Model Systems:
Aristolochic Acid Case Study

Edward J. Kelly

MEDCH/PCEUT 527 –
ADVANCED DRUG METABOLISM
March 8th, 2019

SCHOOL OF PHARMACY
UNIVERSITY of WASHINGTON
Department of Pharmaceutics
Outline

What are “Organs on Chips”?  
“Liver on a Chip”  
“Kidney on a Chip”  
Aristolochic Acid Nephropathy  
Linked Liver>Kidney System to Model Aristolochic Acid Nephropathy
Francis Collins: “Drug development system in the United States is in crisis”

- Molecular basis for ~ 5,000 human diseases understood, but safe effective treatments exist for only ~250

- 80-90% of new drugs fail in clinical trials, 10 – 20% are proven safe and effective in humans

- Phase 2 attrition rates 80%, Phase 3 attrition rates 50%

- Relevance and reproducibility of animal models

Francis Collins at TEDMED 2012
We need better drugs — now

https://www.youtube.com/watch?v=ahKnVXzVb3o
Hearing: FY 2019 Budget - National Institutes of Health
Major drivers for developing \textit{in vitro} models in predicting human toxicological response

- \textbf{High throughput (HT):} based on mechanisms of toxicity (MOT) \textit{ToxCast™} data

- \textbf{High content (HC):} 3D microfluidic models that reflect physiological effects of organs \textit{in vivo/ex vivo}
  - Microphysiological system/organ-on-a-chip
What is a “solution” to overcome the issues of inter-species predictability of animals and the in vitro to in vivo disconnect of cell-based HTS testing?
Examples of Organs on Chips Developed in NCATS Consortium

https://ncats.nih.gov/tissuechip/chip
Examples of Organs on Chips Developed in NCATS Consortium

➔ Vascularized Tumor on a Chip - Chris Hughes, UC-Irvine

Cytochrome C-RFP

Caspase 3-GFP
The DARPA “Vision”
“Human-on-a-Chip”
Short of a “Human on a Chip”
What organ systems do we need for accurate ADME modeling?
The “Holy Trinity” of ADME

- Intestine
- Liver
- Kidney
“Liver on a Chip”
Nortis Microphysiological System (MPS)

Technical details:
- Gas-permeable PDMS silicone, polycarbonate base, collagen type I ECM, with a microscope coverslip
- Diameter of “tubule” is ~120 µm with an internal volume of ~70 nL
- Flow rate of 0.5 µL/min (1 Dyne/cm²)
- A 6 mm tubule contains ~5000 PTECs
MPS Scheme for Liver & Kidney MPS

Collagen type I- base matrix or Matrigel™ overlaid layer

ECM: Type I Collagen (6 mg/mL)
“Liver on a Chip”

Freshly isolated rat hepatocytes (Sprague Dawley®) or cryopreserved human hepatocytes

1. Morphology
2. Viability
3. Functionality

Cell suspension
Flow rate: 5-30 μL/hour

Rat Hepatocyte Morphology

Day 1  Day 5  Day 7

2D

1 Hr  Day 2  Day 5  Day 14

MPS- 1.3 mg/ml Coll

1 Hr  Day 8  Day 14  Day 21

MPS- 3 mg/ml Coll

bar = 100 µm

bar = 50 µm
Human Hepatocyte Morphology

2D

Day 1 | Day 3 | Day 7
--- | --- | ---

MPS

Day 1 | Day 3 | Day 7 | Day 10 | Day 15
--- | --- | --- | --- | ---

bar = 50 μm
Determine the viability over time

- Live/Dead® staining

LIVE/DEAD® assays

Live cells fluoresce bright green (Calcein AM)
Dead cells with compromised membranes fluoresce red-orange (EtBr)

- Measures of lactate dehydrogenase (LDH) and alanine aminotransferase (ALT) release
Viability of hepatocytes by LIVE/DEAD® staining

Rat

2D

Day 6

LIVE DEAD

Day 12

LIVE DEAD

MPS

Day 6

LIVE DEAD

Day 14

LIVE DEAD

Day 28

LIVE DEAD

Human

2D_ Day 8

MPS_ Day 15

bar = 100 μm
Measure LDH releasing from rat hepatocytes

MPS: N=4
2D: N=4

\( t \)-test, 
\#, \ p<0.01
Measure ALT releasing from human hepatocytes

2D: N=3
MPS: N =4~6
t-test, *, p<0.05; #, p<0.01
Enhanced viability, but what about function?

