Chemical Research in Toxicology

Review

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Use of Zebrafish in Drug Discovery Toxicology

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ABSTRACT: Unpredicted human safety events in clinical trials for new drugs are costly in terms of human health and money. The drug discovery industry attempts to minimize those events with diligent preclinical safety testing. Current standard practices are good at preventing toxic compounds from being tested in the clinic; however, false negative preclinical toxicity results are still a reality. Continual improvement must be pursued in the preclinical realm. Higher-quality therapies can be brought forward with more information about potential toxicities and associated mechanisms. The zebrafish model is a bridge between in vitro assays and mammalian in vivo studies. This model is powerful in its breadth of application and tractability for research. In the past two decades, our understanding of disease biology and drug toxicity has grown significantly owing to thousands of studies on this tiny vertebrate. This Review summarizes challenges and strengths of the model, discusses the 3Rs value that it can deliver, highlights translatable and untranslatable biology, and brings together reports from recent studies with zebrafish focusing on new drug discovery toxicology.

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INTRODUCTION

In pharmaceutical drug discovery, the number of compounds discovered in early target- or phenotypic-based screens for drug candidates far outweighs the number that advances as clinical candidates, of which relatively few to none progress to clinical trials.[1−3] This nonclinical attrition is the result of diligent studies conducted in animal models designed to demonstrate bioavailability, efficacy, and safety. During this early phase, depending on the reporting source, between 40 and 80% of the compounds are halted in development due to safety concerns.[4,5] The current practice serves well to provide many safe molecules to clinical studies,[6] yet surprising clinical toxicities are still a reality. An analysis of a pharmaceutical-industry-wide preclinical-to-clinical database[7] showed that overall, the specificity of preclinical toxicology studies is good (>80%), reflecting a low level of false positive predictions of toxicity. However, the sensitivity is low (<50%), reflecting a high false negative rate meaning unpredicted human safety events.

The later they occur during drug development, the more costly unpredicted safety events are in terms of human health and money. Improving preclinical testing practices could mitigate such losses. Advances in investigative toxicology, which aims to derisk safety and reveal toxic mechanisms of discovery candidates, have provided paths toward such improvement.[8,9] Many of these include platforms that are considered alternatives to animal models in line with the 3Rs philosophy toward animal research.[10,11] These include stem cells and other cell cultures,[12,13] 3D tissue models,[14−16] organs on chips,[17] in silico prediction,[18] and machine learning[19] as well as humanized chimeric mouse models for more translatable data[20] and nonmammalian animal testing in animals like the round worm *Caenorhabditis elegans*[21] and teleost fish *Danio rerio* (zebrafish)[22−27] (Figure 1).

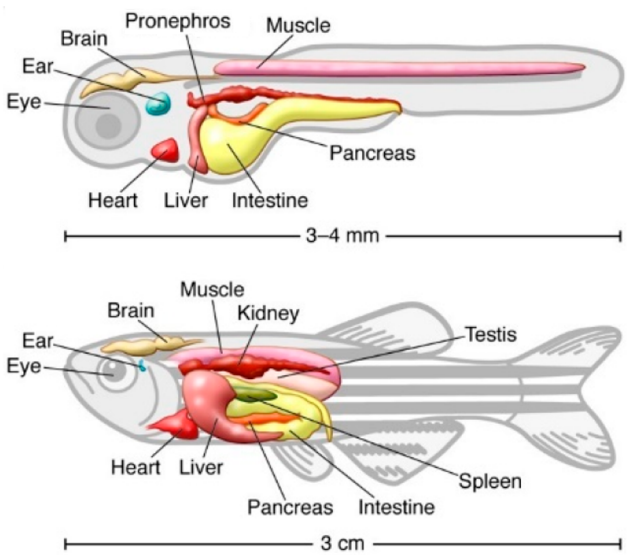

Figure 1. General body plans of a larval (top) and an adult (bottom) zebrafish indicating organ locations. Note the relative lengths of the two stages—approximately 5 days post fertilization for the larva and 3 months for the adult. In body volume, the larval fish is hundreds of times smaller than the adult. Cartoon used with permission from authors of ref 46 and The Journal of Clinical Investigation, copyright 2012.

Low sensitivity for clinical safety issues is the primary focus of efforts to improve preclinical toxicology testing, but mammalian models are also laborious, costly, and fraught with ethical questions concerning animal testing,[28,29] affording other motivations for developing and implementing alternative assays. Regulatory requirements for new drug discoveries are rigorous; thus, acceptance of alternative toxicology assays by government agencies will rely on well-established predictivity of clinical toxicity.[30] Predictivity of an alternative assay can be evaluated independently for each human biological system, organ, tissue, or cell type. This Review brings together literature reporting on potential and actual applications of zebrafish in drug discovery toxicology, sorted into sections based on organ or biological system.

ZEBRAFISH PROVIDE 3RS VALUE TO DRUG DISCOVERY TOXICOLOGY

The 3Rs, replacement, reduction, and refinement of animal studies in research,[10,31,32] has evolved, over time, along with philosophies underlying the use of animals, regulatory directives, and new technologies, the latter of which can be beneficial to science as well as animal welfare. Among new approaches, the zebrafish has arisen as a popular alternative animal model. According to the European Commission Directive from 2010, experiments with the earliest life-stages of some animals are not regulated as animal studies; for zebrafish, independent feeding, which begins around 5 days post fertilization (dpf), is considered the first stage subject to regulation for animal experimentation. Therefore, work with zebrafish embryos/larvae under 5 dpf can be considered an alternative to animal testing. Some controversy exists around this; there are different interpretations of criteria by national and regional authorities, especially around 120−144 h postfertilization (hpf). Supporting the 5 dpf transition, according to a review of the literature and available data,[33] where they include criteria such as yolk consumption, feeding, and swimming behavior, independent feeding becomes evident at 120 hpf.

HOW THE 3 RS APPLY TO ZEBRAFISH

- Replacement: Zebrafish assays using larval zebrafish could be used to replace some animal toxicity studies, first establishing that the larval zebrafish is a relevant model for the system (target, gene, pathway, mechanism, tissue, organ, etc.) with validation studies.
- Reduction: As a first-tier model for toxicity, zebrafish larvae could be used to identify toxic drug candidates, allowing safer molecules to be tested in mammalian models. In the end, this would ultimately reduce the number of animals used in testing.
- Refinement: The embryonic and larval zebrafish model offers refinement to animal study design, because the embryos are fertilized externally and are transparent through the early days of life. This allows for noninvasive observation of toxicities and perhaps recovery.

CHALLENGES OF ZEBRAFISH AS AN ANIMAL MODEL

There are many reasons zebrafish have become a popular laboratory animal, some of which that make them easy to work with are described below. However, this model has several deficiencies compared to mammalian models, making certain

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aspects of toxicology cumbersome to interrogate in zebrafish and raising yet unanswered questions about the translatability of toxic potencies and affected tissues. For example, the most popular method for dosing embryonic and larval zebrafish is through solubilizing chemicals in water (in-water dosing). This poses two issues; one is that chemicals with poor solubility and/or absorption need to be injected into fish to gain sufficient exposure, limiting the pool of chemicals that can be tested in high-throughput screening to those that have good water solubility; the second is that in-water dosing may yield unique exposures compared to typical mammalian routes, as the fish are literally immersed and/or swimming in the treatment solution. How this second issue is manifested, and impacts the translatability of results, needs interrogation and likely varies depending on the biological system and chemicals being investigated.

One of the main strengths of zebrafish as a model organism, their small size, also poses challenges. Nonclinical toxicology studies are relied upon to predict a therapeutic window between efficacious and toxic exposures, guide clinical dosing upper limits based on those exposures, and deliver absorption, distribution, metabolism, and excretion (ADME) data. Toxic in-water dosing levels generated from larval zebrafish studies currently cannot be decidedly related to mammalian plasma levels; more work is needed in this area and, like the questions around unique exposures driven by in-water dosing, will likely depend on the system being interrogated. Methods for measuring plasma levels and ADME from larval zebrafish are currently being pursued but are in their early stages.[34,35]

Finally, because of phylogenetic distance manifested in anatomy and physiology differences, zebrafish will, in general, generate less translatable data to clinical toxicities compared to data from mammalian models. Strategic studies that employ zebrafish to interrogate systems and chemicals that have the highest likelihood of translatability will make the most of this model. Beyond considering gene similarity, congruence of cellular mechanisms, and comparable tissue biology, toxicology studies with zebrafish need to consider the regenerative capacity of the model. Zebrafish can regenerate multiple tissues including the fin, brain, retina, spinal cord, and heart.[36,37] This regenerative capacity may impact translational toxicity end points as has been demonstrated for the retina.[38]

■ ENVIRONMENTAL PARAMETERS AND HUSBANDRY PRACTICES

An inherent advantage of zebrafish is their broad tolerance of environmental conditions.[39] This trait makes it possible to rear and breed these animals for research using a wide array of protocols. Accordingly, there are very few standards for husbandry and management of the fish and their environment, and conventional approaches have long centered around the simple and straightforward goal of producing fish that are free from visible disease, breed well, and survive at rates high enough to complete experiments.[40]

While this flexibility has been a major driver in the growth of the zebrafish model overall, it somewhat paradoxically limits its adoption by the pharmaceutical industry. This is because other animal models (mice, rats, rabbits, dogs) already accepted and widely employed by drug companies for preclinical toxicology studies are, by comparison, well-established models, with commercially available standardized breeds/strains accompanied by robust historical control data. This allows researchers employing these models to exert a degree of control over a number of variables (e.g., diet, genetic background, pathogen status) that they are not able to achieve with fish. Without this level of standardization, preclinical studies using fish may be less reproducible and more challenging to undertake, especially when they involve collaborations among multiple groups at multiple sites.

