P160

Toxicity profiling using a battery of assays in zebrafish embryos including teratogenicity, behavior, cardiotoxicity, ototoxicity and hepatotoxicity

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OBJECTIVE

Screening of 32 compounds of interest to the NTP with known or hypothesized developmental toxicity or neurotoxicity for an overall assessment of systems toxicity in zebrafish embryos

INTRODUCTION

Human health is impacted by lifetime exposure to chemicals in the air, food and water. The U.S National Toxicology Program (NTP) is an interagency program whose mission is to evaluate agents of public health concern by developing and applying tools of modern toxicology and molecular biology. The NTP is interested in evaluating alternative methods that can be used to screen compounds to prioritize further testing in vivo. As part of this effort, the NTP contracted with Biobide to evaluate the utility of zebrafish in screening using a set of 32-compounds: 18 with suspected developmental toxicity, 6 with suspected developmental neurotoxicity/neurotoxicity, 5 with unknown effects and 3 negative controls.

WORK FLOW

1. **Developmental toxicity (internal dosing)**
2. **Cardiotoxicity**
3. **Behavior alterations**, **Hepatotoxicity**
4. **Ototoxicity**

MATERIAL AND METHODS:

•**Developmental toxicity assay**: 3-4 hours post fertilization (hpf) embryos were treated with 8 concentrations per compound chosen based on a previous MTC (Maximum Tolerated Concentration) experiment. Detailed analysis of embryo morphology and lethality was performed at 2 and 4 dpf. Percentage of altered and dead embryos was used to calculate the half maximum effective concentration (EC50) and lethal concentration (LC50). A teratogenic Index (TI) was estimated as the ratio between the LC50 and EC50 values. Once analyzed, embryos treated at the highest concentration without effect and at the concentration/s that induced malformations were washed and frozen for internal dosing analysis. 5 different techniques were used: Liquid Chromatography and Mass Spectrometry, Gas Chromatography and Mass Spectrometry, Head Space Gas Chromatography, UV Vis Spectroscopy and Atomic Spectroscopy.

•**Cardiotoxicity:** 48-54 hpf embryos expressing CopGFP in the heart were treated with five concentrations per compound (1, 3, 10, 30 and 100 µM generally, with some exceptions due to solubility limitations). After 3 hours at 28.5ºC, embryo heartbeat was recorded over 15 sec (Zeiss Axiovert 200 microscope) and analyzed using the non-commercial Cardio v3.0.0.5 software.

•**Behavior assay**: embryos at 3 dpf were exposed to 5 concentrations per test item chosen based on results in the developmental toxicity assay. After two days of incubation at 28.5 ºC, plates were placed in the Daniovision automated tracking system powered by Ethovision (Noldus). After 10 minutes of habituation, tracking (two rounds of 10-minutes light and 10-minutes dark phases) started. Total distance moved is shown as representative of locomotor activity.

•**Hepatotoxicity**: After the evaluation of behavior, plates were recovered from Daniovision and embryos were analyzed under the stereoscope. When liver opacity was observed, images of the liver region were taken and optical density of a central area inside the liver quantified with ImageJ software.

•**Ototoxicity:** 5 dpf embryos were exposed to 5 concentrations per chemical (1, 3, 10, 30 and 100 µM). After 24 hours of incubation, neuromasts were stained with DASPEI (2-(4-dimethylaminostyryl)-N-ethylpyridinium iodide), pictures were taken and neuromasts present in the lateral line were quantified.

RESULTS

1 Developmental Toxicity

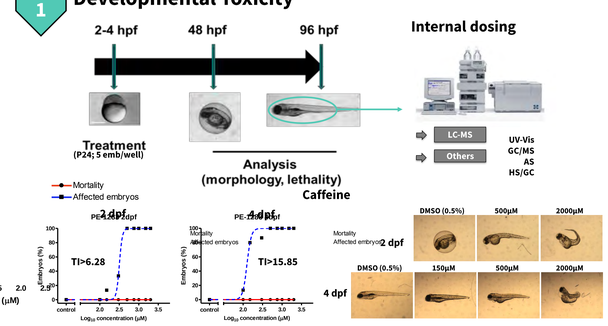

Figure 1: Graphs showing the regression curves obtained for affected and dead embryos treated with caffeine at 2 and 4 dpf.

