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

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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

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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

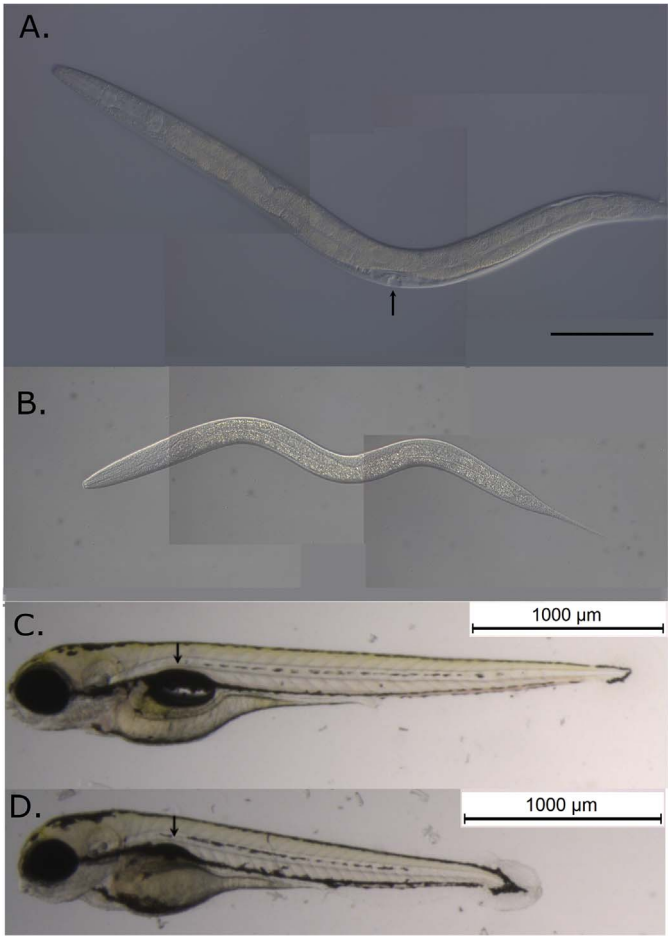

Fig. 1. Phenotypic effects in nematodes and zebrafish exposed to Piperazine reveal mild effects in development.
Nematodes and zebrafish both show a mild developmental delay when exposed to high concentrations of Piperazine. While 100% of the nematode larvae developed to L4 stage in the control after 25 h exposure at 20 °C (A), 10^{-2} M Piperazine exposure revealed 71% L3 and 29% L2 stage animals (B) (also shown in Table S2). Fig. B shows an L3 stage animal. The arrow in A indicates the vulva, a developmental marking point of L4 stage. Scalebar: 100 µm. Panel C and D show zebrafish embryos and show delayed zebrafish development (D) compared to the control (C). In zebrafish in 60% of the cases the swim bladder appeared undeveloped at 4dpf after Piperazine exposure, indicative for developmental delay effects (position of swim bladder is indicated by an arrow). Also tail length and head-trunk angle are affected in the animal in D and are indicative for developmental delay.

completely mapped and development is traceable within precision of hours using the state of organ development as a reference point in relation to the total developmental time (development of young larvae only starts when they receive food; $t = 0$). Both vulva development as well as gonadogenesis can be scored in L4 larvae to monitor developmental progression. Clear synchronous stages of the developing vulva can be observed during time, like early divisions of the vulva precursor cells (VPCs) in L3 stage of development (starting after 29 h at 20 °C), appearance of the initial vulva cleft (34 h at 20 °C), Christmas tree (40 h at 20 °C), and vulva lip formation (50 h at 20 °C). In case of developmental delay, all parameters should have the features that are representative for younger animals than expected according to experimental duration. Because of these easy set of scorable characteristics, affected development can be monitored in a precise manner and can be separated from organ specific effects.

