Immediate and long-term consequences of vascular toxicity during zebrafish development


A R T I C L E   I N F O

Article history:
Received 8 April 2014
Received in revised form 21 May 2014
Accepted 27 May 2014
Available online 4 June 2014

Keywords:
Adverse outcome pathway
EGFR
Interssegmental vessel
VEGFR2

A B S T R A C T

Proper formation of the vascular system is necessary for embryogenesis, and chemical disruption of vascular development may be a key event driving developmental toxicity. In order to test the effect of environmental chemicals on this critical process, we evaluated a quantitative assay in transgenic zebrafish using angiogenesis inhibitors that target VEGFR2 (PTK787) or EGFR (AG1478). Both PTK787 and AG1478 exposure impaired intersegmental vessel (ISV) sprouting, while AG1478 also produced caudal and pectoral fin defects at concentrations below those necessary to blunt ISV morphogenesis. The functional consequences of vessel toxicity during early development included decreased body length and survival in juvenile cohorts developmentally exposed to inhibitor concentrations sufficient to completely block ISV sprouting angiogenesis. These data show that concentration-dependent disruption of the presumed targets for these inhibitors produce adverse outcomes at advanced life stages.

1. Introduction

The vascular system is the first functional organ system to develop in the embryo [1]. The process occurs by de novo synthesis of blood vessels termed vasculogenesis and by angiogenic sprouting from preexisting blood vessels. Vascular development relies on a complex array of biological processes including endothelial cell differentiation, migration and proliferation, extracellular matrix remodeling, and tube formation [2]. These processes are governed by a constellation of molecular signaling pathways [3], many of which have been shown to be susceptible to disruption by environmental chemicals [4–6].

Computational models have identified in vitro signatures for vascular disruption that predict adverse developmental outcomes in mammalian studies, including prenatal lethality, fetal weight reduction, and skeletal defects [7,8]. Using data generated in the initial phase of the U.S. EPA ToxCast™ program, which captures data from hundreds of cell-based and biochemical assays following exposure to 309 environmental chemicals, a number of compounds were predicted to exert toxicity in developing blood vessels by disruption of key molecular signaling pathways that control angiogenesis [7]. Over 90% of these compounds exhibited mammalian developmental toxicity based on in vivo guideline studies in the ToxRefDB database [7]. We extended these predictions to >700 chemicals with relevant ToxCast™ assay data but lack guideline study data in traditional animal models. Alternative testing platforms such as embryonic stem cells [9], Caenorhabditis elegans [10], and zebrafish [11,12] may provide surrogates to evaluate model predictions. Thus, there is an outstanding need to evaluate predictions generated by the computational vascular toxicity model in a relevant in vivo experimental system that examines vascular development. The goal of the research described here was to examine the longer-term consequences of embryonic...
vascular disruption in zebrafish using a detailed concentration response curve of chemicals know to disrupt vascular development. From a chemical screening perspective, this is essential to understand the functional relevance of vascular toxicity during development.

In the current study, we used embryonic zebrafish, a transparent vertebrate model of developmental toxicity with conserved genomic synteny and substantial genetic homology with mammals [13–17] as an experimental model of vascular development. Zebrafish possess orthologs for 70% of human genes [13] and 86% of 1318 human drug targets [18]. Here, transgenic zebrafish that express enhanced green fluorescent protein (EGFP) in blood vessels were used to visualize and quantify blood vessel formation during early development [19]. Intersegmental vessels (ISVs) were selected as a phenotypic readout of angiogenic vessel formation and used to generate a quantitative in vivo model of developmental vascular toxicity. While previous studies have employed automated image based phenotypic evaluation of ISV sprout length, assay sensitivity is unclear. Tran et al. screened 1280 phamacologic compounds and identified one novel ISV hit in addition to two control compounds [20]. Zoyo et al. screened 10 known cardiovascular toxicants through an image analysis pipeline that included ISV sprout length quantitation and reported no chemical-mediated effects, but significant inter-plate variability in ISV length [21]. In comparison, Vogt et al. deployed automated ISV quantitation to identify concentration dependent decrements in a number of ISV metrics following exposure to a known anti-angiogenic reference compound, but the assay was not expanded to test pharmacological agents or environmental chemicals [22]. We also previously reported a quantitative assay to detect ISV sprout growth, but the assay relies on confocal imaging of individual larvae and is not amenable for screening purposes [23,24]. Here, we generated a quantitative assay capable of detecting relatively subtle changes (∼8%) in ISV length relative to control sprouts.