The problem for the zebrafish model with respect to experimental variability is twofold; the inaccurate and/or incomplete reporting of husbandry and environmental conditions is compounded by the overall lack of available standards for researchers to follow. Reporting can be improved now by using tools like ARRIVE guidelines,[41] protocols.io,[42] or benchmark toxicity concentrations,[43] but the overall lack of standards for diet, health monitoring, and genetic maintenance is a larger, more complicated puzzle to solve.[44] Standards can be developed through improving scientific understanding of these factors to the point where broad recommendations for standards can be made with confidence. At the same time, commercial platforms are needed to support these efforts, for example, in the production of specialized diets and genetically defined and specific-pathogen-free (SPF) fish. At present, the lack of this infrastructure is limiting.

■ VALUE OF ZEBRAFISH AS AN ANIMAL MODEL

Conserved vertebrate biology, ease of husbandry, high fecundity, small size, rapid development, and transparent young are some of the main attractions of zebrafish as an alternative to mammals for toxicology studies. Although there are some major differences related to anatomy and physiology associated with an aquatic species, most zebrafish organs perform the same functions as their human counterparts and exhibit well-conserved physiology.[45,46] Hundreds of adult zebrafish, each only about 4 cm long, can be easily housed in a standard aquarium, and there are several commercial options for automated water quality control and feeding. Successful mating of a single pair results in hundreds of externally fertilized embryos that develop rapidly. During early development, between 48 and 72 hpf, the development of most organs is nearly complete, except for organs in the gastrointestinal (GI) tract. After 76 hpf, the liver, pancreas, and gut are fully developed, and at 96 hpf, the GI tract is completely developed.[47] As at this stage they are small enough to fit into individual wells of multiwell plates, toxicology can be conducted on hundreds of whole organisms in a hand-held platform using an amount of test article that would be required to dose only one or two mice.

The transparency of young zebrafish allows for noninvasive examination of organ development and toxic end points. This, along with the ease of generating transgenic models, lends itself to gene expression and cell-specific reporter assays, offering powerful options for real-time in vivo studies of toxic mechanisms.[48] Biological effects of chemicals have been studied extensively in the zebrafish.[49,50] Exposure to xenobiotics is typically accomplished by simply dissolving a chemical in the water after which phenotypic changes and/or toxic effects can be monitored. Eight small molecules discovered in zebrafish have been advanced into clinical trials,[51−58] illustrating the ability to move fundamental discoveries from zebrafish to humans. The zebrafish genome has been sequenced, and comparison to that of humans reveals that 70% of human genes have a zebrafish homologue, and 82% of human genes associated with disease have a zebrafish homologue.[59] This allows for strategic application of this

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model when homology is high, and molecular players can be expected to interact with xenobiotics in translatable ways. For the reasons above, there is a long history of zebrafish being studied for genetics, cell biology, embryology, and environmental toxicology. Most toxicological studies using zebrafish have focused on environmental contaminants, but an increasing number are emerging in the field of pharmaceutical toxicology.[48,60]

LITERATURE SURVEY

The number of scientific publications mentioning zebrafish has grown steadily (Figure 2); in 2018, there were well over 3000, roughly 3 times the amount from 10 years ago. A similar trend is observed in those publications around zebrafish toxicity, with latest yearly reports being 4 times those from 10 years ago (Figure 2).

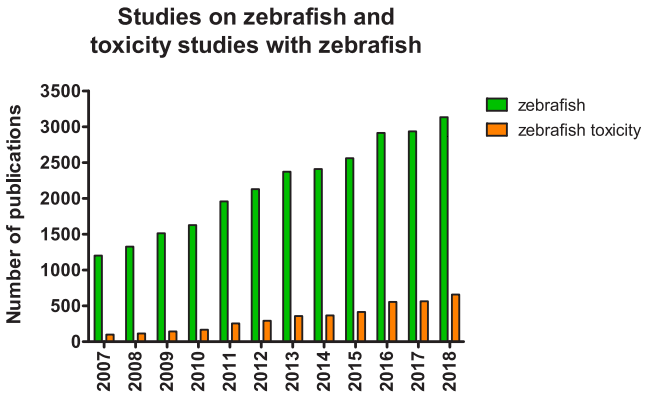

Figure 2. Publications on zebrafish for toxicology have grown over 4-fold in the last 10 years.

These publications originate not only from universities, hospital research centers, and regulatory or governmental entities but also pharma, biotech, chemical, cosmetic, tobacco, and nutraceutical companies. In recent years, the developmental, hepatic, and nervous systems are the main systems being interrogated for toxicity using zebrafish (Figure 3).

SYSTEMS PHARMACOLOGY

Given that it is important to understand the capabilities and limitations of mammalian models, the need is even greater for animal models that are phylogenetically further removed from humans like zebrafish.[45] Interspecies translation will improve if toxicologists move from an empirical to a mechanistic approach, in which multiple components of a biological system and their interactions can be monitored. Systems pharmacology is such an approach, combining the strengths of systems biology and pharmacometrics.[47,61,62] It integrates modeling and simulations with preclinical and clinical data. Combining omics data with mechanistic and computational models can improve prediction of toxicity. Data that contribute to basic understanding of a toxicity can be gathered from in vitro experiments; however, as organ toxicities are complex, in vivo whole organism experiments, such as those using zebrafish larvae, deliver more detail to the understanding of the system. Combining all approaches will improve interspecies translation by understanding biological processes in a model organism and understanding how these systems differ among species.

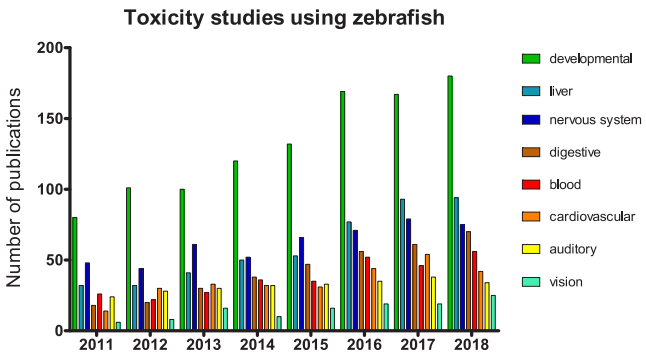

Figure 3. Numerous systems can be interrogated for toxic end points using zebrafish, and the number of publications is trending upward, as indicated by these examples.

EMBRYO TOXICITY

Zebrafish embryos are increasingly used for developmental toxicity screening of candidate drugs and chemicals (Figure 3). The zebrafish embryo model is already accepted as a validated alternative assay to assess fish acute toxicity (OECD, No. 236),[63] and currently, the zebrafish embryo is also being explored as a potential replacement for one of the regulatory in vivo mammalian embryofetal developmental toxicity studies in view of the upcoming third revision of the ICH S5 guideline on detection of the toxicity to reproduction for human pharmaceuticals.[64] In the 2015 final concept paper of this guideline, it is stated that in vitro, ex vivo, and nonmammalian in vivo assays are considered not to be the default approach for developmental toxicity testing but might be considered for regulatory purposes under limited circumstances.[65] The 2017 draft of the revised guideline also includes a section on the qualification of alternative test systems for regulatory acceptance.[64] The zebrafish embryo is of particular interest, as effects are assessed in a whole vertebrate organism during the entire period of organogenesis, in contrast to other alternative assays such as the whole embryo culture and embryonic stem cell test. Additionally, zebrafish embryo assays are less time-consuming and costly than the embryofetal development studies in rats and rabbits. This increases throughput for pharmaceutical and chemical companies, and if accepted as a regulatory test, this could also lead to replacement and/or reduction of animal studies in line with the 3Rs principle in animal research.

