Toxicity Screening using Zebrafish Embryos: Form and Function

Stephanie Padilla

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Zebrafish Screening

• Integrated, highly conserved model of development
• Applicable to both human and eco toxicology
• “Form”: basic developmental toxicity screening
  – Padilla et al, Reprod. Toxicol., 2011
• “Function”: more subtle, functional endpoints
Zebrafish Development
practical considerations

• Rapid development
• Transparent embryo
• Developmental homology
• Easy to manipulate genome
• Inexpensive maintenance
• Hundreds of embryos
• Duration of experiment: 6 days

From Airhart et al (2007)

Courtesy of J. Olin, A. Tennant, and K. Jensen
ToxCast_320 Chemicals

- 309 chemicals: primarily pesticides and pesticide metabolites
- Intra- and inter-plate duplicates and triplicates
- Many of the chemicals have gone through testing in mammals, including guideline tests for developmental toxicity in rat and/or rabbit. (ToxRef Database; http://www.epa.gov/ncct/toxrefdb)
## Malformation Assessment

<table>
<thead>
<tr>
<th>Irregular Fins</th>
<th>Enlarged Organs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Missing Fin</td>
<td>Grumular Organs</td>
</tr>
<tr>
<td>Stunted Fin(s)</td>
<td></td>
</tr>
<tr>
<td>Acardia</td>
<td>Hemorrhage</td>
</tr>
<tr>
<td>Bradycardia</td>
<td></td>
</tr>
<tr>
<td>Tachycardia</td>
<td>Lumps/Tumors</td>
</tr>
<tr>
<td>Cardiac enlargement</td>
<td></td>
</tr>
<tr>
<td>Tube Heart</td>
<td>Jaundice</td>
</tr>
<tr>
<td>Brachycephalic</td>
<td>Kink in Tail</td>
</tr>
<tr>
<td>Dolichocephalic/Beak Face</td>
<td>Lordosis</td>
</tr>
<tr>
<td>Microcephalic</td>
<td>Scoliosis</td>
</tr>
<tr>
<td>Microphthalmia</td>
<td>Eel-Like Body</td>
</tr>
<tr>
<td>Ocular Edema</td>
<td>Emaciated</td>
</tr>
<tr>
<td>Under-Developed Jaw</td>
<td>Stunted Growth</td>
</tr>
<tr>
<td>Enlarged Otoliths</td>
<td></td>
</tr>
<tr>
<td>Distended Thoracic Region</td>
<td>Not Moving/Can't Swim</td>
</tr>
</tbody>
</table>
6-8 hr post fertilization

6 days post fertilization

Normal

Malformed
Distribution of Potencies

49% had AC$_{50}$ above 10 µM
80% had AC$_{50}$ above 1 µM

Number of Chemicals in Each Range

Range of AC$_{50}$s (µM)
Example Dose-Response Curves

**Milbemectin**

- AC50 = 0.1519
- AC10 = 0.0019

**Methyl Isothiocyanate**

- AC50 = 2.9635
- AC10 = 0.4966

**Butafenacil**

- AC50 = 0.0059
- AC10 = 0.0059

**Cypermethrin**

- AC50 = 0.3253
- AC10 = 0.0935
Concordance Among Replicates

- 3-lodo-2-propynylbutylcarbamate
- Bensulide
- Chlortrifos (ethyl)
- Chlorsulfuron
- Dibutyl phthalate
- Diclofop-methyl
- EPTC
- Fenoxaprop-ethyl
- Prolsulfuron

Office of Research and Development
National Health and Environmental Effects Research Laboratory, Integrated Systems Toxicology Division
Comparison of the present data with the zebrafish embryo toxicity data in the ECOTOX database as well as recently published papers on the toxicity of triazole derivatives (Hermsen, SA et al, 2011) □ = chemicals that were positive in the ECOTOX Database and in the present study; ● = chemicals that were positive in the ECOTOX Database, but negative in the present assay; ▼ = triazole derivatives tested in the above publications. The correlation line (dashed line) fit to the positive chemicals resulted in a slope 1.07 and $R^2=0.79$. 

Concordance with Previous Data
A. % Positive vs LogP Bin

-3 to 0  
0 to 1  
1 to 2  
2 to 3  
3 to 4  
4 to 5  
5 to 6  
6 to 8

% Positive Chemicals

B. AC50 vs LogP Bin

-3 to 0  
0 to 1  
1 to 2  
2 to 3  
3 to 4  
4 to 5  
5 to 6  
6 to 8

AC50 (uM)
Relationship between LogP and body burden of chemical in zebrafish embryos/larvae. These data are taken from Berghmans et al, 2008 (■ = mean of 3 dpf and 7 dpf measures); Gustafson et al, 2012 (△); and Thomas et al, 2009 (grey circle). Linear regression (—) of these combined data gives an equation relating the concentration in the embryo to the LogP of the chemical: % nominal concentration in the embryo/larva = antilog[-0.089 + 0.725(LogP)]. The r² of this linear regression is 0.81. The dashed line is the relationship between LogP and BioConcentration Factor (BCF) calculated by Petersen and Kristensen, 1998 where Log BCF= -0.46 + 0.86 (LogP) for a group of lipophilic compounds tested in zebrafish embryos and larvae.
Maximum likelihood phylogenetic tree of all zebrafish and human CYPs