In the current study, embryonic zebrafish were exposed to two mechanistically diverse reference chemicals that target molecular signaling pathways controlling ISV angiogenesis. We investigated concentration-dependent vascular toxicity in embryonic zebrafish exposed to human VEGFR2 or EGFR inhibitors. Both receptors have highly conserved tyrosine kinase domain sequences with higher order vertebrates suggestive of evolutionary conservation of receptor structure and function. In comparison to previous reports examining the effects of these compounds that relied on more qualitative metrics like the percent of normal vessels [25,26], the current assay was tested over a broad range of inhibitor concentrations and used to identify overt toxicity and quantitative vascular endpoints at both early developmental (i.e., embryonic and larval) stages and longer term time points (i.e., juvenile) following embryonic exposure.

We hypothesized that concentration-dependent disruption of the presumed targets for these inhibitors would impact development in such a way as to translate into phenotypic outcomes at advanced life stages. Our results show that, in general, embryonic vascular malformations persist in 5-day post fertilization (dpf) larvae and that severe vascular toxicity during embryogenesis is accompanied by overt malformations that result in increased mortality at the transition to independent feeding.

2. Materials and methods

2.1. Fish care and husbandry

Tg(kdrl:EGFP)∼843/+ (AB) strain zebrafish (Danio rerio) embryos were obtained from the Zebrafish International Resource Center and reared and bred in an Association for Assessment and Accreditation of Laboratory Animals (AAALAC) accredited facility at the U.S. EPA according to approved Institutional Animal Care and Use Committee (IACUC) protocols. Tg(kdrl:EGFP)∼843/+; mitfa~b602/b602 strain adults were generated and maintained at the University of Houston and reared according to IACUC protocols reviewed and approved at that institution. Adult fish were maintained on a 14 h light/10 h dark schedule on a recirculating water system at 28 ± 1 °C with a pH of 7.0 ± 0.2. For juvenile studies, Tg(kdrl:EGFP)∼843/+ (AB) strain zebrafish were reared at the Gulf Ecology Division of the U.S. EPA according to approved protocols. Adults were housed on a 14 h light/10 h dark schedule on a flow-through system in which water was maintained at 26 ± 1 °C. Mating tanks were set up the day prior to embryo collection using basic approved breeding protocols specific to each facility.

2.2. Chemical exposures

Unless otherwise noted, embryos were collected at U.S. EPA facilities on day 0 and bleached at 3–4 h post fertilization (hpf) according to the Zebrafish Book [27]. Bleached embryos were housed in 100 mm Petri dishes at 26 °C overnight. At 24 hpf, embryos were dechorionated in Proteinase K [27]. Stock solutions of PTK787 (Selleck Chem; CAS #: 212141-51-0) and AG1478 (Tocris Bioscience; CAS #: 153436-53-4) were prepared in dimethyl sulfoxide (DMSO; Sigma). Following dechorionation, groups of embryos were waterborne exposed to 0.07–1.25 μM PTK787 or 0.173–8.0 μM AG1478 in 10% Hanks’ solution [27] or 10% Hanks’ with 0.4% DMSO in 20 ml glass vials sealed with Teflon-lined lids to prevent volatilization. Exposures were initiated following dechorionation at 24 hpf in order to directly precede ISV sprouting and purposefully exclude the initial 24 hpf period to avoid earlier, non-vessel related teratogenesis. Each exposure group/vial was considered a single biological replicate and contained 500 μl of exposure solution per embryo. Each experiment was comprised of three independent groups per concentration with 6 or 8 embryos per vial for PTK787 or AG1478 exposures, respectively.

