inhibitors of Oatp1d1

SUBSTRATES	Oatp1d1
Hormones	
Estrone -3- sulfate [H^3]	K_m = 1.96 µM
Dehydroepiandrosterone sulfate (DHEAS)	K_i = 0.58 µM
Estradiol-17ß-glucuronide	K_i = 1.91 µM
Thyroxine	no interaction
Bile acids	
Taurocholate	Inhibition of E3S uptake
Taurochenodeoxycholate	K_i = 6.98 µM
Cholate	no interaction
Other	
Bromosulfophtalein (BSP)	K_i = 0.439 µM
INHIBITORS	
Probenecid	K_i = 22.99 µM
CyclosporineA	K_i = 2.993 µM
Gemfibrozil	K_i = 0.527 µM

- Oatp1d1 is responsible for the uptake of numerous environmental pollutants from blood into the liver and subsequent elimination of these compounds into the bile (Fig 5.)

Fig. 5. Interaction of zebrafish Oatp1d1 with high - priority environmental contaminants

Group	Subgroup	Chemical	DrOatp1d1 interaction
Industrials	Anti-oxidants	butylated hydroxytoluen	weak inhibition
	Perfluorates	perfluorooctanic acid (	strong inhibition(Ki=0.96 µM)
	Phenols	nonylphenol**	weak inhibition
		bisphenol A	strong inhibition (Ki=54.2 µM)
	Phtalates	diethyl phtalate	weak activation
		bis(2-ethylhexyl) phtalate	strong activation
Pesticides	Carbamates	carbaryl	moderate inhibition
	Chloroacetanilides	metolachlor	weak inhibition
	Organophosphates	chlorpyrifos	strong inhibition (Ki=1.73 µM)
		diazinon***	moderate inhibition
		malathion	strong inhibition (Ki=3.61 µM)
	Organochlorines	dieldrin***	no interaction
		endosulfan	no interaction
	Other	diuron**	strong inhibition (Ki=40.6 µM)
		isoproturon**	weak inhibition
Pharmaceuticals and personal care products	Analgesics	Acetaminophen***	no interaction
	Anticonvulsants	Carbamazepine***	weak induction
	Antimicrobials	Erythromycin	moderate inhibition
		Triclosan***	moderate-strong inhibition
	Polycylic musks	1,1,3,4,4,6,-hexamethyl-1,2,3,4-tetrahydronaphtalene)	no interaction
	Non-steroidal anti-inflammatory drugs (NSAIDs)	Diclofenac	strong inhibition (Ki=1.36 µM)
			no inhibition
	Synthetic hormones	17ß-estradiol (ßE2)***	strong inhibition (Ki=1.91 µM)
		17a-ethinylestradiol (Et	strong inhibition (Ki=9.73 µM)
	Other	DEET (N,N-diethyl-meta	very weak inhibition
		Caffeine	no inhibition
	UV filters	Benzophenone-3 (BP3)	weak inhibition
		Benzophenone-4 (BP4)	moderate inhibition

- Fish Pgp (Abcb1a) shows similar, but not identical pattern and substrate/inhibitor affinities in comparison to mammalian Pgp (Fig. 6)
- We have identified testosterone as a weak physiological substrate of fish Pgp (Abcb1a) (Fig. 7)
- Abcb1a is strongly inhibited by numerous pharmaceuticals (Fig. 7), but also environmental contaminants like eritromycin and pesticide endosulfan that can lead to impairment of normal efflux of endobiotics and xenobiotics through bile (Fig. 7).

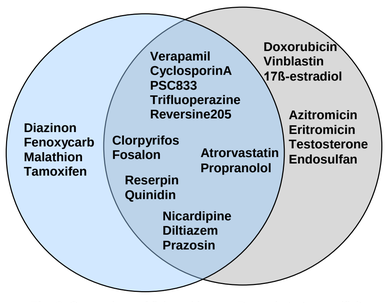


Fig. 6. Comparison of fish and human Pgp substrate specificity using calcein-AM assay (Venn diagram showing the specific and overlapping interaction between human and fish Pgp).

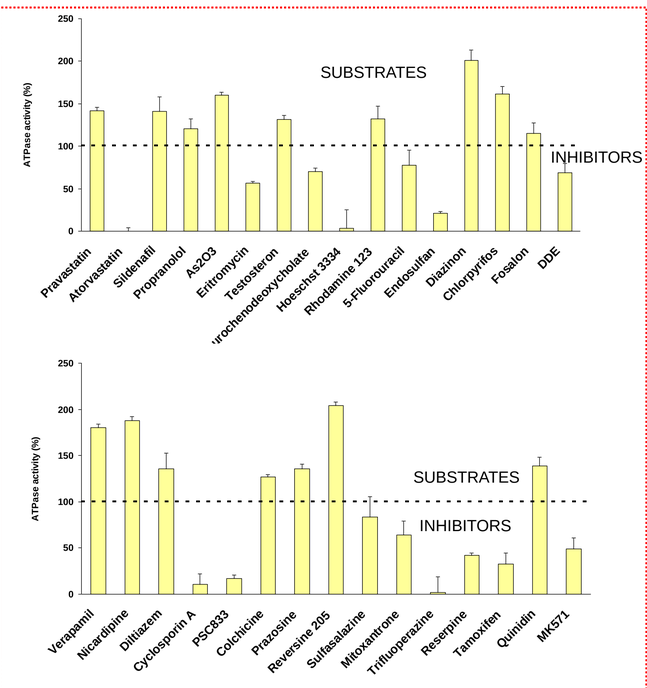


Fig. 7. Identification of fish Pgp substrate and inhibitors using ATPase assay. Baseline ATPase activity is set to 100%. The values represent mean + SD (n=3).

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