Significance of MDR3 and BSEP Inhibition in DILI

Kan He
President, Biotranex LLC
Why BSEP and MDR3

- Bile salts and phosphatidylcholines are the primary components in bile
- **BSEP** is responsible for export of bile salts
- **MDR3** is responsible for transport of phosphatidylcholine
- Bile salts and phosphatidylcholine forms mixed micelles in bile
- Genetic mutations of BSEP and MDR3 in humans can lead to liver failure
Why BSEP and MDR3

- **BSEP** is the primary mechanism for export of bile salts
  - The driving force for bile salt enterohepatic recirculation and bile flow
  - “Vacuum cleaner” to maintain low intracellular level of bile salts
- **Interference of BSEP function leads to liver diseases**
  - **Progressive Familial Intrahepatic Cholestasis 2 (K903X, Intron 4 (+3)A>C)**
    - Liver failure, death
  - **Benign Recurrent Intrahepatic Cholestasis type 2 (E297G, R432T, V444A)**
  - Associated with intrahepatic cholestasis of pregnancy
  - **Drug induced liver injury (DILI)**

- **MDR3** is the primary mechanism for transport of phosphatidylcholine (PC)
  - PC forms mixed micelles with Bile salts
    - Solubilize cholesterol
    - Reduce cytotoxicity of high concentration of bile salts
  - Maintain lipid asymmetry of hepatocytes
- **Interference of MDR3 function leads to liver diseases**
  - **Progressive Familial Intrahepatic Cholestasis 3 (loss-of-function mutations)**
    - Liver failure, death
  - Associated with intrahepatic cholestasis of pregnancy
  - Several mutations increase risk of liver diseases
  - **Drug induced liver injury**

DILI accounts for >50% acute liver failures, and is the leading cause of failures in drug development, boxed warnings on marketed drugs, and market withdrawals of approved drugs. Inhibition of BSEP and MDR3 is one of the underlying mechanisms for DILI.

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MDR3 and Phosphatidylcholine

- **MDR3 (ABCB4)**
  - ABCB4 gene
  - 1279 amino acid glycoprotein
  - 86% similarity to ABCB1 (p-gp)
  - Functional partner with ABCB11 (BSEP)
  - Mdr2 in mouse
  - Mainly in liver
    - Hepatocyte canalicular membrane
  - **Transport phosphatidylcholine from inner to outer leaflet of hepatocytes**
    - floppase

- **Phosphatidylcholine of hepatocytes**
  - Primarily transported from inner to outer by MDR3
  - ~98% of phospholipids in bile
  - Forms mixed micelles with bile salts and cholesterol
    - Prevents bile salt toxicity
    - Solubolizes cholesterol
  - Maintain hepatocyte membrane lipid asymmetry

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MDR3: Mechanism of Action

Groen, et al. 2011

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MDR3 Mutations for PFIC3: An Example

MDR3 Mutations and Liver Diseases:
Large-scale whole-genome sequencing of the Icelandic population

We imputed these variants into 104,220 individuals down to a minor allele frequency of 0.1% and found a recessive frame shift mutation in MYL4 that causes early-onset atrial fibrillation, several mutations in ABCB4 that increase risk of liver diseases and an intronic variant in GNAS associating with increased thyroid-stimulating hormone levels when maternally inherited.

Gudbjartsson et al, 2015

**Supplementary Table 13.** The association of variants in ABCB4 with liver traits and diseases. Effects and odds ratios (OR) are given for the minor allele. Effects are given in standard deviations. The $r^2$ between the markers is below $6.0 \times 10^{-6}$ in Iceland and no sequenced individual carried both mutations. aSplice region variant.
MDR3 and DILI

• Can drugs interfere MDR3 activity?
• Is MDR3 inhibition associated with DILI?
In Vitro Models for MDR3 Inhibition

- Non-hepatocyte based systems
  - MDR3 transfected cells
    - LLC-PK1, HEK293
  - MDR3-transfected Sf9 insect cell membrane vesicles
    - Doesn't work

- MDR3cyte®
  - Primary hepatocyte
    - Efflux
    - In situ metabolism
  - Pooled or individual donor hepatocytes
  - Human and animal species
Development of MDR3cyte® in Hepatocyte Suspension

- Primary hepatocytes
  - Human, monkey, dog, rat, mouse
- D9-choline
- LC-MS/MS analysis
- Validation

\[ \text{R1} = \text{saturated or unsaturated C16-C22 fatty acid} \]
\[ \text{R2} = \text{saturated or unsaturated C16-C22 fatty acid} \]

