Pharmaceutica Analytica Acta
ISSN: 2153-2435

Bhusnure et al., Pharm Anal Acta 2015, 6:10
DOI: 10.4172/2153-2435.1000426

Review Article Open Access

Drug Target Screening and its Validation by Zebrafish as a Novel Tool

Omprakash G Bhusnure[1*], Jyoti M Mane[1], Sachin B Gholve[1], Sanjay S Thonte[1], Padmaja S Giram[1] and Jaiprakash N Sangshetti[2]

[1]*Department of Quality Assurance, Channabasweshwar Pharmacy College, Latur, India*
[2]*Department of Quality Assurance, Y. B. Chavan College of Pharmacy, Aurangabad (MS), India*

Abstract

Presently, the research using Zebrafish is expanding into areas such as pharmacology, clinical research as a disease model and interestingly in drug discovery. 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. The use of Zebrafish in pharmaceutical research, drug discovery and development is mainly target screening, target identification, target validation and drug toxicity study. Zebrafish have recently entered the fray as a model animal for some human diseases. It has numerous attributes in toxicology studies and high throughput screening. The fish are more affordable, easier to keep, and faster to rise than mammals, giving a higher-throughput system. Perhaps surprisingly, genes that cause disease in zebrafish are similar to those in humans. Zebrafish being a non-mammalian, drugs can also be tested for toxicity and their potential therapeutic activity against the target more easily than in mammals. The Zebrafish embryo has become an important vertebrate model for assessing drug effects. It exhibits unique characteristics, including ease of maintenance and drug administration, short reproductive cycle and transparency that permits visual assessment of developing cells and organs. Using Zebrafish it is possible to obtain results quickly at lower costs. "Reducing failures early in development is far more important than filling a pipeline with poorly chosen late-stage products likely to fail, and fail expensively."

Keywords: Drug discovery; Animal model; Zebrafish; Target screening; Target validation

Introduction

Drug discovery involves a complex iterative process of biochemical and cellular assays, with final validation in animal models, and ultimately in humans. The physiological, cellular, and/or genetic basis of a disease is studied to identify potential therapeutic targets. New compounds are isolated and purified from natural sources or synthesized [1].

Drug discovery and development is a very long and expensive process (Figure 1). It takes, on average, 10-15 years and $800 million to research develop and introduce a completely new drug. This is due, in large part, to the high attrition rate throughout the drug development process, i.e. the number of compounds that fail at different points in the development process. Using the *in vitro* laboratory assay, 5,000-10,000 new and previously developed compounds are tested for biological activity. It is estimated that only five out of 250 compounds selected from *in vitro* drug discovery programs that enter pre-clinical testing (i.e., animal testing) will be approved for clinical trials.

Thus, 98% of compounds tested in animals are eventually abandoned. In the majority of cases this is because either the compound did not show sufficient therapeutic activity (efficacy) *in vivo* or it had adverse effects and was considered unsafe. The majority of this animal testing is currently performed using rodents and/or higher mammals. Therefore, assays that allow a more accurate prediction of either efficacy or safety can have a significant impact in reducing the number of animals subsequently used in regulatory testing.

- Pre-Clinical Trials and Clinical Trials are the processes by which scientists test drugs and devices to see if they are safe and effective.

- Preclinical trial- a laboratory test of a new drug or a new medical device, usually done on animal subjects, to see if the hope for treatment really works and if it is safe to test on humans.

Steps involved in doing a pre-clinical trial

Step one: Get an idea for a drug target: Drugs usually act on either cellular or genetic chemicals in the body, known as targets, which are believed to be associated with disease (Figure 2).

Scientists use a variety of techniques to identify and isolate individual targets to learn more about their functions and how they influence disease.

Compounds are then identified that have various interactions with the drug targets that might be helpful in treatment of a specific disease.

- Drugs target specific points in biochemical pathways

- Biochemical pathways are series of chemical reactions occurring within a cell. In each pathway, a principal chemical is modified by chemical reactions.

Examples of different types of biochemical pathways:

In a biochemical pathway, each step usually involves either adding atoms (synthesis) or taking certain atoms away (degradation)

***Corresponding author:** Omprakash G Bhusnure, Department of Quality Assurance, Channabasweshwar Pharmacy College, Kava Road, Latur-413512, Distt. Latur (MS), India, Tel: (+91)-2382-641008 (O), (+91)-2382-240008(O); (+91) 9765360611, 02382-241611; Fax: +91-2382-243855; E-mail: omprakashbhusnure@gmail.com

Received August 21, 2015; **Accepted** September 22, 2015; **Published** September 25, 2015

Citation: Bhusnure OG, Mane JM, Gholve SB, Thonte SS, Giram PS, et al.(2015) Drug Target Screening and its Validation by Zebrafish as a Novel Tool. Pharm Anal Acta 6: 426. doi:10.4172/21532435.1000426

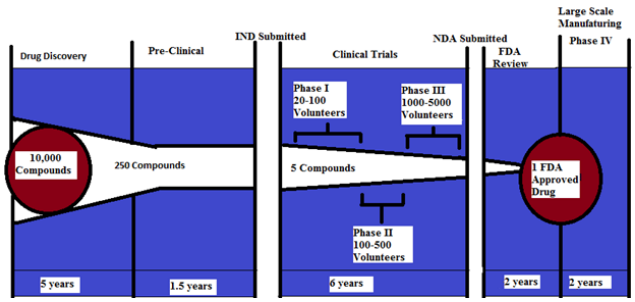


Figure 1: Drug discovery and development process [2].

Figure 2: Steps involved in doing a pre-clinical trial.

to produce a different compound. These steps are often enabled by enzymes that are specific for that step. Drug development can be aimed at blocking or enhancing the enzyme or directly reacting the drug with the compounds in the pathway. Any step in the pathway, for example from A to B, or B to C, might be a target for the right drug.

