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REVIEW ARTICLE

Zebrafish as model organism for drug discovery and toxicity testing: A review

V.N. Sarvaiya, *K.A. Sadariya, M.P. Rana and A.M. Thaker

Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand-388001, Gujarat, India.

*Corresponding Author:

K.A. Sadariya

Email: dr_kasadariya@yahoo.co.in

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Abstract

The zebrafish (*Danio rerio*) has become a widely used vertebrate model organism for drug discovery because of its high fecundity, transparent embryos and larvae, morphological and physiological similarity to mammals, allowing *in vivo* analysis of embryogenesis and organogenesis combined with ease of use and low cost. This review contain background on zebrafish as model organism, fields of applications, advantages and limitations of this vertebrate model system and then cited the utility of this model in the drug discovery and toxicity studies. The zebrafish promises to contribute to several aspects of the drug development process, including target identification, disease modelling, lead discovery and toxicology. The numerous advantages of this whole animal approach provide new promise for the discovery of safe, specific and powerful new drugs. The zebrafish as model organism provides vast new opportunities in future.

Key words: Zebrafish, model organism, drug discovery, toxicity testing.

Introduction

Model organism is a non-human species that is extensively studied to understand particular biological phenomena, with the expectation that discoveries made in the model organism will provide insight into the workings of other organisms (Fields and Johnston, 2005). Biomedical research depends on the use of animal models to understand the pathogenesis of disease at a cellular and molecular level and to provide systems for developing and testing new therapies. Studying model organisms should be informative, but care must be taken when extrapolating from one organism to another. *In vivo* models are widely used to research human and animal diseases. Broadly speaking there are three main types of model organism:

- Genetic model organisms: They are use for genetic analysis. They have short generation times so large scale crosses can be followed over several generations like Baker's yeast (*Saccharomyces cerevisiae*), the fruit fly (*Drosophila melanogaster*) and a nematode worm (*Caenorhabditis elegans)*.

- Experimental model organisms: They are widely used in research of developmental biology. Species used for this purpose produce robust embryos that can be easily manipulated and studied like African clawed frog (*Xenopus laevis*).

- Genomic model organisms: They are used particularly in genome research. Species used for this purpose have manageable genome sizes or have a genome similarities with the human or other vertebrate large animal genome like zebrafish and mouse.

Model organisms are chosen on the basis of characteristics such as short life-cycle, techniques for genetic manipulation (inbred strains, stem cell lines and methods of transformation) and non-specialist living requirements. *In vivo* studies represent an essential step in drug development and rely largely on mice. But there are several limitations of mammalian models which motivated the search for complementary vertebrate model systems. So now it is focused on Zebrafish (*Danio rerio*) the facile model system to study human and animal disease and drug responses. The Zebrafish (*Danio rerio*) has been a popular pet for decades. The Zebrafish is a tropical freshwater fish and is an important vertebrate model organism in scientific research. It is particularly notable for its regenerative abilities and has been modified by researchers to produce several transgenic strains. Synonyms of Zabrafish are *Barilius rerio, Brachydanio rerio, Cyprinus rerio, Danio frankei, Danio lineatus, Nuria*

rerio, Perilampus striatus etc. Scientific classification of the Zebrafish is presented below:

- Kingdom : Animalia
- Phylum : Chordata
- Class : Actinopterygii
- Order : Cypriniformes
- Family : Cyprinidae
- Genus : *Danio*
- Species : *Danio rerio*

There are five uniform, pigmented, horizontal, blue stripes on the side of the body which are similar to the zebra's stripes and extend to the end of the caudal fin, so it is given name as zebrafish (Fig 1). Its shape is fusiform and laterally compressed, with its mouth directed upwards. Adult zebrafish measures 4-5 cm in length.

Fig 1: Adult zebrafish having characteristic stripes running along the body and the fins

