Human \textit{in silico} trials for drug cardiac safety and efficacy evaluation

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Acknowledgements

Computational Cardiovascular Science Group
http://www.cs.ox.ac.uk/ccs

- Blanca Rodriguez
- Alfonso Bueno-Orovio
- Oliver Britton
- Ana Mincholé
- Francesc Levrero
- Xin Zhou
- Linford Briant
- Jenny Wang
- Jakub Tomek
- Hector Martinez
- Cristian Trovato
- Peter Marinov
- Polina Mamoshina
- Francesca Margara
- Patricia Benito
- Kevin Burrage
- Ernesto Zacur
- Vicente Grau
INTRODUCTION

• Computer Models of Cardiac Electrophysiology:
  ✓ Technology mature (almost 60 years of history)
  ✓ Multiscale
  ✓ Human data -> Human models

• Computer Models in Drug Development:
  ✓ Facilitate the translation to HUMANs
  ✓ Early prediction of drug cardiotoxicity

A fast, cheap and potentially effective alternative to animal experiments!

• Major challenges:
  ✓ High variability in drug response
  ✓ Diseased conditions and concomitant medications
From Ion Channels to Whole Heart...

MULTISCALE

Tissue/Organ

Whole-Organ models

Single Cell

Action Potential models

Ion Channel

Ionic current models

Image credits: Doregan (cardiac MRI), Coppini et al. Circ Res. 2013, Carusi et al. AJP 2012

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Cardiac Action Potential (AP)

- Electrical signal of the heart at the **single cell level**
Torsade de Pointes (TdP) Arrhythmia

Image Credit: www.hopkinsmedicine.org (ECG), Jer5150 (TdP), Fauci AS et al. Harrison's Principles of Internal Medicine (EAD)
A Human Ventricular Action Potential Model

  - novel experimental data from more than 150 human hearts

• ODEs cannot be solved analytically
✓ numerical methods can help!!!
Is ONE model ok for the whole population?

6 AP traces from the same region of 6 different human hearts

We are all different!!!

Image Credit: Marylou Pausewang Gelfer and Sara L. Denor, AJSLP 2014

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**Variability and Populations of Models**

- O’Hara-Rudy (ORd) model as baseline
- Same equations, different parameters
- Calibration with human data

Image Modified from Britton et al. Front Physiol. 2017

Experimentally calibrated population of models predicts and explains intersubject variability in cardiac cellular electrophysiology

Oliver J. Britton\(^a\), Alfonso Bueno-Orovio\(^b\), Karel Van Ammel\(^c\), Hua Rong Lu\(^d\), Rob Tawara\(^e\), David J. Gallacher\(^f\), and Blanca Rodriguez\(^*\)

Britton et al. PNAS 2013
Passini et al. JMCC 2016
Liberos et al. Heart Rhythm 2016
Britton et al. Front Physiol 2017
Passini et al. Front Physiol 2017

n = 62 experiments
Original ORd model
Population of Models

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Variability in Drug Response

- **Single AP model**
  - single drug response

- **Population of models**
  - inter-subject variability in drug response

Phenotype I
- changes in APD, \( \frac{dV}{dt_{MAX}} \), …

Phenotype II
- repolarisation abnormalities

Phenotype III
- depolarisation abnormalities
Recent development on Multi-Scale Modelling of Inherited Disease Conditions

Phenotype I
✓ changes in APD, \(dV/dt_{\text{MAX}}\), ...

Phenotype II
✓ repolarisation abnormalities

Phenotype III
✓ depolarisation abnormalities

I\(_{\text{Kr}}\) and I\(_{\text{KS}}\) mutations leading to acquired LTQ
(Itoh et al. Eur Heart J 2015)

I\(_{\text{NaK}}\) importance in repolarisation:
(Bueno-Orovio et al. Pflugers Arch 2014
Niemeijer et al. Heart 2015)
In Silico Drug Trials:

Possible drug identified → Animal testing → Clinical trials → Drug approved for sale → Drug withdrawn from the market

In Silico drug trials
What do Pharma think about this?

Image Credit: Max Pixel
In Silico Drug Trials:

Possible drug identified → Animal testing → Clinical trials → Drug approved for sale

In Silico drug trials

Retrospective studies

Drug withdrawn from the market
Recent development on Multi-Scale Modelling of Inherited Disease Conditions

SCCSOT Annual Meeting

Human in silico trials for drug cardiac safety and efficacy evaluation

Elisa Passini

7th November 2019

Virtual Assay Software

- *In Silico* predictions of inter-subject variability in drug response

Virtual Assay

http://www.cs.ox.ac.uk/ccs/virtual-assay

Academic Licence and Commercial Licence available!
**In Silico Drug Trials: Design**

**INPUTs**
- Drug effects on ion channels

**OUTPUTs**
- Drug-induced changes in AP

**Virtual Assay**

**INPUTs**
- Current %
- Current % = \( \frac{1}{1 + \left( \frac{x}{IC50} \right)^h} \)

**OUTPUTs**
- Generating a population
  - 1,000 HUMAN models
  - < 30 minutes

- HUMAN in silico Drug Trials
  - 300 models
  - (1 drug, 1 dose)
  - < 5 minutes

---

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**Prediction of Clinical TdP Risk**

**In silico Models:**
- 1,213 human AP models
- 62 compounds
- TdP risk

<table>
<thead>
<tr>
<th>Sensitivity: 87%</th>
<th>Specificity: 92%</th>
<th>Accuracy: 89%</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRUE + 32</td>
<td>TRUE - 23</td>
<td></td>
</tr>
<tr>
<td>FALSE + 2</td>
<td>FALSE - 5</td>
<td></td>
</tr>
</tbody>
</table>

**Animal Models:**
- rabbit Langendorff heart
  - 64 compounds
  - TdP risk
  - 75%
- in vivo dog
  - 19 compounds
  - QT prolongation
  - 85%

Population of Models

{Valentin et al. J Pharmacol Toxicol Methods 2009}
Recent development on Multi-Scale Modelling of Inherited Disease Conditions

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Towards Replacement of Animals

Repolarisation Abnormalities

<table>
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<th>TRUE + 32</th>
<th>TRUE - 23</th>
</tr>
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</table>

Sensitivity: 87%
Specificity: 92%
Accuracy: 89%

Human In Silico Drug Trials Demonstrate Higher Accuracy than Animal Models in Predicting Clinical Pro-Arrhythmic Cardiotoxicity

Elisa Passini1, Oliver J. Britton1, Hua Rong Lu2, Jutta Rohrbacher3, Ann N. Hermans4, David J. Galleher5, Robert J. H. Greig6, Alfonsa Bueno-Grovio6 and Blanca Rodriguez7

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http://www.cs.ox.ac.uk/insilicocardiotox

NC3Rs Infrastructure for Impact Award (2016-2021)

Academia
Industry
Clinical
Regulators
Recent Development of NEW Cellular Models

- **NEW Human Ventricular model**
  - Tomek et al. Under Review

- **NEW Human Cardiac Purkinje model**
  - Trovato et al. Under Review

Both models have been developed through an extensive CALIBRATION and VALIDATION based on HUMAN experimental data, both in control and under drug action.
Recent development on Multi-Scale Modelling of Inherited Disease Conditions

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MULTISCALE

From Ion Channels to Whole Heart...

Tissue/Organ

Single Cell

Ion Channel

Whole-Organ models

Action Potential models

Ionic current models

Image credits: Doregan (cardiac MRI), Coppini et al. Circ Res. 2013, Carusi et al. AJP 2012

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From Ion Channels to Whole Heart...

**MULTISCALE**

- **Tissue/Organ**
- **Whole-Organs**

**Single Cell**

- **Action Potential**

**Ion Channel**

- **Ionic current**

*Image credits: Doregan (cardiac MRI), Coppini et al. Circ Res. 2013, Carusi et al. AJP 2012*
Beyond Single Cell Simulations

1 heart beat: 60 minutes 600 cores

Ana Mincholé, Ernesto Zacur, Hector Martinez, Francesc Levrero, Louie Cardone-Noott

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In Silico Drug Trials in Acute Ischemia

Control
Nilotinib

Towards Personalised Heart Models

Clinical Images from Patients

High Performance Computing Simulations

Different Anatomical models

Mincholé and Zacur et al. Front Physiol 2019
Simulations of the Purkinje-Ventricular Junction

Carpio et al. Front Physiol 2019
Towards Electro-Mechanical Models

- Ionic currents
- Potential
- Calcium in troponin
- ODEs for cell electrophysiology
- Calcium
- Strain
- PDEs for electrical diffusion
- PDEs for nonlinear solid mechanics
- Stretch and stretch rate
- ODEs for excitation-contraction coupling

Alya
HPCM

Barcelona Supercomputing Center
Centro Nacional de Supercomputación

Levrero et al. Under Review
Margara et al. In Preparation
CONCLUSIONS

♥ Computer models of cardiac electrophysiology
✓ human-based and multiscale
✓ population of models to take into account variability

♥ Human *in silico* drug trials
✓ early prediction of clinical risk of drug-induced arrhythmias
✓ potential to replace pre-clinical animal experiments

♥ 3D whole-heart simulations
✓ cardiac diseases (e.g. acute ischemia)
✓ contractility

Thank You!

Questions?
Applications of Artificial Intelligence in Drug Efficacy and Safety Testing

Mahnaz Maddah, PhD
Managing Member
Dana Solutions
Computer Science and Artificial Intelligence Lab

- Co-founded with Kevin Loewke in 2016
- Focused on AI in Life Sciences Applications
AI: “The science and engineering of creating intelligent machines that can achieve goals like humans do” (J. McCarthy, computer scientist, 1955).
Deep Learning is inspired by how the human brain learns. Deep neural networks find patterns and learn features during the training mode.

Images of female face

Images of male face

A model which has learned to classify face images to male/female
Example Image Classification, Input and Output

Class Probabilities

Male Face  (0.9)
Female Face (0.1)

Deep Learning (Inference)
Deep Learning has revolutionized computer vision

ImageNet Challenge
1000 classes
Over 1 million images for training

Top 5 Error (%)

- Large Error Reduction Due to Deep Learning
- AlexNet
- ResNet
- Human

*Data from Sze et al, Proceedings of the IEEE, 2017*
Applications of Artificial Intelligence in *In Vitro* Drug Efficacy and Safety Testing

1) Automated QC in high-throughput

2) Early in-vitro drug testing

3) In-vitro drug-induced structural toxicity testing

4) Single-cell phenotype discovery

5) All the above, not on images!
**Problem statement:** Can we build robust QC models for high-throughput imaging?

**Example 1:** Automated detection of out-of-focus images in high-throughput

**Example 2:** Flagging poor-quality iPSC colonies
Application #1 – Collecting Training Data

Out-of-focus Images

In-focus Images
Application #1 – Training

Train a Deep Neural Network

Out-of-focus Images
80% training 20% validation

In-focus Images
80% training 20% validation

Deep Neural Network Focus Classifier

choosing the network with best performance on validation

Achieved accuracy of 100% on training and 99.8% on validation
Application #1 – Inference

In-focus or Out-of-focus?

Deep Neural Network Focus Classifier

Out-of-focus (0.8)
In-focus (0.2)
Applications of Artificial Intelligence in In Vitro Drug Efficacy and Safety Testing

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Application 2: In-Vitro Drug Testing

*With Tenaya Therapeutics*

**Problem statement:** Using an iPSC model of dilated cardiomyopathy (DCM), can we find drug candidates that reverse the disease phenotype?
Application 2 (Training) – Building a Disease Classifier

Train a Deep Neural Network

Achieved model accuracy of **98%** on validation
Application 2 (Inference) – Compound Testing

- Identified compounds that correct disease phenotype back to healthy
- Results were validated with replicate experiments, functional contractility assays and animal models

Deep Neural Network
DCM Classifier

DCM cells treated with a library of 5120 compounds (1 replicate, single dose)
Applications of Artificial Intelligence in *In Vitro* Drug Efficacy and Safety Testing

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Application #3: In Vitro Structural Toxicity Testing (PhenoTox)

In collaboration with CDER, FDA

**Problem statement:** Using in-vitro cell models and imaging, can we capture and quantify drug-induced structural changes?

At which drug dose does structural change happen?
Application #3: Structural Toxicity Testing – Plate Setup

Vehicle | Dose 1 | Dose 2 | Dose 3 | Dose 4 | Dose 5 | Dose 6 | Dose 7
Application #3: PhenoTox Method of Training and Inference

Train a Deep Neural Network

Output for Dose 1

Accuracy & Z-Factor for Val Images
Application #3: Structural Toxicity Testing – Plate Setup
Application #3: PhenoTox Method of Training and Inference

Train a Deep Neural Network

Accuracy & Z-Factor for Val Images

Output for Dose 2

Vehicle

Dose 2

Training  Validation

Training  Validation

Accuracy of 90% and Z-Factor close to 1    Strong change

Accuracy of 60% or Z-Factor < -1    No change relative to control
Application #3: PhenoTox on iPSC-derived Hepatocytes

Imaging data from A. Ribeiro (CDER, FDA)

Cells were fixed and stained after 48 hours of drug treatment.

