The Zebrafish: A Powerful Platform for *In Vivo*, HTS Drug Discovery

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ABSTRACT

The zebrafish (Danio rerio) 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. This review will begin with a discussion on the background, suitability, and advantages of this vertebrate model system and then, citing specific examples, will describe the utility of zebrafish at specific stages in the drug development pipeline. We will end with a synopsis of recent drug screens based on morphological disruptions, genetic disease models, fluorescent markers, behavioral changes, and specific targets. The numerous advantages of this whole animal approach provide new promise for the discovery of safe, specific, and powerful new drugs.

INTRODUCTION

With the advent of high-throughput screening (HTS), the excitement and anticipation of strong pipelines for new pharmaceuticals was high. However, despite the explosion of new leads, the number of marketable drugs produced was relatively small, and in recent years these numbers have dwindled further.[1] Although HTS *in vitro* has scale and throughput advantages, and *in cellulo* screening provides necessary cofactor requirements, mimics physiological conditions of a particular cell type, and selects for membrane permeability, the overwhelming majority of leads obtained by these approaches fail in humans. These failures are due primarily to issues such as metabolic inactivation, failure to reach target tissues, and off-target toxicity.[2,3] A paradigm shift in the way new and safe compounds are discovered is thus warranted, and the diminutive zebrafish may lead the way.[4]

The rapid development and transparency of zebrafish embryos has already made them an ideal model for the study of vertebrate-specific developmental processes.[5] Since zebrafish are also amenable to genetic screens, a plethora of data has emerged concerning the roles of specific genes, most of which function similarly in human development.[6] Many of these genetic perturbations have also shown tremendous utility for modeling human diseases such as cancer,[7,8] renal disorders,[9] cardiovascular disease,[10] hearing loss,[11] blood disorders,[12] muscular dystrophies,[13] as well as neurological diseases such as Alzheimer's and Huntington's,[14,15] among others.

Although whole-animal small molecule screens are not a novelty, with numerous screens having been performed in simple invertebrate model organisms such as *Drosophila melanogaster*[16] and *Caenorhabditis elegans*,[17] the use of zebrafish for drug identification is growing at a rapid rate.[18] Given its much closer genetic, morphological, and physiological relationship to humans, it is becoming the model of choice to discover and assess new potential leads.[19]

In this review, we discuss the zebrafish model, including technical considerations along with both the advantages and limitations of the system as a drug discovery tool. We will focus on how the zebrafish model fits into the drug discovery pipeline with some highlighted examples of recent zebrafish drug screening studies and platforms. For a summary of studies not mentioned here, such as reviews on genetic screens performed in zebrafish, the reader is directed to several articles.[20–23]

THE ZEBRAFISH MODEL SYSTEM

Traditionally used in research laboratories to study development and embryogenesis, the zebrafish is now emerging as an important drug discovery tool with the potential to alter the way new blockbuster pharmaceuticals are discovered. Although zebrafish diverged from humans ~450 million years ago,[24] genome sequencing projects have shown that the synteny and sequence similarity between zebrafish and human genes is very high.[25] Along with the many advantages of the model, there also exist several disadvantages that must be considered when establishing hypotheses and designing screens. These are summarized in *Table 1* and elaborated upon in several of the sections that follow.[26–30]

As alluded to in Table 1, there are some differences between zebrafish and human physiology that are important to consider when designing screens or interpreting data. That said, the large number of compounds active in both zebrafish and humans is impressive. Some examples include steroids,[31–33] statins,[34] compounds that affect heart rate,[35] and compounds that affect angiogenesis.[36] Also, just because certain organs or tissues may differ between fish and humans does not necessarily mean that drugs that affect them cannot be discovered or tested in fish. For example, a drug useful for treating prostate

ABBREVIATIONS: ADMET, absorption, distribution, metabolism, excretion, and toxicity; BMP, bone morphogenetic protein; dpf, day postfertilization; ED_{50}s, median effective doses; FSH, Flag-Strep-His; GFP, green fluorescent protein; GSK3β, glycogen synthase kinase 3 beta; hpf, hours postfertilization; HTS, high-throughput screening; LT, ligand trap; NR, nuclear receptor; PAS, Per-Arnt-Sim; SAR, structure–activity relationship; TRIAC, triiodothyroacetic acid; TRβ, thyroid receptor beta.

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Table 1. Advantages and Disadvantages of Zebrafish as a Model Organism for High-Throughput Screening Drug Discovery

Advantages	Disadvantages
Ex vivo development and optical clarity of the embryo.	Several mammalian organs are not present in the zebrafish, including breast tissue, lungs, and prostrate. Skin lacks some specific cellular components found in humans.
Embryogenesis is completed by 72 hpf and most organs are fully developed by 96 hpf.	Adult zebrafish are not as suitable for high-throughput screens due to their large size.
Small size of embryos and hatchlings make them amenable for arraying into multiwell plates—zebrafish can survive in as little as 50 μL of water in a 384-well plate.	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.[30]
The cost to maintain zebrafish is a fraction of that of rodents or other mammalian organisms (1 cent per fish versus 1 dollar per mouse).	Teleost fish possess two copies of many mammalian genes due to an evolutionary gene duplication event.
Sexual maturation is reached in ∼3 months.	
Dimethyl sulfoxide tolerant up to 1%, allowing for direct delivery of drugs to the surrounding water, with subsequent absorption through the skin, gills, and mouth.	
The genome of zebrafish is sequenced. Molecular biology tools are available for genetic manipulation and forward/reverse genetic screens, including morpholinos for translational "knockdown,"[26] transposon-mediated gene insertion,[27] TILLING,[28] and zinc-finger-mediated recombination.[29]	
In vivo screens allow the identification of bioactive compounds with good permeability, uptake, stability, and delivery.	
In vivo screens allow the detection of prodrugs and tissue-selective modifiers.	

hpf, hours postfertilization.

cancer was recently discovered in zebrafish despite the fact that zebrafish do not possess prostates.[37]

MANIPULATION OF ZEBRAFISH EMBRYOS FOR HTS

There are four major methodological steps to consider when undertaking a chemical library screen in zebrafish: (1) adult pair mating and embryo collection, (2) embryo sorting or arraying into multiwell plates, (3) chemical library administration, and (4) data acquisition and analysis. There are several variations to the methods and details described below, and detailed material descriptions can be found in protocol reviews.[38,39] That said, the basic steps are outlined below.