Step two: Develop a Bioassay (Figure 3). A Bioassay is a "live" system that can be used to measure drug effect. It may be a culture of cells or organs or a whole animal. For example: Zebrafish embryos-effects of drugs on bone density, blood vessel growth and many other systems of the zebra-fish can be seen.

Step three: Screen the drug in the Bioassay. This is the actual test of the drug on the chosen bioassay. This will determine if the drug is SAFE and if it is effective in the bioassay (before it is ever tested on humans!)

Step four: Establish what dosage amount of the drug is safe and what dosage amount of the drug is toxic. Most drugs have a toxic level or an amount at which the drug will become harmful instead of helpful.

Step five: Application is made to the Food and Drug Administration (FDA) as an Investigational New Drug (IND) (Figure 4). Since Zebrafish can be used at multiple stages in the drug discovery, they offer an alternative to rodent assays and also the potential to reduce time and costs in the process. Furthermore, they offer the potential to obtain *in vivo* data on both efficacy and safety at the earliest possible opportunity. Study demonstrates that the good correlation exists between Zebrafish and rodent data, and therefore the potential of the Zebrafish model to replace that of the rodent. Also, the ability to perform safety assessments on unlicensed animals allows only the selection of compounds with good safety profiles for further development [4].

Problems in drug discovery and development process

- The pharmaceutical industry is short of new drugs. In the 2nd part of the 20th century, about 50-60 new drugs (NCEs) were approved by the FDA every year. In contrast, in 2002, a historical low of 18 NCEs were approved (in 2001, 24 NCEs, in 2000, 27 NCEs, in 2003, 21 NCEs).

- Conversely, research costs for a new drug are estimated to be in the $1-1.5 Bi. range. Considering all high-profile failures in recent drug discovery, this figure is unlikely to drop substantially.

- 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.

- Mammalian models of absorption, distribution, metabolism, excretion and efficacy are expensive, laborious and consume large quantities of precious compounds.

- Lengthy process: takes 10-15 years to develop

- Also, results are unguaranteed.

- The current processes by which drugs are discovered are long and expensive.

Many compounds still fall out of the discovery pipeline due to lack of efficacy and mechanism-based toxicity. Central to these reasons is a failure to understand properly all of the biological roles of potential drug targets in normal and disease processes. This knowledge failure results in ignorance of the many potential unpleasant consequences that could be rendered by compound modulation of the target's activity *in vivo* [5].

Pharmacogenomics and clinical trials

Pharmacogenomics in clinical trials is a relatively new area in which considerable hesitation is shown by pharmaceutical companies. Incorporation of pharmacogenomic testing with clinical trials has multiple advantages. The two most important concerns for new drug development are efficacy and safety. Before the advent of pharmacogenetic tools, the predictability of both these factors was very low. This translated into heavy financial loss due to attrition of the drug compound during clinical trials. In current times, the scenario has changed and with the availability of sophisticated pharmacogenetic tools, the attrition rate can be significantly reduced. This translates into reduction in loss of financial resources for drug development. With*in vitro* methods, it can be identified during preclinical studies, if the drug is metabolized by polymorphic enzymes, and a decision regarding continuation of the trial can be made. Also, this information can help in selecting appropriate patients with normal metabolizing enzymes in phase I clinical trial; it can also help prevent adverse events. It must be noted that pharmacogenetic principles can be used for inclusion or exclusion criteria only when the metabolic pathway of the drug is known. In cases of exploratory studies, where knowledge regarding the metabolism of the drug is not known, pharmacogenetic principles cannot be applied for selection of subjects in the early phases of studies. However, the acquisition of pharmacogenetic data in the early phases of clinical trial can be useful for the later phases.

The FDA has made submission of a complete pharmacogenetic data report mandatory, if these results have been used for decision making

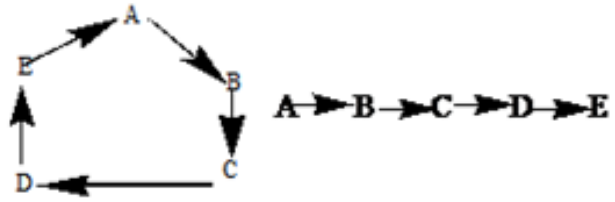


Figure 3: Step two-develop a bioassay.

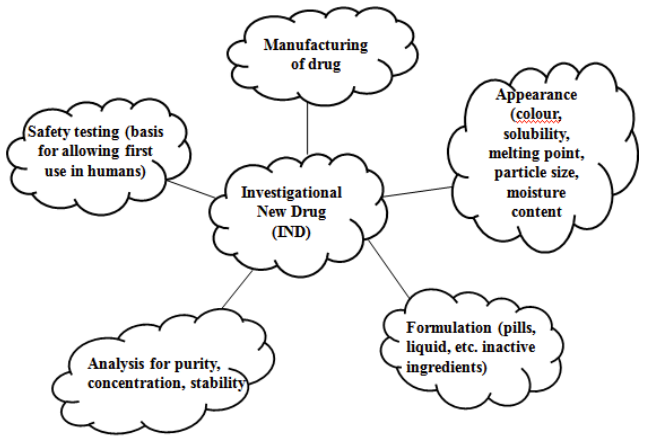


Figure 4: Drug characteristics shown by IND [3].

in the animal study, to support the safety of the drug, or in clinical trials, for the selection of subjects, dose range or its modification. The complete data is also required in cases where the sponsor uses the pharmacogenetic test results to validate safety, efficacy, dosage selection and mechanism of action in the clinical trials. However, in cases where such pharmacogenetic test results are not being used by the sponsor to support the results of the trial, but the test is a valid biomarker for that drug, an abbreviated report of the pharmacogenetic test has to be submitted to the FDA. In cases where the pharmacogenetic testing has been done as an exploratory study or for research, it is not mandatory to submit such data, as they cannot be considered as valid biomarkers. However, the FDA encourages voluntary submission of such exploratory pharmacogenetic test data. In future, as more information becomes available, exploratory pharmacogenetic test data will also need to be submitted to the FDA.

