Expert Opinion

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Zebrafish assays for drug toxicity screening

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Zebrafish are vertebrate organisms that are of growing interest for preclinical drug discovery applications. Zebrafish embryos develop most of the major organ systems present in mammals, including the cardiovascular, nervous and digestive systems, in < 1 week. Additional characteristics that make them advantageous for compound screening are their small size, transparency and ability to absorb compounds through the water. Furthermore, gene function analysis with antisense technology is now routine procedure. Thus, it is relatively simple to assess whether compounds or gene knockdowns cause toxic effects in zebrafish. Assays are being developed to exploit the unique characteristics of zebrafish for pharmacological toxicology. This review discusses assays that may be used to assess *in vivo* toxicity and provides examples of compounds known to be toxic to humans that have been demonstrated to function similarly in zebrafish.

Keywords: cardiotoxicity, cytochrome P450, neurotoxicity, teratogenesis, zebrafish

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1. Introduction

Many drugs developed by the pharmaceutical industry fail in clinical trials because of unanticipated toxic side effects. Toxicity may be caused by a compound interacting with a molecular target that is distinct from the target selected for therapeutic benefit, defined as an off-target effect. An example of this is the large number of compounds that interfere with the cardiac potassium ion channel human ether a-go-go (hERG), which can cause a fatal arrhythmia [1].

Alternatively, severe side effects may result from blockage of a molecular target in a nontargeted tissue, defined as an on-target effect; for example, NSAIDS derive therapeutic benefit from inhibiting COX-1 and/or -2. However, inhibition of COX-1 in the gastrointestinal system has been shown to cause gastric ulcers and more recently, specific inhibition of COX-2 has been potentially linked to cardiovascular complications [2].

Drugs are usually tested for *in vivo* toxicity in mammalian models, such as mouse, rat and dog. Toxicity testing is a time-consuming and expensive part of the modern drug discovery process. In the last few years, many investigators have begun to explore the use of zebrafish as an alternative model for toxicity testing of pharmaceuticals in order to provide an *in vivo* assessment of compound effects at an earlier stage in drug discovery. Because they are small, easy to care for, inexpensive to maintain, and produce large numbers of transparent embryos that develop outside of the mother, zebrafish have been used to study almost every aspect of vertebrate biology, including the development and function of the cardiovascular system, the CNS and the digestive system. The ability to culture large numbers of zebrafish embryos and larvae in small volumes of media [3] facilitates rapid testing of compounds for toxicity, while using a minimal amount of compound (nanograms or less per animal). Compounds in the media are absorbed by the zebrafish through the skin and gills at embryonic stages and through the digestive system during later larval stages. Embryonic, larval and adult zebrafish have all been used for many years for environmental toxicity testing [4]. Although this review focuses on assays that use the more

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experimentally tractable embryos and larvae, adult zebrafish may also play a role in toxicity testing, complementing studies in embryos and larvae.

Reliable antisense technology affords the opportunity to rapidly assess the consequences of gene knockdown. The most common method used to knock down genes in zebrafish uses morpholinos – oligomers made up of nucleic acid bases attached to a morpholine backbone that renders the molecules resistant to endogenous nucleases. Morpholinos can be designed to block either translation [5] or splicing [6]. Splice site morpholinos provide the advantage of allowing knockdown to be monitored by reverse transcriptase polymerase chain reaction. Although small interference RNA has been reported to be effective for knocking down some genes in zebrafish [7,8], morpholino technology remains the gold standard for reproducible gene knockdown in zebrafish. Peptide nucleic acids have also been shown to be effective for antisense applications in zebrafish [9]. Thus, genes that are potential targets for drug development can be rapidly knocked down in zebrafish to assess the effects of gene inactivation in both target and nontarget tissues. The zebrafish genome is being sequenced by the Sanger Institute [101], which has greatly simplified the process of identifying zebrafish orthologues of human gene targets.

Although any organ or tissue can be monitored in response to a compound or a gene knockdown, this review focuses on a few zebrafish assays that have been most widely used for toxicity evaluation. Transgenic zebrafish with fluorescent tissues and organs that can be useful for assay development are summarised in **Table 1**. Because the effects of toxins are dependent on metabolic enzymes produced by the zebrafish, the current state of knowledge regarding zebrafish metabolic enzymes, such as CYPs, is also reviewed.

2. Teratogenesis

Because zebrafish embryos develop outside of the mother and are transparent, they are ideal for analysis of drugs that are potential teratogens. In addition, the developmental biology of zebrafish has been well studied and > 1000 mutations in developmental pathways have been described [10,11]. Known teratogens have also been studied in zebrafish; for example, the effects of ethanol, a well-known teratogen, have been extensively studied in zebrafish embryos. Ethanol has been shown to cause cyclopia when embryos are exposed during gastrulation [12], a phenotype also observed in human babies that were exposed to high levels of alcohol during gestation. When zebrafish embryos are exposed to nonlethal levels of ethanol during development they exhibit craniofacial abnormalities as well as deficits in learning and memory later in life [13], phenotypes known to be associated with fetal alcohol syndrome in humans. Zebrafish larvae exposed to alcohol exhibit defects in visual function [14], a phenotype also associated with exposure to alcohol during human development.

To examine whether zebrafish could be used for standard testing of potential environmental toxicants, several chemicals used in the rubber industry were screened for teratogenicity in three species: zebrafish, rainbow trout and chick [15]. Comparison of the median effective concentration (EC_{50}) (for fish experiments) with the median effective dose (ED_{50}) (for chick experiments) revealed a close correlation in the dose required to cause toxic effects. EC_{50} calculations were based on the concentration of compound in the media, whereas ED_{50} calculations were based on the amount of compound injected into chick embryos.

Valproic acid (VPA), an epilepsy drug that causes birth defects when taken by women during pregnancy, induces multiple developmental defects in zebrafish embryos bathed in the compound [16]; for example, VPA causes a shortened body axis, cardiac oedema and reduced pigmentation. VPA is a strong inhibitor of histone deacetylase (HDAC). Other HDAC inhibitors, such as sodium butyrate and trichostatin A (TSA), were shown to cause similar defects, suggesting that the VPA-mediated defect is likely to be due to HDAC inhibition [16]. Cardiac and neural defects caused by HDAC inhibitors have been phenocopied in zebrafish carrying mutations in the *HDAC-1* gene or by knocking down HDAC-1 with antisense morpholinos [17,18], thus confirming HDAC-1 as the likely target for VPA. Antisense knockdown may also be an effective way of assessing whether modulation of a genetic target by a potential drug will have unanticipated on-target side effects.

