Zebrafish: A Predictive Model For Assessing Developmental Neurotoxicity

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Abstract

Conventional assessment of neurotoxicity in mammalian models relies on histological, neurophysiologic, and behavioral studies that are laborious and time consuming. The zebrafish (*Danio rerio*) is an increasingly attractive alternative model for developmental neurotoxicity, in part due to its transparency, rapid development, and simplicity of chemical delivery. Previous studies at Phylonix demonstrated a high correlation between developmental neurotoxicity observed in zebrafish and findings in mammalian models (Ton *et al.* 2006. Birth Defects Research (Pt. A) 76: 553-567; Parng *et al.* 2005. Meths. Cell Biol. 76: 75-85). In these studies, we characterized brain apoptosis and tail motor neuron defects and correlated results with behavioral defects. To further establish the validity of a zebrafish model for assessing developmental toxicity, we recently surveyed 120 environmental toxicants selected from the Voluntary Children's Chemical Evaluation Program (VCCEP) list and the EPA Emergency Planning and Community Right-to-Know Act (EPCRA). We assessed several quantitative parameters of neurotoxicity, including: 1) lethality, 2) brain apoptosis, 3) axon tract disruption in the brain and tail, 4) motor neuron formation in the tail, 5) catecholaminergic neurotoxicity, and 6) motility. We identified several compounds that showed effects on one or more of the neurotoxicity parameters examined and some compounds showed broad toxicity across all neuroanatomical endpoints. We also identified compounds that increased or decreased motor activity in day 6 zebrafish. These results combined with our previous research show that the zebrafish model has predictive value as an alternative model for developmental neurotoxicity screening (Parng et al. 2007. J. Pharm. Tox. Meth. 55: 103-112). Adoption of zebrafish developmental neurotoxicity assays can speed characterization of environmental toxicants and streamline pre-clinical drug development. This work was supported by National Science Foundation SBIR award # 0548657.

Introduction

The scope of agricultural and industrial pollutants: Every year, billions of pounds of toxic chemicals are released by industrial facilities and agricultural practices, much of which ends up in air or groundwater; ¾ of these chemicals are known or suspected neurotoxins (Schettler *et al.* 2000). Currently, more than 85,000 industrial chemicals are produced in the US every year, with an additional 2000-3000 new chemicals registered each year. More than 70% of these chemicals have little or no toxicity data (Claudio *et al.* 2000).

Zebrafish as a new model for developmental neurotoxicity: Conventional neurotoxicity assessment in mammalian models using histological, neurophysiologic, and behavioral studies are expensive, laborious, and time consuming. Due to the large number of compounds that require testing, a more rapid yet informative model would facilitate screening of potential toxicants. The zebrafish (*Danio rerio*) has previously been shown to be a useful model for assessing drug and developmental toxicity (Parng *et al.* 2002; Zhang *et al.* 2003). Since the embryo is transparent and develops rapidly, visualizing development of the central and peripheral nervous system is possible. Behavioral assays are also being developed which look at motor activity and startle response. Zebrafish assays can be performed relatively quickly on a large number of animals.

Developing a zebrafish developmental neurotoxicity model: To demonstrate the utility of zebrafish screens for developmental neurotoxicity, we initiated a pilot screen of 120 environmental toxicants. We focused on measuring lethality, apoptosis/necrosis in the brain, as well as surveying axon tracts, motor neurons, and the catecholaminergic system. We also developed a high-throughput screen for motor activity.

Materials and methods

LC_{50} Determination: Zebrafish (n=20) were exposed by static immersion from 6-96 hours post fertilization (hpf) at compound concentrations of 1, 10, 100, and 500 µM. Zebrafish treated with 0.1% DMSO were used as controls. Determination of LC_{50} values was accomplished through logistic regression using JMP 7.0 (The SAS Institute, Cary, NC) based on total mortality throughout the entire 96hr treatment period.