Pharmacogenomics: the application of genome science (genomics) to the study of human variability in drug response. Pharmacogenomics can be used to improve drug discovery and drug development in at least two ways: development of new drugs to overcome drug resistance or target new drug targets, and optimization of drug metabolism and pharmacokinetics (DMPK) to minimize variations in drug levels. Genotype: Genotype refers to the genetic makeup of a cell. For each individual trait (such as hair or eye color), a cell contains instructions on two alleles which are alternative forms of the gene obtained from the mother and the father.

Pharmacogenomics research is based on the 'genotype to phenotype' principle, i.e., correlation of the genetic information and clinical information. Thus it finds practical applications in:

- Understanding and Validating drug target/metabolic pathways.
- Identification of optimal dosage.
- Improving drug safety and understanding adverse side effects.
- Identification of patients benefiting from personalized medicine.

Applications of pharmacogenomics across the drug development process

Phenotype: Phenotype refers to a trait that can be observed, such as morphology or behavior (Figure 5).

Animal models in drug discovery: Traditionally, the pipeline for preclinical drug discovery includes a first step of in silico and biochemical affinity assays, which aim is to sort out drugs regarding their binding to target molecules. This step is followed by cell culture assays designed to address how efficient are these molecules when confronted to the target biological process, i.e., angiogenesis, inflammation, etc. Both procedures help to reduce the number of initial molecules based fundamentally on their possible biological function. In a later stage, it is essential to use mammalian models, primarily rodents, to fully understand the properties of new drugs and avoid any possible adverse effects on humans. However, this conventional pipeline has certain disadvantages: first, some molecules brought forward from the *in vitro* stage have serious toxicity effects when administered to mammals; and second, by focusing only on the affinity of the screened drugs to target molecules, other compounds with interesting properties might get discarded. Thus, it becomes clear the need for new model organisms such *as D. melanogaster, C. elegans or D. rerio* in the preclinical pipeline to fill the gap between *in vitro* assays and expensive screenings using mammals.

Biomedical research depends on the use of animal models to understand the pathogenesis of human disease at a cellular and molecular level and to provide systems for developing and testing new therapies. Mammalian models, such as the mouse, have been pre-eminent in modelling human diseases, primarily because of the striking homology between mammalian genomes and the many similarities in aspects spanning from anatomy to cell biology and physiology. However, a range of factors must be considered in addition to evolutionary proximity and anatomical similarity when selecting an animal disease model (Table 1) [6-8]. High reproduction rate, low maintenance cost and embryo development outside the mother's body are some of the Zebrafish's advantages.

For example, larger mammals such as rats or sheep can have physiologies and organ sizes that are more similar to humans, which are advantages when developing surgical therapeutic interventions. On the other hand, the surprising degree of functional conservation in basic cell biological processes between mammals and invertebrates suggests that diseases that result from the disruption of these conserved cellular processes can be accurately modelled at a genetic level. Strategies that are available in invertebrate systems have been extraordinarily successful in molecular level in flies and worms. In this regard, the large-scale 'forward-genetic' mutagenic determining gene functions, providing considerable insight into how orthologous human disease genes function in similar processes. Despite these advantages, invertebrates lack many structures and organ systems that are involved in human disease pathogenesis and their role in modeling human disease will therefore be limited. Conversely, although forward-genetic

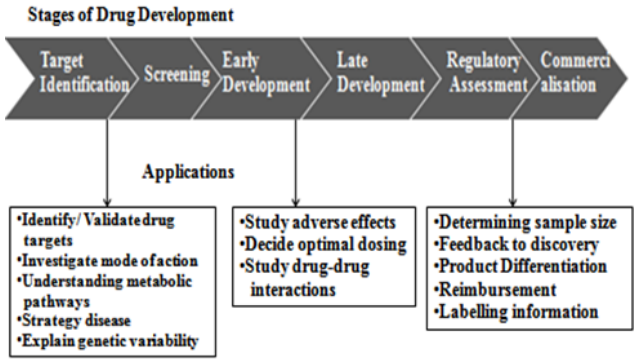


Figure 5: Applications of pharmacogenomics in the drug development process [6].

screens and random mutagenesis-based reverse genetics are feasible in the mouse and are currently underway, they cannot be done on a scale that is possible in invertebrates because they require considerable staff and infrastructure support. Hence, such approaches in mice are limited to a few large projects, often operating as screening consortia. In this context, the Zebrafish (*Danio rerio*) has come to attention recently as a genetically tractable vertebrate model system. As early as the 1930s, the Zebrafish was being used as a classical developmental and embryological model. Early studies drew on the unique combination of the optical clarity of the embryos and larvae (allowing the *in vivo* visualization of cell-biological events) and embryological manipulability to make several important observations.

Pathway conservation between humans and fish

A common ancestor between humans and Zebrafish lived roughly 400 million years ago, which at times has raised the question of whether the similarities between the two species are outnumbered by the differences (Figure 6).