**Functional Measures**

a. Enzyme activities of cytochrome P450s (CYPs)
b. Expression of Hepatocyte Nuclear Factor 4 alpha (HNF4 alpha)
c. Albumin production
Methods for Measuring CYPs Activity

Cells were pre-treated with beta-naphthoflavone (BNF), rifampin (Rif), and 0.1% DMSO vehicle control for 72 hours prior to performing the enzyme activity assays.

- **CYP1A1/2**: EROD confocal kinetic assay
- **CYP3A4**: 1. CYP3A4 Glo (Promega)
  2. Midazolam metabolites by GC-MS

Figure 1. Contribution of enzymes to the metabolism of marketed drugs

*Pharmacology & Therapeutics* 138 (2013) 103–141
EROD kinetic confocal assay

7-ethoxyresorufin → CYP1A → Resorufin
EX/EM = 530/580 nm

MPS_DMSO

MPS_BNF 25μM
CYP1A1/2 activity in primary human hepatocytes cultured in MPS using EROD kinetic confocal assay

2D: N=3
MPS: N =3

\[ \text{t-test} \quad * \quad p<0.01 \]
Measure of CYP3A4 activity in human hepatocytes by using CYP3A4 Glo substrate

**CYP3A4 activity in human hepatocytes**

- **Y-axis**: $10^{-2}$ fmole/1x10$^4$ cells
- **X-axis**: Day 5, Day 9, Day 15

**Groups**
- 2D
- 2D_Rif Day6-9
- MPS
- MPS_Rif Day6-9/Day13-15

**Legend**
- Light grey: 2D
- Dark grey: 2D_Rif Day6-9
- Black: MPS
- Grey: MPS_Rif Day6-9/Day13-15

**Statistics**
- **t-test**: * p<0.05; # p<0.01
- **Sample Size**: 2D: N=3, MPS: N =3
Measure of CYP3A4 activities in human hepatocytes cultured in MPS by analysis of MDZ metabolite (1-OH MDZ) using GC-MS

100 µM MDZ for 4h
before and after rif 5 µM for 72 hour

<table>
<thead>
<tr>
<th>MPS sample</th>
<th>1-OH (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#2660_before</td>
<td>0.243221</td>
</tr>
<tr>
<td>#2660_after</td>
<td>0.748300283</td>
</tr>
<tr>
<td>#2658_before</td>
<td>0.1876532</td>
</tr>
<tr>
<td>#2658_after</td>
<td>0.596139907</td>
</tr>
<tr>
<td>#2659_before</td>
<td>0.0854364</td>
</tr>
<tr>
<td>#2659_after</td>
<td>0.224148425</td>
</tr>
</tbody>
</table>

MPS: N =3
t-test, # p<0.01
Measure of albumin production in human hepatocytes cultured in 2D and MPS over 15 days

2D: N=2
MPS: N=3~4
t-test, * p<0.05; # p<0.01
Why “Kidney-on-a-chip”? 
Liver and Kidney on Chips: Microphysiological Models to Understand Transporter Function.

<table>
<thead>
<tr>
<th></th>
<th>2D Culture</th>
<th>3D Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>High throughput</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Cost</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Polarity</td>
<td>Variable</td>
<td>Yes</td>
</tr>
<tr>
<td>Flow</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Sensitivity - Injury</td>
<td>Standard</td>
<td>Higher</td>
</tr>
<tr>
<td>Drug Metabolism</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Transport</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Proximal Tubule Epithelial Cells (PTEC) Self-assemble and correctly polarize in 3D

ZO-1 (apical)  Na/K ATPase (basolateral)
Development of a microphysiological model of human kidney proximal tubule function