3. Cardiotoxicity

The molecular mechanisms underlying the development and function of the cardiovascular system have been well conserved in vertebrate evolution. The zebrafish has a two-chambered heart and a vascular network that develops in the first few days after fertilisation. Cardiomyocytes isolated from the zebrafish embryonic heart have been shown to have voltage-gated sodium currents, L-type calcium currents, T-type calcium and potassium currents, similar to other vertebrate hearts [19].

Several zebrafish mutations have been described that cause phenotypes similar to human diseases [20]; for example, molecular analysis has revealed that mutations in the zebrafish orthologues of genes such as cardiac troponin T and titin cause syndromes similar to those caused by mutations in the human orthologues [21,22]. Antisense morpholinos directed against the zebrafish orthologue of EYA4 cause cardiac dysfunction in zebrafish, similar to humans that carry mutations in EYA4 [23].

Measurement of the heart rate in zebrafish has been used as an indicator of cardiac toxicity for many years; for example, the environmental toxin tetrachlorodibenzo-*para*-dioxin (TCDD) was shown to reduce the heart rate in zebrafish embryos and larvae [24]. Compounds known to increase heart rate, such as adrenaline, have been shown to increase the heart

Table 1. Transgenic zebrafish for toxicity assays.

Tissue or organ labelled	Promoter	Transgene	Reference
Heart	Cardiac myosin light chain 2	GFP	[31,32]
Heart	Cardiac myosin light chain 2	GRCFP	**Figure 1**
Cranial motor neurons	Islet-1	GFP	[45]
Neurons (brain and spinal cord)	Gata-2 neuronal enhancer	GFP	[43]
Neurons (brain and spinal cord)	Elav/HuC	GFP	[44]
Notochord, pineal gland, spinal cord motor neurons	Floating head	GFP	[46]
Pancreas	Insulin	GFP	[60]
Pancreas	PDX-1	GFP	[60]
Intestine, liver, pancreas, hepatic duct, pancreatic duct, oesophagus, swim bladder	Unknown	GFP	[61]

GFP: Green fluorescent protein; GRCFP: Green reef coral fluorescent protein; PDX: Pancreas duodenum homeobox.

rate of zebrafish embryos [25]. Compound-induced changes in heart rate in zebrafish embryos could also be a result of developmental defects.

Recent work has shown that TCDD causes several defects in heart development, including a decrease in the overall size of the heart as well as a defect in the positioning of the atrium relative to the ventricle [26]. Pericardial oedema is another indicator of cardiac toxicity that is frequently observed in response to both compounds and gene mutations in zebrafish.

A compound and a gene mutation may cause similar defects in the development of the heart, suggesting that the compound affects a particular molecular pathway. For example, the compound concentramide causes the atrium to form within the ventricle, a phenotype that is replicated by a mutation in protein kinase C-λ [27].

3.1 QT prolongation

Analysis of the QT interval on electrocardiograms is now a requirement for drugs in clinical trials because of the association of QT prolongation with Torsade de Pointes (TdP), a serious heart arrhythmia that often leads to death [1]. Thus, it is vital to determine the likelihood that new drugs will cause QT prolongation and/or TdP at an early point in preclinical drug development. Drug-induced QT prolongation is usually the result of drugs binding to the hERG potassium channel. At present, new compounds are screened in assays that measure hERG potassium channel activity in cell culture or in mammalian models (reviewed in [28,29]). However, each of these assays has its limitations; for example, electrophysiological measurement of hERG channel activity in single cells may not be an accurate representation of hERG activity in whole organisms. Mammalian models are expensive and require large amounts of drugs for sufficient evaluation.

Two studies have shown a correlation between drugs that cause QT prolongation in humans and those that cause changes in heart rate in zebrafish [25,30]. Furthermore, both studies showed that antisense knockdown of zebrafish orthologues of hERG caused similar changes in heart rate.

In one study, the heart rate of zebrafish embryos was recorded after several hours of exposure through the embryo media to each compound in a set of 100 compounds [25]. Of these, 36 compounds caused a statistically significant decrease in the heart rate, and 3 compounds increased the heart rate. Out of 24 compounds that were known to cause QT prolongation in humans, 18 were shown to cause bradycardia in zebrafish. Of the six that did not, subsequent microinjection of five demonstrated that they also caused bradycardia, suggesting that they were poorly absorbed through the skin. This experiment exposed one limitation of testing compounds in zebrafish. The authors found that compounds with an octanol:water partition coefficient (logP) of < 1 were frequently poorly absorbed by zebrafish embryos [25].

One compound in this study, erythromycin, did not affect heart rate when administered to embryos by itself, but did cause bradycardia when combined with cisapride, thus recapitulating a well-known drug–drug interaction that occurs in humans. Thus, this study also demonstrated the potential for using zebrafish to rapidly analyse drug–drug interactions.

Many compounds (18 in this study) that reduce the zebrafish heart rate do not induce QT prolongation in humans, suggesting that blockade of the hERG channel is not the only mechanism that can result in bradycardia in zebrafish, just as is the case in mammals. It is also possible that developmental defects induced by the compounds could affect the heart rate.