Adult Mating and Egg Collection

Adult zebrafish lines are typically housed in high efficiency rack systems that hold dozens of tanks with interconnected plumbing (described in the *Zebrafish Book*[40]). Mating crosses are set up in the early evening and females begin to lay eggs when the lights are turned on in the morning. A successful mating can yield 200–300 embryos per day per adult pair, which are then collected and incubated at 28°C in embryo media for the desired length of time depending on the assay of interest. To overcome the limitations of pigmentation when observing internal organs in older larvae or fish, the media can be supplemented with the tyrosinase inhibitor 1-phenyl-2-thiourea. Alternatively, transparent *casper* lines developed by White *et al.*, which have no pigmentation,[41] can be crossed with any mutant or transgenic fish of interest.

Embryo Sorting

Embryo sorting is typically done manually using wide-bore pipette tips and arrayed into multiwell plates. Automation of the process can be carried out using the COPAS XL system (Union Biometrica, Holliston, MA), which can array both embryos and hatchlings in 96- and 384-well plates. This system was recently used by Makky *et al.* in a large-scale screen that measured metabolic rate in zebrafish larvae.[42] It has not yet, however, seen widespread use, due presumably to its significant purchase price and the limited throughput required in screens performed thus far. Once deployed into multiwell plates, embryos develop until the desired time point for drug delivery. Food must be provided at and beyond 6 day postfertilization (dpf).

Compound Delivery

Compounds can be delivered manually with multichannel pipettes or with the use of robotic liquid-handling machines that can rap-

idly deliver precise levels of compounds from stock library plates. Chemicals are typically dissolved in dimethyl sulfoxide. Screening concentrations must be determined carefully, but are typically done in the initial range of 1–10 μM. If compound application is to be performed before day 2/3, dechorionation may be necessary to ensure the efficient and uniform absorbance of all compound types. Dechorionation also allows the embryos to be arrayed in a lateral position for more efficient visual analysis. Dechorionation is readily achieved in high throughput using Pronase.[39] After compound delivery, plates are stored at 28°C and incubated for the desired length of time.

Image Acquisition and Data Analysis

Each well of a multiwell plate can be manually scored for phenotype in low-throughput assays. Images are recorded and compounds are then reconfirmed in secondary screens. However, with the use of transgenic reporter fish that express fluorescent proteins, either in a desired tissue or as a reporter of activity for a target protein, the opportunity for automation increases. Fluorescent microscopes with automated stages allow high-throughput examination of zebrafish embryos in microtiter plates. Automated laser cytometers such as the Isocyte device (Molecular Devices, Sunnyvale, CA) can be used similarly. These facilitate whole-well scanning through a relatively large depth (400 μm), conversion of signal to pixels, and pixel quantification. The first such study used transgenic zebrafish embryos expressing green fluorescent protein (GFP) in blood vessels to screen for antiangiogenic compounds.[43] Clearly, there is a great deal of potential here for new pattern recognition programs that can automate the documentation of novel morphological variations. As described below, real-time processes such as heart beat, blood flow, and swim behavior can also be documented by video, and analyzed for typical and atypical patterns. Taken together, the acquisition and analysis of these types of static and dynamic images potentiates drug discovery in a huge variety of processes that could never be screened for *in vitro*, *in cellulo*, or in simpler animal models.

USES FOR ZEBRAFISH IN THE DRUG DISCOVERY PIPELINE

Zebrafish fit into multiple stages of the drug discovery pipeline, from lead and target identification to lead optimization and absorption, distribution, metabolism, excretion, and toxicity (ADMET) studies (*Fig. 1*). Studies focusing on different phases of pharmaceutical development are outlined below to show the utility of this model throughout the drug discovery process.

Toxicology Studies

To date, zebrafish have been used most successfully and extensively for the testing of promising compounds in toxicology screens (reviewed in[44–46]). Most compounds that reach preclinical or clinical trials fail due to safety and toxicity reasons, usually because of off target effects.[47] These high attrition rates are a grave cause for concern in the pharmaceutical industry due to the exponential rise in time and costs of clinical trials, and the consequences of missed complications. Hence, predictive models that can be used in high-throughput to reveal toxicity issues early on have the potential to save money, time to market, and subsequent medical issues. Although traditionally used in environmental toxicology studies, zebrafish models have begun to integrate themselves early in the drug discovery process where small focused libraries of potential lead compounds identified in prior screens are assayed for dose-dependent toxic effects. Many specific categories of toxicity can also be assessed in the zebrafish, including developmental toxicity, cardiotoxicity, and neurotoxicity (including ototoxicity, locomotor effects, gastrointestinal, and visual toxicity).[48,49] Based on these relatively efficient and insightful assays, a number of companies have been established that work on contract, not only to assess toxicity, but also compound absorption, metabolism, half lives, as well as other more specific assays (see *Table 2*).

