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Authors
Tal, Tamara
Yaghoobi, Bianca
Lein, Pamela J

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Translational toxicology in zebrafish

Tamara Tal[1], Bianca Yaghoobi[2] and Pamela J. Lein[2]

Abstract

A major goal of translational toxicology is to identify adverse chemical effects and determine whether they are conserved or divergent across experimental systems. Translational toxicology encompasses assessment of chemical toxicity across multiple life stages, determination of toxic mode of action, computational prediction modeling, and identification of interventions that protect or restore health after toxic chemical exposures. The zebrafish is increasingly used in translational toxicology because it combines the genetic and physiological advantages of mammalian models with the higher-throughput capabilities and genetic manipulability of invertebrate models. Here, we review the recent literature demonstrating the power of the zebrafish as a model for addressing all four activities of translational toxicology. Important data gaps and challenges associated with using zebrafish for translational toxicology are also discussed.

Addresses

[1] Department of Bioanalytical Ecotoxicology, Helmholtz Centre for Environmental Research – UFZ, Permoserstraβe 15, 04318, Leipzig, Germany

[2] Department of Molecular Biosciences, University of California, Davis School of Veterinary Medicine, 1089 Veterinary Medicine Drive, Davis, CA 95616, USA

Corresponding authors: Lein, Pamela J (pjlein@ucdavis.edu); Tal, Tamara (tamara.tal@ufz.de)

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Keywords

Gut microbiome, Hazard identification, Interventions, Life stages, Mode of action, Predictive toxicity.

Abbreviations

AhR, aryl hydrocarbon receptor; BPA, bisphenol A; BPAF, bisphenol AF; BPB, bisphenol B; BPF, bisphenol F; BPS, bisphenol S; CYP1A1, cytochrome P450, family 1, subfamily A, polypeptide 1; DOHaD, developmental origins of health and disease; dpf, days post-fertilization; DMSO, dimethylsulfoxide; GenX, ammonium salt of hexafluoropropylene oxide dimer acid fluoride; eGFP, enhanced green fluorescent protein; gper-1, G protein–coupled receptor 1; hpf, hours post-fertilization; LELs, lowest effect levels; *mrp1*, multi-drug resistance-associated protein 1; PFAS, per- and polyfluoroalkyl substance; PFOS, perfluorooctane sulfonate; SARs, structure–activity relationships; *slincR*, *sox9b* long intergenic noncoding RNA; *sox9b*, SRY-box transcription factor 9b; QSARs, quantitative SARs; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; VHL, von Hippel-Lindau syndrome.

Introduction

A main goal of toxicology is to determine the potential for and the mechanisms by which xenobiotic agents cause harm to biological systems. Although the human is a predominant target species of interest for most xenobiotics, there are limited human data. To address this data gap, it is often necessary to extrapolate data integrated from diverse species across varying levels of organization, including computational, biochemical, *in vitro*, and *in vivo* systems. *In vivo* models offer a distinct advantage by enabling assessment of integrative effects across organ systems and across different life stages. However, all experimental models fail to recapitulate some aspects of human biology, so it is important to understand and account for the limitations of any given model (Figure 1a).

Due to genetic and physiologic conservation between zebrafish and humans (Box 1) and the relevance of this small aquatic vertebrate to translational toxicology (Box 2), the zebrafish has become a widely used model for toxicological research [1,2] that is increasingly being used to address long-standing challenges in toxicology. For example, zebrafish are a powerful model for studying the toxicity of chemical mixtures, as exemplified by a recent study in which gene expression changes and lethality were quantified in embryonic zebrafish exposed to multiple concentrations of three different pesticides, either individually or as binary or tertiary mixtures [3]. The authors concluded that the quantitative and qualitative effects of the mixtures would not have been predicted based on changes elicited by exposure to individual chemicals. Another long-standing challenge — sex differences in toxic outcomes — was recently studied in zebrafish exposed to perfluorooctane sulfonate (PFOS). Transcriptomic analysis of multiple organs revealed that PFOS altered expression of genes associated with fatty acid metabolism and neural function in a manner that varied not only according to the target organ and concentration and duration of PFOS exposure but also according to sex [4]. In a separate study, wild-type female zebrafish were found to be significantly more sensitive to the behavioral effects of chronic ethanol exposure than long-fin striped females or males [5], suggesting that sex and the genetic background interact to determine toxic outcomes. These