Elijah J. Weber\(^1,\)\(^7\), Alenka Chapron\(^1,\)\(^7\), Brian D. Chapron\(^1,\)\(^7\), Jenna L. Voellinger\(^1\), Kevin A. Lidberg\(^1\), Catherine K. Yeung\(^2,\)\(^6\), Zhican Wang\(^1,\)\(^8\), Yoshiyuki Yamaura\(^1,\)\(^9\), Dale W. Hailey\(^3\), Thomas Neumann\(^4\), Danny D. Shen\(^1,\)\(^2\), Kenneth E. Thummel\(^1\), Kimberly A. Muczynski\(^5\), Jonathan Himmelfarb\(^5,\)\(^6\) and Edward J. Kelly\(^1\)

\(^1\)Department of Pharmaceutics, University of Washington, Seattle, Washington, USA; \(^2\)Department of Pharmacy, University of Washington, Seattle, Washington, USA; \(^3\)Department of Biological Structure, University of Washington, Seattle, Washington, USA; \(^4\)Nortis Inc., Seattle, Washington, USA; \(^5\)Department of Medicine, University of Washington, Seattle, Washington, USA; and \(^6\)Kidney Research Institute, University of Washington, Seattle, Washington, USA

- Glucose Reabsorption
- Glutathione Reclamation
- Vitamin D Homeostasis
- Ammoniagenesis
- Organic Solute Secretion
MPS Scheme for Liver & Kidney MPS

Collagen type I- base matrix or Matrigel™ overlaid layer

ECM: Type I Collagen (6 mg/mL)
Sources of Renal Epithelial Cells & Cell Seeding Into Chip

- Human kidney epithelial cell isolation
- 2D culture growth
- Injection into 3D microphysiological system
Cell Viability and Basic Functionality
Self-Assembly and Tight Junction Formation of PTECs in MPS

E-Cadherin
Nuclei

ZO-1
Nuclei

Merged

Nature Medicine News and Views, 2017
Proximal Tubule MPS Phenotyping

Proximal Tubule Marker
Aquaporin 1
Distal Tubule Marker
Aquaporin 2

Polarity-Basolateral Marker
Na+/K+ ATPase

Cilium Marker-Mechanosensation
Nuclei
Acetylated tubulin

Polarity-Apical Marker
ZO-1
Kidney Tubule Functional Characterization

- Glucose Reabsorption
- Ammoniagenesis
- Glutathione Reclamation
- Organic Solute Secretion
- Vitamin D Homeostasis


Development of a microphysiological model of human kidney proximal tubule function
Glucose Reabsorption

A

SGLT-2

B C D E

F

Normalized 2-NBDG Fluorescence

2-NBDG (0.6 mM)
2-NBDG (0.6 mM) Apigenin (50 uM)
2-NBDG (0.6 mM) Dapagliflozin (0.5 uM)

*
Aristolochic Acid Nephropathy
• Aristolochic acid (AA) is found in *Aristolochia* plants

• Banned by the FDA in 2001 due to nephrotoxicity and carcinogenicity

• Widely used as an herbal remedy throughout Asia
After the incision, prepare silphium juice a drachma in weight, grate Aristolochia to the amount of a deer’s vertebra, and sift a half-choinix each of parched lentils and vetches...”
Outbreak of kidney failure in Belgium


• ~100 otherwise healthy young women living in Brussels diagnosed with end-stage kidney disease

• All women attended the same weight reduction clinic, where they were treated with “slimming” agents, including Chinese herbs

*Aristolochia fangchi*
Association with Upper Urinary Tract Cancers

The New England Journal of Medicine

UROTHELIAL CARCINOMA ASSOCIATED WITH THE USE OF A CHINESE HERB (ARISTOLOCHIA FANGCHI)

JOËLLE L. NORTIER, M.D., Ph.D., MARIE-CARMEN MUNIZ MARTINEZ, M.D., HEINZ H. SCHMEISER, Ph.D., VOLKER M. ARLT, CHRISTIAN A. BIeler, Ph.D., MICHEL FETEIN, M.D., Ph.D., MICHEL F. DEPIERREUX, M.D., LUC DE PAUW, M.D., DANIEL ABRAMOWICZ, M.D., Ph.D., PIERRE VEREERSTRAETEN, M.D., Ph.D., and JEAN-LOUIS VANHERVEGHEM, M.D., Ph.D.