2.3. Zebrafish

Zebrafish experimental procedures were conducted in accordance with local and international regulations and followed the guidelines on the protection of experimental animals by the Council of Europe, Directive 2010/63/EU reduction, replacement and refinement strategy. Zebrafish were handled and maintained according to standard protocols ("The Zebrafish Model Organism Database," ZFIN www.zfin.org). Zebrafish larvae were collected from laboratory cultures. All tests were undertaken at 28 °C under a 14 h:10 h dark-light cycle. Controls and tests solutions were prepared in 'egg water' (60 µg/ml Instant Ocean™ sea salt, Sera Marin in distilled water). Individual larvae were raised in a separate well in a 24 well polypropylene plates containing 2 ml of the test substance (10^{-7} M, 10^{-6} M, 10^{-5} M, 10^{-4} M, 10^{-3} M, 10^{-2} M). Larvae were exposed in the static way. For phenotypic observation bright-field, Leica M165C stereomicroscope was used at various magnification (2 ×-16 ×) equipped with a DFC420C digital colour camera (Leica Microsystems).

20 newly fertilized zebrafish eggs were selected per replicate, between 2 and 64 cell stage (before blastulation) and exposed to test chemicals for a period of 96 h. The development of the embryos was followed on a daily basis. After 96 h, lethality was assessed on the basis of either/or: (I) coagulation of fertilized eggs, (II) lack of somite formation, (III) lack of heartbeat. At the end of the 96 h exposure period, fish larvae behavior in response to mechanical stimuli and phenotypic changes were recorded. Unresponsive behavior of the test is indicative of abnormal development or destruction of the nervous system and/or abnormality of the muscle contraction. Phenotypic examinations were undertaken on 20 larvae per concentration using relevant endpoints identified during the NC3R Crack it PREDART project (Table 1 and Table S4). The procedure was performed in duplicate. 10% deviation from zero incidents was accepted for the internal control fish (4 per 24 well plate) similarly to what has been agreed as acceptable in the Fish Embryo Acute Toxicity (FET) Test (OECD/OCDE 236). The phenotypic assessments, which were considered to be indicative of teratogenicity, included observation of abnormalities in organ development (Table 1). Acute toxicity (lethality) and delayed development were also scored on day 4 post fertilization. Spontaneous incidents in the untreated control group were scored as well. In the case of low occurrence (< 10%) in the experiment, the results were normalized to the untreated control group and the score of the spontaneous events were subtracted from the result. In the case of higher percentage of spontaneous death or malformation in the control group (> 10%), the test became invalid and was discarded. Characterization of normal development of the embryo was followed in the untreated control group and was identified based on the standard developmental timeline (Kimmel et al., 1995). Developmental delay was based on three main phenotypic appearances: head-trunk angle, tail length and occurrence of the swim bladder. When effects in at least two characteristics were scored, this was indicated as developmental delay. Note: delayed development might be a secondary effect of abnormal organ development and conclusions regarding developmental delay should, therefore, be treated with caution.

3. Results & discussion

Nematodes and zebrafish larvae were exposed to a range of concentrations of Piperazine and three Piperazine analogs. Developmental effects were scored by analyzing organ development and a set of other parameters (see Table 1 and Tables S2 and S4).

No chemical analysis was undertaken to assess exposure concentrations. However, based on their physicochemical properties, including water solubility, all the compounds are expected to be soluble and well absorbed (Lipinski, 2004). Furthermore, as Piperazine appears to be well absorbed (with peak plasma concentrations attained 1 h after oral administration according to the REACH dossier), it can be assumed that nematodes and zebrafish have been exposed significantly systemically.

Nematodes exposed to Piperazine and PIP-A, showed a slight delay in development compared to untreated animals but only at high