Aspirin
Vehicle (.01 to 30 µM)

Tamoxifen
Vehicle (.01 to 30 µM)
Application #3: PhenoTox Results for Tamoxifen and Aspirin

- **Aspirin**
  - No structural change
  - Graph showing Z-Factor vs. Dose (μM)
  - Data points for DAPI, Phalloidin, and Phase

- **Tamoxifen**
  - Graph showing Z-Factor vs. Dose (μM)
  - Data points for different concentrations
Application #3: PhenoTox is more sensitive than CYP384 for Tamoxifen-treated hepatocytes

At 5µM, changes are detected in structure by PhenoTox, where no change is detected in function by CYP384 assay.
Applications of Artificial Intelligence in *In Vitro* Drug Efficacy and Safety Testing

1) Automated QC in high-throughput

2) Early in-vitro drug testing

3) In-vitro drug-induced structural toxicity testing

4) Single-cell phenotype discovery

5) All the above, not on images!
Problem statement: Can we do “single-cell” level phenotypic discovery?

Only interested in screening single-nucleus/non-touching cells
Application #4 – Segmentation of DAPI Images

Create a training data by manually labelling cells of interest
Application #4 (Training) – Building an Image Object Segmentor

Train a U-Net Deep Neural Network

Neural Network Single-Cell Segmentor
Application #4 – Phenotype Discovery, Single-Cell Level

With a biotech startup

DAPI image → Neural Network Single-Cell Segmentor

Composite Single-Cell Sub-Images for Phenotype Discovery

9 months old
18 months old
24 months old

Keeping the Single-Cells (segmented in Red by the DNN)
Applications of Artificial Intelligence in *In Vitro* Drug Efficacy and Safety Testing

1) Automated QC in high-throughput

2) Early in-vitro drug testing

3) In-vitro drug-induced structural toxicity testing

4) Single-cell phenotype discovery

5) All the above, not on images!
Application #5: Drug efficacy and safety testing with MEA/Patch-Clamp/Impedance Data

**Problem statement:** Can we apply similar techniques to signals?

**Example for Quality Control**

Poor recording  
Good recording

Patch clamp (1-dimensional signal)

**Example for Automated Segmentation**

MEA Signal Annotation – Detection of Spikes and T-Waves

Signals plotted for visualization only; models trained and deployed on raw signals
Limitations/Cautions

• Black box

• Amount of training data

• Garbage in, garbage out

• Collaboration is the key to success
Dana Solutions AI Platforms

AutoQC:  https://aiforqc.com

PhenoLearn: https://www.phenolearn.com/

PhenoTox: https://danasolutionsllc.com/phenotox/

Pulse:  https://www.pulsevideoanalysis.com

DeepMEA: coming soon

Thank You

Questions?
Nonclinical safety assessment of T cell targeting bispecifics aka CD3 Bispecific Antibodies
Acknowledgements

- Many thanks to all the researchers that supported the projects/data represented in the slides
- Special thanks to Cris Kamperschroer
Outline

• Background and brief history of T cell targeting bispecifics
• Key safety concerns and nonclinical strategy to support IND
• Project case study
• Emerging risk management strategies
“Redirecting” T cells against tumor cells

‘Normal’ recognition

- Bypasses usual mode of tumor cell recognition
- Allows any T-cell to recognize and kill tumors

T cell bispecific

- Bypasses usual mode of tumor cell recognition
- Allows any T-cell to recognize and kill tumors
Brief history of T cell bispecific therapeutics

- 1970
  - Identified a role for the immune system in spontaneous regression in melanoma

- 1980
  - First study with IL-2
  - IFN-α approved

- 1983
  - First treatment with tumor specific mAb

- 1986
  - Characterization of human tumor associated antigens

- 1991
  - BLINCYTO approved

- 1998
  - IL-2 approved

- 1999
  - First T-cell–engaging bsAb approved in Europe

- 2002
  - BiTE® constructs are being investigated in multiple cancer indications

- 2005
  - First BiTE® is synthesized

- 2015
  - Imlygic® and combination of Opdivo® and Yervoy® approved

Adapted from Yuraszeck et al. 2017 Clin Pharmacol Ther
A renewed interest in T cell bispecifics

Clinical success with blinatumomab (Blincyto)


Activity in solid tumors (CRC)

Tabernero et al. J Clin Oncol 35 (15_suppl) 3002

Phase I studies of the novel carcinoembryonic antigen CD3 T-cell bispecific (CEA-TCB) antibody as a single agent and in combination with atezolizumab: preliminary efficacy and safety in patients with metastatic colorectal cancer (mCRC)


Many T cell bispecifics in clinical development

Adapted from Yuraszeck 2017 Clin Pharmacol Ther. doi: 10.1002/cpt.651
Key toxicities with CD3 bispecifics

1) Cytokine release

2) Damage to normal cells that express target

3) Neurotoxicity

Toxicities appear to be related to pharmacologic activation of T cells
CRS as a safety issue with CD3 bispecifics

Common CRS-related Toxicities

<table>
<thead>
<tr>
<th>Organ System</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constitutional</td>
<td>Fever +/- rigors, malaise, fatigue, anorexia, myalgias, arthralgias, nausea, vomiting, headache</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>Tachycardia, hypotension, widened pulse pressure, increased (early) or decreased (late) cardiac output</td>
</tr>
<tr>
<td>Respiratory</td>
<td>Dyspnea or tachypnea, hypoxemia</td>
</tr>
<tr>
<td>Coagulation</td>
<td>Elevated D-dimer, hypofibrinogenemia, bleeding</td>
</tr>
</tbody>
</table>

Adapted from Lee 2014 Blood 124:188.
Generic nonclinical safety strategy

In Vitro Studies
- Characterization of potential for effector function
  - Complement binding
  - FcγR binding and ADCC assay
- Tissue cross-reactivity assay and *understanding of target expression*
- Cytokine release assay (slide 11)

In Vivo Studies
- Cynomolgus monkey is often most relevant species
- Exploratory/Dose Range Finding repeat-dose (slide 12)
- 1-Month GLP Repeat Dose Studies
In vitro cytokine release assays

1. Human whole blood ‘soluble’ CRA
   Cytokine (IFN-γ, TNF-α, and IL-6) at 24 hr
   Human blood

2. ‘Solid phase’ CRA with human PBMC and test article coated onto plate
   Cytokine at 24 hr
   hPBMC

3. CRA with PBMC and soluble test article with target cells spiked in
   Target(+) cells
   Cytokine at 24 hr
   hPBMC

<table>
<thead>
<tr>
<th>Format</th>
<th>Cytokine release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble phase</td>
<td>-</td>
</tr>
<tr>
<td>Solid phase</td>
<td>↑↑</td>
</tr>
<tr>
<td>With TA+ cells</td>
<td>↑↑↑↑↑↑↑</td>
</tr>
</tbody>
</table>

→ Value of CRA??
Addressing risk of normal tissue targeting

- Major challenge for solid tumor indications
- Pick ‘clean’ targets – Good luck!
- Make molecules more tumor selective – affinity/avidity of binders, masking, etc
- Expression data – IHC (ISH, other, for other targets)
  - IHC useful, but may not be sensitive enough (eg low expression)
- Mouse target validation study? – Not typically used due to model limitations
- Early in vitro screening

- In vivo monkey studies
  - Key decision-maker

**In vitro killing of tumor vs normal cells (P-cadherin LP DART)**

<table>
<thead>
<tr>
<th></th>
<th>Target levels (MFI)</th>
<th>CTL activity EC50 (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor cells</td>
<td>163</td>
<td>2.5</td>
</tr>
<tr>
<td>Normal cells</td>
<td>62</td>
<td>118</td>
</tr>
<tr>
<td><strong>Fold difference</strong></td>
<td>2.6</td>
<td>47</td>
</tr>
</tbody>
</table>

More potent killing of tumor cells than normal cells
Notes on design of early in vivo toxicity studies

- No ‘standard’ design, stress flexibility

  Design often used (for long half-life)

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 8</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dose $x_1$</td>
<td>$3x_1-5x_1$?</td>
<td>necropsy</td>
</tr>
<tr>
<td>2</td>
<td>Dose $x_2$</td>
<td>$3x_2-5x_2$?</td>
<td>necropsy</td>
</tr>
</tbody>
</table>

- Staggered starts for each group (n=2/group)
- Decide on dose $x_2$ based on tolerability of dose $x_1$
- Primary goals: push doses to characterize potential organ tox, ID GLP tox doses

- Not necessarily doing dose escalation in GLP toxicity studies
Case Study: CD3 Bispecific with extended half-life

P-cadherin
- Mediates Ca++-dependent cell-cell adhesion
- Promotes tumor invasion, metastasis, and stem cell-like properties
- Overexpressed in multiple tumor types

P-cadherin
- Mediates Ca++-dependent cell-cell adhesion
- Promotes tumor invasion, metastasis, and stem cell-like properties
- Overexpressed in multiple tumor types

Adapted from Root, et al. 2016 Antibodies

bispecific heterodimeric diabody Fc-fusion

Albergaria, et al. 2011 Int J Dev Biol
P-cadherin In vivo nonclinical safety studies

- Studies with candidate P-cadherin CD3 bispecific in monkey, the only cross-reactive species
- Evidence of species relevance (prior to in vivo study)
  - Binding to both cyno CD3 and cyno P-cadherin
  - CTL activity with cyno T cells and on cyno P-cad+ cells
- Exploratory toxicity studies in monkey
- Multiple dose FIH-enabling GLP toxicity study in monkey
  - Cytokine release, expected toxicity related to target expression
- Key goal: to characterize potential toxicities (MABEL starting dose)
Target-dependent cytokine release in monkey

Negative control confirms target dependence, and suggests cytokine release is related to intended pharmacology
T cell effects in monkey confirm pharmacology

- **CD8 T cells**
  - Study Day: 1x 3x 3x 3x 1x 3x 3x 3x
  - Ratio to baseline

- **% CD25+ CD8 T cells**
  - Study Day: 1x 3x 3x 3x 1x 3x 3x 3x
  - Ratio to baseline

- **% Ki-67+ CD8 T cells**
  - Study Day: 1x 3x 3x 3x 1x 3x 3x 3x
  - Ratio to baseline

- **Soluble CD25**
  - Study Day: 1x 3x 3x 3x 1x 3x 3x 3x
  - Ratio to baseline
P-cadherin: Clinical starting dose

MABEL approach, based on EC20 of most sensitive measure of pharmacology

<table>
<thead>
<tr>
<th>Approach</th>
<th>Assay</th>
<th>Readout</th>
<th>MABEL</th>
</tr>
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<tbody>
<tr>
<td>PK-driven</td>
<td>In vitro CRA</td>
<td>Cytokine release</td>
<td>Cmax &lt; EC20</td>
</tr>
<tr>
<td>PK-driven</td>
<td>In vitro CTL assay</td>
<td>Cytotoxicity</td>
<td>Cave &lt; EC20</td>
</tr>
<tr>
<td>Receptor occupancy</td>
<td>In vitro binding</td>
<td>RO</td>
<td>Cmax &lt; EC10</td>
</tr>
<tr>
<td>PK/PD-driven</td>
<td>In vitro kinetic CTL assay</td>
<td>Cytotoxicity</td>
<td>Max tumor synapse conc &lt; EC20</td>
</tr>
</tbody>
</table>

Adapted from Chen et al, 2016. Clinical Pharmacology and Therapeutics.

- Recent paper: Saber et al, Regul Toxicol Pharmacol. 2017;90:144-152
  - Conclude HNSTD/NOAEL, ≥10% RO approaches unacceptable
  - Suggest MABEL approach: 10-30% “pharmacologic activity”

- MABEL approach + high potency = very low doses
Cytokine risk mitigation strategies

- Early clinical data point to CRS as DLT
- Can cytokine mitigation strategies decouple desired T cell effects and unwanted cytokine release

1) Molecular engineering
2) Dosing strategies
3) Blocking cytokine release or cytokine signaling
Engineering T cell activity with less toxicity

1. Altered CD3 affinity, epitope, and/or formats

Hernandez-Hoyos G et al. 2016 Mol Cancer Ther 15:2155

2. Masking

Beyond affinity?