Renal proximal tubules leading to renal failure and fibrosis

Upper urinary tract urothelium leading to urothelial cell cancer

AA Targets

Cortex

Medulla

Pelvis

Ureter
Balkan Endemic Nephropathy (BEN)

- Occurs only in farming villages in Croatia, Bosnia, Serbia, Romania and Bulgaria.
- Geographical distribution unchanged after 50 years.
- Affects adults - often in the same household - but never children < age 18.
- Upper urinary tract cancer incidence 20x higher in endemic regions.
Farming practices in Kaniza, an endemic village

*Aristolochia* seeds and wheat grain

*Aristolochia* plant in a wheat field

Communal baking

Village mill

*Hranjec et al* (2005)
Aristolochia herbal remedies used for centuries in traditional Chinese medicine

- Guang-Fang-Ji (广防己): *Aristolochia fangchi*
- Guan-Mu-Tong (关木通): *Aristolochia manshuriensis*
- Quing-Mu-Xiang (青木香): *Aristolochia debilis*
- Tian-Xian-Teng (天仙藤): *Aristolochia debilis or A. contorta*
- Ma-Dou-Ling (马兜铃): *Aristolochia contorta*
- Xun-Gu-Feng (寻骨风): *Aristolochia mollissima*
- Zhu-Sha-Lian (朱砂莲): *Aristolochia cinnabarina*

140,000 lbs Quing-Mu-Xiang imported annually by Taiwan
Longdan Xiegan Wan

Main Ingredients: Dragon胆, Chai Hua, Huang Zhi, Dang Gui, Ze Xie.

Functions and Indications: Clear liver gallbladder, resolve heat, used for liver gallbladder wet heat, blurry eyes, redness, itching, ear ringing, ear pain, gingival bleeding, bitter taste, redness of tongue, wet heat in the lower abdomen.

Usage and Dosage: Oral, once 3-6 g, twice a day.

Notes: Pregnancy should be used with caution.

Specifications: Each 100 granules contain 6 g, every bag contains 3 g. Store in a cool, dry place to prevent moisture.
The frequency of urinary cancer in Taiwan is among the highest in the world. Between 1997 and 2003, one of three Taiwanese were prescribed herbal medicines containing *Aristolochia*. 

*Chen, Dickman et al.*, *PNAS* (2012)
Growing Awareness of AAN/UUC

- 100 AAN/ UUC....1800 at risk
- ~100,000 at risk
- ~100,000,000 at risk (NTP)
- ~8,000,000 at risk

(c) www.kinabaloo.com
Human ‘Liver-on-a-chip’ + Human ‘Kidney-on-a-chip’

Can they be coupled to identify toxicologically important ‘organ-organ interactions’ that might occur *in vivo*?
Linked Liver>Kidney System to Model Aristolochic Acid Nephropathy

Aristolochic Acid –I (AA-I)

Bioactivation

metabolism

Or

Detoxification
Glatt, et. al., *Int. J. Cancer*, 2006. Human sulfotransferases are involved in the activation of aristolochic acids.....

Schmeiser, et. al., *Environmental and Molecular Mutagenesis*, 2011. The human carcinogen aristolochic acid I is activated to form DNA adducts......without the contribution of acetyltransferases or sulfotransferases

---

**Aristolochic acid (AA) → N-hydroxy aristolactam (AL-NOH) → N-sulfonyl aristolactam (AL-NOSO3) → Cyclic nitrenium intermediate → DNA adducts**

**Attaluri, Johnson, et al., Chem Res Tox, 2014**

**Sidorenko, et. al., Carcinogenesis, 2014**
Study Design

LIVE/DEAD® assays

Liver

Kidney
What role does liver play in bioactivation of AA-I?

Demethylation = Detoxification
Nitroreduction = Bioactivation

Morphology and viability of PTECs after AA treatment

<table>
<thead>
<tr>
<th></th>
<th>PTEC (Kidney only)</th>
<th>PTEC (Liver→Kidney)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat AA 0 μM</td>
<td><img src="AA_0muM.png" alt="Image" /></td>
<td><img src="AA_0muM.png" alt="Image" /></td>
</tr>
<tr>
<td>Rat AA 5 μM</td>
<td><img src="AA_5muM.png" alt="Image" /></td>
<td><img src="AA_5muM.png" alt="Image" /></td>
</tr>
<tr>
<td>Rat AA 10 μM</td>
<td><img src="AA_10muM.png" alt="Image" /></td>
<td><img src="AA_10muM.png" alt="Image" /></td>
</tr>
<tr>
<td>Rat AA 25 μM</td>
<td><img src="AA_25muM.png" alt="Image" /></td>
<td><img src="AA_25muM.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Human