This is a question of particular relevance to those who use Zebrafish as an entry point to learn about vertebrate physiology and human disease, but has less relevance to those who study fish development and biology in their own right. There are a number of themes surrounding the issue of conserved function between fish and humans. In Zebrafish and other teleosts one finds, in 20-30% of cases, two homologous genes compared with the mammalian counterpart. Apparently, this stems from partial genome duplication or duplication of the entire genome with subsequent loss of much of the duplicated material. The resulting paralogs vary in function and expression pattern, which can complicate the comparison with mammalian equivalents. Eighty percent of the Zebrafish and human genomes appear to be syntenic, which is very helpful in determining homology relationships in cases where members of the same protein family are to be compared. A reasonably precise assessment of the exact extent of genome duplication will have to await completion of the Zebrafish genome sequencing and annotation effort, which is expected to be finished in 2005.

A seemingly attractive way to address the question of conserved gene function is to compare fish mutants in a particular gene with mouse mutants in the corresponding gene. At present, there are roughly 150 Zebrafish mutants that have been cloned but this number is not nearly high enough to allow a meaningful comparison. Only about half of these mutants exhibit a well-described phenotype and there is not a mouse mutant counterpart for all of them [9].

Zebrafish and preclinical drug discovery

The modern drug discovery process can be divided into four major components: screening of lead compounds, target identification, target validation and assay development. Target identification describes to the process of identifying gene or protein (target gene product) that, when modulated by a drug, can have a positive impact on disease state progression. Once a possible target is identified which is very promising, the target validation process begins by determination of protein function and assessment of the 'druggability' of the target.

Furthermore, validated targets for their ability can be tested along with the small molecule compounds to modulate the function of the protein. Compound screening can also be useful in disease models when the target is unknown. The Zebrafish has the importance in each of these areas of drug discovery (Figure 7).

Drug toxicity study: Toxicity plays a major role in drug development. The several new molecular entities submitted to the FDA for the approval of new drugs. But FDA has declined about half of the molecules due to their toxicity problem. In a statement, the FDA point out to technological difficulties in toxicology as one of the principal causes of this 'pipeline problem'. Drug toxicity can result from the inhibition or activation of a therapeutic target by a drug or from an interaction between a drug and a target protein different from the therapeutic target of the drug. In the former case, "on-target" toxicity, such as excessive bleeding from high doses of warfarin, is observed; in the latter case, "off-target" toxicity, such as statin-induced myopathy, takes place. All genetic factors that influence drug response-drug targets, drug-metabolizing enzymes, drug transporters, and genes indirectly affecting drug action can modulate drug toxicity and contribute to its individual variability. However, new animal models are needed to test the safety of novel drug candidates. The FDA reports that an estimated 10% improvement in predicting failures before clinical trials would save US $100 million per drug in development costs. To evaluate the toxicity of a drug, it is essential to identify the endpoints of toxicity and the dose-response relationships, elucidate the mechanisms of toxicity, and determine the toxicodynamics of the drug. In addition to outdated technologies, toxicology frequently suffers by being divorced from the drug discovery process-efforts to discover leads and improve their potency often occur independently from the assessment of toxicity. Some efforts are being made to involve toxicology earlier in the drug discovery process, such as eliminating compounds with problematic chemical moieties from screening libraries or prioritizing leads on the basis of performance *in vitro* toxicity assays. However, much more progress is needed to develop better animal models for toxicological assessment and to involve toxicology earlier in the drug discovery process. The Zebrafish is rapidly gaining acceptance as a promising animal model for toxicology. The ability to efficiently assess the toxicity of a large number of compounds enables whole libraries to be prescreened for potential toxicity. In this way, compounds with obvious toxicity can be eliminated from libraries before HTS. Alternatively, preliminary hits from HTS can all be tested for toxicity as a means of prioritizing compounds for further development. Significantly, toxicological studies in Zebrafish also require compound quantities only in the microgram or milligram ranges, whereas mammalian assays frequently require a few grams to several hundred grams of a given compound. Most Zebrafish toxicity studies so far have focused on environmental contaminants, including pesticides. Zebrafish is not as well established as a model for drug toxicology, and questions remain about how relevant fish toxicity is to humans. Nevertheless, studies have begun to show that many toxic responses are well conserved

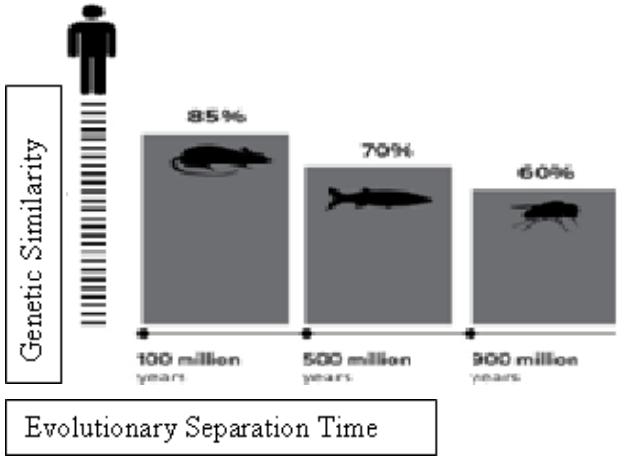


Figure 6: Comparison with human beings.

between fish and mammals. Toxic-response similarities between Zebrafish and mammals have been noted for small molecules that cause endocrine disruption, reproductive toxicity, behavioural defects, teratogenesis, carcinogenesis, cardiotoxicity, ototoxicity liver toxicity and so on. Several Zebrafish assays have been developed specifically to monitor toxicities of significance to drug development. For example, Amanuma et al. developed a sensitive Zebrafish assay for detecting small-molecule-induced mutagenesis. To examine significant toxicities to drug development, some Zebrafish assays have been developed specifically [10-16].

Zebrafish embryos were utilized to compare the developmental toxicity resulting from either ethanol or acetaldehyde exposure. Toxicity of diclofenac, anti-rheumatic drug, was evaluated by using Zebrafish model [17,18].