Ellerman D Methods 2019 154:102-117

CD123

V1 (34nM)

V2 (323nM)

V3 (223nM)

Cleavable linkers

Masking peptides
‘Primming’ dose to mitigate cytokine release

Nonclinical monkey studies with P-cadherin LP DART

- $\frac{1}{3}$ x dose 1 wk prior
- 1x dose
- 1x dose (no ‘prime’)

Increasing serum cytokine

IL-6

Hrs post dose

Primming dose used in the clinic with other molecules:

- EpCAM x CD3 BITE (Fiedler MWW et al. *Clin Oncol* 30, 2012 (suppl; abstr 2504))
- CD123 x CD3 (Jacobs KG et al, ASH 2017)
- gp100 TCR x CD3 (Middleton MR et al. ASCO 2017)

Similar results for IFN-γ and IL-2

A lower ‘primming’ dose reduces cytokine release
SC dosing to mitigate cytokine release

Nonclinical monkey studies with P-cadherin LP DART

**Cytokine: IL-6**

- Serum conc (pg/mL) vs Hrs post
- Day 1 dose vs Day 10 dose
- IV (10→20 ug/kg) vs SC (10→30 ug/kg)

**T cell proliferation**

- % CD8+Ki-67+
- Study Day vs Day 1 dose vs Day 10 dose
- IV (10→20 ug/kg) vs SC (10→30 ug/kg)

SC dosing: similar T cell responses but less cytokine release

Target tissue tox similar with SC and IV
Pharmacological Blocking cytokines or cytokine responses

Key Question
If we block cytokines or cytokine responses to prevent CRS, will we impact the intended T cell activity against target-expressing cells?
Blinatumomab: mitigating CRS

Steps to mitigate CRS
1. Continuous infusion
2. “Step” dosing
3. Corticosteroid pretreatment – at infusion start and if dose increases

Approved clinical dosing scheme

BSA-based dosing for patients weighing < 45 kg:

<table>
<thead>
<tr>
<th>CYCLE 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting dose</td>
</tr>
<tr>
<td>5 mcg/m²/day</td>
</tr>
<tr>
<td>Full dose</td>
</tr>
<tr>
<td>15 mcg/m²/day</td>
</tr>
</tbody>
</table>

20 mg Dex 1 hr predose

14-day interval

<table>
<thead>
<tr>
<th>CYCLES 2–9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subsequent doses</td>
</tr>
<tr>
<td>START AT</td>
</tr>
<tr>
<td>(Not to exceed 28 mcg/day)</td>
</tr>
</tbody>
</table>

Fixed dosing for patients weighing ≥ 45 kg:

<table>
<thead>
<tr>
<th>CYCLE 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting dose</td>
</tr>
<tr>
<td>9 mcg/day</td>
</tr>
<tr>
<td>Full dose</td>
</tr>
<tr>
<td>28 mcg/day</td>
</tr>
</tbody>
</table>

20 mg Dex 1 hr predose

14-day interval

<table>
<thead>
<tr>
<th>CYCLES 2–9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subsequent doses</td>
</tr>
<tr>
<td>START AT</td>
</tr>
</tbody>
</table>

14-day* interval
Testing cytokine blockade in monkey

- Investigative study done in cyno to test effects of cytokine blockade
- Used CD3 bispecific against solid tumor target “Z” (in several defined tissues)
  - Induces CRS
  - Induces killing of Z+ cells in tissues (intended pharmacology; ‘efficacy’ surrogate)

**Z+ cell**
- Solid tumor target
- Constitutive expression on epithelial and serosal cells of certain tissues
- Monkey cross-reactive

**Diabody**
- Half-life extension
- Effector function null

**CD3**
Blocking cytokines in monkey: study design

Key Questions
1) Do we block CRS? – Clin signs, C-reactive protein (CRP; downstream of cytokines)
2) Do we retain T cell activity? – T cell activation, killing of Z+ cells (histopath)
### CRS-associated clinical signs on Day 1

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>CD3 x Z alone</th>
<th>IL-6 mAb</th>
<th>JAKi</th>
<th>Dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emesis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decreased Activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hunched Posture</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Clinical signs observed
- No clinical signs

**CRS-associated clinical signs**

- Prevented by JAK inhibitor and dexamethasone
- Not prevented by anti-IL-6 mAb
Serum cytokines

- **CD3 bispecific** induced cytokines

- When compared to CD3 x Z alone...
  - **anti-IL-6 mAb** – cytokine response similar
  - **JAK inhibitor** – Inhibited cytokines
  - **Dexamethasone** – inhibited cytokines
**T cell activation**

### Blood % CD69+ CD8 T cells

- **Vehicle**
- **CD3 x Z alone**
- **+ anti-IL-6**
- **+ JAKi**
- **+ Dex**

### Spleen % CD69+ CD8 T cells

**Percent positive**

<table>
<thead>
<tr>
<th>Cytokine Blocker</th>
<th>Effect on T cell Activation (CD69)</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-IL-6</td>
<td>Little/none</td>
</tr>
<tr>
<td>Dex</td>
<td>Little/none</td>
</tr>
<tr>
<td>JAKi</td>
<td>↓</td>
</tr>
</tbody>
</table>

**Similar effects with CD4 T cells**
## Effects on target-expressing cells in tissues

<table>
<thead>
<tr>
<th>Group</th>
<th>Histo severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>N/A</td>
</tr>
<tr>
<td>CD3 x Z alone</td>
<td>Inflammation &amp;/or epithelial cell damage in serosa/mucosa</td>
</tr>
<tr>
<td>anti-IL-6</td>
<td>No consistent difference (from CD3 x Z alone)</td>
</tr>
<tr>
<td>JAKi</td>
<td>Slightly less severe in 2 tissues, same in others</td>
</tr>
<tr>
<td>Dex</td>
<td>Slightly less severe in 2 tissues, same in others</td>
</tr>
</tbody>
</table>
## Blocking cytokines in cyno: summary

<table>
<thead>
<tr>
<th>CRP</th>
<th>CYTOKINES</th>
<th>CLIN SIGNS</th>
<th>T CELL ACTIVATION</th>
<th>CELL KILLING</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CRP</td>
<td>IL-6</td>
<td>IFN-γ</td>
<td>sCD25</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3 x Z alone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-IL-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JAKi</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Undesired CRS-associated effects**
- **Desired T cell activities**

Dexamethasone appears to be the best option

Cyno data suggest we can block cytokines prior to dosing and retain T cell activity and killing of target cells

→ Expect to retain tumor cell killing
Future directions for T cell bispecifics

What will the future bring for T cell bispecific therapies?

• New molecular constructs to increase tumor selectivity and/or reduce tox
  o Multivalent constructs
  o Altered CD3 vs target affinities
  o Other?

• Improved strategies for identifying and managing toxicities
  o More accurate/sensitive expression data
  o Optimized dosing schemes
  o Blocking cytokines

• Combination therapies – checkpoint inhibitors, costimulatory agonists, small molecule immune stimulators, etc
Toxicity limits T cell engager therapy

- Success likely requires decreasing toxicity (esp. solid tumors)
- How to decrease toxicity and widen TI?

How?

- Cleaner targets
- Increase tumor selectivity (affinity tuning, masking, etc)
- Clinical management of tox

Safe but no clinical benefit!

Dose

- Higher safe starting dose
- Rapid safe dose escalation
Redirected tumor cell killing

P-cadherin expression

CTL activity

Killing correlates with P-cad expression

- In Vivo activity in PDX models
- Mice reconstituted with human T cells
Serum CRP

- **CD3 bispecific** induced CRP
- When compared to CD3 x Z alone...
  - **anti-IL-6 mAb** – blocked CRP (suggesting pharmacology)
  - **JAK inhibitor** – Inhibited CRP
  - **Dexamethasone** – inhibited CRP
Efficacy

Cytokines

Killing

CRS

Dexamethasone

JAKi

??

Efficacy

Cyno data suggest we can block cytokines prior to dosing and retain T cell activity and killing of target cells → Expect to retain tumor cell killing
Potential Attributes of a Platform Technology: How Best to Capitalize on Cumulative MOE Oligonucleotide Safety Data

Scott P. Henry, VP, Preclinical Development, Isis Pharmaceuticals, Inc.
Advantages and Challenges of Working with ASO Platform Technology

• Platform technologies offer the promise of increased efficiency throughout the development process

• Safety profiles within a Chemical class are similar
  • But the magnitude of effects does vary widely with sequence
  • Differences between chemical classes can be profound

• Challenge is that ON drugs are becoming quite diverse
  • Chemistry, structure, mechanism, formulations, conjugates

• Yet, generalizations within classes can be made if disciplined and sufficient experience exists
  • Improves quality and efficiency of nonclinical development
  • Allows more confident interpretation of data from small number of animals and humans
  • Greatly improves the confidence in translational safety assessment
# Distinct Chemical Classes of Oligonucleotide Therapeutics

<table>
<thead>
<tr>
<th>Antisense</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single-Strand</td>
<td>Phosphorothioate</td>
</tr>
<tr>
<td>RNase H Mechanism</td>
<td>2’-MOE, 2’-Me, cEt, LNA</td>
</tr>
<tr>
<td>Saline vehicle</td>
<td></td>
</tr>
</tbody>
</table>

## siRNA

<table>
<thead>
<tr>
<th>RNA</th>
<th>Structured RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double-strand</td>
<td>Cas expression vector or mRNA</td>
</tr>
<tr>
<td>RISC Mechanism</td>
<td></td>
</tr>
<tr>
<td>Delivery vehicle for</td>
<td></td>
</tr>
<tr>
<td>systemic use</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CRISP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Guide RNA and Cas9 enzyme</td>
<td></td>
</tr>
</tbody>
</table>

**Antisense Strand**

**Sense Strand**
Distinct Oligonucleotide-Based Therapeutic Mechanisms of Action
<table>
<thead>
<tr>
<th></th>
<th>2’-F RNA</th>
<th>2’-OMe RNA</th>
<th>2’-MOE RNA</th>
<th>cEt BNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affinity</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Stability</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++++</td>
</tr>
<tr>
<td>Hydrophobicity</td>
<td>++</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Hydrophilicity</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td></td>
</tr>
</tbody>
</table>
2’-O-Methoxyethyl Chimeric Antisense Drugs RNase H Mechanism

**Chimeric RNase H Oligo Design**

↑ affinity  
↑ stability  
↑ tolerability  

**RNase H Substrate**

↑ affinity  
↑ stability  
↑ tolerability

**MOE**  
**DNA**  
**MOE**

**2’-O-methoxyethyl (MOE)**

- Compared to first generation P=S ODNs, Chimeric MOE ASOs:
  - Increase potency
  - Increase duration of action
  - Decrease unwanted side effects

**Clinical Experience**

- > 5000 subjects dosed
- > 100 clinical studies
- Multiple therapeutic indications
- > 200 patients dosed for > 1 year
- > 100 patients dosed for > 2 years
Non-Clinical Safety Assessment of 2’-MOE ASOs Closely Follows CDER Guideline

• IND Toxicology Studies for more than 30 ASOs
  • Toxicity testing in 2 species: mice and monkeys
  • Simple formulation: PBS or water for injection
  • Full dose response in male and female animals
    • High dose in subchronic studies is typically 100 mg/kg/wk in mice and 30 to 40 mg/kg/wk in monkeys
  • Weekly dosing by IV infusion or SC injection
  • Duration of 6 and 9 months in mice and monkeys
  • Carcinogenicity study with 1st and 2nd generation drugs
  • Reprotox and Gentox batteries negative
  • Both human and species-specific oligos used in Tox Studies when scientifically justified
Non-Clinical Development Experience is Very Broad

- Experience covers many therapeutic areas
  - Oncology, inflammation, diabetes, cardiovascular disease, anti-viral
- Non-Clinical data submitted to multiple countries
  - US, Europe, Canada, Russia, South America, Australia
- Tolerability and exposure documented for multiple routes of exposure
  - IV/SC – Up to 6 months in mice and 1 yr in monkeys
  - Oral – 6-week studies in mice and dogs
  - Inhalation – 13-week studies in mice and monkey
  - Ocular – 26-week studies in rabbit and monkey
  - CNS (ICV and IT) – 13-week studies in rat and monkey
  - Intradermal – 4 week studies in rats, rabbits and monkey
Safety Evaluation Package Must Address Potential for Hybridization Dependent and Independent Effects

• Hybridization - dependent
  • Exaggerated Pharmacology-
    • Requires cross-species homology or use of animal surrogate
    • Utilize information from knock-out mice if available
  • Hybridization to unintended targets- low probability
    • Addressed using bioinformatic and in vitro-expression profiling approach