MDR3cyte® Protected by US patent No 10,280,401

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MDR3cyte®: Phosphatidylcholine Species Transported from Human Hepatocytes to Extracellular Media

-Similar Profile to Human Bile

Major PC in bile: 1-palmitoyl 2-linoleyl (34:2) and 1-palmitoyl 2-oleol (34:1)

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## MDR3cyte®: MDR3 Inhibition by Drugs Associated with DILI

<table>
<thead>
<tr>
<th>Drug</th>
<th>DILI Type</th>
<th>BSEP (IC50, µM)</th>
<th>MDR3 (IC50, µM)</th>
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<td></td>
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<td>Human</td>
<td>Human</td>
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<td>&gt;1000</td>
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<td>5.6</td>
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MDR3cyte®: MDR3 Inhibition by 125 Drugs

- MDR3 inhibition was determined using MDR3cyte® in human hepatocytes
- MDR3 inhibition is associated with DILI
- Inhibition of both BSEP and MDR3 is associated with most severe DILI


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MDR3 Inhibition Summary

• MDR3 function preserved in primary hepatocyte suspension with MDR3cyte® assay format
• Phosphatidylcholines are transported primarily by MDR3 in human hepatocytes
• MDR3 inhibition
  – Associated with DILI
  – Species difference
Why BSEP and MDR3

- **BSEP** is the primary mechanism for export of bile salts
  - The driving force for bile salt enterohepatic recirculation and bile flow
  - “Vacuum cleaner” to maintain low intracellular level of bile salts
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  - **Drug induced liver injury**

DILI accounts for >50% acute liver failures, and is the leading cause of failures in drug development, boxed warnings on marketed drugs, and market withdrawals of approved drugs. Inhibition of BSEP and MDR3 is one of the underlying mechanisms for DILI. It has been an important task to develop physiologically relevant, reliable, higher throughput assays to determine the inhibition of BSEP and MDR3.

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Bile Salt Synthesis and Biology

- Multiple enzymes and steps
  - Hydroxylation by CYP7A1 is the rate limited step
- Multiple subcellular organelles
  - ER, cytosol, mitochondria, peroxisomes
- Majority of bile salts reabsorbed
- **Important biological process**
  - ~500 mg/day in human
    - ~95% re-absorption
  - Osmotic bile flow
  - Mixed micelles with phospholipids
  - Absorption of fat and vitamins
- Bile salts for gene regulation
  - Ligands for FXR and TGR5
- **Transporters involved in bile salt disposition**
  - BSEP for bile salts transport to bile
  - NTCP for uptake transport
  - Others: ASBT, OST, MRP2/3/4, OATP1B1/2
- Other pathways
  - Secondary bile salts
  - Sulfate and glucuronide conjugates of BA

**Chemical Structures:**
- CA: \( R_1 = \text{OH} \)
- CDCA: \( R_1 = \text{H} \)
- GCA/GCDCA: \( R_2 = \text{NHCH}_2\text{COOH} \)
- TCA/TCDCA: \( R_2 = \text{NHCH}_2\text{CH}_2\text{SO}_3\text{H} \)
Bile Salt Export Pump (BSEP)

- Encoded by ABCB11 gene
  - 1321 AA
  - 12 transmembrane domains
  - Glycoprotein, 160 kDa
- Expressed in canalicular plasma membrane of hepatocyte
- Responsible for ATP-dependent bile salt transport
  - Predominant
    - <1% bile salts in bile of PFIC-2 patient vs normal
    - Like “vacuum cleaner” to keep low intracellular level of bile salts
  - Drive force for enterohepatic circulation of bile salts
    - Rate limited step
- Substrate specificity for bile salts
  - A few exceptions

Physiological Bile Acid Conjugation Reactions

- CA
- GCA
- TCA
- CDCA
- GCDCA
- TCDCA
- DCA
- GDCA
- TDCA
- LCA
- GLCA
- TLCA

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Assay Formats for BSEP Inhibition

• BSEP inhibition assay formats
  – BSEP-transfected Sf9 insect cell membrane vesicles
    • Artificial membrane uptake system
    • Incapable of evaluating BSEP inhibition by metabolites
    • Questionable physiologically relevance
  – Human sandwich-cultured hepatocytes
    • Technical challenges
    • Low throughput
  – Others
    • BSEP and sodium taurocholate transporter co-transfected kidney cells
    • Xenopus laevis oocytes
    • Canalicular membrane vesicles (CMV)

