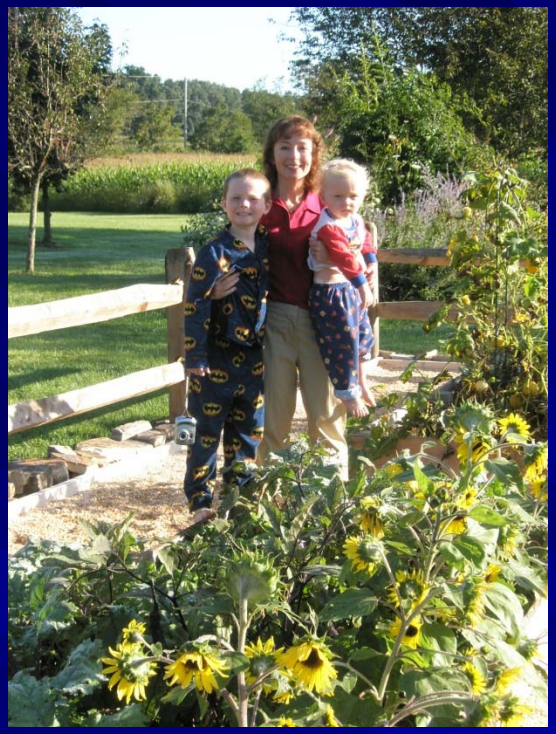
Generation and Preliminary Assessment of a Zebrafish Teratogenicity Assay

An Overview of How BMS Generated the Assay



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Zebrafish as a Developmental Toxicology Model

Intriguing Attributes:

- Relatively good conservation of embryological processes and molecular pathways
- Genotype-phenotype evaluation has been extensively evaluated-promising for “on and off-target” teratogenic assessment
- Fertilized eggs/embryos can be easily grown in vitro for several days
- Organogenesis completed within a couple days/various structures and organ systems clearly evident by 4-5 days in the larva stage
- Embryos and larva are relatively transparent facilitating morphological assessment
- Aligns well with the 3 R’s
- Relatively inexpensive husbandry and assay costs

As such, various laboratories have been exploring the use of zebrafish embryos as model for development toxicology assessment

How Assay Design Was Approached: *Basic Criteria*

- Used AB strain wild types
- Eliminated a potential barrier for compound uptake:
  - Chorion-off embryos (pronase treatment & manually dissected)
- Onset of compound administration:
  - 5-8 hr post fertilization (hpf) embryos *i.e.* similar developmental stage (gastrulation-stage) as in in vivo EFD studies
- Test concentration:
  - Wide range of concentrations tested (typically up to 1000uM) tailored to LC25/LC50 profiles and compound solubility limitations
- Compound selection
  - ~31 compounds were assessed in total
  - Similar ratio of non teratogens and teratogens
  - Represented diverse target pharmacology classes/chemotypes
    - Included compounds from the ECVAM list of definitive teratogens and non teratogens
    - Marketed compounds that were either not teratogenic in at least 2 test species or teratogenic in at least 1 test species
    - About half were BMS proprietary compounds that were evaluated in in vivo teratogenicity studies

How Assay Design was Approached:
Variables Evaluated

- Embryonic vs. larval stage for morphological assessment:
  - Initially examined morphology over 5 day period
    - Selected 2 stages that would support up to 2-3 assays per week with no weekend requirement
    - 30hpf and 5 day post fertilization (dpf) selected
  - Identified stage that provided the best concordance for correctly classifying teratogens and non teratogens
- Classification of Teratogenic Potential- toxicity: teratogenicity ratios
  - Explored the utility of a surrogate cell line (NIH3T3) for establishing an IC50 endpoint to represent “adult toxicity”
  - Explored the utility of using a LD25 and/or LD50 endpoint as a measurement of embryo-larva general toxicity
- Explored a battery of morphological and functional endpoints
- Evaluated mode of evaluating dysmorphology calls
  - +/- call
  - Numerical score system based upon severity of dysmorphology

Initial Assessment

- Worked with 15 compounds- majority were teratogens due to early emphasis on identifying endpoints for sensitivity
- Determine zebrafish NOAEL concentrations assessed at 30 hpf and 5 dpf
- Compared NIH3T3 IC50:zebrafish NOAEL ratios vs. LC50 /LC25 : Zebrafish NOAEL ratios on both evaluation days
  - Ultimately pursued LC25:Zebrafish Embryo-Larvae NOAEL ratios in comparative assessment due to limitations in achieving LC50 for various compounds (compound solubility limitations, etc)