In another study, 12 drugs known to cause QT prolongation, including the antihistamine terfenadine, were shown to cause a 2:1 atrioventricular block, in which the ventricle beats half as often as the atrium [30]. Application of another antihistamine, epinastine, which does not block hERG, did not result in a 2:1 atrioventricular block. Bradycardia was observed at lower concentrations of the QT prolonging drugs, in agreement with the first study. Very high concentrations of

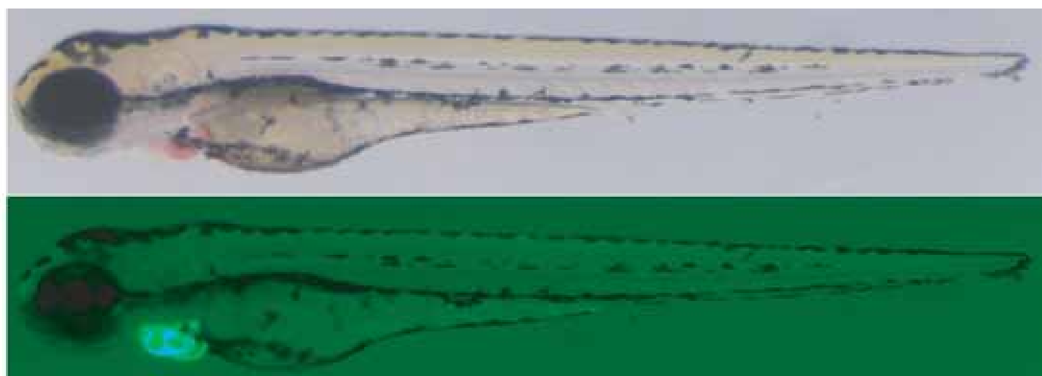

Figure 1. Zebrafish embryo at 3 days postfertilisation. Light microscope image (top panel) and fluorescent image (bottom panel) of a transgenic fish that expresses green reef coral fluorescent protein (GRCFP, Clontech) under the control of the cardiac myosin light chain 2 promoter.

the drugs caused severe bradycardia and arrhythmia, and in some cases, complete cardiac arrest.

In summary, the zebrafish heart rate assay may be a useful addition to *in vivo* preclinical toxicity testing. Assay automation can be facilitated through the use of a transgenic zebrafish with a fluorescent heart (**Figure 1**, **Table 1**) [31,32]. Furthermore, secondary assays in the zebrafish are currently being developed that will improve the ability to identify drugs that cause TdP (C MacRae, pers. commun.).

4. Neurotoxicity

The zebrafish brain and CNS have been extensively studied and shown to exhibit many similarities to the mammalian nervous system (reviewed in [33-35]). In recent years, several studies have described assays that may be useful for assessing the neurotoxicological properties of compounds.

4.1 Dopaminergic neurotoxicity

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is known to cause a Parkinsonian syndrome in humans by selectively destroying dopaminergic neurons in the substantia nigra [36]. MPTP neurotoxicity varies by species: many rodents, including the rat and some strains of mice, are relatively insensitive to MPTP, whereas primates are highly sensitive [37]. Several studies have shown that MPTP specifically damages dopaminergic neurons in zebrafish embryos and larvae [38-40]. Dopaminergic neurons have been identified in the diencephalon, the olfactory bulb, the retina and the hindbrain of zebrafish embryos and larvae [41,42]. Other toxins, such as rotenone and paraquat, failed to induce a specific neuronal phenotype in zebrafish [38].

Analysis of dopaminergic neurons in zebrafish was performed by whole mount *in situ* hybridisation using probes to either the tyrosine hydroxylase or the dopamine transporter genes. Quantification of MPTP-induced neuronal loss was accomplished by either counting the number of neurons [38] or measuring the total area occupied by dopaminergic neurons [40] in control and drug-treated embryos. These studies show that specific groups of neurons can be analysed for their response to neurotoxic compounds. Transgenic fish with fluorescent neurons (**Table 1**) [43-46] could help facilitate analysis of neurons in response to toxin-induced damage.

4.2 Behavioural assays

Zebrafish embryos and larvae exhibit stereotypical behaviours early in their lifecycle, including spontaneous contractions, movement in response to touch, and swimming [47]. Assays have been developed to exploit the behaviours observed in zebrafish as a measure of neurotoxicity. For example, potential toxicity to dopaminergic neurons was assessed by analysis of locomotor activity, which was reduced in MPTP-treated larvae [38-40]. In another study, larval motility was shown to be reduced in acetylcholinesterase mutants as well as embryos exposed to acetylcholinesterase inhibitors [48].

Domoic acid, a neurotoxin produced by the diatom genus *Pseudo-nitzschia*, induced several behavioural phenotypes, including rapid and constant movement of the pectoral fins, defective response to touch and reduced swimming behaviour in zebrafish larvae [49]. In addition, many mutations have also been identified that affect zebrafish locomotion [50]. In all of the studies described above, it should be noted that changes in swimming behaviour could be caused by either damage to neurons or to musculature.

Domoic acid also caused tonic–clonic convulsions, consisting of a whole body contraction and shuddering motion when zebrafish embryos were treated with the toxin [49]. Similar behaviours were observed when zebrafish larvae were treated with the seizure-inducing drug pentylenetetrazole (PTZ) [51]. PTZ caused larvae to swim rapidly in circles prior to exhibiting clonus-like convulsions. PTZ-induced behavioural changes were accompanied by changes in electrical activity measured by

inserting a glass electrode directly into the optic tectum of the zebrafish brain. This data suggests that seizure-like activity can be monitored as a sign of neurotoxicity. However, it would be useful to perform a blind study with a large number of compounds to determine whether seizure-like behaviour is a common occurrence in zebrafish larvae and whether observation of this effect can reliably predict a similar effect in humans.

4.3 Toxicity of Alzheimer's disease drugs

Since the discovery that the amyloid precursor protein associated with Alzheimer's disease is processed by β- and γ-secretases, many groups have tried to develop γ-secretase inhibitors that could be used to halt the progression of Alzheimer's disease. However, the important signalling protein Notch is also processed by γ-secretase and it quickly became apparent that many γ-secretase inhibitors blocked the Notch pathway as well, leading to unacceptable toxicity for these compounds. For example, γ-secretase inhibitors have been shown to cause gastrointestinal toxicity in rats, manifesting as an increase in intestinal goblet cells [52,53]. In mice, chronic treatment with a potent γ-secretase inhibitor also caused an increase in the number of intestinal goblet cells as well as defects in the thymus and spleen. Treated mice lost weight and 40% died after 15 days of treatment at the highest dose [54].