The majority of fish toxicology studies to date have focused on acute toxicity to chemicals, pesticides, and pharmaceuticals.[46] Examples include testing of the antirheumatic drug diclofena,[50] endocrine disrupters such as estrogens and the antiarrhythmic agent Amiodarone,[51,52] as well as ethanol and acetaldehyde.[53–55] In all cases, zebrafish larvae exhibited similar xenobiotic, genetic, and physiological responses as documented in mammalian systems.[56–58] These findings are consistent with the conservation of phase 1 and 2 xenobiotic genes and their expression patterns in fish and mammals.[57]

Several recent studies have evaluated larger sets of compounds, with well-established toxicity profiles in mammals, to try and gain a broader idea of the extent of usefulness of zebrafish for toxicology screening.[35,57–60] In all cases, the majority of responses were the same or similar as those documented in humans. It was noted, however, that a few responses or metabolites differed, and that many of the response

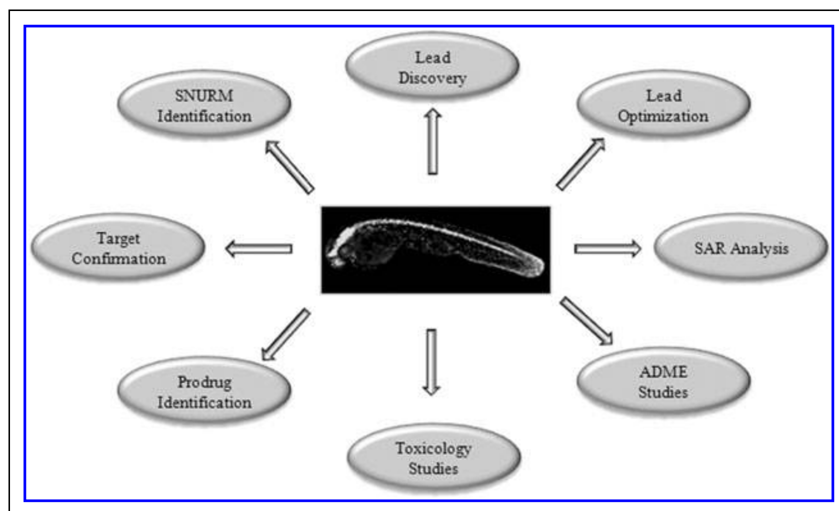

Fig. 1. Various assays and functions of the zebrafish model in drug discovery.

genes and enzymes differ in expression levels and localization over the course of development, with xenobiotic responsiveness generally increasing over time. Thus, developmental staging, methods of compound delivery, and duration of exposure all need to be carefully considered when drawing conclusions from such studies. Further large-scale screens using compounds with known toxicity profiles will help determine the full effectiveness of the zebrafish model in predicting human toxicity.

Target Confirmation

After the identification of a lead compound, the vast arsenal of molecular biology and genetic approaches available for zebrafish can be harnessed for rapid target identification or confirmation. An example is the work of Ito *et al.*,[61] who used zebrafish to verify that the teratogenic compound thalidomide, acts *in vivo* via the *in vitro*-identified target protein Cereblon (CRBN). The authors chose zebrafish because of its rapid development, its ability to absorb compounds from its aqueous environment, and the convenient use morpholinos to reduce expression of a desired target gene. As expected, embryos treated with thalidomide at 2 hours postfertilization (hpf) exhibited developmental defects similar to those observed in humans (pectoral fin malformations). The authors then showed that morpholino knockdown of *zcbrn* recapitulates the effects of the drug.[61] Molecular mechanisms could also be delineated, pointing to the power of zebrafish models to not only confirm drug targets but also dissect their modes of action and downstream effects.

Structure–Activity Relationship Studies

The discovery of a lead compound is generally followed by structure–activity relationship (SAR) studies to generate related entities with different side groups and decorations that lead to greater potency, and minimal off-target effects. Zebrafish provide a powerful *in vivo* route to simultaneously assess the effects of these modifications on both the efficacy and toxicity of the new compounds. Hao *et al.*[62] used this strategy to modify their previously described small molecule dorsomorphin, a selective bone morphogenetic protein (BMP) inhibitor that causes abnormal dorsoventral patterning. Dorsomorphin also has significant inhibitory effects on vascular endothelial growth factor signaling, which prevent its use as a BMP therapeutic.[63] Using synthetic chemistry around dorsomorphin, the authors created a small library of 63 related molecules, which were then administered to 12 hpf embryos to assess their effects on angiogenesis. Among these, several highly selective BMP signaling inhibitors, with no observable vascular endothelial growth factor inhibition, were found. Additionally, it was noted that there were discrepancies between observed *in vitro* and *in vivo* activities, which showed once again that test tube-based assays can be poor predictors of *in vivo* activity.[62] Compounds that show low activity *in vitro* often exhibit greater activity *in vivo*, and vice versa.

Lead Compound Discovery

Small molecule screens conducted with zebrafish embryos are too numerous to list here, but have been extensively reviewed and summarized elsewhere.[30,64] Small molecule screens in zebrafish began with wild-type animals, observing the effects of these compounds on the development, morphology, and function of specific organs and tissues. They then made use of newly available mutant lines that render disease like models that can be enhanced or suppressed genetically and chemically. The past decade has also seen the development of many transgenic lines that express fluorescent markers within numerous tissues that greatly enhance screening throughput and functionality. For example, the Z-Tag system used by Zygogen has been adapted for high-throughput antiangiogenic compound screening using fluorescently marked blood vessels.[43] This system has tremendous advantages over previously used *in vitro* and cell-based assays that lack the biological complexity associated with blood vessel development and function in a whole animal. Screening the LOPAC1280 compound library, two known antiangiogenic compounds were identified, along with one additional compound with no previously known antiangiogenic activity.[43] Similarly, Burns *et al.*[65] developed an automated microwell assay for heart rate using automated fluorescence microscopy of transgenic embryos that express GFP in myocardium. This system measures heart rates efficiently and accurately over a large linear dynamic range, and rapidly characterizes dose dependence and kinetics of small molecule-induced changes in heart rate.