3R'S implications of use of the Zebrafish for disease modelling and drug discovery

Additionally, the Zebrafish has been considered as an alternative model organism for disease modeling and drug discovery and has further been applied for the reduce, refine, replace (3R) concept.

Replacement: The ASPA regulates the use of vertebrates in scientific procedures which may cause pain, suffering, distress or lasting harm. A licence 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 d.p.f. 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.

The evolutionary strategy of Zebrafish is to develop extremely rapidly to attain an adult-like stage within 72 hours, in order to be able to escape predation. Thus, by 4 d.p.f. they are already able to see and to swim to escape predation. As a consequence, at unlicensed stages, non-neuronal organs such as the heart are well developed, allowing for functional assessments to be performed (as described below), but the central nervous system (CNS) remains relatively primitive. Indeed, complex behaviours such as responses to visual and auditory stimuli are only apparent from 5 d.p.f. onwards, suggesting that higher brain development is delayed relative to that of other organs. Thus, Zebrafish can be used at unlicensed stages to generate *in vivo* data in certain organs as a replacement for use of mammals at more sentient, licensed stages.

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: Large numbers of animals are used in the drug discovery process as compounds identified as having activity *in vitro* are subsequently tested in animal models. There is often a failure to reproduce *in vitro* results *in vivo* due to problems with absorption, distribution, metabolism and excretion (ADME) in the whole organism that cannot be predicted in cell-based models. 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 [19].

Zebrafish embryo in drug screening

After scaling up, it is possible, in principle, to reach high-throughput (1,000-10,000 assays per day; or even ultra-high throughput (100,000 assays per day; Dove 1999). Such large numbers of replicates increase the reliability of the statistics and allow rare (idiosyncratic) responses to be identified. Rare responses are most readily detected using 'wild type' (pet shop) Zebrafish with high genetic variability. Several Zebrafish-embryo assays can help to predict drug safety in humans, and therefore Zebrafish disease models have been developed.

Zebrafish embryos and early larvae can serve as invaluable screening tools in the pre-regulatory, preclinical phase of drug discovery. They can be used as kind of filter that reduces the number of compounds passing through to testing on the much more expensive rodent models. The Zebrafish can never replace rodents in the later phases of drug discovery, but may be complementary to rodent or cell-based assays at earlier stages [20].

There are two main categories of genetic screens, forward genetic screens and reverse genetic screens. In a forward genetic screen, you would mutagenize Zebrafish and then screen fish carrying the mutations for defects in eye development. In a reverse genetic screen, you might start with a gene that you know is expressed in the eye, cause a reduction or absence in the expression of this gene, and then see if the resulting fish have anything wrong in their eyes.

Rationale for small molecule screens in zebrafish

The strengths and limitations of genetic screens: Of all the virtues of the Zebrafish as a model organism, its suitability for large scale screening is paramount. In no other vertebrate has it been possible to screen for mutations so readily and on such a scale as has been achieved using Zebrafish. The earliest genetic screens captured the imaginations of many as wondrous mutant phenotypes were discovered, from the dramatically disrupted to the dramatically subtle. In many cases, these mutants have allowed connections to be drawn between specific genes and their functions, especially for early developmental processes and organogenesis. Finally, the suppressor and enhancer screens that are valuable tools for identifying upstream and downstream components of genetic pathways in *Drosophila melanogaster, Caenorhabditis elegans* and *Saccharomyces cerevisiae* have not been practical in the Zebrafish.

Advantages of small molecule screens

Many of the limitations of traditional genetic screens outlined

above can be overcome when genetic screens are complemented with small molecule screens. In fact, Drosophila and *C. elegans* are well suited for genetic screening but are not as tractable for small molecule screening because of difficulties in access of small molecules to tissues in these organisms. Zebrafish are amenable to both genetic and small molecule screening, and the ability to combine these approaches in Zebrafish is particularly promising. Like genetic mutations, small molecules are a classical means of disrupting biological processes and serve to link genes or gene products with their molecular functions. This approach has different strengths and weaknesses from the genetic one, so chemical and genetic screens are complementary (Figure 2). For example, small molecules are excellent conditional biological probes, can overcome gene redundancy, and facilitate suppressor and enhancer screens as described below. Furthermore, small molecule screens are generally simpler than are genetic screens.

Conditionality: Most Zebrafish mutations identified to date are non-conditional and have fixed allele strength. In an effort to overcome these limitations, some screens for temperature sensitive alleles have been performed (Johnson and Weston, 1995), but conditional mutants remain the exception. In contrast, small molecules are the ultimate conditional disruptors, allowing both the timing and dosage of pathway disruption to be regulated.

Redundancy: Zebrafish chemical screens have identified many phenotypes that are similar to those previously identified using genetic screens, but some of the small molecule-induced phenotypes are unlike any identified by genetic screening. One potential explanation for this expansion of phenotypes is functional redundancy in the Zebrafish genome. When multiple isoforms of a protein play overlapping roles in a biological process, mutation of one isoform may be insufficient to cause an observable phenotype. In contrast, a small molecule may bind to and inhibit multiple isoforms simultaneously, and thereby reveal the importance of those proteins in the biological process.

Suppressors/enhancers: No genetic suppressor or enhancer screen has ever been reported in a vertebrate. Small molecule screens make it possible to identify suppressors and enhancers of existing mutations as described in section III.

Ease: One final advantage of chemical screens is that they are much easier to perform than genetic screens. Whereas to reach any degree of saturation, genetic screens conventionally require large Zebrafish facilities for the maintenance of thousands of Zebrafish strains and lines, chemical screens typically require at most a few Zebrafish lines. And, while the mutagenized fish used for genetic screens are often less fertile, the fish used for chemical screens can be selected in part for fertility.