Two groups have demonstrated that γ-secretase inhibitors can induce a phenotype in zebrafish embryos that resembles mutations in the Notch pathway [55,56]. For example, the γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine t-butyl ester causes defects in somitogenesis that are also found in zebrafish Notch pathway mutants [55]. Defects in somitogenesis are easily visualised in the transparent zebrafish embryo by either morphological observation or by *in situ* hybridisation with a probe to the *myoD* gene. Another study compared several γ-secretase inhibitors by their ability to interfere with expression of the gene encoding the Notch ligand Delta C in zebrafish embryos [56]. These studies suggest that zebrafish can be used to rapidly assess whether γ-secretase inhibitors developed to treat Alzheimer's disease will also interfere with the Notch pathway and thus cause Notch-associated toxicity.

4.4 Ototoxicity

Drug-induced hearing loss is a common side effect of many drugs, including antineoplastic agents such as cisplatin. The zebrafish counterpart of inner ear hair cells are known as neuromasts and are used to detect changes in the motion of the water (reviewed in [57]). A recent study has shown that zebrafish hair cells are sensitive to several drugs, including cisplatin, gentimicin and quinine [58]. Zebrafish hair cells are easily visualised by staining with a fluorescent vital dye, allowing the investigators to quantify the loss of hair cells in response to toxic compounds.

5. Digestive system

By 5 days postfertilisation, zebrafish larvae have developed a fully functioning digestive system, including liver, intestine, pancreas and oesophagus [59]. Morphological analysis of digestive system organs can be performed to assess organ-specific toxicity. Although some organs, such as the liver and the intestine, can be observed with a light microscope, other organs, such as the pancreas are less easily visualised. Transgenic fish with a fluorescent pancreas have been developed that enable morphological evaluation of the pancreas in zebrafish embryos and larvae (**Table 1**) [60]. Another transgenic fish with several fluorescent organs, including the liver, pancreas and intestine, has also facilitated observations of gut morphology (**Table 1**) [61].

Functional analysis of the digestive system may also be performed in zebrafish. For example, zebrafish larvae have been shown to swallow, absorb and process fluorescent lipids added to the water, resulting in distinctive illumination of the intestine and gall bladder [62]. Drugs such as atorvastatin disrupt this process, which is likely due to a reduction in cholesterol-derived biliary emulsifiers in their intestinal tracts. However, it is possible that compounds with adverse effects on the digestive system could also be detected using this assay.

Histological examination of the liver in zebrafish larvae has been used to analyse the toxic effects of the fungicide triphenyltin acetate (TPTA) [63]. In addition to other toxic effects, TPTA caused numerous changes in the subcellular structure of hepatocytes, including increases in the number of nucleoli, changes in mitochondrial structures, and depletion of large glycogen fields within liver cells.

6. Genotoxicity

Genotoxic properties of compounds must be assessed during drug development because of the potential for mutagenic drugs to cause cancer. At present, both *in vitro* tests such as the Ames test, as well as *in vivo* tests in rodents are used to assess genotoxicity. A zebrafish assay has been developed that uses a transgenic fish carrying the *Escherichia coli* gene *rpsL* as a mutational target [64]. The known mutagen ethylnitrosourea (ENU) was shown to increase the number of mutations in the *rpsL* gene, as expected.

In a more recent study, adult *rpsL* transgenic zebrafish exposed to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) exhibited an increase in mutation frequency, particularly in the gills and hepatopancreas [65]. Embryos exposed to MNNG also exhibited an increase in mutational frequency at the *rpsL* locus. Although this transgenic fish has mainly been used to analyse environmental toxicants, it is possible that it could also be useful for analysis of the mutagenicity of pharmaceuticals in development. Both ENU and MNNG have been shown to cause tumours in adult zebrafish that were exposed to the toxins as embryos or larvae [66,67]. However, a wider range of mutagenic drugs, particularly those that are not detected by the Ames test, must be tested in this model to assess whether it will provide any advantages over the standard battery of tests already available.

7. Microarrays

There has been increasing interest in using gene expression profiles as a way of predicting drug toxicity. Because it is relatively easy and inexpensive to expose thousands of embryos to drugs, the microarray approach in zebrafish may provide useful data in a time- and cost-effective way.

Microarrays are beginning to be used to analyse gene expression changes caused by drug treatment; for example, microarray analysis of zebrafish embryos exposed to the acetylcholine esterase inhibitor chlorpyrifos showed that 45 genes were upregulated at a high concentration and 15 genes at a lower concentration, using a microarray containing > 16,000 oligonucleotides [68]. A microarray containing > 15,000 zebrafish genes was used to analyse differences in expression patterns between zebrafish embryos exposed to the structurally distinct HDAC inhibitors VPA and TSA [16]. Similar changes in gene expression were observed when embryos were treated with either VPA or TSA, thus suggesting that microarray patterns could be used to screen for HDAC inhibitor-induced toxicity. It is possible that microarray studies could help to identify toxicity associated biomarkers. However, it should be noted that the use of microarrays for predictive toxicology is in its infancy, and their predictive potential is at present unclear.

Smaller microarrays containing specific sets of genes are also being constructed; for example, a microarray containing zebrafish genes encoding proteins involved in apoptosis was designed to study gene expression changes in embryos treated with apoptosis inhibitors [69]. In another study, a cDNA microarray containing 682 brain-specific gene fragments was used to analyse the effects of chlorpromazine, an antidepressant that acts through dopaminergic pathways [70]. More than 50 genes were found to be differentially regulated in drug-treated and control adult zebrafish. Further analysis of the genes identified will be necessary to determine the significance of this differential regulation.

8. Zebrafish metabolic enzymes

Enzymes of the CYP family are responsible for the metabolism of most drugs in mammals. CYP3A is the most important of these enzymes, itself metabolising ~ 50% of all drugs in humans. Zebrafish orthologues of CYP3A have been identified and characterised in two recent studies [71,72]. In one study, a CYP3A orthologue designated CYP3A65 was shown to be expressed in the liver and intestine of both larval and adult zebrafish [71]. CYP3A65 expression was upregulated by treatment with both rifampicin and dexamethasone, similar to the response of CYP3A4 in humans. However, at very high doses, dexamethasone actually repressed expression of CYP3A65. The environmental toxin TCDD also upregulated CYP3A65.