The zebrafish is native to the streams of the south-eastern Himalayan region and is found in parts of India, Pakistan, Bangladesh, Nepal and Burma. Zebrafish have been introduced to parts of the United States, presumably by deliberate release or by escape from fish farms. Zebrafish are omnivorous, primarily eating zooplankton, insects, insect larvae and phytoplankton. The approximate generation time for *Danio rerio* is three to four months. Adult females are able to laying 200-300 eggs in each clutch. Upon release, embryonic development begins, growth stops after the first few cell divisions (Fig 2). Fertilized eggs immediately become transparent, a characteristic that makes *Danio rerio* a convenient research model species (Spence *et al.*, 2007). Development progresses very rapidly. Precursors to all major organs appear within 36 hours of fertilization and hatching takes place 12-36 hours later, depending on the embryo's internal conditions and the external temperature, which is ideally 28.5 °C (83.3 °F). Swimming and feeding behavior begin about 36 hours later. In late 2003, transgenic Zebrafish that express green, red and yellow fluorescent proteins became commercially available in the United States. The fluorescent strains are trade named GloFish; other cultivated varieties include 'golden', 'sandy', 'longfin' and 'leopard'. The Zebra Fish Information Network (ZFIN) provides up-to-date information about current known wild-type strains of *D. rerio*, some of which includes AB (AB), AB/C32 (AB/C32), AB/TL (AB/TL), AB/Tuebingen (AB/TU), C32 (C32), Cologne (KOLN), Darjeeling (DAR), Hong Kong (HK), India (IND), Indonesia (INDO), Nadia (NA), RIKEN WT (RW), Singapore (SING) and Ekkwill (EKW).

Advantages of Zebrafish as a model organism

Fish are the most widely used non-mammalian vertebrates in risk assessment and regulation. Among them, Zebrafish has special characteristics, which expedite its use as a model organism. The tiny size of the larval and adult Zebrafish greatly reduces cost as it enables the reduction of housing space and husbandry cost. In toxicological and pharmacological studies (i.e. investigating drugs, potentially toxic compounds, environmental samples etc.) can be performed in a miniaturized format, which minimizes the quantities of chemicals to arrange and reduces the volumes of potentially hazardous waste (Hill *et al.*, 2005; Spitsbergen and Kent, 2003). Zebrafish has versatile system, offering many molecular and genetic tools to model human disease and development to study gene function during normal development and disease. More recently its utility in the identification of lead compounds by drug screening has proven to be cost and time effective (Chitramuthu, 2013).

The small size of eggs and juveniles allows the operation of tests in high-throughput screenings, i.e. in multi-well plates and thus a sufficient database from many replicate samples can be gained for statistical evaluation and validation of results. Zebrafish embryo and larvae are relatively tolerant to dimethylsulphoxide, a commonly used solvent in *in vitro* assays. Another advantage of this species is its high fecundity. One pair of adult fish is capable of laying 200 eggs a day and depending on the conditions of maintenance, this yield can be expected every 5-7 days. In addition, the rapid maturation of Zebrafish also enables the performance of trans-generational studies. Thus, with sexual maturation after around 100 days, Zebrafish can be utilized e.g. in mutagenesis analyses (Hill *et al.*, 2005). Zebrafish embryonic development has been well characterized (Kimmel *et al.*, 1995). Zebrafish eggs are transparent, as well as the embryos themselves during their first days of life (Fig 3). Pigmentation in the embryos starts only about 30-72 hours post fertilization (Hill *et al.*, 2005). Therefore, -

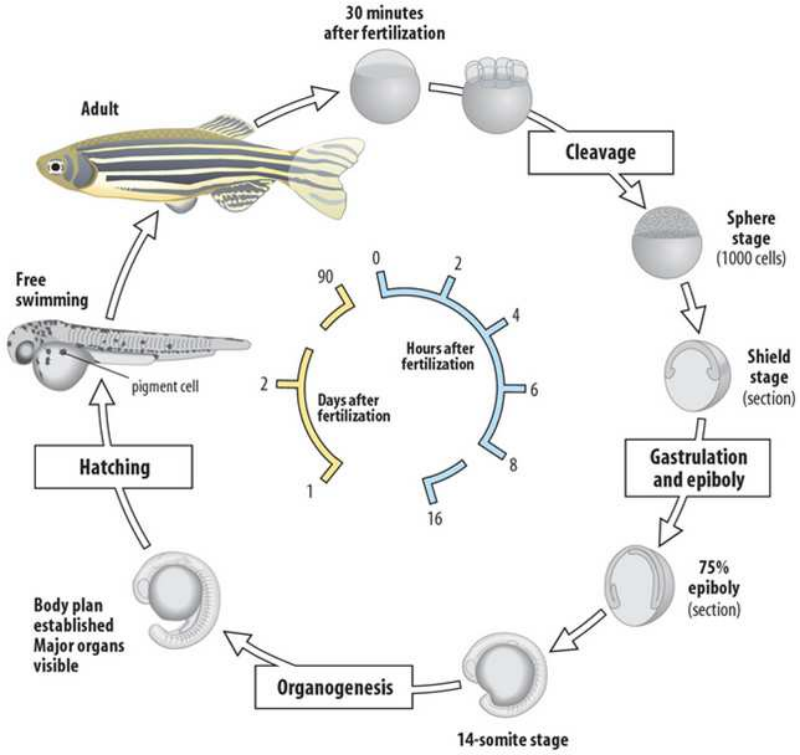

Fig 2: Developmental stages of zebrafish (*Danio rerio*) (Kimmel *et al.*, 1995).

