Assessment of the Zebrafish Model for Developmental Toxicology Screening

Karen Augustine, Ph.D.
Discovery Toxicology
Bristol-Myers Squibb

Disclaimer

Zebrafish Model

- Aligns well in context of the 3Rs
- Inexpensive husbandry and assay costs
- Embryos/larvae can be cultured
- Short organogenesis process
- Transparent embryos and larva
- Cross-species conservation of developmental processes and pathways
- Extensive evaluation of genotype / phenotype, applied to evaluate teratogenic mechanism

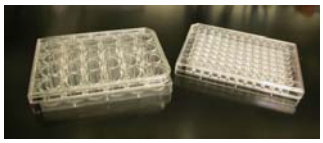
(Gustafson et al. Poster in Teratology Conference June, 2010)

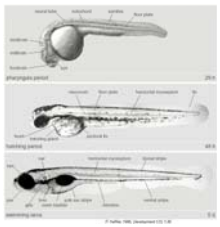
Zebrafish Developmental Toxicity Screens

Fish embryos

Add embryos to multi-well plates containing medium + compound

One or more fish embryos per well





Incubate at 28.5°C
For designated period

Evaluate embryo development at desired stages

How BMS Zebrafish Assay Design was Approached: B*asic Criteria*

- Strain: AB wild type
- Embryo chorion condition: Off
- Compound administration time: 4-6 hours post fertilization
- Compound test concentrations: 0.1~1000μM (or depends on LC25 and solubility)
- Compound selection:
  - Training set: 31compounds with in vivo teratogenicity data
  - Relatively balanced ratio of teratogens vs. non-teratogens
  - Diverse targets of pharmacologic classes / diverse chemotypes

How Assay Design was Approached (cont.): (*Variables Evaluation)*

- Selection of Morphological evaluation stages
  - Embryonic vs. larval stage / day 1 vs. day 5 evaluation
- Measurement of toxicity
  - IC50 – surrogate cell line (NIH3T3)
  - LC25 & LC50 – embryo-larvae general toxicity
- Establishment of morphological and functional endpoints
- Evaluation the mode of dysmorphology calls
  - Positive / negative calls
  - Numerical score system

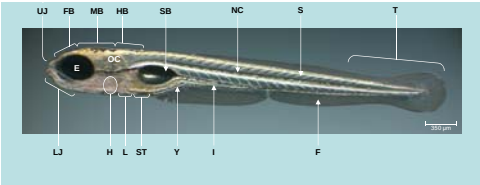
Morphological Score System

Morphological endpoints:

Heart, spinal cord, somite, notochord, brains, arches, jaws, tail, fins, face, stomach, liver

Growth Measurements:

Body length, body shape, somite numbers, yolk ball, swimming bladder, pigmentation



Morphological Score Guideline

5 = Structure is entirely normal for developmental stage

4 = Subtle variation / anomaly, recoverable developmental delay or anomaly

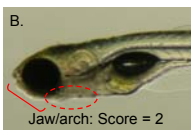
3 = Structure has one mild abnormality

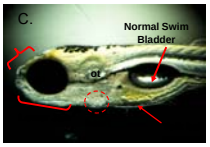
2 = Moderate malformations with 2 or more abnormalities

1 = Severe malformations with multiple abnormalities.

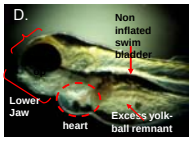
0.5 = structure is not evident by gross morphology assessment

Examples of Craniofacial and Visceral Dysmorphology and Assigned Scores





Structural Scores: 5 (normal)



Structural Scores: 2 or 1
(moderate or severe dysmorphology)

Birth Defect Research (Part B) 98, 2010

Initial Assessment

- Evaluation of endpoints
  - Worked with 18 compounds
  - Analyzed number varied (15-18) due to compound precipitation which led to undetermined results
- Determination of zebrafish NOAEL
  (no observed adverse effect level)
  - Assessed on both 30 hpf and 5 dpf
- Selection of approaches for toxicity identification
  - NIH 3T3 IC50 (fibroblast cell line)
  - Zebrafish embryo/larvae LC25 / 50
- Teratogenic Index for classification of *in vivo* teratogenic potential
  - 3T3 IC50 : NOAEL
  - Zebrafish LC25/LC50 : NOAEL

Initial Results of Toxicity Assessment

NOAEL Assessment	Toxicity Assessment	Total Concordance of correct classification
30 hpf	IC50 (NIH3T3 cells)	80%
30 hpf	LC25 zebrafish embryo viability	87%
5 dpf	IC50 (NIH3T3 cells)	86%
5 dpf	LC25 zebrafish embryo-larvae viability	93%