2.3. Larval imaging for quantitative and non-quantitative analyses

Following chemical exposures from 24 to 72 hpf, embryos were transferred to 12-well plates with 74 μm mesh inserts (Costar 3477) and washed three times in 10% Hanks’ solution. Embryos were visually assessed for abnormal morphology on an Olympus SZH10 stereomicroscope. Embryos were anesthetized (125 mg/L MS222 and 0.5 g/L of NaHCO₃) and imaged on a Leica M205FA fluorescence stereomicroscope (GFP filter, Planapo 1X objective, and zoom setting at ~21) and a Leica DFC365FX camera. To obtain fluorescent images, a 3.5 s exposure time was used; preliminary experiments determined that this exposure time would enhance ISV contrast but avoid saturation in the trunk region of the fish (data not shown). Transmitted light images were obtained using auto-exposure settings. For non-quantitative comparisons, images were obtained on a Nikon Eclipse Ti fluorescence microscope with a Photometrix Coolsnap HQ camera at 4× magnification using a GFP HQ filter with auto-exposure settings.

2.4. Lateral molds

A lateral orientation tool was developed to allow for reproducible orientation of zebrafish embryos and larvae (Supplementary Fig. S1) [28] and personal communication from Jochen Gehrig, Karlsruhe Institute of Technology, Germany). The lateral orientation tool consists of an 88.9 mm steel dowel pins (1/32 in. × 5/8 in.; McMaster Carr) were placed in the center of each well with 0.8 mm of the pins exposed. To cast
a mold, 96 well lids with evaporation rings (Costar 3931) were inverted and filled with 1% agarose (Sigma). The lateral orientation tool was inverted and gently depressed, and the agarose was allowed to solidify at room temperature. Following tool removal, anesthetized animals (125 mg/L MS222 and 0.5 g of NaHCO₃) were laterally oriented by placing the head of each animal in residual depressions. An additional 25 μl of 1% agarose was used to immobilize embryos or larvae in the agarose depressions. A custom stage adaptor was built to the image Costar 3931 lids on a Nikon Eclipse Ti fluorescence microscope.

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2014.05.014.

2.5. ISV and body length quantification

A uniform region of ISVs, consisting of five ISVs located anterior to the yolk sac extension, was targeted for quantitative analysis. Inter- and intra-vessel length within this region did not differ significantly among unexposed larvae (Supplementary Fig. S2A and B). Briefly, using Nikon Imaging Software (NIS) Elements, blinded fluorescent images were used to measure selected ISVs in 3-day old larvae (Supplementary Fig. S1). These measurements yielded a mean ISV length per larva in pixels. Mean ISV sprout lengths from 3 larvae were averaged to yield a mean ISV length per biological replicate. Each experiment was comprised of three biological replicates. Transmitted light images were used to obtain individual body length measurements with NIS Elements by a user blinded to the exposure groups.

Supplementary Fig. S2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2014.05.014.

