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Zebrafish embryotoxicity test for developmental (neuro)toxicity: Demo case of an integrated screening approach system using anti-epileptic drugs

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ABSTRACT

To improve the predictability of the zebrafish embryotoxicity test (ZET) for developmental (neuro)toxicity screening, we used a multiple-endpoints strategy, including morphology, motor activity (MA), histopathology and kinetics. The model compounds used were antiepileptic drugs (AEDs): valproic acid (VPA), carbamazepine (CBZ), ethosuximide (ETH) and levetiracetam (LEV). For VPA, histopathology was the most sensitive parameter, showing effects already at 60 μM. For CBZ, morphology and MA were the most sensitive parameters, showing effects at 180 μM. For ETH, all endpoints showed similar sensitivity (6.6 mM), whereas MA was the most sensitive parameter for LEV (40 mM). Inclusion of kinetics did not alter the *absolute* ranking of the compounds, but the *relative* potency was changed considerably. Taking all together, this demo-case study showed that inclusion of multiple-endpoints in ZET may increase the sensitivity of the assay, contribute to the elucidation of the mode of toxic action and to a better definition of the applicability domain of ZET.

1. Introduction

Developmental toxicity testing in laboratory animals is required for the preclinical safety assessment of new pharmaceuticals [1]. Although studies with rodents are still the "gold standard" used to study developmental toxicity for regulatory purposes, over the last decades efforts have been made to replace these studies with cell-based alternatives (*e.g.* stem cells) [2–5]. Rodent studies are time consuming, expensive and involve large numbers of laboratory animals. To date, however, the search for alternatives has not been very successful, due to the difficulty of investigating the complexity of developmental processes in relatively simple cellular assays [6]. The zebrafish (*Danio rerio*) is emerging as a candidate lower vertebrate animal model capable of filling the gap between high throughput *in vitro* cellular assays and conventional preclinical animal testing [7–10]. The embryonic/larval zebrafish model offers an intact whole animal model with many of the advantages of *in vitro* systems, making it a suitable model organism for medium/high-throughput screening [11,12].

Currently, one of the most commonly used endpoints for developmental toxicity screening in zebrafish is its morphology, which makes it relatively easy to detect various anomalies [13,14] that correspond quite well with higher mammalian species [15,16]. In a previous study [17], we demonstrated that additional histopathological examination of the larvae contributed to an improved understanding of the mode of toxicity action, thereby increasing the predictability of the zebrafish assay for toxicity screening. The use of zebrafish larvae to test compounds for potential developmental (neuro)toxicity involves various behavioral tests, such as the motor activity test (MA). Tests of this kind are based on measuring the effects on factors such as total distance moved and velocity [18–20]. Recently, an extension of the MA test analysis was proposed, in which a wider range of endpoints can be evaluated from the same collected data set [17]. Furthermore, other endpoints than morphological scoring have been used in the embryonic and larval model to evaluate the potential toxicity of compounds, including proteomics [21] and transcriptomics [22]. Although each endpoint evaluation has its own relevance, in general, they are studied separately,

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so the results of different studies need to be integrated. In addition, the measurement of test compound uptake by zebrafish embryos and larvae has been shown to be a critical factor, one that can influence the predictability of the assay. Various studies have revealed an overestimation [23] or an underestimation of exposure, leading to false positive or false negative assay results.

Here we report on an integrated zebrafish screening approach based on morphology, histopathology, motor activity and kinetics, using anti-epileptic drugs (AEDs) as a group of model compounds.

Some AEDs can exert their effects *via* inhibition of the histone deacetylases (HDAC) [24,25] by binding to retinoic acid (RA) receptor [26]. Using *in situ* hybridization (ISH), we also investigated the gene expression profile of two genes (Aldh1a2 and Cyp26a1) involved in the retinoic acid (RA) signaling pathway and ways in which these are affected by AEDs. Aldh1a2 is involved in the *synthesis* of RA, through the oxidation of retinaldehyde, while Cyp26a1 is involved in the *degradation* of RA, by the conversion of RA into more polar metabolites [27].

It is known that, when administered during pregnancy, most AEDs are capable of inducing developmental (neuro)toxicity [28,29]. The effects of AEDs on the offspring may range from major congenital malformations (MCM) to delayed postnatal cognitive development and growth [30]. The developmental toxic potential of first generation AEDs, such as valproic acid (VPA) and carbamazepine (CBZ), has been clearly demonstrated in humans and in various animal models [31,32]. Only a limited amount of data has been published on the developmental effects of other first generation AEDs such as ethosuximide (ETH) and for newer compounds, such as levetiracetam (LEV). Despite the limited availability of data, teratogenicity and cognitive impairment have been described for ETH in rodents [33,34]. Levetiracetam was found to have developmental effects only at very high dose levels [35]. In addition, it was recently reported that LEV might be a safer alternative to VPA, as it involves a lower risk of major congenital malformations in women with epilepsy who are of childbearing age [36].