Apoptosis/Necrosis Determination: Zebrafish (n=20) were exposed to selected compounds by static immersion from 6-96hpf at ½ LC_{50}. Embryos were stained with acridine orange, washed, and imaged within 1 hour of staining. Quantification of fluorescence was accomplished using an inverse thresholding function and particle counting with ImageJ (NIH, Bethesda, MD). Images were analyzed for total fluorescent area above threshold in an oval area from a line posterior to the developing nasal pits to posterior of the developing ears. The medial edges of the developing eyes defined the boarders of the analyzed area. Fluorescence in compound-treated zebrafish (n=5) was compared with control zebrafish treated with carrier (DMSO) alone. The average fluorescent area was determined and compared between compound treated and controls with a two-sample T-test assuming unequal variances.

Motor Activity Assay: Zebrafish (n=15) were exposed to selected compounds by static immersion from 6-144hpf at ½ LC_{50}. After treatment zebrafish were washed and placed into a 96-well plate. After a 1 hour period of equilibration to the testing room (under constant light), zebrafish activity was recorded over a 1 hour period consisting of alternating 10 min photoperiods. The total distance moved each minute was recorded.

Whole-Mount Neuron Immunostaining: Albino zebrafish (n=10) were exposed to selected compounds by static immersion from 6-96hpf at ½ LC_{50}. 0.1% DMSO treated zebrafish were used as controls. Axon tracts were visualized with fluorescently labeled anti-acetylated tubulin (Sigma). Motor neurons were visualized with fluorescently labeled anti-ZNP1 (Developmental Studies Hybridoma Bank, U. of Iowa). Catecholaminergic neurons were visualized using fluorescently labeled anti-tyrosine hydroxylase (Millipore/Chemicon).

Results

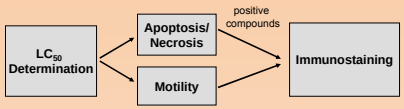

Figure 1: Screening paradigm for environmental toxicants. Compounds with an identifiable LC_{50} between 1 and 500µM were both subjected to apoptosis/necrosis and motility assays. Compounds that tested positive to both assays were subject to immunostaining of motor neurons, axon tracts, and the catecholaminergic system.

I. LC_{50} Determination

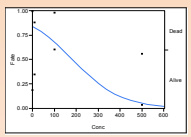

Figure 2: LC_{50} identification. The LC_{50} of a compound was identified through logistic regression, where the probability of a given fate (alive/dead) is equal to the vertical distance underneath the blue line. In this example for p-cresol, the whole-model regression was significant (p<0.0001). Inverse prediction based on the regression equation gave a predicted LC_{50} of 171.5µM.

II. Apoptosis / Necrosis Assay

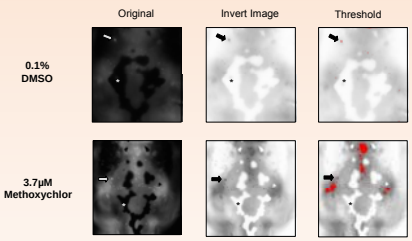

Figure 3: Morphometric analysis of apoptosis/necrosis. Zebrafish (n=5) were stained with acridine orange, and dorsal images of the brain (anterior at the top of the image) were acquired using a fluorescent scope with the same exposure time and gain. Images were inverted and thresholded using ImageJ software. Positive signals were defined by particle size (in pixels) and fluorescent intensity. Arrows indicate apoptotic/necrotic cells or tissue in the brain. An "*" indicates the location of pigment bands that normally occur during development.

III. Motility Assay

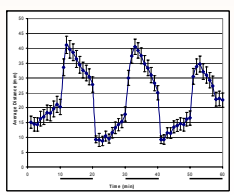

Figure 4: Zebrafish motor activity under alternating photoperiods. Day 6 zebrafish (n=200) were subjected to alternating periods of light and dark (indicated by black bars on the X-axis). The switch to no lighting resulted in increased motor activity, indicated by an increase in the mean distance traveled/minute. Error bars = ± 1 SEM.