In general, concordance with the findings in mammalian developmental toxicity studies is high, reaching up to 85[66] or 87%[67] in some laboratories, but false negative and positive results are reported, and the results for the same compound can vary substantially between laboratories.[66−74] The latter can be explained by the large diversity in protocols, such as study design[70] and calculation of the teratogenic index,[66,67,71−74] indicating a clear need for harmonization of this alternative assay when considering it for more than internal decision making. There may be several other reasons for discordance between laboratories and false negative/positive results in the zebrafish embryo developmental toxicity assay, such as internal concentration (compound uptake), metabolism, and species differences in mode of action. Compound uptake has already been addressed as a challenge earlier in this manuscript. Although the chorion is removed by some groups to ensure adequate exposure of the embryos,[67] the former does not appear to be the major cause of impaired uptake for most small molecules.[66] Solubility can be an issue for some compounds, especially at higher concentrations,[73] and the actual exposure

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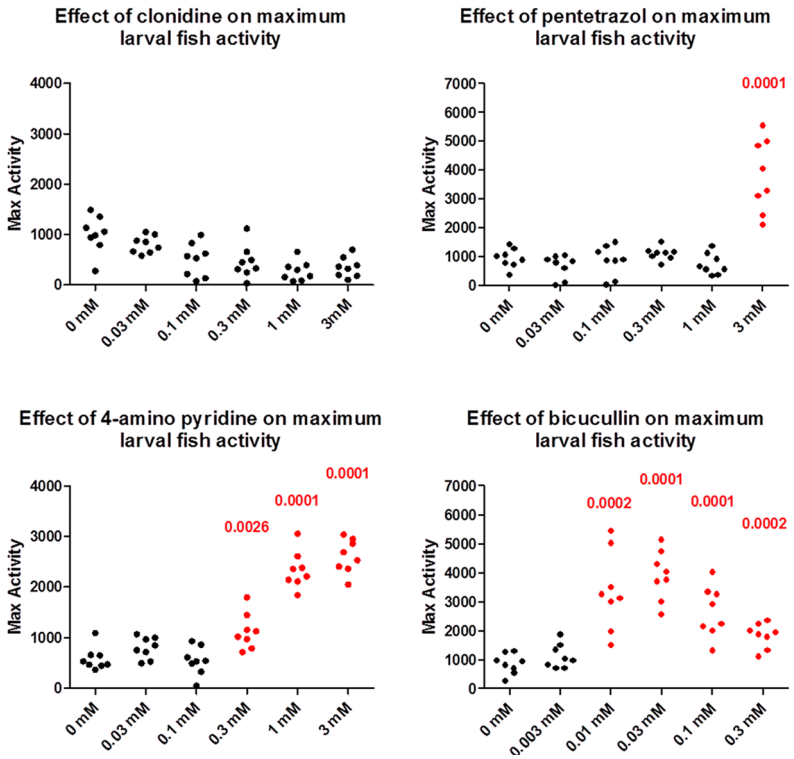

Figure 4. Example results from a zebrafish locomotor assay for seizure liability. Drugs which interact through different mechanisms with the nervous system evoke activity responses with different potencies. Each dot represents the maximum activity for a single larva. Red dots indicate that the group average was significantly higher than that of the vehicle (0 mM) group; *p*-values are given in red. Copied with permission from ref 92 and Elsevier, copyright 2019.

of the zebrafish embryos may be overestimated when assessing the medium concentrations without analysis of the internal concentrations.[68] Recently, efforts have been undertaken to better predict embryotoxic medium concentrations in the zebrafish embryo assay and link them with embryotoxic rat plasma concentrations.[75,76] As such, the assay can be refined and its predictivity enhanced. Regarding metabolism, it is generally accepted that the intrinsic biotransformation capacity of zebrafish embryos is low up to 72 hpf, especially for cytochrome P450 mediated reactions,[77,78] and that exogenous metabolic activation, e.g., by rat liver microsomes, increases the sensitivity of the assay for some compounds.[79] The latter should be kept in mind when negative results are obtained in the zebrafish embryo assay and the compound appears to be highly metabolized in man. Also, species differences in the mode of action of compounds need to be considered when using the zebrafish embryo assay. For instance, ribavirin has been reported as negative for zebrafish embryotoxicity but causes malformations in mammals by accumulation of ribavirin triphosphate in the erythrocytes.[74] As zebrafish have nucleated erythrocytes and no accumulation of ribavirin triphosphate can occur, this may be a reason for the lack of malformations in this species. Finally, although organogenesis in zebrafish is well-characterized,[80] the number of morphological end points evaluated in the zebrafish embryo may need to be extended to increase the sensitivity of the assay. The number of end points that are evaluated in the currently used zebrafish embryo protocols is much more limited compared to the exhaustive list of external, visceral, and skeletal end points in the in vivo mammalian embryofetal development studies.[81]

NEUROTOXICITY AND BEHAVIORAL ANALYSES

The zebrafish is being widely applied to study the mechanisms and pathogenesis of neurological disorders and diseases,[82−84] with great promise for drug discovery and toxicity testing in this realm.[85−87] The central nervous system of the zebrafish is similarly organized to that of other vertebrates and is well-described at multiple life-stages.[88] The main structures, as well as many principal subdivisions of the brain, are found in the zebrafish,[89] and behavioral studies have identified strong associations between the functions of zebrafish and human brain regions.[90] One brain region that zebrafish do not have is a neocortex, so they cannot be used to model cognitive processes that rely on that region.[91] Neurotransmitter systems, such as dopamine, GABA, glutamate, noradrenaline, serotonin, histamine, and acetylcholine, are present in zebrafish[92−96] and can serve as pharmacological and toxicological targets.

Locomotion is a complex behavior that requires an integrated response of the brain function, nervous system, and visual pathway. Given so, this behavioral pattern, in conjunction with automatic tracking methods, is increasingly gaining attention for its use in high-throughput screening of neurotoxic compounds.[97] Embryos show a basic swimming capacity right after hatching, which is then refined to a beat and glide mode of swimming after 4 dpf.[98] The startle reflex in response to tactile, visual, or auditory stimuli appears around 5 dpf.[99] Adult zebrafish are able to display a varied repertoire of complex behaviors including memory and learning, social interactions, or prey hunting.[89]

The photomotor response assay, consisting of the automatic tracking of larval movement in response to alternative illumination conditions, is extensively used for the screening

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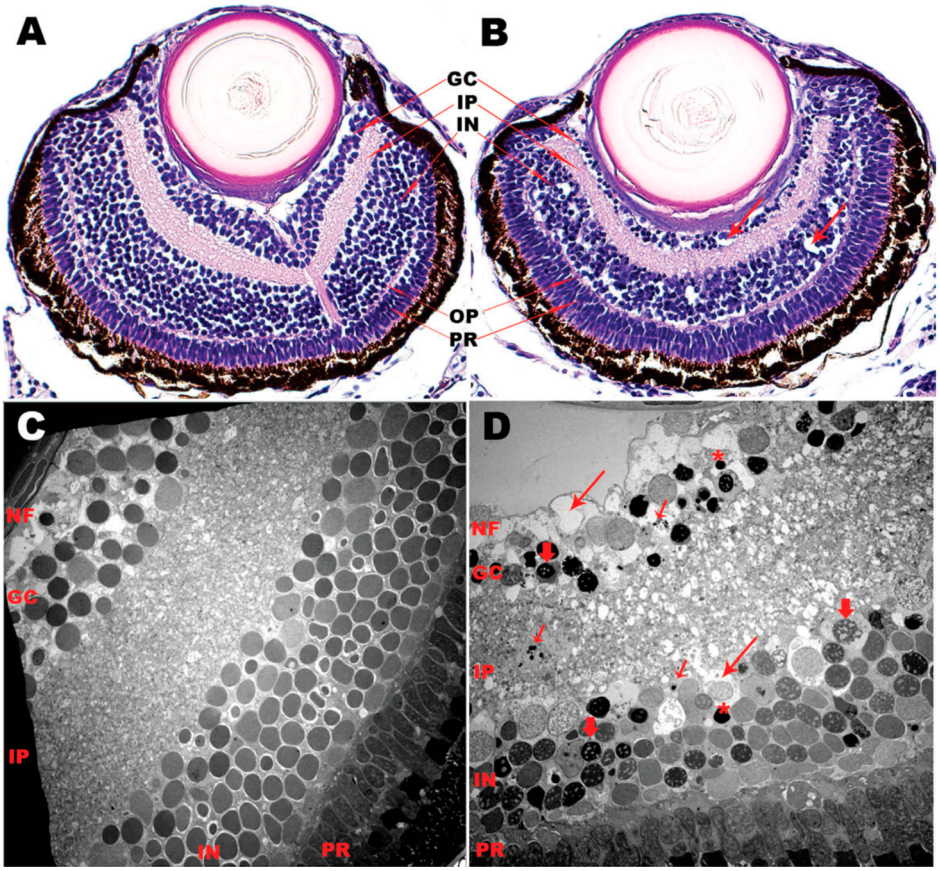

Figure 5. Representative ocular histology (top, 40×) and transmission electron microscopy (2000×) from larval zebrafish treated with vehicle (A + C) or a chemotherapeutic discovery compound (B + D). The vehicle-treated eye (A) depicts normal retinal features with homogeneously spaced cells. The retina from the compound-treated larva has decreased cell numbers in both the ganglion (GC) and inner nuclear (IN) layers (red arrows in B), and the inner plexiform (IP) layer has a disorganized, vacuolated appearance. The electron microscopic images reveal evenly spaced nuclei in the ganglion and inner nuclear layers for the vehicle-treated larva (C) but apoptosis (asterisk and red arrows), nuclear vacuolation/fragmentation (red block arrows), and vacuolation, associated with cell loss, in the retinal layers of the compound-treated larva. Similar effects were found in retinas of rats and dogs treated with that compound. NF, nerve fiber layer; OP, outer plexiform layer; PR, photoreceptor layer. Copied with permission from ref 84 and Oxford University Press, copyright 2014.

of neurotoxic effects.[85,100] The principle of this assay relies on the specific patterns in response to the illumination transition. The light−dark transition increases the locomotor activity, while the dark−light transition decreases locomotor activity. Convulsant drugs, such as the GABA receptor antagonists pentylenetetrazol and picrotoxin cause a dose-dependent locomotor activity increase,[93,101,102] and antiepileptic drugs such as diphenylhydantoin and valproic acid produce a decrease in locomotor activity[103] (for examples of data from other neuronally active drugs, see Figure 4). The locomotor effects induced by these drugs are similar to those observed in rodents. In the case of some environmental hazards such as flame retardants, this assay has shown a similar sensitivity and value for predicting human neurotoxicity.[104] However, non-translatable results have also been reported; for instance, exposure to venlafaxine showed an opposite effect to that observed in rodents.[105]

