IN VITRO AND IN VIVO MODELS TO EVALUATE DRUG INFLICTED CHANGES ON BILE ACID HOMEOSTASIS

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Meet the Experts Transporter Conference

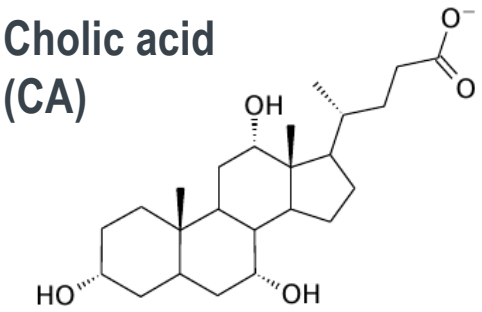
Outline

- Introduction
- Hypothesis
- Characterization of Bsep KD rat model without drug treatment
- Bile acid responses in plasma and liver in Bsep KD rats with drug treatment
- Summary and future directions

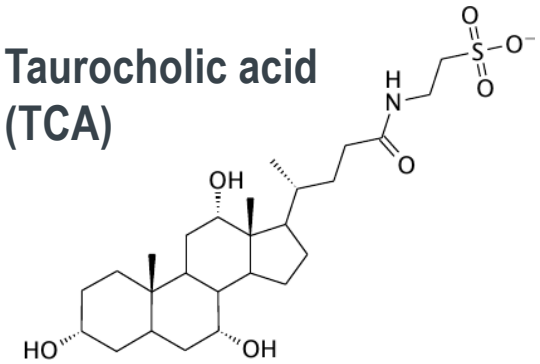
Conjugated bile acids as biomarkers of BSEP/Bsep inhibition

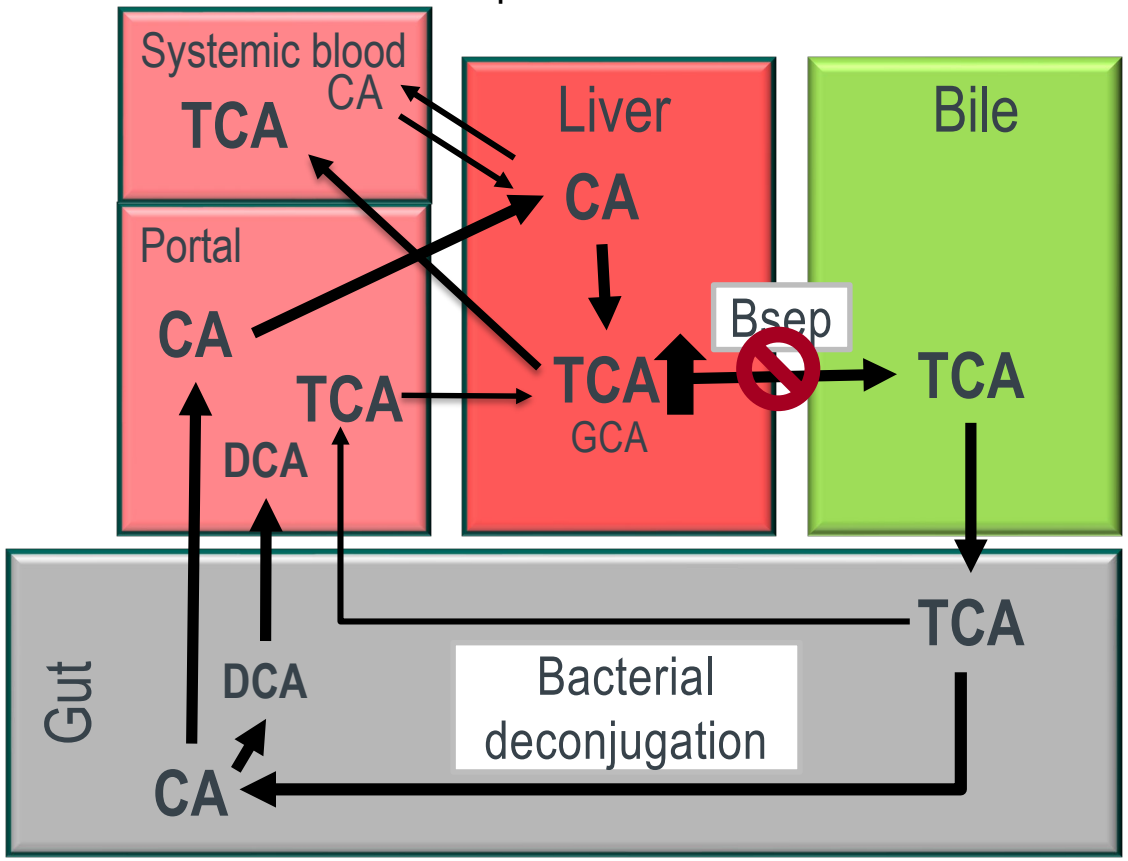
- Drug induced liver injury (DILI) is a major cause of attrition in drug development
- Inhibition of the bile salt export pump (BSEP) may be associated with clinical DILI, but is poorly predicted in animal models
- Inhibition of multiple bile acid (BA) transporters is suspected of increasing risk for hepatotoxicity
- Plasma conjugated bile acid elevation may be used as a biomarker of BSEP/Bsep inhibition in liver

Cholic acid (CA)



Taurocholic acid (TCA)