Breakthroughs in computer programming and imaging software that allow for automated detection of complex patterns and phenotypes are also greatly increasing the throughput and potential of screens, allowing more rapid, comprehensive, and unbiased analyses. As an example of the progress and potential of this approach, Gehrig *et al.*[66] have used fluorescent reporters, either expressed throughout early embryos or driven by tissue-specific promoters, to create reference embryos that delineate morphological features such as yolk cells, the notochord and various other organs. They then screened for novel enhancer trap lines, overlaying the resulting images onto the averaged reference embryos to accurately and automatically detect new tissue-specific patterns. An analogous system has also been reported by Vogt *et al.*,[67] who created a ruleset based on Cognition Network Technology methods to accurately identify intersegmental blood vessels labeled with GFP. Further optimization of these technologies will allow for their application to various zebrafish drug screens, thereby decreasing the bottle neck imposed by manual data assessment.

The combined use of monogenic disease models together with fluorescent biomarkers can dramatically increase the power of zebrafish screens. For example, Paquet *et al.* have recently described a transgenic zebrafish model of tauopathy that can be used for *in vivo* imaging and drug screens.[14] The transgenic lines express a mutant version of TAU protein, which causes frontotemporal dementia in humans, and neuronal death and other pathological phenotypes typical of the human disease when expressed in zebrafish neurons. A DsRed fluorescent protein expressed in the same neurons is used as a marker. The authors were able to test compounds directed against glycogen synthase kinase 3 beta (GSK3β) and found that many molecules that were potent *in vitro* were not active in the transgenic animals.[14] These studies indicate the necessity of early *in vivo*

screening methods to eliminate potential lead compounds that are likely to fail *in vivo*.

The examples provided thus far indicate the utility of zebrafish to discover compounds that regulate the genesis and function of complex organs and tissues. Another major utility, not possible with simpler screening systems, is the ability to screen for compounds that affect complex behaviors that underlay a number of major diseases. Indicative of the potential and sophistication of such drug screens in zebrafish is a recent study by Rihel *et al.* to identify compounds that modulate sleep behavior.[68] By analyzing the movement of the fish (at 4 dpf) in 96-well microtiter plates, the authors were able to cluster the responses into a vast array of common behaviors, consisting primarily of variable periods of rest and wake cycles. Using 5,648 compounds, behavior was altered in ~10% of the cases. The behavioral signature created by each compound allowed for the prediction of biological targets for compounds not previously assigned. For example, it was shown that amitraz, a pesticide that binds to α2-adrenergic receptors, gave similar behavioral profiles to those of the α2-adrenergic agonists guanabenz, guanfacine, clonidine, and others.[68] As expected, amitraz side effects also closely resembled those caused by the other compounds.[68] These, along with other findings, such as connections between the sleep/wake cycle and immune responses, show the power of behavioral screening strategies.

In an analogous study, Kokel *et al.* were able to screen thousands of compounds for neuroactivity.[69] By combining screening methodologies with video image-capturing technologies, the authors analyzed the photomotor response of embryos in response to drug treatment, creating activity "barcodes" for each molecule. As in the above sleep study, these groupings facilitated the prediction of common biological targets. Appropriate secondary screening assays designed to identify targets and optimize activity can now be applied.

MOLECULARLY TARGETED *IN VIVO* SCREENS

One of the many advantages of the above-mentioned screens is that new leads can be found in an unbiased fashion that affect complex processes. Compounds with similar effects may be acting through novel and unique targets. While this opens up drug discovery to new processes and targets, significant follow-up studies are generally required to identify the targets and their mechanisms of action. To date, all zebrafish-based compound screens have been nontarget based. However, with a little ingenuity and effort, known disease-causing or therapeutic targets can also be directly focused upon, thereby substantially decreasing the time required to identify the mechanisms and possible side effects of compounds with interesting outcomes. Knowing the molecular target and the nature of the protein–compound interaction also enhances the ability to refine the design of effective drugs.

A targeted platform that will allow HTS for compounds that target the human nuclear receptor (NR) proteins has recently been described.[70] NRs are responsible for, and capable of ameliorating, many of the most prevalent and debilitating diseases that plague current society. Examples include cardiovascular, autoimmune, and neurological disorders, as well as diabetes and most cancers.[71–73] The small molecule hormones and drugs that target and modulate these proteins are therefore of great interest and potential. However, NR-directed drug discovery efforts have seen a significant decline in the past 5 years. Reasons for this drop in interest include difficulties in identifying new modulators of orphan NRs using current approaches, as well as an inability to predict *in vivo* specificity, efficacy and toxicity of those leads that are obtained.

To overcome the limitations of currently used *in vitro*/cell-based NR drug pipelines, transgenic fish that express human NR fusion proteins and fluorescent reporters that together signal the presence and activity of NR ligands (*Fig. 2*) have been generated.[70] The ligand responses of these transgenic fish vary temporally and spatially, due to the differential trafficking, expression, and metabolism of natural or exogenously added NR ligands and cofactors. The NR fusion proteins expressed are also affinity-tagged, which facilitates the isolation and identification of directly bound small molecules and cofactors.