changes in the morphology within their early-stage development can be easily observed under the microscope. Another important characteristic of zebrafish is that mutant zebrafish embryos, even having strong morphological malformations or displaying organ dysfunction, are still able to survive. In contrast, malformed embryos of rodents mostly die *in utero* (Hill *et al.*, 2005). Because mutants are useful for many kinds of studies, such as human diseases, due to the high homology with humans, hundreds of Zebrafish phenotypic mutants were produced and applied to unravel molecular regulations of ontogeny **(**Stern and Zon, 2003). The availability of the complete genome sequence of Zebrafish allowed the production of commercially available microarrays (Agilent, Affymetrix, Compugen / Sigma-Aldrich, MWG Biotech and Qiagen / Operon) that offer a standardized tool set for Zebrafish transcriptional profiling studies

Fields of applications of Zebrafish

Zebrafish is an emerging vertebrate model for drug discovery that permits whole animal drug screens with excellent throughput, combined with ease of use and low cost (Delvecchio and Tiefenbach, 2011). Zebrafish is a widely used model organism in many different fields of research. In the past, it was a major vertebrate model especially in the developmental and genetic research (Hill *et al.*, 2005). Zebrafish has become a popular model in pharmacological studies for e.g. screening of chemical libraries, mode of action studies, analysis of gene function, predictive toxicology, teratogenicity and pharmacogenomics and toxicogenomics. It was shown that Zebrafish can be used as a suitable model in cancerogenesis studies, i.e. anti-cancer drug investigations, inflammatory processes, as well as for lipid metabolism, since the response to cholesterol blockers is similar to those in mammals (Langenheinrich, 2003). The replacement of mammalian test systems by Zebrafish, as less cost-intensive alternative in late-phase toxicity screening of drugs, was proposed by Rubinstein (2006), because of the many similar biological processes. *Danio rerio* is an excellent system for chemical toxicity testing. The number of chemicals that need to be tested in the field of chemical toxicity and drug discovery is steadily increasing. Therefore, also the need for high-throughput screening methods arises, where the use of Zebrafish embryos was proposed (Hill *et al.*, 2005), because of their small size and thus suitability for studies in multi well plates. Not only toxicity screening

applications are imaginable; also applications for the clarification of mechanisms of toxicity have been reported (Spitsbergen and Kent, 2003). The Zebrafish has been pioneered as a developmental and genetic tool for the study of organogenesis and disease. The Zebrafish model is a very powerful model to study cardiac development (Bakkers, 2011).

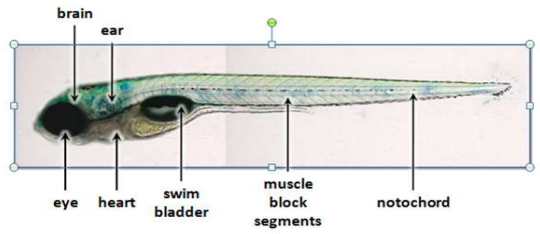

Fig 3: Two days post fertilized (dpf) larvae of zebrafish showing development of major organs

Drug discovery

Drug discovery involves a complex iterative process of biochemical and cellular assays, with final validation in animal models. Mammalian models of absorption, distribution, metabolism and excretion (ADME)/pharmacokinetics and efficacy are expensive, laborious and consume large quantities of precious compounds. There is also increasing pressure to limit animal use to situations in which they are absolutely necessary, such as in preclinical toxicity and safety assessment. Zebrafish can also be useful model organism to study the mechanisms enabling regenerative neurogenesis (Kyritsis *et al.,* 2012). Zebrafish can be used to identify novel compound with selective toxicity against leukemia (Ridges *et al.,* 2012). Zebrafish are beginning to be used at various stages of the drug discovery process and can be a useful and cost-effective alternative to some mammalian models (such as rodents, dogs and pigs). Exploration of the Zebrafish cancer models can be helpful in understanding underlying mechanisms of cancer and also provide platforms for drug discovery (Liu and Leach, 2011; Etchin *et al.,* 2011). Zebrafish embryos and larvae are small, transparent and undergo rapid development *ex-utero*, allowing phenotypic analysis *in vivo*. These physical characteristics have lead to the popularity of this organism to use in drug discovery process. These factors can take advantage for implementations of the 3Rs (Replacement, refinement and reduction), which are protected in the UK Animals (Scientific Procedures) Act 1986 (ASPA). Use of non-mammalian vertebrates can contribute to the 3Rs (Replacement, refinement and reduction).