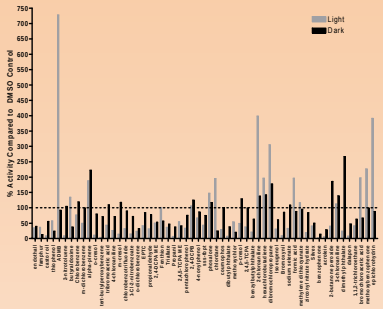

Figure 5: Normalized activity within each photoperiod. The motor activity for each treatment is shown relative to 0.1% DMSO-treated controls. Compounds that increased activity will be greater than 100% (indicated by a dashed line), while those that decreased activity will be lower than 100%. For a compound to be classified has having a motor effect in our assay: 1) A two-sample T-test comparing the compound treated fish to controls in either photoperiod (light or dark) must determine the two populations are significantly different (P<0.05), and 2) the response of the compound treated fish must fall outside the value of the DMSO control ± ¾ the coefficient of variation of the DMSO control population.

IV. Secondary Immunoassays

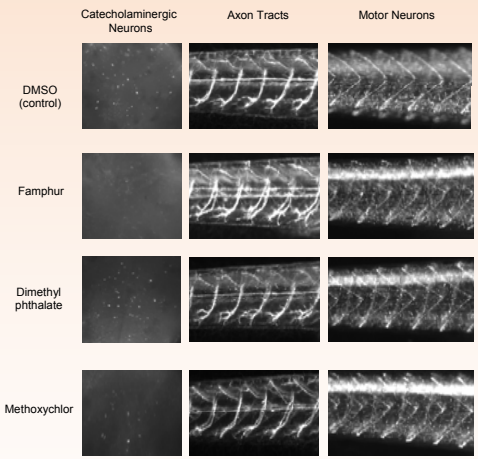

Figure 6: Neurotoxicity assessment by immunostaining. 16x magnification images of catecholaminergic neurons (α-tyrosine hydroxylase), axon tracts (α-acetylated tubulin), and motor neurons (α-ZNP1), for some of the compounds studied. Dorsal images of catecholaminergic neurons (anterior at the top) and lateral images of axon tracts and motor neurons (anterior to the right) were acquired. Peripheral nervous system development appears less sensitive to environmental toxicants than central nervous system development (based on compound effects on catecholaminergic neurons).

Table 1: Effects of selected compounds on secondary immunoassay endpoints.

Compound	*Concentration (µM)*	*Catecholaminergic Neuron Toxicity*	*Axon Tract Toxicity*	*Motor Neuron Toxicity*
DMSO (negative control)	0.1%	-	-	-
Famphur	27.45	+/-	-	-
Dimethyl phthalate	46.1	-	-	-
Methoxychlor	3.7	+	-	+/-
Cu Sulfate Pentahydrate	5.2	+	+	+
Tribromoacetic acid	9.5	+	-	+

Table 2. Summary of Compound Effects on Zebrafish Developmental Neurotoxicity. Of the 200 compounds assayed, 60 compounds had an identifiable LC_{50} between 1 and 500µM, 9 of those compounds resulted in significant increases in brain apoptosis/necrosis, while 11 compounds resulted in motility defects. Three compounds were "hits" with an identifiable LC_{50}, brain apoptosis/necrosis, and motility defects.

