POSTER Nº 9:

Zebrafish Embryo Developmental Toxicity Assay (DTZ) for weak and non teratogen compounds.

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ABSTRACT

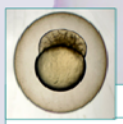
When testing the teratogenicity of chemical compounds, it is sometimes difficult to distinguish between weak and non teratogen compounds. This can lead to false negatives, reducing the reliability of the test. Thus, seeking the combination of endpoints which correctly separate weak from non teratogens is important in order to predict developmental toxicity of new drug candidates (*Chapin R.E. et al, 2006*).

Preliminary results with the Zebrafish Embryo Developmental Toxicity Test (DTZ) developed by ZF Biolabs have shown good results in differentiating between non and weak teratogens. DTZ protocol defines as strong teratogen those compounds with Teratogenic Index (TI) greater than 1,5, while TI data between 1 and 1,5 defines weak teratogen. TI data below 1 or indeterminated defines non teratogen compounds. Based on TI data obtained in our facilities, our endpoint appear to be appropriated to differentiate compounds classified as weak/moderate teratogen in previous developmental toxicity test such as the Embryonic Stem Cell Test.

A battery of 6 compounds was selected for the study: Aspirin, Caffeín, Diphenhydramine, Saccharin, Cupric sulphate and Acetone, for which we had data on their teratogenicity either form scientific references or in house research work.

Complete results of weak and non teratogen compounds using the DTZ protocol will be presented. Applicability of the DTZ test for predicting teratogenicity and to differentiate between non teratogen and weak/doubtful teratogens will be discussed.

Keywords: Zebrafish, developmental toxicity, weak teratogens, non teratogens.



INTRODUCTION

The zebrafish embryo was originally used to study the genetics of development due to its transparency, quick embryonic development, easy collection in high numbers and similarity with human development (Page, 1990). In fact the zebrafish embryo constitutes a complete, developing vertebrate organism, and it allows testing predictability for toxicity not only in the context of cellular function, but also at the level of organ and organism toxicity (Hill *et al*, 2005). Moreover the anatomic and genomic similarity with humans, 80% genetic homology, foresees tests predictability for human toxicity (Carroll PM and Fitzgerald K 2003).

All these characteristics make the Zebrafish especially suitable for the assessment of developmental toxicity and highly prone to yield additional information to the cell-based assays. Humans and fishes share many developmental pathways, organ systems and physiological mechanisms, making conclusions relevant to human biology (Crollius R.H. & Weissenbach J. 2005). The Zebrafish embryo has already been used in the assessment of developmental toxicity, as can be ascertained with nearly 300 publications covering this topic, and recently has gained interest in the pharmaceutical industry as a promising model for the late discovery/early preclinical toxicological studies (Parng, 2005; Rubenstein A. L. 2006).

OBJECTIVES

When testing the teratogenicity of chemical compounds, it is difficult to distinguish between weak and non teratogen compounds. This can lead to false negatives, reducing the reliability of the test. Thus, seeking the combination of endpoints which correctly separates weak from non teratogens is important in order to predict developmental toxicity of new drug candidates.

We have evaluated if the **Zebrafish Embryo Developmental Toxicity Test** (**DTZ**) developed by **ZF Biolabs** (Rodriguez *et al*, 2006) is a reliable test for differentiating between non and weak with a good reproducibility and reliability among different replicates.

DTZ has been designed, developed and validated in house as a reliable alternative method to evaluate the potential developmental toxicity to humans of chemical compounds, following ECVAM (Worth A.P & Balls M. 2004), ICCVAM (NIH Publication No. 03-4508) and OCDE (OECD 1996) recommendations to develop and validate new & alternative methods. DTZ has been developed with the aim of increasing reproducibility and reliability as well as decreasing variability among replicates as much as possible.

METHODS

The DTZ protocol used covers all aspects (biological, toxicological and statistical) concerning the developmental toxicity test with Zebrafish embryos. This protocol includes specific procedures in order to standardize as much as possible all the work and strict quality control measures such as standardized maintenance conditions for zebrafish breeders, controlled embryo production, quality control of spawns, embryos and replicates, all these standardize in an Atlas of Abnormalities of Zebrafish, including all the morphological endpoints to be assessed during the test.

Compounds selected for this study were:

- Aspirin (CAS 50-78-2).
- Caffein (CAS 58-08-2).
- Diphenhydramine (CAS 47-24-0).
- Cupric Sulfate (CAS 7758-98-7).
- Saccharin (CAS 82358-42-0).
- Acetone (CAS 67-64-1).

In order to asses the capacity of the DTZ test to discriminate between non and weak teratogens, a small preliminary battery of 6 compounds was tested. Aspirin, Caffein and Diphenhydramine were classified as weak teratogen during the EST test validation programme (Genschow, 2002) and Saccharin as non teratogen also using the EST test. Cupric Sulfate and Acetone were selected for being clear non teratogen compounds in previous in-house research work.

Embryos were placed with the tested compound in 50 µl of media at different concentrations in 96-well plates (1 embryo per well) at 26ºC throughout the test. Number of embryos used for each concentration and replicate depends on that quality of the spawn and is normally between 20 and 28 embryos (40-56 in the control).