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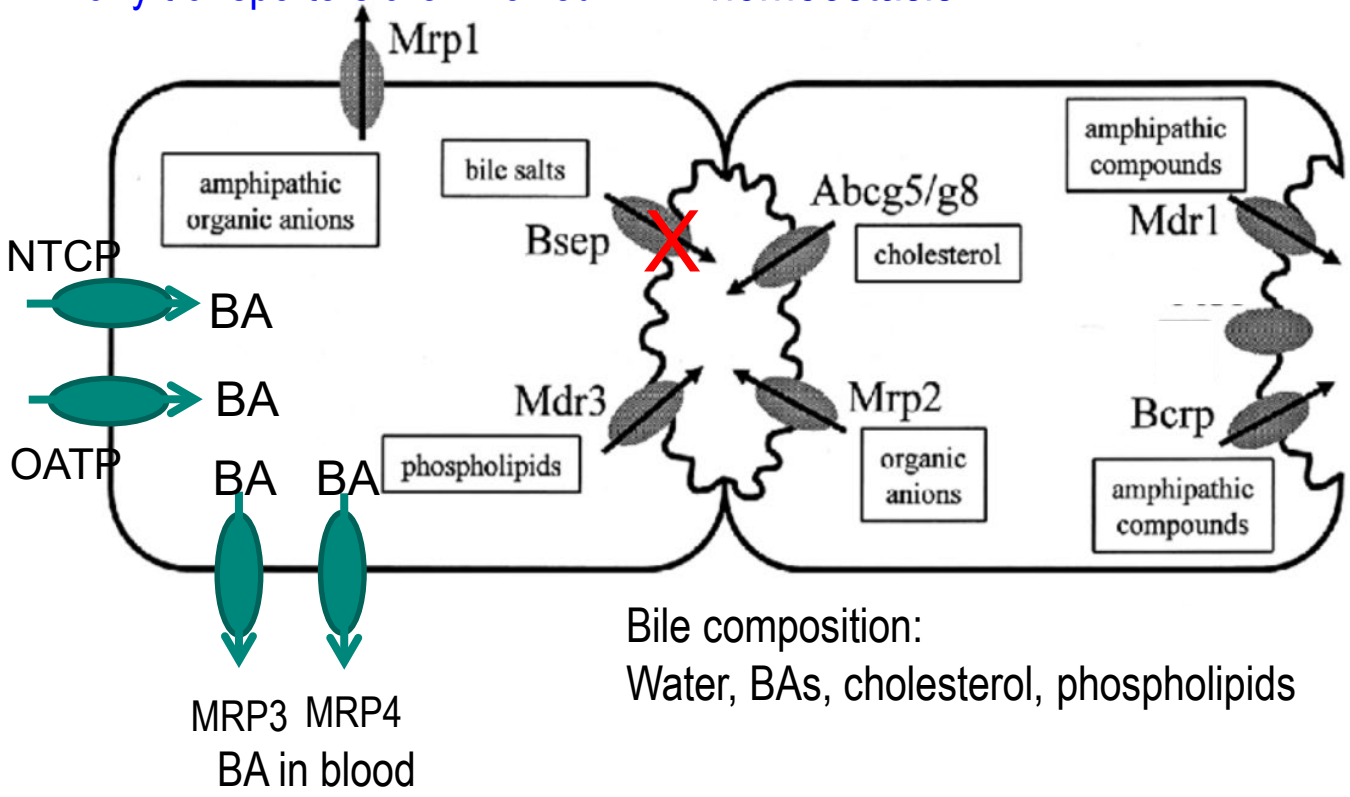
BSEP inhibition by drugs may lead to DILI, but is poorly predicted by animal models (Transporter function & compensatory mechanism differ between rodents and humans)

Many transporters are involved in BA homeostasis



Bile composition:
Water, BAs, cholesterol, phospholipids

- Ntcp: sodium taurocholate co-transporting peptide
- Oatp: organic anion-transporting polypeptide
- Bsep: bile salt export pump (human: BSEP)
- Mrps: multi-drug resistance-associated proteins

Bsep function and compensatory mechanisms

Bsep KO/KD rats/mice: **Normal (**some exception**)**
BSEP mutation in human: **Liver disease**
♦Benign recurrent intrahepatic cholestasis type 2
♦Progressive familial intrahepatic cholestasis type 2

Compensatory Mechanisms:
Uptake: Ntcp and Oatp (human and rodent different)
Efflux: Mrps, Mdr1 (P-gp)

Cytotoxicity ranking:

LCA > DCA >> CDCA > GCDCA > TCDCA > GCA = TCA

Human has more toxic BAs than rat

- **GCDCA is the most abundant BA in human plasma**

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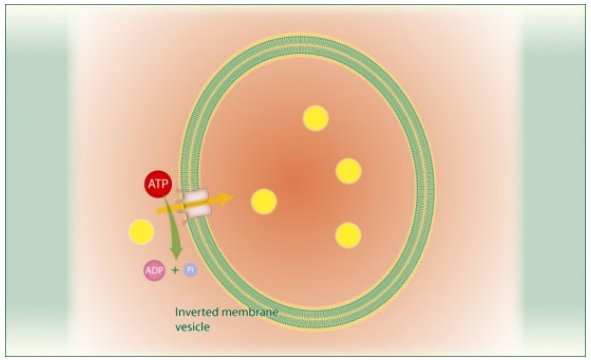
In vitro rat bile acid transporter inhibition assay

Bsep Vesicle assay



Membrane vesicles isolated from Sf9 cells containing rBsep

Uptake transport by inside-out vesicles is driven by ATP.

For inhibition study, transport of a probe substrate (TCA) is measured in the presence of potential inhibitors.

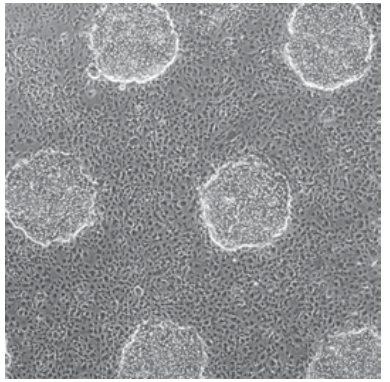
Mrps and Ntcp assay

	Mrp2	Mrp3	Mrp4	Ntcp
Cell line	Vesicles*	Vesicles*	Vesicles*	CHO-K1
Probe substrate	Ethacrynic acid-Glutathione	E-17ßG	Folic acid	3H-TCA

*: membrane vesicles were isolated from sf9 cells containing Mrp2/3/4

For inhibition study, transport of a probe substrate with ATP (or AMP) is measured in the presence of potential inhibitors.

