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Short communication

Evaluating the developmental toxicity of trypanocidal nitroaromatic compounds on zebrafish

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ABSTRACT

Current therapies against African and American trypanosomiasis are problematic and with no immediate prospect of a vaccine there is an urgent need for cheap, more effective treatments. To aid the drug discovery pipeline, we report a novel *in vivo* screening approach using zebrafish (*Danio rerio*) embryos as a means of rapidly assessing a compounds developmental toxicity. This technique, amenable to high-throughput screening, was validated using several trypanocidal nitroaromatic prodrugs including nifurtimox and benznidazole.

1. Introduction

Over 10 million people are infected by *Trypanosoma brucei* and *Trypanosoma cruzi*, the causative agents of African and American trypanosomiasis, respectively. Treatment is restricted to a small number of drugs but their use is controversial (Wilkinson and Kelly, 2009). Against this backdrop there is a need for the development of novel, more effective and safer drugs. One group of compounds shown to have trypanocidal activity are the nitrobenzyl phosphoramide mustards (NBPMs) prodrugs (Hall et al., 2010; Hu et al., 2011). Originally developed as anticancer agents, they consist of a nitrobenzyl group attached to the cytotoxic phosphoramide mustard moiety (Hu et al., 2003) (Fig. 1). In reactions catalysed by type I nitroreductases (NTRs), the electron-withdrawing nitro group on the NBPM is converted to an electron-donating hydroxylamine. This promotes an electronic rearrangement in the structure's backbone resulting in cleavage of the benzylic C—O bond, leading to the release the cytotoxic phosphoramide mustard. Selectivity of NBPMs is via the activation reaction as type I NTRs are absent from most eukaryotes, with trypanosomes being an exception (Wilkinson et al., 2008).

Preclinical drug development requires cell culture and whole organism toxicity screening, the later often being seen as a limiting factor in the high throughput testing of chemicals. Zebrafish (*Danio rerio*), a small freshwater fish, are increasingly being developed and used to provide a cost-effective *in vivo* model for the large scale screening of chemicals (Yang et al., 2009; Zon and Peterson, 2005). Zebrafish lay hundreds of eggs externally, the embryos can be grown in multiwell plates (up to 96-wells), embryonic development is rapid (hatching at 2 days) and organ formation can be easily viewed throughout development. Moreover, some aspects of physiological function (for example, cardiovascular and nervous systems) remain relatively conserved between zebrafish and mammals, providing the potential to study disease (Chico et al., 2008; Nemtsas et al., 2010; Rinkwitz et al., 2011). These and several other factors all make zebrafish particularly useful as a vertebrate model in which to study drug action (Peterson and Macrae, 2012). Using the trypanocidal compounds nifurtimox, benznidazole and two NBPMs (LH34 and LH37) (Fig. 1), we set about establishing a zebrafish toxicity screening assay.

2. Materials and methods

Embryos collected by natural spawning and staged according to Kimmel et al. (1995) were placed in 1 ml embryo medium and

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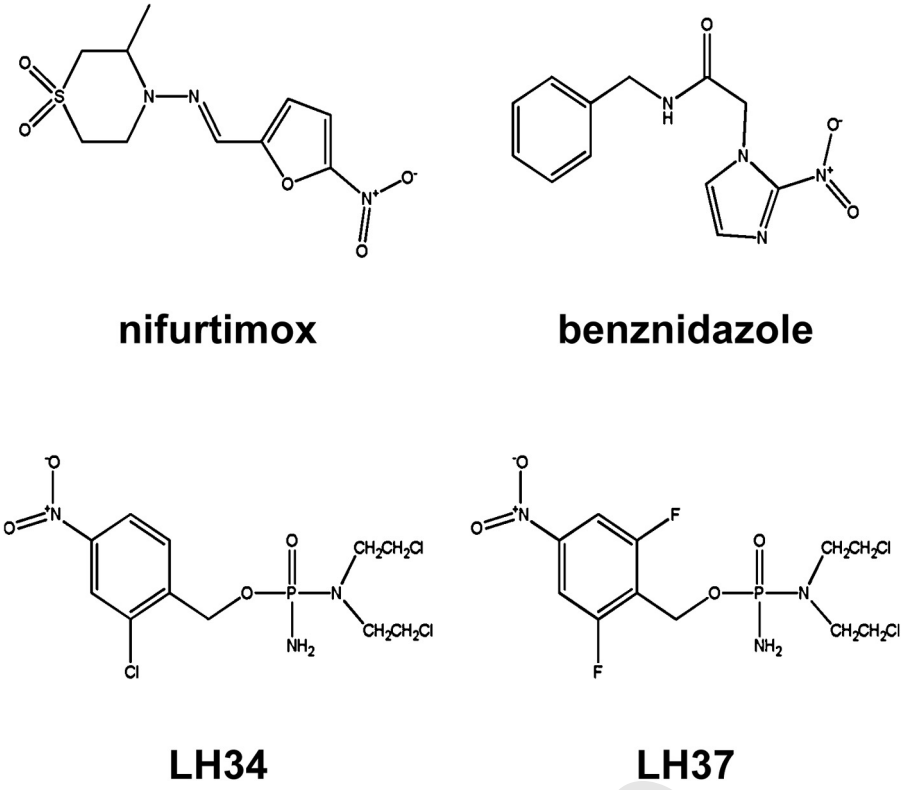