- **Replacement:** The ASPA regulates the use of vertebrates in scientific procedures which may cause pain, suffering, distress or lasting harm. A license is required to conduct regulated procedures on mammals from half-way through the gestation period and on fish from the time at which they become capable of independent feeding (rather than being dependent on the food supply from the yolk), which in the Zebrafish is accepted to be at 5 days post fertilized. Life stages before this time are considered to be not sufficiently aware that they will suffer or otherwise have poor welfare when a procedure is carried out on them.

- **Refinement:** Researchers can take advantage of the size and transparency of Zebrafish larvae to perform similar procedures as those performed in mammals at licensed stages but using less invasive methods.

- **Reduction:** Zebrafish provide a cost-effective model to bridge the "gap" between *in vitro* and *in vivo* work and thereby reduce the attrition rate and hence numbers of animals used in the drug discovery process.

It is not only that Zebrafish be used as a replacement to rodent/mammalian models in numerous assays but that they can be used to obtain *in vivo* data earlier in the drug discovery process. This should dramatically improve the odds of identifying novel therapeutics that are both effective and safe, thereby reducing the total number of animals used throughout the discovery process.

Manipulation of Zebrafish embryos for drug discovery

There are four major methodological steps to consider when undertaking a chemical library screen in Zebrafish:

- Adult pair mating and embryo collection
- Embryo sorting or arraying into multiwell plates
- Chemical library administration
- Data acquisition and analysis.

The modern drug discovery process can be divided into four major components (Handen, 2002):

- Screening of lead compounds
- Target identification
- Target validation
- Assay development.

Screening of lead compounds

The Zebrafish is an outstanding vertebrate system for developing *in vivo* disease-related assays that can be useful for high volume compound screening. Moon *et al.* (2002) used Zebrafish embryos to screen a small library of triazine compounds for their ability to inhibit tubulin polymerization *in vivo* which was a first step toward identification of new anticancer drugs (Moon *et al.*, 2002). Murphey *et al*. (2006) examined a broad range of known cell cycle compounds like the mitotic marker phospho-histone H3 using Zebrafish embryos. The majority of the known compounds exhibited the predicted cell cycle effect in embryos. These findings demonstrate that small molecule screens in Zebrafish can identify compounds with novel activity and thus may be useful tools for chemical genetics and drug discovery. Zebrafish provide a very useful platform for novel therapeutic discovery through chemical screening (Tan and Zon, 2011).

Target identification

Identification of novel drug targets is a bottleneck in drug discovery (Lindsay, 2003). Here only crude morphological defects and behavioral changes have been considered to evaluate the effects of the compounds, but it is also feasible to observe the protein localization, gene expression and metabolic changes into the specific organ where the morphological changes occur. One target identification focused assay was performed where whole-mount in situ hybridization was used to analyze catecholaminergic neurons in mutated fish (Guo *et al.*, 1999). For neurodegenerative and psychiatric disorders, class genes that interrupt the formation of specific neuronal classes may be important targets. A fluorescent lipid assay was used to identify genes that may hinder with normal lipid processing (Farber *et al.*, 2001).

Target validation

Target validation as well as lead compound optimization can be done through a Zebrafish diseases model. Disease model can be created through transgenic line or knockdown line that can help to validate the target (Deiters and Yoder, 2006). Therefore, creation of transgenic line or knockdown line can validate the target as well as lead optimization. Once novel targets are identified, the zebrafish has the added benefit of providing a system for their validation through the rapid analysis of gene function.

Morpholino Oligonucleotide Screens: Morpholinos are chemically modified antisense oligonucleotides. It is designed to hybridize to the translation-initiation or splicing acceptor/donor sites of specific mRNAs. They possess altered backbone linkages compared with DNA or RNA. Morpholinos cause a vigorous knockdown of gene function when injected into Zebrafish embryo (Ekker, 2008). Genetic and morpholino oligonucleotide screens are an efficient means of systematically assessing the roles of individual genes in disease processes in the Zebrafish. This process represents a potential route to the identification and validation of novel drug targets.