• Concerns and issues
  – Physiological relevance
  – Contribution of metabolite
  – Throughput
  – Difference in inhibition of export of different bile salts
  – Species difference
  – Competitive vs noncompetitive inhibition

• BSEPcyte® is a physiologically relevant, hepatocyte-based, higher throughput assay format to address the above concerns and issues


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BSEPcyte® Assay Principle

Bile Acids (pKa: 5-6.5)

Cholesterol

CA: R₁ = OH
CDCA: R₁ = H
GCA/GCDCA: R₂ = NHCH₂COOH
TCA/TCDCA: R₂ = NHCH₂CH₂SO₃H

Bile Salts (pKa: 1-4)

>99% Bile salts exported by BSEP in human liver.

BSEPcyte® Protected by US patent No 9,772,325
BSEPcyte®: Time-Dependent Bile Salt Transport in Human Hepatocytes


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BSEPCyte®: Bile Acid Concentration Dependent Transport of Bile Salt in Human Hepatocytes

BSEPcyte®: BSEP Activity Variation in Human Hepatocytes

Note: Both pooled and individual hepatocytes can be used with BSEPcyte® method

Collaboration with Dr. Albert Li at IVAL

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<th>Drug</th>
<th>Human</th>
<th>Monkey</th>
<th>Dog</th>
<th>Rat</th>
<th>Mouse</th>
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Biotranex – Accurate. Efficient. Innovative
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Total 86 drugs

**BSEPcyte®**
- Detected more severe DILI drugs than MV
  - metabolism-mediated
  - drug accumulation
- Produced physiologically relevant IC50 values
- Produced results highly correlated with cholestatic and mixed liver injury


Biotranex – Accurate. Efficient. Innovative
### BSEP IC50: BSEPcyte® vs Membrane Vesicles (MV)

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>BSEPcyte® (uM)</th>
<th>MDR3cyte® (uM)</th>
<th>MV BSEP (uM)</th>
<th>MRP2 (uM)</th>
<th>MRP3 (uM)</th>
<th>MRP4 (uM)</th>
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**Additional 250 drugs**

- Detected more severe DILI drugs than MV
  - metabolism-mediated
  - drug accumulation
- Produced physiologically relevant IC50 values
- Produced results highly correlated with cholestatic and mixed liver injury

He, et al. (To be published)

Biotranex – Accurate. Efficient. Innovative
BSEP Inhibition: Competitive vs Noncompetitive

- **Troglitazone**
  - Activity remaining (%)
  - Concentration (µM)
  - 3 µM, 10 µM, 30 µM, 100 µM, 300 µM

- **Pioglitazone**
  - Activity remaining (%)
  - Concentration (µM)
  - 3 µM, 10 µM, 30 µM, 100 µM, 300 µM

- **Flutamide**
  - Activity remaining (%)
  - Concentration (µM)
  - 3 µM, 10 µM, 30 µM, 100 µM, 300 µM

- **Cyclosporine**
  - Activity remaining (%)
  - Concentration (µM)
  - 3 µM, 10 µM, 30 µM, 100 µM, 300 µM
Cornish-Bowden Plots

Graphs showing Cornish-Bowden Plots for different drugs:
- Troglitazone concentration vs. 1/Relative BSEP activity
- Concentration vs. 1/Relative BSEP activity for different concentrations of Pioglitazone
- Concentration vs. 1/Relative BSEP activity for different concentrations of Flutamide
- Concentration vs. 1/Relative BSEP activity for different concentrations of Cyclosporine
BSEP Inhibition Summary

• BSEP function preserved in primary hepatocyte suspension with BSEPcyte® assay format

• Bile salts are exported primarily by BSEP in human hepatocytes

• BSEP inhibition
  – Highly associated with cholestatic and mixed DILI
  – Species difference
  – Noncompetitive inhibition may be associated with severe DILI

BSEPcyte®, Protectd US patent 9,772,325
Why BSEP and MDR3

• **BSEP** is the primary mechanism for export of bile salts
  — The driving force for bile salt enterohepatic recirculation and bile flow
  — “Vacuum cleaner” to maintain low intracellular level of bile salts

