# Systematic Approaches to Toxicology in the Zebrafish

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## Abstract

As the current paradigms of drug discovery evolve, it has become clear that a more comprehensive understanding of the interactions between small molecules and organismal biology will be vital. The zebrafish is emerging as a complement to existing in vitro technologies and established preclinical in vivo models that can be scaled for high-throughput. In this review, we highlight the current status of zebrafish toxicology studies, identify potential future niches for the model in the drug development pipeline, and define the hurdles that must be overcome as zebrafish technologies are refined for systematic toxicology.

## INTRODUCTION

Recent advances in small interfering RNA (siRNA) technology, synthetic chemistry, and robotics combined with the development of efficient multiwell plate assays for cellular phenotypes have made it possible to ascertain the biological activity of thousands of genes or chemical compounds in high-throughput screening (HTS) (1–3). Nowhere has this been so widely applied or so successful as in drug discovery. Once a therapeutic target has been identified and validated, HTS based on target binding or function is routinely used to identify novel structures that modify the target protein's activity in vitro (1). In addition, in vitro assays rarely recapitulate even a single target's full range of functions or exhibit the diversity of potential off-target sites for drug toxicity (4). Importantly, the most representative in vitro assays are usually poor surrogates for complex multicellular, multiorgan diseases. This lack of representation in simpler assays is a fundamental driver for the development of complementary in vivo platforms capable of HTS for use in the exploration of disease pathways, drug discovery, or toxicology (1, 5–8).

Animal models are an integral component of disease mechanism discovery. Although they are widely used in the drug discovery pipeline, animal models have rarely been exploited in primary chemical screens. Few organisms are capable of the throughput required for screening large libraries of small molecules (or genes) for their effects on particular traits of interest, whether suppression of disease phenotypes or the identification of potential on-target or off-target toxicities (9, 10). An ideal in vivo model would capture all the complexities of integrated human physiology and pharmacology, while enabling the interrogation of tens or hundreds of thousands of different conditions (chemical or genetic manipulations) (5, 11). This scale of screen has proven feasible in simple organisms such as yeast, *Caenorhabditis elegans*, or *Drosophila*, but until recently was not a viable proposition in a representative vertebrate model (11–14).

An intense effort has advanced the zebrafish as a model for the genetic and genomic study of vertebrate development, and has led to the emergence of this organism as a platform for the annotation of gene function (15–17). More recently, investigators have begun to use high-throughput approaches in the zebrafish for disease modeling and for chemical biology, combining these in drug discovery screens (8, 18–20). In this review, we outline the use of the zebrafish for studies of toxicology, highlighting some of the most promising advances in this field. We describe the potential of the zebrafish model to contribute to our understanding of different aspects of the complex in vivo interface between chemistry and biology (5). Specific examples are used to illustrate the current and future utility of the zebrafish and to define the hurdles that still must be overcome as the organism is developed as a model for the systematic investigation of toxicology.

## ZEBRAFISH AS A MODEL ORGANISM

The zebrafish has emerged as a powerful tool for phenotype-based screening (11). Its genome and body plan are similar to those of other vertebrates, but its transparency and external development make real-time observation of its cell biology and physiology straightforward. Using the zebrafish model, in combination with innovative optical approaches to physiologic measurement, fluorescent markers, or molecular reporters, researchers can investigate integrative biology at high resolution across multiple organ systems and in a completely native context (19, 20). For example, dozens of transgenic zebrafish lines have been created that express fluorescent proteins in specific cellular subtypes or in cells exhibiting particular biologic processes such as apoptosis in any location in the body (21–23). These lines greatly facilitate detection of the anatomical and physiological changes resulting from genetic or chemical perturbation. Numerous zebrafish disease models have

been developed (18–20, 24), and in many areas of investigation, there is compelling evidence that zebrafish genetics, physiology, and pharmacology are remarkably similar to humans (8, 25, 26).

The zebrafish is small for a vertebrate, reaching only 3 cm in length, and during embryonic and larval stages, it is only 2–3 mm long. Larvae can live for days in individual wells of standard 96- or 384-well plates, surviving on nutrients stored in their yolk sacs. Zebrafish are simple and inexpensive to raise, and one pair of adults routinely lays hundreds of fertilized eggs in a single cross. Large-scale breeding chambers allow the efficient and reliable generation of thousands of embryos each day. Consequently, even a small laboratory can generate many thousands of embryos per day, making possible large-scale screens for essentially any phenotype that can be imagined (27, 28). As zebrafish have become more widely used, additional technologies have been developed that have further increased the utility of this model.

The zebrafish genome project is nearly complete, and DNA microarrays have been generated for expression-profiling studies (29, 30). Antisense morpholino oligomers have proven an effective means of "knocking down" any gene or for excluding specific exons during RNA processing during the first few days of development (31). The advent of reverse-genetic approaches, including TILLING and, more recently, germline gene-targeting using zinc finger nucleases, has enabled researchers to generate mutations in virtually any gene of interest. These advances have also spurred the concept of a zebrafish phenome project to generate null alleles for each gene in the genome, and then to annotate gene function across a broad range of physiologic phenotypes (32–34). These genomic and phenotyping tools are changing the scope of potential in vivo screens.

The unique attributes of the zebrafish embryo and larvae allow HTS technologies to be applied in models of complex disease and other physiological perturbations (**Figure 1**). Unlike yeast, flies, and worms that are generally resistant to small-molecule permeation, zebrafish embryos readily absorb small molecules from the surrounding medium (5, 10, 11, 35). Furthermore, their transparency and small size enable screening on a scale that would be prohibitive for mice or other vertebrate model organisms (36). The development of phenotypes that can be adapted for HTS is in its infancy. However, several groups have already successfully used zebrafish in screening modes to identify genetic or chemical modifiers of different aspects of vertebrate development (28, 37–39) and to discover novel compounds that suppress disease phenotypes (40–42). The careful derivation and validation of relevant phenotypes for HTS in the zebrafish is currently a focus of intense activity and is central to the success of efforts in genetics, systems biology, drug discovery, and toxicology.

