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Review Article

ZEBRAFISH: AN EMERGING MODEL SYSTEM FOR DRUG DISCOVERY

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ABSTRACT

The zebrafish is no doubt a powerful model organism with a combination of forward and reverse genetics, low cost, amenable high throughput, and rapid *in vivo* analysis. With these unique features, it can be expected that the zebrafish will become more frequently used for drug discovery. This review outlines the potential of zebrafish to contribute to drug discovery through the identification of novel drug targets, validation of those targets and screening for new therapeutic compounds and assay development.

Keywords: Zebrafish, Drug screening, Drug target, Development.

INTRODUCTION

The zebrafish (*Danio rerio*) is a small tropical fish, family of Cyprinidae and native to the river of India and South Asia. Zebrafish has been an important model organism in genetics and toxicology. A number of unique features have contributed to its attraction such as it is a very small vertebrate that can be kept in captivity in large number easily. Its generation time is short and most important a single spawning can produce hundreds of offspring and that is fertilized outside of the mother and can be easily collected from the breeding tank. Furthermore, the entire body plan established by 24 hrs postfertilization (hpf) and most of the internal organ such as heart, liver, kidney, and intestine totally developed by 96 hpf [1]. A direct comparison of the zebrafish and human protein coding genes reveals a number of interesting features. 70% of protein coding human genes are related to genes found in the zebrafish and that 84% of genes known to be associated with human disease have a zebrafish counterpart [2]. The 9th assembly of the zebrafish genome (Zv9) reports 1.41 billion base pairs with ~24,000 protein coding genes present in zebrafish. Due to above advantages, the zebrafish has become an established model for genetics, development biology, neuroscience, cardiovascular, behavior science, toxicology, and human diseases [1,3-8]. Recently, it has crossed the border and metamorphosised into a promising tool for drug discovery and development.

THE UNIQUENESS OF THE ZEBRAFISH

The ability to culture large numbers of zebrafish embryos and larvae in small volumes of media [9] 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 [10-12]. These features combine to create an ideal *in vivo* model suitable for medium throughput phenotypic screening in microtiter plates. Now zebrafish is used in various pharmacological studies including screening and investigation of mechanisms of action of biologically active substances, pharmacogenomics, and toxicogenomics [13].

In this study, we highlighted the potential of zebrafish to contribute to drug discovery and development through the identification of novel drug targets, validation of those targets and screening for new therapeutic compounds.

ZEBRAFISH IN DRUG DISCOVERY

Drug discovery is a complex process and normally involves expensive, laborious, and time-consuming tests. To tackle these limitations, people tend to look for a "shortcut" by which to reach their desired result. Modern drug discovery involves a wide variety of approaches for the identification and validation of new therapeutics, including both *in vitro* and *in vivo* assays. *In vivo* assays have been performed in cultured cells and yeast, as well as in whole animals, both invertebrates, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, and mammals such as mice, rats, and primates [14].

The modern drug discovery process can be divided into three major components: Target identification and target validation; drug screening and clinical trials (Fig. 1). A conventional drug discovery has recently employed systematic, target based high throughput screening (HTS) in purified proteins or cells as primary screens with *in vivo* models as tertiary screens in the cascade after more mechanistic cell assays. While the *in vivo* screens have been successful at identifying small molecules affecting known mechanisms, there is still the need to identify modulators of the complex *in vivo* phenotypes in the whole organism for less well-understood pathways or those that only occur in a physiological perspective. The advantage of larval zebrafish is it will allow high throughput *in vivo* screening [15,16]. However, that uptake of compound into the zebrafish can be variable and should be measured for accurate interpretation of results and particularly to avoid false negative, and the larval stages of the zebrafish may not be appropriate in all disease [7]. Peterson and colleagues were done first chemical screen in zebrafish. They have screened 1100 compounds in 96 well-plates for small molecules that caused developmental phenotypes during the first 3 days of development [9]. This was an important study showing that small molecule screening in zebrafish could identify genetic mutations, developmental disorder and timing of the chemical action during development.