Final Study Design for Preliminary Assessment

- Compound test concentrations:
  - 0.1, 1, 10, and 100 µM
- Viability and morphological assessment stage:
  - 5 day post fertilization
- Teratogenicity prediction method:
  - LC25 : NOAEL (Zebrafish larva)

  If the ratio are:
  - LC25 : NOAEL ≥ 10, → teratogenic
  - LC25 : NOAEL < 10, → non teratogenic
  - LC25 and NOAEL > 100 µM → non teratogenic
- Morphological evaluation method
  - Used score system to define NOAEL

Summary of Assay Performance

	Classified as Non-teratogen	Classified as Teratogen
True Non-Teratogen	acebutolol acrylamide ascorbic acid bisphenol A BMS-1 camphor clozapine glybenclamide isoniazid penicillin G saccharin	dimethyl phthalate BMS-2
True Teratogen	BMS-10 valproic acid	5-fluorouracil hydrocortisone 9-cis-retinoic acid ATRA hydroxyurea phenobarbital Retinol BMS-3 BMS-4 BMS-5 BMS-7 BMS-8 BMS-11 BMS-12 BMS-13 BMS-14

Non Teratogen Concordance: 11/13 = 85%
Teratogen Concordance: 16/18 = 89%
Total Concordance: 27/31 = 87%

Comparison of Zebrafish Wild Types

Classification	A/B strain	Pond Fish
Correct Classification of Non Teratogens	13 Non-teratogen 11/13 Correct = 85%	13 Non-teratogens 10/13 Correct = 77%
Correct Classification of Teratogens	15 Teratogens 13/15 Correct = 87%	16 Teratogens 15/16 Correct = 94%
Total Concordance	28 Compounds 24/28 Correct = 86%	29 Compounds 25/29 Correct = 86%

Summary of Preliminary Zebrafish Teratogenicity Assay

- **Promising concordance**
- **Follow up studies needed**:
  - *Expand the compound set*
  - *Test different strains of wild types*
  - *Compare chorion off vs. on condition*
  - *Optimize / simplify the assay*
  - *Conduct inter-laboratory assessments*

Zebrafish Consortium Effort

- **Goals:**
  - Establish a harmonized zebrafish developmental toxicology assay
  - Identify an alternative prediction model to simplify the assay
  - Adapt at least part of assay to an automated screening platform

Zebrafish Consortium Phase I Study

Phase I: Ring Test 1

- Based upon protocols used by consortium members
  - Chorion on assay
  - Test concentrations: 0.1, 1, 10, 100 and 1000µM; pH adjustment of compound stocks as needed
  - 12 embryos/concentration
    - 1 embryo/well,
    - 12 larva read for viability
    - 8 viable larva randomly selected for scoring
  - Inter-experimental controls: All-trans-retinoic acid (positive), Saccharine (negative)
  - Viability and score assessment performed on day 5 post fertilization
    - Used BMS morphological score system and LC_{25}/NOAEL for teratogenic classification
    - LC_{25}/NOAEL ≥10: positive for teratogenic liability)

Ring Test 1: Interlaboratory assessment

- 20 non-proprietary compounds evaluated as blinded
- Run at 4 labs
- 3 wild types included –pond fish (Labs A and B), WIK (Lab C) and proprietary strain (Lab D)

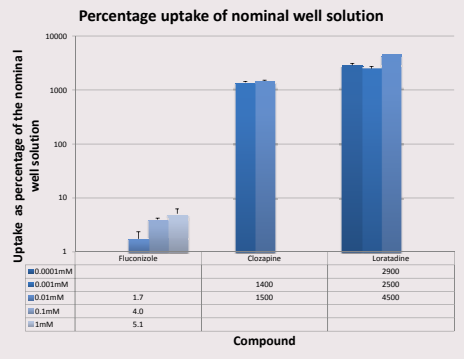
Ring Test I Results

Compound	Mammalian classification	Classification based on result from zebra fish assay			
		Lab A	Lab B	Lab C	Lab D
Chlorathalideone	N	N	N	N	T
Ascorbic Acid	N	N	N	N	T
Lovastatin	T	T	T	T	T
Thalidomide	T	N	T	T	T
Clozapine	N	T	T	T	T
Fluconazole	T	N	N	N	T
Methotrexate	T	T	N	T	T
Glybenclamide	N	N	T	N	N
Hydrochlorothiazide	N	N	N	N	N
5-Azacitidine	T	T	T	T	T
Cefotaxime	N	N	N	N	T
Dexamethasone	T	T	T	T	T
Dimethadione	T	N	T	N	N
Loratadine	N	T	T	T	T
Metoclopramide	N	N	N	T	T
Odansetron	N	N	N	T	N
Penicillamine	T	N	N	N	N
Sulfasalazine	N	N	T	N	N
Topiramate	T	T	N	T	T
Warfarin	T	T	T	T	T
Correctly predicted		14/20	12/20	12/20	12/20
Number of false positives		2	4	4	6
Number of false negatives		4	4	4	2

