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Original article

Early safety assessment of human oculotoxic drugs using the zebrafish visualmotor response

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ABSTRACT

Introduction: Many prescribed drugs can adversely affect the eye by causing damage to the function of visual pathways or toxicity to the retina. Zebrafish have the potential to efficiently predict drugs with adverse ocular effects at pre-clinical stages of development. In this study, we explore the potential of using a semi-automated visual behaviour assay to predict drug-induced ocular toxicity in wild-type zebrafish larvae. **Methods:** 3 dpf larvae were treated with six known oculotoxic drugs and five control drugs in embryo medium containing 0.1% DMSO. After 48 h, larvae were assessed using the visualmotor response (VMR), an assay which quantifies locomotor responses to light changes; the optokinetic response (OKR), a behavioural assay that quantifies saccadic eye responses to rotating stimuli; and the touch response, a locomotor response to tactile stimuli. **Results:** 9 of 10 negative control drugs had no effect on zebrafish visual behaviour. 5 of the 6 known oculotoxic drugs (digoxin, gentamicin, ibuprofen, minoxidil and quinine) showed adverse effects on zebrafish visual behaviour assessed by OKR or the more automated VMR. No gross morphological changes were observed in treated larvae. The general locomotor activity of treated larvae, tested using the touch response assay, showed no differences with respect to controls. Overall the VMR assay had a sensitivity of 83%, a specificity of 100% and a positive predictive value of 100%. **Discussion:** This study confirms the suitability of the VMR assay as an efficient and predictive pre-clinical approach to evaluate adverse ocular effects of drugs on visual function in vivo.

1. Introduction

Adverse drug reactions can negatively impact upon patient welfare and can curtail further development of promising drugs. Indeed, of drugs entering clinical development, drug-induced organ toxicity is the leading factor associated with failure to reach the market (Kola & Landis, 2004). The toxic effects of drugs on vision are significant, with ~6.8% of drugs removed from clinical trials because of visual toxicity (Richards et al., 2008). Notably, adverse effects associated with drug-induced ocular toxicity are difficult to manage once they occur, and even though cardiovascular and gastrointestinal toxicity are of higher incidence, ocular toxicity has the highest negative influence on drug development (Redfern et al., 2008; Verdugo-Gazdik, Simic, Opsahl, & Tengowski, 2006). Thus, there is a need for efficient and predictive pre-clinical assays of ocular toxicity that can eliminate drugs that induce visual toxicity at earlier stages in development.

Many prominent FDA/EMA approved drugs adversely affect vision by functional or morphological damage to tissues in the visual pathway (Fraunfelder & Fraunfelder, 2004; Santaella & Fraunfelder, 2007). Here, we test the toxicity of cisplatin, digoxin, gentamicin, ibuprofen, minoxidil and quinine, on zebrafish visual behaviour. Cisplatin cross-links DNA and is a chemotherapeutic prescribed for solid tumours (Plummer et al., 2011). However, cisplatin can also produce irreversible oculotoxic effects including optic neuritis and retinal ischemia (Caraceni, Martini, Spatti, Thomas, & Onofrj, 1997; Kwan, Sahu, & Palexes, 2006). Optic neuritis is caused by immune-mediated destruction of the myelin sheath surrounding the optic nerve, leading to disrupted signal transmission between the eye and brain (de Seze, 2012). Cisplatin can also cause papilledema; a swelling of the optic disc caused by increased intracranial pressure (Schmid, Kornek, Scheithauer, & Binder, 2006). Cisplatin accumulation in the macular area initiates damage to the optic nerve and results in irreversible blindness (Al-Tweigeri, Magliocco, & DeCoteau, 1999). Although not a first-choice drug, digoxin is used to treat several cardiac dysfunctions by inhibiting the sodium/potassium ATPase pump (Lawrenson, Kelly, Lawrenson, & Birch, 2002). Digoxin is also known to inhibit this exchanger in photoreceptor cells and administration of digoxin is associated with altered colour perception and blurred vision (Lawrenson et al., 2002). The aminoglycoside antibiotic, gentamicin, is linked with conjunctivitis, in which accumulated drug irritates the conjunctival epithelial cells causing ocular burning (Thomas, Galiani, & Brod, 2001). Retinal

Abbreviations: dpf, days post fertilisation; ERG, electroretinogram; hpf, hours post fertilisation; OKR, optokinetic response; OMR, optomotor response; VMR, visualmotor response.