The touch-evoked response test, which tracks behavior of zebrafish larvae in response to a tactile stimulus given to the head or tail, is an indicator of the integration of sensory and motor function.[99] For instance, larvae exposed to endosulfan sulfate and fipronil showed a decreased reactiveness in this assay.[106,107] Assays based on the startle response to other stimuli such as visual and acoustic stimuli have also been useful to screen for neurotoxic effects.[85,108,109]

Likewise, adult zebrafish can be used to identify the anxiety-like effects of new pharmaceuticals in preclinical screenings. The most common models are the novel tank test (NTT) and the light−dark test (LDT). The NTT principle is similar to the rodent open field test. It relies on instinctive diving behavior in response to an unfamiliar environment, which diminishes with time.[110] The LDT is based on fish scototaxis (innate preference for dark vs light areas). This behavior is associated with the natural tendency of wild zebrafish to prefer dark environments to avoid detection by potential predators.[111]

Recent advances of zebrafish in the field of neurology show great promise for future utility in studying disease. Neurologic-disease-associated genes are conserved in the zebrafish, enabling identification of molecular drug targets.[112−114] The ex vivo development of the zebrafish embryos allows observation of these conserved proteins with fluorescent tags, and the ease of genetic manipulation in the zebrafish has led to the creation of several zebrafish neurologic transgenic models. These models permit studies interrogating neurodevelopmental disorders such as autism,[115] neuropsychiatric disorders including depression and anxiety,[116,117] and neurodegenerative diseases such as Alzheimer's and Parkinson's disease.[118−121]

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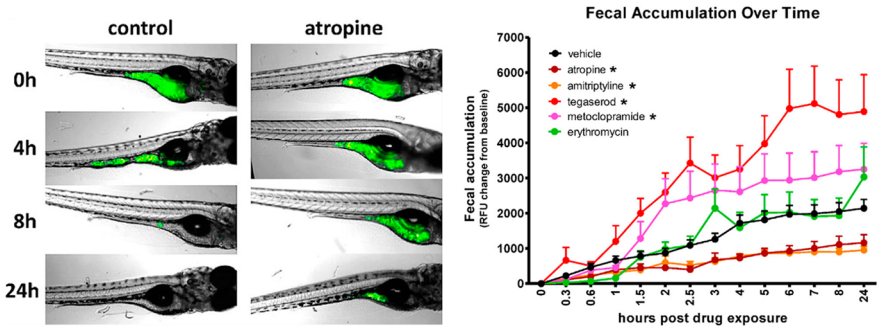

Figure 6. Fluorescent food transit can be detected and measured as a loss of signal over time from microscopic imaging (left) or, in a high-throughput fashion, from a corresponding gain in signal by plate-based spectrophotometry (right). The latter method allows the measurement of intestinal transit from dozens of larvae simultaneously in a microwell plate. Modified with permission from ref 143 and Elsevier, copyright 2015.

Similar to other disease pathways, promise of the application of the CRISPR-Cas9 system to generate zebrafish knock-in and knock-out mutations is now set to rapidly expand the availability of transgenic models for drug discovery for neurological diseases and disorders.[122]

OCULAR TOXICITY

The vertebrate eye is highly conserved;[123] thus, zebrafish offer an excellent model for studying ocular toxicity. Besides obvious anatomical similarities, including the cornea, lens, choroid, and retina as well as vascularization and innervation, the zebrafish and human eye have conserved gene expression, cellular makeup, and tissue architecture.[124,125]

Vision develops rapidly in zebrafish embryos, demonstrated by the reliance of 4 dpf larvae on visual cues for predation and evasive behavior.[126] Primary retinal cell types are organized into recognizable layers, and the optic tectum is innervated by axons from the ganglion layer by 3 dpf.[127] Homologous retinal layers and cell types are found in zebrafish for all mammalian counterparts;[127−129] these include the nerve fiber, ganglion, inner and outer plexiform, inner and outer nuclear, photoreceptor, and the pigmented epithelium.[127,129] Zebrafish are diurnal and have color vision owing to a cone-dense retina, like that of humans; comparatively, rats and mice have relatively fewer cones and weak color vision.[128]

Even though the zebrafish retina is cone-dense, they lack a fovea, or a cone-concentrated area. Some other differences in zebrafish compared to mammalian retinas are that there are more cones than rods in zebrafish retinas, and they have cones that are sensitive to ultraviolet radiation as well as double cones that consist of a red-sensitive (principle) compartment and a green-sensitive (accessory) compartment.[129] A key difference pertaining to the use of zebrafish as a model of toxicity is that the zebrafish retina (larval and adult) can regenerate. Retinal progenitor cells with the capacity to differentiate into any of the primary retinal neurons are derived from dividing Müller glial cells in response to tissue injury.[130]

Overall, the zebrafish retina is vascularized similarly to that of mammals, but there are some differences. Like mammals, angiogenesis from a central retinal artery forms the vasculature. And, initially, hyaloid vasculature is found in the lens but diminishes with age. The ganglion cell layer is vascularized throughout adulthood.[131] However, unlike mature mammalian retinal vascularization, the inner and outer plexiform layers of the zebrafish retina are not vascularized. It has been proposed that less vascularization is needed for the thinner zebrafish retina, because it can rely on diffusion from surface vessels (hyaloid, choroid).[131]

Given the above-mentioned similarities between zebrafish and mammalian eyes, it is no surprise that several investigators have evaluated zebrafish as a model to interrogate pharmaceutical ocular toxicities. These studies have demonstrated concordance between results from zebrafish vision assays and human ocular reactions to drugs,[132−134] including chlorpromazine, cisplatin, gentamicin, quinine, deferoxamine, minoxidil, thioridazine, and vardenafil, among others. By testing drugs with no established human ocular effects as well as the oculartoxic drugs, those authors reported that the zebrafish assays were sensitive 68−83% of the time and specific 75−100% of the time, demonstrating utility for detecting oculartoxic chemicals. The optokinetic[135,136] and optomotor response[137] assays are two common ways to evaluate vision in adult and larval zebrafish. In the optokinetic assay, dark and light alternating vertical stripes are passed around an immobilized fish, and eye saccades are counted as an indicator of a healthy eye response to moving stimuli; in the optomotor assay, the fish are free swimming and allowed to respond to temporal or spatial changes in light. This latter assay often uses a second, comparator, stimulus (sound or touch) to assess general mobility. Both assays typically rely on video recording to help assess response. Recently, zebrafish larvae were used for detecting retinal toxicity in pharmaceutical pipeline discovery compounds, replacing studies in mammalian models[85] and demonstrating clear value from zebrafish for drug discovery toxicology (Figure 5).

INTESTINE, PANCREAS, AND HEPATOBILIARY TOXICITY

The postesophageal digestive system in zebrafish consists of the intestine, pancreas, liver, and gall bladder. Among these organs, there are similarities and differences between zebrafish and mammals that should be considered when planning studies with, and analyzing results from, zebrafish. Numerous reports have highlighted the utility of zebrafish for interrogating drug toxicities on the digestive system.

Intestine. Zebafish are agastric throughout their lives, but the anterior portion of the intestine, referred to as the intestinal bulb, has an enlarged lumen compared to the rest of the intestine and can act as a food reservoir; however, the intestinal bulb lacks gastric glands and thus has a neutral pH.[138] Other similarities and differences concerning intestinal anatomy and physiology are reviewed in Brugman.[139] Important similarities for drug toxicology are that, like mammals, intestinal

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movement relies on smooth muscles controlled by the enteric nervous system[140] and that the intestine consists of an epithelium containing villi, enterocytes, goblet cells, and enteroendocrine cells. The epithelial cell maturation, differentiation, and turnover are like those of mammals.[141] The key differences between zebrafish and mammalian intestines lie in the absence of structures or cell types in zebrafish. Missing from zebrafish are intestinal crypts, Peyer's patches, Paneth cells, and a submucosal layer; smooth muscles connect directly to the mucosal layer.[139]

Zebrafish have been used extensively to model intestinal disorders, including investigations into microbiome influences, congenital disorders, intestinal inflammation, enteric nervous system/motility disorders, and intestinal tumorigenesis.[142] Methods for imaging gut movement and measuring gut transit time in zebrafish have been recently improved[143,144] allowing for higher sensitivity and throughput for toxicology (Figure 6). However, reports demonstrating the use of zebrafish for detecting potential intestinal toxicities from compounds in drug development are uncommon. Two reports, focused on assessing the predictive value of zebrafish for intestinal/gut toxicities, have been published. Those reports[132,143] tested marketed drugs with and without GI effects in humans for effects on zebrafish gut contractility or transit time, respectively. Gut contractility was reportedly highly variable among the larval fish tested. This variability may have been caused by the poor/inconsistent bioavailability of some of the drugs, according to the authors. The gut transit time was also highly variable; however, by testing more (up to 24) larvae per treatment group, the sensitivity of the assay was improved. Both reports indicate that false negative results (i.e., no toxicity detected, where it was expected) are more common than false positive results. This may be due to low absorption for some compounds but also may be driven by the lack of certain cell types/structures in the zebrafish intestine or the agastric nature of the model. However, as a first-tier screen for intestinal toxicities, based on these reports, the zebrafish would be expected to identify >50% of toxic discovery compounds, allowing for prioritization of safer compounds for mammalian testing.

