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Assessment of developmental delay in the zebrafish embryo teratogenicity assay

E. Teixidó, E. Piqué *, J. Gómez-Catalán, J.M. Llobet

GRET-CERETOX and Toxicology Unit, Public Health Department, School of Pharmacy, University of Barcelona. Av. Joan XXIII s/n, E-08028 Barcelona, Spain

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ABSTRACT

In this study we analyzed some aspects of the assessment of developmental delay in the zebrafish embryotoxicity/teratogenicity test and explored the suitability of acetylcholinesterase (AChE) activity as a biochemical marker and as a higher throughput alternative to morphological endpoints such as head–trunk angle, tail length and morphological score. Embryos were exposed from 4 to 52 h post-fertilization (hpf) to a selection of known embryotoxic/teratogen compounds (valproic acid, retinoic acid, caffeine, sodium salicylate, glucose, hydroxyurea, methoxyacetic acid, boric acid and paraoxon-methyl) over a concentration range. They were evaluated for AChE activity, head–trunk angle, tail length and several qualitative parameters integrated in a morphological score. In general, the different patterns of the concentration–response curves allowed distinguishing between chemicals that produced growth retardation (valproic and methoxyacetic acid) and chemicals that produced non-growth-delay related malformations. An acceptable correlation between the morphological score, AChE activity and head–trunk angle as markers of developmental delay was observed, being AChE activity particularly sensitive to detect delay in the absence of malformations.

1. Introduction

Animal testing will increase dramatically over next decade as a consequence of implementation of the new EU regulation for the Registration, Evaluation and Authorization of Chemicals (REACH) (Pedersen et al., 2003; Van der Jagt et al., 2004). One of the toxic responses that must be evaluated is the effect on development, principally teratogenesis. Reproductive and developmental toxicity studies will use by far the most animals and resources within REACH (particularly 23% of animals and 32% of resources in developmental studies). Thus, the introduction of valid alternatives and research into new alternative methods for developmental toxicity testing is specifically urgent in order to reduce the number of animals used (Piersma, 2006). Furthermore, medium-to-high throughput assays of developmental toxicity would be valuable during the screening phase of new drugs research.

In the last decade assays using embryonic stages of the vertebrate zebrafish (Danio rerio) have attracted the attention of toxicologists due to their several advantages. In particular, fish embryos are considered as non-protected life stages and –similar to in vitro assays- an alternative to animal testing (Directive, 2010/63/EU), although they use whole organisms. The fish embryo test with zebrafish (FET) has been suggested as replacement of the acute tests performed with juveniles or adults (Braunbeck et al., 2005) and a draft for an OECD guideline is currently under review (OECD, 2006). Approval of this OECD guideline would prompt the application of the fish embryo test for the chemical safety evaluation on an international scale. The FET is also employed for effluent testing in different countries replacing adult fish tests (DIN, 2001). Analysis of acute toxicity in embryos can also include the screening for developmental disorders as an indicator of teratogenic effects.

Zebrafish are easy to maintain and produce large numbers of embryos that develop outside the mother. The transparency of their embryos allows the scoring of teratological and embryotoxic effects easily. In addition, the development is fast and has been well characterized, including morphological, biochemical and physiological information at all stages of early development (Hill et al., 2005). The development process is highly conserved across vertebrates and the zebrafish genome is completely characterized. Hence, zebrafish embryos represent an attractive model allowing reduction and refinement of animal use in research (Yang et al., 2009).

Numerous studies have been reported exploring the capacity of zebrafish assays for the assessment of the teratogenic potential of chemicals showing a good concordance with in vivo results in mammals (Brannen et al., 2010; Hermsen et al., 2011; Nagel, 2002; Selderslaghs et al., 2009; Van den Bulck et al., 2011). They focused on three manifestations of deviant development: death,

Abbreviations: AChE, acethylcolinesterase; DMSO, dimethyl sulfoxide; TMS, total morphological score; WEC, whole embryo culture; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); hpf, hours post-fertilization.

* Corresponding author. Tel./fax: +34 93 403 58 16.
E-mail address: esterpique@ub.edu (E. Piqué).

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malformation and growth retardation (Wilson and Fraser, 1977). However, they applied different experimental protocols and the number and the variety of assayed substances were limited. Currently there is no consensus about the optimal procedure in some basic features as the specific endpoints and scoring systems to use, the time of exposure and the stage of embryonic/larval development to do the observations.

General retardation of development is a phenomenon often observed in teratogen-exposed embryos (Liang et al., 2010; van den Brandhof and Montforts, 2010). Developmental delay is usually considered as a reversible and unspecific effect. However, it might lead to persistent delays or deficits in function (Daston et al., 2010) and permit teratogens to act for a longer time during sensitive stages, and thus intensify the severity of the produced anomalies (Weis and Weis, 1987). Some studies have aimed to distinguish between growth retardation and other developmental effects in the zebrafish assays. Nagel (2002) considered that "growth-retardation" at 24/48 hpf and tail length at 120 hpf were "teratogenic endpoints", whereas defects on development of somites, eyes or blood circulation were "development endpoints". Brannen et al. (2010) proposed an extensive scoring system that takes into account the severity of the effects, but those endpoints more related to growth retardation were finally eliminated because they were non-discriminating or not cost-effective. More recently, Van den Bulck et al. (2011) considered "growth retardation" as a teratogenic endpoint. However, Hermsen et al. (2011) split the assessment in two endpoint categories and two respective scores, a general morphology score and a teratogenic score, suggesting that the first score gives a "semi-quantitative assessment of (mal) development".

