Application of Rapid In Vivo Approaches to Green Chemistry

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Challenges and Opportunities

- Opportunities to integrate recent advances in toxicology to promote green chemistry
- Traditional whole animal-based studies present barriers
- Low throughput and expensive
- We need to more rapidly identify hazards and mechanisms of toxicity
- Develop predictive models to proactively design inherently safer products
- We have new tools to dramatically transform the way we proceed
How can modern toxicology help advance green chemistry?
Where are we going?

- 1-3/year
- 10’s/year
- 1000’s/day
- 10,000’s/day
- 100,000’s/day

High Throughput Molecular mechanism

Immediate Human Relevance

Adapted from the National Academy
Toxicity Testing for the 21st Century
Exposures and Biological Responses

We need to look earlier for responses that predict adverse outcomes

We need phenotypic anchoring...for now

Adapted from the National Academy Toxicity Testing for the 21st Century
Biology is Highly Dynamic

Entry Point = Toxicity Pathway

Biologic Inputs

Exposure
  ↓
Tissue Dose
  ↓
Biologic Interaction
  ↓
Perturbation
  ↓

Higher yet

Normal Biologic Function

Early Cellular Changes

Adaptive Stress Responses

Cell Injury

Morbidity and Mortality

Adapted from the National Academy Toxicity Testing for the 21st Century
Toxicity Pathways

“Toxicity Pathway: A cellular response pathway that, when sufficiently perturbed, is expected to result in an adverse effect.”

National Academy Toxicity Testing for the 21st Century

• We need to identify these toxicity pathways
• Determine if chemicals or NP perturb them
• Develop predictive quantitative relationships between molecular response and toxicity
Biological Assessments

• *In vitro* – *rapid, can be cost effective*
  – Continuous cell culture system
  – Primary cell culture system

• *In vivo* - *traditionally slow, costly*
  – Rodents, fish, flies and worms
Limitations to Cell-based Studies
-Lack complexity-

Response

Proliferation
Cell death
Metabolism
Gene expression
Phenotypic change

"There are blind spots"
What blind spots?

• Different cell-cell interactions not easily evaluated

• Indirect effects cannot be evaluated

• Cells in culture can only respond using their unique repertoire of expressed gene products – limited potential targets

• Practical problem…what cells do you choose?

• What assays? Is the data informative? Predictive?

• Tremendous potential for missed data

• Rapid, in vivo models may help…….
Collecting Biological Response Data

We need to pick up the pace…

But….  

High throughput ≠ high content

For example:

If an assay is developed for a specific response, that is the only data that can be obtained.

i.e. Apoptosis, proliferation, ROS, Calcium influx,
Data interpretation

Cultured cells

Expose and collect “omics data”

Observe robust gene expression changes following exposure
   -Difficult to have a benign response

Are these gene expression changes related to an adverse outcome? Do they represent an adaptive response?

What decisions can be made based solely on this information? What would trigger concern?

We need phenotypic ANCHORS..at least right now we do
Why Whole animal Studies?
-A Systems Biology Approach-

Criteria

- Share developmental, anatomical, and physiological characteristics with mammals (fish>flies>worms)
- Conserved molecular signaling and networks
- Must have inherent technical advantages of cell culture
- Amenable to high throughput phenotypic and behavioral screens
- Amenable to rapid whole animal mechanistic evaluations
  - Sequenced genome
  - Transgenics
  - Mutants
  - Genetic and chemical screens
Models currently well suited for rapid throughout assessments

<table>
<thead>
<tr>
<th></th>
<th>Time to Maturity</th>
<th>Genome Sequenced</th>
<th>Phenotypic Screens</th>
<th>Behavioral Screens</th>
<th>Automation Implemented</th>
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<td><strong>Drosophila</strong></td>
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<td><strong>Zebrafish</strong></td>
<td>60 days</td>
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<td>yes</td>
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**Systems Biological Approach**
- early embryonic development -

Why?

- Generally more responsive to insult... because
  
  Most dynamic life stage... and the full signaling repertoire is expressed and active, therefore fewer blind spots.
  
  Highest potential to detect adverse interactions

- If a chemical or nanomaterial is developmentally toxic it must influence the activity of a molecular pathway or process.. i.e. hit or influence a “Toxicity Pathway”

- Use the biological response to identify the “Toxicity Pathway”
Phenotypic Based Structure Activity Relationships (PBSAR)

i.e. Don’t run from biological complexity; exploit it!