Hepatocyte sandwich Assay



- Micropatterned plates contain tiny colonies of organized hepatocytes surrounded by supportive fibroblasts.
  - Multiple transporters
  - Metabolism and binding
- Sinusoidal uptake affected
- Canalicular efflux affected

Why do we seek an in vivo approach for DILI derisking of Bsep liability along with in vitro efforts

- To verify in vivo dose-exposure-margins achieved in animals and humans when BSEP concern is flagged in vitro
- To account for effects on enterohepatic recirculation and gut bile acid metabolism
- To understand species difference on regulation of bile acid homeostasis: rat, dog, monkey, human
  - Select in vivo species: costs of drug materials and large animals
- To address clinical toxicity that occurs weeks/months after drug treatment
- To develop a susceptible rodent model to crisp mechanistic biomarkers (conjugated BAs) in normal rodents.

Bsep KD rat offers potential to assess Bsep inhibition risk in vivo

Hypothesis

- Knockdown of Bsep will reduce the rat liver's normal BA export capacity resulting in enhanced BA levels in liver and plasma.

- This model is expected to enhance our understanding of the utility of mechanistic liver tissue (mRNA and BAs) and translational biomarkers (plasma BAs and a stable label BA tracer) that could inform disruption by drugs of BA homeostasis.

- Because rats have much less toxic BA profiles as compared to humans, the model may or may not be expected to present a conventional DILI phenotype.

Test compounds for evaluation of Bsep KD model were selected based on in vitro transporter inhibition data

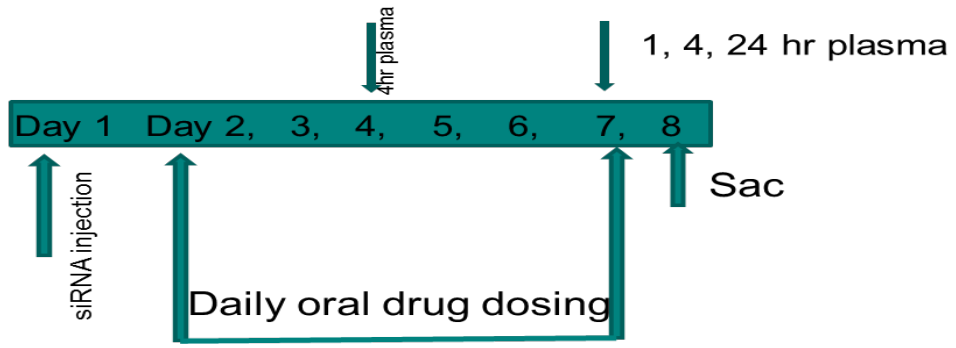
Drug Name	Abbreviation	*In vitro* rat vesicle or cell line assay, Transporters IC_{50} (μM)				
		Bsep	**Mrp2**	**Mrp3**	**Mrp4**	**Ntcp**
Asunaprevir	ASN	3.2	11	12	5	0.57
TAK-875	*TAK-875*	21.3	9	>25	12	4.3
Benzbromarone	BBR	7.9	>25	>25	5	1.6
Lopinavir	LPN	13.7	>25	>25	~25	1
Simeprevir	SMP	1.3	16	14	>25	0.05
Bosentan	BST	25	>25	>25	<1	0.4
Cyclosporine A	CSA	0.7	6	>25	>25	1.5
Ritonavir	RTN	3.9	>25	>25	>25	1.6
MK-0974	MK-0974	>25	>25	>25	24	17.9
Acetaminophen	APAP	>25	>25	>25	>25	>25
Clarithromycin	CTM	>133	>25	>25	>25	>25

In-vitro assay
- Short 10 min incubation
- No metabolism

In-vitro data indicated that all 8 drugs are potent Bsep and Ntcp inhibitors

Rat Bsep siRNA knockdown (Bsep KD) model for investigating DILI risk from drug-induced Bsep inhibition. All drugs given at maximum tolerable dose (MTD)

Study Design

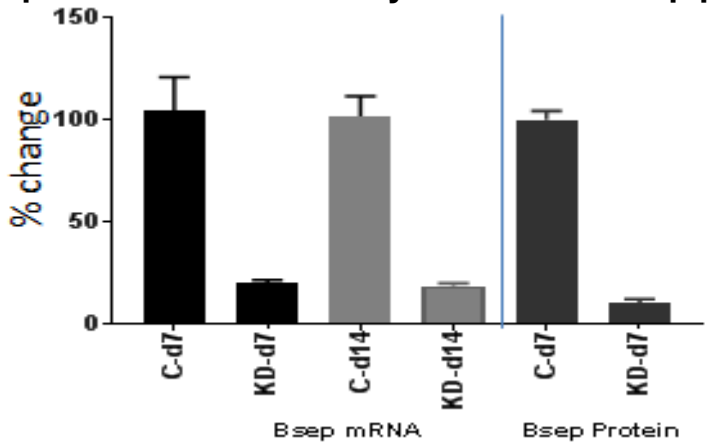


Dose Group	siRNA Treatment	Drug Dose (mkd)	Treatment Group
1	C*	0	C
2	C	100-800	C+Dr
3	Bsep KD**	0	KD
4	Bsep KD	100-800	KD+Dr

*: scrambled siRNA was injected in the control groups
**: siRNA targeting rat Bsep was injected