Another study examined the regulation of three genes, zebrafish orthologues of CYP3A, the pregnane X receptor (believed to regulate CYP3As in many species), and multi-drug resistance protein 1 (known to be regulated by CYP3A) [72]. In this study, drugs known to affect CYP3A in humans were studied in zebrafish. The synthetic steroid pregnelone 16α-carbonitrile upregulated all three genes, whereas nifedipine did not, which is similar to studies in humans. Injection of zebrafish with the antibiotic clotrimazole, an inducer of CYP3A in humans, resulted in an increase in the expression of all three genes, but the increases were not statistically significant. Thus, it is possible that some differences exist between the regulation of CYP3A in humans and zebrafish.

The observation that zebrafish CYP3A was upregulated by rifampicin [71] is intriguing, as it is known that rifampicin upregulates human CYP3A, but not rat CYP3A. This finding suggests that the zebrafish, in some cases, may be a better model for human toxicity than some commonly used mammalian models. However, a much wider range of compounds must be tested to determine how similar the regulation of the fish CYP3A is to regulation of the human enzyme.

Inhibition of CYP3A is a common cause of toxic drug–drug interactions in humans. Compounds that are metabolised by CYP3A have been shown to shift the ED_{50} of terfenadine in the zebrafish bradycardia assay discussed above [25]. This result suggests that the zebrafish bradycardia assay may be useful for detecting drugs that modulate the activity of CYP3A and thus help predict which drugs may contribute to toxic drug–drug interactions. Studies on a wider range of known human CYP3A inhibitors and non-CYP3A inhibitors will be necessary to further validate this assay.

Several other CYPs have been cloned and characterised in zebrafish, including CYP1A, -2K, -26 and -19. CYP1A has been shown to be required for metabolism of the developmental toxin TCDD [73]. Antisense knockdown of CYP1A prevented TCDD-induced developmental abnormalities, including pericardial oedema and blood flow. CYP2K1 was shown to metabolise aflatoxin B1 in zebrafish [74]. CYP19 was shown to be expressed in neurons and upregulated by estrogen, similar to its human counterpart [75]. Genes encoding CYP26 enzymes, which metabolise retinoic acid, have also been described in the zebrafish [76].

Upregulation of CYPs is another cause of toxic drug–drug interactions. Transgenic fish that express fluorescent proteins under the control of relevant CYP promoters may be useful for developing quantitative assays for CYP upregulation in response to drugs. A similar assay, involving transgenic fish that expresses luciferase under the control of environmental pollutant response elements, is being developed to detect aquatic environmental toxins [77].

Zebrafish orthologues of other metabolic enzymes have also been identified; for example, a monoamine oxidase (MAO) with similarities to both MAO-A and -B in humans has been characterised [78]. Zebrafish MAO activity in brain and liver extracts was shown to be inhibited by both clorgiline (an MAO-A inhibitor) and deprenyl (an MAO-B inhibitor).

9. Conclusion

Fish have been used for years to assess the toxicology of environmental agents. This work has recently been extended to testing pharmaceuticals for toxicity. In support of this application, zebrafish biology has been shown to be highly similar to that of mammals. The body of work reviewed here suggests that certain known pathways leading to drug toxicity in mammals are conserved in zebrafish. Assays have been developed to analyse the cardiovascular, nervous and digestive systems. Furthermore, zebrafish orthologues of mammalian genes encoding enzymes required for drug metabolism have been identified. This work has been a necessary first step toward developing zebrafish as a model to predict toxicity of compounds in humans.

10. Expert opinion

Because so many drugs in development fail in clinical trials due to unacceptable toxicity, predictive assays for toxicity are a crucial part of drug development. Due to the cost and labour-intensive nature of testing compounds in mammals, toxicity testing is usually performed relatively late in the development process, after selection of a limited number of compounds. Thus, a need exists for assays that retain the high value of *in vivo* toxicity testing, but can be rapidly performed on a large number of compounds. Zebrafish embryos and larvae are small vertebrates that live for days in a low volume of water, thus making them an excellent choice for compound screening when only limited amounts of each compound are available. Only nanogram quantities of compound are required for each animal tested and most assays can be performed in < 1 week.

Most of the proof-of-concept studies performed so far have shown that known drug-induced toxicity in humans can be recapitulated in the zebrafish. It remains to be proved, however, whether drugs with unknown toxicity characteristics will cause effects in zebrafish that will allow toxicologists to predict with reasonable assurance that such effects are likely in humans. It will be difficult to assess the predictability of zebrafish assays until a wider range of compounds has been tested and zebrafish assays begin to be integrated into drug toxicity screening programmes. Furthermore, because compounds are not all absorbed equally well by zebrafish embryos, it may be difficult to correlate the concentration of compound in the media required to cause toxic effects in zebrafish to the plasma concentration of compound that would cause the same effect in humans. Determination of the amount of compound actually absorbed by zebrafish as well as a better understanding of compound metabolism in zebrafish will be necessary to make useful correlations. Potential differences in the physiology of embryos, larvae and adults should also be considered.

Because zebrafish assays are less costly than similar studies in mammals, it may be possible to begin adding zebrafish assays to standard late-stage preclinical toxicity screens. As data accumulates to validate the predictivity of the assays further , they can then be incorporated earlier in the drug discovery process, where the real power of using zebrafish will be realised. Because hundreds of embryos and larvae can be obtained for every pair of adult zebrafish, compounds can be tested on a much larger number of individual organisms than is possible with mammalian models. Selecting compounds for development that have the least likely probability of causing toxicity in humans at an early stage in drug discovery would have a significant impact on attrition rates of drugs in clinical trials and should ultimately lead to the development of safer drugs.