• Hybridization - independent
  • Sequence-independent or class-related tox account for the majority of tox observed
  • Sequence-dependent – Examples of Toxicity arising from specific motifs that define a receptor interaction
    • e.g. TLR-9 interaction with CpG motifs
    • Avoided in development compound by diligent screening
Nature of Various Class-Related Toxicities

- **Effects Related to Oligo-Protein Interactions**
  - Interaction with plasma proteins
    - Increase in APTT: Inhibition of Intrinsic Tenase complex
    - Activation of Complement: Interference with Factor H function
  - **Proinflammatory effects**
    - Interaction with receptors of innate immune system
    - Produces Inflammatory cell infiltrate and splenomegaly
    - Typically occurs to greater degree in mice than monkey

- **Effects Related to Oligonucleotide Accumulation**
  - Uptake is greatest in Kupffer cells and renal proximal tubular epithelium
    - Results in basophilic granules – not toxicologically significant
    - Cytoplasmic vacuoles related to lysosomal compartmentalization
    - Renal tubular epithelial cell degeneration at high doses
Basis for Class Related Toxicities

• **Common Biophysical Properties**
  • All ASO are water soluble polyanions with similar charge-to-mass ratio

• **Common PK/ADME Properties**
  • Plasma kinetics, tissue distribution, metabolism and clearance are largely independent of sequence

• **Common Toxicologic Properties**
  • Class effects qualitatively similar in general tox studies
  • Uniformly negative in GeneTox studies

• **But, There are Exceptions to Every Rule**
  • Proinflammatory effects are influenced by sequence
  • Small fraction of PS oligos tend to be hepatotoxic
  • Sequence that are proinflammatory in monkey have been observed
Implication of Platform Technology on Safety Assessment

• Best development compounds are the product of thorough screening and selection process

• Certainly are efficiencies in many areas:
  • Manufacturing process and CMC analysis
  • Pharmacokinetic characterization and bioanalytical methods
  • Efficient design of toxicology studies and end points
  • Absence of genetic toxicity

• Every compound still needs thorough safety evaluation
  • Monkey still clearly the most relevant species
  • Rodent tolerability has limited direct relevance to human safety, but serves as a good filter for outliers

• One should not extrapolate class effects between chemistries
Summary
Toxicology Properties of 2’-MOE ASOs

• Class- and chemistry-related effects predominate:
  • Tox profile usually predictable from sequence to sequence
  • Similarity is a function of consistent PK and protein binding properties
  • Effects occur at high doses relative to clinical efficacy

• Target organs for toxicity have been identified
  • Effects related to oligonucleotide accumulation or interaction with plasma/cellular proteins
    • Animal models are predictable of exposure/effect relationship

• Numerous potential toxicities not observed

• Similarities among compounds combined with clinical experience with other ASOs provides a basis for relating Tox and Clinical data
  • Over ~10,000 patients dosed and cataloged in database
Class-Related Effects for 5-10-5 MOE ASO at Toxicologically Relevant Doses

- **Rodents (Mice and Rats)**
  - Pro-inflammatory effects
    - Lymphohistiocytic infiltrates in various tissues at $\geq 10$ to 20 mg/kg
      - Lymphoid hyperplasia
    - Slight increase in AST and ALT at $\geq 25$ mg/kg
  - Endosomes accumulate oligo in basophilic granules in Kupffer and proximal convoluted tubules at $\geq 10$ mg/kg
  - Rats are more sensitive to renal effects, especially male rats
    - Increased proteinuria at $\geq 10$ mg/kg

- **Monkey**
  - Proinflammatory effects not prominent
    - Minimal SC injection site reaction
  - Complement activation at doses $\geq 10$ mg/kg
  - Transient ↑ in APTT at high doses during the first 4hr
  - Minimal proximal tubular epithelial cell degeneration at $\geq 10$ mg/kg/wk
Attributes to 2’-MOE ASO Platform Technology in Safety Assessment

• Many tactical benefits that make our job easier
  – Standardized manufacturing and CMC
  – Standardized bioanalytical methods
  – Consistent and predictable PK/ADME properties

– First toxicology studies are often 13-week studies
  • Enables dose selection and collection of key data points
  • Generally predictable target organ effect and NOAEL
  • Negative Safety Pharm, GeneTox and Reprotox
  • Supports clinical testing through Phase 1 and early Phase 2 trials
Where are the Potential Regulatory Efficiencies in Safety Assessment of MOE ASO?

- With traditional small molecule drugs:
  - Every compound is different and unpredictable
  - Biggest challenge is the human predictivity of data generated from small number of animals

- With MOE ASO:
  - We do not start development in a vacuum of knowledge on safety or PK
  - Within a structural class, one can generalize and build on the ‘translational’ relationship between animals and human subjects

- If done well, the cumulative experience can be used to improve safe clinical development of MOE ASO therapies
Examples where subtle differences within a class can alter properties
MOE Modified ASO

- Avoid CpG motifs
- Include 5-me-C and 2’-MOE modifications
- Produce little to no IL-6 and IL-12
- Mild splenomegaly at higher doses
- Produce 10-fold less chemokine
- Multi-organ cell infiltrate still present
Sequence Can Greatly Affect Magnitude of Effect

• Sequence dependence of inflammatory effects in mice are well known

• ALT can show similar spectrum of sequence-dependent differences in magnitude
  • Some ALT effects related to inflammation
  • Other mechanisms being investigated
Selection Strategy for 2’-MOE ASO Development Compound (MOE 20-mer)

- **Potency dose-response in mice**
  - Optimize activity in normal or transgenic mouse

- **6 week CD-1 Mice Tolerability at 100 mg/kg**
  - 17 ASOs lost to high LFTs, Histology, Hematology & Organ weights

- **8 week SD Rat-Renal Tolerability 80 mg/kg**
  - 14 ASOs lost to high LFTs, high spleen weight

- **Evaluated in 12 week Cyno PK/PD Study**
  - Human PBMC

~100 ASOs

---

IONIS
ASO Producing Poor Tolerability are Eliminated in Screening Studies

- CD-1 Mice treated with 100 mg/kg/wk for 4 weeks
- 404161 Eliminated for elevation in ALT
  - 2- to 4-fold increase in ALT expected at this dose level
  - 409998 and 409975 have border line acceptable ALT/AST levels

<table>
<thead>
<tr>
<th>ISIS</th>
<th>Chemistry</th>
<th>Walk Position</th>
<th>ALT</th>
<th>AST</th>
</tr>
</thead>
<tbody>
<tr>
<td>409988</td>
<td>2-13-5</td>
<td>-5</td>
<td>5.1</td>
<td>2.1</td>
</tr>
<tr>
<td>409821</td>
<td>5-10-5</td>
<td>-1</td>
<td>2.7</td>
<td>1.5</td>
</tr>
<tr>
<td>404176</td>
<td>5-10-5</td>
<td>0</td>
<td>2.5</td>
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<td>2.6</td>
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<td>1.3</td>
<td>0.8</td>
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<td>5-10-5</td>
<td>0</td>
<td>1.2</td>
<td>0.9</td>
</tr>
</tbody>
</table>
Attributes of 2’-MOE ASO Platform Technology in Safety Assessment

- Data on Class Toxicity profiles enhances our overall Safety/Risk assessment
- Any new MOE ASO can be compared/benchmarked relative to broader experience
  - Multiple disease states, compounds, doses and greater patient number
- Phase 1 have the benefit of dozens or hundreds of subjects treated with similar compounds
  - Can compare nonclinical behavior in rodent and monkey
Preclinical Monkey Toxicology Database Established for ISIS MOE ASOs

- GLP Toxicology Studies for Development ASOs
  - Dose response 1 to 30/40 mg/kg
  - Discovery Pharmacology Screening Toleration Studies (non-GLP)
    - 13-week high dose 30/40 mg/kg
  - Investigative Toxicology Studies (non-GLP)
- Preclinical and clinical data in similar format to support integrated queries

### Composition of Monkey DB

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Studies</td>
<td>35 (16 GLP)</td>
</tr>
<tr>
<td>Compounds (multiple chemistry)</td>
<td>&gt;100 (85, 2′-MOE 20mers)</td>
</tr>
<tr>
<td>Animals (multiple sources)</td>
<td>Placebo (~400); ASO (~1500)</td>
</tr>
<tr>
<td>CROs</td>
<td>4</td>
</tr>
<tr>
<td>Endpoints (Clin path, BW, Tissue Wt,</td>
<td></td>
</tr>
<tr>
<td>Cytokines, Complement, TK, PD)</td>
<td>&gt;150</td>
</tr>
</tbody>
</table>
Clinical Safety Database Established for Isis MOE ASO

- Clinical studies conducted or initiated for 8 PS ODN oligonucleotides and 28 MOE ASOs
- 16 treatment populations; 100 studies
- IV/SC doses up to 1200 mg / week (17 mg/kg/wk)
- > 650 patients treated ≥ 12 weeks, > 330 treated ≥ 6 months, > 140 treated ≥ 1 year

<table>
<thead>
<tr>
<th>Number of Subjects Treated (est.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; Gen</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>IV &amp; SC</td>
</tr>
<tr>
<td>Local / Oral</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

- ISIS MOE ASOs
  - 16 treatment populations; 100 studies
  - IV/SC doses up to 1200 mg / week (17 mg/kg/wk)
  - > 650 patients treated ≥ 12 weeks, > 330 treated ≥ 6 months, > 140 treated ≥ 1 year
Utilize Database to Understand Translational Effects of Standard Target Organ Effects - Kidney

- No trend for a treatment affect on Renal Function in Monkey or Humans

<table>
<thead>
<tr>
<th>Human Parameter</th>
<th>Placebo (N= )</th>
<th>ASO Treated (N= )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine &gt; 30% Increase vs. Baseline</td>
<td>6.2% (534)</td>
<td>6.6% (1775)</td>
</tr>
<tr>
<td>BUN &gt; 2X Baseline</td>
<td>1.7% (472)</td>
<td>1.6% (1953)</td>
</tr>
<tr>
<td>GFR &gt; 30% decrease vs. Baseline</td>
<td>3.3% (509)</td>
<td>3.7% (1797)</td>
</tr>
<tr>
<td>Urine Protein (qualitative)</td>
<td>In Progress</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Monkey Parameter</th>
<th>Placebo (N=306)</th>
<th>ASO Treated (N=1142)</th>
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</thead>
<tbody>
<tr>
<td>Creatinine &gt; 30% decrease vs. Baseline</td>
<td>7.8%</td>
<td>10.2%</td>
</tr>
<tr>
<td>BUN &gt; 30% Increase vs. Baseline</td>
<td>17.3%</td>
<td>20.0%</td>
</tr>
<tr>
<td>Urine P/C Ratio &gt; 50% Increase vs. Baseline</td>
<td>9.2%</td>
<td>10.6%</td>
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</tbody>
</table>

- Confirms adequate safety margins for common class effects
Utilize Database to Understand Species Specific Platform Effects – Complement Activation in Monkey

• Monkey: Complement activation is a common PS ON effect at high doses
• Humans: No trend for a treatment affect on C3a, C5a

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>C3a &gt; 2-fold Increase vs. Baseline</td>
<td>9.6%</td>
<td>52</td>
<td>12.1%</td>
<td>149</td>
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<tr>
<td>C5a &gt; 2-fold Increase vs. Baseline</td>
<td>9.7%</td>
<td>257</td>
<td>9.8%</td>
<td>775</td>
</tr>
</tbody>
</table>

[1]:# Subjects (%) that meet criteria; [2]:Total # Subjects where data is available

• Helps support interpretation of species specific toxicities
Low Incidence of Thrombocytopenia in Monkey With MOE ASOs is Not Observed in Humans

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>PLT &lt; 75K/mm³</td>
<td>Monkey</td>
<td>0.0%</td>
<td>350</td>
<td>2.9%</td>
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<tr>
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<td>Human</td>
<td>0.4%</td>
<td>560</td>
<td>0.5%</td>
<td>2032</td>
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</table>

[1]: % Subjects that meet criteria; [2]: Total # Subjects where data is available

- Low incidence of severe PLT reduction in monkey
- Occur within 6-8 weeks of treatment
- Present spuriously anytime
- Low incidence (1-2 animals/study)
- No clear dose-dependent effect
- Not reproducible from study to study for any given ASO
- Nature of response suggestive of individual animal susceptibility

• Useful in understanding low incidence events and translating findings to clinic
Toxicities Not Observed for Systemically Administered MOE ASOs in Animals

Numerous target organ systems not affected

- Bone marrow
- Skeletal muscle
- CNS
- Mitochondrial
- Cardiac
- Gastrointestinal
- Respiratory
- Ocular