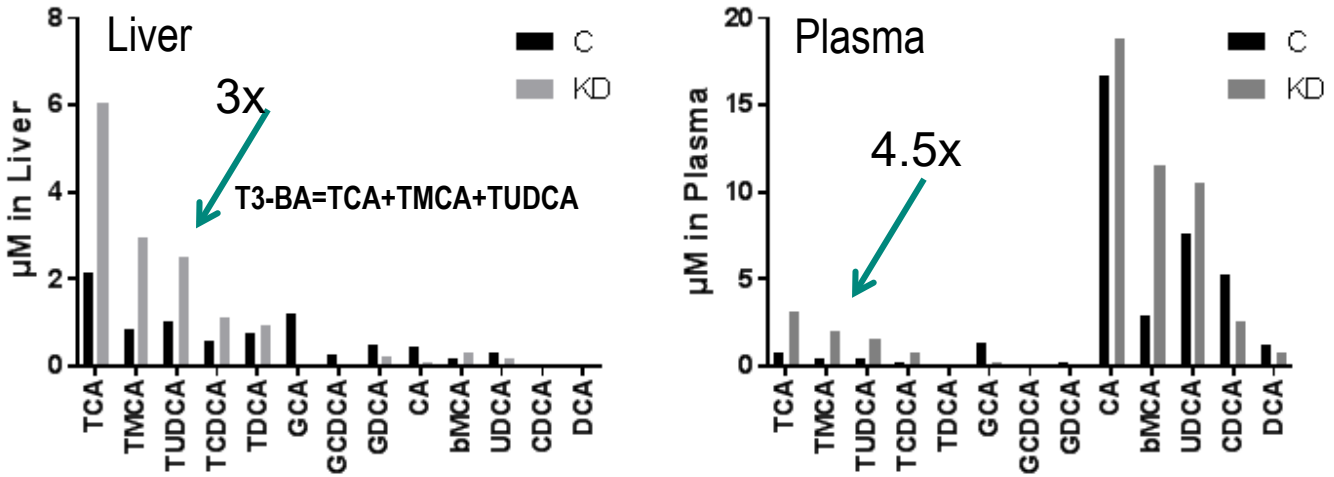
Model Characterization (Bsep KD alone)

- Bsep KD was confirmed by mRNA and Bsep protein



80% Bsep KD (mRNA)
90% Bsep KD (protein)

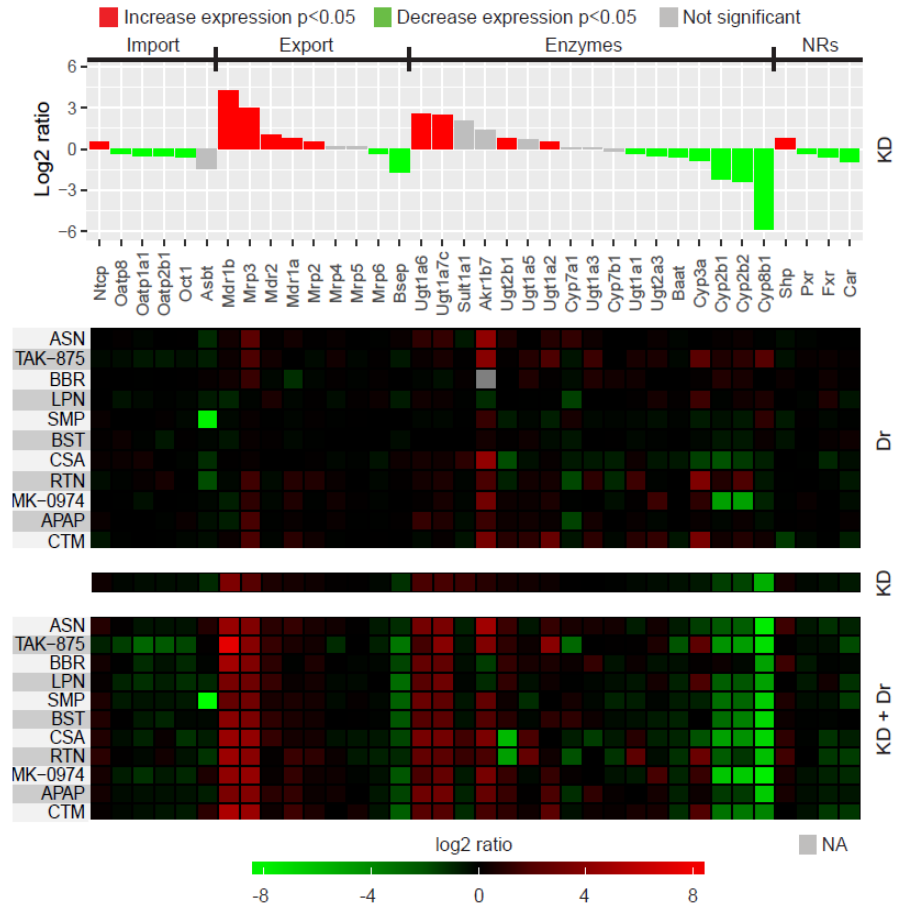
- Conjugated bile acids were increased in liver and plasma after Bsep KD