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Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. RODEN DM: Drug-induced prolongation of the QT interval. *N. Engl. J. Med.* (2004) **350**:1013-1022.
   • **Review of the issues surrounding drug-induced QT prolongation and TdP.**
2. KONSTANTINOPOULOS PA, LEHMANN DF: The cardiovascular toxicity of selective and nonselective cyclooxygenase inhibitors: comparisons, contrasts, and aspirin confounding. *J. Clin. Pharmacol.* (2005) **45**:742-750.
   • **Review of toxicity associated with COX inhibitors.**
3. PETERSON RT, LINK BA, DOWLING JE, SCHREIBER SL: Small molecule developmental screens reveal the logic and timing of vertebrate development. *Proc. Natl. Acad. Sci. USA* (2000) **97**:12965-12969.
   •• **First published paper describing a compound screen in zebrafish embryos.**
4. SPITSBERGEN JM, KENT ML: The state of the art of the zebrafish model for toxicology and toxicologic pathology research-advantages and current limitations. *Toxicol. Pathol.* (2003) **31**:62-87.
5. NASEVICIUS A, EKKER SC: Effective targeted gene 'knockdown' in zebrafish. *Nat. Genet.* (2000) **26**:216-220.
   •• **First paper describing the use of antisense morpholinos for reproducible knockdown in zebrafish.**
6. DRAPER BW, MORCOS PA, KIMMEL CB: Inhibition of zebrafish fgf8 pre-mRNA splicing with morpholino oligos: a quantifiable method for gene knockdown. *Genesis* (2001) **30**:154-156.
7. DODD A, CHAMBERS SP, LOVE DR: Short interfering RNA-mediated gene targeting in the zebrafish. *FEBS Lett.* (2004) **561**:89-93.

8. LIU WY, WANG Y, SUN YH *et al.*: Efficient RNA interference in zebrafish embryos using siRNA synthesized with SP6 RNA polymerase. *Dev. Growth Differ.* (2005) **47**:323-331.
9. URTISHAK KA, CHOOB M, TIAN X *et al.*: Targeted gene knockdown in zebrafish using negatively charged peptide nucleic acid mimics. *Dev. Dyn.* (2003) **228**:405-413.
10. HAFFTER P, GRANATO M, BRAND M *et al.*: The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. *Development* (1996) **123**:1-36.
11. DRIEVER W, SOLNICA-KREZEL L, SCHIER AF *et al.*: A genetic screen for mutations affecting embryogenesis. *Development* (1996) **123**:37-46.
12. BLADER P, STRAHLE U: Ethanol impairs migration of the prechordal plate in the zebrafish embryo. *Dev. Biol.* (1998) **201**:185-201.
13. CARVAN MJ, LOUCKS E, WEBER DN, WILLIAMS FE: Ethanol effects on the developing zebrafish: neurobehavior and skeletal morphologenesis. *Neurotoxicol. Teratol.* (2004) **26**:757-768.
14. BILOTTA J, SASZIK S, GIVIN CM, HARDESTY HR, SUTHERLAND SE: Effects of embryonic exposure to ethanol on zebrafish visual function. *Neurotoxicol. Teratol.* (2002) **24**:759-766.
15. VAN LEEUWEN CJ, GROOTELAAR EMM, NIEBEEK G: Fish embryos as teratogenicity screens: a comparison of embryotoxicity between fish and birds. *Ecotoxicol. Environ. Saf.* (1990) **20**:42-52.
16. GURVICH N, BERMAN MG, WITTNER BS, GENTLEMAN RC, KLEIN PS, GREEN JB: Association of valproate-induced teratogenesis with histone deacetylase inhibition *in vivo*. *FASEB J.* (2005) **19**:1166-1168.
17. CUNLIFFE VT: Histone deacetylase 1 is required to repress Notch target gene expression during zebrafish neurogenesis and to maintain the production of motoneurones in response to hedgehog signalling. *Development* (2004) **131**:2983-2995.
18. PILLAI R, COVERDALE LE, DUBEY G, MARTIN CC: Histone deacetylase 1 (HDAC-1) required for the normal formation of craniofacial cartilage and pectoral fins of the zebrafish. *Dev. Dyn.* (2004) **231**:647-654.
19. BAKER K, WARREN KS, YELLEN G, FISHMAN MC: Defective 'pacemaker' current (*I*h) in a zebrafish mutant with a slow heart rate. *Proc. Natl. Acad. Sci. USA* (1997) **94**:4554-4559.
20. SEHNERT AJ, STAINIER DY: A window to the heart: can Zebrafish mutants help us understand heart disease in humans? *Trends Genet.* (2002) **18**:491-494.
21. SEHNERT AJ, HUQ A, WEINSTEIN BM, WALKER C, FISHMAN M, STAINIER DY: Cardiac troponin T is essential in sarcomere assembly and cardiac contractility. *Nat. Genet.* (2002) **31**:106-110.
22. XU X, MEILER SE, ZHONG TP *et al.*: Cardiomyopathy in zebrafish due to mutation in an alternatively spliced exon of titin. *Nat. Genet.* (2002) **30**:205-209.
23. SCHONBERGER J, WANG L, SHIN JT *et al.*: Mutation in the transcriptional coactivator EYA4 causes dilated cardiomyopathy and sensorineural hearing loss. *Nat. Genet.* (2005) **37**:418-422.
24. HENRY TR, SPITSBERGEN JM, HORNUNG MW, ABNET CC, PETERSON RE: Early life stage toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in zebrafish (Danio rerio). *Toxicol. Appl. Pharmacol.* (1997) **142**:56-68.
25. MILAN DJ, PETERSON TA, RUSKIN JN, PETERSON RT, MACRAE CA: Drugs that induce repolarization abnormalities cause bradycardia in zebrafish. *Circulation* (2003) **107**:1355-1358.
•• **This study examined 100 compounds in a bradycardia assay and showed that most drugs that cause QT prolongation in humans cause bradycardia in zebrafish.**
26. ANTKIEWICZ DS, BURNS CG, CARNEY SA, PETERSON RE, HEIDEMAN W: Heart malformation is an early response to TCDD in embryonic zebrafish. *Toxicol. Sci.* (2005) **84**:368-377.
27. PETERSON RT, MABLEY JD, CHEN JN, FISHMAN MC: Convergence of distinct pathways to heart patterning revealed by the small molecule concentramide and the mutation heart-and-soul. *Curr. Biol.* (2001) **11**:1481-1491.
• **This study showed that a small molecule and a genetic mutation could cause identical phenotypes in zebrafish embryos, providing evidence that small molecules can be used to analyse genetic pathways in zebrafish.**
28. WITCHEL HJ, MILNES JT, MITCHESON JS, HANCOX JC: Troubleshooting problems with *in vitro* screening of drugs for QT interval prolongation using HERG K^+ channels expressed in mammalian cell lines and Xenopus oocytes. *J. Pharmacol. Toxicol. Meth.* (2002) **48**:65-80.
29. ECKARDT L, HAVERKAMP W, BORGREFFE M, BREITHARD G: Experimental models of Torsades de Pointe. *Cardiovasc. Res.* (1998) **39**:178-193.
30. LANGHEINRICH U, VACUN G, WAGNER T: Zebrafish embryos express an orthologue of HERG and are sensitive toward a range of QT-prolonging drugs inducing severe arrhythmia. *Toxicol. Appl. Pharmacol.* (2003) **193**:370-382.
•• **This study showed that QT-prolonging drugs cause a 2:1 atrioventricular block in zebrafish embryos.**
31. BURNS CG, MILAN DJ, GRANDE EJ, ROTTBAUER W, MACRAE CA, FISHMAN MC: High-throughput assay for small molecules that modulate zebrafish embryonic heart rate. *Nat. Chem. Biol.* (2005) **1**:263-264.
32. HUANG CJ, TU CT, HSIAO CD, HSIEH FJ, TSAI HJ: Germ-line transmission of a myocardium-specific GFP transgene reveals critical regulatory elements in the cardiac myosin light chain 2 promoter of zebrafish. *Dev. Dyn.* (2003) **228**:30-40.
33. MOENS CB, PRINCE VE: Constructing the hindbrain: insights from the zebrafish. *Dev. Dyn.* (2002) **224**:1-17.
34. LEWIS KE, EISEN JS: From cells to circuits: development of the zebrafish spinal cord. *Prog. Neurobiol.* (2003) **69**:419-449.
35. WILSON SW, BRAND M, EISEN JS: Patterning the zebrafish central nervous system. *Results Probl. Cell Differ.* (2002) **40**:181-215.
36. LANGSTON JW, BALLARD P: Chronic parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* (1983) **219**:979-980.
37. FORNAI F, VAGLINI F, MAGGIO R, BONUCCELLI U, CORSINI GU: Species differences in the role of excitatory amino acids in experimental parkinsonism. *Neurosci. Biobehav. Rev.* (1997) **21**:401-435.
38. BRETAUD S, LEE S, GUO S: Sensitivity of zebrafish to environmental toxins