Figure 2: Representative pictures of embryos treated with Caffeine at the concentrations and stages indicated.

2 Cardiotoxicity

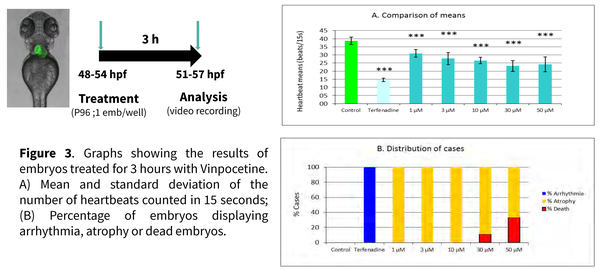

Figure 3. Graphs showing the results of embryos treated for 3 hours with Vinpocetine. A) Mean and standard deviation of the number of heartbeats counted in 15 seconds; (B) Percentage of embryos displaying arrhythmia, atrophy or dead embryos.

3 Behavior alterations and Hepatotoxicity

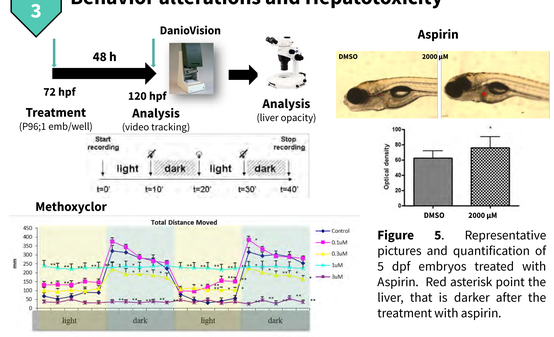

Figure 4. Graph showing the distance moved by 5 dpf control embryos and embryos treated with Methoxyclor during a tracking of 40 min.

Figure 5. Representative pictures and quantification of 5 dpf embryos treated with Aspirin. Red asterisk point the liver, that is darker after the treatment with aspirin.

4 Ototoxicity

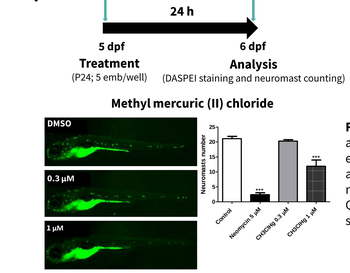

Figure 6. Representative pictures and quantification of 6 dpf embryos stained with DASPEI after treatment with Methyl mercuric (II) chloride. Quantification of neuromasts is shown in the graph.