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detachment and lamellar liposomal inclusions have been identified in animals receiving gentamicin treatment because of abnormal deposition of lysosomal cells in the retinal pigment epithelium (RPE) (D'Amico et al., 1985). The anti-inflammatory agent ibuprofen, a non-selective cyclooxygenase inhibitor, causes optic neuritis and visual disturbances, perhaps by altering retinal blood flow (Gamulescu, Schalke, Schuierer, & Gabel, 2006; Haefliger, Meyer, Flammer, & Luscher, 1994). Minoxidil, a prostaglandin I2 inhibitor, can cause bilateral optic neuritis. A report suggests that this is mediated by induction of peroxides in the retinal vasculature (Gombos, 1983). Finally, the anti-malarial agent quinine, can cause blurred vision, optic neuritis and night blindness by directly damaging photoreceptor and "ON" type ganglion cells (Dyson, Proudfoot, Prescott, & Heyworth, 1985).

Zebrafish (*Danio rerio*) are freshwater fish belonging to the family Cyprinidae. These vertebrate model organisms are routinely used for research in genetics and developmental biology (Fadool & Dowling, 2008). Zebrafish also possess many attributes advantageous for pharmacological and toxicological research. These include; uncomplicated husbandry, small size, simplicity of generating offspring, high fecundity, external fertilisation and rapid development (Taylor, Grant, Temperley, & Patton, 2010). The established efficacy of several small molecules in zebrafish embryos provides *proof-of-principle* for efficient, medium-scale pharmacological screens (Richards et al., 2008; Taylor et al., 2010; Yin et al., 2012). As, in vitro and in vivo results often poorly correlate, zebrafish offer the advantage of a complex physiological environment that is absent in in vitro systems (Barros, Alderton, Reynolds, Roach, & Berghmans, 2008). Many of the major organs of zebrafish are formed within 24 h (Phillips et al., 2011). In relation to vision, the larval and adult zebrafish eye shows similar morphology to the human eye (Goldsmith & Harris, 2003). Development of the zebrafish eye commences as early as 11 hour post fertilisation (hpf) and by 3 day post-fertilisation (dpf) the morphology of the eye has the main characteristics of an adult eye (Goldsmith & Harris, 2003). The large eye size compared to the rest of the body reflects the importance of vision in zebrafish. Nascent visual behaviour responses are first seen in 3 dpf larvae and these mature significantly by 5 dpf (Easter & Nicola, 1996; Yin et al., 2012). Behavioural tests have long been used to assess vision and to screen for visual deficits (Brockerhoff et al., 1995). An optomotor response assay (OMR) examines the position of zebrafish placed in elongated chambers to moving black and white stripes presented underneath the transparent chambers (Neuhauss et al., 1999; Richards et al., 2008). The optokinetic response (OKR) assay is commonly used assay to examine larval eye movements to a rotating black-and-white striped drum (Brockerhoff, 2006; Fleisch & Neuhauss, 2006). Recently, semi-automated systems for quantifying the locomotor movements of zebrafish larvae in response to light stimuli have been developed. The visualmotor response (VMR) assay allows for the simultaneous monitoring of individual larvae in wells of a 96-well plate in response to lights being turned ON or OFF (Emran, Rihel, & Dowling, 2008; Yin et al., 2012).

This study supports previous studies reporting that zebrafish are an appropriate model to assess drug-induced visual toxicity (Richards et al., 2008). Here, we compare the OKR and VMR activity of 5 dpf wildtype larvae treated with drugs known to cause visual toxicity in humans. Five of the six tested drugs, resulted in altered visual locomotor behaviour in zebrafish, without effects on a tactile locomotor response. In addition, we demonstrate the utility of the semi-automated VMR assay to more efficiently identify drugs inducing adverse visual effects.