TARGET IDENTIFICATION

Forward and reverse genetics screening have been successfully applied in zebrafish [17,18]. Forward genetics, characterized as "phenotype to genotype," first involves the identification and characterization of a specific phenotype, followed by the identification of the underlying genetic mutation. Reverse genetics, "genotype to phenotype," takes advantage of molecular biology techniques. In these cases, a gene of interest is selected and targeted by the morpholino oligonucleotide knockdown, Targeting Induced Local Lesions IN Genomes, or zinc finger nucleases and CRISPR associated protein-9 nuclease (Cas9) to discover the function of the genetic mutation within the fish [19,20]. The novel genes that underlie the zebrafish disease phenotypes might lead directly to the identification of novel drug targets. Alternatively, the disease phenotypes might form the basis of further screens to

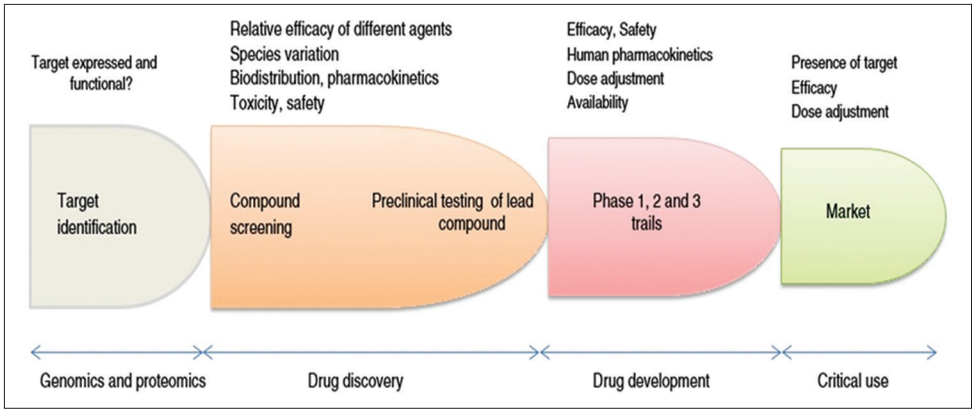

Fig. 1: Drug discovery and development process

find genes or chemicals that can correct the phenotype. Despite an appreciable history of pharmacological investigation dating back over half a century, it is only within the past decade that large-scale screens of small molecules have been performed using zebrafish-based assays. Initial compound screens have focused on the analysis of combinatorial chemistry libraries encompassing up to 20,000 molecules for their ability to induce a variety of developmental phenotypes [9,21,22]. While many currently used clinical drugs have no known target [18], target identification remains an important aspect of drug discovery. So far, only crude morphological defects [9] and behavioral changes [23] have been considered to evaluate the effects of the compounds, but it is also feasible to observe the gene expression, protein localization and metabolic changes into the specific organ. The numbers of assays are being used for target identification; one assay was used to analyze catecholaminergic neurons in mutated fish using whole mount *in situ* hybridization (ISH) [24]. Another experiment was performed that directly examined the behavior of zebrafish that was exposed to cocaine. In this experiment, identification of mutants was performed with the reduced sensitivity to cocaine [23]. In a third screen, zebrafish mutations with defects in blood clotting were identified by measuring time to clotting prolonged by copper chloride. This assay can analyze mutations with defects in blood clotting that may help identify drug targets [25]. Fourth, a fluorescent lipid assay was used to identify genes that may hinder with normal lipid processing [26]. A triazine-based combinatorial library of small molecules was screened in zebrafish to identify compounds that produced interesting phenotypes. Genes that are expressed specifically in a tissue affected by a disease process may also represent novel targets for that disease. The ability to generate tissue-specific fluorescent transgenic fish can lead to the production of exquisitely tissue specific cDNA libraries through the use of fluorescence activated cell sorting for tissue collection [27].