Identification of “Toxicity Pathways” using the whole animal response as a path
Example acute exposures -early responses in zebrafish-

- Expose

- Multiple Levels of Interrogation
- Challenge the complex system as soon as possible
- Embryonic development serves as a “biological sensor and amplifier
- Look for “any” difference related to exposure
- The more we measure, the higher the sensitivity and resolution

5 days
Development Stages of Assessments

1. 25hr

3 min

4 hr

6 hr

19 hr

24 hr

120 hr

48 hr
Toxicity Testing (First Steps) Process

A large adult colony is required to support testing laboratory

Remove Chorions

1 Embryo/well

Multiple Replicates
Multiple Concentrations
QA/QC
- Negative
- Controls

Test Materials

Screening for responses 1-5 days
High Content Endpoints
(Assessed between 24 and 120 hpf)

Morphological
Malformations
i.e. pericardial edema, yolk sac edema, body axis
fin malformations, eye diameter

Circulation
Heart rate
Developmental progression

Omics

Behavioral
spontaneous movement (18-24 hpf)
touch response (27 hpf)
Motility, learning and memory
Example of Toxicity Endpoints

TCDD

- SHORTENED SNOUT
- PERICARDIAL EDEMA
- HEAD EDEMA
- UNINFLATED GAS BLADDER

CONTROL

- GAS BLADDER
- YOLK SAC EDEMA
Early Life Stage
Responses to Dithiocarbamates

Control

Exposed

Yolk

N

Yolk

N
Ethanol Induced Cell Death
Live Animals Acridine Orange
Alterations in gene expression

*In situ hybridization*

α-col2a

Control

Thiuram
Consider Startpoints - Not Endpoints

- Signaling pathways and molecular events are conserved
- But fish, flies and worms are not humans
- Consequences of disrupted signaling often species specific
- In other words, the mechanism by which a “target” is hit is likely conserved, but the consequence of the “hit” may be species or life stage specific
Interpreting Common Endpoints

Common 5 day endpoints in zebrafish revealed by chemical, nanomaterial and genetic screening

- Pericardial edema
- Yolk sac edema
- Reduced growth
- Bent body axis
- Lack of swim bladder inflation
- Behavioral changes

Common nonspecific
Repression of ARNT1 or AHR2 Makes Fish Non-responsive to TCDD

TCDD

AHR2

ARNT1

DRE TCDD-Responsive Genes

No Toxic Response

TCDD

AHR2

ARNT1

DRE TCDD-Responsive Genes

No Toxic Response

TCDD

AHR2

ARNT1

DRE TCDD-Responsive Genes

lethal
Automation: To Increase Throughput

Automation has been implemented in flies, worms and fish. Throughput is not longer a barrier.

_Zebrafish_

- Embryo Production – unlimited
- Embryo handling
- Chorion removal
- Microinjections
- Plate reader based assays
- Behavioral assays – multiple platforms
Zebrafish Enhanced Spawning Tanks

Maximize embryo production
Automated Embryo Handling
Automated Imaging and Scoring
Making the data useful

• DATA SHARING

• Need widely used knowledgebases

• New methods for whole animal informatics are needed
Take Home Message

• non-mammalian model offer a number of powerful advantages

• Models best applied to identify hazards and toxicity mechanisms, and to drive green chemistry choices (not directly for risk assessments)

• Managing uncertainty, false negatives and false positives
  • physiological and ADME differences
  • No maternal contribution during exposure
  • Molecular signaling functionally well conserved
  • Primary sequence not necessarily conserved
Acknowledgements

The Group

Dr. Tamara Tal, PhD
Michael Simonich, PhD
Siba Das, PhD
Kitae Kim, PhD
Tamara Tal, PhD
Katerine Saily
Jill Franzosa, MS
Lisa Truong, MS
Jane LaDu
Britton Goodale
Galen Miller
Hao Truong
Joe Fisher

David Mandrell, MS
Margaret Corvi, MS
Aaron Moore
Andrea Knecht
Leah Wehmas
Fei Chen
Cari Buchner
Carrie Barton
Greg Gonnermann
Eric Johnson, MS

Air Force Research Laboratory
#FA8650-05-1-5041

RC4ES019764
P42ES016465
P30ES000210