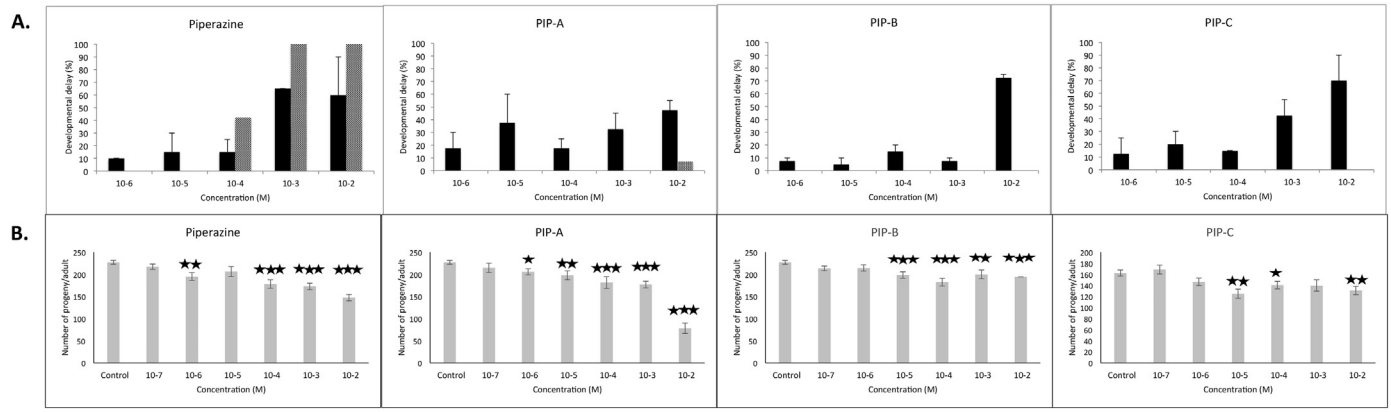

Fig. 2. Only high concentrations of Piperazines affect developmental rate and reproduction in nematodes and zebrafish.
The four top panel graphs indicate developmental delay in nematodes and zebrafish after exposure to different concentrations of Piperazines (Piperazine, PIP-A, PIP-B and PIP-C). Nematode bars are in light grey, zebrafish bars in black. PIP-B and PIP-C do not cause any developmental delay in nematodes while Piperazine at high concentrations causes the strongest developmental delay. Only a trend could be scored in Piperazine and PIP-C in zebrafish as unlike the effects in nematodes, developmental delay could be caused by a range of secondary effects like: acute toxicity, organ malformation and effects on the rate of development. All plotted samples are normalized against the control. As the test criteria for valid nematode tests is that control nematodes always develop according to a fixed time schedule (without variation), all light grey bars represent therefore the deviation from the control (A). The lower four panels show the average number of offspring per nematode hermaphrodite larvae after exposure to the different Piperazine analogs. Only high concentrations of Piperazine analogs cause effects on the number of offspring. Piperazine and PIP-A show the strongest effects. Significance was determined by an unpaired *t*-test with 95% confidence interval (*p < 0,05; **p < 0,01, ***p < 0,001) (B). Error bars are indicating the standard error (standard deviation/√n).

concentrations (starting at 10^{-4} M and 10^{-2} M, respectively), whereas PIP-B and PIP-C did not show any delay effects (Figs. 1 and 2 and Table S2). Total brood size of the nematodes was also reduced when exposed to Piperazine and PIP-A at 10^{-6} M and higher, for PIP-B and PIP-C at 10^{-5} M and higher (Fig. 2B and Table S3). There was no effect on larval mortality in nematodes after any of the treatments.

In zebrafish an increased dose of Piperazine and analogs indicated an increase in the percentage of fish with developmental delay.

Table 1
Scoring table of potentially affected organ development & reproduction.

A broad range of developmental and reproductive effects were scored after Piperazine exposure in nematodes and zebrafish. Both species were only mildly affected by the Piperazines. The percentage of the affected organisms at the highest test concentration (10^{-2} M) are indicated in the table for the individual compounds. All data is normalized to the control. Brood size in nematodes was significantly affected. Only the hemorrhage that was observed in PIP-A appeared to be significant in zebrafish. Significance values are indicated as followed: < 0.05 (*), < 0.01 (**) and < 0.001 (***). The number of incidences and statistics can be found in Tables S2, S3, S4 and S5.