TARGET VALIDATION

The process of target validation identifies and assesses whether a molecular target merits the development of pharmaceuticals for therapeutic application. Target validation as well as lead compound optimization can be done through zebrafish system. Disease model can be created through transgenic line or knockdown line that can help to validate the target [28]. The zebrafish has the added benefit of providing a system for their validation through the rapid analysis of gene function.

DRUG SCREENING

Drug screening assays using zebrafish becoming increasingly popular and used as a platform for *in vivo* HTS drug discovery [29,30]. Zebrafish has been more used for toxicological assessment, particularly cardiotoxicity, hepatotoxicity, neurotoxicity, and developmental toxicity [31,32]. The most promising use of drug screens in the zebrafish is to screen genetically modified zebrafish embryos instead of wild-type embryos. These genetically modified fish may be screened with large panels of drugs for reversion of the phenotypes. The targets of the chemical compounds will include factors downstream of the mutant gene and may include the expressed transgene as well. These types of screens provide a direct means to screen for chemical compounds that act in disease pathways. Examples of human disease gene which have been identified in the zebrafish are presented in Table 1.

ASSAY DEVELOPMENT FOR DRUG DISCOVERY

Four major classes of whole organism chemical screening assays are commonly used: Morphological screens, behavioral screens, fixed time point/labeling assays, and fluorescence assays [37]. Morphological assays encompassing basic qualitative observations to automated imaging, manipulation, and data processing systems provide whole organism to subcellular levels of detail. Behavioral screens extend chemical screening to the level of complex systems. In addition, zebrafish based disease models provide a means of identifying new potential therapeutic strategies. Automated systems for handling/sorting, high-resolution imaging and quantitative data collection have significantly increased throughput in recent years.

Morphological screens

Visual observation of arrayed fish treated with chemicals is the simplest possible assay and can be performed easily at a dissecting microscope. Morphological screens utilizing zebrafish assessed specific processes such as organogenesis [38], fin regeneration [39], or bone morphogenetic proteins signaling [40]. Researchers have developed a number of mutant and transgenic lines with disease relevant phenotypes that can be utilized for chemical screening purposes. Chemical suppression of such mutant phenotypes is a powerful method that can give insight into the effect of a compound on specific disease relevant pathways. Similarly, the heart rate of zebrafish can be visually assessed by 1.5 dpf and has been used to screen for drugs that affect heart rate [41]. This assay was later extended to a voltage-gated potassium channel mutant that exhibits a long QT syndrome phenotype. To improve throughput and remove subjectivity, advances in high content imaging (HCI) techniques have provided. HCI entails the rapid, automated capture of a collection of images, such as those from each well of a multi well-plate. These images can then be used for quantification of parameters such as the effects from compound exposure in a chemical screen. The visual system is especially amenable to study in the zebrafish, due to the large size of the eyes and the ease with which visual assays can be established [42,43].

Behavioral assays

Zebrafish swimming kinetics is a commonly used parameter in behavioral studies, for instance in assays to quantify the effects of single compounds [44]. Due to commercial availability of devices that record and quantify swimming behavior like mean velocity, active velocity, and percent time moving in a 96 well-plate format are also greatly increased in this type of assay. Rihel *et al*. developed an automated rest/wake behavioral assay that quantified several parameters (rest bouts, rest

Table 1: Selected zebrafish model of human disorder [33,34-36]