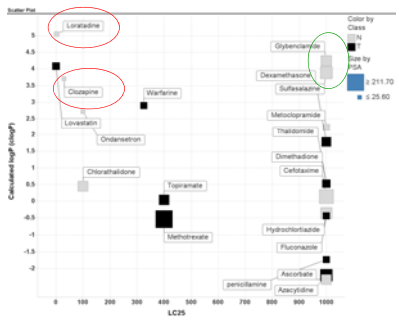
Ring Test I Results

- Total concordance was 60-70% for correct classification of teratogens/ non-teratogens
  - Results agreed at a rate of 75 – 100% between the 4 laboratories with the exception for metoclopramide (50%).
  - 15% compounds misclassified by all labs
  - 25% compounds correctly classified by all labs
- Precipitation and drug induced pH shifts were observed at the top concentration (1000µM) could have influenced the assay performance.

Compound uptake may influence viability and embryonic development



Compound logP values did not correlate with in vivo teratogenicity



Protocol Optimization

Potential gaps identified in first study:

- Concentration range/Teratogenic Index
  - Cases of too high and not low enough
  - Is there a need to incorporate concentration adjustments in some cases?
- Compound instability
  - Thalidomide/dimethadione known to be unstable
  - Formulation with pH adjustment rate limiting
- Variations in compound uptake
  - Reconsider chorion permeation
- Lack of replicate experiments within each lab
  - Would replicate experiments improve classification?

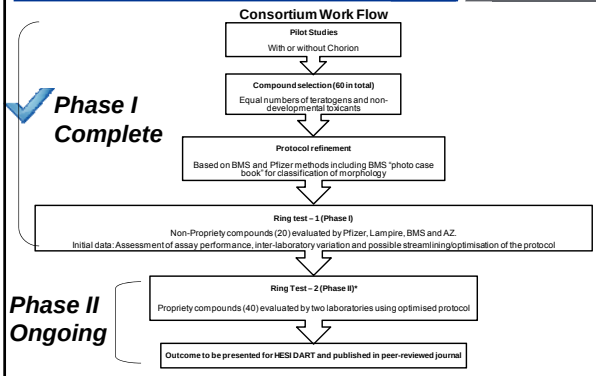
Optimized Protocol

Same framework as original protocol with the following adjustments:

• Concentration range:	• 0.1 – 100 µM
• pH adjustment:	• No
• Compound administration:	• within 30 minutes after embryo plating
• Concentration adjustment:	• when there is a steep lethality curve
• Chorion condition:	• protease treatment of intact chorion
• Biological replicates:	• 2 - 3

Compound	Replicate 1	Replicate 2	Replicate 3	Final Classification
Hydrochlorothiazide	N	N		N
Metoclopramide	N	N		N
Sulfasalazine	T	N	T	T
Warfarin	T	T		T
Dimethadione	T		T	T
Fluconazole	T		T	T
Ascorbic Acid	N	T	N	N
Chlorathalideone	N	T	N	N
5-Azacytidine	N	T	T	T
Cefotaxime	N	N		N
Clozapine**	T	T	T	T
Dexamethasone	T	T		T
Glybenclamide	N	N		N
Loratadine*	T	T	T	T
Lovastatin*	T	N	T	T
Methotrexate	T	T		T
Odansetron	N	N		N
Penicillamine	N	T	T	T
Thalidomide	N	T	T	T
Topiramate***	T	T	T	T
Total Correct	14/20	12/18	9/12	17/20

Ongoing Activities



Summary

- The zebrafish embryo/larva is promising as an animal alternative that could be used in developmental toxicology assessment
- Multiple studies suggest good to very good concordance for predicting in vivo teratogenic outcome
- *Existing challenges:*
  - Achieving complete automation for HTS
  - Overcoming compound solubility/uptake limitations
  - Keeping the assay classified as "in vitro" under evolving animal welfare regulations

Acknowledgements

BMS Zebrafish Assay Development:

- Kimberly Brannen; Tracy Danberry; Julie Panzica-Kelly; Karen Augustine

Zebrafish Consortium Participants:

- **Abbott**
  Belen Tornesi, Brian Enright
- **AstraZeneca:**
  Anne-Lee Gustafson, Malcolm Hetheridge, Jon Ball
- **BMS:**
  Cindy Zhang, Julie Panzica-Kelly, Karen Augustine, Lois-Lehman McKeeman
- **Pfizer:**
  Don Stedman, Aleasha M. Coburn, Bob Chapin
- **Lampire Biological Laboratories:**
  Jedd Hillegass, Annette Flood, Lampire In Vitro Developmental Toxicology Team