Database can be queried to confirm the absence of target organ effects
Despite the Consistency – Exceptions Will Occur

• Exceptions could be related to pharmacologic effect or patient population
  • Increased ALT present for Kynamro
    • Secondary to lowering apoB
• Exceptions need to be interpreted in the context of their screening process and specific chemical class
  • Renal effect of PCSK9 - LNA ON gapmer
  • DMD splicing ASO – Full 2’-Me
Conclusions on Consistency and Translatability of MOE ASO Safety Profile

• Generalization of ASO Class Effects can be made, but best done on defined set of criteria
  • Consistent chemical class
  • Defined set of performance criteria
  • Exceptions to the rule will be found
• Optimized MOE ASO drugs have consistent behavior
  • Need to stay vigilant for exceptions
• Overall Safety Assessment is facilitated by consistency
  • Focuses attention on key class effects
  • Greater confidence in safety margins for class effects
  • Can be used to confirm presence or absence of certain species specific effects
  • Facilitates the identification of compounds with suboptimal tolerability
Functional Characterization of Neural Network Activity in Human iPSC-Derived Neuron/Glia Co-Cultures for *In Vitro* Neurotoxicity Assessment

SoCal Regional SOT Conference

November 7th, 2019
J.L. Jonathan Davila, Ph.D.
Human iPSC-Derived Neural In Vitro Approaches

- Direct neuronal reprogramming of iPSCs by expression of transcription factors (TF) ⇒ induced neurons (iNs)
- Separate and robust generation of defined neural cell types

- 2D differentiation
- 3D differentiation (spheroids/organoids)

- Direct reprogramming
  - Homogenous population
  - Defined cell types
  - Fast maturation

- Physiological cell type specification
- Recap. development

- Homogenous population
- Defined cell types
- Fast maturation

- Tissue structure/Microenvironment
- Multiple cell types
- Recap. development

U.S. Patent No. 9,057,053
12.11.2019 3
HiPSC-Derived Neural *In Vitro* Platforms

- Generation of specified neuronal and glia cells types
- Cell-specific genetic modification (e.g. cell type labels, structural reporters, functional reporters)
- Well-defined highly functional *in vitro* co-cultures for purpose
- Biologically relevant assays with multimodal readouts

**In vitro model**

- Assays for multiple endpoints
- Compound exposure
- Complementary data for neurotoxicity assessment

---

**iPS cells**

- Reprogram
- Reprogram or differentiate

**Genetically defined subject (population)**

- Pure populations of different neural subtypes and glia cells

- Defined neuron/glia co-cultures

- Glutamatergic neurons
- GABAergic neurons
- Motor neurons
- Dopaminergic neurons
- Astrocytes
- Microglia

**Genetic modification**
SynFire® Neural Cells

- Combining highly functional defined neuronal cells types with human primary astroglial cells
- Suitable for versatile readouts (HCI, life-cell imaging, electrophysiology)

**iN/astroglial co-culture**

- Combining highly functional defined neuronal cells types with human primary astroglial cells
- Suitable for versatile readouts (HCI, life-cell imaging, electrophysiology)

**TUJ-1 / GFAP / Hoechst**
SynFire® Neural Cell Characterization

- Show principal neurophysiological properties
- Firing of action potentials
- Functional synapses
- Spontaneous bursting and network activity
- Fast maturation

Voltage-dependent K⁺ and Na⁺ currents

- Evoked single action potential
  - 40 mV
  - 10 ms

- Evoked action potential firing
  - 1 nA
  - 400 pA
  - 100 ms

- Large postsynaptic currents
  - -70 mV
  - 100 pA
  - 100 ms
  - 10 sec

- Membrane resting potential
  - 12.11.2019

Patch clamp analysis of iN cultures
- Exclusive glutamate- of GABA-mediated postsynaptic currents in monocultures
- NMDA currents
- Extra-synaptic GABA currents

Patch clamp analysis of iN cultures

**EPSCs in pure glutamatergic iN cultures**

**IPSCs in pure GABAergic iN cultures**

**Robust NMDA currents**

- +Glutamate (300 nM) +Glycine (10 µM)
- +Glutamate +Glycine +D-APV (100 µM)

**Tonic GABA current**
SynFire® Neural Cell Characterization

- Mature synapses
- Complex network
- Spine-like structures
- Mature neuronal marker expression

β3-Tub | GABA | Dapi | Merge
---|---|---|---
MAP2 | GFAP | Dapi | Merge
Dapi/tdTomato/GFP

Mature synapses, complex network, spine-like structures, and mature neuronal marker expression are characterized using Immunofluorescence Imaging.
Characterization of SynFire® Neural Activity on MEAs

- Extensive QC of starting iPSC lines
  - exome sequencing, copy number variations, karyotype, pluripotency
- QC of iN batches
  - cell-intrinsic properties, network development, responsiveness
- iN/glia co-cultures show robust activity
  - little variability in firing behavior across batches and iPSC lines
  - High uniformity of network development across experiments and batches

![Stable synchronized network activity](image)

![Network activity frequency distribution (+/- STD)](image)

Variability across batches/experiments

Inter-individual variability on MEA

Network development across batches

Variability between replicates (#active electrodes)

12.11.2019
Structural readouts using live-cell imaging:
- Neurons expressing genetically encoded fluorescence markers are co-seeded with unlabeled neurons and astrocytes
- Time course acquisition of fluorescence images
- Cell type-specific labeling and tracking (e.g. neurite outgrowth or cell ratios)
- Semi high throughput scale (96-well)

Functional readouts using live-cell imaging:
- \( \text{Ca}^{2+} \) flux
- Autophagic flux
Co-seeding with few labeled iNs

**Structural readouts using live-cell imaging:**
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**Functional readouts using live-cell imaging:**
- Ca2+ flux
- Autophagic flux

Functional reporter for autophagy

Live-cell imaging system (IncuCyte)

Lantrunculin A modulation neurite growth

Neurite length per cell body cluster (% change over time)

Neurite length per cell body cluster (% change over time)
Structural readouts using high-content imaging:
- Neurons expressing genetically encoded fluorescence reporters (e.g. PSD95, vGLUT2, GEPHYRIN) are co-seeded with unlabeled neurons and astrocytes
- Qualitative and quantitative Imaging of structures (e.g. spine morphology, synapse type/density)
- Cell type-specific labeling
- high throughput scale (384-well)
Structural readouts using high-content imaging:

- Neurons expressing genetically encoded fluorescence reporters (e.g. PSD95, vGLUT2, GEPHYRIN) are co-seeded with unlabeled neurons and astrocytes
- Qualitative and quantitative Imaging of structures (e.g. spine morphology, synapse type/density)
- Cell type-specific labeling
- high throughput scale (384-well)
Functional readouts on microelectrode arrays (MEAs)
- Unlabeled neurons co-seeded with astrocytes
- Readout of electrical signals (local field potentials) by electrodes
- Quantitative parallel measurement of multiple activity parameters including complex synchronized network activity
- Medium throughput scale (48-well)

Co-seed on electrode grid

Excitatory iNs
Inhibitory iNs
Astroglia

Multiwell MEA reader
Parallel electrode recording
Complex neural network activity
Analysis of neural activity parameters

Mean firing rate (MFR)
# of Spikes per burst
Burst duration
Inter-burst interval (IBI)
ISI
Burst frequency
% of Spikes in burst
Characterization of SynFire® Neural Activity on MEAs

- Rapid maturation with increasing neural activity
- Complex bursting and network bursting
- Development of synchronized neural network activity

Valiullina et al., 2016, Moore et al., 2011, Peinado 2001

Makinen et al., 2018
Characterization of SynFire® Neural Activity on MEAs

- Rapid maturation with increasing neural activity
- Complex bursting and network bursting
- Development of synchronized neural network activity

<table>
<thead>
<tr>
<th>Week</th>
<th>1 week</th>
<th>2 weeks</th>
<th>3 weeks</th>
<th>4 weeks</th>
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<tr>
<td></td>
<td>1 week</td>
<td>2 weeks</td>
<td>3 weeks</td>
<td>4 weeks</td>
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</tbody>
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- PTX (GABA\(_A\) Antagonist Picrotoxin): 10µM; CBX (gap junction blocker carbenoxolone): 25µM

PTX (GABA\(_A\)R Antagonist Picrotoxin): 10µM; CBX (gap junction blocker carbenoxolone): 25µM
Characterization of SynFire® Neuron/Glial Co-Cultures

- Comprehensive RNA-seq Analysis of complete neural co-cultures:
  - Increase of neuronal-specific transcripts along with in vitro maturation (DPP 7, 14, 21, 28, 35, 42)
  - Broad representation of neuronal ion channels

<table>
<thead>
<tr>
<th>Gene</th>
<th>7 days</th>
<th>14 days</th>
<th>21 days</th>
<th>28 days</th>
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<th>Potassium channels</th>
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Comprehensive RNA-seq Analysis of complete neural co-cultures:

- Increase of neuronal-specific transcripts along with *in vitro* maturation (DPP 7, 14, 21, 28, 35, 42)
- Broad representation of neuronal signaling pathways

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Cholinergic (nicotine)

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<th>21 days</th>
<th>28 days</th>
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Histamine (Muscarinic)-Opioid Purinergic

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<tr>
<th>Gene</th>
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<th>28 days</th>
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<th>42 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTR1A</td>
<td>0.97</td>
<td>0.16</td>
<td>0.16</td>
<td>0.16</td>
<td>0.16</td>
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<td>HTR1B</td>
<td>2.57</td>
<td>2.71</td>
<td>2.41</td>
<td>1.96</td>
<td>1.67</td>
<td>1.46</td>
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<tr>
<td>HTR1D</td>
<td>0.67</td>
<td>0.04</td>
<td>0.07</td>
<td>0.06</td>
<td>0.05</td>
<td>0.03</td>
</tr>
<tr>
<td>HTR1E</td>
<td>0.25</td>
<td>0.92</td>
<td>1.67</td>
<td>1.50</td>
<td>1.54</td>
<td>1.59</td>
</tr>
<tr>
<td>HTR1F</td>
<td>0.04</td>
<td>0.03</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>HTR2A</td>
<td>1.03</td>
<td>1.47</td>
<td>1.90</td>
<td>2.16</td>
<td>2.77</td>
<td>2.06</td>
</tr>
<tr>
<td>HTR2B</td>
<td>0.14</td>
<td>0.08</td>
<td>0.20</td>
<td>0.12</td>
<td>0.11</td>
<td>0.06</td>
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<tr>
<td>HTR2C</td>
<td>0.02</td>
<td>0.08</td>
<td>0.14</td>
<td>0.28</td>
<td>0.45</td>
<td>0.63</td>
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<tr>
<td>HTR3</td>
<td>0.79</td>
<td>2.08</td>
<td>2.33</td>
<td>2.87</td>
<td>2.50</td>
<td>2.80</td>
</tr>
<tr>
<td>HTR4</td>
<td>24.29</td>
<td>12.17</td>
<td>16.22</td>
<td>14.83</td>
<td>5.14</td>
<td>3.78</td>
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<tr>
<td>HTR5</td>
<td>0.25</td>
<td>0.33</td>
<td>0.07</td>
<td>0.10</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>HTR6</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
<td>0.01</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>HTR7</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
<td>0.01</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>HTR8</td>
<td>0.04</td>
<td>0.09</td>
<td>0.30</td>
<td>0.35</td>
<td>0.44</td>
<td>0.48</td>
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<tr>
<td>HTR9</td>
<td>0.04</td>
<td>0.12</td>
<td>0.36</td>
<td>0.68</td>
<td>1.42</td>
<td>1.78</td>
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<tr>
<td>HTR10</td>
<td>0.17</td>
<td>0.21</td>
<td>0.37</td>
<td>0.79</td>
<td>0.47</td>
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</table>

Dopamine

<table>
<thead>
<tr>
<th>Gene</th>
<th>7 days</th>
<th>14 days</th>
<th>21 days</th>
<th>28 days</th>
<th>35 days</th>
<th>42 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRD1</td>
<td>0.88</td>
<td>0.49</td>
<td>0.47</td>
<td>0.38</td>
<td>0.44</td>
<td>0.48</td>
</tr>
<tr>
<td>DRD2</td>
<td>15.37</td>
<td>8.63</td>
<td>8.47</td>
<td>8.00</td>
<td>10.48</td>
<td>11.57</td>
</tr>
<tr>
<td>DRD3</td>
<td>0.53</td>
<td>0.25</td>
<td>0.13</td>
<td>0.13</td>
<td>0.10</td>
<td>0.10</td>
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<tr>
<td>DRD4</td>
<td>4.95</td>
<td>3.33</td>
<td>3.74</td>
<td>3.85</td>
<td>4.75</td>
<td>3.83</td>
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<tr>
<td>DRD5</td>
<td>0.80</td>
<td>0.95</td>
<td>0.58</td>
<td>0.06</td>
<td>0.52</td>
<td>0.43</td>
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</table>