Figure 1

a Relative advantages and disadvantages for translational toxicology

	Advantages	Disadvantages
Rat (*R. novergicus*) and Mouse (*M. Musculus*) 	• High genetic conservation with human genome (~80–90%)[a,b] • Large availability of genetically modified and disease-specific models • Microbiota composition similar to humans[c,d]	• Diet differs from humans • Low throughput for toxicity testing[b,c,e] • Mouse genomic inflammatory response different from humans[f]
Zebrafish (*Danio rerio*) 	• Considerable genetic conservation with human genome (~70%)[g] • Large availability of genetically modified models • High fecundity, over 100 eggs per female released every one-to-two weeks • Optically transparent during early development • Rapid development and generation time • Medium-high throughput model for toxicity testing • Cardiac electrophysiology and regulation of telomeres more similar to human than rodents[h,i] • Majority of epigenetic machinery conserved between zebrafish and humans[j]	• Diet and environment significantly differ from humans[b] • Lacks stomach, lungs, lymph nodes, splenic germinal centers and Peyer's patches[k] • Sex determination delayed and not chromosomally determined[g] • Microbiota composition markedly different from human[c] • Distinct differences in epigenetic programming between humans and zebrafish[j]
Nematode worm (*C. elegans*) 	• Highly fecund, eggs released every day • Rapid development and generation time • Transparent until adulthood • Genetically tractable • All 302 neurons mapped and correlated to specific behaviors[l] • High-throughput model for toxicity testing[m]	• Low conserved homology with human genome (~35%)[n] • Lacks most vertebrate organs and systems (contains simple nervous and gastrointestinal system and reproductive organs • Microbiota composition typically consists of a single bacterial species, often *E. coli*[m]

b Comparative timelines for zebrafish and human development[o–r]

	Developmental stages	Zebrafish	Human
Life Stages	Embryo	0 – 2 dpf	0 – 56 days
	Larva	3 – 29 dpf	N/A
	Juvenile	30 – 90 dpf	13– 17 years
	Adult	Starting at 90 dpf	Starting at 18 years
Developmental Events	First cell division	0.75 hpf	1 day
	Blastula/blastocyst	2–5 hpf	4–6 days
	Gastrulation	5–9 hpf	13–19 days
	Neural plate formation	10 hpf	17–19 days
	First somite	10–11 hpf	19–21 days
	10 Somite stage	14 hpf	22–23 days
	Neural tube formation	18–19 hpf	22–30 days
	First neuromuscular junction	17 hpf	54 days
	Organogenesis	10–96 hpf	21–56 days
	First heartbeat	24 hpf	22 days
	Functionally mature liver	4–6 dpf	56 days
	Birth/hatching	48 – 72 dpf	253 days
	Microbial colonization of GI	3.5–5 dpf	1-3 days after birth
	Sex determination	20–30 dpf	At conception
	Onset of adaptive immunity	28–30 dpf	15–17 wk gestation
	Onset of sexual maturity	90–120 dpf	7–15 years

Comparisons of model organisms with humans. **(a)** Strengths and weaknesses of widely used translational toxicology animal models. **(b)** Comparative timelines for zebrafish and human development (https://zfin.org/zf_info/zfbook/stages/). The timing of all developmental events in zebrafish is influenced by temperature. dpf = days post-fertilization; hpf = hours post-fertilization; wk = weeks. [a]Keane et al., 2011 *Nature* 477:289-294; [b]Fritz et al., 2013 *Microbiome* 1:14; [c]Kostic et al., 2013 *Genes Dev* 27:701-718; [d]Nagpal et al., 2018 *Front Microbiol* 9:2897; [e]Bedell et al., 1997 *Genes Dev* 11:11-43; [f]Seok et al., 2013 *Proc Natl Acad Sci U S A* 110:3507–3512; [g]Howe et al., 2013 *Nature* 496:498-503; [h]MacRae and Peterson 2015 *Nat Rev Drug Discov* 14:721-731; [i]Cayuela et al., 2018 *Front Cell Dev Biol* 6(178); [j]Aluru et al., 2018 *Environ Epigenet* 4:dvy005; [k]Goldsmith and Jobin (2012) *J Biomed Biotechnol* 2012: 817,341; [l]Bargmann 2006 *Worm Book* 1–29; [m]Clark and Walker 2018 *Cell Mol Life Sci* 75:93-101; [n]Kim et al., 2018 *Genetics* 210:445-461; [o]Kimmel et al., 1995 *Dev Dyn* 203:253-310; [p]O'Rahilly et al., 1979 *Anat Embryol (Berl)* 157:167-176; [q]Phelps et al., 2017 *Sci Rep* 7:11,244; [r]Rawls et al., 2007 *Proc Natl Acad Sci U S A* 104:7622-7627.

studies suggest the potential for using zebrafish to identify specific gene × environment interactions that influence individual susceptibility to adverse outcomes [6].

The zebrafish is also proving to be a strong model for addressing emerging questions in toxicology, such as the influence of xenobiotics on the developmental origins of health and disease (DOHaD), epigenetic mechanisms of toxicity [7], and the role of the microbiome in modifying toxic effects of xenobiotics [8,9]. For example, the effects of bisphenol A (BPA) or the replacement chemicals bisphenol AF (BPAF), bisphenol B (BPB), bisphenol F (BPF), or bisphenol S (BPS) on developmental toxicity and the microbiome community structure were recently studied in zebrafish [10]. Chemical potency was conserved in a zebrafish developmental toxicity assay when compared with previously reported estrogen receptor activity in both human *in vitro* and zebrafish reporter systems [10]. However, an inverse relationship between zebrafish developmental toxicity and chemical-dependent microbiome disruption was observed, indicating that traditional toxicology tests fail to capture microbiome-dependent effects. Through the use of colonized, microbe-free axenic, and conventionalized zebrafish, recent work has shown that host-associated microbes biotransform xenobiotic agents into metabolites with unknown toxicity profiles [11,12].