## PHENOTYPES AND PHENOTYPE ANCHORING

The most obvious "sweet spot" for the zebrafish as a model organism is at the nexus between throughput and vertebrate representation (5, 6, 8). The balance between these two competing factors is optimal when zebrafish can be studied in multiwell plates: essentially, during the first 4–5 days post fertilization. For many biological processes of relevance to disease or toxicology, dynamic changes may occur during the first few days of life in the zebrafish that must be accounted for as phenotypic assays are established and validated across species. The success of this approach is dependent on the extent to which the nuances of the relationship between zebrafish and mammalian phenotypes are understood. This phenotypic anchoring requires zebrafish phenotypes of comparable resolution to those in human or mammalian models and also mandates substantial effort to define the precise extent of the biological parallels. Although there are decades of experience with such comparative studies in rodents and other animal models, these correlations are only beginning to be defined for the zebrafish. Nevertheless, the throughput of the zebrafish should accelerate these comparisons, and for those phenotypes best studied to date, the zebrafish

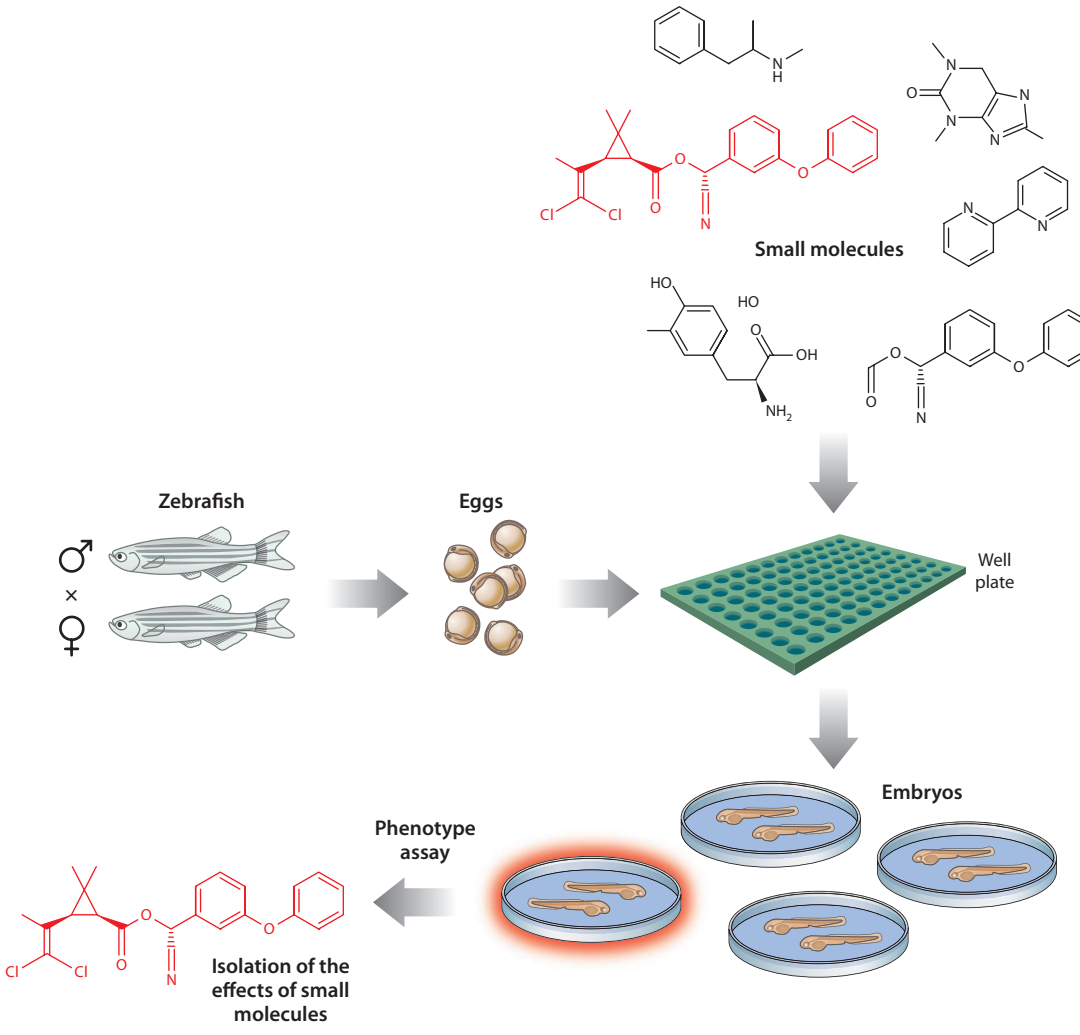


**Figure 1**

Schema of chemical genetic screens in zebrafish. Adult zebrafish are mated to generate thousands of fertilized eggs, which are distributed into the wells of 96- or 384-well plates. Compounds from a structurally diverse, small-molecule library are added to the water surrounding the embryos. After the appropriate incubation time, phenotypes are scored visually or using an automated assay. This approach allows for the discovery of small molecules that produce the desired effect in an integrated physiological context.

has proven at least as representative as existing models. For example, within 48 h post fertilization, zebrafish cardiac electrophysiology and contractile physiology are remarkably similar to those of the adult human (26). Thus, the initial dependence of normal zebrafish cardiac electrophysiology on calcium currents is replaced within 1–2 days by a more comprehensive repertoire of ionic currents that is highly representative of adult human cardiac electrophysiology (26, 43). The basal physiology and its responses to a large number of drugs at 48 h post fertilization are closer to those of the adult human than any existing rodent model employed in preclinical studies of drug efficacy or toxicity (44). Importantly, although it appears highly accelerated, the developmental sequence of events at the cardiomyocyte membrane is also directly representative of the

same sequence in human cardiac maturation. Although such homology is unlikely to be universal, these data suggest that the compressed developmental timeline of the zebrafish may render many more adult human phenotypes accessible during the larval stages when the fish is amenable to HTS (5).

