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. 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. A paradigm shift in the way new and safe compounds are discovered is thus warranted, and the diminutive zebrafish may lead the way.

The rapid development and transparency of zebrafish embryos has already made them an ideal model for the study of vertebrate-specific developmental processes. 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. Many of these genetic perturbations have also shown tremendous utility for modeling human diseases such as cancer, renal disorders, cardiovascular disease, hearing loss, blood disorders, muscular dystrophies, as well as neurological diseases such as Alzheimer’s and Huntington’s, 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 and Caenorhabditis elegans, the use of zebrafish for drug identification is growing at a rapid rate. 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.

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.

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, genome sequencing projects have shown that the synteny and sequence similarity between zebrafish and human genes is very high. 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.

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, statins, compounds that affect heart rate, and compounds that affect angiogenesis. 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; ED50s, 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.
cancer was recently discovered in zebrafish despite the fact that zebrafish do not possess prostates.\textsuperscript{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—zebrafish can survive in as little as 50μL of water in a 384-well plate. 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). Sexual maturation is reached in \( \sim 3 \) 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,"\textsuperscript{26} transposon-mediated gene insertion,\textsuperscript{27} TILLING,\textsuperscript{28} and zinc-finger-mediated recombination.\textsuperscript{29}

**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 \textit{et al.} in a large-scale screen that measured metabolic rate in zebrafish larvae.\textsuperscript{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-

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<th>Table 1. Advantages and Disadvantages of Zebrafish as a Model Organism for High-Throughput Screening Drug Discovery</th>
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<tr>
<td><strong>Advantages</strong></td>
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<tr>
<td>Ex vivo development and optical clarity of the embryo.</td>
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<td>Embryogenesis is completed by 72 hpf and most organs are fully developed by 96 hpf.</td>
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<tr>
<td>Small size of embryos and hatchlings make them amenable for arraying into multiwell plates—zebrafish can survive in as little as 50μL of water in a 384-well plate.</td>
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\textit{In vivo} screens allow the identification of bioactive compounds with good permeability, uptake, stability, and delivery. \textit{In vivo} screens allow the detection of prodrugs and tissue-selective modifiers. hpf, hours postfertilization.
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. 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. 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 toxicity screens (reviewed in). Most compounds that reach preclinical or clinical trials fail due to safety and toxicity reasons, usually because of off target effects. 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). 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. Examples include testing of the antirheumatic drug diclofenac, endocrine disrupters such as estrogens and the antiarrhythmic agent Amiodarone, as well as ethanol and acetaldehyde. In all cases, zebrafish larvae exhibited similar xenobiotic, genetic, and physiological responses as documented in mammalian systems. These findings are consistent with the conservation of phase 1 and 2 xenobiotic genes and their expression patterns in fish and mammals.

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. 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...
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., 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 zcbn recapitulates the effects of the drug. 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. 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. 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. 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. 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. 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. Similarly, Burns et al. 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. 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., 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 monogenetic 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. 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. 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 2-adrenergic receptors, gave similar behavioral profiles to those of the 2-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 ED₅₀8. 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 (TRβLT) fish with the human hormones thyroxine or triiodothyronine, or with the synthetic TR compound triiodothyroacetic acid (TRIAC), results in unique GFP response patterns. 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, 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 prodrg activity. Undoubtedly, numerous unpredictable leads will arise due to these effects. With the LT system

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<td>Phylonix</td>
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<tr>
<td>Uniservices</td>
<td>Auckland, New Zealand</td>
<td><a href="http://www.uniservices.co.nz">www.uniservices.co.nz</a> (wholly owned by the University of Auckland)</td>
<td>Tissue-specific expression of fluorescent proteins</td>
<td>Contract research services, Tox screens</td>
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<td>Biobide</td>
<td>San Sebastian, Spain</td>
<td><a href="http://www.biobide.es">www.biobide.es</a></td>
<td>Fully automated Cardio Tox screens</td>
<td>Multiple tox screens, high-throughput screening, target validation, efficacy</td>
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ZF, zebrafish.
described above, the identity of the prodru 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 insights 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. PAS domains bind small molecules such as NADH and heme, and control important processes such as the circadian clock. 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). 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 produgs, 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.

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