implicated in Parkinson's disease. *Neurotoxicol. Teratol.* (2004) **26**:857-864.
- **This study examined the effect of several toxins on zebrafish dopaminergic neurons.**

39. LAM CS, KORZH V, STRAHLE U: Zebrafish embryos are susceptible to the dopaminergic neurotoxin MPTP. *Eur. J. Neurosci.* (2005) **21**:1758-1762.
- **This study showed that MPTP-induced toxicity could be prevented by the MAO inhibitor L-deprenyl.**

40. MCKINLEY ET, BARANOWSKI TC, BLAVO DO, CATO C, DOAN TN, RUBINSTEIN AL: Neuroprotection of MPTP-induced toxicity in zebrafish dopaminergic neurons. *Brain Res. Mol. Brain Res.* (2005) **141**:128-137.
- **This study showed that MPTP-induced toxicity could be prevented by both pharmacological inhibition of and antisense knockdown of the dopamine transporter.**

41. GUO S, WILSON SW, COOKE S, CHITNIS AB, DRIEVER W, ROSENTHAL A: Mutations in the zebrafish unmask shared regulatory pathways controlling the development of catecholaminergic neurons. *Dev. Biol.* (1999) **208**:473-487.

42. HOLZSCHUH J, RYU S, ABERGER F, DRIEVER W: Dopamine transporter expression distinguishes dopaminergic neurons from other catecholaminergic neurons in the developing zebrafish embryo. *Mech. Dev.* (2001) **101**:237-243.

43. MENG A, TANG H, ONG BA, FARRELL MJ, LIN S: Promoter analysis in living zebrafish embryos identifies a cis-acting motif required for neuronal expression of GATA-2. *Proc. Natl. Acad. Sci. USA* (1997) **94**:6267-6272.

44. PARK HC, KIM CH, BAE YK *et al.*: Analysis of upstream elements in the HuC promoter leads to the establishment of transgenic zebrafish with fluorescent neurons. *Dev. Biol.* (2000) **227**:279-293.

45. HIGASHIJIMA S, HOTTA Y, OKAMOTO H: Visualization of cranial motor neurons in live transgenic expressing GFP under the control of the Islet-1 promoter/enhancer. *J. Neurosci.* (2000) **20**:206-218.

46. GAMSE JT, THISSE C, THISSE B, HALPERN ME: The parapineal mediates left-right asymmetry in the zebrafish diencephalon. *Development* (2003) **130**:1059-1068.

47. SAINT-AMANT L, DRAPEAU P: Time course of the development of motor behaviors in the zebrafish embryo. *J. Neurobiol.* (1998) **37**:622-632.

48. BEHRA M, ETARD C, COUSIN X, STRAHLE U: The use of zebrafish mutants to identify secondary target effects of acetylcholine esterase inhibitors. *Toxicol. Sci.* (2004) **77**:325-333.

49. TIEDEKEN JA, RAMSDELL JS, RAMSDELL AF: Developmental toxicity of domoic acid in zebrafish. *Neurotoxicol. Teratol.* (2005) **27**:711-717.
- **This study showed that the diatom toxin domoic acid causes several abnormal behaviours in zebrafish.**

50. GRANATO M, VAN EEDEN FJM, SCHACH U *et al.*: Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva. *Development* (1996) **23**:399-413.

51. BARABAN SC, TAYLOR MR, CASTRO PA, BAIER H: Pentylenetetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. *Neuroscience* (2005) **131**:759-768.
- **This study showed that the convulsant pentylenetetrazole can cause seizure-like behaviour in zebrafish.**

52. SEARFOSS GH, JORDAN WH, CALLIGARO DO *et al.*: Adipsin, a biomarker of gastrointestinal toxicity mediated by a functional γ-secretase inhibitor. *J. Biol. Chem.* (2003) **278**:46107-46116.