Evaluating zebrafish for translational toxicology research

Translational toxicology broadly refers to the determination of toxicological effects as conserved or divergent across different experimental systems. Four activities are proposed to comprise translational toxicology [13]: (1) assessing chemical toxicity across multiple life stages, (2) identifying chemical mode of action and relevance of key events across models, (3) using data from one model to predict chemical toxicity in other

Box 1. Relevance of zebrafish to human biology and disease.

The zebrafish expresses gene orthologs for >70% of human genes, 82% of human disease-causing proteins, and 85% of known human drug targets [71]. Zebrafish proteins resemble their human counterparts, particularly within functional domains. For example, while the zebrafish glucocorticoid receptor is only 54% identical to the human glucocorticoid receptor, the ligand binding domain is 74% identical and its pharmacologic properties closely resemble those of the human [72]. There is also considerable anatomic and physiologic conservation between zebrafish and humans. The zebrafish possesses counterparts of most human organ systems, and zebrafish organs largely perform the same functions as their human analogs. Physiologic mechanisms are well conserved at the molecular and cellular levels, and some cases (e.g., cardiac electrophysiology), the zebrafish is a better model of the human than rodents (reviewed in [72]). The zebrafish has many of the same sensory modalities as humans, including vision, olfaction, taste, touch, balance and hearing, and it exhibits an extensive behavioral repertoire, ranging from simple stimulus-response behaviors to complex behaviors such as sleep, pain, affective and depressive-like behavior, locomotion, social interactions and cognitive behaviors [16].

Zebrafish are widely used to model diverse human diseases. Because targeted gene mutations can be generated and phenotyped more efficiently in zebrafish than in rodents, there is significant interest in using zebrafish to investigate rare genetic disorders. For example, using a *scn1lab* mutant zebrafish that recapitulates critical clinical features of Dravet syndrome, a chemical library screen identified the 5-HT2B receptor as a novel therapeutic target for this rare genetic seizure disorder [73], In a second example, zebrafish expressing human type I collagen gene mutations were engineered to investigate human genetic skeletal dysplasias because unlike mouse models, zebrafish bone mutants survive into adulthood [74]. Using micro-computed tomography (μCT) for detailed and rapid skeletal phenotyping of zebrafish mutants and systematic collagen analysis by SDS/PAGE and mass-spectrometry, the authors demonstrated that zebrafish and human type I collagen are compositionally and functionally related, and that expression in the zebrafish of select human mutations in type I collagen gives rise to phenotypic variability that mirrors the clinical variability associated with the human disease [74].

systems, and (4) deploying models to develop and evaluate interventions to protect or restore a healthy status after chemical exposure. Here, we discuss recent evidence collected in zebrafish that encompasses these four activities (Figure 2).

Assessing chemical toxicity across the zebrafish life span

Zebrafish pass through four major life stages: embryonic, larval, juvenile, and adult. Zebrafish are considered embryos from fertilization until hatching, which can occur between 48 and 96 h post-fertilization (hpf), at which point they are considered larvae. Zebrafish transition to the juvenile stage at ~30 days post-fertilization (dpf) (https://zfin.org/zf_info/zfbook/stages/), which corresponds to the age when many laboratory-bred strains have determined their sex. Sexual maturity and the ability to produce offspring signals the adult stage, which occurs by ~90–120 dpf. Compared with humans, the key molecular and cellular transitions that occur during the development and maturation of most major organ systems are similar in terms of sequence, but occur more rapidly in zebrafish (Figure 1b).

Zebrafish embryos and larvae are widely used for developmental toxicology studies for theoretical reasons — the molecular and cellular mechanisms of early development are among the most conserved between zebrafish and humans [14] — and practical considerations – zebrafish develop rapidly and external to the mother, and for the first 7 dpf, they obtain most of their nutrients from the yolk sac. Zebrafish are therefore readily adapted to higher-throughput formats that deploy 96- or 384-well plates and automated tools for image acquisition, processing, and associated analyses. Because of its relatively short life cycle, the zebrafish offers significant advantages for assessing transgenerational (e.g. epigenetic) effects [7] and differential vulnerability to toxic effects across the life span. With regard to the latter, a recent evaluation of embryonic (3 hpf), larval (3 dpf), juvenile (30 dpf), and adult (3 month old) zebrafish exposed to varying concentrations of four different strobilurin fungicides revealed that the larval stage was the most susceptible [15]. Whether this reflects toxicokinetic or toxicodynamic mechanisms is yet to be determined.