The potential for zebrafish - based drug discovery

In addition to their utility for dissection of essential biological processes, Zebrafish small molecule screens may be useful for discovering novel therapeutic compounds and drug targets. By modeling human diseases in Zebrafish, it may be possible to screen directly for compounds that modify the disease phenotype. Compounds that ameliorate the disease phenotype may serve as lead compounds for drug development, and identification of the compound's protein binding partner may effectively identify novel drug targets for traditional drug discovery efforts.

Many Zebrafish models of human diseases have already been developed and are reviewed elsewhere. The majority of these are single gene mutations that cause Zebrafish phenotypes reminiscent of some aspect of human disease. In a number of cases where the genes underlying the human and Zebrafish disease are known, orthologous genes are responsible for both conditions. Recently, it has become possible to identify mutations in virtually any Zebrafish gene by target-selected resequencing or to 'knock down' the function of a gene using antisense morpholino oligonucleotides. Therefore, it should be possible to generate Zebrafish models for many of the human diseases resulting from a known single-gene mutation. Therapies for many of these human diseases have not been developed because of the difficulty in predicting a priori which proteins should be targeted to reverse the disease phenotype. Significantly, unbiased screening in Zebrafish may allow discovery of compounds that reverse the disease, even without knowing what protein is being targeted.

In addition to diseases caused by genetic mutation, it may be possible to discover novel drugs for treating infectious diseases. Several Zebrafish models of infection have been developed, including models of tuberculosis and Salmonella typhimurium infection. Screening in Zebrafish may allow assays to be performed on microbes that cannot be cultured outside of a whole organism. And by screening in the context of a whole organism, it should be possible to identify compounds with antimicrobial activities that have no undue toxicity to the host. Two of the infection models developed thus far use fluorescently-labeled microbes for infection, so the efficacy of a small molecule could be measured by quantitating the number of pathogens or by assessing survival of the host.

Will small molecules that reverse a disease phenotype in Zebrafish have similar effects in humans? While that question has not been answered, it is clear that many drugs with known effects in humans cause analogous effects in Zebrafish. For example, Milan et al. treated Zebrafish with 23 drugs known in humans to lengthen the QT interval on the electrocardiogram, often a harbinger of arrhythmogenesis, and an undesirable drug side-effect. Of the 23 drugs, 22 also caused an analogous prolongation of the cardiac cycle in Zebrafish. Other drugs that have similar effects in humans and fish include angiogenesis inhibitors, vasodilators, opiates, cholesterol synthesis blockers and anticoagulants. Therefore, tissue access, drug binding sites, and pharmacodynamic effects seem to be generally well conserved between Zebrafish and humans [21,22].

Target validation- A door to drug discovery: An animal model is described as valid if it "resembles the human condition in aetiology, pathophysiology, symptomatology and response to therapeutic interventions".

Validity is broken down into three aspects: predictive validity (performance in the test predicts performance in the modelled condition), face validity (phenomenological analogy with the modelled condition) and construct validity (the model has a sound theoretical rationale) [23]. However, when the results of an animal study are intended to be translated into human treatments (preclinical research), the ultimate proof of a model's value is its predictive validity.

The validation of animal models potentially carries monetary as well as ethical costs. Validation is time consuming (2-6 years for the alternative methods), costly, and financial returns may be more difficult to secure, since intellectual property rights over animal models are more restricted than they are for alternative methods. Ethical concerns may also arise over the use of animals for the sole purpose of validation.

Process of validation - the alternative methods approach: The predictive validity of an animal model can be tested by systematic examination of the data from animal model studies, and by comparing

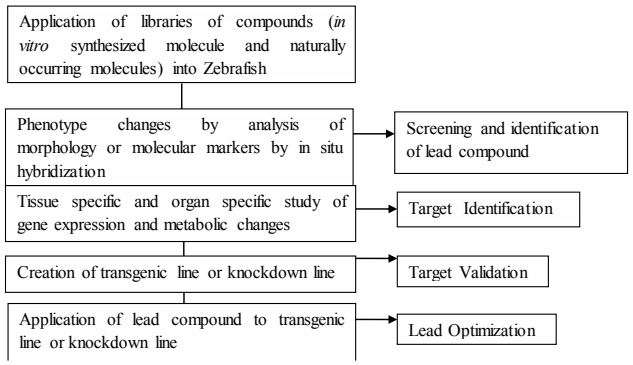


Figure 7: A flowchart that describes the Zebrafish has the potentiality in each of the areas of drug discovery [8].

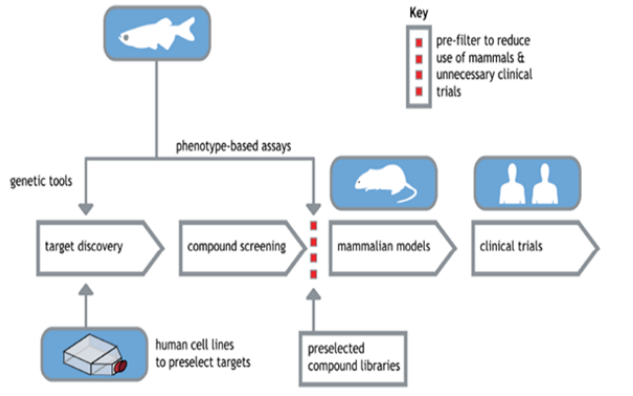


Figure 8: Drug discovery pipeline involving novel Zebrafish models.

these data with reference data obtained in humans. One way of doing this would be to follow the validation process for alternative methods. The process described here is used by the European Centre for the Validation of Alternative Methods (ECVAM); a similar system has been adopted by OECD and North American organizations, which have harmonized their validation processes.