One of the most obvious advantages of in vivo modeling using the zebrafish is the ease of interrogation of a broad range of biologic pathways. The repertoire of pathways displayed is particularly rich during the dynamic processes of normal development. Discrete morphogenetic phenotypes were identified for specific pathways during the original genetic screens in zebrafish, and they have already been exploited for the discovery of novel chemical probes (5, 38). For example, the dorsalization phenotype observed with bone morphogenetic protein (BMP) pathway inhibition was used to identify the specific BMP antagonist dorsomorphin, and subsequent medicinal chemistry has generated a range of reagents that are specific inhibitors of different BMP receptors (41, 45, 46). Such phenoclustering approaches have been exploited in lower model organisms, but they are only just beginning to be adapted for the annotation of genes or small molecules in the zebrafish (47, 48). This strategy will require considerable investment to be systematized, but it is one way in which the rigorous annotation of small-molecule activities across a broad range of both on-target and off-target toxicity pathways may be achieved (5, 49).

As noted above, the transparency of the zebrafish offers many advantages for assay development, including the dynamic range and temporal precision inherent to optical endpoints. This has been exploited for the tracking of labeled or unlabeled cells in vivo. Tissue- or cell-type-specific reporters enable the characterization of aspects of cell biology that are difficult to access in many other models (50, 51). The fluorescent labeling of specific cell types enables these to be isolated through mechanical disruption and cell sorting, each of which can be undertaken at scale (52). The resultant cells can then be characterized using functional genomics or other technologies (30). FRET-based reporters, as well many molecular dyes, are readily adapted for in vivo use in the zebrafish (53–55). These tools enable cell-, organelle-, and pathway-specific assays to be constructed and then scaled for use in higher throughput, thereby bringing many of the most recent advances in cell biology to bear on integrated physiology, pharmacology, and toxicology in the intact organism.

The small size of the zebrafish can make it difficult to obtain sufficient tissue for some traditional assays. This is a particular challenge for histology or ex vivo cell biology, unless large numbers of zebrafish are used in aggregate. The use of microarray technologies in the larval zebrafish has often been limited by cell numbers, but the advent of next-generation sequencing technologies in samples as small as a single cell will facilitate gene expression profiling (56). Other functional-genomics technologies including metabolomics and lipidomics are being adapted for use in the zebrafish (57, 58). Because the molecules assayed by these technologies are identical in higher vertebrates, cross-species comparisons are extremely efficient.

Even though the administrative and husbandry barriers to studying zebrafish are less stringent than are those for rodents, the challenges of physiologic measurement in such a small organism are far from trivial. In many cases, technical issues have led to disappointing results. The simple standardization of basic husbandry protocols that has characterized work in other experimental animals has not yet been developed in the zebrafish field (59). Nutrition, lighting, vibration, and commensal microbes, to name but a few parameters, are only beginning to be defined at the required level of resolution. Technical aspects of each assay can also be demanding, and variation in simple parameters such as temperature gradients across a multiwell plate or pH changes over time can confound results (5). As noted above, the timing of assays with respect to developmental stage is also a critical variable. When designing complex multistage screening assays at any scale, but especially at high throughput, considerable expertise is required to ensure the appropriate

controls are utilized. In many instances, parallel assessment of cofounders and normalization strategies are required to overcome these issues, especially when screening using automation (5, 60). Many of these factors have been extensively addressed in rodent and other toxicology models, and it will take time to standardize each aspect of zebrafish toxicology at a similar resolution. Ultimately, the goal of phenotype anchoring is to establish the areas in which the zebrafish can complement existing models through improvements in sensitivity, specificity, cost, or efficiency. Assays that fail to satisfy these basic criteria are unlikely to offer any meaningful advantage, and they may be better undertaken in conventional model systems.

Despite these hurdles, several investigators have developed innovative zebrafish assays for physiologic parameters that have been scaled to allow the screening of hundreds or thousands of genes or compounds for their effects on complex physiologic or disease pathways (14, 61). Automation as well as the process of scaling up a validated toxicity assay for higher throughput can present unique challenges. These include reevaluation of the specificity and sensitivity of the assay at the requisite throughput. Here, it is important to consider carefully the second- or higher-round assays that will be used for subsequent confirmation of the initial results from the screening mode (62, 63). The appropriate staging of complementary assays in a rigorously tested sequence is an opportunity to optimize sensitivity and specificity. Objective assessment of the robustness of the complete assay is a central feature of any experiment, but there are particular statistical concerns when dealing with high-throughput assays (14, 64).

## PARALLELS WITH HIGHER VERTEBRATES

A central question in the use of the zebrafish in toxicology studies, as in many other areas of investigation, is the degree to which the biology observed is representative of higher organisms, particularly humans. The larval zebrafish develops rapidly and organogenesis is complete within approximately 48 h post fertilization. Physiologic integration is initiated prior to this developmental time point, and it progresses over the next few days to weeks as humoral and neural communication is established and different cell populations migrate into each organ. For example, peripheral innervation by the somatic and autonomic nervous systems is not complete until weeks after fertilization (65). In common with other vertebrates, there is significant functional plasticity during development, and this can be observed in each organ system. The accessibility of these aspects of normal biology is a distinctive attribute of the zebrafish model, but care is necessary during assay development to ascertain that the relevant biology is present at the specific developmental stages under evaluation (8).

Importantly, the results of prior zebrafish genetic or chemical screens have proven highly representative for other organisms including humans. Genes identified in the original zebrafish organogenesis screens have been consistently validated in mice or humans (11, 40). In many instances, zebrafish and human gene discovery have occurred side by side (66, 67). Similarly, in annotative chemical screening where wild-type embryos are exposed to libraries of small molecules, the correlations between the observed effects in zebrafish and human exposures to the same compounds are strong (37, 38). The remarkable pharmacologic homologies with humans extend to modifiers of disease phenotypes identified in screening mode (25, 26). For example, screening the zebrafish arterial fate mutant *gridlock* against a library of 5000 compounds identified a novel class of compounds capable of suppressing the mutant phenotype (40, 68). Beyond their ability to suppress the *gridlock* phenotype in zebrafish, these compounds promote tubulogenesis in cultured human endothelial cells, and they improve the collateralization of ischemic hind limbs in mice, strongly suggesting that the compounds are vasculogenic, not only in teleosts, but also in adult mammals (40, 69). There is also evidence, outlined below, that these homologies extend across

multiple organ systems. These findings offer a rationale for efficient translation from fish to other model organisms. Small molecules discovered in such screens could be used as pathway probes or perturbogens. They could also be used as leads for conventional drug development (8, 70).