Assay development

The disease relevant assay development is very necessary to understand the potential of Zebrafish for both target validation and drug screening. Assays have been designed to test for hearing defects, by examining either defects in normal swimming behavior, or the response of Zebrafish to loud sounds (Bang *et al.*, 2002).

Structure activity relationships (SAR) of drugs

Study of a structure-activity relationship (SAR) of drugs using Zebrafish is the process by which chemical structure is quantitatively correlated with a well defined process, such as biological activity or chemical reactivity. For example, biological activity can be expressed quantitatively as in the concentration of a substance required to give a certain biological response.

This principle is called structure-activity relationship (SAR) (Patani and LaVoie, 1996). The zebrafish is highly amenable to the study of structure–activity relationships (SARs). During the above screen for compounds that alter phospho histone H3 (pH3), several compounds were identified. SARs have been established for several of these compounds. Several derivatives of parent compound were generated and tested for their ability to alter pH3 levels in intact Zebrafish. Three compounds induce the Zebrafish phenotype at concentrations similar to those of the parent compound, four compounds induce the phenotype only at a fivefold higher concentration, and three compounds have no activity (Zon and Peterson, 2005). One advantage of performing SAR studies in Zebrafish is that they couple the analysis of binding affinity and ADME/toxicity. In a traditional *in vitro* SAR study, structural changes that improve potency are pursued, but these changes might have detrimental effects on absorption, toxicity and so on. By performing SAR studies in whole Zebrafish, structures can be identified that improve potency without increased toxicity or loss of *in vivo* efficacy.

Table 1: Toxic effect of various drug preparations on zebrafish (Kari *et al*., 2007)

Preparation	Pharmacological activity	Concentration	Observed effect/toxicity
Camptothecin	Anticancer agent (topoisomerase I inhibitor)	500 nM	Growth retardation
Gentamycin	Antibiotic	5 μM	Hair cells loss
Neomycin	Antibiotic	10 μM	-
Cisplatin	Anticancer agent	50 μM	-
Vinblastine	Plant antitumor preparation	100 μM	-
Quinine	Antimalarial drug	200 μM	-
Doxorubicin	Anticancer drug (anthracyclin antibiotic, topoisomerase II inhibitor)	30 mg/l	Teratogenicity, nephrotoxicity, hepatotoxicity,cardiotoxicity
Dexamethasone	Corticosteroid	324 mg/l	Nephrotoxicity,hepatotoxicity, gastrointestinal tract lesion
Methotrexate	Antimetabolite cytostatic(folic acid antagonist)	454 mg/l	Teratogenicity, nephrotoxicity, hepatotoxicity, cardiotoxicity, gastrointestinal tract lesion
Fluorouracil	Antimetabolite cytostatic	3.3 mg/l	Nephrotoxicity,hepatotoxicity
Cyclosporin A	Immune suppressor	69 mg/l	Teratogenicity, nephrotoxicity, hepatotoxicity, cardiotoxicity
Caffeine	Methylxanthine alkaloid(phosphodiesterase inhibitor), CNS stimulator	108 mg/l	Change of locomotor activity, muscular spasticity

Table 2: Zebrafish toxicity testing compared with mammalian models (http://www.phylonix.com)

Compound tested	Zebrafish		Mammalian models	
	LC50/Log LC50 (mg/l)	Specific toxicity observed	LC50/Log LC50 (mg/kg)	Specific toxicity
Geldanamycin	3.13/0.49	Liver	1.0/0.4 (mice, i.p.)	Liver
Doxorubicin	30.3/1.51	Teratogen, liver, cardiovascular, kidney	21/1.35 (mice, i.v.)	Liver, cardio vascular
Cyclosporin A	69/1.83	Teratogen, kidney, cardiovascular, liver	170/2.23 (mice, i.v.)	Kidney, ureter, bladder
Ibuprofen	5.56/0.74	Liver, kidney, gastrointestinal	495/2.69 (mice, i.p.)	kidney, gastro intestinal
Dexamethasone	324/2.51	Liver, gastrointestinal	410/2.61 (mice, i.p.)	Liver, heart, gastrointestinal
Aspirin	100.9/2.0	keratogen, kidney, muscle contraction erratic movements	167/2.22 (mice, i.p.)	kidney, ureter, cardiovascular, musculoeskeletal
Naproxen	13.2/1.12	Liver, gastrointestinal	435/2.63 (mice, i.v.)	Gastrointestinal
Acetaminophen	252/2.4	Liver	500/2.69 (mice, i.p.)	Liver, kidney, gastrointestinal

FDA point out to technological difficulties in toxicology as one of the principal causes of this 'pipeline problem'. To evaluate the toxicity of a drug, it is essential to identify the endpoints of toxicity and the dose-response relationships, determine the toxicodynamics of the drug, in which Zebrafish has numerous attributes (Pritchard *et al*., 2003).