Pancreas. The basic structure and function of the pancreas is conserved between zebrafish and mammals. An exocrine compartment secretes enzymes into the digestive tract, and an endocrine compartment produces insulin, glucagon, somatostatin, and ghrelin. Polypeptide-producing cells (PP cells) have not been positively identified in zebrafish pancreas.[145] Most studies on pancreas using zebrafish have focused on the endocrine compartment, examining islet damage/protection for the sake of diabetes research.[146] As an indicator of potential to translate chemical toxicity in the zebrafish pancreas to mammalian models/humans, these studies use challenge chemicals to damage zebrafish islets that are commonly used in mammalian models to drive the same ends.[147−150] Reports on the chemical toxicity of zebrafish exocrine pancreas are focused on embryo developmental toxicity driven by environmental contaminants;[151,152] these are being used to expose sensitivities of the developing pancreas to common environmental pollutants as well as to shed light on cellular mechanisms involved in those toxicities.

Liver. Drug-induced liver injury (DILI) is a major concern in the drug development industry.[153,154] The most common assays for hepatotoxicity testing in drug development use human hepatocytes or HepG2 cells in culture. Two-dimensional (2D) and 3D in vitro human tissue models, including multiple cell types, are becoming popular, as they may more closely reflect what happens in the whole organ.[155] The zebrafish liver performs the same functions as those of the human liver[156] and is fully functional 5 days post fertilization, so larval zebrafish can be used to interrogate xenobiotics for hepatotoxicity in an in vitro format, providing whole organ/whole animal data from multiwell plates.[157] Gene expression analysis after exposure to established human hepatotoxins revealed similar gene changes among in vitro models (human hepatocytes, mouse hepatocytes, rat hepatocytes, and zebrafish embryos) and in vivo models (mouse liver, rat liver, and zebrafish embryos), highlighting that zebrafish embryos have in vitro- and in vivo-like properties.[158] As reported to date, there is good overlap in the metabolic capacity of the zebrafish liver and that of humans.[159,160] The common metabolic pathways indicate a potential to predict hepatotoxic metabolites using zebrafish. The tissue architecture of the zebrafish liver differs from mammalian livers in that it is less organized, but it contains all the same cell types.[156,160,161] In zebrafish, hepatocytes are arranged in tubules, rather than bilayered plates, as in mammals. Small bile ducts are found within the hepatocyte tubules, and these function to transfer bile to the gall bladder.[161,162]

Based on the biological similarities given above, and the relative ease for whole liver toxicology, the zebrafish offers an attractive model.[163] Fluorescent markers for various cell types make it possible to visualize and quantify cell-specific compound effects in the zebrafish liver in real-time.[156,164,165] More simpler techniques employ lipid stains, like oil-red-O, to visualize gross liver changes in the larval zebrafish.[166] A study conducted by two major pharmaceutical companies demonstrated added predictive value from zebrafish liver toxicity assays for predicting DILI when they were used in conjunction with high-content cellular (hepatocyte) toxicity assays.[167] Other studies have also shown congruence between zebrafish and human DILI using marketed drugs with known hepatotoxicity, like tetracycline, aspirin, erythromycin, cyclosporin A, amiodarone, and acetaminophen.[25,27,158,168,169]

Zebrafish have delivered popular models for demonstrating, or investigating mechanisms of, the hepatotoxicity from traditional medicines[170−172] and environmental contaminants, including those of pharmaceutical[173−175] and of other industrial origin.[176−178] In addition to these applications, zebrafish hepatotoxicity assays are being directly employed in early drug discovery toxicology. Evidence for this can be found in recent reports on studies designed to discover liver-protective compounds against known hepatotoxins,[179] improve formulations or therapeutic indices for promising chemotherapies,[180,181] and demonstrate novel compound efficacy in a zebrafish autoimmune disease model, which was coupled with an assessment of hepatotoxicity.[182]

Gall Bladder. Lipophilic dyes can be used to visualize both lipid metabolism and transport in vivo in larval zebrafish.[183] Lipid uptake, enzymatic metabolism, and transport in zebrafish have high homology to the human processes.[184] Recent efforts at AbbVie have focused on using the reporter dye, PED6 (*N*-([6-(2,4-dinitro-phenyl)amino]hexanoyl)-1-palmitoyl-2-BODIPY-FLpentanoyl-*sn*-glycerol-3-phosphoethanolamine)[183] to monitor for biliary toxicities from internal discovery compounds in zebrafish. PED6, as well as fluorescent transgenic zebrafish expressing reporter tags in gall bladder cells, have been used to identify biliary toxins and their mecha-

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nisms.[185−188] Although there appear to be no reports yet on the use of zebrafish for early drug discovery biliary toxicity, the utility is evident, and this is likely an underutilized application of the model.

■ NEPHROTOXICITY

The kidney is especially vulnerable to xenobiotic toxicity, because its function to remove toxic molecules from circulation relies on higher blood exposure than other organs. Thus, monitoring biomarkers that track kidney failure and kidney histology are primary end points in preclinical toxicology.[189] Based on tractability of zebrafish larvae for research into vertebrate biology, they have recently been used to model kidney disease or nephrotoxicity.[190−193]

The higher complexity of the mammalian kidney compared to that of zebrafish renders comprehensive modeling of kidney toxicity in zebrafish unpractical. However, nephrotoxicity can be reasonably interrogated with this model. The zebrafish larval pronephros has a simpler anatomy than that of the mammalian metanephros; it consists of only two nephrons with fused glomeruli. Despite the simple anatomy, similarities between the cellular constitution and function of the zebrafish pronephros and those of the mammalian metanephros make the zebrafish attractive. The zebrafish glomerulus contains fenestrated capillary endothelial cells and podocytes. Also, polarized epithelial cells with primary cilia line the nephron tubules, which are segmented into specialized regions for differential secretion from, and reabsorption into, the blood.[194]

Several investigators have demonstrated proof-of-concept that larval zebrafish can be used to monitor for drug-induced nephrotoxicity. Treatment with puromycin or knockdown of either CD2-associated protein or podicin caused effacement of podocytes and proteinuria;[195] these authors also introduced a fluorescent tracer (FITC-labeled 70 kDa dextran) for live quantification of podocyte failure. More recently, electron microscopy was used to visualize the glomerular injury induced by puromycin in larval zebrafish.[196] Three drugs known to cause tubulopathy in humans (paracetamol, gentamicin, and tenofovir) were shown to induce functional, morphological, and histopathological kidney changes in zebrafish larvae.[192] Metabolic changes in biomarkers (purines, glutathione, and amino acids) associated with acute kidney injury were detected in larval zebrafish exposed to those same three drugs.[197] Beyond proof-of-concept for detecting nephrotoxicity, the larval zebrafish are being used in phenotypic screens to discover compounds with a potential to repair acute kidney injury.[198]

■ ENDOCRINE TOXICITY

The activity of the endocrine system in vertebrates is mostly controlled by the hypothalamic−pituitary axis. The hypothalamus controls the activity of the adenohypophyseal through the release of neuroendocrine peptides, to which adenohypophyseal cells respond by secretion of specific hormones to evoke peripheral organ responses.[199] Although their regulation and effects are tightly interconnected, the hypothalamic−pituitary axis can be functionally subdivided into the hypothalamic−pituitary−adrenal (HPA) axis, related mainly with stress and immune responses; the hypothalamic−pituitary−thyroid (HPT) axis, correlated with metabolism; and the hypothalamic−pituitary−gonadal (HPG) axis, related to reproduction.[200]

The structure and function of the endocrine system are strongly conserved among vertebrates, even if some differences can be observed between humans and zebrafish.[201] Terrestrial vertebrates present a hypothalamic−pituitary portal system. Consequently, neuroendocrine peptides produced in the hypothalamus are secreted to the blood vessels and reach the adenohypophysis through the bloodstream. In contrast, in zebrafish, no hypothalamic−pituitary portal system is present, and the neurosecretory fibers enter the pituitary and release their hormones directly onto the adenohypophyseal cells.[202] Similarly, zebrafish lack a distinctive adrenal gland but present a functionally comparable interrenal gland. This organ has no separation between the adrenal cortex and the medulla, containing both steroidogenic and chromaffin cells.[201] Nevertheless, development of the steroidogenic cell lineage is well-conserved. For instance, both mammalian NR5A1 and its zebrafish homologue nr5a1a are essential for adrenal and interrenal gland development, respectively,[203,204] as well as for activation of the side-chain cleavage enzyme cyp11a1, which is the rate-limiting enzyme in steroid biosynthesis.[205]

Most of the endocrine system in zebrafish develops during the first 5 dpf.[201] Pituitary hormone gene expression starts at 48 hpf.[206] Estrogen receptors are already present at 24 hpf,[207] and aromatase gene expression, coding for the enzyme that catalyzes the biosynthesis of estrogens from precursor androgens on the brain, can be detected from 24 hpf.[208] Key steroidogenic gene expression of the interrenal organ originates at 2 dpf.[209] Thyroxin production of the thyroid gland starts at 3 dpf.[210]

Endocrine-disrupting chemicals (EDCs) are of high relevance for human and wildlife health, since endocrine signaling controls many essential physiological processes that impact the individual's health, such as growth and development, stress response, and ultimately reproduction and population development.[201] Studies on endocrine disruption in fish have focused mainly on the estrogen, androgen, thyroid, and steroidogenesis (EATS) pathways, in the context of environmental risk assessment of new chemical substances.[211]

Specific chemical biomarkers can be measured in zebrafish to screen for the endocrine activity of xenobiotics. For instance, one of the most commonly assessed responses is the induction of vitellogenin (vtg), an egg-yolk protein precursor produced in the liver and induced by estrogenic exposure.[211] VTG is strongly induced after exposure to 10 ng/L of ethinylestradiol.[212] In the same way, other markers related to endocrine activity such as thyroidal[213,214] or steroidal hormones[215] may be of interest but not amenable to high-throughput assays.