## IN VIVO SCREENS FOR THERAPEUTICS

An important context for the emerging role of the zebrafish in toxicology is its use in drug discovery. The identification of ENU-induced alleles for human disease genes, the generation of transgenic models, and, more recently, zinc finger nuclease knockout models all have enabled the precise recapitulation of many human diseases (20, 34, 71). These genetically modified lines have subsequently been used for chemical screens to identify suppressors or modifiers of disease phenotypes (40, 72). Several successful screens have been completed with "hit" rates that are considerably higher than those detected in traditional drug discovery screens focused on a single molecular target in a single cellular context (6). These high hit rates reveal the utility of targeting multiple pathways in concert and raise the possibility that this approach, as opposed to more stringent modification of a single pathway, may offer a strategy for disease therapy with distinctive profiles in efficacy and toxicity. Some compounds identified in zebrafish screens are now progressing through structure-activity studies, which may also be performed directly against the disease itself in vivo in the zebrafish, and are moving toward clinical use (5, 6). Currently, the rate-limiting steps for several of these efforts include target identification and deconvolution of absorption, metabolism, and distribution in the zebrafish. Although outside the scope of this review, such therapeutic screens highlight the advantages of in vivo screening and raise the possibility of combining drug discovery and toxicology in screens designed to identify the optimal balance between specific therapeutic and toxic endpoints in simultaneous quantitative assays.

## TERATOGENICITY

A major form of clinically relevant drug toxicity is embryopathy or teratogenicity. Numerous examples exist of mammalian toxicities that have been recapitulated in the embryonic or larval zebrafish (73–76). These include very specific effects on individual cell types, such as the effects of various heavy metals on neurogenesis or of alcohol on the central nervous system and muscle. Small studies of teratogenicity in a range of models have suggested good correlations between the effects in zebrafish and those observed in other models including humans (76). However, much larger studies would be required to enable rigorous characterization of the predictive utility of the zebrafish in this context (77).

There are elements of mammalian development that are not directly represented in fish biology, such as placentation. Nevertheless, many of the fundamental pathways required for successful implantation and placentation are present in the zebrafish and could be captured in appropriately designed assays. Similarly, the phenotypic parallels for defects as diverse as cardiac outflow tract patterning and lung surfactant production in zebrafish may not be immediately apparent, even if they exist. Even when the phenotypic comparisons appear tangential, it may be possible to explore the pathways at high throughput in the zebrafish before moving to a more directly relevant model for second-round validation and subsequent study (70). Thus, the zebrafish may offer some advantages simply through the efficiencies of scale. However, for the zebrafish to emerge as a bona fide organism for the study of embryopathy, systematic comparison with other models across a wide range of chemical structures is required.

## ORGAN-SPECIFIC TOXICOLOGY

Organ-specific toxicities remain the most frequent reason for the failure of drugs late in development or for withdrawal of drugs from the market. New models that may improve our ability to identify such toxicities earlier in the drug development process or in ways that would enable more precise estimation of the risks of toxicity have been a focus of work in pharmacology for decades. In the past few years, several investigators have pursued such modeling in the zebrafish.

### Cardiotoxicity

Cardiac repolarization abnormalities, often leading to life-threatening arrhythmias, represent one of the most important types of drug toxicity (78–80). Drugs that cause clinical arrhythmias are often associated with prolongation of the QT interval measured on the electrocardiogram. The majority of drugs that result in clinical problems interfere with the human Ether-à-go-go Related Gene (hERG) channel, blocking the rapid component delayed rectifier potassium current ($I_{Kr}$). However, in vitro hERG inhibition has been limited in its predictive value (81–86). The lack of utility of current assays and the failure of these assays to detect drug effects on other targets which may modulate hERG indirectly are a result of the intrinsic complexity of the myocardial substrate and the involvement of extracardiac factors in the genesis of actual toxic arrhythmic events (86–89).

Predicting the effect of a drug on an organism remains extremely difficult (90). In vitro systems enable high-throughput testing of cytotoxicity, but they fail to recapitulate the complexity of the intact organism. Traditional in vivo models are low throughput and expensive; as such, they are usually employed late in the discovery process (91). In silico predictive algorithms are limited in their applicability, partly as a result of the few comprehensive data sets that are available (92). One potential solution for many of these problems is the use of empirical testing of a large number of variables in an intact organism. This approach is capable of modeling much of the true complexity of drug absorption, metabolism, excretion, and toxicity but has not been feasible in traditional model organisms (25). Partly because of the feasibility of characterizing the toxicity at every level—from the individual ion channels through to integrated physiology—this area has been one of the most extensively studied in zebrafish toxicology.

Because larval zebrafish had shown promise as a tool for the investigation of drug toxicity (26), for annotation of small molecule function (38), and for empirical pathway discovery in genetic disease models (40), several groups have explored its utility for the prediction of cardiotoxicity. Using simple heart-rate responses, investigators have been able to establish an excellent correlation with known adult human repolarization cardiac toxicity and recapitulate clinically relevant, drug-drug interactions (26). Within 48 h post fertilization, the developing zebrafish has a beating heart with a complex repertoire of ion channels and a functioning metabolism (26, 93). Even dramatic effects on cardiac function are well tolerated by the larvae, which can survive for 4–5 days without an active circulation. Early patch-clamp recordings documented the presence of the majority of known cardiac currents in larval and adult zebrafish myocytes (94); thus, assays have been designed to measure basic zebrafish physiologic parameters. These results have then been validated using a comprehensive panel of cardioactive drugs. It also proved feasible to define a range of sophisticated second-tier assays with higher resolution and lower throughput that have been used in series to optimize the overall sensitivity and specificity of the approach. These assays include calcium imaging and optical voltage mapping that enable characterization of integrated myocardial electrophysiology at a resolution comparable to that in current "state of the art" canine or rabbit models. Using the zebrafish, investigators have also been able to define action potential prolongation in the setting of QT-prolonging drugs, known ion channel mutations, or novel genes recently implicated in cardiac repolarization. The zebrafish mutant *breakdance* carries a missense