2. Methods

2.1. Zebrafish husbandry and maintenance

Wild-type stocks of the Tuebingen (Tu) and AB strains of zebrafish were maintained on a 14 hour light/10 hour dark cycle at 28 °C according to standard procedures (Westerfield, 2000). Male and female adults were placed in breeding tanks following their afternoon feed or 1–2 h before the end of the light period. Embryos were obtained by natural spawning and developmental stages were determined by morphology and development time (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). Embryos were raised in embryo medium (0.137 M NaCl, 5.4 mM KCl, 5.5 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 4.2 mM $NaHCO_3$) containing methylene blue on a 14:10 light: dark cycle at 28 °C. Media was changed daily. All experiments were carried out under ethical approval granted by the UCD Animal Research Ethics Committee.

2.2. Drug preparation & treatment

Drugs were obtained from the following suppliers: digoxin (Tocris Biosciences 20830-75-5) minoxidil, (Tocris Biosciences 38304-91-5), gentamicin (Gibco Life Technologies 15750–045), ibuprofen (Cayman Chemicals 15687-27-1), cisplatin (Sigma Aldrich 15663-27-1), quinine (Sigma Aldrich 6119-70-6), kanamycin (Sigma Aldrich 246-933-9), streptomycin (Sigma Aldrich 223-286-0), chloramphenicol (Sigma Aldrich 200-708-1), penicillin-G (Sigma Aldrich 113-98-4) and ampicillin (Sigma Aldrich 200-708-1). Drugs solutions were prepared using 0.1% v/v DMSO/Embryo media as solvent. Stock solutions of 10 mM were stored at −20 °C. On the day of treatment, unless stated otherwise, 10 μM dilutions of the drugs were prepared. 0.1% v/v DMSO/Embryo media was used as the vehicle control. Larvae were treated with drugs in a volume of 400 μl, from 3 to 5 dpf, in 48-well plates with 5 larvae per well. The fish were removed from drug in a fume hood before being assayed.

2.3. Optokinetic response assay

Single larvae were immersed in a Petri dish containing 9% methylcellulose (Sigma Aldrich #9004-65-3) to retard swimming behaviour and placed inside a drum with black and white stripes comprising 18° per stripe and a contrast of 99% (Brockerhoff, 2006). The drum was rotated at 18 rpm for 30 s clockwise and then 30 s anti-clockwise. The number of eye saccades produced in response to the rotation of the drum was manually recorded and the average number of saccades per minute quantified.

2.4. Visualmotor response assay

The VMR assay quantifies the locomotor behaviour of zebrafish larvae to light changes using an infrared tracking system (Emran et al., 2008). Individual larvae are placed in wells of a 96 well clear polystyrene plate (Whatman #7701-1651 square, flat bottom wells of 650 μl volume) and immersed in 600 μl of embryo medium. Unless stated otherwise, 12 larvae were used per treatment group. The plate was then placed in a Zebrabox recording chamber (Viewpoint Life Sciences, France) and the light driving parameters were set to 1 h and 40 min. Within this time period, the light is set to ON for the first 30 min to allow a period of settling. Following this, there are four periods where the light changes from ON to OFF and vice versa in twenty-minute intervals. The detection sensitivity is set to 10, the activity burst threshold set to 25 and the activity freeze threshold set to 3. The activity burst threshold corresponds to a given surface change by more than 25 pixels from one image to the next and activity above this threshold is regarded as burst activity. The activity freezing threshold corresponds to a given surface changing by less than 3 pixels from one image to the next and is regarded as having no activity. Locomotor activity between the two threshold values is regarded as normal activity. The activity of individual larvae is measured in milliseconds per second (ms/s). The analysis of the complex data produced by the VMR assay was achieved by customised MS Excel macro sheets available upon request from the authors. The "*overall activity*" of larvae within a treatment group for the 1 hour 40 minute assay is plotted as an activity trace. The average ON and OFF