Gene expression analysis has been a useful method to identify specific pathways involved in the biological effects of EDC on zebrafish embryos. For instance, exposure to fadrozole led to a downregulation of vitellogenin (vtg) and brain aromatase (cyp19a1b) transcript levels in 96 hpf embryos.[216] This key event produced an apical adverse effect of the sex ratio shifting toward male during sexual differentiation, indicating that the existing adverse outcome pathway (AOP) for aromatase inhibition in fish can be translated to the life-stage of sexual differentiation. A microarray study demonstrated the compensatory induction of androgen-pathway-related genes, sult2st3 and cyp2k22, after exposure to steroidal hormones.[217] In the same way, expression of HPT axis-related marker genes thyroperoxidase (tpo), transthyretin (ttr), thyroid receptor α (trα), and deiodinase 2 (dio2) were altered

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in zebrafish embryos after exposure to perfluorinated compounds,[218] polybrominated diphenyl ethers (PBDEs),[219] and triazole fungicides,[220] all well-established human endocrine disruptors.[221,222]

Multiple fluorescent reporter lines have been developed for testing of xenobiotics for endocrine activity. The EASZY assay makes use of the tg(cyp19a1b-GFP) transgenic zebrafish line to screen for estrogen active substances. This line expresses GFP in a concentration-dependent manner under the control of the gene encoding for the cyp19a1b brain aromatase.[223] The model is highly sensitive to natural estrogens such as E2 (17ß-estradiol) as well as synthetic estrogens such as 17a-ethinylestradiol or diethylstilbestrol at nanomolar concentrations. The EASZY assay has been recently used to monitor estrogenic activities of waste and surface waters sampled across Europe.[224] Another estrogen-responsive model was developed in a pigment-free "Casper" phenotype to identify the specific target tissues and quantify the response in whole fish.[225] In the same way, different reporter lines for thyroid[226,227] and glucocorticoid[228] pathways are available. Those reporter lines are fast and cost-effective methods for the detection of the endocrine activity of xenobiotics and hence have considerable potential for both a high-content and high-throughput screen of endocrine disruptors.[27]

■ HEMATOLOGIC TOXICITY

Zebrafish have many blood cell types that are consistent with human peripheral blood cells. This includes red blood cells, neutrophils, monocytes, macrophages, T and B cells, and dendritic cells. The equivalent of a platelet is the thrombocyte. Reporters for each of these lineages have been generated as tissue-specific promoters driving fluorescent proteins. It is therefore possible to visualize all the blood cell types in different colors and evaluate toxicity.

Zebrafish hematopoietic stem cells have been discovered based on GFP reporters.[229] It is possible to transplant the cells into irradiated adult fish, and the graft shows long-term stability for more than a year. There are many studies evaluating the trafficking of blood stem cells and their developmental biology. Most of the processes are conserved with those of humans.

A number of zebrafish mutants exist that represent hematologic disorders. In the original screen, there were 26 mutant complementation groups, and five of those genes proved to be novel genes, which later became associated with human diseases.[230] It is possible to use zebrafish models and undertake genetic or chemical suppressor screens. This would potentially lead to novel therapeutics.

A few studies on hematologic toxicology have been undertaken using zebrafish. The hematopoietic system can be assayed in many ways.[231] Using a double transgenic fish that has a GATA-1 promoter driving dsRED and globin LCR driving GFP, the stages of erythroid development can be studied.[232] Adding drugs of the phenyl hydrazine family leads to lysis specifically of the mature red blood cells. This establishes a facile system for the evaluation of toxicity of chemicals and drugs.

■ CARDIOVASCULAR TOXICITY

Cardiovascular physiology is conserved between humans and zebrafish at anatomical, cellular, and membrane-biology levels. Zebrafish have been shown to provide a good model for cardiotoxicity. Many human cardiovascular drugs have shown comparable effects on zebrafish physiology,[233] and numerous human cardiovascular disorders have been recapitulated in zebrafish genetic models.[234] Systematic studies of drugs that cause QT prolongation in humans show a >95% conservation of effect in zebrafish.[45] QT prolongation is a common cardiotoxicity discovered during new drug development.

Milan et al.[235] developed an automated, high-throughput assay for bradycardia in zebrafish embryos, which was shown to correlate with QT prolongation in humans. They tested 100 compounds in the assay and showed that 22 of 23 drugs known to cause QT prolongation in humans cause bradycardia in zebrafish. In addition, the assay was able to detect drug–drug interactions that lead to QT prolongation, such as the well-known synergistic interactions between erythromycin and cisapride and between cimetidine and terfenadine. These interactions result from the physiological effects of one compound influencing the metabolism of the second compound and can only be detected in a whole organism. These results highlight the value of performing toxicity studies in zebrafish—zebrafish assays can achieve the scale and throughput of in vitro assays, but they occur in a relevant physiological setting, in which complex pharmacokinetic and pharmacodynamic processes remain intact.[60] In early work, it was possible to show that more than 90% of drugs that cause repolarization toxicity in humans result in cognate electrophysiological effects in the zebrafish even as early as 48 hpf. Based on those data from 100 drugs, the specificity for reporting on repolarization toxicity in humans was 76%; the sensitivity was 80%, which increased to 96% when poorly absorbed drugs were injected.[235]

Letamendia et al.[236] also showed how zebrafish is a model amenable for the automation of cardiotoxicity screenings. They developed an automated high-throughput platform for in vivo chemical screening on zebrafish embryos that includes automated methods for embryo dispensation, compound delivery, incubation, imaging, and analysis of the results. A validation of this platform with known positive and negative compounds was successfully carried out based on the rationale of compounds inhibiting the zERG channel, similar to the hERG channel in humans. This produces a 2:1 atrio:ventricular arrhythmia that resembles the QT prolongation in humans.

In vitro hERG binding and patchclamp assays are accepted by both the pharmaceutical industry and regulatory bodies as suitable methods for investigating the potential of compounds to cause QT prolongation. However, these assays only measure the affinity of compounds for the hERG channel. By comparison, the distinctive 2:1 atrial:ventricular arrhythmia observed in larval zebrafish can be used to identify compounds that block not only the ERG channel but also other cardiac ion channels. While this could be argued as advantageous, because the zebrafish assay can identify cardiotoxicity arising from the blockage of myriad ion channels, it raises problems when investigators attempt to compare their results with those from single target in vitro assays. Data indicating cardiotoxic effects in zebrafish from compounds that are apparently safe according to in vitro data sets might be interpreted as false positive results from the zebrafish; however, they may be caused by the compound acting on a target not represented in in vitro models.[237]

Moreover, zebrafish can regenerate heart muscle. Several research groups are working to discover factors involved in this process to help develop methods of repairing heart tissue in

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humans. One group revealed the role of TGFβ signaling in the regenerative capacity of the zebrafish heart after myocardial infarction using a cryoinjury procedure.[238] Understanding the key healing processes after myocardial infarction in zebrafish may result in identification of the barriers to efficient cardiac regeneration in mammals and enable the design of novel therapeutic strategies for improved regeneration of the infarcted mammalian heart.

■ OTOTOXICITY

Drug-induced ototoxicity is often reversible but sometimes is not, leading to a severe impact on the quality of life for patients faced with no alternative treatment, as is with aminoglycosides for life-threatening infection and cisplatin for cancer.[239] Screening for ototoxicity in mammalian preclinical models is very difficult, and given few regulatory requirements, this is rarely done.[240] A zebrafish model for ototoxicity may fill this gap in preclinical safety testing. Zebrafish and human auditory systems most likely share an evolutionary origin with those of all vertebrates. This is indicated by homologous hair cell physiology,[241,242] similar auditory anatomy, and neuronal signaling[243] and is further supported by apparently conserved genetic regulation.[244−246] A large part of our understanding of the genetics of human hearing and balance has come from studies with zebrafish.[247−249]

The zebrafish ear is composed of three semicircular canals arranged orthogonally to one another for sensing the direction and the speed of movement. It also contains two otoliths that help transmit vibrations to associated maculae (the utricular macula for sensing balance and the saccular macula for hearing) and a third macula (lagenar) that has both balance and hearing functions; these work together to stimulate anterior, posterior, and lateral cristae containing hair cells, which transduce signals to the brain.[250−252] This parallels the basic anatomy and physiology of the mammalian inner ear[245,250,253] except that there is no cochlea in zebrafish. Zebrafish lack homologous structures for the mammalian outer ear (visible part), middle ear (ear drum and tympanic bones), and cochlea. The cochlea, in mammals, helps to amplify sounds.[254] Sound amplification in zebrafish is accomplished via the Weberian apparatus, a series of bones which convey vibrations from the resonant and relatively large swim bladder to the lagenar macula in the ear.[252]