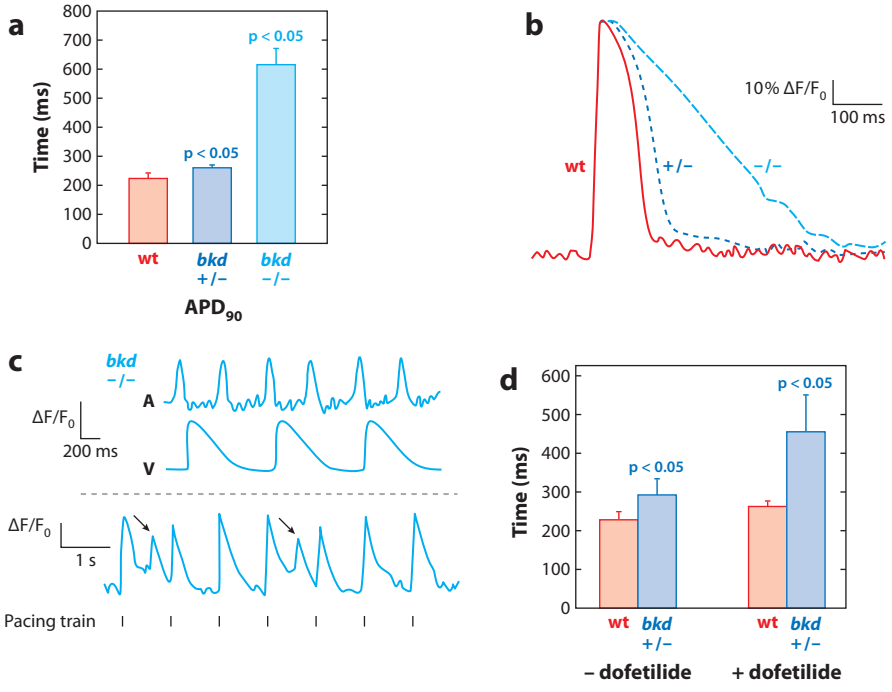


**Figure 2**

Parallels between zebrafish and human disease physiology and pharmacology. (*a*) Ventricular action potential (AP) durations in wild-type (*red*) and *breakdance* heterozygotes (+/−; *dark blue*) and homozygotes (−/−; *light blue*) at 6 days post fertilization. (*b*) Typical ventricular APs are displayed for wild-type and *breakdance* heterozygote and homozygote embryos. The heterozygote AP is subtly prolonged, whereas the homozygote shows marked AP prolongation. Vertical calibration bar denotes 10% $\Delta F/F_0$; horizontal bar denotes 100 ms. (*c*) (*Upper panel*) Simultaneous atrial and ventricular voltage recordings from *breakdance* (−/−) heart showing the mechanism of 2:1 atrioventricular block. APs are so prolonged in the ventricle that alternate atrial impulses encroach on the refractory plateau of the previous ventricular repolarization. (*Lower panel*) Early afterdepolarizations (*small black arrows*) in *breakdance* (−/−) embryos during ventricular pacing. The pacing train is shown below the AP recording. (*d*) Heterozygote *breakdance* embryos display increased sensitivity to 10-nM dofetilide. Abbreviations: A, atrial; *bkd*, *breakdance*; V, ventricular; wt, wild-type.

mutation in the cardiac *KCNH2* gene, the major subunit of the potassium channel responsible for $I_{Kr}$ (95, 96), and thus mimics one form of the long QT syndrome (LQTS). Using high-resolution optical mapping, it was possible to demonstrate that *breakdance* homozygotes exhibit prolonged action potentials with "triangulation" or prolongation of the $APD_{25-75}$, similar to those observed in cognate human repolarization disorders (97) (**Figure 2*b*,*c***). It has even been possible to identify spontaneous early afterdepolarizations, the postulated triggers of fatal arrhythmias in both inherited and acquired repolarization disorders (**Figure 2*c***) (79, 98). Finally, the fidelity of the zebrafish model has been confirmed through reduction in repolarization reserve with the specific $I_{Kr}$ blocker dofetilide at 10 nM (**Figure 2*d***) and with loss of function of the novel repolarization gene NOS1AP (99).

Subsequently, elegant chemical screens have been able to identify compounds that suppress the phenotypes associated with genetic or chemical blockade of the hERG channel (72). These studies raise the possibility of performing chemical counterscreens to explore the mechanisms of drug toxicities and to identify potential suppressors of these adverse effects. This strategy may nicely

complement more traditional structure-activity relationship studies in efforts to retain therapeutic efficacy while eliminating adverse effects.

Using these same assays, a blinded screen of FDA-approved compounds predicted 22 of 23 known agents that cause the drug-induced arrhythmia torsades de pointes and also successfully reproduced a broad range of relevant drug-drug interactions (26). The recapitulation of these canonical multiorgan interactions, which are inaccessible in vitro, demonstrates a major advantage of in vivo modeling (8). As noted, these data suggest robust functional conservation between humans and zebrafish at multiple levels, and they have formed the basis of commercial assays for cardiotoxic drugs using screening technologies in larval fish. Similarly, panels of drugs known to perturb contractility and/or vasomotor tone have been tested and found to recapitulate very accurately the physiologic effects observed with these same agents in humans (100).

## Hepatotoxicity

Several mammalian hepatotoxicants cause hepatic injury in the larval or the adult zebrafish. In a number of cases, very specific patterns of injury or dysfunction have been directly replicated, but in others, the specificity of the zebrafish responses and their representation of human toxicity are not entirely clear. For example, exposure of zebrafish to mercury elicits hepatic transcriptional responses that closely parallel those of human Hep2 cells (101). As such, more extensive evaluation will be required to document the full extent of pathophysiologic parallels.

Many of the existing discrepancies may reflect the developmental timing or more comprehensive cellular and molecular context of the zebrafish liver. However, for some of these studies, the phenotype resolution used in the zebrafish to date may preclude direct comparisons between fish and human responses. Systematic approaches to phenotype anchoring will be vital if the utility of the zebrafish in hepatotoxicity, as in other organ-specific toxicities, is to be fully realized. Ultimately, the development of heterologous reporter systems for specific hepatotoxicity pathways may be the most efficient means of detecting hepatotoxicity at the scale where zebrafish may have an advantage over existing models.