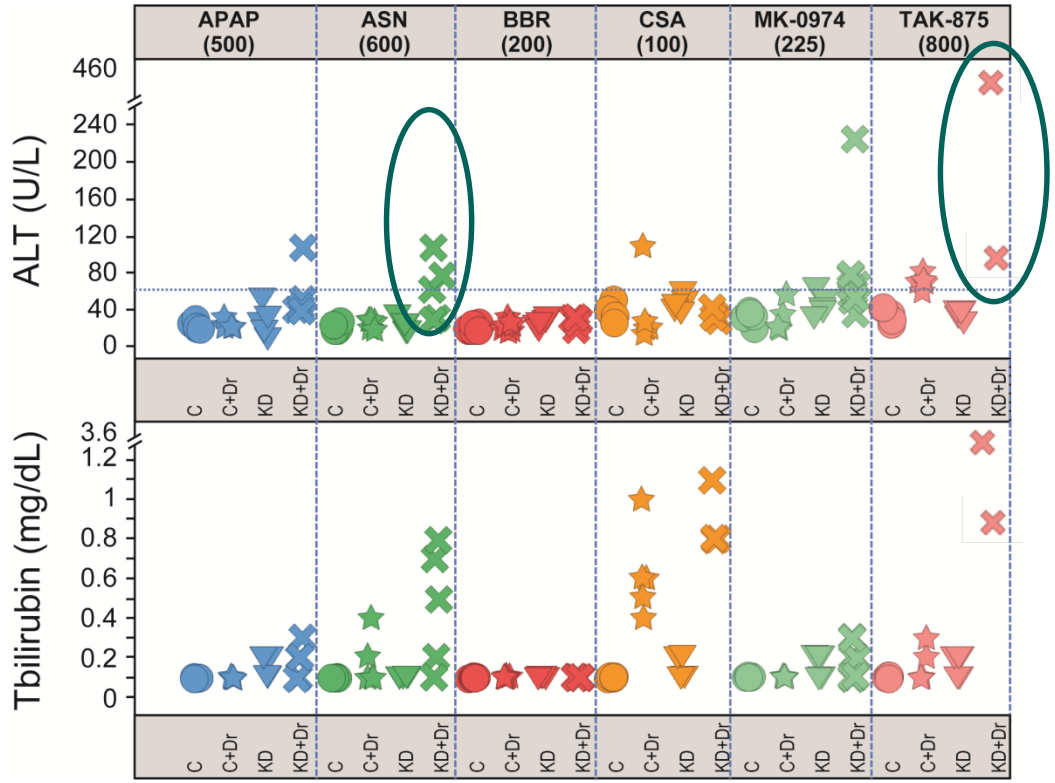
- Histo: Bsep KD resulted only in centrilobular hepatocellular vacuolation

While drug treatment combined with Bsep KD did not alter transcriptional responses beyond KD alone; Only ASN and TAK-875 show signs of toxicity in this sensitized model

- No consistent gene expression pattern in drug treatment in WT
- Bsep KD resulted in robust regulation of certain genes to protect liver
  - Induction: Mdr1b (23x), Mrp3 (8x), Ugt1a6/Ugt1a7c (6x)
  - Down regulation: Cyp8b1 (53x) Cyp2b1/2b2 (4x)
- No further impact on Bsep KD +Drug group



- Bsep siRNA KD alone did not result in elevation of ALT & bilirubin
- When drugs combined with Bsep KD, consistant synergistic ALT ↑ and total bili ↑ were seen only with ASN and TAK-875



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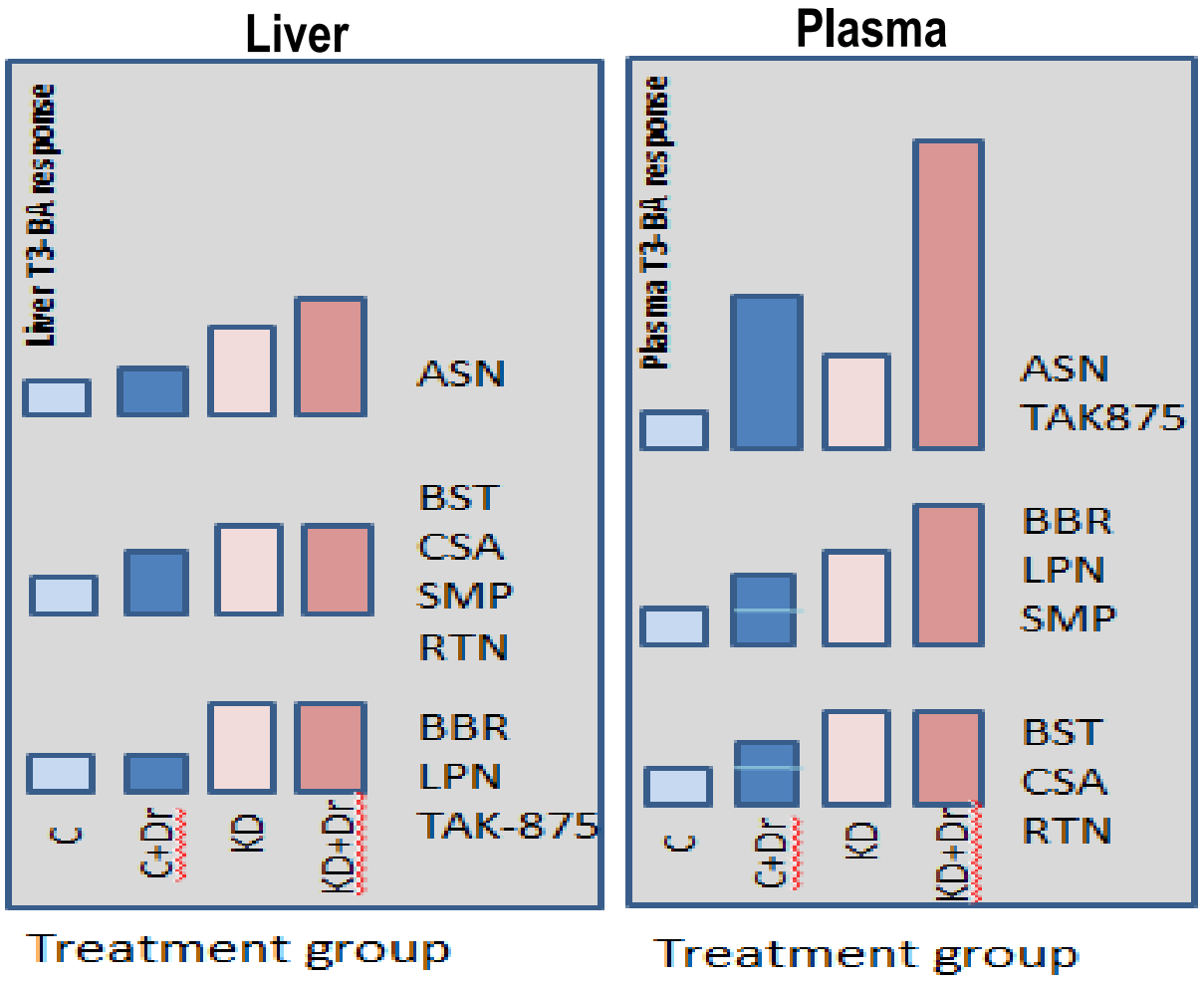
Bsep KD model showed differential susceptibility to Bsep inhibitors (KD vs KD+Drug groups)