In contrast, there are significantly fewer examples of juvenile and adult zebrafish being used for toxicology research. This may be because unlike embryonic and larval zebrafish, juvenile and adult zebrafish cannot be maintained in multi-well format plates for prolonged periods of time, and they are not optically transparent. Despite these limitations, juvenile and larval zebrafish are advantageous for toxicological studies of phenotypes not exhibited at earlier life stages, such as sex, reproductive function, and adaptive immunity (Figure 1b), and behaviors that cannot be readily assessed in younger fish, including learning, memory, social, and anxiety-like behaviors [16]. For example, adult zebrafish were recently used to evaluate the therapeutic and toxic effects of the antidepressant amitriptyline [17]. Adult zebrafish are also gaining traction as models for studying chemical effects on phenotypes and diseases associated with aging, including various cancers [18]. An adult zebrafish model was used to screen novel small molecule therapeutics for liver cancer to identify compounds with a better therapeutic index than the standard of care, sorafenib [19]. Adult zebrafish were also recently validated as a model for evaluating drug-induced kidney injury [20]. Larval zebrafish have only one pair of nephrons, whereas the adult zebrafish kidney has several hundred nephrons with a similar histological structure and physiological function as the mammalian kidney

Box 2. Zebrafish is a powerful model for toxicology research.

The zebrafish model is particularly well suited for molecular and developmental toxicology studies. Key advantages include: (*i*) the zebrafish genome has been completely sequenced and is highly homologous to the human genome [71]; (*ii*) powerful gene-editing techniques continue to be developed and optimized for use in zebrafish [75]; and (*iii*) zebrafish embryo and larvae are optically transparent, which enables visualization of the dynamic changes in individual cells and organs *in vivo* across a broad range of developmental stages [76].

Another significant advantage of zebrafish is that toxic outcomes can be measured at the molecular (e.g., mRNA or protein expression), structural (e.g., cells and organs) and systems level, including structural [76] and electrophysiological [77] parameters of neural circuitry and behavior. This enables molecular effects to be anchored to phenotypic outcomes.

In contrast to cell-based assays that provide limited toxicokinetic information, zebrafish can reveal critical insights about the absorption, distribution, metabolism, and excretion (ADME) of xenobiotics. Zebrafish have a functional liver, kidneys, and blood-brain barrier, expression conserved tissue-specific transporters, and exhibit both phase I and II metabolism (reviewed in [72]).

Zebrafish are easily exposed by direct addition of chemicals to the house media (referred to as water-borne exposures). This is a significant advantage for screening studies, especially with the availability of robotics that automate delivery of compound into multi-well plates. However, nominal media concentrations are not necessarily representative of internal dosimetry [78,79], and this remains an important challenge in the use of zebrafish for toxicity testing. Compounds that do not readily dissolve in aqueous solutions represent another challenge for using waterborne exposures; however, this can be overcome by injecting chemicals directly into zebrafish [80].

(reviewed in the study by Kato et al. [20]). The renal pathology observed in adult zebrafish exposed to nephrotoxic levels of gentamicin or doxorubicin was similar to that seen in mammals, and a screen of 28 chemicals with known nephrotoxicity and 14 with no known nephrotoxicity in humans demonstrated that 16 of the nephrotoxic chemicals and none of the negative controls caused drug-induced kidney injury in adult zebrafish.

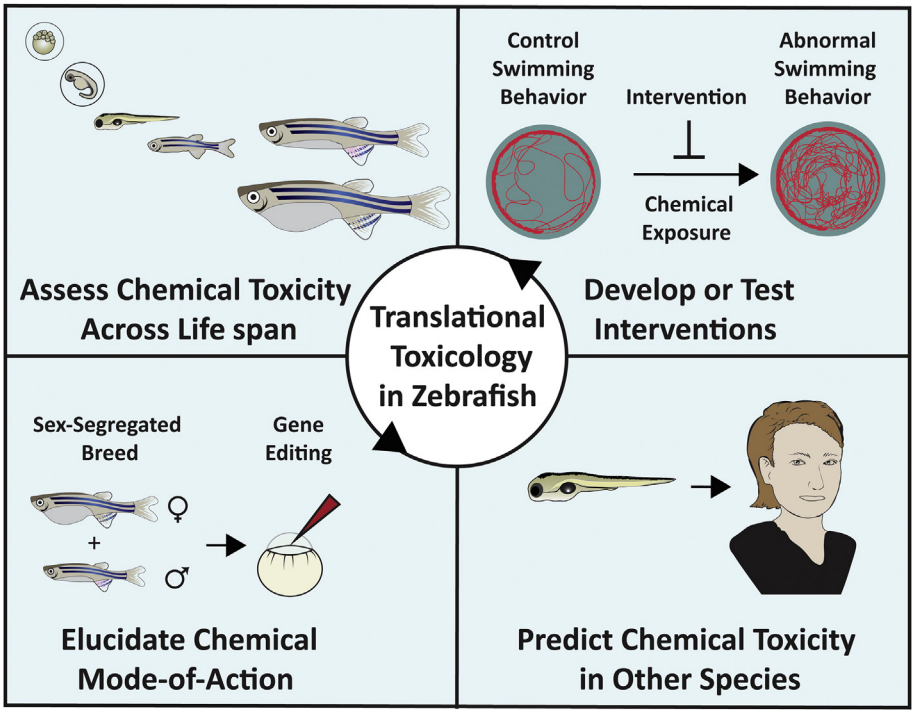
Using zebrafish to define chemical mode of action