## Nephrotoxicity

One of the most common forms of adverse drug effect is nephrotoxicity. Specific tubular toxicities directly limit the utility of many highly effective drugs including multiple classes of antibiotics, antineoplastic agents, and a wide variety of other efficacious drugs. To date, only acute toxicities have been directly explored in the zebrafish kidney. Investigators have confirmed the tubular toxicity of gentamicin, a commonly used nephrotoxic antibiotic, establishing both histological and functional effects on extracellular volume homeostasis that parallel aminoglycoside toxicity in higher organisms (102). Existing approaches have been adapted to the quantitation of renal function for use in the larval zebrafish, demonstrating a decline in glomerular filtration rate after gentamicin exposure. Similarly, cisplatin, another drug whose utility is limited by kidney toxicity, causes typical histological changes and a decline in renal function in larval zebrafish (102). Interestingly, a specific inhibitor of Omi/HtrA2, a serine protease implicated in cisplatin-induced apoptosis, prevents renal failure and increases survival. This protective effect was subsequently confirmed in a mouse model of cisplatin-induced nephrotoxicity. These preliminary studies suggest that the zebrafish will be of great use in exploring the prediction and prevention of drug-induced nephrotoxicity. Once again, it will be important to establish innovative assays of renal function in the larval stages that are capable of scaling to higher throughput so that the relationship between zebrafish and human kidney injury can be validated across a broader chemical "space."

## Neurotoxicity

Drug-induced neurological effects are remarkably common and are among the most frequent reasons for poor drug compliance, even in the context of well-established benefits. The sheer complexity of the nervous system makes in vivo modeling attractive, and several groups have pioneered neurological assays in the zebrafish. Behaviors including feeding, seizures, involuntary movement, flight responses, sleeping, and addiction can be directly observed and quantified in the zebrafish (103–106). Similarly, genetic and chemical effects on each of these behaviors in humans can be recapitulated in the zebrafish. However, large-scale chemical screens exploiting these assays have only just begun to be undertaken (106). An alternative approach, focusing on a single neurological circuit, but adapting this for much higher throughput, has led to the characterization of the neurological effects of tens of thousands of small molecules (14). This strategy suggests not only that effectively clustering neuroactive drugs using robust quantitative metrics is possible, but also that such clustering will extend to potential neurotoxicity. Ongoing work in screens of truly integrated behaviors will shed additional light on the utility of the zebrafish for the study of neurotoxicity.

## Muscle Toxicity

Myotoxicity from several drugs has been studied in the zebrafish. Perhaps the most widely explored is statin-related myopathy. Expression profiling studies identified substantial upregulation of skeletal muscle atrophic signaling molecule atrogin-1 in the muscles of those with statin myopathy. Subsequently, it was shown that the atrogin ortholog is also upregulated in zebrafish skeletal muscle on statin exposure and that knockdown of atrogin-1 markedly attenuates the associated myopathy (107). More recent work has established that polymorphism in an organic anion transporter required for statin hepatic uptake is a substantial contributor to the drug-related myopathy. Ongoing work is exploring both the role of interaction between these pathways in statin myopathy and the roles of other genes potentially involved. Importantly, although the zebrafish has proven useful in modeling diverse toxicity pathways, it remains unclear what the predictive utility of the zebrafish may be for myopathies of unknown mechanism.

## Gastrointestinal Toxicity

Perturbation of gut motility is a common adverse effect with many drugs. Gut contractility emerges around day 4–5 post fertilization in zebrafish and is relatively straightforward to assay at scale in the zebrafish. Several groups have designed assays that allow the effects of drugs on gut motility to be estimated quantitatively (108). Higher-throughput assays have been developed, but they have not been used for toxicity screening (109).

Assays for other gut functions including absorption, secretory function, and endocrine function can be imagined, and in some instances, they have been realized (53). Innovative assay design is required for some of these physiologic endpoints, but the benefits of genetic or chemical screening make such development efforts worthwhile.

## Other Toxic Effects

Many other toxic effects have been recapitulated in the zebrafish. Virtually every organ system has been represented, ranging from thyroid toxicity to the immune effects of arsenic (110, 111). Although a publication bias in favor of studies reproducing human observations is likely, there

are also reports of toxicities that have not been observed in higher animals. Many of the factors highlighted in this review are also potential contributors to these discrepancies. Great care must be taken to understand the parallels between phenotypic assays in zebrafish and other species, as well the biological and technical confounders, when interpreting these findings. As the study of toxicity in the zebrafish matures, the correlates across species will become better defined. Of note, the scalability of the zebrafish may facilitate the collection of a large matrix of small-molecule phenotypes around which a systems-level comparative toxicology may be built (70).

## CARCINOGENICITY

Several zebrafish tumor models have been described, and new models are being generated in a large number of laboratories with a view to the large-scale screens for genetic and chemical modifiers that the zebrafish facilitates (24, 112). To date, no direct (112) assays of chemical tumorigenicity have been developed for the zebrafish, and given the likely time course of tumor formation, the advantages of using the zebrafish are less obvious in this arena (113). However, investigators have exploited the tractability of the zebrafish to explore a host of molecular and cellular pathways implicated in drug-induced tumorigenesis (113).

The zebrafish may also provide a useful model for the discovery of tumorigenicity through the development of chromosomal injury reporters in different sensitized strains or through global assays of chromosomal disruption based on next-generation sequencing technologies. Here again, the complex native context that the zebrafish provides allows investigators to capture the full repertoire of metabolites, organ-specific toxicities, and other biological nuances (including genetic modifiers) with efficiencies of scale approaching those of cell cultures.

## ADSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

A rate-limiting step in using the zebrafish to explore drug-organism interactions is the difficulty in characterizing absorption, distribution, metabolism, and excretion (ADME) in such a small organism. There are multiple examples of canonical drug-drug interactions dependent on various ADME effects that have been reproduced in the zebrafish, but the systematic characterization of ADME for even modest numbers of small molecules remains challenging (26). These hurdles are less relevant in the initial screening mode for drug discovery where the therapeutic efficacy of the "hits" identifies small molecules that have penetrated the zebrafish and have presumably distributed to the relevant tissues. In this construct, false negatives due to ADME issues can be ignored. However, in subsequent studies of toxicity, detailed ADME is less straightforward than in traditional model systems. Although it can be argued that studies in traditional preclinical models will be necessary, ADME on a scale comparable with that of the primary efficacy/toxicity screens, or at least at an order of magnitude higher efficiency than is currently feasible, would be extremely powerful. Such an advance would allow the natural variation in these processes to be explored at a relevant throughput.

Drug absorption from aqueous media by the larval fish is highly dependent on pH, so care must be taken to ensure that the media for chemical screens are suitably buffered. The physicochemical properties of most small molecules can be used to predict absorption, and these properties correlate with the usual attributes of successful drugs. The objective measurement of drug absorption as well as drug distribution can be readily assessed using fluorescence or radioactive assays (53, 114). If these approaches cannot be used, standard bioanalytic techniques can be adapted for the zebrafish, ideally in combination with tissue/organ sorting based on traditional fluorescent reporters (52). Modern LC-MS/MS (liquid chromatography–mass spectrometry) techniques and other unbiased

technologies are proving useful in specific cases, but to date, there is no universal scalable approach to ADME in the zebrafish.

Studies of drug metabolism in the zebrafish have been limited, but there is extensive genomic work on the cytochrome gene family and the genes encoding many other relevant protein families including drug transporters and modifying enzymes (115). Once the functional distinctions between zebrafish and other models have been better defined, more systematic efforts in ADME are likely to emerge. Defining the effects of ADME on drugs and their metabolites in the zebrafish will be vital as the organism becomes more widely used in toxicology.

## MECHANISM OF ACTION STUDIES

Small molecule toxicity can be an obstacle, but also an opportunity to learn about the biological mechanisms underlying the toxicity. Therefore, mechanism of action (MOA) studies are often an integral element of toxicology. MOA studies of compounds and their toxicities can be among the most useful outgrowths of pharmacology and toxicology. The history of biological discovery is filled with examples of small-molecule MOA studies bringing to light important biological phenomena. As examples, MOA studies of the opiates led to discovery of the opioid receptors and to a fundamental understanding of nervous system function (116). MOA studies of trapoxin also led to discovery of the histone deacetylases and to a fundamental understanding of chromatin modification (117).

Both computational and experimental approaches have been successful in determining compound MOAs, and both are being applied to small-molecule effects observed in zebrafish screens. Computational approaches typically seek to identify patterns of similarity between a compound of interest and previously characterized compounds. For example, the similarity ensemble approach is a chemoinformatic technique that compares the structure of a given compound to a large database of compound structures with previously determined MOAs (118). It then predicts MOAs for the novel structure on the basis of similarity to known structures. Other computational approaches look for similarity, not of compound structures, but of biological effects. For example, phenoclustering has been used to predict MOAs for compounds by comparing detailed, quantitative phenotypic descriptions of a compound's effects with a collection of similar phenotypic descriptions for well-characterized compounds (14). The primary limitation of these computational methods is that they require the compound of interest to share something (e.g., structural features or biological effects) with existing compounds of a similar mechanism.

There are several experimental approaches for determining compound MOAs, many of which use affinity between the small molecule and its biological binding partners as a means of purifying and identifying the compound's molecular targets. Unlike computational approaches, these affinity-based approaches do not require a priori assumptions about a compound's MOA. The techniques of affinity purification have been previously described (37, 117, 119–123). Most published examples conduct affinity purification using mammalian lysates, but a few examples have used zebrafish lysates. Notably, several tagged triazine molecules have been used to purify binding partners directly from embryo lysates (37, 124, 125).

An important challenge associated with MOA studies is that small molecules often bind to multiple proteins, and the beneficial or toxic effects of the compound may result from activity at multiple targets. Modern proteomic techniques are likely to improve greatly the process of affinity-based MOA identification by simultaneously identifying all the binding partners for a small molecule. Such proteomic techniques such as SILAC and iTRAQ have proven particularly powerful in this regard (126, 127). Once these techniques identify a list of candidates, the relevant targets must still be verified experimentally, and the ability to rapidly knock down target candidates

in zebrafish can accelerate the process. As these techniques evolve, the process of determining a small molecule's MOA is likely to become much easier.

## ENVIRONMENTAL TOXICOLOGY

Considerable effort has been undertaken to exploit the zebrafish for the study of environmental toxicants. This work has encountered all the difficulties discussed in this review. To date, the dominant efforts have been focused on the early life-stage toxicities of a limited range of environmental chemicals where the zebrafish offers outstanding access to the very earliest stages of vertebrate development (128). There have not been any systematic approaches to the study of large numbers of environmental agents, although such projects are likely in the future (14, 129, 130). Ultimately, the rate-limiting steps in such efforts are the scope and resolution of phenotyping. Although the success of simple visual screens in limited libraries bodes well, in cases where the phenotypic endpoints are unknown, large-scale automation is considerably more difficult. If the field is to move beyond these constraints, in pharmaceutical as well as environmental toxicology, robust new phenotyping technologies with the capability of identifying novel toxicities will be vital (130).

## TOXICOGENETICS AND PHARMACOGENETICS

A central tenet of modern pharmacology is the promise of genomics to unravel the basis of individual responses to drugs or environmental toxicants (131). Most drugs reach market with testing across only a very limited range of genotypes. In many instances, fewer than 1000 animals have been exposed to a drug during routine preclinical testing, and many of these are inbred, thus restricting the numbers of independent genotypes that have been tested. It is not surprising, therefore, that many genotype-dependent or "idiosyncratic" adverse drug events are not apparent until late in the clinical trial process or during postmarketing surveillance. The outbred nature of the zebrafish complements existing preclinical models, thereby offering not only the scale, but also the genetic repertoire required for the identification of such toxicities (59, 132).