Goldstone et al, 2010
<table>
<thead>
<tr>
<th>Zebrasfish</th>
<th>Human</th>
<th>Zebrasfish</th>
<th>Human</th>
<th>Zebrasfish</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A</td>
<td>CYP1A1/1A2</td>
<td>CYP3A65</td>
<td>CYP3A-se1,-se2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CYP19A1,2</td>
<td>CYP19A1</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>CYP1B1</td>
<td>CYP3C1-4</td>
<td>CYP3A3,4,7</td>
<td>CYP20A1</td>
<td>CYP20A1</td>
</tr>
<tr>
<td>CYP1C1,2</td>
<td>-</td>
<td>CYP4F43</td>
<td>CYP4F</td>
<td>CYP21A1</td>
<td>CYP21A2</td>
</tr>
<tr>
<td>CYP1D1</td>
<td>CYP1D1P</td>
<td>CYP4V7,8</td>
<td>CYP4V2</td>
<td>CYP24A1</td>
<td>CYP24A1</td>
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<tr>
<td>CYP2Ks</td>
<td>CYP2W1</td>
<td>CYP4T8</td>
<td>-</td>
<td>CYP26A1</td>
<td>CYP26A1/C1</td>
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<tr>
<td>CYP2N13</td>
<td>CYP2J2</td>
<td>CYP5A1</td>
<td>CYP5A1</td>
<td>CYP26B1</td>
<td>CYP26B1</td>
</tr>
<tr>
<td>CYP2Ps</td>
<td>CYP2J2</td>
<td>CYP7A1</td>
<td>CYP7A1</td>
<td>CYP26C1</td>
<td>-</td>
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<tr>
<td>CYP2R1</td>
<td>CYP2R1</td>
<td>CYP7B1</td>
<td>CYP7B1</td>
<td>CYP27A3-7</td>
<td>CYP27A1</td>
</tr>
<tr>
<td>CYP2U1</td>
<td>CYP2U1</td>
<td>CYP7C1</td>
<td>-</td>
<td>CYP27B1</td>
<td>-</td>
</tr>
<tr>
<td>CYP2V1</td>
<td>CYP2J2</td>
<td>CYP8A1</td>
<td>CYP8A1</td>
<td>CYP27C1</td>
<td>-</td>
</tr>
<tr>
<td>CYP2X1-10</td>
<td>-</td>
<td>CYP8B1-3</td>
<td>CYP8B1</td>
<td>CYP39A1</td>
<td>CYP39A1</td>
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<tr>
<td>CYP2Y3,4</td>
<td>CYP2A/B/F/S</td>
<td>CYP11A1,2</td>
<td>CYP11A1</td>
<td>CYP46A1</td>
<td>CYP46A1</td>
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<tr>
<td>CYP2AA1-12</td>
<td>-</td>
<td>CYP11C1</td>
<td>-</td>
<td>CYP46A2,4,5</td>
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<tr>
<td>CYP2AD2,3,6</td>
<td>CYP2J2</td>
<td>CYP17A1,2</td>
<td>CYP17A1</td>
<td>CYP51A1</td>
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<tr>
<td>CYP2AE1,2</td>
<td>-</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> pseudogene (single remnant exon)
Figure 4 Expression of 88 CYP genes in zebrafish during the first 48 hours of development. Single color microarray analyses of CYP gene expression throughout early development (3-48 hours post fertilization, hpf) shows that while some CYP genes are expressed in the whole embryo at high levels, most CYP genes are expressed at levels significantly above background (~5 fluorescent units; see Additional File 2, Table S3). Strong temporal signals are apparent.
Figure 7 Expression of four CYP genes in unfertilized oocytes. The maternal contribution to transcript abundance of selected CYPs was determined using qPCR on unfertilized oocytes. Eggs were expressed from gravid female zebrafish (n = 3) by gentle squeezing of anesthetized fish. Data was normalized using ARNT2.
<table>
<thead>
<tr>
<th>Parent</th>
<th>Metabolite</th>
<th>Outcome</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl Phthalate</td>
<td>Methyl Hydrogen Phthalate</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Atrazine</td>
<td>6-Deisopropylatrazine</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Methoxychlor</td>
<td>2,2-Bis(4-hydroxyphenyl)-1,1,1-Trichloroethane (HPTE)</td>
<td>+ 2.63</td>
<td>+ 24.68</td>
</tr>
<tr>
<td>Metam-Sodium</td>
<td>Methyl Isothiocyanate</td>
<td>+ 21.63</td>
<td>+ 2.96</td>
</tr>
<tr>
<td>Diethylhexyl Phthalate</td>
<td>Monoethylhexyl Phthalate</td>
<td>—</td>
<td>+0.5665</td>
</tr>
<tr>
<td>Metiram-Zinc</td>
<td>Ethylenethiourea</td>
<td>+ 1.44</td>
<td>—</td>
</tr>
<tr>
<td>Maneb</td>
<td>Ethylenethiourea</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Mancozeb</td>
<td>Ethylenethiourea</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Dibutyl Phthalate</td>
<td>Monobutyl Phthalate</td>
<td>+ 1.46</td>
<td>—</td>
</tr>
<tr>
<td>Malathion</td>
<td>Malaoxon</td>
<td>+ 23.5</td>
<td>—</td>
</tr>
<tr>
<td>Diazinon</td>
<td>Diazoxon</td>
<td>—</td>
<td>+ 28.99</td>
</tr>
<tr>
<td>Chlorpyrifos (Ethyl)</td>
<td>Chlorpyrifos-oxon (Ethyl)</td>
<td>+ 8.5</td>
<td>+ 0.4</td>
</tr>
</tbody>
</table>
Main Conclusions