Compound Name	*LC_{50} (µM)*	*Brain Apoptosis/Necrosis Result*	*Motility Result*
a-Pinene	147	Negative	**Positive**
Chlorobenzene	632	Negative	Negative
m-Dichlorobenzene	489	Negative	Negative
Benzylbutylphthalate	4.3	Negative	Negative
Tioxaphene	6.6	Negative	**Positive***
4-Nonylphenol	2.6	Negative	Negative
2,4-Dichlorophenoxybuteric acid	2.6	Negative	Negative
Chlordane	7.9	Negative	Negative
Phosalone	2.7	Negative	Negative
2,4,5-Trichlorophenoloxyacetic acid	3.7	Negative	Negative
S,S,S-Tributylphosphorotrithioate	5.3	Negative	Negative
Methoxychlor	7.4	**Positive**	**Positive**
Dibutyl phthalate	2.6	Negative	Negative
Coumophos	12.2	Negative	Negative
Trialiate	8.9	**Positive**	Negative
Fenthion	5	Negative	Negative
2,4-Dichlorophecyoxyadetic acid, methyl ester	6.4	Negative	Negative
Pentachlorophenol	0.7	Negative	Negative
2,4,5-Trichlorophenoxyacetic acid methylester	0.99	Negative	Negative
Propanil	13.1	Negative	**Positive**
Tribromoacetic acid	18.9	Negative	Negative
2-Hydroxy-4-methoxybenzophenone	14.4	Negative	Negative
Copper(II) sulfate pentahydrate	10.3	Negative	**Positive***
4-Chlorobenzotrifluoride	34.1	Negative	Negative
4-methylphenol (p-cresol)	171.5	Negative	Negative
3-methylphenol (m-cresol)	14.7	Negative	Negative
4-chloroaniline	66.8	Negative	Negative
tert-Butyl peroxybenzoate	22.8	Negative	Negative
2-methylphenol (o-cresol)	179.6	Negative	Negative
1-chloro-2-nitrobenzene	17	Negative	Negative
Bromochloroacetic acid	238.9	Negative	Negative
Dimethyl Phthalate	92.2	**Positive**	**Positive**
Epichlorohydrin	227.1	Negative	Negative
3-Chloroaniline	1.9	Negative	Negative
1,1,2-trichloroethane	113.8	**Positive**	Negative
Silvex	25.2	Negative	**Positive**
Benzophenone	46.8	Negative	**Positive**
acrolein	5.4	Negative	**Positive**
2-chloroaniline	180.9	Negative	Negative
Hexachloro-1,3-butadiene	151.3	Negative	Negative
2-butanone peroxide	14.4	Negative	Negative
Dalapon	206.3	Negative	Negative
1,2-dibromo-3-chloropropane	210	Negative	Negative
Zirconyl nitrate hydrate	296.6	Negative	Negative
butyraldoxime	17.8	Negative	Negative
formic acid	347.6	Negative	Negative
methylene dithiocyanate	5.5	Negative	Negative
Thiophenol	1.9	Negative	**Positive**
sodium selenate	206.2	Negative	Negative
2-nitrotoluene	144.1	**Positive**	Negative
4-Allyl-1,2-dimethoxybenzene	39.4	**Positive**	Negative
isoeugenol	25.3	Negative	Negative
castor oil	17.2	**Positive**	Negative
diphenyltriazine	32	Negative	Negative
endothall	242.2	Negative	Negative
bromoxynil	2	Negative	Negative
o-dichlorobenzene	175.1	Negative	Negative
propionaldehyde	90.7	**Positive**	Negative
famphur	54.9	**Positive**	**Positive**
eptc	233.8	Negative	Negative

Conclusions

- Zebrafish are amenable to developmental toxicity assessment, in particular neurotoxicity.
- While the peripheral nervous system may not show evidence of malformation, endpoints in the central nervous system may be more sensitive indicators of toxicant exposure.
- Future research will refine secondary immunoassays and continue screening compounds to categorize the extent of neuronal malformation observed during toxicant exposure.

Literature cited

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For further information

Please contact *louis.damico@phylonix.com*. All studies were conducted in accordance with institutional animal care protocols consistent with the AVMA's panel on euthanasia. Phylonix offers a wide range of zebrafish assays for drug/compound screening. Please see www.phylonix.com for more information.

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