Zebrafish – an *in vivo* model for drug screening

Various characterisics of the zebrafish make it an ideal tool for drug screening.

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Drug screening assays in diverse formats have been developed using zebrafish, including visual assessment of effects on organs in the transparent embryo and quantitative assays using microplates

The increasing number and diversity of compounds made available by rapid synthesis techniques such as combinatorial chemistry, combined with high throughput or ultra-high throughput *in vitro* drug screening assays, generate large numbers of preliminary "hits". However, validating these preliminary hits by mammalian animal models is very slow and costly, resulting in a gap in the drug development process. The zebrafish is a vertebrate model organism that can bridge this gap.

Zebrafish-based assays combine the advantages of higher throughput analysis (compared with mammalian models) and higher relevance to humans (compared with *in vitro* and invertebrate models). Drug screening assays in diverse formats have been developed using zebrafish, including visual assessment of effects on organs in the transparent embryo and quantitative assays using microplates. The microplate-based quantitative assays can be performed in a similar fashion to cell-based assays, and can be used for primary drug screening at high throughput. In this article, we review the recent advances in applying *in vivo* zebrafish assays for testing drug toxicity and drug effects on angiogenesis and apoptosis.

The zebrafish as a model organism

The zebrafish (*Danio rerio*) is a small fresh-water teleost well suited for preclinical drug screening (Figure 1). Zebrafish are easy to maintain and breed: large numbers can be housed in a small space, and the generation time (around three months) is relatively short. Mating is not seasonal, and each female can produce 100-200 eggs per mating,

Figure 1. The zebrafish is a small vertebrate animal suitable for drug screening. (A) A zebrafish embryo at 120 hpf. Note the transparency that permits visualisation of the internal organs. (B) An adult zebrafish.

The optical clarity of the zebrafish embryo enables a thorough assessment of drug-induced changes in morphology and colour of the internal organs, without the complicated surgery or other procedures that are required in studies using mammals

enabling high throughput assays that require a large number of animals. Zebrafish embryogenesis is rapid, with the entire body-plan established by 24 hours post fertilisation (hpf). Most of the internal organs – including the heart, liver, intestine and kidney – are fully developed by 96 hpf. This rapid development is comparable with three months of development in the human embryo.

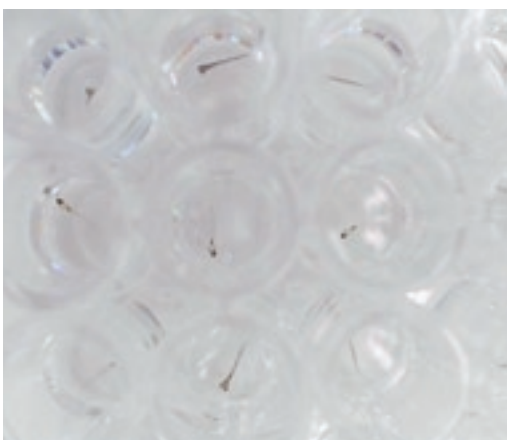
The zebrafish embryo is transparent and develops externally, enabling an easy and thorough assessment of drug effects on internal organs in the live organism. This is a great advantage over mammalian model organisms, where embryonic development occurs *in utero*. The zebrafish embryo is small, requiring a small amount of compounds per assay; this small size also enables drug screening in the 96-well microplate format (26, 27) (Figure 2). Embryos can be raised for five days in individual wells of a 96-well plate, in as little as 100 microliter of fish water. Because the zebrafish embryo has an attached yolk that provides nutrients, no feeding is needed for the first week. Drug administration is also simple – small molecule compounds can be dissolved in fish water and diffuse into the embryo.

The zebrafish is the only vertebrate species for which large-scale forward genetic screens have been carried out (9, 17). Many mutants obtained from the genetic screens display phenotypes resembling human diseases, and many zebrafish orthologues of mammalian genes have been cloned and found to have similar functions, validating use of the zebrafish to model human diseases.