As is the case for all other zebrafish-based platforms, this *in vivo* approach has the ability to identify compounds with promising target and tissue-specificity, while at the same time excluding compounds with poor ADMET properties, as the latter either fail to produce a signal or kill the fish. Fluorescence intensity can also be monitored both spatially and quantifiably to determine potential indications

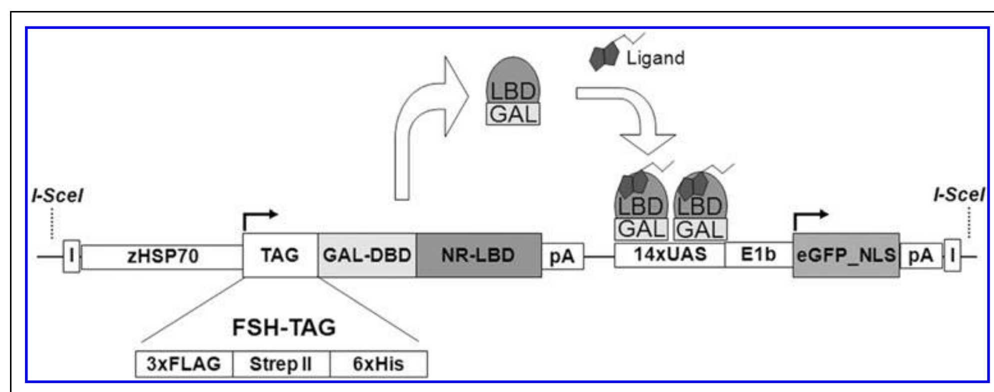

Fig. 2. The ligand trap system. Schematic diagram of the ligand trap construct showing the heat-inducible zHSP70 promoter that drives expression of the Gal4DBD-nuclear receptor (NR) fusion that has a triple affinity Flag-Strep-His (FSH) Tag at its N-terminus. In the presence of ligand and appropriate cofactors, the Gal4-NR fusion binds to the tandem upstream activating sequence (UAS) response elements resulting in green fluorescent protein (GFP) expression.

and ED_{50}s.[70] Details of some of the advantages of this type of target-specific approach are elaborated upon below.

Selective Modulators

One of the largest efforts within the current NR drug discovery community is to identify selective NR modulators. These compounds are expected to have fewer side effects as a result of more tissue- and target gene-selective activities. This selectivity can arise due to (1) unique ligand-induced NR structures by variably contoured ligands, (2) different tissue distributions and expression levels of the cofactor proteome, and (3) differential stability, delivery, or metabolism of compounds within different tissues. The ability to identify such molecules by *in vitro*/cell-based screens is clearly limited, and only apparent upon the design and completion of additional lengthy screening steps. However, initiating the screening process with an appropriate whole-animal platform has the capacity to identify compounds that function differentially in any or all developing or adult tissues.

Treating thyroid receptor beta ligand trap (TRβ LT) fish with the human hormones thyroxine or triiodothyronine, or with the synthetic TR compound triiodothyroacetic acid (TRIAC), results in unique GFP response patterns.[70] Triiodothyronine and thyroxine elicit strong GFP expression in muscle, whereas TRIAC does not. Conversely, TRIAC is uniquely capable of inducing GFP expression in the spinal cord. Coupling these findings to imaging technologies such as those described above would allow for the identification of tissue-selective compounds in a high-throughput manner. These findings illustrate the power of zebrafish for uncovering drugs that can selectively modulate a specific subset of the target's activities and biological functions.

Identification of Prodrugs

Many of the leads of *in vitro*/cell-based screens fail subsequently due to metabolic conversion into inactive and/or secretable compounds. Conversely, compounds that may be converted from inactive to active compounds do not register in these screens. The latter can be grouped within a category of compounds referred to as prodrugs. Prodrugs that are converted by enzymes within various tissues into active compounds have been successfully designed and used, based on prior knowledge of tissue-specific enzyme expression or activity,[74] or by accident. A much more efficient and unbiased approach would be to let the animal do the work. This would allow all potential metabolic enzymes to work on all available classes of small molecules in all tissues and cell types. All *in vivo* screening systems have the capacity to detect prodrug activity. Undoubtedly, numerous unpredictable leads will arise due to these effects. With the LT system

Table 2. Companies Using Zebrafish Models and Their Unique Offerings and Services

Company	Location	Web site	Unique Technology/ Offering	Products/Services
North America				
Phylonix	Cambridge, MA	www.phylonix.com	Multiple patents on methodologies for ZF screening	Tox screens, drug discovery, drug reprofiling, custom work
Novartis	Cambridge, MA	www.novartis.com	Angiogenesis assays	Drug discovery
InDanio Bioscience, Inc.	Toronto, Canada	www.indanio.com	Ligand trap system	Drug discovery
Europe/Australia				
Evotec	Hamburg, Germany	www.evotec.com	ADMETox screening, ZF disease models	Tox screening, custom services
ZF BioLabs	Tres Cantos, Spain	www.zfbiolabs.com	Synchronous ZF embryos	Ready-to-use ZF kits, research contracts
Uniservices	Auckland, New Zealand	www.uniservices.co.nz (wholly owned by the University of Auckland)	Tissue-specific expression of fluorescent proteins	Contract research services, Tox screens
Biobide	San Sebastian, Spain	www.biobide.es	Fully automated Cardio Tox screens	Multiple tox screens, high-throughput screening, target validation, efficacy

ZF, zebrafish.

described above, the identity of the prodrug metabolite can also be determined by purifying the responding NR fusion protein and determining the identity of the copurified small molecule, as described below. Subsequent SAR studies can then be used to improve or diversify the original lead.