A significant strength of the zebrafish is that molecular insights into chemical mode of action can be obtained using diverse approaches ranging from chemical screens to elucidate structure–activity relationships (SARs) to genetic manipulation that identifies molecular targets of xenobiotics. Chemical screens in zebrafish have revealed novel mechanistic information about compounds with unknown modes of action via phenotypic mapping to compounds with known modes of action. For example, in a screen of 14,000 compounds, automated behavior testing of zebrafish coupled with a barcoding-based computational approach was used to identify novel neuroactive compounds that shared behavioral profiles with compounds with known modes of action [21]. This strategy has since been applied to identify chemicals that regulate zebrafish sleep–wake cycles [22], passive and active threat response [23], addiction [24], and psychosis [25]. Phenotypic SARs have been identified for the developmental toxicity of oxygenated, hydroxylated, or heterocyclic polycyclic aromatic hydrocarbon (PAH) derivatives [26]. In the same study, a relationship between behavior phenotypes and specific PAH substitutions was not apparent [26]. More recently, a smaller-scale comparison of alkyl sulfonic acid, alkyl carboxylic acid, or branched or ether containing per- and polyfluoroalkyl substances (PFASs) showed that exposure to alkyl sulfonic acid PFASs with more than four fluorinated carbons caused hyperactivity and that the potency for this structural subclass of PFAS correlated with fluorinated carbon chain length [27]. Quantitative SARs (QSARs) have also recently been used to predict zebrafish acute toxicity for neutral [28] or ionizable [29] organic chemicals.

Medium- to high-throughput zebrafish screens coupled with automated morphological and behavioral phenotyping represent a powerful strategy to identify SARs that illuminate phenotypic readouts particularly sensitive to chemical disruption. Subsequent unbiased pathway-level assessment and gene editing can then be used to solve mode of action *in vivo*. Unbiased [30–37] or targeted RNA sequencing [38] is routinely used to identify chemical-dependent perturbations in zebrafish at the level of genes and pathways. As an example of the translational potential of this approach, changes in gene expression after exposure to three hepatotoxic compounds were compared across whole zebrafish, mouse and rat livers, *in vitro* mouse and rat hepatocytes, and primary human hepatocytes [39]. Although specific changes in gene expression were not generally conserved across models, shared pathway-level perturbations were identified, demonstrating that the zebrafish has the capacity to identify pathway-level transcriptomic disruptions that indicate liver toxicity in a suite of mammalian models [39]. The observed lack of concordance on the gene level likely stems from comparing profiles obtained from whole zebrafish homogenates versus liver-specific human cells or tissues. Future studies should consider isolating specific cell types via cell sorting or microdissecting specific tissues from zebrafish to enable cross-species transcriptomic comparisons based on similar cell or tissue types.

Once key phenotypes and pathway-level perturbations have been identified, zebrafish can easily be used for mechanistic research, with the goal of identifying causative events that link chemical exposure to phenotypic outcomes. Alternatively, molecular toxicology

Figure 2



Translational toxicology in zebrafish. Zebrafish can be used to assess the four components of translational toxicological research, including assessment of chemical toxicity across life stages, delineation of chemical mode of action, development or testing of interventions that block chemical-dependent toxicity outcomes and restore health, or prediction of chemical toxicity in other systems, including humans.

approaches can be used to disprove dogma related to assumed or predicted modes of action. One recent example was the demonstration of the nonessentiality of peroxisome proliferator-activated receptor gamma (PPARγ) for ciglitazone-dependent dorsoventral patterning defects in early zebrafish development [40]. Injection of an antisense oligonucleotide morpholino into single-cell stage zebrafish to transiently suppress the generation of PPARγ protein revealed that defects in patterning elicited by ciglitazone exposure occurred via a PPARγ>-independent mechanism [40]. Although some researchers have argued that morpholino knockdown phenotypes can be more severe than mutant phenotypes because of off-target effects [41], lack of concordance between morpholino knockdowns and stable gene knockouts may be more complex and involve genetic compensatory mechanisms specific to gene knockouts, but not knockdowns, at least at certain loci [42]. Nevertheless, gene editing approaches are now widely used to discover mutations that cause phenotypes and define toxicological modes of action. This endeavor is aided by a wide array of mutant zebrafish available via the Zebrafish Mutation Project [43] and the generation of cell type–specific mutant zebrafish lines [44]. Although concerns regarding off-target effects of Clustered Regularly Interspaced Short Palindromic Repeats–CRISPR-associated protein 9 (CRISPR-Cas9)–based gene editing have been raised [45], recent whole-exome sequencing evidence obtained across two generations of zebrafish derived from the same founding mutant pair failed to show evidence of off-target, *de novo* mutations [46], supporting the use of CRISPR–Cas9–based gene editing to uncover mechanisms by which toxicants elicit adverse outcomes.