Organism	Phenotype	Effect	Affected organisms (%) at highest test concentration (10^{-2} M)			
			Piperazine	PIP-A	PIP-B	PIP-C
N	Reproductive organs (gonad, vulva)	Organisation, shape, size and absence of the organs; multi vulva	0	0	0	0
N	Nervous system	Movement, egg laying, behavoir	0	0	0	0
N	Intestine	Organisation, shape, size and presence of the organs	0	0	0	0
N	Cuticle	Molting problems, protruding/burst through vulva, dumpy, blistered	0	0	0	0
N	Muscles	Movement, egg laying, uncoordinated movements	0	0	0	0
ZF	Fin		0	0	0	0
ZF	Heart	Acardia - absence of heart	0	0	0	0
		Pericardial oedema	7.5	10.0	2.5	0
		Tube heart formation (heart has no chamber)	0	0	0	0
		Cardiac enlargement	0	0	0	0
ZF	Brain (head)	Brachycephalic (short broad head)	0	0	0	0
		Dolichocephalic (long narrow head)	0	0	0	0
		Reduced development the nose and the jaw	0	0	5	0
ZF	Spine	Bent tail, bent head-trunk angle	0	0	2.5	2.5
ZF	Eye	Cyclopia (one eye)	0	0	0	0
		Eye oedema	0	0	0	0
N	Clear	Often correlated to defects in FGF signaling pathway	0	0	0	0
N	Chromosomal instability	High incidence of males	0	0	0	0
N	Variably abnormal	Often correlated with cell-cell contact problems in epithelial cells	0	0	0	0
N	Size	Often correlated with cell division problems	0	0	0	0
N	Reduced number of progeny	Percentage	35.1***	65.7***	14.6***	19.3**
N	Dauers	Often correlated with problems in metabolism or eating problems	0	0	0	0
ZF	Hemorrhage	Blood collection in abnormal places	0	10***	2.5	2.5
ZF	Larvae movement	Partial hatch/no reaction to touch stimulus	0	0	0	0
ZF	Excessive opercular movement	(Absence of oxygen)	0	0	0	0
ZF	Abnormal hatching		0	0	2.5	0
ZF	Pigment formation	Abnormal pattern	2.5	2.5	0	0
		Absence of pigmentation	0	0	0	0

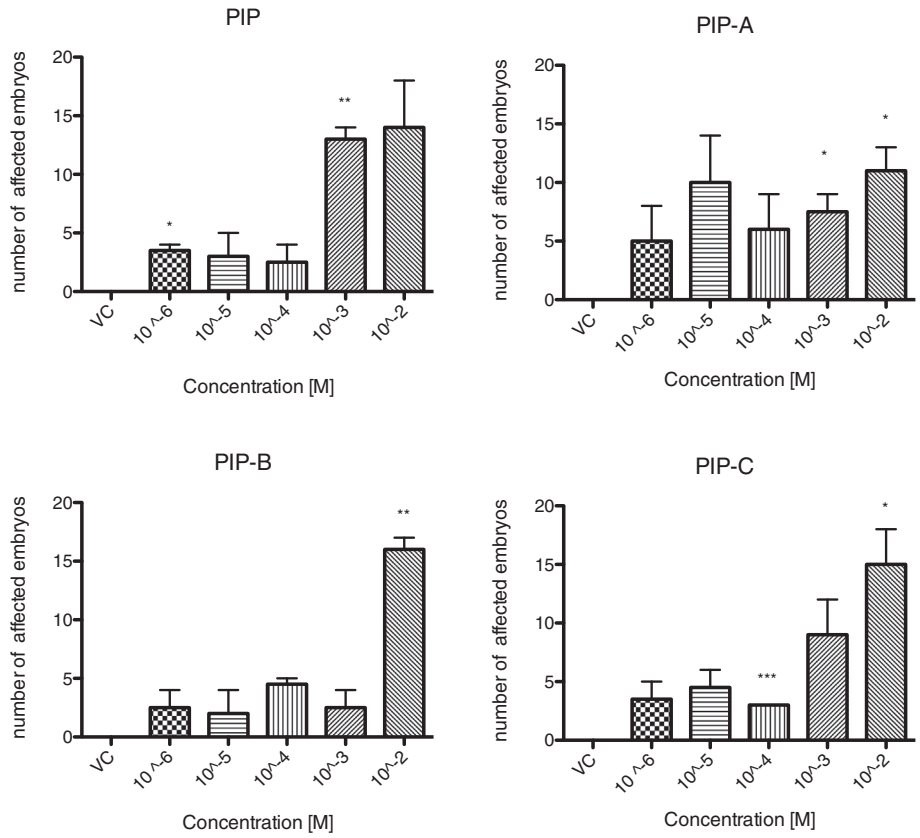

Fig. 3. The number of affected zebrafish embryos upon treatment with Piperazine and its analogos.
Malformation occurrence was registered based on a CrackIT scoring table (y-axis represents the number of affected embryos). The bars represent the mean and standard deviation of the experiments (n = 2 experiments). The malformation occurrence was normalized to controls, and acute toxicity effects were corrected. A significant number of fish with malformations can be observed at Piperazine concentrations of 10^{-6} M. The number of incidences increases at higher concentrations in all analogs (see also Tables S4 and S5). Statistical values were calculated with an unpaired *t*-test with 95% confidence interval (*p < 0,05; **p < 0,01, ***p < 0,001).