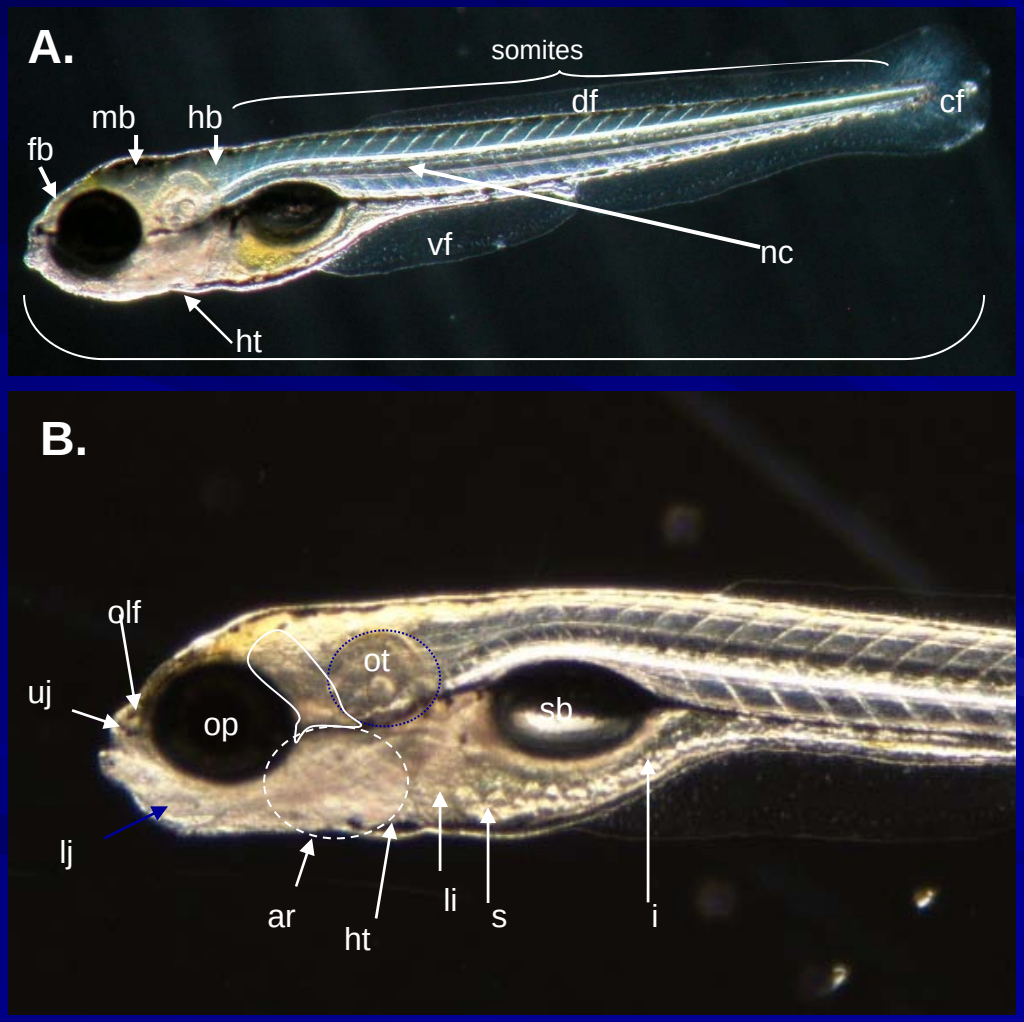
Initial Assessment Results

Zebrafish Stage of NOAEL Assessment	Surrogate Adult Toxicity/ General Toxicity Assessment	Total Concordance: Correct Classification of Teratogens and Non Teratogens
30 hours post fertilization	IC50 (NIH3T3 cells)	80%
30 hours post fertilization	LC25 zebrafish embryo viability	87%
5 days post fertilization	IC50 (NIH3T3 cells)	86%
5 days post fertilization	LC25 zebrafish embryo-larvae viability	92%

Final Study Design for Preliminary Assessment

- At least 4 test concentrations per compound
- Viability and morphological assessment conducted on day 5
- LC25: Zebrafish Larva NOAEL ratio used in analysis
- Morphological score system utilized
  - Found to increase sensitivity for identifying some teratogens
  - Individual calls vs. group averages found to better define NOAELs and identify concentration-response relationships
  - Score data also used for statistical model generation
- LC25: Zebrafish NOAEL ratio $\geq$10: positive classification for teratogen; <10: classification for non teratogen
- LC25>100µM/Zebrafish NOAEL >100µM : default to non teratogen classification