Sound is transmitted to the brain via mechanosensory hair cells.[241] In zebrafish, these cells are either bathed in the endolymph of the ear or positioned along the lateral line system, which is a series of neuromasts (bundles of hair cells), located on, or near, the body surface in canals, for sensing water flow changes.[255,256] Hair cells transduce sound (or water) waves into electrical nerve signals and transmit them to the brain through associated afferent nerve endings.[242,257,258] The synapses between vertebrate hair cells and neurons are highly specialized to allow fast transduction of mechanical stimulation.[259] Most hearing loss in humans (90%) stems from a loss of function during this transition and is referred to as sensorineural hearing loss.[260] Zebrafish and human hair cells are homologous at the cellular and molecular levels and synapse in the same way with afferent neurons.[257,258,261] Recent research has exposed a plethora of shared genetic involvement in hearing, comparing zebrafish to humans,[244,246,248,262−267] including proof-of-concept that gene knock-out studies in zebrafish can identify genes that cause nonsyndromic deafness in humans.[249]

Hair cell functionality is well-developed in zebrafish in advance of 5 dpf, as demonstrated by tests for auditory and somatosensory responses.[252,268,269] The superficial location of hair cells in zebrafish allows for the ability to monitor hair cell changes in vivo in a high-throughput manner. This has made zebrafish a very popular model for ototoxicity interrogation and protection.[247] Many drugs that are ototoxic to humans are ototoxic to developing, larval, and adult zebrafish; these include aminoglycoside antibiotics, carbonic anhydrase inhibitors, platinum-based chemotherapies, and environmental metal contaminants.[247,270−272] These data indicate that this model is a promising tool to screen novel chemical entities for ototoxicity.

Several screening platforms were recently tested and proven useful for detecting hair cell damage.[273−279] Using platforms such as these, many potentially protective cotreatments have been discovered in zebrafish that may mitigate human ototoxicity of useful, and yet unimproved, therapeutics. Some methods, through live imaging and forward genetic screens, can provide insight into the chemical and genetic mechanisms of ototoxicity as well as information about whether a protective cotreatment interferes with the therapy.[280] The following are some examples of protective cotreatments discovered using this model: for radiotherapy for head and neck cancer, p38 inhibition;[281] to mitigate cisplatin-induced ototoxiticy, quercetin,[282] curcuminoids,[283] CDK2 inhibition,[284] or sirtuin 1 activation;[285] for neomycin ototoxicity, melatonin,[286] astaxanthin nanoemulsion;[287] and recently discovered potential aminoglycoside-protective cotreatments.[55,288] The latter work resulted in repurposing a preapproved drug into clinical trials for aminoglycoside protection, demonstrating the utility for zebrafish to help advance discoveries that circumvent human ototoxicity.

■ PINEAL/CIRCADIAN RHYTHM

Sleep disturbances are induced by many classes of pharmaceuticals, including beta-blockers, benzodiazepines, opioids, and amphetamines. Understanding the nature and severity of the sleep disturbance is necessary to assess the drug safety; for instance, reduced sleep is detrimental to human health and can contribute to diabetes, hormonal deficiencies, and neurological disorders. Impacts on sleep may be desirable for sleep aids and acceptable for severe indications such as cancer but unacceptable for drugs to treat less severe conditions; in any case, understanding effects on sleep early in the development process is important.

In zebrafish, sleep/wake behavior based on locomotor activity can be easily determined in a high-throughput manner.[289] An advantage of the diurnal zebrafish over nocturnal rodent models is the greater similarity of sleep regulation to other diurnal vertebrates including humans. In both zebrafish and humans, melatonin production by the pineal organ (Figure 7) regulates the circadian regulation of sleep, downstream of the light-entrained circadian clock.[290] The hypothalamic hypocretinergic system is also conserved between zebrafish and mammals.[291] Clinically used hypnotics and stimulants have similar effects on the sleep/wake cycle in zebrafish as they do in humans, further demonstrating the translatability of effects on sleep/wake behavior,[292] with occasional exceptions, e.g., dopamine D1 agonists, which increase rest in zebrafish but wakefulness in mammals.[289]

Zebrafish larvae have been successfully used for identifying previously unappreciated effects of pharmaceuticals with

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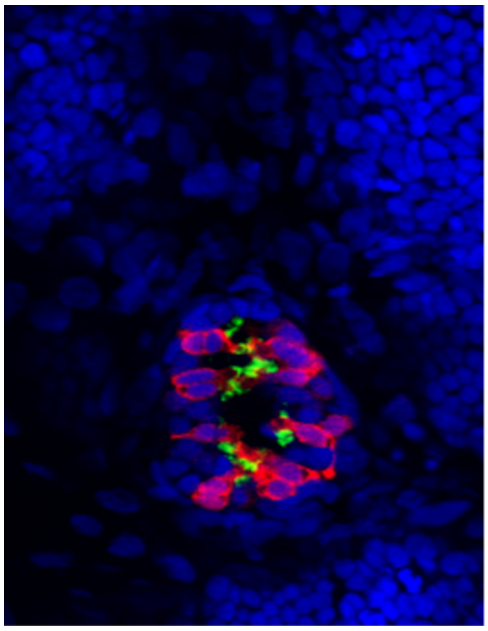

Figure 7. Photoreceptive pineal organ of a 4 dpf larval zebrafish, medially located in the forebrain (top of image = rostral). Nuclei are blue (DAPI), cone photoreceptors are red (antiarrestin 3a antibody), and rod photoreceptors are green (antirhodopsin antibody). Previously unpublished, provided by J. Gamse. Photo credit: Joshua Clanton.

known targets on sleep/wake behavior, indicating the utility of this system for identifying potential sleep-disturbing effects of new pharmaceuticals in preclinical screening.[289] Moreover, using fine-grained analysis of locomotor behavior in combination with the statistical power available with zebrafish, relatively subtle effects on sleep/wake behavior can be teased out[292] and then confirmed in other preclinical models such as cynomolgus monkeys with telemetered polysomnography.[293]

■ LATEST ADVANCES AND FUTURE DIRECTION

In general, zebrafish are a useful tool for pharmaceutical toxicity testing based on the evidence for translatable toxicology from zebrafish to mammals and augmented by the tractable nature of zebrafish for testing a wide spectrum of toxicities. New and innovative applications of zebrafish in relation to pharmaceutical toxicology appear regularly in the literature. These include studies interrogating toxic mechanisms, adverse outcome pathways (AOPs), drug abuse liability, endocrine disruption, as well as pertinent studies around metabolism, bioavailability, transcriptomics, and proteomics.

Mechanistic evidence can be used as a key to help escape toxicities that are encountered during new drug development. The mechanism through which toxicity is enacted can be driven through the therapeutic target (on-target toxicity) or another protein/molecule (off-target). Also, the precise molecular interaction between a xenobiotic and the therapeutic target may drive the desired therapeutic effect and a toxic effect based on the site and nature of the interaction. Knowledge about the toxic mechanism can help guide structure−activity relationship (SAR) studies and allow for faster discovery of less toxic drug candidates. The concept of using zebrafish for interrogating toxic mechanisms of environmental pollutants is not new,[294,295] and in that arena, publications have steadily grown over the past 10 years to about 300 per year. Although the application of zebrafish in pharmaceutical toxicity mechanism studies[296,297] has lagged, the numbers have recently begun to grow (Figure 8) to nearly 100 per year.

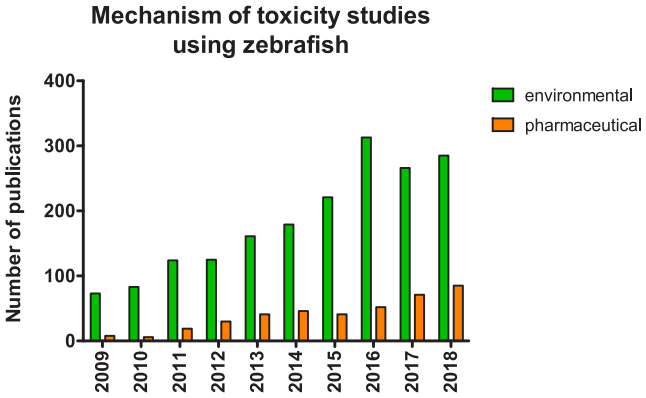

Figure 8. Number of publications reported from PubMed using search phrases "zebrafish (environmental or pharmaceutical) toxicity mechanism of action".

The pharmaceutical mechanism studies have revealed cell types, pathways, and genes involved in cardiovascular,[298,299] neuromuscular,[300] neuronal,[301−303] ocular,[85] auditory,[55,281−288] and embryo-development[304−307] toxicities. Those same studies introduce models by which safer compounds can be discovered by conducting SAR within or around a chemical series as has been lately shown for several chemotherapies.[308−311] Equally important, those models can be used to interrogate species-specific mechanisms of toxicities, which is of great value for predicting clinical toxicity. For example, promising immunomodulatory (IMiDS) chemotherapies, thalidomide, lenalidomide, and pomalidomide, all have teratogenic properties but with different potencies, depending on the species. Recent studies including knockdown and transgene expression in zebrafish, mice, and human stem cells[312−314] have shed light on the mechanisms and molecular players that make a developing embryo sensitive or insensitive to those drugs.