This process has five basic steps. The first is test development. The fifth is formal regulatory acceptance. Actual validation, in the sense of generating, analyzing and assessing data, takes place in steps two, three and four:

Pre-validation: An inter-laboratory pre-validation study is conducted to optimize the protocol and assess its performance over three phases: phase I, where the protocol is refined in a single laboratory; phase II, assessing the transferability of the method to a second laboratory; and phase III, where the relevance and reliability of the test are assessed under blind conditions in two or more laboratories.

Validation: The formal validation study can be thought of as an extended version of the phase III stage of pre-validation in which an inter-laboratory blind trial (involving at least three laboratories) is conducted to assess whether tests can be shown to be relevant and reliable for one or more specific purposes. This inter-laboratory trial is followed by data analysis and an evaluation of the outcome of the study in comparison with predefined performance criteria.

Validation has two principal aspects: how well a test method compares with itself when repeated under identical as well as different conditions (e.g., with and how well a test method compares with a reference method. These two aspects present somewhat different challenges in terms of data required, but there is no theoretical obstacle to their application to animal models in biomedical research.

Rational for validation of model: An exciting paradigm for drug discovery is evolving. The integration of model systems into the drug discovery process, the speed of the tools and the amount of *in vivo* validation data that these models can provide will clearly help to define better the disease biology and thereby result in better validated targets. Better targets will lead to high efficacy and less toxic therapeutic compounds. The future will see a merging of the genetics of model systems with proteomics, bioinformatics, structural biology and compound screening, creating the exciting new framework of drug discovery for the 21st century [24].

In vitro assays typically rely on simple interactions of chemicals with a drug target, such as receptor binding or enzyme activity inhibition. However, *in vitro* results often poorly correlate with *in vivo* results because the complicated physiological environment is absent in the *in vitro* testing system. Although cell-based assays can provide some information, cultured cells still do not provide physiological conditions and complex interactions among different cell types and tissues.

Moreover, cell lines are usually transformed, exhibiting different gene expression and cell cycle profiles than those of cells in the living organism. There is a growing trend of using human tissues for drug discovery research. Tissues, however, only provide an isolated *ex vivo* condition, which is not completely representative of *in vivo* response because drug action often involves metabolism and interplay among different tissues. For example, the effects of a drug on muscle may involve absorption by the intestine and metabolism by the liver. Therefore, results in animal studies are essential to validate HTS (high-throughput screening) hits and exclude compounds with unfavorable ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties, which are responsible for more than half of compound attrition in costly clinical trials. Zebrafish is the only vertebrate species for which large scale forward genetic screens have been carried out, and many mutants obtained from these genetic screens display phenotypes that mimic human disorders, including cardiovascular disease, neurodegeneration, cancer, and blood disease. These mutants not only identify genes that may be involved in diseases but also can be used for drug screening [25] (Figure 8).

This schematic illustrates a potential drug discovery pipeline showing the incorporation of novel approaches using cell-based and Zebrafish assays into target discovery and zebrafish behaviour- based assays into compound screen.

The Zebrafish model will never replace mammalian models in the drug development pipeline, particularly at later stages when the regulatory authorities demand studies in mammalians and clinical trials. Rather, Zebrafish model can serve as an invaluable screening tool in the pre-clinical phase, before rodent models, in the drug pipeline. Zebrafish (*Danio rerio*) is the best described and most popular vertebrate model species in developmental genetics and ecotoxicology. Zebrafish is a highly valid model for studying gene function and drug effects in humans. 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).

Advantages

The Zebrafish (*Danio rerio*) is small, cheap to keep, fast to develop and has high fecundity. Its early-stages embryos have a transparent body, making it relatively easy to collect numerous data points using high-quality imaging (including the fluorescent imaging of transgenic lines) [25].

Annual maintenance costs for adult Zebrafish are somewhat lower than those for rodents. However, this cost advantage is hugely multiplied when the test animal is a Zebrafish embryo, because a female Zebrafish can lay as many as 10,000 eggs per annum. Zebrafish embryo may be able to address the unmet need in biomedical research for low cost, high-throughput whole-animal assays and models. *In vitro* assays offer the advantages of low cost, of being less prone to legal and ethical restrictions and of having the ability to be scaled-up. By contrast, whole-animal assays provide data that are more easily extrapolated to humans and allow complex organismal functions (e.g., behavior and development) to be studied. Compounds on various organs, including the heart, brain, intestine, pancreas, cartilage, liver, and kidney, were observed in the transparent animals without complicated processing, demonstrating the efficiency of toxicity assays using Zebrafish embryos [26].

Application

High throughput screening: High-throughput screening (HTS) is the process of testing a large number of diverse chemical structures against disease targets to identify 'hits'. Compared to traditional drug screening methods, HTS is characterized by its simplicity, rapidness, low cost, and high efficiency, taking the ligand-target interactions as the principle, as well as leading to a higher information harvest. As a multidisciplinary field, HTS involves an automated operation-platform, highly sensitive testing system, specific screening model (*in vitro*), an abundant components library, and a data acquisition and processing system. Various technologies, especially the novel technologies such as fluorescence, nuclear-magnetic resonance, affinity chromatography and DNA microarray, are now available, and the screening of more than 100,000 samples per day is already possible [27]. HTS-based *in vitro* drug screening assays are widely applied to pharmaceutical companies because of the increasing number and diversity of compounds made available by rapid synthesis techniques such as combinatorial chemistry. However, validating these *in vivo* preliminary hits made by *in vitro* drug screening by mammalian animal models is slow and costly, resulting in a gap in the drug development process. The Zebrafish is a vertebrate model organism that holds a great potential to bridge this gap. In fact, Zebrafish represent as one of the most ideal animal models for *in vivo* high-throughput screening [28] (Figure 9).