Media used to prepare different concentrations of the tested compounds contains 1% DMSO in order to increase embryo permeability and solubility of certain compounds. Media is changed in 75% every 24hrs. Lectures of the plates are obtained using a optical microscope and all endpoints are assessed "*in vivo*". These endpoints are qualitative aspects (presence/non presence) of certain morphological characteristics of the embryo and one quantitative data: embryo growth at 72 hours post fertilization (hpf) (see tables below).

Endpoint	LC50/EC50	Time of observation
Embryo coagulation	LC50	24 h
No somites	LC50	24 h
Tail not detached from yolk	LC50	24 h
Embryo coagulation	LC50	48 h
No heartbeat	LC50	48 h
No somites	LC50	48 h
Tail not detached from yolk	LC50	48 h
Blood circulation abnormalities	EC50	48 h
Brain malformation	EC50	48 h
Eye malformation	EC50	48 h
Head malformation	EC50	48 h
Median fins malformation	EC50	48 h

Endpoint	LC50/EC50	Time of observation
Notochord malformation	EC50	48 h
Otic vesicle malformation	EC50	48 h
Pigmentation abnormalities	EC50	48 h
Presence of edema	EC50	48 h
Tail malformation	EC50	48 h
Yolk malformation	EC50	48 h
Embryo coagulation	LC50	72 h
No heartbeat	LC50	72 h
Tail not detached from yolk	LC50	72 h
Embryo growth retardation	EC50	72 h
Hatching success	EC50	72 h

Four different lectures of the plates are carried out during the test:

- At 4 hpf for the 2nd. tier quality control of the embryos,
- At 24 hpf, to assess the quality of the replicates
- At 48 hpf is the main lecture of the test where the 15 endpoints are assessed and results used to determine LC50, EC50 and Teratogenic Index (TI).
- At 72 hpf is an optative final lecture that can provide further data on growth and embryo development delays.

Results of the test are expressed as:

- **EC50** which is the concentration of the compound that can be expected to cause 50% malformation of the embryos at 48 hpf.
- **LC50**: concentration that is expected to be lethal to 50% of the embryos at 48 hpf.
- **Teratogenic Index**: ratio of LC50 and EC50

At least 3 valid replicates should to be used to calculate these values, and must be based on the following selection criteria:

- % of malformations and % of mortality in the control group cannot be over 10% in both cases.
- The number of viable embryos has to be at least 10 for each concentration and 20 for the control.
- Analysis capacity of the PROBIT 2.1 programme, used to calculate EC50 and LC50 (USEPA. 1993).

DTZ protocol defines as strong teratogen those compounds with Teratogenic Index (TI) over 1.5 while TI data between 1 and 1.5 defines weak/doubtful teratogens. TI data below 1.0 or indeterminate defines non teratogen compounds.

RESULTS AND DISCUSSION

LC50 and EC50 (at 48 hpf) were estimated following the DTZ protocol for the tested compounds and the TI calculated.

Results obtained for the 6 compounds tested for this study are shown in the table bellow:

Compound	Teratogen Index (TI)	DTZ Status	EC50 (mg/L)
Caffeine	10.8	Strong Teratogen	460.4
Diphenhydramine	4.9	Strong Teratogen	12.1
Aspirin	1.2	Weak /Doubtful Teratogen	865.7
Saccharin	1.0	Doubtful	23888.5
Cupric Sulphate	n.a.*	Non Teratogen	n.a.*
Acetone	n.a.*	Non Teratogen	n.a.*

* Non applicable

DTZ protocol is a valid method to asses the teratogenic effect of chemical compounds as it clearly identifies those compounds that are no teratogen from those that produce clear and precise malformations in zebrafish embryos (strong teratogens) and those for which the effect is not clear or strong enough to be able to classify them as teratogens (weak or doubtful teratogens).

With the preliminary data obtained with the 5 compounds tested we are not able to assure the capacity of the DTZ to clearly differentiate between weak and doubtful teratogens. These results can be partially explained by the fact that assessing teratogenic effects is a complex task as many biological processes take place and there is a great variability between different species.

As an example, compounds such as aspirin which with our test have been classified as weak/doubtful teratogen have shown different effect in different organisms: Aspirin is not teratogenic in rabbits as opposed to rats (Cappon, 2003). Caffeine which is strong teratogen using DTZ, has clearly shown teratogenic effects in rodents and Xenopus but traditionally has not considered teratogen for humans (Nehlig et al. 1994), although recently epidemiology studies (Bech *et al*, 2005) have reported different conclusion.

The capacity of the DTZ test, gained through an intensive evaluation of the zebrafish embryo during all its development, to at least establish that a compound is a weak or doubtful teratogen and is not classified as non teratogen (false negative), can also be considered of clear value in order to anticipate possible effects of the tested compound when using mammalian models.

In order to increase our knowledge on this complex, but very important topic, more research should be performed in different directions. Aspects such as the capacity of the zebrafish embryo to bioconcentrate most of the *drug-like* compounds including the influence of the chorion permeability, the influence of the activation due to the zebrafish metabolic enzymes, comparative studies between different species, etc. need further research. The use of embryo microinjection to study non water soluble compound or improved data analysis tools to better analyze this complex biological processes of embryo development, can also be of great help to understand the process of teratogenicity and improve the reliability of the DTZ test.

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