Developmental delay could occur as secondary effects of malformations or acute toxicity and thus possibly represents an accumulative effect.

Malformations in individual fish were therefore also scored (Fig. 3 and Table S4). A significant number of fish with malformations could be seen at Piperazine concentrations of 10^{-6} M. Yet, a huge increase in the number of incidences took only place at higher concentrations in all analogs (Fig. 3, Table S4, Table S5). PIP-A and PIP-B required an even 1000 × higher dose before malformations were statistically relevant. Thus, Piperazine seemed the most toxic compound in zebrafish with only high increase in incidence numbers at high dose.

None of the tested compounds showed effects on heart function in zebrafish (bradycardia, tachycardia and arrhythmia). In addition, there was no indication of neurological functional defects as all of the exposed zebrafish larvae, even in the highest concentrations, responded to the touch stimulus test (Table 1, data not shown). A very weak effect was found when mortality was assessed in the highest concentration of Piperazine (Table S1).

In summary, from the Piperazines tested in the current study, Piperazine itself was the most potent toxicant to induce both reproduction toxicity and developmental delay in nematodes and malformations and mortality in zebrafish. Piperazine and PIP-A showed the highest sensitivity in affecting brood size in nematodes (10^{-6} M). These results are in alignment with the reported test data for rats and rabbits on Piperazine, where indications of reproductive effects were observed at high test concentrations (Cross et al., 1954; Ridgway, 1987; Risk et al., 2005). Furthermore, the observed responses are considered to be a consequence of maternal toxicity rather than a direct developmental or reproductive effect per se. Therefore, based on all of the above it is concluded that the Piperazine analogs tested are unlikely to be developmental toxicants.

An important consideration from the outset was the speed and cost of the alternatives in comparison to longer term 'traditional' DART studies. The tests have been compared in Table 2.

These data demonstrates that in comparison to the conventional DART studies the alternative methods are rapid, far less time consuming and could significantly reduce animal use. At the moment nematodes and zebrafish tests are not yet suitable to make translational statements on effective concentration levels in higher systems nor in other aspects of risk assessment. This study indicates however that there is a relevance to use these assays as alternative screening tests to identify potential developmental and reproductive toxicity effects of compounds early in the product developmental pipeline.

Further evidence of the value of these assays comes from NC3Rs CrackIT PREDART challenge project in which a DART hazard assessment of a whole group of 31 well-known positives was analyzed using *Dictyostelium discoideum* (slime mould), nematodes and zebrafish embryos. For a selected group of compounds, the molecular response of the three different species were assessed using RNAseq analyses. Despite the fact that different phenotypic outcomes were observed, the

Table 2
Overview of different test methodologies for assessment of developmental and reproduction toxicity.
Golden standard OECD protocols are compared with 3R nematodes and zebrafish test models. These latter two models show that testing is fast, low cost and 3R proof.

	Nematode	Zebrafish	OECD 414	OECD 416/ 443
Indicative cost	Low	Low	Moderate	High
Study duration	1 week	1 week	3 weeks[b]	30 weeks[b]/ 21 weeks[b]
Exposure	Buffer	Water	Gavage	Gavage
3Rs issues	None	Vertebrate	Rats & rabbits	Rats
Number of animals used	None	None (until 5dpf)	~900 rats, ~500 rabbits	~2600/ 1400[a] rats
Regulatory acceptability	No (screen WoE)	No (screen WoE)	Yes	Yes

[a] Basic design, i.e. no cohorts and extension to F2.
[b] In-life portion of the study.

toxicogenomic profile identified potential molecular mechanisms with human relevance and was shared across the test species (https://www.nc3rs.org.uk/integrative-dictyostelium-c-elegans-and-zebrafish-approach-assess-dart, manuscripts in prep).

4. Conclusions

The fact that the results of the nematodes and zebrafish assays are in alignment with data obtained from mammalian toxicity studies indicate that these have potential as developmental and reproductive toxicity screens without the need to use significant numbers of animals. The results of these studies also provide indication that none of the Piperazine analogs tested are likely to have any significant developmental issues to humans when used in commercial applications.

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