Human disorder/disease	Genes
Neurological disorders	
Alzheimer's disease	*Presenilin-1*
Huntington's disease	*Presenilin-2*
Attention-deficit hyperactivity disorder	*Acetyl cholinesterase*
X-Linked neurodevelopmental disorder	*Amyloid precursor protein*
	ApoE
	Huntingtin
	Per1b
	RPL10
Blood disorder	
Hypochromic microcytic anemia	*SLC11A2*
Hereditary elliptocytosis	*EBP41*
Erythropoietic protoporphyria	*Ferrochelatase*
Congenital dyserythropoietic anemia type 2	*SLC4A1*
Hemochromatosis type 4	*Ferroportin 1*
Thrombocytopenia	*Gata 1*
Leukemia	*Run×1*
Thrombosis	*Cbfb*
	Factor VII
	COX-1
	COX-2
Heart disease	
Cardiomyopathy	*Titin*
Holt-oram syndrome	*Tnnt2*
Long QT syndrome	*ACTC*
	Myh6
	Tb×5
	KCNH2
Musculo-skeletal/skin disorder	
Muscular dystrophy	*Dystrophin*
Brody myopathy	*Dystroglycan*
Campomelic dysplasis	*Dp71*
	ATP2A1
	So×9a
Neuro-organ disorder	
Deafness	*Myo6b*
Pituitary anomalies	*Gli2a*
Kidney/Pancreas/endocrine	
Polycystic kidney disease 2	*PKD2*
Glomerulocystic kidney disease	*nek kinase 8 (nek8)*
Diabetes mellitus	*Tcf2*
	Insulin
	Ptf1a
	IA-2 autoantigen
	IA-2β autoantigen
Inherited disorder	*Nf1*

latency, and waking activity) to create a "behavioral profile" for each assayed drug [45]. However, some of the important factors affecting the throughput of behavioral screens are require replicates for each assayed; the amount of time for a single assay.

Fixed time point/labeling assays

Procedures involving either fixation of samples, or labeling compounds that compromise biological function, represent "fixed time point" assays. Labeling methods can yield sample sets that can be repeatedly analyzed, and possibly even archived. The simplest of these methods is histochemical staining. ISH of endogenous mRNA has also been utilized in chemical screens. Furthermore, automation of immunolabeling and ISH screens through the use of mesh bottomed micro well-plates greatly increased throughput assays. Incubation of treated larvae in vital dyes is often a simple protocol that requires little processing compared with fixed embryo assays. In addition, a quantifiable signal is often quickly available, an advantage that increases throughput. Chemical screens that utilize hair cell specific vital dyes [46], and the commonly used acridine orange stain of apoptotic cells have been performed; as these dyes are fluorescent, they can be quantified by analysis of pixel intensity in captured images [47]. Other vital dye assays do not label specific cell types but have been used in screens as a reporter of biological processes such as fat content [48]. Similarly, an assay involving chemiluminescent substrate conversion to reveal drug metabolizing enzymes activity has been developed for use in toxicology studies.

Fluorescence assays

Many advantages brought to the forefront with this approach of fluorescence assays. Fluorescence-based screens are highly quantifiable and subtle cellular phenotypes can often be achieved. Furthermore, through expression of multiple, different colored fluorescent proteins within the same transgenic fish, several cell types or organs can be assayed simultaneously. Fluorescent zebrafish has also been utilized in multiple screens for potential cancer therapeutics including screens for antileukemia drugs [49] and anti angiogenics [50]. Angiogenesis was measured using either endogenous alkaline phosphatase staining of blood vessels or microangiography. Transgenic lines of zebrafish with fluorescent blood vessels have been developed, which simplifies the process by which blood vessels are visualized [51].

CRISPR/Cas9 system for genome editing

Several studies have employed the CRIPR/Cas9 system in zebrafish with high precision. By employing this technique targeted single nucleotide polymorphism modifications were done that could be transferred roughly to 10% of the germline. With such high precision, polymorphisms that drive disease condition could be modeled and screened for potential therapy and understand molecular pathways. In zebrafish the genes *gol*, *ntl*, and *kra* have been disrupted, *th*, *fam46c*, and *smad5* have been added among the multiple others modified [52].

CONCLUSION

The zebrafish has already provided a wealth of fundamental information about embryonic development and disease [14,53]. With the completion of the Zv9 project and the establishment of a robust infrastructure for genetic and physiological studies, the zebrafish system sits poised to take on a larger role in the field of drug development. 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 disease. However, the development of disease relevant assays and disease models in the zebrafish is still in infancy and should be more. With the improvement of modern technology, the zebrafish might be able to important substitute of other mammalian models for the pharmaceutical discovery.

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