Modeling Zebrafish Developmental Toxicity using a Concurrent *In vitro* Assay Battery

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Introduction

Goal: Determine mode of action (MOA) or molecular initiating event (MIE) for chemicals causing zebrafish developmental toxicity

Approach

- Used data from zebrafish developmental assay on 1060 chemicals
- Combined zebrafish data with *in vitro* data from hundreds of assays
- Added information from the literature on known molecular targets or MOA of chemicals
- Identified candidate MOA where a large fraction of MOA-associated chemicals are active in the zebrafish assay (ZF+)
- Determined which chemicals may be ZF+ solely because of generalized cell stress or cytotoxicity

Methods

Zebrafish assay data: AC50s (uM concentration at which the endpoint reaches 50% of its maximum value) from a zebrafish teratogenicity assay (Padilla, Corum et al. 2012, Deal, Wambaugh et al. 2016) was determined where significant activity occurred at concentrations below the maximum concentration tested (80 uM). Otherwise chemicals were called negative (no AC50 value determined).

***in vitro* assay data**: ToxCast data (Judson, Houck et al. 2016) was used to identify active chemical-assay relationships. This includes a set of cell stress and cytotoxicity assays. AC50s were determined for active chemical-assay combinations. A median log concentration for cytotoxicity for each chemical was determined from a set of 33 cytotoxicity assays. For each active combination, a Z-score was determined, equal to (log(AC50)-median log(AC50, cytotoxicity))/ 0.28. High Z-scores indicate that activity occurs below cytotoxicity / cell stress, so is not confounded by those effects.

ToxCast gene targets: ToxCast assays (Judson, Houck et al. 2016). were classified by their gene target, and target groups were combined by subtype (e.g. estrogen receptors ESR1 and ESR2 were both included in the ER group). Target groups with at least 3 assays were included. For a chemical to be called positive against the target group, greater than half of the target group assays had to be positive with Z-score>2. If a chemical was positive against a target group, the median log(AC50) values from the active assays was calculated.

Stress % determination: Assays for cytotoxicity, oxidative stress, mitochondrial disruption, microtubule disruption, ER stress, apoptosis and heat shock were compiled, and the % of these assays active for a chemical was designated as the stress%. The total number of assays included in the stress% metric was 60. See Figure 2.

Reference chemical targets: We developed a database of chemical targets and MOA from public sources including DrugBank, the Therapeutic Target Database TTD (TTD), the Open Targets Database (OTD), and in-house manual curation efforts. As above, targets were grouped into target classes.

Determination of candidate MOA: For each ToxCast or Reference target or MOA, we calculated the fraction of chemicals with MOA activity (MOA+) that were also ZF+. MOA with fraction>75% were considered candidates (overall there was a 47% ZF+ rate).

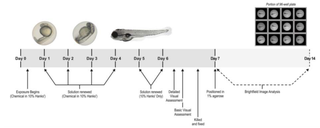


Figure 1: Schematic of zebrafish dosing schedule

References

1. Padilla, Corum et al. 2012, Reproductive Toxicology, **33**, 174-187
2. Judson, Houck et al. 2016, Toxicological Sciences, **152**, 323-339
3. Deal, Wambaugh et al. 2016, J Applied Toxicology, **36**, 1214-1222
4. DrugBank: https://www.drugbank.ca/
5. TTD: Therapeutic Target Database: http://bidd.nus.edu.sg/group/cjttd/
6. OTD: Open Targets: https://targetvalidation.org/

Stress Observations

We observed a strong trend between logP and both cell stress / cytotoxicity and zebrafish embryotoxicity (Figures 3 and 4). This indicates that some embryotoxicity may be caused by non-specific cell stress effects which may be modulated by lipid solubility or bioavailability. By modeling zebrafish potency, we can see chemicals with "excess toxicity", (those with effects at concentrations much lower than where cell stress is seen) (Figure 4). These are candidates for finding specific MOAs.