2.6. Time-lapse microscopy

Tg(kdr:EGFP)p843/+; mitfa6962/b692 embryos were collected at the University of Houston on day 0 and placed in 6-well plates at a density of 25 embryos per well. Embryos were exposed to 0.22, 0.39 or 0.7 μM PTK787 or 0.17, 1.76 or 3.14 μM AG1478 in E3 medium [27] at 3.0–3.5 hpf (equivalent to 3.5–4.1 hpf at 26 °C) [27] and stored at 26 °C overnight. The control group was treated with 0.1% DMSO. The following day, embryos were selected for GFP fluorescence. At ~21 hpf at 26 °C, embryos were manually dechorionated and anesthetized with 0.13 μM eugenol [29]. Embryos were mounted on MatTek glass bottom dishes (Matek Corporation) in 0.3% low melting agarose supplemented with 0.13 μM eugenol (Sigma) and concentrations of PTK787 or AG1478 identical to the original exposure solutions. After low melt agarose solidification, E3 medium containing 0.13 μM eugenol and PTK787 or AG1478 was added. An Olympus Fluoview 1000 confocal microscope was used to obtain images in 15 min intervals over a span of 20 h. 100 z-plane optical slices were obtained with a 1.5 μm step size. Images were rendered by Olympus Fluoview software and projections were generated into a time-lapse video using NIH ImageJ (http://rsweb.nih.gov/ij/). Two animals per concentration were imaged simultaneously.

2.7. Quantification of time-lapse images

Vascular growth depicted in time-lapse image stacks was quantified using image analysis as described previously [23,30]. Briefly, stacks of images were smoothed with Gaussian filter. Next, threshold values were computed on maximum intensity projection (MIP) using the Li entropy method [31]. A MIP projection includes the brightest voxel along the z-axis of a 3D stack and is thus represents 2D mapping of 3D morphology. Both MIPs and stacks were binarized using the same threshold value. For quantification, the number of pixels, which represent zebrafish ISVs, were measured for each image at time point (t) and compared to the subsequent time point (t + 1). ISV quantification was performed on 2–3 embryos per concentration, except for the 0.17 μM AG1478 exposure (1 embryo). The growth rates (slopes) were calculated using linear regression trendline (Excel).

2.8. Juvenile stop-exposure study

At 24 hpf, groups of dechorionated embryos were exposed to aqueous concentrations of 0.07, 0.12, 0.22, or 0.7 μM PTK787; 0.17, 0.98, 1.76, or 3.14 μM AG1478 or 0.4% DMSO in 10% Hanks’ Solution as previously described. Each study was comprised of three biological replicates containing 15 fish per replicate. At 72 hpf, larvae were washed three times in 10% Hanks’ and transferred to 4 in. glass Carolina bowls containing 150 mL of 10% Hanks’ Solution and maintained at 26.5 °C under a 14 h light/10 h dark photoperiod. 50% water changes were performed daily beginning at 4 days post fertilization (dpf). Marine rotifers (Brachionus plicatilis; Reed Mariculture) and Brine shrimp (Artemia nauplii; Brine Shrimp Direct) were reared as a food source and enriched using the algal supplement N-Rich PL Plus (Reed Mariculture) following manufacturer instructions. Marine and freshwater invertebrates and dry larval diet #2 were fed to animals according to Supplemental Table S1. At 15 dpf, larvae were imaged and transferred to 8 in. Carolina dishes containing 500 ml of fish water (60 μg/ml; Instant Ocean® Sea Salts). At 30 dpf, surviving zebrafish were imaged then sacrificed in 300 mg/mL MS222, weighed, and fixed in 10% buffered formalin for later histological examination. Length measurements were determined using NIH ImageJ. Morbidity and mortality assessments were conducted daily. Dishes were aerated to maintain a minimum dissolved oxygen concentration of ≥60% saturation (4.86 mg/L at 26 °C).

Supplemental Table S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2014.05.014.