Cannabinoid

<table>
<thead>
<tr>
<th>Gene</th>
<th>7 days</th>
<th>14 days</th>
<th>21 days</th>
<th>28 days</th>
<th>35 days</th>
<th>42 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNR1</td>
<td>6.26</td>
<td>9.52</td>
<td>18.24</td>
<td>20.01</td>
<td>23.93</td>
<td>20.81</td>
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<tr>
<td>CNR2</td>
<td>0.62</td>
<td>0.35</td>
<td>0.46</td>
<td>0.49</td>
<td>0.60</td>
<td>0.55</td>
</tr>
<tr>
<td>CNR3</td>
<td>14.59</td>
<td>8.64</td>
<td>5.77</td>
<td>5.74</td>
<td>17.88</td>
<td>10.48</td>
</tr>
<tr>
<td>CNR4</td>
<td>0.54</td>
<td>0.99</td>
<td>1.06</td>
<td>1.11</td>
<td>1.39</td>
<td>1.29</td>
</tr>
<tr>
<td>CNR5</td>
<td>0.82</td>
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<td>0.24</td>
<td>0.27</td>
<td>0.36</td>
<td>0.43</td>
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<td>CNR6</td>
<td>84.79</td>
<td>68.91</td>
<td>63.77</td>
<td>54.43</td>
<td>69.03</td>
<td>61.49</td>
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<td>CNR7</td>
<td>1.56</td>
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<td>CNR8</td>
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<td>0.85</td>
<td>0.85</td>
<td>1.23</td>
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</table>

NMDA

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<th>Gene</th>
<th>7 days</th>
<th>14 days</th>
<th>21 days</th>
<th>28 days</th>
<th>35 days</th>
<th>42 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMDA</td>
<td>4.67</td>
<td>40.68</td>
<td>88.88</td>
<td>32.50</td>
<td>29.65</td>
<td>28.05</td>
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<tr>
<td>NMDB</td>
<td>0.60</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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</tbody>
</table>

Opioid Purinergic
Modulation of GABA\textsubscript{A} Receptor activity:

- **Single cell patch clamp:**
  - Normal inward currents upon agonist dosing
  - Current is rapidly blocked by selective antagonist

- **Network activity on MEAs:**
  - Dose-dependent response in activity to agonist/antagonist
  - Distinct bursting pattern in response to agonist/antagonist
Modulation of AMPA Receptor activity:

- Single cell patch clamp:
  - Normal inward currents upon agonist dosing
  - Current is blocked by selective antagonist

- Network activity on MEAs:
  - Biphasic effect of agonist dosing on spiking and network bursting
  - Dose-dependent inhibition of network activity in response to antagonist
Modulation of NMDA Receptor activity:

- Single cell patch clamp:
  - Isolated synaptic NMDA-receptor currents
  - Extrasynaptic persistent currents are blocked by antagonist

- Network activity on MEAs:
  - Biphasic effect of agonist dosing on spiking and network bursting
  - Broad inhibition of neuronal activity only at high antagonist dose

10 μM CNOX + 50 μM Picrotoxin (no TTX)

100 μM NMDA + 10 μM Glycine

12.11.2019
Functional *In Vitro* Neurotoxicity: Assay for Seizure Liability Testing

- **Identification of seizure liabilities**
  - GABA<sub>A</sub> receptor antagonists (e.g. bicuculline, BIC) induce tonic-clonic seizures *in vivo*.*
  - Evoked seizure-like events (SLEs) *in vitro* based reflected by alteration of neural network activity (firing rate and network bursting characteristics)
  - Timepoint optimization for maximal effect size: 23 Days Post Plating (DPP)

- **Confirmation of biologically relevant phenotype**
  - SLEs appearance can be rescued by antiepileptic drug dosing (phenytoin, lamotrigine)

*Olsen et al., PNAS 2006, Chen et al., PNAS 2006*
Human vs. rodent comparison:
- Rat primary cortical cultures
- Human iPSC-iN/glia co-cultures
- Overall concordance
- Different effective concentrations
- AEDs: Phenytoin (P), Lamotrigine (L)

Functional *In Vitro* Neurotoxicity: Assay for Seizure Liability Testing
Comprehensive assessment of anti-seizure effects:
- Human iPSC-derived iN/glia co-cultures at day 23
- SLE induction by PTX [10μM] → dosing with AED
- Multiparameter readout on MEAs → dose-response curves
High correlation between *in vitro* and *in vivo* efficacy

Coefficient (linear fit):
- $r^2 = 0.79$
- $r^2 = 0.87$ when excluding Stiripentol (GABA pathway involved to produce full efficacy)

Safinamide: A drug approved for PD treatment but showing strong anticonvulsant efficacy in human trials
High correlation between *in vitro* and *in vivo* efficacy

- Coefficient (linear fit):
  - $r^2 = 0.79$
  - $r^2 = 0.87$ when excluding Stiripentol (GABA pathway involved to produce full efficacy)
  - Safinamide: A drug approved for PD treatment but showing strong anticonvulsant efficacy in human trials
Testing drugs with described seizurogenic effects (HESI NeuroTox Group):

- Training set: 10 proconvulsant compounds and 3 controls (5 conc., 6 replicates)
- Multiple modes of action, multiparameter readouts on MEAs
- Identification of endpoints of neuronal network alteration to predict seizure liability

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Solvent</th>
<th>Concentrations (uM)</th>
<th>Mode of Action</th>
<th>Seizurogenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Picrotoxin (PTX)</td>
<td>DMSO</td>
<td>0.3, 1, 3, 10, 30</td>
<td>antagonist of GABA$_{\alpha}$ receptors</td>
<td>Yes</td>
</tr>
<tr>
<td>Pentylenetetrazol (PTZ)</td>
<td>DMSO</td>
<td>10, 30, 100, 300, 1000</td>
<td>antagonist of GABA$_{\alpha}$ receptors</td>
<td>Yes</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>DMSO</td>
<td>0.3, 1, 3, 10, 30</td>
<td>muscarinic receptor agonist</td>
<td>Yes</td>
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<tr>
<td>Linopirdine</td>
<td>DMSO</td>
<td>1, 3, 10, 30, 100</td>
<td>selective KCNQ channel blocker</td>
<td>Yes</td>
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<tr>
<td>4-Aminopyridine (4-AP)</td>
<td>DMSO</td>
<td>0.3, 1, 3, 10, 30</td>
<td>potassium channel blocker</td>
<td>Yes</td>
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<tr>
<td>Chlorpromazine</td>
<td>DMSO</td>
<td>0.1, 0.3, 1, 3, 10</td>
<td>D2 receptor antagonist</td>
<td>Yes</td>
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<td>Amoxapine</td>
<td>DMSO</td>
<td>0.3, 1, 3, 10, 30</td>
<td>blocks dopamine receptors, …</td>
<td>Yes</td>
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<tr>
<td>Phenytoin</td>
<td>DMSO</td>
<td>1, 3, 10, 30, 100</td>
<td>sodium channel blocker</td>
<td>No</td>
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<tr>
<td>Maprotiline</td>
<td>DMSO</td>
<td>0.1, 0.3, 1, 3, 10</td>
<td>inhibits reuptake of norepinephrine</td>
<td>Yes</td>
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<tr>
<td>Clozapine</td>
<td>DMSO</td>
<td>0.1, 0.3, 1, 3, 10</td>
<td>5-HT$_{2A}$ receptor antagonist, induce glutamate and serine release from astrocytes (NMDAR activation)</td>
<td>Yes</td>
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<tr>
<td>Amoxicillin</td>
<td>DMSO</td>
<td>1, 3, 10, 30, 100</td>
<td>inhibitor of bacterial wall biosynthesis</td>
<td>No</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>DMSO</td>
<td>1, 3, 10, 30, 100</td>
<td>COX-1 and 2 inhibitor</td>
<td>No</td>
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<tr>
<td>Bicuculline (BIC)</td>
<td>DMSO</td>
<td>3</td>
<td>antagonist of GABA$_{\alpha}$ receptors</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Viability testing in iN/glia co-cultures upon dosing of seizurogenic compounds:

- Lactate dehydrogenase (LDH) release and resazurin (CellTiter Blue®) staining
- No significant neuronal cytotoxic effects (structural neurotoxicity)
Evaluation of Assay to Identify Seizure Liabilities of Chemical Compounds

- Acetaminophen → negative control
  - No alteration of neuronal network activity
- Bicuculline (BIC) → positive control
  - Increase in spiking, bursting, and network bursting
- Pentylenetetrazol (PTZ) → GABA_A Receptor antagonist
  - Dose-dependent increase in spiking, bursting, and network bursting

**Acetaminophen (control)**

- No alteration of neuronal network activity

**PTZ**

- Dose-dependent increase in spiking, bursting, and network bursting

12.11.2019
Evaluation of Assay to Identify Seizure Liabilities of Chemical Compounds

- **Grouping of neuroactive effects:**
  - Cluster analysis of MEA Parameter
  - Groups form by compound mode of action
  - Negative controls form separate group
Combination of pre-patterning and direct reprogramming (induced motor neurons, iMNs)

- Scalable protocol, ~21 days duration
- Upregulation of LMN markers, downregulation of cortical markers

### Lower motor neuron markers expression (qRT-PCR)

<table>
<thead>
<tr>
<th>Marker</th>
<th>NeuCyte iMN</th>
<th>Commercial (std. protocol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB9</td>
<td>8 ± 2</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>LHX3</td>
<td>4 ± 1</td>
<td>3 ± 0.5</td>
</tr>
<tr>
<td>vCHAT</td>
<td>6 ± 2</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>ISL1</td>
<td>4 ± 0.5</td>
<td>2 ± 0.5</td>
</tr>
</tbody>
</table>

### Cortical transcription factor expression (qRT-PCR)

<table>
<thead>
<tr>
<th>Factor</th>
<th>NeuCyte iMN</th>
<th>Commercial (std. protocol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTIP2</td>
<td>0.8 ± 0.2</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>FEZF2</td>
<td>0.6 ± 0.3</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>LHX2</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.05</td>
</tr>
<tr>
<td>OTX1</td>
<td>0.1 ± 0.05</td>
<td>0.05 ± 0.02</td>
</tr>
</tbody>
</table>

LMNs – standard protocol (commercial) (day 21 on astrocytes)

NeuCyte iLMN protocol (day 21 on astrocytes)
Outlook: HiPSC-Derived Astrocytes

- Direct reprogramming of astrocytes from iPSCs (→ induced astrocytes, iAs)
  - Scalable protocol, 14 days duration
  - Expression of mature astroglial markers, support of synaptogenesis
Summary

- **SynFire® HiPSC-derived iN/glial co-culture system for *in vitro* assays**
  - Fast maturation and low variability
  - Specified cell composition and robust readouts
  - Cell-type specific modification for flexible assay design
  - High level of functionality (electrophysiology)

- **Neural network activity in iN/glial co-cultures**
  - Rapid developing complex synchronized network activity
  - Coordination of firing is mainly driven by chemical synapses
  - Responsive to a broad range of neurotransmission and pathway modulation

- **Mixed excitatory/inhibitory iN/glial co-cultures for seizure liability testing**
  - Quantitative assessment of neural network activity alteration (multiparameter readout on MEAs)
  - SLE responses to K+ channels blockers, GABA<sub>A</sub> receptor antagonists, and 5-HT2A/NMDA antagonists
  - Not suitable for distinct MoAs like D2R modulation, norepinephrine modulation, mAChR agonists
  - Additional pharmacoprofiling required to refine applicability
  - Extended compound set needed to develop predictive assay
THANKS TO

- **NeuCyte Team**
  - Lorena Saavedra
  - Daniel Haag
  - Touhidul Molla
  - Thomas Portmann
  - Ray Hsieh
  - Alex Lloret
  - Adrian Featherstone
  - Weiwei Zhong
  - Peng Zou
  - Cristopher Noel
  - Atossa Shaltouki
  - Chengyun Lee
  - Angela carter
  - Shane McGillis

- **U.S. Environmental Protection Agency**
  - Timothy Shafer
  - Kathleen Wallace
  - Theresa Freudenrich

- **HESI NeuTox Group**
Contacts:

Jonathan Davila, PhD  Daniel Haag, PhD
jdavila@neucyte.com    dhaag@neucyte.com

1561 Industrial Road, San Carlos, CA 94070
Chemical Elements and Metals in Aerosols from Three Generations of Electronic Cigarettes: Do They Cause VAPI?