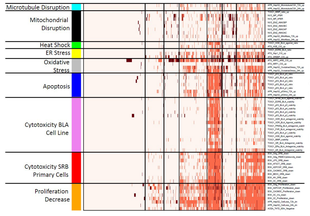


Figure 2: Stress and cytotoxicity activity across the 1060 chemicals. From (Judson, Houck et al. 2016). The 1060 chemicals are arrayed on the x-axis. An active chemical-assay pair is colored red, with more potent pairs being darker red.

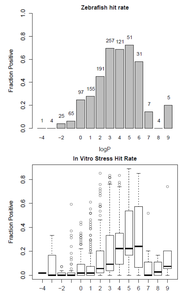


Figure 3: Trends with zebrafish activity (top) and cell stress (bottom) as a function of logP, showing similar peaks in activity at intermediate values of logP.

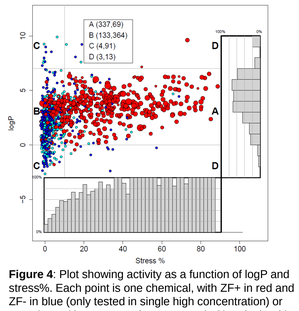


Figure 4: Plot showing activity as a function of logP and stress%. Each point is one chemical, with ZF+ in red and ZF- in blue (only tested in single high concentration) or cyan (tested in concentration-response). Chemicals with intermediate logP values and high levels of stress are mostly ZF+. A, B, C and D are regions with ranges of stress and logP indicated by the gray lines

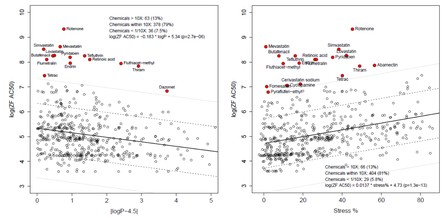


Figure 5: Results of linear models of zebrafish potency vs. logP (left) and stress% (right), indicating outliers, showing "excess toxicity" (active concentration is significantly below that expected from just stress or logP). One highlighted class of chemicals are statins (HMGCR antagonists), which are known developmental toxicants in zebrafish. Y-scale is negative log(micromolar), so 4 is equivalent to 100 uM, 6 to 1 uM, 8 to 0.01 uM, etc.

Candidate MOA

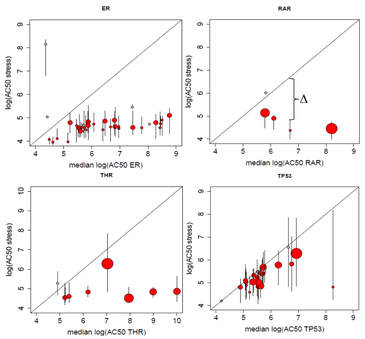
Table 1: Candidate MOA from the reference database. These MOA have 75% or greater ZF+ rate. Examples of negative associations are chemicals targeting cholinergic nicotinic receptors, HIV targets and phosphodiesterases. Chemicals can have more than one candidate MOA. [***] p<0.001, [**] p<0.01, [*] p<0.05 in a chi-squared test. Ordered alphabetically by target.

Target	Description	Target-active chemicals	Zebrafish and Target active chemicals	Fraction positive
ACCase	Plant Acetyl CoA Carboxylase (lipid synthesis inhibitors)	11	10	0.91[**]
ACHE	Acetylcholinesterase	55	41	0.75 [***]
AR	Androgen receptor	14	12	0.86 [**]
ER	Estrogen receptor	29	25	0.86 [***]
HMGCR	HMG-coA reductase	8	6	0.75
HTR2A	Serotonin receptor 2A	5	4	0.80
ion channel	General ion channels	33	27	0.82 [***]
ion channel (Na)	Sodium ion channels	22	19	0.86 [***]
lipid synthesis	Lipid synthesis targeting (includes sterol synthesis)	38	30	0.79 [***]
microtubule	Microtubule-targeting	20	18	0.90 [**]
mitochondria	Mitochondria targeting	21	21	1.00 [***]
PGR	Progesterone receptor	5	4	0.80
PPO	Plant Protoporphyrinogen Oxidase (lipid membrane disruption)	13	11	0.85 [**]
sterol synthesis	Sterol synthesis targeting	24	23	0.96 [***]
THR	Thyroid hormone receptor	4	4	1.00 [*]
tubulin	Tubulin (microtubule) targeting	7	7	1.00 [**]

Table 2: Candidate MOA from the in vitro database. These MOA have 75% or greater ZF+ rate. Examples of negative associations are chemicals targeting adenosine receptors and caspases.
[***] p<0.001, [**] p<0.01, [*] p<0.05 in a chi-squared test. Ordered alphabetically by target.