The aim of this study was to improve the predictability of the zebrafish model and to extend its applicability domain by using an integrated screening strategy, including a wide-range of endpoints (modules): morphology, behavior (motor activity parameters), histopathology, kinetics and phenotyping (*in situ* hybridization). Four well-known AEDs (valproic acid (VPA), carbamazepine (CBZ), ethosuximide (ETH) and levetiracetam (LEV)) with different developmental (neuro)toxic potencies were chosen as model compounds.

2. Material and methods

2.1. Fish husbandry and egg production

Adult wild type AB line Zebrafish (Zebrafish International Resource Center, Eugene, OR, USA) were kept in a 12 h dark/12 h light cycle at 28 °C in self-regulating aquaria (Tecniplast, Tecnilab-BMI, The Netherlands) at pH 7.5 and a water conductivity of 500 μS/cm. The animals were fed twice a day with dry flake food (SDS) and once a day with live food (Artemia), as recommended by Westerfield [37] and in accordance with the OECD draft guideline on the use of zebrafish for chemical testing [38]. Eggs and larvae used in this study were derived from a zebrafish colony held in the animal facility of TNO. Housing and management of the zebrafish colony fully comply with EU/2010/63 regulations [39]. For egg production, fish of between 7 and 11 months old were used. On the evening prior to the day of breeding, two male fish and one female fish were placed in a breeding tank containing a partition (Techniplast) and egg traps to prevent egg predation. Next morning, spawning was triggered by turning the light on and removing the partition. Eggs were collected using an 800 μm mesh and transferred to a Petri dish containing aquarium water spiked with 0.05% methylene blue. Staining indicates that eggs are either non-fertilized or that they have a damaged membrane. Any such eggs were discarded. Only batches in which more than 80% of the eggs were fertilized were used in the experiments.

Table 1
Concentration range from the DRF and main experiments.

Compound	VPA (μM)	CBZ (μM)	ETH (mM)	LEV (mM)
DRF	30–1500	30–720	2–20	0.5–150
Main	30–730	30–480	3–18	20–140

Abbreviations: VPA, valproic acid; CBZ, carbamazepine; ETH, ethosuximide; LEV, levetiracetam.

2.2. Compounds

Valproic acid sodium salt (VPA, CAS No. 1069-66-5), carbamazepine (CBZ, CAS No. 298-46-4) and ethosuximide (ETH, CAS No. 77-67-8) were purchased by Sigma-Aldrich (St. Louis, MO, USA). Levetiracetam (LEV, CAS No. 102767-28-2) was purchased by TCI Europe N.V. (Zwijndrecht, Belgium). First, a stock solution of each compound was prepared in dimethyl sulfoxide (DMSO). The stock solution was then further diluted to the desired concentration, in aquarium water. The final concentration of DMSO in the water supplemented with the compound was 0.2%. Therefore, 0.2% DMSO in aquarium water was used as negative control.

2.3. Experimental design

2.3.1. Dose range finding (DRF) experiments

In the DRF experiments, a wide range of concentrations (partly based on studies from Berghmans et al. [40] and Weigt et al. [41]) was selected for each test compound. Embryos were exposed to the selected concentrations to narrow down the relevant concentration range for further testing in main experiments. In the DRF studies, the embryos were assessed for lethal embryotoxic and/or developmental toxic effects at 24, 48, 72 and 96 hours post fertilization (hpf) (results not shown). The concentration ranges of the various compounds used in the DRF and subsequent main experiments are shown in Table 1.

2.3.2. Main experiments

The main experiments were performed after the concentration range of interest, which were the concentrations around the EC_{50} for morphological endpoints, had been established in the DRF studies. Selected embryos were at first placed in a Petri dish containing the test concentrations of interest (see Table 1). Subsequently, each embryo was transferred to a 12-well plate containing 3 ml of test medium per well, one embryo per well, unless otherwise indicated. Embryos were kept in an incubator (Binder Germany, Type BD115) at 26.5 ± 1 °C with a 12 h/12 h dark/light cycle and relative humidity of 100% (to prevent evaporation). For all experiments, we used intact embryos, the embryos were not dechorionated before exposure. Test solutions were not refreshed during the exposure period. The embryos were subjected to morphological evaluations at 24 and 48 hpf, primarily for lethal endpoints (data not shown). Evaluations at 72 and 96 hpf were used for detailed morphological developmental endpoints (see Section 2.4). Following a motor activity assessment (see Section 2.5) the larvae were euthanized, and processed for histopathological investigations (see Section 2.6). For morphology and motor activity analysis, three independent experiments were performed per compound, unless otherwise indicated. In each independent experiment, 12 embryo/larvae per concentration were used. Histopathology was