Morphology of a Day 5 Post Fertilization Zebrafish Larva with Assessed Structures



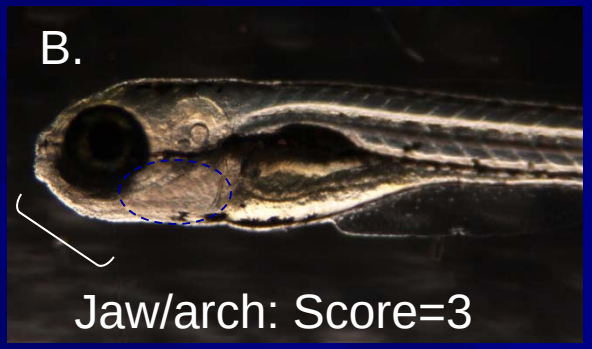
Morphological Endpoints Evaluated in Assay

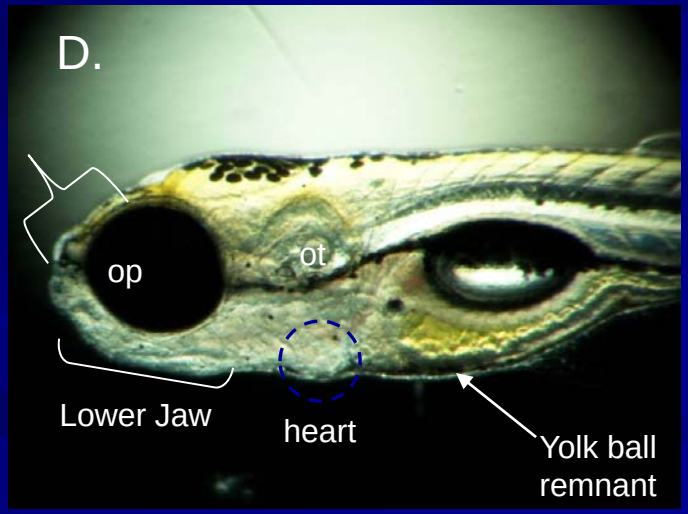
Morphological Endpoint	Method of Assessment
body length*	Measured in millimeters
body shape	Morphological score and description
viability	% incidence in treatment group
head-trunk angle	Measured in degrees
otic vesicle length	Estimation of distance between eye and otic vesicle
somite number	Somite pairs counted
somite morphology	Morphological score and description
notochord morphology	Morphological score and description
tail morphology	Morphological score and description
fin morphology	Morphological score and description
heart morphology	Morphological score and description
facial structure morphology	Morphological score and description
brain morphology	Morphological score and description
jaw & pharyngeal arch morphology	Morphological score and description
motility	Normal/abnormal and description
pigmentation *	Normal/abnormal and description
swim bladder*	Normal/abnormal and description
stomach*	Normal/abnormal and description
intestine	Normal/abnormal and description
liver*	Normal/abnormal and description
heart rate	Counted in beats per minute

Red text: endpoints ultimately not used In determining Zebrafish Larva NOAEL

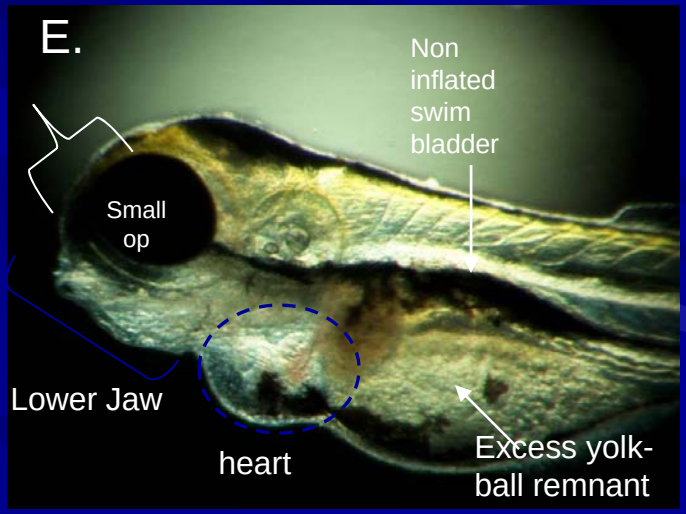
*Endpoints continued to be Tracked during assessment As a means to cross-reference Staging or to track for potential Hepatic/gastric toxicity