<table>
<thead>
<tr>
<th></th>
<th>PTEC (Liver→Kidney)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA 0 μM</td>
<td><img src="AA_0muM.png" alt="Image" /></td>
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<tr>
<td>AA 10 μM</td>
<td><img src="AA_10muM.png" alt="Image" /></td>
</tr>
<tr>
<td>AA 25 μM</td>
<td><img src="AA_25muM.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Quantitative results of AA-induced cytotoxicity

**Rat**

<table>
<thead>
<tr>
<th>Rat Cells</th>
<th>IC$_{50}$ (µM)</th>
<th>95% CI (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEC (Kidney only)</td>
<td>66.52</td>
<td>38.30 to 115.5</td>
</tr>
<tr>
<td>PTEC (Liver→Kidney)</td>
<td>5.35#</td>
<td>2.792 to 10.24</td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>32.34*</td>
<td>23.53 to 44.44</td>
</tr>
</tbody>
</table>

**Human**

<table>
<thead>
<tr>
<th>Human Cells</th>
<th>IC$_{50}$ (µM)</th>
<th>95% CI (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEC (Kidney only)</td>
<td>77.52</td>
<td>50.25 to 119.6</td>
</tr>
<tr>
<td>PTEC (Liver→Kidney)</td>
<td>17.84#</td>
<td>10.67 to 29.84</td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>38.04*</td>
<td>27.72 to 52.21</td>
</tr>
</tbody>
</table>

$t$-test, *, $p < 0.05$, #, $p < 0.01$ compared to PTEC (Kidney only)

N= 5-6
Liver *does* play a role in bioactivation of AA-I.

**AL-1 DNA adducts measured by IHC**

<table>
<thead>
<tr>
<th>AA-I</th>
<th>Kidney</th>
<th>Liver → Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DAPI</td>
<td>DAPI</td>
</tr>
<tr>
<td>0 µM</td>
<td>AL-1-</td>
<td>AL-1-</td>
</tr>
<tr>
<td></td>
<td>DNA adduct</td>
<td>DNA adduct</td>
</tr>
<tr>
<td>10 µM</td>
<td>DAPI</td>
<td>DAPI</td>
</tr>
<tr>
<td></td>
<td>AL-1-</td>
<td>AL-1-</td>
</tr>
<tr>
<td></td>
<td>DNA adduct</td>
<td>DNA adduct</td>
</tr>
</tbody>
</table>

AA 0 µM

AA-I 25 µM
Attenuation of AA-I Nephrotoxicity by Dicumarol
AL-I-NOSO$_3$ (Sulfate conjugated AL-I metabolite): Nephrotoxicity in MPS
Inhibition of AL-I-NOSO₃ Nephrotoxicity by Blocking Uptake: Probenecid-OAT inhibitor
MRP3/4 Efflux of AL-I-NOSO$_3$
OAT1/3/4 Uptake of AL-I-NOSO₃
Conclusions

Hepatic metabolism led to overall bioactivation of AA rather than to detoxification.

- NQO1 – primary role in activation
- Hepatic SULT – route of Phase II conjugation
- AL-I-Sulfate – circulating toxic metabolite
- OAT4 – lumenal uptake transporter in kidney
• High concentrations of AA-I are directly toxic to PTECs
  • Not inhibitable by dicoumarol (NQO1 inhibitor)
• In patients suffering from Balkan Endemic Nephropathy, *NQO1*2*2* expressors (null) have an increased risk of developing uroepithelial cancer
• *CYP3A5*1 carriers (functional) more likely to develop Balkan Endemic Nephropathy
• Supersomes expressing CYP3A5 can bioactivate AA-I and increase the number of AA-DNA adducts
Questions?
ARISTOLOCHIACEAE

ARISTOLOCHIA CALIFORNICA
CALIFORNIA PIPEVINE
CALIFORNIA
The life cycle of a California pipevine swallowtail butterfly

https://www.vox.com/2016/7/6/12098122/california-pipevine-swallowtail-butterfly-population