A model for toxicity testing

The zebrafish has been extensively used to study the toxic effects of environmental pollutants (2, 3, 10, 24). These studies examined lethality, embryo survival rate, behaviour and organ malformation as general assay parameters, and demonstrated that zebrafish exhibit good dose-responsiveness to toxicity and are a suitable animal model for toxicity screening.

Figure 2. Whole-animal high throughput screens can be performed using zebrafish embryos in 96-well microtiter plates. A portion of a 96-well microtiter plate is shown in this picture, with individual zebrafish embryos at 120 hpf being raised in the individual wells. The embryos can be maintained in 100 microliter of fish water for the first week without feeding.



Recently, zebrafish-based assays have been developed for testing toxicity of drug candidates, including acute toxicity (LC_{50}), organ-specific toxicity and developmental toxicity (26, 30). The optical clarity of the zebrafish embryo enables a thorough assessment of drug-induced changes in morphology and colour of the internal organs, without the complicated surgery or other procedures that are required in studies using mammals. Necrosis can be readily detected as abnormal opacity, and circulation defects such as haemorrhage can be easily assessed.

Zebrafish also exhibit similar responses to xenobiotic chemicals as mammals, including induction of xenobiotic enzymes and generation of oxidative stress (7, 36). These attractive characteristics make zebrafish a useful preclinical model organism for predicting drug toxicity in humans. For example, Milan *et al.* screened 100 small molecules for their effects on zebrafish embryonic heart rate, and found that compounds known to cause cardiac QT prolongation and torsades de pointes in humans consistently caused bradycardia in zebrafish (23).

Organ toxicity, such as cardiotoxicity, is a major problem with many candidate drugs and even some drugs on the market (12). To reduce attrition rates and drug development costs, it is important to identify these risks as early as possible. Zebrafish assays can serve as such an early screen, before testing on mammalian models or in clinical trials. The zebrafish has a prototypic vertebrate heart comprised of a single atrium and a single ventricle. The molecular mechanisms governing the patterning of the zebrafish heart have been shown to be similar to those in higher vertebrates (13, 35).

In addition, zebrafish and mammalian hearts have been shown to exhibit similar characteristics, including valves that direct blood flow, specialised endocardium musculature that drives a high-pressure system, an electrical system that regulates rhythm, and pacemaker activity that is associated with the heart-beat (1, 31). The patterns of zebrafish and human electrocardiograms (ECGs) are also similar, consisting of a PR interval (activation of atrial action potential (AP) and conduction to the ventricle), the QRS complex (activation of the ventricle) and the QT interval (duration of ventricular action potentials) (18).

The zebrafish heart develops rapidly; a beating heart forms within 26 hours of fertilisation and has a complex repertoire of ion channels and functional metabolism (1). Transparency of the zebrafish permits rapid measurement of heart rate (HR), and examination of cardiac rhythm and contractility. In addition, because the zebrafish embryo receives

Figure 3. Internal organs of the zebrafish embryo can be highlighted by whole-mount staining with specific markers. Staining in the liver and intestine shown here is based on the presence of biotinylated enzymes, and involves the application of HRP-conjugated streptavidin.

nutrients by diffusion, and can survive for 4-5 days without blood circulation, dramatic effects on cardiac function can be studied (34).

Zebrafish can also be used to assess toxicity in other organs, such as the liver, intestine and pancreas. The morphology of these organs can be assessed directly in the transparent embryo, and the expression of specific enzymes in these organs can be visualised in the whole animal using specific dyes or antibodies (Figure 3).