There are several recent examples of using gene editing to solve toxicological mode of action in zebrafish. In an elegant study that integrated human hepatocellular carcinoma samples, human hepatocyte culture, and zebrafish, CRISPR–Cas9–dependent knockout of G protein–coupled receptor 1 (gper-1) was sufficient to block liver growth in 17-beta estradiol–exposed zebrafish [47]. This work identified gper-1 as a fundamental hepatic estrogen sensor. Given data from human studies demonstrating a causal link between gper signaling and atherosclerosis, heart failure, reproduction, metabolic disorders, cancer, and menopause (reviewed in the study by Zimmerman [48]), this research broadly illustrates that zebrafish can be used to elucidate human-relevant toxicity mechanisms [47]. CRISPR–Cas9–dependent gene knockout was also effectively deployed to show that the efflux transporter multi-drug resistance-

associated protein 1 (*mrp1*) functions to efflux both cadmium and benzo[a]pyrene [49]. Increased compound accumulation, mortality, and, at lower concentrations, increased incidence of pericardial edema and failure to hatch were observed in *mrp1*-mutant zebrafish exposed to either compound [49]. Perturbation of aryl hydrocarbon receptor (*ahr*)—dependent signaling represents a well-studied molecular mechanism by which xenobiotic exposure triggers adverse outcomes. Mutant zebrafish lines have revealed the essentiality of *ahr2* [50] and *sox9b* [51] in mediating 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)—dependent effects on zebrafish heart development. Morpholino-mediated knockdown of the long non-coding RNA *slincR* was used to demonstrate that *slincR* repressed *sox9b* expression as part of the mechanism by which TCDD-induced vascular hemorrhage in zebrafish [52]. Mutant *ahr2* zebrafish have also been leveraged to reveal the essentiality of the receptor for monosubstituted isopropylated triaryl phosphate, a component of Firemaster 550, to cause a heart looping defect [53].

Retrofitting zebrafish toxicity data to build or evaluate predictive toxicity models

Historically, zebrafish toxicity data have been used for hazard identification and chemical prioritization [54–59]. To fully leverage available zebrafish toxicity data, its ability to predict toxicity in humans must be defined. An early key article calculated overall concordances between developmental toxicants in zebrafish and rat (52%) or rabbit (47%) guideline studies [60]. Interestingly, the percentage of concordant chemicals identified between rat and rabbit studies was similar (58%), indicating that at least for the evaluated set of chemicals, zebrafish toxicity data were generally as predictive as the calculated concordance between two widely used mammalian models [60]. A subsequent meta-analysis compared zebrafish toxicity data of 443 chemicals, 19 aggregated toxicity phenotypes (e.g. cardiovascular), and 57 individual toxicity phenotypes (e.g. pericardial edema) with guideline toxicity data collected from rats, mice, and rabbits [61]. Zebrafish LC_{50} values were highly correlated with acute mammalian inhalation toxicity, with zebrafish LC_{50} values roughly 180% more sensitive than their mammalian counterparts [61]. From a developmental perspective, zebrafish hatching rate, pericardial edema, and decreased heart rate positively correlated with rabbit lowest effect levels (LELs) for prenatal loss [61]. In an interesting twist, the authors incorporated human exposure values to rank chemicals based on the integration of zebrafish toxicity data and human exposure estimates [61]. The resulting hazard index identified 14 chemicals where exposure levels in humans occur at concentrations that cause toxicity in zebrafish and therefore deserve further scrutiny [61].

A refinement of toxicity concordances that considers SARs is critical to understanding chemical blind spots in the zebrafish test system. For example, several compounds identified as reference compounds for developmental neurotoxicity because of documented human developmental toxicity were negative hits in a screen of 91 compounds for teratological and behavioral effects in larval zebrafish [54]. These included lead acetate trihydrate, valproic acid sodium salt, and toluene. Whether these compounds are true negatives or the lack of toxic effect is due to toxicokinetic (e.g. reduced bioavailability due to minimal uptake of the compound, lack of metabolic activation, or photoinactivation of the compound) or toxicodynamic (e.g. deficient target expression) differences in developing zebrafish versus mammalian models remains to be determined.

These observations are relevant to a second challenge in establishing concordance between zebrafish and mammalian toxicity data, which is that most of the zebrafish data used in these analyses compare nominal media concentrations with phenotypic outcomes. Tissue dose in zebrafish, as a result of waterborne exposure, is affected by diverse physicochemical properties [28,29,56]. If a compound fails to provoke phenotypic effects in zebrafish, paired analytical chemistry data are necessary to demonstrate chemical uptake and confirm the assumption that a chemical is negative for the measured toxicity outcome. For example, a recent study showed that GenX, an emerging PFAS of public health concern, was unstable in dimethylsulfoxide (DMSO), a solvent widely used in zebrafish chemical screening studies. Without tissue dose measurements, this compound would have been assumed to be negative for a number of developmental toxicity and developmental neurotoxicity endpoints [27].