By contributing to target identification and validation, drug lead discovery and toxicology, the Zebrafish might provide a shorter route to developing novel therapies for human and other mammal diseases. Organ specific toxicities remain the most frequent reason for the failure of drugs late in development or withdrawal of drugs from the market. A large body of scientific references is available for validity of Zebrafish embryo as a model to evaluate toxicity of various chemical compounds. Major types of toxicity studies reported in the Zebrafish are acute toxicity (Lammer *et al.*, 2009), carcinogenesis (Parng, 2005), cardiotoxicity (Mittelstadt *et al*., 2008), developmental toxicology (Chapin *et al.*, 2008), digestive system toxicity (Berghmans *et al*., 2008), endocrine disruptors (Segner, 2009), organ toxicity (Winter *et al.*, 2008), genotoxicity (Grisolia *et al*., 2009), hepatotoxicity (Du *et al*., 2009), nanotoxicity (Cheng *et al*., 2008), nephrotoxicity (Tsay *et al.*, 2007), neurotoxicity

(Peterson *et al*., 2008), ototoxicity (Chiu *et al*., 2008), and toxicogenomics (Liedtke *et al.*, 2008). In all cases, Zebrafish larvae exhibited similar xenobiotic, genetic and physiological responses as documented in mammalian systems (Wiegand *et al.*, 2000).

In recent years, the Zebrafish has also proven to be a promising model for safety pharmacology assessment in drug discovery and screening (Redfern *et al.*, 2008; Barros *et al.*, 2008; Peter *et al.*, 2013; Genri and Louis, 2013; Diekmann and Hill, 2013). All the advantages of the zebrafish model for toxicity assessment are also valid for safety pharmacology. Various comparison of larval Zebrafish assays are available and suitable for safety pharmacology, with nearest equivalent mammalian cell based assays. Various compounds were used to study LC50 and liver and kidney toxicity in the Zebrafish and there was good correlation with mammalian models. These data provide strong preliminary validation of Zebrafish as a model for toxicity testing.

Limitations of Zebrafish as a model organism

Several mammalian organs are not present in the zebrafish, including breast tissue, lungs, and prostrate. Skin lacks some specific cellular components found in humans. Adult Zebrafish are not as suitable for high-throughput screens due to their large size. The metabolizing enzymes of the liver (e.g., CYP450s) are not fully characterized in the Zebrafish with an unclear understanding of the relevance to human drug metabolism (Wheeler and Brandli, 2009). Teleost fish possess two copies of many mammalian genes due to an evolutionary gene duplication event. The problem of skewed sex ratios in cohorts of Zebrafish, because it can interfere with natural breeding and can complicate studies such as carcinogen or other toxicant bioassays where balanced sex ratios in control groups are desired. Microsporidiosis and mycobacteriosis are two infectious diseases that commonly occur in well-managed Zebrafish colonies.

Conclusions

The Zebrafish occupies an important role between more traditional representative animal models and *in vitro* systems. The Zebrafish has been pioneered as a developmental and genetic tool for the study of organogenesis and disease. Therefore, small molecules identified in whole-organism screens might be more relevant than those identified by *in vitro* and cell-culture-based screens. The small size of Zebrafish embryos and their ability to be cultured during the first week of life make this system ideal for drug discovery and safety testing compare to the rodent. The high degree of conservation between Zebrafish and human genes and cellular processes can be use early in the drug discovery process. With the completion of the Zebrafish genome project and the establishment of a robust infrastructure for genetic and physiological studies, the Zebrafish system able to take good position in the field of drug development. It is possible to add Zebrafish assays in late stage preclinical toxicity screens. Relatively low cost of Zebrafish experiments (compared with experiments on mammals) and improvement of modern technology allows using Zebrafish as an inexpensive alternative to rodent test systems in next few years.

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