Affinity Purification of Interacting Molecules

One of the important features of the LT system, and one that can be applied to other targeted platforms, is the ability to affinity purify active fusion proteins from responding tissues. Each LT fusion protein contains three different affinity tags, which when used in succession, provide a potential combined purification factor of up to a billion fold. This allows sufficient levels of purity and enrichment for the identification of low abundance, nonstoichiometric ligands, and cofactors using various types of mass spectrometry. This potentially efficient method for ligand and cofactor identification can provide new means for monitoring ligand and target activities, new insights into target function, and potential new targets (cofactors) for small molecule screens.

Application to Other Drug Targets

Although ideal for NR-specific drug discovery, the LT approach is readily adaptable to most other potential small molecule targets. A good example is the Per-Arnt-Sim (PAS) domain family of transcription factors, of which there are ~35 in humans.[75] PAS domains bind small molecules such as NADH and heme, and control important processes such as the circadian clock.[76] Fusing the PAS domains of these proteins to a heterologous DNA binding domain such as GAL4 would produce chimeric transcription factors that can also be modulated by small molecules. The same is true for any motif that has the potential to function as a transcriptional activation or repression domain. There are, of course, numerous other ways to generate fusion proteins that, when active, lead to the production of fluorescent proteins (*e.g.*, split luciferase or GFP fusion proteins[77]). These could be used to target other classes of potential drug targets such as kinases, membrane receptors, transporters, and so forth. Adding highly efficient affinity tags would also provide the ability to isolate the activated proteins together with directly bound small molecules and protein cofactors.

CONCLUSIONS

The zebrafish as a tool for drug discovery is rapidly growing in use, and it is clear that, in most cases, the advantages appear to outweigh the limitations. The high degree of conservation between zebrafish and human genes and cellular processes can be leveraged for highly rich and relevant data early in the drug discovery process. Subsequently, or simultaneously, the model can be further utilized to assess ADMET characteristics, to uncover otherwise missed prodrugs, to optimize lead compounds in SAR analyses, and to assess toxicity. The ability to focus in on specific organs, molecular targets, or the whole organism provides vast new opportunities. Although certain caveats must be considered (see disadvantages in *Table 1*), the systems biology approach made possible by zebrafish will lead to new and safer drugs as well as reduced time to market, resulting in an acceleration in novel and useful lead compounds, time and cost savings, and safer more useful drugs.

DISCLOSURE STATEMENT

J.T. and H.M.K. own shares of InDanio Biosciences, Inc., which uses the LT system for drug discovery and analysis.

REFERENCES

1. Paul SM, Mytelka DS, Dunwiddie CT, *et al.*: How to improve R&D productivity: the pharmaceutical industry's grand challenge. *Nat Rev Drug Discov* 2010;9:203–214.
2. Keseru GM, Makara GM: The influence of lead discovery strategies on the properties of drug candidates. *Nat Rev Drug Discov* 2009;8:203–212.
3. Macarron R: Critical review of the role of HTS in drug discovery. *Drug Discov Today* 2006;11:277–279.
4. Kari G, Rodeck U, Dicker AP: Zebrafish: an emerging model system for human disease and drug discovery. *Clin Pharmacol Ther* 2007;82:70–80.
5. Holder N, Xu Q: The zebrafish: an overview of its early development. *Methods Mol Biol* 2008;461:483–491.
6. Golling G, Amsterdam A, Sun Z, *et al.*: Insertional mutagenesis in zebrafish rapidly identifies genes essential for early vertebrate development. *Nat Genet* 2002;31:135–140.
7. Amatruda JF, Shepard JL, Stern HM, Zon LI: Zebrafish as a cancer model system. *Cancer Cell* 2002;1:229–231.
8. Stern HM, Zon LI: Cancer genetics and drug discovery in the zebrafish. *Nat Rev Cancer* 2003;3:533–539.
9. Drummond IA: Kidney development and disease in the zebrafish. *J Am Soc Nephrol* 2005;16:299–304.
10. Milan DJ, Macrae CA: Zebrafish genetic models for arrhythmia. *Prog Biophys Mol Biol* 2008;98:301–308.
11. Hughes I, Thalmann I, Thalmann R, Ornitz DM: Mixing model systems: using zebrafish and mouse inner ear mutants and other organ systems to unravel the mystery of otoconial development. *Brain Res* 2006;1091:58–74.
12. Amatruda JF, Zon LI: Dissecting hematopoiesis and disease using the zebrafish. *Dev Biol* 1999;216:1–15.
13. Ingham PW: The power of the zebrafish for disease analysis. *Hum Mol Genet* 2009;18:R107–R112.
14. Paquet D, Bhat R, Sydow A, *et al.*: A zebrafish model of tauopathy allows *in vivo* imaging of neuronal cell death and drug evaluation. *J Clin Invest* 2009;119:1382–1395.
15. Paquet D, Schmid B, Haass C: Transgenic zebrafish as a novel animal model to study tauopathies and other neurodegenerative disorders *in vivo*. *Neurodegener Dis* 2010;7:99–102.
16. Chang S, Bray SM, Li Z, *et al.*: Identification of small molecules rescuing fragile X syndrome phenotypes in Drosophila. *Nat Chem Biol* 2008;4:256–263.
17. Petrascheck M, Ye X, Buck LB: A high-throughput screen for chemicals that increase the lifespan of *Caenorhabditis elegans*. *Ann N Y Acad Sci* 2009;1170:698–701.
18. Giacomotto J, Segalat L: High-throughput screening and small animal models, where are we? *Br J Pharmacol* 2010;160:204–216.
19. Zon LI, Peterson RT: *In vivo* drug discovery in the zebrafish. *Nat Rev Drug Discov* 2005;4:35–44.
20. Fishman MC: Zebrafish genetics: the enigma of arrival. *Proc Natl Acad Sci U S A* 1999;96:10554–10556.