For the purpose of attain a better characterization of the developmental retardation in the zebrafish embryo assays we delineated a scoring system based on some qualitative morphological features characterizing the zebrafish stages described by Kimmel et al. (1995) during the 52 hpf. This score has a similar design as the scoring system developed for mammalian whole embryo culture assays (WEC) by Klug et al. (1985). Furthermore, we measured the length of the tail and head–trunk angle as quantitative morphological markers of development (Bachmann, 2002; Kimmel et al., 1995). Finally, we determined the acetylcholinesterase (AChE) activity in whole 52 hpf embryos. In zebrafish, AChE is expressed in a variety of tissues, including non-cholinergic cells (Hanneman and Westerfield 1989). Its expression starts early before synapse formation (Layer, 1990) and increases with age along the embryo development (Behra et al., 2002; Bertrand et al., 2001). Therefore, AChE activity was a reasonable candidate as a sensitive biochemical marker of developmental delay with a medium–high throughput potential. Embryos were exposed to a selection of eight compounds characterized by diverse known embryotoxic/teratogen activities (Table 1). Most of these compounds belong to the set of chemicals tested in the ECVAM international study of validation on the three in vitro embryotoxicity tests namely embryonic stem cells test, limb bud micromass test and post-implantation whole-embryo culture test (Genschow et al., 2004). The concentration–response curves obtained for the different endpoints were analyzed and compared in order to assess their relative performance as markers of developmental delay and in connection with the teratogenic effects. In addition, an irreversible AChE inhibitor was tested so as to compare AChE inhibitors and compounds that decrease AChE activity since they induce developmental delay.

2. Materials and methods

2.1. Chemicals and test media

All the selected chemicals were purchased from Sigma–Aldrich (St. Louis, MO). Danieau's buffer (58 mM NaCl; 0.7 mM KCl; 0.4 mM $MgSO_4{\cdot}7H_2O$; 0.6 mM $Ca(NO_3)2$; 5 mM HEPES; pH 7.4) was used as the medium for all solutions during the experiments to keep the pH stable and constant between assays due to the different pKa of the chemicals tested.

2.2. Zebrafish maintenance and egg production

Adult female and male zebrafish were obtained from a commercial supplier (Pisciber, Barcelona) and housed separately in a closed flow-through system in standardized dilution water as specified in ISO 7346–1 and 7346–2 (ISO, 1996; 2 mM $CaCl_2{\cdot}2H_2O$; 0.5 mM $MgSO_4{\cdot}7H_2O$; 0.75 mM $NaHCO_3$; 0.07 mM KCl). Fish were maintained at 26 ± 1 °C on a 14-h light and 10-h dark cycle and were fed with commercial dry flake food and live brine shrimp. The day before eggs were required, males and females were placed in breeding tanks (Aquaneering, San Diego, California) with a 2:1 male:female ratio. On the next morning, the eggs could be collected 30 min after the light had been turned on. Eggs were collected and successively cleaned with dilution water corresponding to the reconstituted water according to ISO-standard 7346, which was diluted 1:5 using deionized water.

Table 1
Test substance – overview. LC_{50}, EC_{50} with confidence intervals. Slopes and TI values of the test substances. EC_{50} for sodium salicylate was calculated taking into account embryos with brain hemorrhages and swimming disorders. – No effect.

Test substance	Cas no.	Characterization	LC_{50} (mM)	Slope LC_{50}	$EC_{50\ teratogenic}$ (mM)	Slope EC_{50}	TI (LC_{50}/EC_{50})
Valproic acid	1069–66-5	Antiepileptic drug	1.74 (1.68–1.75)	5.0×10^{-2}	0.52 (0.48–0.57)	0.15	3.3
Caffeine	58–08-2	Xanthine alkaloid, psychoactive substance	5.0 (4.8–5.2)	0.36	0.8 (0.7–1.0)	0.69	6.2
All-trans retinoic acid	302–79-4	Vitamin A metabolite	5.1×10^{-5} (4.99×10^{-5}–5.71×10^{-5})	4.15×10^{-6}	1.9×10^{-6} (1.7×10^{-6}–2.2×10^{-6})	1.96×10^{-6}	26.8
Methoxyacetic acid	625–45-6	Glycol ether alkoxy acid metabolite	32.8 (32.1–36.4)	2.68	11.3 (10.0–12.0)	3.54	2.9
Salicylic sodium salt	54–21-7	Aspirin metabolite	41.0 (39.2–47.1)	4.15	12.5 (11.7–13.4)	7.26	3.2
Hydroxyurea	127–07-1	Antineoplasic drug	42.6 (40.2–43.3)	3.09	31.6 (28.3–32.0)	2.03	1.3
Boric acid	10043–35-3	Antiseptic, insecticide, flame retardant	53.4 (52.7–57.3)	4.07	18.3 (16.7–20.0)	13.24	2.9
Paraoxon-methyl	950–35-6	AChE inhibitor	8.8×10^{-2} (8.4×10^{-2}–9.2×10^{-2})	1.99×10^{-2}	1.0×10^{-2} (9.4×10^{-3}–1.08×10^{-2})	1.26×10^{-3}	8.8
D-(+)-glucose	50–99-7	Sugar	–	–	–	–	–