The zebrafish also contributes to translational toxicology as a tool for evaluating computational models developed using *in vitro* and biochemical data generated with human cells or receptors. A computational model predicting xenobiotic disruption of blood vessel development [62] was subsequently validated using a transgenic zebrafish assay for evaluating chemical-dependent effects on vessel development [63]. Comparison of human amino acid sequence similarities for members of the predictive signature in the computational model with the zebrafish analog demonstrated biological domain-specific differences in protein sequence conservation [64], and the zebrafish assay proved zebrafish are particularly adept at detecting vascular disruptors associated with chemokine and/or extracellular matrix disruption in human *in vitro* assays [64].

Testing interventions in zebrafish

Zebrafish are increasingly used in phenotypic screens to identify compounds that reverse or suppress adverse effects of genetic mutations. For example, a zebrafish expressing a germline mutation in the *vhl* gene was used

to identify pharmacologic approaches for reversing the loss of vision associated with von Hippel-Lindau (VHL) syndrome [65], a rare disease characterized by vision loss associated with retinal capillary hemangioblastomas (tumors of retinal blood vessels). Zebrafish nullizygous for *vhl*, which were developed because *Vhl* knockout is embryolethal in mice, exhibit ectopic ocular blood vessels and aberrant eye development associated with an absent optokinetic response and significantly reduced visual motor response [65]. Sunitinib malate, an anti-angiogenic compound approved for cancer treatment, was found to reverse the ocular behavioral and morphological phenotypes in the *vhl*-knockout zebrafish [65]. The methods used in this study were not high throughput; however, an automated system for histological analyses in zebrafish was recently described, in which a commercially available platform that automates the transfer of zebrafish larvae from multi-well plates was combined with a customized spinning disk confocal microscope interfaced to software for high-resolution image acquisition and analysis [66]. Using this system to screen 175 chemicals in *Tg*(*mbp:eGFP*) larvae, a transgenic zebrafish line that expressed enhanced green fluorescent protein (eGFP) in myelinating oligodendrocytes, three novel compounds that significantly altered myelination were identified [66].

Zebrafish are also being leveraged to screen for compounds that mitigate the adverse effects of xenobiotics. A screen of 2271 small molecules identified 120 compounds that prevented cardiotoxicity in doxorubicin-exposed zebrafish, and subsequent SAR and target enrichment analyses of the seven most effective compounds identified CYP1A1 as a putative target [67]. This was corroborated by showing that *cyp1a*-knockout zebrafish larvae were resistant to doxorubicin-induced cardiotoxicity [67]. In a separate study, the age-dependent sensitivity of zebrafish to cyanide was leveraged to identify novel therapeutic targets for cyanide poisoning [68]. Initial studies revealed that zebrafish embryos are highly resistant to cyanide during the first 3 dpf but become progressively more sensitive as the larvae mature. Unbiased transcriptomic and metabolomic analyses revealed age-dependent differences in energy metabolism during cyanide exposure [68]. This observation led to the identification of compounds that modulate the pyruvate dehydrogenase complex and the small molecule sodium glyoxylate as potential prophylactic treatments for modulating sensitivity to cyanide poisoning [68].

Major data gaps and summary

Zebrafish are widely used for hazard identification and chemical prioritization [27,32,33,54,56,58,59,61,64]. To improve the use of zebrafish toxicity data in human risk assessment, several data gaps need to be addressed. First, harmonization of common toxicity assays and assessments is necessary to overcome variability in zebrafish data that is due to differences in testing protocols [69]. In addition, recent advances in developmental toxicity SARs [26,56] and chemical uptake [28,29] need to be expanded, and large-scale SAR analysis of more sensitive behavior end points, such as hyperactivity [27], are needed.

A major hindrance to the identification of relevant target pathways in zebrafish is the widespread use of whole-animal transcriptomic data, in part due to the technical difficulties associated with obtaining organ- or cell type–specific expression data. Future work should capitalize on the ability to sort specific populations of cells from transgenic zebrafish to increase the ability to detect xenobiotic-dependent transcriptional effects on sensitive, but low-abundance, cell types or single cell transcriptomics. In addition, although there are a growing number of examples in zebrafish [47,49,51,70], more studies should consider using gene editing to characterize toxicity mechanisms.

Perhaps, the area ripest for gains is the development of computational models to predict human toxicity from zebrafish toxicity data. Here, toxicokinetic data must be more routinely gathered in phenotypic zebrafish studies, both to identify true negative compounds [27] and to serve as the basis for dose extrapolation to human-relevant exposure scenarios.

In summary, the zebrafish is an exceptional model for the illumination of chemical-dependent toxic effects that are conserved or divergent across different experimental systems. Because of the inherent power of the system for medium- to high-throughput chemical-genetic screens, zebrafish represents a powerful experimental system for assessing chemical toxicity across life span, identification of chemical mode of action, generation of data sets for the prediction of chemical toxicity in humans, and rapid assessment of interventions to prevent chemical toxicity in exposed organisms. Researchers who focus on translational research using zebrafish may ultimately have a deep impact on the protection of both human health and the environment.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Using CRISPR-Cas9-dependent gene editing in zebrafish, the authors show that the *mrp1* transporter gene is critical for mitigating the toxic effects of cadmium and benzo[a]pyrene via cell efflux. This study leveraged gene editing techniques to reveal new knowledge about mechanisms of detoxification, and provides a tool to evaluate a key mechanism by which zebrafish, and potentially other organisms, combat xenobiotic toxicity.