21. Pichler FB, Laurenson S, Williams LC, Dodd A, Copp BR, Love DR: Chemical discovery and global gene expression analysis in zebrafish. *Nat Biotechnol* 2003;21:879–883.
22. Shin JT, Fishman MC: From Zebrafish to human: modular medical models. *Annu Rev Genomics Hum Genet* 2002;3:311–340.
23. Warren KS, Fishman MC: "Physiological genomics": mutant screens in zebrafish. *Am J Physiol* 1998;275(1 Pt 2):H1–H7.
24. Liu TX, Zhou Y, Kanki JP, *et al.*: Evolutionary conservation of zebrafish linkage group 14 with frequently deleted regions of human chromosome 5 in myeloid malignancies. *Proc Natl Acad Sci U S A* 2002;99:6136–6141.
25. Barbazuk WB, Korf I, Kadavi C, *et al.*: The syntenic relationship of the zebrafish and human genomes. *Genome Res* 2000;10:1351–1358.
26. Bill BR, Petzold AM, Clark KJ, Schimmenti LA, Ekker SC: A primer for morpholino use in zebrafish. *Zebrafish* 2009;6:69–77.
27. Kawakami K: Transposon tools and methods in zebrafish. *Dev Dyn* 2005;234: 244–254.
28. Moens CB, Donn TM, Wolf-Saxon ER, Ma TP: Reverse genetics in zebrafish by TILLING. *Brief Funct Genomic Proteomic* 2008;7:454–459.
29. Amacher SL: Emerging gene knockout technology in zebrafish: zinc-finger nucleases. *Brief Funct Genomic Proteomic* 2008;7:460–464.
30. Wheeler GN, Brandli AW: Simple vertebrate models for chemical genetics and drug discovery screens: lessons from zebrafish and Xenopus. *Dev Dyn* 2009; 238:1287–1308.
31. Emelyanov A, Parinov S: Mifepristone-inducible LexPR system to drive and control gene expression in transgenic zebrafish. *Dev Biol* 2008;320:113–121.
32. Mathew LK, Sengupta S, Kawakami A, *et al.*: Unraveling tissue regeneration pathways using chemical genetics. *J Biol Chem* 2007;282:35202–35210.
33. Tong SK, Mouriec K, Kuo MW, *et al.*: A cyp19a1b-gfp (aromatase B) transgenic zebrafish line that expresses GFP in radial glial cells. *Genesis* 2009;47:67–73.
34. Thorpe JL, Doitsidou M, Ho SY, Raz E, Farber SA: Germ cell migration in zebrafish is dependent on HMGCoA reductase activity and prenylation. *Dev Cell* 2004;6:295–302.
35. Milan DJ, Peterson TA, Ruskin JN, Peterson RT, MacRae CA: Drugs that induce repolarization abnormalities cause bradycardia in zebrafish. *Circulation* 2003; 107:1355–1358.
36. Chan J, Bayliss PE, Wood JM, Roberts TM: Dissection of angiogenic signaling in zebrafish using a chemical genetic approach. *Cancer Cell* 2002;1:257–267.
37. Wang C, Tao W, Wang Y, *et al.*: Rosuvastatin, identified from a zebrafish chemical genetic screen for antiangiogenic compounds, suppresses the growth of prostate cancer. *Eur Urol* 2010;58:418–426.
38. Hong CC: Large-scale small-molecule screen using zebrafish embryos. *Methods Mol Biol* 2009;486:43–55.
39. Murphey RD, Zon LI: Small molecule screening in the zebrafish. *Methods* 2006; 39:255–261.
40. Westerfield M: The zebrafish book a guide for the laboratory use of zebrafish *Danio (Brachydanio) rerio.* Text] http://zfin.org/zf_info/zfbook/zfbk.html. (Last accessed Dec 23, 2010).
41. White RM, Sessa A, Burke C, *et al.*: Transparent adult zebrafish as a tool for *in vivo* transplantation analysis. *Cell Stem Cell* 2008;2:183–189.
42. Makky K, Duvnjak P, Pramanik K, Ramchandran R, Mayer AN: A whole-animal microplate assay for metabolic rate using zebrafish. *J Biomol Screen* 2008;13: 960–967.
43. Tran TC, Sneed B, Haider J, *et al.*: Automated, quantitative screening assay for antiangiogenic compounds using transgenic zebrafish. *Cancer Res* 2007;67: 11386–11392.
44. Eimon PM, Rubinstein AL: The use of *in vivo* zebrafish assays in drug toxicity screening. *Expert Opin Drug Metab Toxicol* 2009;5:393–401.
45. Hill AJ, Teraoka H, Heideman W, Peterson RE: Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicol Sci* 2005;86:6–19.
46. Scholz S, Fischer S, Gundel U, Kuster E, Luckenbach T, Voelker D: The zebrafish embryo model in environmental risk assessment—applications beyond acute toxicity testing. *Environ Sci Pollut Res Int* 2008;15:394–404.
47. Liebler DC, Guengerich FP: Elucidating mechanisms of drug-induced toxicity. *Nat Rev Drug Discov* 2005;4:410–420.
48. McGrath P, Li CQ: Zebrafish: a predictive model for assessing drug-induced toxicity. *Drug Discov Today* 2008;13:394–401.
49. Rubinstein AL: Zebrafish assays for drug toxicity screening. *Expert Opin Drug Metab Toxicol* 2006;2:231–240.
50. Hallare AV, Kohler HR, Triebskorn R: Developmental toxicity and stress protein responses in zebrafish embryos after exposure to diclofenac and its solvent, DMSO. *Chemosphere* 2004;56:659–666.