- The majority (62%) of ToxCast Phase 1 chemicals were toxic to the developing zebrafish.

- Both toxicity incidence and potency were correlated with chemical class and hydrophobicity (logP)

- Need to understand dose, which is related to logP
  - Inter-and intra-plate replicates showed good agreement.
  - Hepatic metabolism is present.
Measures

- Area
- P2A
- LWR
- Head Tail Distance
- Spine length
- Width
- Straightness
- Convexity
- Curvature

Frady, Houck, Wambaugh, Judson, Radio and Padilla, in preparation
Comparison of Human Visual Assessment with Cellomics Automated Assessment of Larvae

Cellomics Tox Score

Visual Assessment

- Normal
- Abnormal
- Severely Abnormal

p < .0001

p = .006
Lessons Learned and Future Directions

• Larval zebrafish assay has excellent reproducibility even with n=2-3.
• Good correlation between single high dose study and dose response study.
• Larval zebrafish have metabolic capability.
• Larval zebrafish assay may correlate with mammalian assays, but it won’t be simple. (Sipes et al, 2011)
• Future Directions
  – Automated assessment of dysmorphology
  – ToxCast Phase II
Function

- Integration of Development
  - Spatial and temporal aspects of nervous system development
- Functional Assessments
- Sensory Assessments
  - Threshold
- Learning and Memory

Behavior

Take advantage of the whole animal approach
General Experimental Approach

Day 0
Eggs collected, Exposure begins About 6 hours After fertilization

Day 1
Solutions Renewed

Day 2
Solutions renewed

Days 3 & 4
Hatching Solutions renewed

Day 5
Fish removed from chemical solutions

Day 6
Teratological and Behavioral Assessment

All eggs/larvae kept at 26°C with 14:10 hr light:dark cycle.
Decision Tree for Day 6 Larvae

No Alive? Yes

Hatched?

No Terata Score ≤3? Yes

Behavioral Testing

Note: No testing reported on abnormal larvae
Activity in the Light
overall effect of malformation  p=.0002

Activity in the Dark
overall effect of malformation score p=.014

Degree of Malformation

Mean Activity (cm traveled)

<table>
<thead>
<tr>
<th>Degree of Malformation</th>
<th>Mean Activity (cm traveled)</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal</td>
<td>4</td>
</tr>
<tr>
<td>mildly abnormal</td>
<td>3</td>
</tr>
<tr>
<td>obviously deformed</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Degree of Malformation</th>
<th>Mean Activity (cm traveled)</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal</td>
<td>10</td>
</tr>
<tr>
<td>mildly abnormal</td>
<td>15</td>
</tr>
<tr>
<td>obviously deformed</td>
<td>5</td>
</tr>
</tbody>
</table>
Developmental Heptachlor

Elapsed Time (min)

Locomotor Activity (cm/2min)

Control n=48
Developmental Heptachlor

Locomotor Activity (cm/2min)

Elapsed Time (min)

Control n=48

1.2 uM n=24
Developmental Heptachlor

Elapsed Time (min)

Locomotor Activity (cm/2min)

Control n=48
1.2 uM n=24
2.1 uM n=23
Developmental Heptachlor

Elapsed Time (min)

0 10 20 30 40 50 60

Locomotor Activity (cm/2min)

0 5 10 15 20 25 30

Control n=48

1.2 uM n=24

2.1 uM n=23

3.7 uM n=23
Developmental Heptachlor

Locomotor Activity (cm/2min)

Elapsed Time (min)

Control n=48
1.2 uM n=24
2.1 uM n=23
3.7 uM n=23
6.6 uM n=18
Specific Accomplishments

1. We have developed a convenient method for assessing behavior in larval zebrafish in 96-well plates.
   - Zebrafish larvae respond differently to light and dark conditions.
     • Toxic chemicals may have different effects on each
   - An optimal test is at least 50 minutes under both light and dark conditions.
   - Need to take into consideration
     • Dysmorphology

2. Thus far, the behavioral test is capable of identifying developmental neurotoxicants, and non-neurotoxic chemicals.
   With 24 chemicals tested so far we have a Sensitivity of 82% and a Specificity of 80%.