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Using genetic and pharmacologic approaches, the authors demonstrated that *ahr2*, but not *ahr1a* or *ahr1b*, is required for normal development of adult zebrafish fins and craniofacial structures. From a toxicological perspective, the authors confirmed that *ahr2* mediates TCDD-dependent toxicity and demonstrated that loss of *ahr1a* or *ahrb* was not sufficient to block TCDD toxicity. This study used gene editing to elucidate the role of *ahr* genes in the context of TCDD-dependent toxicity and examine their crosstalk with estrogen receptor genes.

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von-Hippel Lindau (VHL) syndrome is a rare, systemic disease in which loss-of-function mutations in the tumor suppressor gene *VHL* leads to vision loss associated with retinal capillary hemangioblastomas (tumors of retinal blood vessels). Genetic knockout of *Vhl* in mice is embryolethal, therefore, in this study, the authors engineered *vhl* knockout zebrafish as an alternative vertebrate model of VHL syndrome. The authors not only demonstrate that *vhl* null zebrafish exhibit pathological lesions in the eye and deficits in vision similar to human VHL patients, but also identify sunitinib malate as a novel therapeutic agent for treating VHL-associated vision loss.

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This paper describes an exciting advance in automating histologic analysis of zebrafish, which has been a significant bottleneck in using histologic endpoints in higher throughput screening assays. The authors demonstrated the feasibility of the system by screening a small library of 175 chemicals for effects on myelination in larvae of transgenic zebrafish that expressed eGFP in myelinating oligodendrocytes Tg(mbp:eGFP). Three novel compounds that strongly enhanced the number of myelinating oligodendrocytes were identified. Importantly, this imaging platform and analysis pipeline appears flexible enough to adapt to high-resolution imaging-based screens of diverse phenotypes.

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Cyanide causes rapid toxicity via inhibition of cytochrome c oxidase-dependent cellular respiration, and while there are approved antidotes for cyanide poisoning, these are effective only when administered during the acute phase of cyanide poisoning. In this study, the authors leveraged their observation that zebrafish embryos are highly resistant to cyanide during the first 3 dpf but become progressively more sensitive to identify significant age-dependent differences in energy metabolism during cyanide exposure that led to the identification and validation of small molecules that modulate the tricarboxylic acid cycle and related metabolic processes as potential antidotes for cyanide poisoning.

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The authors generated a zebrafish *ahr2* null line using the CRISPR-Cas9 system to demonstrate that *ahr2* is required for zebrafish reproduction and fertility, development of adult fins and skeletal structures, as well as larval and adult behavioral responses. The *ahr2* null line was also resistant to TCDD-dependent toxicity, confirming that disruption of AhR2-dependent signaling is a key event in the mode of action for TCDD.

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Type I collagenopathies are a heterogenous group of connective tissue disorders, caused by genetic defects in type I collagen. Inherent to these disorders is a large clinical variability, for which the underlying molecular basis remains undefined. By systematically analyzing skeletal phenotypes in a large set of type I collagen zebrafish mutants, the authors show that zebrafish phenocopy different forms of human type I collagenopathies, suggesting a similar pathogenetic basis. This study illustrates the potential of zebrafish as a tool to further dissect the molecular basis of phenotypic variability in human type I collagenopathies, to improve diagnostic strategies, and enhance the discovery of novel therapeutic targets for treating these disorders.

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Dynamic tracking of neural activity across integrated neuronal networks at the level of the brain is critical to understanding how seizures initiate and propagate. The authors addressed this data gap by using a well-established larval zebrafish model of seizure activity and fast confocal imaging of larvae expressing genetically encoded calcium indicator (GCaMP). They found that seizure activity rapidly propagates from anterior-to-posterior brain regions in the zebrafish brain, and that neuronal subpopulations are active during interictal-like periods in a manner similar to that seen in human EEG recordings. Collectively, this work suggests the potential for non-invasive optical imaging approaches to advance understanding of the network basis underlying seizures and facilitate the development of methods to suppress these events.

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While zebrafish are increasingly used to study seizure disorders, most studies rely on locomotor assays to assess seizurogenic activity, which has raised significant questions about the zebrafish as a valid model of human seizure disorders. In this study, the authors demonstrated that GABA type A receptor antagonists known to cause status epilepticus in mammalian models, also caused sustained locomotor responses and seizure-like electrical activity in the brain of larval zebrafish. The authors also showed that these chemical-induced responses were attenuated by positive allosteric modulators of the GABA-A receptor. These data validate the zebrafish as a tool for screening chemical libraries for both pro- and anti-seizure activity.

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