Recently, mechanistic studies using zebrafish have discovered personalized therapies for rare genetic diseases. Full exome next generation sequencing and refined gene editing tools combined with a rapidly developing tiny vertebrate with strong genetic homology to humans creates a proving ground for repurposing drugs to cure, or suppress symptoms of, life-threatening diseases.[315] After patient-specific rare alleles are discovered, they are engineered into the genome of zebrafish, and if disease symptoms are recapitulated, a high-throughput platform is created for discovering personal therapies. Moreover, these models can deliver novel insight about mechanisms through which rare diseases are manifested and may lend themselves to our understanding of more common diseases.[316] Rare diseases for which therapies have been discovered using zebrafish include pediatric diseases involving the following systems: musculoskeletal, blood, endocrine, neuronal, genitourinary, lymphatic, congenital malformations,[52,317] and cancer.[318] In this new application of zebrafish lies great potential for a positive impact on human health and medicine.

The impact of industrial waste on the environment is a growing concern keeping pace with the growing human population, and pharmaceutical waste is a major component of that pollution. Ecopharmacology is a new field that is

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concerned with the presence, persistence, and effects of parent drugs and metabolites in/on the environment.[319] Pharmaceutical waste enters the environment through three ways; patient excretion, improper disposal, and manufacturing discharge. Pharmaceutical pollution is especially dangerous to life, because drugs are designed to be biologically active; therefore, all life forms are potentially at risk of being harmed or changed by this type of waste. Some ecopharmacological findings are microbial drug resistance, gene expression changes, epigenetic effects, genotoxicity, carcinogenicity, endocrine and immune disruption, and biofilm formation.

Environmental risk assessment is requested of all new drug products marketed for human consumption by the U.S. Food and Drug Administration and the European Medicines Agency. Those assessments compare the predicted environmental concentration to the acute toxic potency of the new drug on various species, which can include an algal species, an aquatic invertebrate, and fish. Zebrafish are being used regularly in those studies due to the ease of raising them in a laboratory environment.[320] Increasing awareness of compounded chemical mixture effects on biological systems[321−323] and recent discoveries of effects of endocrine-disrupting chemicals in the environment[324−327] are heralding times for different and more detailed interrogation into potential environmental impact of new drugs.[328] Recent zebrafish work that is pioneering the way toward such ends includes studies on transcriptomics,[321,329−331] chemical mixture effects,[322,332] lipid metabolism,[333,334] reproduction,[335] and development.[336]

When evaluating drug safety, attention is frequently directed toward identifying cellular and organ toxicities, but there are important safety considerations that go well beyond toxicity. Prominent among these is the potential for drug abuse liability. As the current opioid abuse epidemic highlights, highly efficacious and relatively nontoxic medications can be unsafe if they exhibit potential for abuse. Testing drug candidates for abuse liability remains expensive and time-consuming because of the complexity of the rat and primate models used. Published reports offer some hope that zebrafish could be developed as a model for testing drug abuse liability, thereby enabling screening for abuse liability earlier, more efficiently, and on a larger scale. The earliest demonstration of zebrafish responsiveness to a drug of abuse was by Darland et al., who demonstrated that zebrafish conditioned place preference for cocaine almost 2 decades ago.[337] Since then, several noncontingent (passive) behavioral assays have demonstrated zebrafish conditioning to opioids, amphetamines, alcohol, nicotine, and other drugs with abuse liability.[338−344] More recently, contingent models such as an opioid self-administration model have been developed for zebrafish.[345] In this model, zebrafish can be conditioned in as few as 5 days to self-administer opioids, and their motivation to seek additional doses can be quantified. Because zebrafish perform so well in both noncontingent and contingent models of drug seeking, it may be possible in the future to use zebrafish to test new drugs for their abuse liability.

■ CONCLUSION

Zebrafish can be used to assess the toxicity of drug candidates in early screening assays, sometimes in a high-throughput manner. Due to their small size and transparency, such testing requires a small mass of test article, very little lab space, and data can be collected noninvasively over time in vivo. These data can help prioritize safer compounds for mammalian testing, disclose mechanisms of toxicity, and identify cotherapies that may mitigate toxicity of promising therapeutics. Employed to interrogate xenobiotics around which evidence points to conserved vertebrate biology, zebrafish toxicity assays can quickly and easily provide translatable data on a spectrum of tissues, organs, and systems.

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The authors declare the following competing financial interest(s): L.I.Z. is a founder and stockholder of Fate Therapeutics, CAMP4 Therapeutics, and Scholar Rock. He is a consultant for Celularity.

Biographies

Steven Cassar is an Investigative Toxicologist for AbbVie Pharmaceutical Research and Development. For several years, studies with zebrafish at AbbVie have helped prioritize the development of less toxic compounds and have provided insights into mechanisms of toxicity. Steven received his BS in Biology from Alma College in Michigan and his MS in Mycology from Louisiana State University. Steven has over 20 years of experience in drug discovery and development, specifically in translational medicine, pharmacology, and genetics.

Isaac Adatto is an active participant of the zebrafish community. He joined the Zon lab at Boston Children's Hospital in 2007. He has published his work on zebrafish husbandry in several journals, presented his research findings nationally and internationally, and has served as president of the Zebrafish Husbandry Association. Adatto currently works at Harvard's Department of Stem Cell and Regenerative Biology as the Senior Zebrafish Research Manager supporting and assisting in research for multiple investigators. He holds an M.S. from the University of Massachusetts Intercampus Marine Science Program.

Dr. Jennifer L. Freeman is an Associate Professor of Toxicology in the School of Health Sciences, a faculty affiliate in Environmental and Ecological Engineering, and member of the Public Health Graduate Program at Purdue University. She received her doctorate in Environmental Toxicology and Molecular Cytogenetics from the University of Illinois at Urbana-Champaign and was a postdoctoral fellow at Harvard Medical School and Brigham and Women's Hospital, Boston. The interests of the Freeman laboratory are to define the underlying genetic and epigenetic mechanisms of toxicity. All projects are currently utilizing the zebrafish vertebrate model system as a tool to investigate toxicity.

Joshua Gamse is a Senior Research Investigator in the Developmental and Reproductive Toxicology group within Drug Safety Evaluation at Bristol-Myers Squibb, where he has worked since 2014. Prior to Bristol-Myers Squibb, he was an Associate Professor in the Department of Biology at Vanderbilt University, where his laboratory's research focused on development and function of the epithalamus in zebrafish.

Iñaki Iturria has a Bachelor's degree in Pharmacy by the University of the Basque Country and a Master's Degree in Biotechnology by the Autonomous University of Barcelona. He obtained his Ph.D. in 2016 by developing a model to evaluate the probiotic activity of lactic acid bacteria in zebrafish. He actually works as a Study Director at Biobide, a CRO specialized in toxicity and efficacy assays in zebrafish.

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Christian Lawrence is currently the Manager of the Aquatic Resources Program (ARP) at Boston Children's Hospital (BCH). ARP administers the zebrafish program at BCH, which is one of the largest and most active of its kind in the world. Mr. Lawrence also serves as a faculty member for the Health and Colony Management of Laboratory Fish course at the Mount Desert Island Biological Laboratory and was a Fulbright Specialist in Israel in 2014. He is co-author of The Laboratory Zebrafish and has written a number of scientific publications on zebrafish biology and culture.

Arantza Muriana achieved her MPharm from Navarra University, with an MBA from San Pablo CEU University (Madrid, Spain) added to her scientist background, improving her project management and business skills. She began working in zebrafish after training in zebrafish biology at the Salk Institute (California, US). She has worked in clinical and preclinical CROs for 16 years, with more than 13 years of experience working in zebrafish with several papers, especially in the toxicology and neurodevelopmental areas. She has been part of several consortiums with regulatory agencies (EPA, FDA, NIH-NIEH, or OECD) for the validation and standardization of zebrafish as an alternative model.

Randall T. Peterson, Ph.D., is a chemical biologist seeking to discover new drug candidates for cardiovascular and nervous system disorders. The Peterson lab uses chemical screens in living zebrafish to identify novel compounds that modify organismal processes in vivo. Several of the compounds discovered by the Peterson laboratory have become widely used research tools or are in clinical development. After two decades as a student and faculty member at Harvard University, Randy has recently moved to the University of Utah, where he serves as dean of the College of Pharmacy and L. S. Skaggs Presidential Endowed Professor.

Steven Van Cruchten graduated as DVM at Ghent University in 1999 and obtained his Ph.D. at Ghent University in 2004. He then joined Johnson & Johnson in Belgium as a reproductive toxicologist and he also became involved into the field of juvenile toxicology. In 2008, he joined AstraZeneca in Sweden as a Toxicology Project Leader. He also kept his expert role in reproductive and juvenile toxicology. S. Van Cruchten returned to Belgium in 2011 to take up the position of associate professor at the University of Antwerp. His research focuses on alternative and animal models for juvenile and reproductive toxicology.

Dr. Zon is the Grousbeck Professor of Pediatric Medicine at Harvard Medical School, an Investigator at Howard Hughes Medical Institute, and the Director of the Stem Cell Program at Boston Children's Hospital. He is internationally recognized for his pioneering work in stem cell biology and cancer genetics and has been the pre-eminent figure in establishing zebrafish as an invaluable genetic model for the study of the blood and hematopoietic development.

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