These genetic attributes may also be exploited in formal studies of the genetics of drug responses: a rigorous empirical approach to pharmacogenetics. To date, this has been undertaken only for cardiac repolarization toxicity where the staged assays can be scaled to reach the throughput necessary for the rapid evaluation of novel candidate genes as well as efficient large-scale screens for new genes that modify the effects of specific drugs. For example, in a shelf screen of 340 insertional mutants, investigators identified 15 genes with major effects on repolarization, none of which had previously been implicated in this process (62). The majority of these genes appear to belong to an integrin-associated network modulating channels and their adaptor proteins (**Figure 3**). Recently, in collaboration with investigators in the KORA and Framingham studies performing genome-wide association studies of the QT interval, one of these genes was found to modify human repolarization, confirming the utility of zebrafish screens for gene identification in studies of physiologic pathways (62).

## SYSTEMATIC IN VIVO APPROACHES TO TOXICOLOGY

The examples presented thus far strongly support the use of the zebrafish as a tool in the armamentarium used to explore drug toxicity. At present, the zebrafish is used sparingly in drug discovery, as pharmaceutical companies build experience and comfort with the organism as a model (5, 6, 8). Nevertheless, zebrafish are increasingly used for preclinical toxicology, in vivo studies

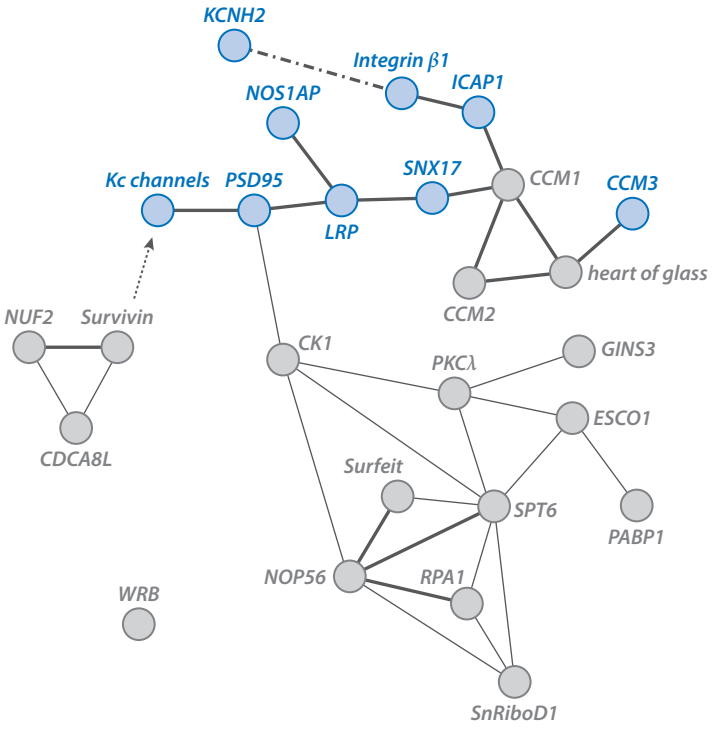


**Figure 3**

A simple interaction diagram depicting interactions between known repolarization genes (*blue symbols*) and the genes identified in a zebrafish shelf screen. Thin lines indicate genetic interactions supported by data from multiple model organisms. Thick lines show direct physical interactions. A dashed line represents a physical interaction that may not be direct. The dashed arrow represents a downstream regulatory effect, the mechanism of which is unknown. This network is conserved in systems ranging from *Drosophila* to humans. Adapted from Reference 62.

of structure-activity relationships, and studies of the mechanisms of drug toxicity (6). The ease with which genes can be manipulated in the zebrafish allows for rapid testing of large numbers of hypotheses generated by functional genomics or other technologies and enables the cost-effective prioritization of experiments for traditional larger-animal models.

Ultimately, the most effective use of the zebrafish may be earlier in the drug discovery pipeline, albeit once the predictive utility of high-throughput zebrafish toxicology assays has been extensively validated and accepted at a regulatory level. The parallel evaluation of chemical libraries for therapeutic effects in zebrafish disease models and toxic effects in a panel of organ-specific toxicity reporter strains would enable the empirical optimization of therapeutic and toxic effects along a spectrum dictated by the disease in question. Irrespective of how the zebrafish is used, the organic growth of our understanding of the biological effects of small molecules in complex systems is likely to prove useful in drug discovery.

## FUTURE DIRECTIONS

The zebrafish occupies an important niche between more traditional representative animal models and tractable lower organisms or in vitro systems. Although often viewed as a cheap alternative to rodents, the zebrafish offers comprehensive vertebrate pathway and cellular context on a scale

hitherto feasible only in cell culture. If we are to maximize the utility of the zebrafish in discovery mode, we will require a much more nuanced understanding of the parallels between the zebrafish and higher organisms in health and in disease. It will be essential to develop more global screening assays, focused not just on previously described toxicities, but also on detecting the unforeseen. An unbiased phenoclustering strategy combining multichannel organ-specific reporters, functional genomics, and automated image analysis may be feasible in the near future, and this would offer significant advantages over current low-throughput toxicity studies in inbred rodent strains. The resultant prospect of systematic exploration of gene-drug, drug-drug, and drug-environment interactions is highly attractive. The ability to predict specific toxicities and to define the modifying effects of genome, microbiome, epigenome, and environment are major milestones in realizing the vision of personalized medicine. As these goals are attained, the role of the zebrafish as a tool for annotation of chemical libraries is likely to expand, moving its use to earlier in the drug discovery pipeline. It is conceivable that with parallel efforts in zebrafish disease modeling that drug discovery and predictive toxicology may eventually occur in parallel, thus enabling direct optimization of therapeutic efficacy and toxicity for each disease context. A robust zebrafish screening platform may be one of the few approaches able to deliver on these promises.



## DISCLOSURE STATEMENT

The authors are founders of Carrick Pharmaceuticals, a company that provides toxicology screening services and discovers novel drugs for important human diseases. R.T.P. is an advisor at Zephase Therapeutics, a company that provides zebrafish toxicology screening. C.A.M. is a consultant for Atlas Ventures. R.T.P. and C.A.M. are supported by the CounterACT Program, Office of the Director, National Institutes of Health (OD), and the National Institute of Neurological Disorders and Stroke (NINDS), NS 063733 and NS 063563.

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**Errata**

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