53. MILANO J, MCKAY J, DAGENAIS C *et al.*: Modulation of Notch processing by γ-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation. *Toxicol. Sci.* (2004) **82**:341-358.

54. WONG GT, MANFRA D, POULET FM *et al.*: Chronic treatment with the γ-secretase inhibitor LY-411,575 inhibits β-amyloid peptide production and alters lymphopoiesis and intestinal cell differentiation. *J. Biol Chem.* (2004) **279**:12876-12882.

55. GELING A, STEINER H, WILLEM M, BALLY-CUIF L, HAASS C: A γ-secretase inhibitor blocks Notch signaling *in vivo* and causes a severe neurogenic phenotype in zebrafish. *EMBO Rep.* (2002) **3**:688-694.
- **This paper shows that the effect of γ-secretase inhibitors on the Notch pathway can be examined in zebrafish.**

56. PETIT A, PASINI A, ALVES DA COSTA C *et al.*: JLK isocoumarin inhibitors: Selective γ-secretase inhibitors that do not interfere with notch pathway *in vitro* or *in vivo*. *J. Neurosci. Res.* (2003) **74**:370-377.

57. GHYSEN A, DAMBLY-CHAUDIERE C: Development of the zebrafish lateral line. *Curr. Opin. Neurobiol.* (2004) **14**:67-73.

58. TON C, PARNG The use of zebrafish for assessing ototoxic and otoprotective agents. *Hear. Res.* (2005) **208**:79-88.

59. WALLACE KN, PACK M: Unique and conserved aspects of gut development in zebrafish. *Dev. Biol.* (2005) **255**:12-29.
- **This is a comprehensive study of the development of the zebrafish digestive system.**

60. HUANG H, LIU N, LIN S: *Pdx-1* knockdown reduces insulin promoter activity in zebrafish. *Genesis* (2001) **30**:134-136.

61. FIELD HA, OBER EA, ROESER T, STAINIER DYR: Formation of the digestive system in zebrafish. I. Liver morphogenesis. *Dev. Biol.* (2003) **253**:279-290.

62. FARBER SA, PACK M, HO SY *et al.*: Genetic analysis of digestive physiology using fluorescent phospholipid reporters. *Science* (2001) **292**:1385-1388.
- **This study describes a functional lipid metabolism assay in zebrafish.**

63. STRMAC M, BRAUNBECK T: Effects of triphenyltin acetate on survival, hatching success, and liver ultrastructure of early life stages of zebrafish (*Danio rerio*). *Ecotoxicol. Environ. Saf.* (1999) **44**:25-39.

64. AMANUMA K, TAKEDA H, AMANUMA H, AOKI Y: Transgenic zebrafish for detecting mutations caused by compounds in aquatic environments. *Nat. Biotech.* (2000) **18**:62-65.

65. AMANUMA K, NAKAMURA T, AOKI Y: MNNG-induced mutations in the adult gill and hepatopancreas and in embryos of *rpsL* transgenic zebrafish. *Mut. Res.* (2004) **556**:151-161.

66. BECKWITH LG, MOORE JL, TSAO-WU GS, HARSHBARGER JC, CHENG KC: Ethylnitrosourea induces neoplasia in zebrafish (Danio rerio). *Lab. Invest.* (2000) **80**:379-385.

67. SPITSBERGEN JM, TSAI HW, REDDY A, *et al.*: Neoplasia in zebrafish (Danio rerio) treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by three exposure

routes at different developmental stages. *Toxicol. Pathol.* (2000) **28**:716-725.

68. LINNEY E, UPCHURCH L, DONERLY S: Zebrafish as a neurotoxicological model. *Neurotoxicol. Teratol.* (2004) **26**:709-718.

69. PICHLER FB, LAURENSON S, WILLIAMS LC, DODD A, COPP BR, LOVE DR: Chemical discovery and global gene expression analysis in zebrafish. *Nature Biotech.* (2003) **21**:879-883.

70. VAN DER VEN K, DE WIT M, KEIL D *et al.*: Development and application of a brain-specific cDNA microarray for effect evaluation of neuro-active pharmaceuticals in zebrafish (Danio rerio). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* (2005) **141**:408-417.

71. BRESOLIN T, REBELO MF, BAINY ACD: Expression of PXR, Cyp3A and MDR1 genes in liver of zebrafish. *Comp. Biochem. Physiol. Part C* (2005) **140**:403-407.

72. TSENG HP, HSEU TH, BUHLER DR, WANG WD, HU CH: Constitutive and xenobiotics-induced expression of a novel CYP3A gene from zebrafish larva. *Toxicol. Appl. Pharmacol.* (2005) **205**:247-258.

73. TERAOKA H, DONG W, TSUJIMOTO Y *et al.*: Induction of cytochrome P450 1A is required for circulation failure and edema by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in zebrafish. *Biochem. Biophys. Res. Commun.* (2003) **304**:223-228.

74. WANG-BUHLER JL, LEE SJ, CHUNG WG *et al.*: Cloning, mapping, developmental/tissue expression, and aflatoxin B1 activation by baculovirus expressed enzyme. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* (2005) **40**:207-219.

75. CALLARD GV, TCHOUDAKOVA AV, KISHIDA M, WOOD E: Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of cyp19 genes in teleost fish. *Steroid Biochem. Mol. Biol.* (2001) **79**:305-314.

76. ZHAO Q, DOBBS-MCAULIFFE B, LINNEY E: Expression of cyp26B1 during zebrafish early development. *Gene Expr. Patt.* (2005) **5**:363-369.

77. CARVAN MJ 3rd, DALTOM TP, STUART GW, NEBERT DW: Transgenic zebrafish as sentinels for aquatic pollution. *Ann. NY Acad. Sci.* (2000) **919**:133-147.

78. SETINI A, PIERUCCI F, SENATORI O, NICOTRA A: Molecular characterization of monoamine oxidase in zebrafish (Danio rerio). *Comp. Biochem. Physiol. B* (2005) **140**:153-161.

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101. http://www.ensembl.org/Danio_rerio/index.html

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