Monique Williams

Southern California Chapter of the Society of Toxicology
Annual Meeting
November 7, 2019
• Conflict of Interest: None

• Disclosure: I have been supported by the Tobacco-Related Disease Research Program of California and the National Institutes of Health
Presentation Outline

I. Background – Introduction to the Electronic Cigarettes
II. Part 1 – Anatomy and Evolution of Electronic Cigarettes
III. Part 2 – Evaluation of Metal Emissions in Electronic Cigarettes
IV. Part 3 – Strategies to Reduce Metal Emissions
V. Part 4 – Health Effects Associated with Electronic Cigarette Use
VI. Summary, Caveats, and Future Directions
VII. Acknowledgments
Electronic cigarettes (EC) are tobacco-free nicotine delivery devices that have gained popularity around the globe.

- Alternative to smoking conventional cigarettes and to aid in smoking cessation.

Three major components: battery, atomizing unit, cartridge with nicotine solution.

- The atomizers are comprised of metal components.

Fluids contain: solvent(s), flavor chemicals, nicotine, metals, and contaminants.
Evolution of Electronic Cigarettes

- During the 15 years since their introduction, EC have evolved to include larger batteries, atomizers, and e-liquid reservoirs.

- The atomizers contain metal components that can transfer into the aerosol and could cause adverse health effects.

Williams, Talbot 2019 IJERPH
The purposes of this study

1. Characterize the anatomy and evolution of EC atomizing components from three generations of EC,
2. Identify and quantify the elements/metals in EC aerosols,
3. Establish how metal concentration in the aerosol is affected by puffing topography,
4. Determine the source of the elements/metals in the aerosol, and
5. Evaluate the relationship between metal concentrations in EC aerosols and adverse health effects.
Anatomy of the Atomizing Unit of Cig-a-likes

- Cartomizers/Disposables have a mouthpiece, gasket, air-tube, atomizing unit (with a filament and thick wire), wick, inner mesh fibers, and outer Poly-fil fibers.

Williams et al 2013, PlosOne 8(3); 1-11. e57987
Anatomy of Clearomizers/Mods

- Consisted of larger batteries and higher capacity reservoirs for refill e-liquid.

- The replaceable dripping atomizers (RDA) had the fewest components with just a filament and wick.
Comparison of Atomizing Unit Components Across Three Generations of EC

- Components such as: filament, air-tube, wick were conserved across all generations of EC.

- In the evolution from cig-a-like to clearomizer/mod, many of the components in the atomizer were removed.
Elemental Analysis of Atomizer Components in Cig-a-likes

- The thin filament was mainly nichrome (nickel, chromium), some brands had iron as well.

- The thick wire was made of copper coated with silver, some were coated with tin or the thick wire was nickel.

- Manufacturers use different methods to join the wires of the atomizer: solder, brazing, and brass clamps.

Williams et al 2015 PlosOne 10(9): e0138933
Anatomy of Disposable EC

- The atomizing unit had similar composition as the cartomizer (nichrome filament, tin-coated copper wire, brass clamps, solder joints).
- One brand used a kanthal wire (chromium, iron, aluminum).
- Two brands had tin-lead solder joints. Lead is used to stabilize solder.

These devices are manufactured in China, and lead was banned in the use of solder.

Composition of the Atomizing Unit in Tank Style EC

- The atomizing units differed from the earlier EC models as they rarely had a thick wire, lacked fibers, and the wires were brazed/welded together.

- Filaments were mainly nichrome, but some brands were elinvar, or kanthal.

- Shell of the atomizing unit was a brass core, nickel-tin plated. Some had iron in the shell as well.

Example of a filament from Protank

Elinvar filament

Williams, Bozhilov, Talbot, 2019, Environmental Research
First Generation Atomizing Unit
Cig-a-like

**Filament:** Nickel, Chromium, Iron, Aluminum

**Thick Wire:** Silver/Tin coated Copper, Nickel, Iron

**Wire-Wire Joint:** Tin/(Lead) solder, Copper/Zinc clamp, brazed, coiled, welded

**Wick:** Silicon, Oxygen, Calcium, Magnesium, Aluminum

**Air-Tube:** Copper, Nickel, Iron

**Air-Tube:** Copper, Nickel, Iron

**Wire-Air-Tube Joint:** Tin/(Lead) solder

**Connect to wall of mouthpiece**

Williams, Bozhilov, Talbot, 2019, Environmental Research; Williams, Talbot 2019 IJERPH
Second & Third Generation Atomizer Units

**Clearomizer/Mod**

- **Filament:** Nickel, Chromium, Iron, Aluminum
- **Air-Tube:** Copper/Zinc (Brass), Nickel, Tin, Iron
- **Wick:** Silicon, Oxygen, Carbon, Calcium, Magnesium, Aluminum
- **Thick Wire:** (when present) Nickel
- **Wire-Wire Joint:** (when present) Brazed Nickel, Chromium,

**Clearomizers/Mods** have fewer components than the Cig-a-likes

Williams, Bozhilov, Talbot, 2019, Environmental Research; Williams, Talbot 2019 IJRPH
Elements of Interest

- Screened for 36 different elements, and 34 were detected in the aerosols.
- Seven elements of interest: chromium, nickel, copper, zinc, selenium, tin, and lead.

http://periodictable.com/index.html
Methods for Elemental Analysis of Aerosol

Cold Trap Method
Generated aerosol using a smoking machine and ice bath

Impinger Method
Generated aerosol using a smoking machine at room temperature

Samples were analyzed using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP–OES) (Optima 7300 PV)

Made a 6TPE (60 puffs/10 mLs 10% HNO₃, 3% HCl, 87% dH₂O) which were stored in 15 mL conical vial

Made a 6TPE (60 puffs/10 mLs 2% HNO₃, 98% dH₂O) and stored in 15 mL conical vial
Frequency of Elements Present in EC Aerosols

- Four elements (silicon, tin, zinc, copper) were detected in over 60 different aerosols across three generations.

- The top 10 most frequently found elements have been identified in the components of the atomizing units.
Overall **Total Concentration** of Elements in EC Aerosols

- All data for each type of EC was pooled independent of collection method and brand.
- Very little variation in disposable EC.
- When looking at the 7 elements of interest (chromium, nickel, copper, zinc, selenium, tin, and lead), tank style EC delivered the most.

<table>
<thead>
<tr>
<th>EC Models</th>
<th>Concentration (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cartomizer max</td>
<td>Greensmoke 6,947 µg/L</td>
</tr>
<tr>
<td>Tank Max</td>
<td>Kanger T3S 2,525 µg/L</td>
</tr>
</tbody>
</table>

**Note:**
- Williams et al 2013 PlosOne
- Williams et al 2017 PlosOne
- Williams et al 2015 PlosOne
- Williams, Li, Talbot 2019 Scientific Reports
Comparison of Individual Elements by EC

- Pooled all brands within a class of EC independent of collection method.
- Copper was higher in the disposable EC than in the cartomizers and tanks.
- In general, tank style EC delivered the highest concentration of individual elements, due to operating at higher voltages.
Cold Trap vs Impinger Method for Tank EC

- Cold trap had an overall higher concentration of elements in the aerosol.

- Tin, lead, and copper were higher in the cold trap samples.

- Chromium, nickel, selenium, and zinc were higher in the impinger samples.

Williams, Li, Talbot 2019 Scientific Reports
Effects on Metal Concentrations with Varying Topography

**Impinger Method**

**Puffing Parameters Altered**

**Puff Interval**

- **Continuous Puffing**
  (consecutive puffs with no breaks)

- **Interval Puffing**
  (10 puffs with 5-20 minute breaks)

Williams, Li, Talbot 2019 Scientific Reports
Comparison **Continuous vs Interval Puffing Using Impinger Method**

- Interval puffing delivered a higher total and individual concentration of elements.

- This is important because interval puffing best represents how EC consumers use the devices.

![Graph showing comparison between Continuous and Interval puffing for various elements](chart.png)

*Williams, Li, Talbot 2019 Scientific Reports*
Comparison of Elemental Concentrations Across Three Generations

- Silicon was most often found in higher concentrations across all ECs.
- Selenium, zinc, and tin were found in all EC aerosols at relatively high concentrations.
- Some elements (tin, lead, zinc) were detected in high concentrations in one or a few products, but not in any others.
Relative Abundance of Elements Across All Three Generations of EC

- Silicon was the most dominant element, and was found in the highest concentration in EC aerosols.

- Across a whole population, tin, copper, lead and zinc were detected in relatively high concentrations.

- Nickel and chromium transfer at much lower concentrations despite being part of the heated filament.

Atomizer Design Can influence Concentration and Number of Elements in Aerosol

- **Clone** had the fewest metal components in its atomizer and the fewest number of elements in its aerosol.

- **Smok** had more metal components in its atomizing unit and more elements in its aerosol.

- Reducing the number of metal components in the atomizing units can reduce the number of elements delivered in the aerosol.

Williams, Li, Talbot 2019 Scientific Reports; Williams, Talbot 2019 IJERPH
Recap: Concentration of Elements in Three Generations of EC Aerosols

- Seven elements (chromium, nickel, copper, zinc, selenium, tin, and lead) were detected in all three generations of EC and originated from the components of the atomizing unit.

- Silicon was most often the dominant element in EC aerosols, with zinc, selenium, and tin also detected frequently and in relatively high concentrations.

- Depending on your element of interest the method of collection and the puffing topography will effect the concentration of elements in the EC aerosol.
Vaping-Associated Pulmonary Injury (VAPI)

- The mysterious and potentially deadly lung illness that hundreds of people nationwide have contracted as a result of vaping has become a full-blown public health crisis.

- VAPI causes damage to the respiratory and digestive systems.
  - Over 1,800 reports, and over 40 deaths associated with VAPI.

- VAPI may include more than one disease and could have more than one cause.

Could metals contribute to VAPI?

Henry et al 2019 NEJM
Spot urine samples were collected from three categories of users in the USA:

- Non-smokers
- Smokers
- E-Cigarette Users

Urine samples were aliquotted into eppendorff tubes.

In a 1:25 dilution using 2% Nitric Acid, urine samples were further processed.

Metals were analyzed using an Agilent 7900 ICP MS with an ASX-500 series autosampler. Data is analyzed using Masshunter software.

Three Biomarkers:
- Metallothionein (effect; metal exposure)
- 8-isoprostane (lipid peroxidation)
- 8-OHdG (DNA oxidation)

Biomarkers of effect and potential harm were analyzed using ELISA kits.

Work was done in collaboration with Roswell Park Cancer Institute in Buffalo NY, and UCR Dept. of Earth Sciences.

Sakamaki-Ching, Williams, et al 2019 BMJ; In Revision
Evaluation of Biomarkers of Effect and Potential Harm

• We compared the levels of three biomarkers of effect and harm (Metallothionein, 8-isoprostane, 8-OHdG) between three smoking groups (Nonsmokers, Smokers, EC Users).

• Metallothionein, 8-OHdG, 8-isoprostane were all significantly elevated in EC users compared to non-smokers.

Sakamaki-Ching, Williams, et al 2019 BMJ; In Revision
Comparison of Metal Concentrations in Three Smoking Groups

- Two elements, selenium and zinc, were elevated in the urine of EC Users.

![Selenium Concentrations, Smoking Groups](image)

![Zinc Concentrations, Smoking Groups](image)

Sakamaki-Ching, Williams, et al 2019 BMJ; In Revision
Evaluation of Biomarkers of Effect and Potential Harm

- For selenium, there was no correlation between concentration of element and oxidative stress in any of the three smoking groups.

- There was a correlation between elevated zinc concentrations and oxidative stress in EC users, over Nonsmokers and Smokers.
# Health Effects of Metal Inhalation

<table>
<thead>
<tr>
<th>Element</th>
<th>Metal Conc. (ng/m³)</th>
<th>PEL (ng/m³)</th>
<th>Health Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromium</td>
<td>$2.3 \times 10^5$ – $3.25 \times 10^7$</td>
<td>$5 \times 10^3$</td>
<td>Respiratory irritation, lung function impairment, lung cancer.</td>
</tr>
<tr>
<td>Lead</td>
<td>$2.86 \times 10^6$ – $1.13 \times 10^9$</td>
<td>$0.5 \times 10^6$</td>
<td>Vomiting, diarrhea, constipation, nervous system damage</td>
</tr>
<tr>
<td>Selenium</td>
<td>$3.61 \times 10^5$ – $3.76 \times 10^8$</td>
<td>$2 \times 10^5$</td>
<td>Respiratory irritation, coughing, bronchial spasms</td>
</tr>
<tr>
<td>Copper</td>
<td>$9.7 \times 10^4$ – $5.56 \times 10^8$</td>
<td>$0.1 \times 10^6$</td>
<td>Nausea, vomiting, abdominal pain</td>
</tr>
<tr>
<td>Nickel</td>
<td>$7.4 \times 10^4$ – $3.61 \times 10^8$</td>
<td>$1 \times 10^6$</td>
<td>Nausea, coughing, vomiting, headache, cancer of the lungs, nose, throat</td>
</tr>
<tr>
<td>Tin</td>
<td>$1.25 \times 10^5$ – $6.82 \times 10^9$</td>
<td>$2 \times 10^6$</td>
<td>Skin and eye irritation, stannosis</td>
</tr>
<tr>
<td>Zinc</td>
<td>$4.9 \times 10^5$ – $9.14 \times 10^8$</td>
<td>$5 \times 10^6$</td>
<td>Fever, nausea, chest pain, metal fume fever</td>
</tr>
</tbody>
</table>

Permissible exposure limits (PEL) is the OSHA occupational exposure limit not to exceed 8 hours.