Rats livers maintain BA homeostasis by compensatory mechanisms (FXR, Mrps, BA synthesis)

- T3-BA accumulation (3x) in liver was observed in Bsep KD alone rats (highest BA accumulation without toxicity)



T3-BA=TCA+TMCA+TUDCA

The Bsep inhibitors fell into 3 categories in terms of their plasma profiles

Group1:

- ASN, TAK-875
- larger T3-BA ↑, ALT tbili ↑

Group2:

- BBR, LPN, SMP
- small T3-BA ↑, ALT tbili ↔

Group3:

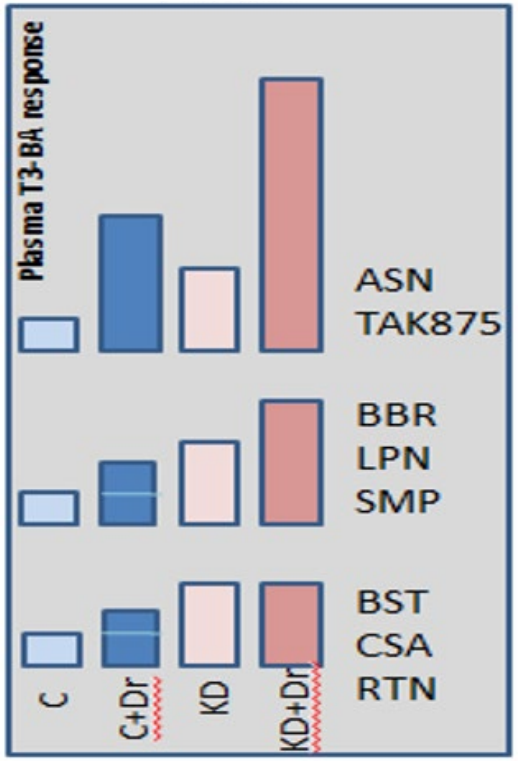
- **BST, CSA, and RTN**
- **T3-BA ↔, ALT tbili ↔**

Dose Group	Drug Dose (mkd)	Treatment Group
1	0	C
2	100-800	C+Dr
3	0	KD
4	100-800	KD+Dr

Differences in the effects on bile acid homeostasis in rats with Bsep inhibitors

T3-BA responses in Bsep KD rat model

	Drug	C+Dr vs C	KD vs C	KD+Dr vs KD	KD+Dr vs C	Response
Grp 1	**ASN**	39.5*	4.13*	**20.45***	84.47*	Enhanced
Grp 1	**TAK-875**	5.57*	4.01*	**13.20***	52.90*	Enhanced
Grp 2	**BBR**	1.58	3.02*	4.26*	12.86*	Enhanced
Grp 2	**LPN**	4.13*	4.55*	2.60*	11.82*	Enhanced
Grp 2	**SMP**	3.13*	3.66*	1.68*	6.14*	Enhanced
Grp 3	**BST**	3.91*	4.34*	1.04	4.53*	
Grp 3	**CSA**	2.97*	4.80*	1.39	6.67*	
Grp 3	**RTN**	1.70	4.80*	0.83	3.97*	
Grp 4	**MK-0974**	3.26*	5.11*	1.02	5.24*	
Grp 4	**APAP**	0.64*	4.75*	0.97	4.61*	
Grp 4	**CTM**	8.58*	6.09*	1.70	10.34*	



T3-BA= sum(TCA+TMCA+TUDCA)

Enhanced: KD+Dr > KD, KD+Dr > C, KD>C

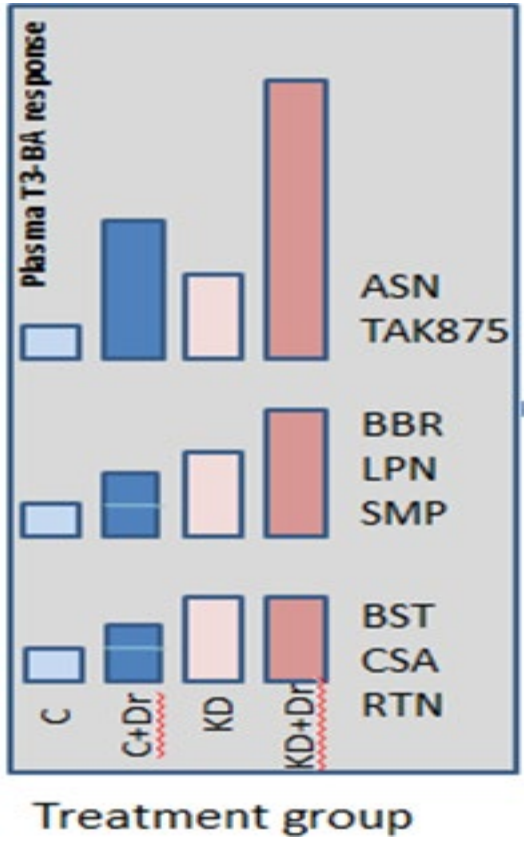
Could drug concentrations be contributing to the toxicity beyond bile acid accumulation?