Target	Description	Target-active chemicals	Zebrafish and Target active chemicals	Fraction positive
ADRB	Beta adrenergic receptors	6	6	1.00 [*]
AR	Androgen receptor	18	15	0.83 [**]
CYP1	CYP450, family 1	4	4	1.00 [*]
CYP2	CYP450, family 2	13	13	1.00 [***]
CYP3	CYP450, family 3	24	22	0.92 [***]
DRD	Dopamine receptors	15	12	0.80 [*]
ER	Estrogen receptors	35	30	0.86 [***]
mitochondria	Mitochondria targeting	4	3	0.75
NR1I3	Constitutive androstane receptor (CAR)	13	12	0.92 [**]
PPARG	Peroxisome proliferating receptor gamma	14	11	0.78 [*]
RAR	Retinoic acid receptor	6	5	0.83
THR	Thyroid hormone receptor	10	9	0.90 [**]
TP53	p53, apoptosis	22	17	0.77 [**]

Figure 6: Specificity analysis for selected ToxCast candidate MOA (ER, RAR, THR, TP53). The x-axis is the median log(AC50) for the active MOA-associated assays, and the y-axis shows the median and upper and lower 90% confidence intervals for the stress assay log(AC50). Active chemicals are red and inactive are gray. The size of the circle is proportional to the potency in the zebrafish assay (larger circle means lower AC50). The value of Δ is the difference between the target log(AC50) and the stress UCI (one example shown). All examples except p53 show a large separation in concentration between the specific effect and cell stress. Note that TP53 is one of the stress targets.



MOA Assignment

Figure 7: Decision tree for designating a specific MOA, a stress MOA or leaving the MOA unassigned. Numbers of chemicals in each MOA class are given in parentheses. UCI=90% upper confidence interval.

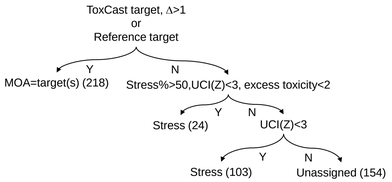


Table 3: Selected counts of chemicals by MOA

Candidate MOA	chemicals
inactive	561
unassigned	154
cell stress	127
ACHE	41
lipid synthesis (sterol synthesis)	30
ion channel	23
mitochondria	21
estrogen receptor	19
androgen receptor	9
estrogen and androgen receptor	8
microtubule	18
lipid membrane disruption (PPO)	11
lipid synthesis (ACCase)	10
lipid synthesis (HMGCR)	6
thyroid hormone receptor	5
lipid processing (PPARA / PPARG)	4
retinoic acid receptor	3
NR1I3 (CAR)	2

Conclusions

- Identified candidate MOA where a large fraction of MOA-associated chemicals are active in the zebrafish embryotoxicity assay (ZF+)
- Classification strategy assigned candidate MOA to 69% of ZF+ chemicals
- Determined which chemicals may be ZF+ solely because of generalized cell stress or cytotoxicity

Frequent MOA are

- Generalized cell stress
- Specific cell stress (microtubule, mitochondria)
- Lipid synthesis disruption that can affect signaling, cell structure and proliferation, and hormone synthesis
- Endocrine pathways (estrogen, androgen, thyroid, progesterone)

Next Steps:

- Review literature for support linking these MOA to developmental toxicity
- Manually review other targets related to development, e.g. sonic hedgehog
- Use CRISPR-Cas9 to knockdown candidate genes (chemical agnostic) and see if loss of gene function causes embryotoxicity