Fig. 1. Structure of nitroaromatic compounds.

incubated at 28.5 °C (Kimmel et al., 1995). At 24 h post fertilisation (hpf), which corresponds to the end of the segmentation stage when the primary stages of organogenesis are complete and the fish has begun to move, the embryos were treated with different concentrations of drug, prepared using DMSO. Drugs were dissolved in DMSO to make a stock solution (100 mM) and a dilution series prepared (300 μM, 100 μM, 33.3 μM, 11.1 μM and 3.7 μM). A vehicle control, equivalent to the concentration of DMSO included in the highest concentration of drug (0.3% DMSO, v/v), was included in each experiment. After 6, 30 and 78 h post-treatment, when the embryos had reached Prim-15 stage (30 hpf), hatching (54 hpf) and larva stages (102 hpf), respectively, their gross embryonic development was checked using a dissecting microscope (Leica) (Westerfield, 1995). At each time point a number of phenotypes were assessed: (i) cardiovascular malformations including any pericardial oedema, alterations to the heartbeat and blood circulation, (ii) swimming behaviour, as assessed by observation of spontaneous events and evoked responses by light touch to the head of the embryo and finally (iii) embryonic death. Additional phenotypic changes were assessed by analysing tissue structure following immunocytochemical staining (Ashworth et al., 2001; Brennan et al., 2005; Westerfield, 1995).

3. Results and discussion

In the absence of drug or in the presence of benznidazole, no significant phenotypic changes were observed to the embryos at any time point examined (Fig. 2A). Analysis of other nitroaromatic agents indicated that several trypanocidal nitrotriazoles NS43 and NS18 behaved similarly (data not shown) (Papadopoulou et al., 2012a,b). In contrast, incubation of embryos in nifurtimox, LH34 or LH37 resulted in weakened heartbeat, pericardial oedema or death (Fig. 2B–D). In the highest concentration (300 μM) at 54 hpf, all nifurtimox-treated embryos exhibited pericardial oedema, in addition other phenotypes including disruption to their swimming behaviour, a reduction in evoked swimming behaviours and reduced pigmentation were observed; the latter is in agreement with the observation that nifurtimox affects melanocyte development in zebrafish (Fig. 2B) (Zhou et al., 2012). By 102 hpf, nifurtimox-treated embryos were dead (300 μM) or displaying pericardial oedema (100 μM). LH34-treated embryos displayed no developmental defects until 54 hpf where ~50% and ~75% of embryos were dead or presenting with pericardial oedema at 100 μM and 300 μM, respectively, (Fig. 2C). Defective movement, slower swimming behaviour and an impaired touch response were also observed (but not quantified) in embryos maintained in drug concentrations as low as 33.3 μM. By 102 hpf, 100% embryonic death was recorded in the 300 μM LH34 treatment group, with cardiovascular impairment noted in the live embryos maintained in 100 μM drug. For LH37-treated embryos, no developmental defects were detected at 30 hpf (Fig. 2D). By 54 hpf, ~90% of embryos in medium containing 100 μM LH37 displayed pericardial oedema and significant embryonic death (~80%) recorded at 300 μM. As for LH34, embryos incubated in 33.3 μM or 100 μM LH37 exhibited an impaired, slowed spontaneous swimming behaviour and were non-response to touch. By 102 hpf, most embryos were dead in the 100 μM and 300 μM LH37-treated groups.

The above phenotypic analysis indicated that high concentrations of nifurtimox, LH34 or LH37 caused disruption to the movement and swimming behaviour suggesting that they may affect neuromuscular tissues. Immunocytochemical staining of drug-treated embryos using sarcomeric myosin heavy chain and actin markers for the slow and fast type of muscle fibres, respectively, (Brennan et al., 2005), revealed defects in the skeletal muscle, with the somites displaying anteroposterior compression and the fibres having a wavy and misaligned appearance (data for LH37 treatment is shown in Fig. 3A). For LH37 but not nifurtimox and LH34 (all at 100 μM), this was accompanied by a significant reduction in the dorsal somite width when compared to controls (Fig. 3B). When the tissue structure of the central nervous system and heart

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