• Interference of BSEP function leads to liver diseases
  — **Progressive Familial Intrahepatic Cholestasis 2** (K903X, Intron 4 (+3)A>C)
    • Liver failure, death
  — **Benign Recurrent Intrahepatic Cholestasis type 2** (E297G, R432T, V444A)
  — Associated with intrahepatic cholestasis of pregnancy
  — **Drug induced liver injury**
  — **BSEPcyte®** assay format
    • Primary hepatocytes in suspension

• **MDR3** is the primary mechanism for transport of phosphatidylcholine (PC)
  — PC forms mixed micelles with Bile salts
    • Solubilize cholesterol
    • Reduce cytotoxicity of high concentration of bile salts
  — Maintain lipid asymmetry of hepatocytes

• Interference of MDR3 function leads to liver diseases
  — **Progressive Familial Intrahepatic Cholestasis 3** (loss-of-function mutations)
    • Liver failure, death
  — Associated with intrahepatic cholestasis of pregnancy
  — Several mutations increase risk of liver diseases
  — **Drug induced liver injury**
  — **MDR3cyte®** assay format
    • Primary hepatocyte in suspension

DILI accounts for >50% acute liver failures, and is the leading cause of failures in drug development, boxed warnings on marketed drugs, and market withdrawals of approved drugs. Inhibition of BSEP and MDR3 is one of the underlying mechanisms for DILI.

**Biotranex – Accurate. Efficient. Innovative**
Acknowledgment

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  – Weida Tong

• IVAL
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  – Qian Yang

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  – Yaofeng Cheng
  – Jinping Gan
  – Williams Humphreys

• Takeda
  – Mingxiang Liao
  – Cindy Xia

• Pfizer
  – A. David Rodrigues
  – Michael Aleo

Accurate. Efficient. Innovative
Backup Slides
Immunofluorescent Detection of Transporters in Cryopreserved Human Hepatocytes

The large majority of transporters (note: including BSEP) seemed to be localized to the plasma membrane, whereas intracellular staining was comparatively weak.


Drug Metabolism and Disposition 2014; 42:448-58.
BSEPcyte® Assay Platform Summary

- BSEP function preserved in primary hepatocyte suspension with BSEPcyte® assay format
- Bile salts are exported primarily by BSEP in human hepatocytes
- BSEP inhibition
  - Highly associated with cholestatic and mixed DILI
  - Species difference
  - Irreversible inhibition may be associated with severe DILI

- BSEPcyte®
  - Physiologically relevant system
    - In vitro-in vivo extrapolation
  - In situ metabolism capability
  - Uptake transport and hepatocyte accumulation
  - Pooled or individual donor hepatocytes
  - Applicable to all bile salts
  - Cross species comparison
  - Large dynamic range
  - Accurate, robust, reproducible, and customizable
  - High throughput

BSEPcyte®, US patent 9,772,325

Biotranex – Accurate. Efficient. Innovative
MDR3cyte® Assay Platform Summary

- MDR3 function preserved in primary hepatocyte suspension
  - Lot variations
  - Timing
  - Components

- Phosphatidylcholines are transported primarily by MDR3 in human hepatocytes

- MDR3 inhibition
  - Highly associated with DILI
  - Species difference

- MDR3cyte®
  - Physiologically relevant holistic system
    - In vitro-in vivo extrapolation
  - In situ metabolism capability
  - Uptake transport and hepatocyte accumulation
  - Pooled or individual donor hepatocytes
  - Cross species comparison
  - Large dynamic range
  - Accurate, robust, reproducible, and customizable

MDR3cyte® Protected by US patent No 10,280,401

Biotranex – Accurate. Efficient. Innovative
Biotranex: A Science-Driven CRO

We Take Pride in Helping You Succeed
Biotranex Core Services

- Drug-drug interaction
  - FDA/EMA guidance
  - CYP, UGT, SULT, others
    » Inhibition, induction, phenotyping
      • Mechanism-based inhibition

- Metabolite ID/synthesis
  - Radioactive or non-radioactive
  - Biosynthesis

- Transporters
  - Efflux and uptake transporters
  - Caco-2 and transfected cells

- Bioanalysis
- Pharmacokinetics

- Hepatocytes
  - Hepatotoxicity
  - CYP induction/inhibition
  - Liver uptake

- New Technology
  - **BSEPcyte**
    • Hepatocyte suspension-based assay platform for BSEP inhibition
  - **MDR3cyte**
    • Hepatocyte suspension-based assay platform for MDR3 inhibition
  - **BILIRUBINcyte™**
    • Hepatocyte-based assay platform to screen drugs for hyperbilirininia potential

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