Pre-clinical screening of trypanocidal drugs

Evaluating the developmental toxicity of trypanocidal nitroaromatic compounds on zebrafish

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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.

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 [1]. 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 [2, 3]. Originally developed as anticancer agents, they consist of a nitrobenzyl group attached to the cytotoxic phosphoramide mustard moiety [4] (Figure 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 [5].

Preclinical drug development requires cell culture and whole organism toxicity screening. One system that is increasingly being viewed as a useful, cost-effective model uses zebrafish (*Danio rerio*) embryos [6], primarily because zebrafish eggs are laid externally and organ formation can be easily viewed throughout development. Moreover, there is a high degree of similarity in physiological function between zebrafish and mammals, for example in their cardiovascular and nervous systems [7-9]. These and several other factors all make zebrafish particularly useful as a vertebrate model in which to study drug action [10].

Using the trypanocidal compounds nifurtimox, benznidazole and two NBPMs (LH34 and LH37) (Figure 1), we set about establishing a zebrafish toxicity screening assay. Embryos collected by natural spawning and staged according to Kimmel *et al* . (1995) were placed in 1 ml embryo medium and incubated at 28. 5°C [11]. At 24 hours 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. After 6, 30 and 78 hours post-treatment, when the embryos had reached Prim-15 stage (30hpf), hatching (54hpf) and larva stages (102hpf), respectively, the embryonic phenotype was assessed and any gross

developmental changes, such as alterations in heart beat, blood circulation, swimming behaviour and embryonic death, recorded. Other phenotypic changes were assessed by analysing tissue structure following immunocytochemical staining [12-14].

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 (Figure 2A). Analysis of other nitroaromatic agents indicated that several trypanocidal nitrotriazoles NS43 and NS18 behaved similarly (data not shown) [15, 16]. In contrast, incubation of embryos in nifurtimox, LH34 or LH37 resulted in weakened heart beat, pericardial oedema or death (Figure 2B, C and D). At the highest concentration (300ï ¡M) and at 54hpf, all nifurtimox-treated embryos exhibited pericardial oedema, disruption to their swimming behaviour, were less responsive to touch and had reduced pigmentation, the latter is in agreement with the observation that nifurtimox affects melanocyte development in zebrafish [17] (Figure 2B). By 102hpf, nifurtimox-treated embryos were dead (300µM) or displaying pericardial oedema (100µM). LH34-treated embryos displayed no developmental defects until 54hpf where ~50% and ~75% of embryos were dead or presenting with pericardial oedema at 100µM and 300µM, respectively (Figure 2C). Defective movement, slower swimming behaviour and an impaired touch response were also observed even in embryos maintained in drug concentrations as low as 33. 3µM. By 102hpf, 100% embryonic death was observed using 300µM LH34, with cardiovascular impairment noted in the live embryos maintained in 100µM drug. For LH37-treated embryos, no developmental defects were detected at 30hpf (Figure 2D). By 54hpf, ~90%

of embryos in medium containing 100µM LH37 displayed pericardial oedema and significant embryonic death (~80%) recorded at 300µM. As for LH34, embryos incubated in 33. 3µM or 100µM LH37 exhibited an impaired, slowed swimming behaviour and were non-response to touch. By 102hpf, most embryos were dead in the 100µM and 300µM 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 [13], 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 Figure 3A). For LH37 but not nifurtimox and LH34 (all at 100µM), this was accompanied by a significant reduction in the dorsal somite width when compared to controls (Figure 3B). When the tissue structure of the central nervous system and heart was assessed using anti-tubulin or anti-cardiac myosin markers, respectively, in 48hpf embryos treated with 100µM LH34 or LH37 no gross morphological difference were observed between drug dosed samples and controls (data not shown).

Our work highlights the potential of a zebrafish embryo-based *in vivo* drug screening/toxicity assay, an approach readily amenable for high throughput analysis. The response to a given agent can be followed by monitoring phenotypic changes using direct, non-invasive observations followed by tissue specific immunocytochemical staining. The assay was initially

validated using nifurtimox and benznidazole with the resultant zebrafish toxicity data showing that the nitrofuran caused more unwanted side effects as compared to the 2-nitroimidazole. Such issues in humans have led the withdrawal of nifurtimox in several Latin countries as an American trypanosomiasis treatment in favour of benznidazole, even though the latter compound does reportedly promote genotoxicity and other of side effects [18, 19]. In relation to NBPMs, we have shown that in this *in vivo* model, LH34 and LH37 both have toxicity issues at high concentrations (> 100ï M) but these problems are offset by their significant trypanocidal activities (IC_{50} <10nM). Our screening data highlights the potential of zebrafish as a reliable reporter system for monitoring toxicity in a whole organism that will aid drug discovery not only against trypanosomal diseases but whole series of other ailments.

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