- Metal concentrations are based off of 60 puffs and may be along the lower limit of puffs taken by a consumer.
- Some EC exceeded all regulatory exposure limits.
In Conclusion

• All EC contain the same basic atomizing components. Through evolution, the tank style EC are larger, operate at a much higher power, and their atomizers have fewer parts.

• EC have metal components in their atomizers and these can transfer to the aerosol.

• Reducing the number of atomizer components, can reduce the number of elements in the aerosol.

• Depending on the element of interest, more than one method of collection could be required for analysis.
In Conclusion

- Increased voltage/power and interval puffing, which best represents an EC user, will increase the concentration of elements that transfer into the aerosol.

- There is a linkage between elevated metal concentrations to biomarkers of effect (metallothionein) and biomarkers of harm (8-OHdG and 8-isoprostane) in electronic cigarette users. Zinc in urine of EC users was significantly correlated with increased oxidative damage.

- Many of the symptoms observed in VAPI patients are ones that can occur with metal exposure.
Caveats and Future Directions

- EC aerosols are complex mixtures of metals and most government safety standards are developed for single metals. Therefore PEL may underestimate risks.

- Health effects associated with inhaling heated metals for prolonged periods of time are not known and future studies should address these concerns in detail.

- The relationship between aerosol metals and VAPI should be further investigated.
Acknowledgements

- Dr. Prue Talbot

- Collaborators: Dr. Jun Li, Dr. Sabrina Lin, Dr. Maciej Goniewicz,

- Metal Analysis in Urine Collaborators (ICP-MS): Andy Robinson, Dr. Steve Bates, and Dr. Tim Lyons

- Metal Analysis in EC Aerosol Collaborators (ICP-OES): Woody Smith and David Lyons

- Team: Shane Sakamaki-Ching and Crystal Hua

- Amanda Villarreal, Shreya Maharana, Malcolm Tran, Avni Parekh, Lindsey Bustos, Victor Camberos, Sarah Leung, Leland Nguyen, Hanna Nguyen, Jazmine Chavez, Jenn Stevens, Michelle Hoa, Jessica Toledo, Sanjay Ghai, Ivana Villarreal, An To, Michael Dang, Vicci Wang

- Talbot Lab
Any Questions?
mRNA-Sequencing Identifies Liver as a Potential Target Organ for Triphenyl Phosphate in Embryonic Zebrafish

Aalekhya Reddam, Constance A. Mitchell, Subham Dasgupta, David C. Volz
Department of Environmental Sciences,
University of California, Riverside, CA, USA
Residential fire statistics in the U.S.

- Roughly 370,000 residential fires per year
- Approx. 2,200 deaths, 10,800 injuries, and $6.6B in property loss per year
- 1 in 5 deaths due to upholstered furniture ignition

California Technical Bulletin 117 (TB117)

- Mandates that all filling materials meet flammability requirements
- Physical barrier or addition of flame retardants
Triphenyl Phosphate (TPHP) is an additive flame retardant

U.S. Production and use: >50 million lbs/year

- 98% Detection in house dust samples
- Range of <150 to 1,798,000 ng/g
  Stapleton et al. 2009
- High detection of diphenyl phosphate, the primary urinary metabolite
  Feng et al. 2016, Mendelson et al. 2016
Zebrafish embryos rapidly develop **ex utero**

- **Heart tube looping** occurs from 30 to 48 hpf
- **Hepatic budding** begins at 24 hpf
- **Hepatic outgrowth** begins after 48 hpf

Villeneuve et al. 2014
TPHP is toxic to developing zebrafish

- Blocks cardiac looping via an AhR2-independent mechanism

McGee et al. (2013)
mRNA-seq reveals that TPHP disrupts RXR-dependent pathways following exposure from 24-72 hpf

Mitchell et al. 2018
mRNA-Seq reveals transcripts associated with cardiotoxicity, hepatotoxicity and nephrotoxicity are significantly affected after TPHP exposure.
Fenretinide (a pan-RAR agonist) reliably mitigates TPHP-induced cardiotoxicity

RAR heterodimerizes with RXR

Mitchell et al. (2018)
Pre-treatment with fenretinide did not mitigate TPHP induced effects on the transcriptome.
Fenretinide was unable to block TPHP-induced effects on cardiotoxicity- and hepatotoxicity-related pathways.
TPHP exposure alters the normal trajectory of liver development – a phenotype that is blocked by pre-treatment with fenretinide.
TPHP exposure as well as fenretinide pre-treatment did not result in hepatocellular toxicity at 128 hpf.
TPHP exposure abolishes lipids within the head and trunk of zebrafish – a phenotype that is not blocked by pre-treatment with fenretinide.
Despite stage dependent differences, total abundance of all metabolites were significantly decreased following exposure to TPHP.
Betaine, the top significant metabolite, is an important osmoprotectant.
Betaine, the top significant metabolite, is an important osmoprotectant.
Conclusions

- mRNA-Seq revealed liver as a target organ for TPHP
- Fenretinide pre-treatment blocked cardiotoxic and hepatotoxic phenotype, but not the respective effects on the transcriptome
- TPHP may be affecting baseline betaine concentration, leading to direct effects on osmoregulation and indirect effects on organ development.
• Fellowship support was provided by UCR’s Graduate Division to A.R
• NRSA T32 Training Program [T32ES018827] to C.A.M
• Research support was provided by a National Institutes of Health grant [R01ES027576] and the USDA National Institute of Food and Agriculture Hatch Project [1009609] to D.C.V.
Questions?

Exposure:
10μM TPHP
24
Exposure:
2μM Fen + 10μM TPHP
30

Betaine Depletion: √
Cardiotoxicity
Hepatotoxicity
48

Pericardial Edema: √
Lipid Depletion: √
Betaine Depletion: √

Increased Liver Area: √
Hepatocellular Toxicity: X

Pericardial Edema: X
Lipid Depletion: √

Increased Liver Area: X
Hepatocellular Toxicity: X

Hours post fertilization
72
128
Analyzing the effect of perfluorobutanesulfonic acid on pancreatic organogenesis in zebrafish using automated image segmentation

Ashley Schwartz
San Diego State University
Department of Mathematics and Statistics

November 7, 2019

In collaboration with
Karilyn Sant, PhD, SDSU, School of Public Health
Uduak George, PhD, SDSU, Department of Mathematics and Statistics
PFBS in our Environment

Perfolorbutanesulfonic acid (PFBS) is an emerging toxicant found in both our environment and biological samples due to increased manufacture and use.

- Atmospheric half-life of $>100$ days
- No evidence of degradation in water
PFBS Replaces PFOS

- The increased utilization of PFBS occurred due to the voluntary phase-out of perfluorooctanesulfonic acid (PFOS) in the early 2000’s.
- In human tissues, PFOS has a half-life of 5.4 years while its half-life in the environment remains unknown.

Figure: PFOS  
Figure: PFBS
The Pancreas

The exocrine pancreas synthesizes and releases digestive enzymes to aid in the breakdown of molecules to fuel metabolism.

Exocrine pancreatic insufficiency results in the inability to digest food properly, or maldigestion.
Determine the toxicity of PFBS in the developing pancreas by using image analysis and automated image segmentation of transgenic zebrafish models to visualize the developmental process invitro.

**Figure:** Zebrafish Development  
**Figure:** Human Development
Animals

Zebrafish are widely used as an embryonic model

- vertebrate animals
- 76% of human genes have homolog in zebrafish
- window of development is a few days
- fertilized externally
- translucent

Figure: *Tg(pft1a:GFP)* zebrafish
Experiment

**Expose Zebrafish**
- 0 (0.01% DMSO v/v)
- 16 μM PFBS
- 32 μM PFBS

**Capture Microscopy Images**
- 4dpf
- 7dpf

**Quantify and Classify Pancreas Area**
- Deformed
- Normal

*Figure*: Overlay captured image

*Figure*: GFP filtered image
A novel image processing MATLAB algorithm was developed that automatically and accurately segments and quantifies pancreas area. The approach shown follows four main steps:

1. Binarize the Image
2. Locate and Label Connected Components
3. Locate and Quantify the Area of the Pancreas
4. Classify the Pancreas as Normal or Deformed
Step 1: Intensitiy Image to Binary Image

The original gray scale image $I$ (960 by 1280) pixels is converted to a black-and-white image by using Otsu’s method for thresholding. Otsu’s thresholding chooses the threshold to minimize the intraclass variance of the thresholded black-and-white pixels.

Figure: $I$: Original Image  
Figure: $I_B$: Binarized Image
Step 2: Locate, Label, and Quantify Connected Components

- Each connected component $S_i$ is identified and uniquely labeled
- $A(S_i)$ is computed where $A(S_i)$ is the number of pixels in the connected component $S_i$ (i.e., the area of $S_i$).

Figure: $I_B$: Binarized Image  
Figure: $I_L$: Labeled Image
Step 3: Locating the Pancreas

**Definition**

Let $S = \{S_1, ..., S_k\}$ be the set of connected components. Then the set $A(S) = \{A(S_1), ..., A(S_k)\}$ is the set of pixel areas for each corresponding connected component. The exocrine pancreas area is then taken to be

$$\max(A(S))$$

**Figure:** $I$: Original Image

**Figure:** $I_P$: The Exocrine Pancreas
A birth defect occurs if a characteristic lies outside of the 90\(^{th}\) or 10\(^{th}\) percentile for what is considered normal.

Letting the control group dictate normality, we obtain the following definition

**Definition (Deformed)**

A pancreas is considered deformed if

\[
\max(A(S)) < P_L \text{ or } \max(A(S)) > P_U
\]

where \(P_L\) represents the 10\(^{th}\) percentile and \(P_U\) represents the 90\(^{th}\) percentile of the control group.
The automated image processing was able to accurately segment, quantify, and classify the pancreas for 100 fish in an average time of 1.50249 seconds, which previously would have taken weeks.
**Statistical Analysis**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Percent Deformed</th>
<th>Sample Size</th>
<th>Statistical Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group</td>
<td>15.38</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>16 µM</td>
<td>30</td>
<td>10</td>
<td>0.617</td>
</tr>
<tr>
<td>32 µM</td>
<td>33.3</td>
<td>12</td>
<td>0.3783</td>
</tr>
</tbody>
</table>

1Fisher’s Exact Test
### Statistical Analysis

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Percent Deformed</th>
<th>Sample Size</th>
<th>Statistical Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group</td>
<td>17.64</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>16 µM</td>
<td>43.75</td>
<td>32</td>
<td>0.03169</td>
</tr>
<tr>
<td>32 µM</td>
<td>48.48</td>
<td>33</td>
<td>0.00959</td>
</tr>
</tbody>
</table>

1. Fisher’s Exact Test
PFBS Contributes to Decreased Pancreas Size

There was a 30.84% increase in deformities 7 dpf, with the majority of those classified as under developed.

The data suggests PFBS is hindering the growth of healthy exocrine pancreas tissue.
Implications

PFBS behaves similarly to PFOS and can contribute to birth defects that may result in

- exocrine pancreatic insufficiency
- maldigestion
- indigestion

Embryonic pancreatic screening may be an important tool in identifying predisposition to disease later in life.
We have created an accurate and efficient means of quantifying pancreas area in the zebrafish model.

PFBS disrupts pancreatic organogenesis, leading to exocrine pancreas insufficiency.
Future Research

- Create a stand-alone image segmentation software for distribution
- Use this software on a wide range of chemicals as a high throughput screening tool
This work was supported by

- Dorris Howell- CSUPERB Research Scholar Program
- George Lab, San Diego State University
- Sant lab, San Diego State University
- San Diego State University, Department of Mathematics and Statistics

Special thanks to Sant lab for zebrafish handling and care
Questions?