Toxicology in Vitro 44 (2017) 11–16

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Application of *Caenorhabditis elegans* (nematode) and *Danio rerio* embryo (zebrafish) as model systems to screen for developmental and reproductive toxicity of Piperazine compounds

Peter I. Racz[a,*,1], Marjolein Wildwater[b,*,1], Martijn Rooseboom[c], Engelien Kerkhof[d], Raymond Pieters[b,e], Elena Santidrian Yebra-Pimentel[a], Ron P. Dirks[a], Herman P. Spaink[f], Chantal Smulders[c], Graham F. Whale[g]

[a] *ZF-screens BV, J.H. Oortweg 19, 2333 CH Leiden, The Netherlands*
[b] *University of Applied Sciences Utrecht, Heidelberglaan 7, 3584 CS Utrecht, The Netherlands*
[c] *Shell Health, Shell International B.V., Carel van Bylandtlaan 16, 2596 HR The Hague, The Netherlands*
[d] *University of Applied Sciences of Arnhem and Nijmegen, Laan van Scheut 2, 6525 EM Nijmegen, The Netherlands*
[e] *Institute for Risk Assessment Sciences, Yalelaan 104, 3584 CM Utrecht, The Netherlands*
[f] *Institute of Biology, Leiden University, Einsteinweg 55, 2333 CC Leiden, The Netherlands*
[g] *Shell Health, Brabazon House, Threapwood Road, Manchester M22 0RR, United Kingdom*

ARTICLE INFO

Keywords:
Piperazine
Zebrafish
Nematode
Danio rerio
Caenorhabditis elegans
Development
Reproduction

ABSTRACT

To enable selection of novel chemicals for new processes, there is a recognized need for alternative toxicity screening assays to assess potential risks to man and the environment. For human health hazard assessment these screening assays need to be translational to humans, have high throughput capability, and from an animal welfare perspective be harmonized with the principles of the 3Rs (Reduction, Refinement, Replacement).

In the area of toxicology a number of cell culture systems are available but while these have some predictive value, they are not ideally suited for the prediction of developmental and reproductive toxicology (DART). This is because they often lack biotransformation capacity, multicellular or multi- organ complexity, for example, the hypothalamus pituitary gonad (HPG) axis and the complete life cycle of whole organisms.

To try to overcome some of these limitations in this study, we have used *Caenorhabditis elegans* (nematode) and *Danio rerio* embryos (zebrafish) as alternative assays for DART hazard assessment of some candidate chemicals being considered for a new commercial application. Nematodes exposed to Piperazine and one of the analogs tested showed a slight delay in development compared to untreated animals but only at high concentrations and with Piperazine as the most sensitive compound. Total brood size of the nematodes was also reduced primarily by Piperazine and one of the analogs. In zebrafish Piperazine and analogs showed developmental delays. Malformations and mortality in individual fish were also scored. Significant malformations were most sensitively identified with Piperazine, significant mortality was only observed in Piperazine and only at the higest dose. Thus, Piperazine seemed the most toxic compound for both nematodes and zebrafish.

The results of the nematode and zebrafish studies were in alignment with data obtained from conventional mammalian toxicity studies indicating that these have potential as developmental toxicity screening systems. The results of these studies also provided reassurance that none of the Piperazines tested are likely to have any significant developmental and/or reproductive toxicity issues to humans when used in their commercial applications.

1. Introduction

New products that are brought to the market have to be proven safe for man and the environment. Hazard assessment of compounds, in close conjunction with exposure characteristics, are therefore essential and mandatory requirements. Accepted regulatory toxicity testing for chemicals currently requires mammalian studies (i.e. rat and rabbit), which are time- and money-consuming and increasingly considered

* Corresponding authors.
E-mail addresses: racz@zfscreens.com (P.I. Racz), marjolein.wildwater@hu.nl (M. Wildwater).
[1] These authors contributed equally, arranged on alphabetical order.

http://dx.doi.org/10.1016/j.tiv.2017.06.002
Received 6 November 2016; Received in revised form 6 May 2017; Accepted 4 June 2017
Available online 26 June 2017

unethical by society. Furthermore, especially when potential hazard for development and reproduction (DART) is considered, these mammalian test systems only show low predictive values to man (Sipes et al., 2011). Proper establishment of alternative testing strategies that are quick, low cost, ethical and predictive are therefore urgently required to reduce, refine and replace (3R principle) mammalian testing.

Historically the focus was set on the use of cell culturing systems to provide promising alternative testing strategies. While these systems have some benefits (e.g. the possibility of using human cells), these systems lack the complexity of a complete organism with different organs and cell-cell and tissue-tissue signaling, organismal defense mechanistic responses towards potential hazardous compounds and as such have their limitations in possible applicability. There is a need for lower cost, more rapid, less animal intensive studies to help screen potential new products to identify those which raise concerns and may require additional assessment. Such tests could also have value to help in the definition of existing product categories under the EU REACH regulations by either 'proving' similar modes of actions and/or identifying products with the highest potential to cause adverse developmental/reproductive effects for longer term animal tests.