Name	Dose (mkd)	Plasma conc. at 4hr (μM)		Plasma Conc. at 24hr (μM)		Liver Conc. at 24hr (μM)		Liver ratio	Plasma 4hr ratio	Plasma 24hr ratio
		C	KD	C	KD	C	KD	KD/C	KD/C	KD/C
ASN	600	56 ± 23	137 ± 32	30.2 ± 22	88.3 ± 71	234 ± 70	335 ± 81	1.4	**2.5***	2.9
TAK-875	*800*	645 ± 90	861 ± 92	---	---	294 ± 275	1044 ± 236	**3.6***	**1.3***	---
TAK-875-AG	*0*	15 ± 4.7	80 ± 23	---	---	15.1 + 8.7	285+340	18.9	**5.2***	---
BBR	200	59 ± 39	235 ± 52	1.2 ± 1.1	8.9 ± 4.8	2.7 ± 2.7	14.5 ± 9.0	**5.4***	**4.0***	7.3
LPN	600	3.6 ± 1.0	4.8 ± 1.0	1.2 ± 0.8	2.0 ± 1.1	6.2 ± 6.5	5.5±4.8	0.9	1.3	1.7
SMP†	750	4.5 ± 0.4	6.0 ± 0.3	---	---	230 ± 30	199 ± 19	0.9	1.3*	---
BST	750	4.7 ± 1.8	13.9 ± 5.7	6.2 ± 2.8	34.5 ± 2.5	114 ± 27	164 ± 21	**1.4***	**3.0***	**5.6***
CSA	100	14.2 ± 3.8	18.1 ± 3.0	9.7 ± 2.2	13.2 ± 2.7	153 ± 39	240 ± 44	1.6*	1.3	1.4
RTN	250	6.9 ± 4.3	9.2 ± 3.1	4.5 ± 4.0	3.9 ± 1.9	93 ± 53	74 ± 26	0.8	1.3	0.87

Total vs free drug concentrations for transporter inhibition (still in debate)
Limitations: free drug concentrations in liver was difficult to measure.

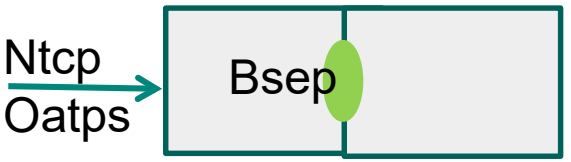
Additional increases of T3-BA in plasma did not seen with BST, CSA, RTN. Why?

Gaps to understand free unbound concentration vs total drug concentration to reach transporter inhibition



Name	Plasma fu	Plasma fu* [I] in, max C	Plasma fu* [I] in, max KD	R value for Ntcp C	R value for Ntcp KD	R value for Bsep C	R value for Bsep KD	Ratio of Liver/plasma 24 hr C	Ratio of Liver/plasma 24 hr KD	Cmax/IC_{50} Bsep† Rat C	Cmax/IC_{50} Bsep† Rat KD
ASN	0.019	19.2	20.7	**34.7**	**37.3**	**7.0**	**7.5**	7.7	3.7	**17.4**	**42.8**
TAK-875	0.01	24.6	26.8	6.7	7.2	2.2	2.3	0.5	1.2	**30.3**	**40.4**
BBR	0.001	0.6	0.8	1.4	1.5	1.1	1.1	2.3	1.7	**7.5**	**29.7**
LPN	0.02	22.8	22.8	23.8	23.8	2.7	2.7	5.2	2.8	0.3	0.4
SMP	0.001	1.2	1.2	25.0	25.0	1.9	1.9	51.1	33.2	3.5	4.6
BST	0.1	162.3	163.2	**406.8**	**409.0**	7.5	7.5	18.4	4.7	0.2	0.6
CSA	0.009	1.0	1.1	1.7	1.7	2.4	2.6	15.8	18.2	**20.3**	**25.9**
RTN	0.14	58.8	59.1	**37.8**	**37.9**	**16.1**	**16.2**	20.8	19	1.8	2.4