Examples of Craniofacial and Visceral Dysmorphology and Assigned Scores





Structural Scores: 5 (normal)



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

Note that you made edits To metrics

Summary of Assay Performance: Preliminary Assessment

- ***Total Concordance***:
  - 28/31X100 = 87%
- ***Non Teratogen Concordance:***
  - 11/13X100 = 85%
- ***Teratogen Concordance:***
  - 16/18x100 =89%
- ***Negative Predictivity:***
  - 11/13x100 = 85%
- ***Positive Predictivity:***
  - 16/18x100 =89%
- ***Tissue-based precision*:***
  - 23/31X100= 74%

	Predicted Non Teratogen	Predicted Teratogen	Total
True Non-Teratogen	11	2	13
True Teratogen	2	16	18

*At least 1 affected structure identified in zebrafish was also reported as a malformation in mammals in vivo

Classification of Compounds in Zebrafish Teratogenicity Assay

	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 all-trans-retinoic acid BMS-3 BMS-4 BMS-5 BMS-7 BMS-8 BMS-11 BMS-12 BMS-13 BMS-14 hydroxyurea phenobarbital Retinol

* BMS-6 and BMS-9 could not be classified due to compound solubility limitations

Summary

- Preliminary assessment suggests the present assay design holds promise as a relatively robust in vitro teratogenicity assay
- However, study needs to be followed up with more extensive evaluation to fully characterize predictive power of the assay
  - Expand the test set
  - Evaluate consistency in results between strains as well as laboratories
  - Explore compound uptake and general necessity of manual removal of the chorion
    - This step limits automation of the assay
  - Explore ways to modify/reduce extent of morphological assessment
    - Further investigation into statistical prediction models

Zebrafish Consortium: Background

- A number of laboratories have been exploring the zebrafish as a model for developmental toxicology assessment using assays developed in-house or run by biotech companies
- A cross-pharma consortium (BMS, Pfizer, Amgen and AstraZeneca) has been established to conduct some exploratory studies to develop an optimized assay and evaluate assay performance
- The goal is to establish a harmonized zebrafish developmental toxicology assay that could be executed across pharma
  - Intent for early screening and internal decision making (that target and chemistry looks clean with the limitations of the assay)
- Intend to use the BMS protocol, perhaps adjusted slightly, and simply adding many chemicals to it
- Results from this assessment are planned to be shared with HESI next year
- From the regulatory perspective, the long-term intent is to build a deeper knowledge of the zebrafish assay, in context of
  - Building familiarity of the assay's predictivity (such as concordance and precision metrics) based upon a rigorous dataset
  - How the assay is applied in proactive safety assessment
  - The context of justification for inclusion or lack of inclusion of WoCBP populations in such exploratory studies based upon assay results and other assessments.

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- Tracy Danberry
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Consortium Members:

- Amgen:
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- AstraZeneca:
  - Anne-Lee Gustafson, Malcolm Hetheridge
- BMS:
  - Karen Augustine, Lois-Lehman-McKeeman, Julie Panzica-Kelly, Cindy Zhang
- Pfizer:
  - Bob Chapin, Don Stedman
- Lampire Biological Laboratories:
  - Gregory Krug, Annette Flood, Lampire In Vitro Developmental Tox Team

Supplemental Slides

Consortium Plans (2009-2010)

- 1. *Comparative data assessment and assay design generation:*
  - Evaluate study designs/concordance metrics of zebra fish developmental tox assays executed by the respective groups
  - Develop an experimental study design and identify a test set of compounds.
- 2. *Execute small pilot study to address compound uptake questions/assess benefit of removing the chorion:*
  - Using the consensus experimental design, conduct a small pilot study that includes duplicate compound treatment groups that includes chorion-on and chorion-off embryos.
  - Have content analysis conducted to determine whether the presence of the chorion leads to mis-classfication due to compound exclusion.
- 3. *Begin consortium validation effort using optimized experimental design:*
  - Complete a final list of compounds to enroll in the zebrafish assay and begin screen assessment.
  - Studies will be conducted at Lampire, Pfizer, AZ and BMS to execute validation as well as test inter-laboratory variability of a selected subset of compounds.
  - Further evaluate data by statistical prediction model assessment to determine feasibility of a cut-down morphological assessment design
- 4. *Report results of study*
  - At this time bring the results to HESI DART for discussion, possibly consider some engagement/opinion from regulatory agencies, as such studies may have increasing presence in INDs or IBs.
  - Generate a manuscript that describes the optimized procedure and the results of the consortium study.