Recently two alternative in vivo model systems, *Caenorhabditis elegans* (nematode) and *Danio rerio* (zebrafish) which were well known and used in the field of Developmental and Molecular Biology became noticed as potential promising test systems for hazard assessment (Avila et al., 2012; Ballatori, 2002; Boyd et al., 2016, 2010; Brannen et al., 2010; Hermsen et al., 2011; Leung et al., 2008; Panzica-Kelly et al., 2010; Dhawan et al., 1999; Selderslaghs et al., 2009, 2012). Both species share high genetic homology to man (~60% for nematodes and 70% for zebrafish), show cell biologically conserved molecular responses (like organ development, cell and tissue signaling etc.) and have proven their translational value (for example, the Nobel prize for the discovery of apoptosis and miRNAs was rewarded to nematode researchers (Fire et al., 1998) and both systems are commonly used in medical research (Ordas et al., 2015; Phillips and Westerfield, 2014; Poureetezadi and Wingert, 2013; Stewart et al., 2014).

Both nematodes and zebrafish embryos until 5-day post-fertilization (5dpf) are not considered animals according to relevant animal welfare acts and regulations. As nematodes and zebrafish are optically transparent small animals with a high reproductive and developmental turnover they can be considered as an alternative test species for DART assessment. Because of the high number of progeny each nematode is able to produce around 250 eggs within 3 days, and one zebrafish animal can produce up to 300 eggs in a week, these organisms have the potential for high throughput screening. Nematode progeny is furthermore genetically tractable as nematodes are self-fertilizing hermaphrodites of only 1 mm in size that have shown highly reproducible predictive developmental timing (Sulston and Horvitz, 1977; Sulston et al., 1983). Young nematode larvae develop within 3 days to reproductive hermaphrodites. In zebrafish, development is also rapid as most organs are formed during early embryo development within 3 days post fertilization. Thus, these systems show high potential to be properly validated as alternative 3R DART test systems.

In the research project, CRACKIT PreDART funded by the NC3Rs (UK's national organisation which leads the discovery and application of new technologies and approaches for 3R purposes), the methodology for implementation of nematodes and zebrafish as alternative 3R test models for developmental and reproductive toxicity was set up (publications in progress). Out of 31 well characterized DART compounds tested in nematodes and zebrafish, respectively 27 and 23 were properly predictive for DART. Interestingly, the ones that were missed by one of the two systems were picked up as DART compounds by the other system and thus all compounds were scored correctly by combinatorial testing using nematodes and zebrafish.

In this study a number of Piperazine analogs for commercial application have been evaluated in an experimental screen for reproductive and developmental toxicity using nematodes and zebrafish embryos. The screening studies are being evaluated for their potential to detect developmental toxicity (e.g. intrauterine death including preimplantation loss, structural abnormalities, altered growth and functional deficits) while avoiding significant use of animals.

In these initial investigations, compounds were tested to assess if the 'screening' studies could detect differences in their potential to cause developmental/reproductive effects. The amines selected were Piperazine (CAS: 110-85-0) and the Piperazine analogs A, B and-C. (PIP-A; PIP-B and PIP-C) One advantage of these substances was that these are stable and water soluble thereby mitigating any concerns regarding their exposure to the organisms.

Piperazine has been classified as a category 2 repro-toxicant under the EU's Classification, Labelling and Packaging (CLP) regulations (EC) No 1272/2008 and was used as a positive control in the studies described, whereas the Piperazine analogs have not been tested and currently have not been classified. In rodents Piperazine is a weak class-2 toxicant as it causes embryotoxic effects as resorptions, retardation of ossification, reduced foetal weights and malformations only at high doses. These effects are considered to be a secondary effect of maternal toxicity, rather than a direct developmental or reproductive toxicity effect (Cross et al., 1954; Ridgway, 1987; Risk et al., 2005).

2. Materials & methods

2.1. Materials

Piperazine (95% purity) was obtained from Sigma-Aldrich (P45907), Piperazine analogs (95% purity) where provided by Shell.

2.2. Nematodes

Nematodes of the N2 strain were synchronized using hypochlorite and hatched L1 larvae were exposed to the compound that was dissolved in nematode growth medium (NGM). L1 larvae were allowed to develop into adults and subsequently transferred daily to fresh medium. The range of exposure concentration was the same for all compounds, i.e. 10^{-7} M, 10^{-6} M, 10^{-5} M, 10^{-4} M, 10^{-3} M, 10^{-2} M. Brood size was determined by daily passage of adult nematodes onto new plates and subsequent counting of offspring. The sum of all progeny on all subsequent wells was used to calculate the total brood size per nematode. Developmental progression was scored by analyzing stage-specific parameters (organ development rate) as shown in Fig. 1, Fig. 2, Table 1, Tables S2 and S3 using the published cell lineage papers (Sulston and Horvitz, 1977; Sulston et al., 1983). Note: Control populations should never show any deviation in developmental progression (developmental delay). If they do, experiments are aborted.

Four days before the start of the experiment, nematodes are grown to bulk quantities on normal food and media (20 times a 5 cm NGM plate with bacterial OP50 food) to ensure sufficient animals to enable the compound test assay. One day before the start of the experiment (the start of exposure), these nematode cultures were bleached to synchronize progeny for the assay. In the absence of food bleaching results in a synchronous population of L1 staged animals ready for the test the next day.

On the first day of the test (day 0) hatched L1 larvae were placed onto the NGM agar containing compound and grown at 15 °C for 72 h to become L4 larvae. Then they were checked under the microscope for developmental age and morphological effects as listed in Table 1.

Additionally, reproduction effects were scored by exposing 30 individual L4 animals in three 12 wells plates. For a period of 4 additional days, these nematodes were transferred each day to a new well leaving any progeny left on the old plate to grow for one more day before counting and assessing the viability of the progeny (hatched eggs) and total brood size.

Proper development of the offspring was assessed by examining them under a Zeiss Axio Imager M2. The nematode cell lineage is