➢ Compounds with suspected developmental toxicity

LogP	COMPOUND NAME	DEVELOPMENTAL TOXICITY 2 dpf EC_{50}/LC_{50}	DEVELOPMENTAL TOXICITY 4 dpf EC_{50}/LC_{50}	TI class.	Internal dose	CARDIOTOXICITY Effect	CARDIOTOXICITY Conc	BEHAVIOR Effect	BEHAVIOR Conc	HEPATOTOXICITY Effect	HEPATOTOXICITY Conc	OTOTOXICITY Effect	OTOTOXICITY Conc
4.68/5.08	Methoxyclor	9.34/>100	1.01/3.28	1	>100%	-	*3*	neuroactive	from 0.1	-	*10*	-	*1*
4.1/5.14	Vinpocetine	0.42/8.70	0.36/4.96	1	>100%	cardiotoxic	from 1	hypoactivity	from 1	-	*10*	-	*1*
4.50/4.72	Dibutyl phthalate	2.09/5.29	1.81/2.93	1	>100%	-	*30*	neuroactive	10	-	*10*	-	*10*
4.47	Bisphenol AF	10.44/27.97	4.12/8.53	1	>100%	bradycardia	from 10	hypoactivity	from 3	-	*10*	-	*10*
5.8	Di-n-Pentyl phthalate	7.54/18.14	4.27/4.75	1	13-18%	cardiotoxic	from 30	-	*10*	hepatotoxic	10	ototoxic	100
4.9	HPTE	20.01/42.41	7.97/15.43	1	>100%	bradycardia	from 10	hypoactivity	10	-	*10*	-	*10*
3.2	Linuron	18.23/41.77	15.05/19.16	1	>100%	bradycardia	from 10	-	*10*	-	*10*	-	*30*
2.9*	Trypan blue	*(2000)*	1064/1558	2	N.D.	-	*100*	hypoactivity	from 100	-	*1000*	-	*100*
-0.07	Caffeine	318.6/>2000	126.2/>2000	1	~100%	-	*100*	hypoactivity	from 300	-	*1000*	-	*100*
2.75/2.8	Valproate	289.8/1961	173.2/744.6	1	4-10%	-	*100*	-	*300*	-	*300*	-	*100*
1.46	Prednisone	*(1000)*	*(750)*	-	0.1%	-	*100*	neuroactive	200	-	*2000*	-	*100*
0.23	4-Methylimidazole	*(2000)*	1518/>2000	3	~100%	-	*100*	neuroactive	2000	hepatotoxic	1000	-	*100*
1.19	Aspirin	1656/>2000	1555/1726	2	1-2%	-	*100*	-	*2000*	hepatotoxic	2000	-	*100*
0.175/-1.9	Boric acid	*(2000)*	*(2000)*	-	>100%	-	*100*	-	*2000*	-	*2000*	-	*100*
0	Trimethadione	*(2000)*	*(2000)*	-	1%	-	*100*	-	*2000*	-	*2000*	-	*100*
-1.1	Gabapentin	*(2000)*	*(2000)*	-	0.5%	-	*100*	-	*2000*	-	*2000*	-	*100*
0.1	Dimethadione	*(2000)*	*(2000)*	-	3%	-	*100*	-	*2000*	-	*2000*	-	*100*
-0.31/-0.32	Ethanol	*(2000)*	*(2000)*	-	N.D.	-	*100*	-	*2000*	-	*2000*	-	*100*

N.D.: not detected; Italic numbers indicate the maximum concentration tested without effect
Effect described in other animal models or humans

Classification:
1=TI>2: Likely Teratogenic
2=TI≤2: Toxic but Not Teratogenic
3=Inconclusive

Differences in assay SENSITIVITY considering or not compound uptake:
- **All the 18 compounds: 50%**
- **Only compounds with at least 10% uptake (11): 82%**
- **Only compounds with logP>1 (11): 72.7%**

➢ Compounds with suspected developmental neurotoxicity/ neurotoxicity

LogP	COMPOUND NAME	DEVELOPMENTAL TOXICITY 2 dpf EC_{50}/LC_{50}	DEVELOPMENTAL TOXICITY 4 dpf EC_{50}/LC_{50}	TI class.	Internal dose	CARDIOTOXICITY Effect	CARDIOTOXICITY Conc	BEHAVIOR Effect	BEHAVIOR Conc	HEPATOTOXICITY Effect	HEPATOTOXICITY Conc	OTOTOXICITY Effect	OTOTOXICITY Conc
4.1	Rotenone	0.053/0.199	0.056/0.10	1	>100%	bradycardia	1	-	*0.1*	-	*1*	-	*0.1*
6.2/5.4	Dieldrin	*(30)*	0.301/3.10	1	>100%	bradycardia	from 10	neuroactive	from 0.1	hepatotoxic	0.1	-	*1*
0.41	Methyl mercuric (II) chloride	*(1)*	0.336/0.520	2	N.D.	-	*1*	hypoactivity	from 0.3	-	*1*	ototoxic	1
?	Lead acetate (II) trihydrate	548.9/629.1	2.82/>10	1	>100%	-	*100*	hypoactivity	30	-	*30*	ototoxic	100
3.32	Bisphenol A	56.58/109.5	21.37/56.58	1	>100%	bradycardia	from 30	hypoactivity	from 30	-	*100*	-	*30*
3.9	n-hexane	*(2000)*	*(2000)*	-	>100%	-	*100*	-	*2000*	-	*2000*	-	*100*