51. Fushimi S, Wada N, Nohno T, *et al.*: 17beta-Estradiol inhibits chondrogenesis in the skull development of zebrafish embryos. *Aquat Toxicol* 2009;95:292–298.
52. Liu YW, Chan WK: Thyroid hormones are important for embryonic to larval transitory phase in zebrafish. *Differentiation* 2002;70:36–45.
53. Bradfield JY, West JR, Maier SE: Uptake and elimination of ethanol by young zebrafish embryos. *Neurotoxicol Teratol* 2006;28:629–633.
54. Gundel U, Benndorf D, von Bergen M, Altenburger R, Kuster E: Vitellogenin cleavage products as indicators for toxic stress in zebra fish embryos: a proteomic approach. *Proteomics* 2007;7:4541–4554.
55. Reimers MJ, Flockton AR, Tanguay RL: Ethanol- and acetaldehyde-mediated developmental toxicity in zebrafish. *Neurotoxicol Teratol* 2004;26:769–781.
56. Dong W, Teraoka H, Kondo S, Hiraga T: 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin induces apoptosis in the dorsal midbrain of zebrafish embryos by activation of arylhydrocarbon receptor. *Neurosci Lett* 2001;303:169–172.
57. Jones HS, Panter GH, Hutchinson TH, Chipman JK: Oxidative and conjugative xenobiotic metabolism in zebrafish larvae *in vivo*. *Zebrafish* 2010;7:23–30.
58. Wiegand C, Pflugmacher S, Giese M, Frank H, Steinberg C: Uptake, toxicity, and effects on detoxication enzymes of atrazine and trifluoroacetate in embryos of zebrafish. *Ecotoxicol Environ Saf* 2000;45:122–131.
59. Berghmans S, Butler P, Goldsmith P, *et al.*: Zebrafish based assays for the assessment of cardiac, visual and gut function—potential safety screens for early drug discovery. *J Pharmacol Toxicol Methods* 2008;58:59–68.
60. Ton C, Lin Y, Willett C: Zebrafish as a model for developmental neurotoxicity testing. *Birth Defects Res A Clin Mol Teratol* 2006;76:553–567.
61. Ito T, Ando H, Suzuki T, *et al.*: Identification of a primary target of thalidomide teratogenicity. *Science* 2010;327:1345–1350.
62. Hao J, Ho JN, Lewis JA, *et al.*: *In vivo* structure-activity relationship study of dorsomorphin analogues identifies selective VEGF and BMP inhibitors. *ACS Chem Biol* 2010;5:245–253.
63. Yu PB, Hong CC, Sachidanandan C, *et al.*: Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. *Nat Chem Biol* 2008;4:33–41.
64. Kaufman CK, White RM, Zon L: Chemical genetic screening in the zebrafish embryo. *Nat Protoc* 2009;4:1422–1432.
65. Burns CG, Milan DJ, Grande EJ, Rottbauer W, MacRae CA, Fishman MC: High-throughput assay for small molecules that modulate zebrafish embryonic heart rate. *Nat Chem Biol* 2005;1:263–264.
66. Gehrig J, Reischl M, Kalmar E, *et al.*: Automated high-throughput mapping of promoter-enhancer interactions in zebrafish embryos. *Nat Methods* 2009;6: 911–916.
67. Vogt A, Cholewinski A, Shen X, *et al.*: Automated image-based phenotypic analysis in zebrafish embryos. *Dev Dyn* 2009;238:656–663.
68. Rihel J, Prober DA, Arvanites A, *et al.*: Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. *Science* 2010;327:348–351.
69. Kokel D, Bryan J, Laggner C, *et al.*: Rapid behavior-based identification of neuroactive small molecules in the zebrafish. *Nat Chem Biol* 2010;6:231–237.
70. Tiefenbach J, Moll PR, Nelson MR, *et al.*: A live zebrafish-based screening system for human nuclear receptor ligand and cofactor discovery. *PLoS One* 2010;5:e9797.
71. Gurnell M, Chatterjee VK: Nuclear receptors in disease: thyroid receptor beta, peroxisome-proliferator-activated receptor gamma and orphan receptors. *Essays Biochem* 2004;40:169–189.
72. Nagy L, Schule R, Gronemeyer H: Twenty years of nuclear receptors: Conference on Nuclear Receptors: from Chromatin to Disease. *EMBO Rep* 2006;7:579–584.

73. Nilsson M, Dahlman-Wright K, Gustafsson JA: Nuclear receptors in disease: the oestrogen receptors. *Essays Biochem* 2004;40:157–167.
74. Swanson HI, Njar VC, Yu Z, *et al.*: Targeting drug-metabolizing enzymes for effective chemoprevention and chemotherapy. *Drug Metab Dispos* 2010;38:539–544.
75. McIntosh BE, Hogenesch JB, Bradfield CA: Mammalian Per-Arnt-Sim proteins in environmental adaptation. *Annu Rev Physiol* 2010;72:625–645.
76. Ukai H, Ueda HR: Systems biology of mammalian circadian clocks. *Annu Rev Physiol* 2010;72:579–603.
77. Paulmurugan R, Massoud TF, Huang J, Gambhir SS: Molecular imaging of drug-modulated protein-protein interactions in living subjects. *Cancer Res* 2004;64:2113–2119.

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