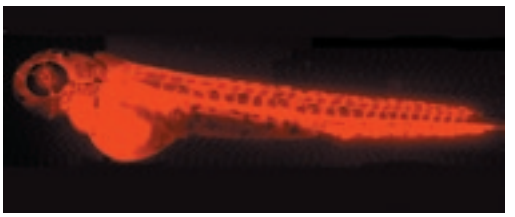
A model for angiogenesis screening

Angiogenesis is the sprouting of new blood vessels from existing ones. Because angiogenesis is required for cancer growth and metastasis, anti-angiogenic drugs are promising cancer therapeutics (14, 25). Angiogenesis is also involved in other diseases, such as diabetic retinopathy and macular degeneration (4). The zebrafish embryo is an excellent model for studying angiogenesis. During early zebrafish embryogenesis, the pattern of angiogenesis is simple, primarily occurring in the head and between somites in the trunk. The intersomitic vessels (ISVs) sprout from the aorta and form between each pair of somites (5). At the molecular level, angiogenesis in zebrafish is also similar to other vertebrates. Several important genes – including VEGF, Flk-1/KDR, Fli-1, Flt-1, Tie-1 and Tie-2 – have been cloned in zebrafish and shown to express in patterns similar to those in mammals (15, 21, 22).

The zebrafish embryo is an excellent model for studying angiogenesis. During early zebrafish embryogenesis, the pattern of angiogenesis is simple, primarily occurring in the head and between somites in the trunk

The zebrafish embryo has a simple blood vessel system and can survive for several days without blood circulation. Angiogenic vessel development can be monitored by endogenous alkaline phosphatase (EAP) activity, which is present primarily in vessels during early development (before 72 hpf) (26, 29). After the lumen is formed, the blood vessels can also be visualised by microangiography, which is obtained by injecting fluorescent microbeads into the circulatory system (20) (Figure 4).

Figure 4. The vasculature of the zebrafish embryo can be visualised by microangiography. A 72-hpf zebrafish embryo is shown with its vasculature labelled with fluorescent microspheres, which were injected into the circulation.



A high-throughput screening format has been developed for assessing drug effects on blood vessel growth, using a 96-well microplate to quantify EAP activity in individual embryos (26). Primary hits can then be further validated by microangiography. The results of these studies showed that drug effects on vessel inhibition in zebrafish correlated well with effects in mammals, suggesting that the zebrafish is a predictive model for testing angiogenesis modulators (26).

A model for apoptosis screening

Apoptosis, or programmed cell death, is an important mechanism for morphogenesis and homeostasis. Abnormalities in apoptosis are involved in many diseases, including cancers and neurodegenerative diseases (32). The apoptotic processes in zebrafish and mammals are similar, and zebrafish homologues of most of the mammalian apoptosis-related genes have been identified, including Bcl-2 family members, caspases, Ced-4-like molecules, IAP (inhibitor of apoptosis), death receptors and ligand, apoptosis-related kinases and transcriptional factors (19).

There is a stereotypic pattern of normal apoptosis during zebrafish embryogenesis (6, 26). Alteration of these normal apoptosis events can serve as an assay for apoptosis modulators. In addition, there are many zebrafish mutants that display abnormal apoptosis that can serve as models for anti-apoptotic drug screening (16, 28). Apoptosis-inducing drugs themselves can also be used to induce apoptosis, which can then be used as a basis for screening anti-apoptotic drugs (26).

Apoptosis can be easily detected in live zebrafish embryos, using fluorescent labelling techniques such as acridine orange and fluorescence-conjugated caspase substrate (for example, PhiPhiLuxG1D2) (26). Quantitative assays have also been developed to quantify the level of apoptosis in the 96-well microplate format: acridine orange can be extracted from whole embryos in individual wells and quantified using a fluorescence microplate reader (26). The quantitative assay can be used as a primary screen to identify compounds that modulate apoptosis, followed by detailed analysis to identify specific sites of apoptosis by whole-mount staining.

Perspective

In addition to the assays described above, zebrafish models for many other human diseases are also available or under development, including neurological, haematopoietic, immunological and metabolic disorders (8, 11, 33, 37). Many complementary

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techniques – such as transgenesis and "gene knock-down" – are also available for zebrafish, enhancing the power of this model for drug discovery. Transgenic zebrafish expressing disease-related human proteins can serve as drug screening models for specific human diseases. Morpholino antisense oligos can be injected into zebrafish embryos to knock down target genes, and the resulting effects can be thoroughly assessed in the transparent embryo, providing an excellent tool for target validation. Since the zebrafish genome project is nearly complete, and diverse new assays are being developed by increasing numbers of zebrafish researchers, this model organism will become an increasingly important drug screening tool.

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