N.D.: not detected; Italic numbers indicate the maximum concentration tested without effect
Effect described in other animal models or humans

➢ Compounds with unknown effect

LogP	COMPOUND NAME	DEVELOPMENTAL TOXICITY 2 dpf EC_{50}/LC_{50}	DEVELOPMENTAL TOXICITY 4 dpf EC_{50}/LC_{50}	TI class.	Internal dose	CARDIOTOXICITY Effect	CARDIOTOXICITY Conc	BEHAVIOR Effect	BEHAVIOR Conc	HEPATOTOXICITY Effect	HEPATOTOXICITY Conc	OTOTOXICITY Effect	OTOTOXICITY Conc
9.07	Isopropylated phenyl phosphate	7.34/189.9	1.70/14.65	1	>100%	bradycardia	from 100	hypoactivity	from 0.1	-	*10*	-	*10*
4.59	Triphenyl phosphate	6.92/14.31	1.27/8.52	1	>100%	cardiotoxic	from 10	hypoactivity	from 1	hepatotoxic	3	ototoxic?	3
4.46	Phenanthrene	35.64/276.2	7.59/30.58	1	>100%	bradycardia	from 30	hypoactivity	from 1	-	*10*	-	*30*
?	Firemaster 550	2.22/112.4	1.49/11.93	1	>100%	bradycardia	from 100	hypoactivity	from 3	hepatotoxic	from 6	-	*10*
1.65	Bisphenol S	*(2000)*	1223/>2000	3	7-11%	-	*100*	-	*1000*	-	*1000*	-	*100*

Italic numbers indicate the maximum concentration tested without effect
Effect described in other animal models or humans

➢ Negative controls

LogP	COMPOUND NAME	DEVELOPMENTAL TOXICITY 2 dpf EC_{50}/LC_{50}	DEVELOPMENTAL TOXICITY 4 dpf EC_{50}/LC_{50}	TI class.	Internal dose	CARDIOTOXICITY Effect	CARDIOTOXICITY Conc	BEHAVIOR Effect	BEHAVIOR Conc	HEPATOTOXICITY Effect	HEPATOTOXICITY Conc	OTOTOXICITY Effect	OTOTOXICITY Conc
2	Kaempferol	8.08/29.02	3.00/8.69	1	>100%	-	*100*	-	*10*	-	*10*	-	*100*
0.91	Saccharin Sodium salt hydrate	*(2000)*	*(2000)*	-	0.2%	-	*100*	slight effect	500	-	*2000*	ototoxic?	100
-2.15/-1.85	L-ascorbic acid	*(2000)*	*(2000)*	-	0.3%	-	*100*	-	*2000*	-	*2000*	-	*100*

Italic numbers indicate the maximum concentration tested without effect

CONCLUSIONS

- **This screen demonstrates the usefulness of zebrafish assays to detect compound induced toxicity in different organs.**
- **A good correlation was observed between the toxicity previously described for the tested compounds and the toxicity results detected in zebrafish embryos.**
- **A clear dependence between compound hydrophobicity/hydrophilicity and embryo uptake was observed.**
- **This study confirms that one limitation of zebrafish embryo toxicity assays is the low uptake of hydrophilic compounds (logP<1) and highlights the importance of conducting internal dosing assays for proper test item classification.**

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