HTS uses some well-designed models or assays to screen large quantity of compounds in relatively short time. In assays, the activities of compounds are visualized: images (in Forward CG) or fluorescent Signals (in Reverse CG).

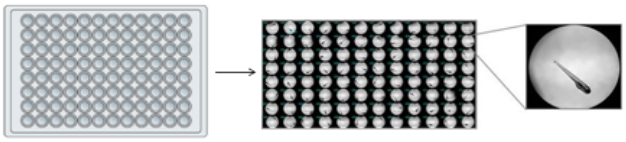
The transparency of the embryo, external development, and the many hundreds of mutant and transgenic lines available add to the allure. Now it appears, Zebrafish can be used for high-throughput screening (HTS) of drug libraries in the discovery process of promising new therapeutics. High-content screening (HCS) has been available for cell-based screens for some time and, very recently, HCS is being adapted for the Zebrafish. This will allow analysis, at high resolution, of drug effects on whole vertebrates; thus, whole body effects as well as those on specific organs and tissues may be determined [29].

The technical advantages of using an animal model such as Zebrafish for drug screening are numerous: (i) drugs can be administered directly in the swimming water. This feature has two main advantages: it is quicker and easier than injecting drugs into mice and it could eventually help to determine how a molecule behaves in terms of ADME (Absorption, Distribution, Metabolism and Excretion) when exposed to a whole living animal. But it has also two caveats: first, some molecules are not water-soluble and this might have a direct impact in the stability and amount of absorbed drug by the treated embryos. To enhance solubility, compounds are first dissolved in organic solvents or carriers such as DMSO, methanol, acetate or cyclodextrin. Second, a universal ADME profiling has proven difficult to perform in Zebrafish embryos; however, advances in the detection of radio and fluoro-labeled molecules, combined with organ and cell sorting procedures, plus a higher knowledge on Zebrafish drug metabolism might contribute to reach this important goal. (ii) The prolific egg laying and small size of Zebrafish embryos allow the parallel and reproducible testing of several drugs and dosages in simple multiwell plates. (iii) Zebrafish has a high genetic conservation with higher vertebrates, also analogous organs (heart, liver, pancreas, brain) and many important aspects of human physiological processes; however, it is important to notice that some organs with importance in cancer studies such as lung, prostate or breast are absent. But clearly, Zebrafish shares with mammals most of the molecular mechanisms governing embryonic development.(iv) Zebrafish embryos are transparent, which combined with a growing battery of fluorescent tissue specific transgenic lines, and novel advances in imaging capture and analysis, allow the visualization and analysis *in vivo* of the effects of drugs in groups of cells or whole tissues. Some of the advantages of using Zebrafish embryos (small size, high number of progeny, easy drug administration or high-throughput analysis) are comparable to the benefits of using invertebrates. However, Zebrafish is a vertebrate, making it a more suitable candidate to fill the gap between "easy, but incomplete" *in vitro*/in silico screenings and "necessary, but costly and time consuming" mammalian drug screens. Nevertheless, given the complexity of the Zebrafish genome, compared with the more compact and simpler invertebrate genomes, the ideal would be to use a mix of both invertebrates and Zebrafish in the drug-screening pipeline before entering studies with mammals [30,31].

Future with zebrafish: The pharmaceutical industry has gone through a significant amount of R&D restructuring as a result of mergers and the desire to increase R&D productivity. Outsourcing early-stage discovery is a growing trend, Danio is considered to be part of that outsourcing industry. Zebrafish transgene technology will benefit pharmaceutical clients by increasing the accuracy and efficiency of their nuclear receptor drug screening programs, reducing the time and cost of discovery work and increasing the hit-to-lead success rate, which involves moving the best drug candidates from screening to preclinical development. The end result will be safer and more effective medicines being brought to market sooner (Figure 10).

Conclusion

New model organisms such as *D. melanogaster, C. elegans* or *D. rerio* in the preclinical pipeline to fill the gap between *in vitro* assays and expensive screenings using mammals. Zebrafish embryos have been proposed as an *in vitro* animal model which could bridge the gap between simple assays based on cell or tissue culture, and biological validation in whole animals such as rodents. It is not only that Zebrafish be used as a replacement to rodent/mammalian models in numerous



96 -well plate 384- well plate

Figure 9: Images (in Forward CG) or fluorescent signals (in Reverse CG).

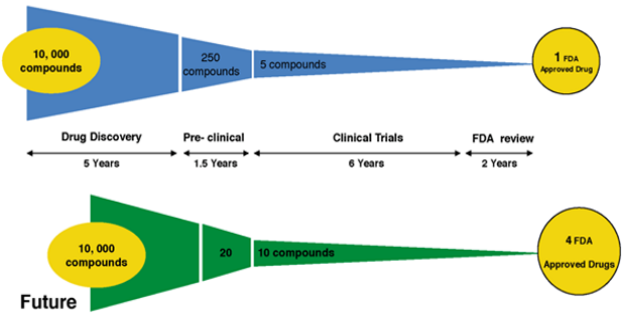


Figure 10: Using an *in vivo* screening platform earlier in the discovery process can save time and money, and increase the clinical success rate.

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. Zebrafish are amenable to both genetic and small molecule screening. It is not the perfect model system for humans in each and every single case investigated. But, it is perfect model system if one considers individual cases (or genes), where it turns out that the genetic pathways between Zebrafish and mammals have been conserved and the function of genes within those pathways has not changed. Examples of this are plentiful and, as long as one is willing to 'embrace the differences and cherish the similarities' between Zebrafish and humans, Zebrafish offer a powerful experimental and genetic system for the understanding of vertebrate biology and disease.

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