$R=1+(fu * [I]_{in, max}/IC_{50})$



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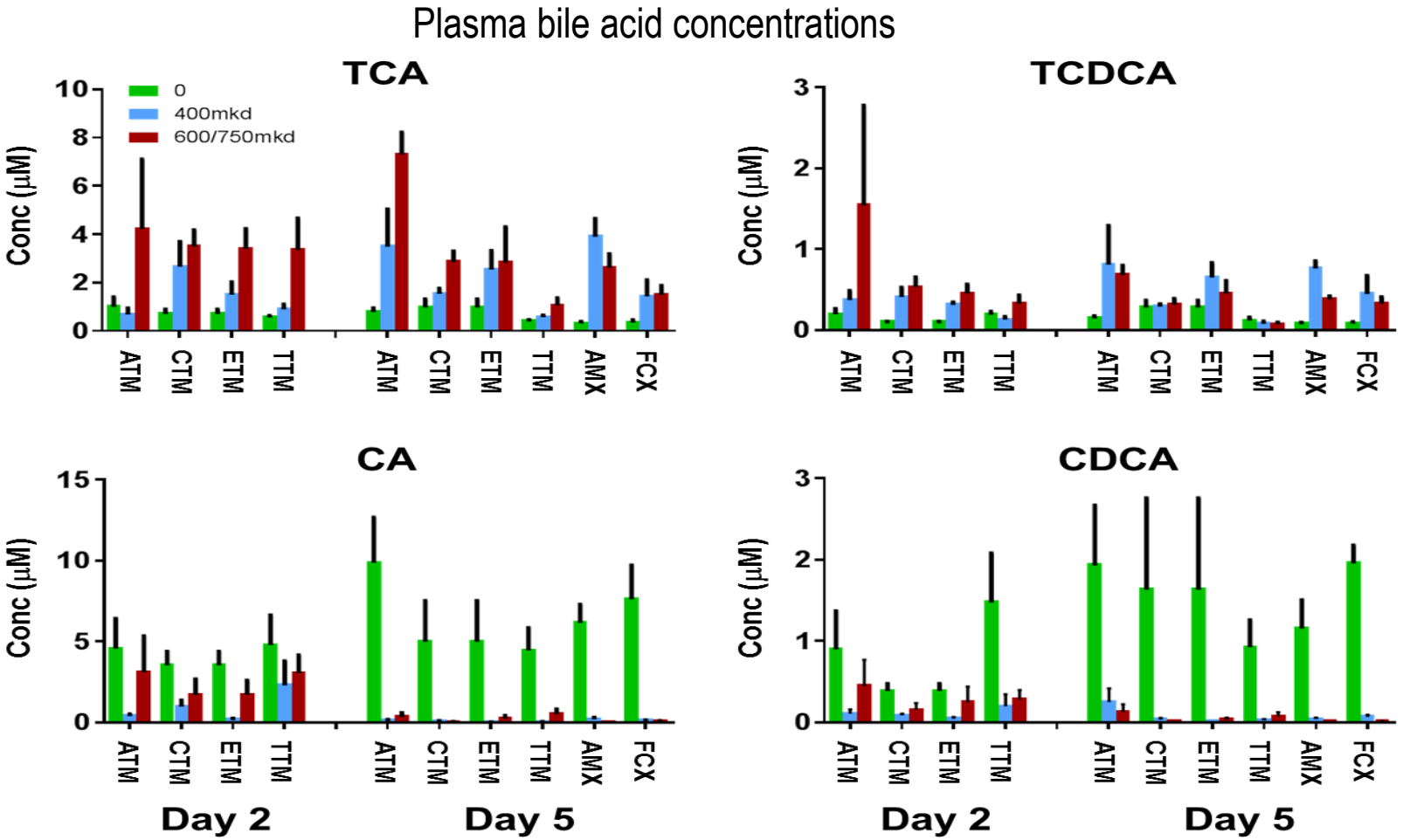
Unconjugated BAs can be used to differentiate Bsep inhibition vs antibiotics effect that both have elevated conjugated BAs

Antibiotics Reduce Ability of Gut Microbes to Deconjugate Bile Acids, Resulting in Increased Conjugated Bile Acids in Plasma



Compounds	Abbv*	Mechanism
Azithromycin	ATM	Prevents bacteria from growing by interfering with their protein synthesis (macrolides)
Clarithromycin	CTM	
Erythromycin	ETM	
Telithromycin	TTM	
Amoxicillin	AMX	Inhibits cell wall biosynthesis, causes death of bacteria (β-lactam)
Flucloxacillin	FCX	

Dosing: Daily
Duration: 5 days
Doses: 0, 400, 600/750 mg/kg/d
Plasma: Days 2 and 5

Unconjugated ↓ are not seen in Bsep inhibition

Ref: **Yutai Li** et al. 2017. Antibiotic-induced Elevations of Plasma Bile Acids in Rats Independent of Bsep Inhibition. Tox Sci. 157:30-40

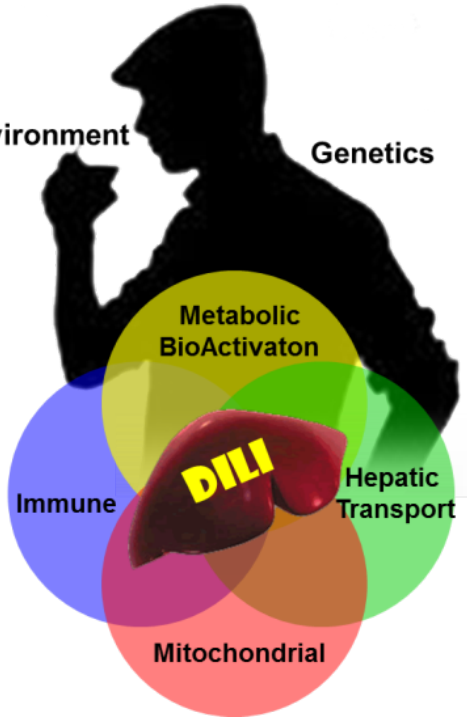
Summary

- The rat is resistant to direct cytotoxicity from elevation of endogenous BA levels in the liver, but alteration in BA homeostasis can still be reflected in plasma by compensatory mechanism
- Conjugated BAs can be used as translational biomarkers to assess BSEP inhibition in vivo.
  - Interpretations can be complicated by many factors
- The Bsep KD rat model has demonstrated the value, albeit imperfect, of measuring T3-BA in rat plasma as molecular biomarkers to rank the *in vivo* risk potential for altered BA homeostasis in rat by drugs that inhibit Bsep *in vitro.*
- Bsep KD in rats can significantly alter liver and/or plasma levels of certain drugs and/or metabolites, presumably by indirectly altering liver biliary elimination.

Future Directions

Further experiments can be conducted to fill the following gaps that are lacking IVIVC

- Protein binding and free liver drug exposures
- Drugs inhibit other transporters besides Bsep
- Bile acid and drug concentrations in bile, bile flow, and rate of drug elimination
- Other mechanisms (bioactivation, mitox etc.)
- Translational PBPK model for better human risk assessment



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