2.9. Juvenile histology

To evaluate persistence of treatment effects on vascular morphology, a subset of zebrafish from the 30 dpf PTK787 and AG1478 juvenile studies were evaluated by histopathology. Three fish per replicate vial from control, 0.07, 0.12, and 0.22 μM groups and one fish per replicate vial from the 0.70 μM group (given limited survival) were sampled for histology. From the AG1478 study, three fish per replicate vial from control, 0.17, 0.98, 1.76, and 3.14 μM groups were sampled. Fish were transferred to labeled cassettes, processed into paraffin blocks, sectioned at 8 μm, and mounted on glass slides. For evaluation of ISVs and other vascular structures, a minimum of four parasagittal sections from the lateral margin of the left eye to the midline were selected and stained with Harris hematoxylin and eosin (H&E) using standard histological procedures. H&E-stained slides were reviewed by a certified study pathologist and imaged using an Infinity2 digital camera (Lumenera). A subset of slides was also read by a peer review pathologist at Experimental Pathology Laboratories Incorporated. To confirm endothelial continuity throughout ISVs tracts, six sections/fish from three control and three high-dose PTK787 fish were also stained for tomato lectin using standard procedures [32]. Briefly, sections were deparaffinized, rehydrated, rinsed in phosphate buffered saline containing 0.1% Triton X-100 (PBSTx) at pH 7.4 for 10 min, and then incubated in PBSTx with cations for 30 min. Slides were blotted, dried, and treated with tomato lectin (10 μg/ml from Lycopersicon esculentum; Vector Laboratories) conjugated with a fluorescent dye (DyLight 488) and counterstained...
with 4′,6-diamidino-2-phenylindole (DAPI) in PBSTx with cations overnight at 4°C in the dark. Following incubation, slides were rinsed in PBS and coverslipped. Fluorescent-labeled sections were imaged using a Nikon Eclipse Ti confocal microscope with GFP and DAPI filters and NIS Elements software.

2.10. Statistical analysis

Unless indicated otherwise, one-way analysis of variance (ANOVA) with a Newman–Keuls multiple comparison test were used to evaluate differences between control and treated groups; values of \( P < 0.05 \) were considered statistically significant. Because we are comparing two or more samples, the data are presented with inferential error bars (i.e., SEM) rather than with descriptive error bars (i.e., SD)[33]. For juvenile survival data, statistical comparisons between exposure groups and DMSO vehicle control were determined by a paired two-way ANOVA to assess associations between treatment, mortality, and dpf. Survival differences between groups were evaluated each day by a Bonferroni post hoc test (* \( P \leq 0.05 \)). Effective concentrations reported in Table 1 were determined by visual inspection of the graphs.

3. Results

3.1. PTK787 exposure produces concentration-dependent malformations in embryonic zebrafish

PTK787 was used to pharmacologically inhibit VEGFR2 activity by blocking autophosphorylation of the TK domain and subsequent receptor activation[34]. To assess the effect of VEGFR2 inhibition on ISV development, embryos were exposed to 0.07–1.25 \( \mu \)M PTK787 or 0.4% DMSO alone from 24 to 72 hpf (Fig. 1A). We observed a concentration-dependent increase in overt toxicity, including pericardial edema, curved body axis, and microphthalmia at 72 hpf (Fig. 1B and C). These defects coincided with a decrease in body length (Fig. 1D). Concentrations of PTK787 \( \leq \) 0.22 \( \mu \)M failed to produce trunk defects or other visible malformations.

3.2. Developmental exposure to PTK787 triggers persistent angiogenic vessel toxicity

To determine whether ISV toxicity is observable in the absence of gross structural malformations, tg(kdrl:EGFP)s843/+ zebrafish were imaged post-exposure at 72 hpf. Concentration-dependent decrements in ISV length were observed following developmental exposure to PTK787 (Figs. 2A and B). Blunting of ISV sprout length was detectable at water concentrations of PTK787 \( \geq \) 0.12 \( \mu \)M PTK787 (Fig. 2B). Additionally, nominal concentrations of PTK787 \( \geq 0.22 \mu \)M appeared to interfere with primary vasculogenesis in the caudal vein region (Fig. 2A). To measure ISV...
5.3. Exposure to AG1478 induces fin and vessel malformations in zebrafish

Embryos were exposed to 0.17–8.0 μM AG1478 or vehicle control from 24 to 72 hpf (Fig. 1A). At 72 hpf, concentration-dependent effects on caudal fin formation (≥0.17 μM), body length (≥1.76 μM), and curved body axis (≥3.14 μM) were observed (Fig. 4A–C). At 72 hpf, blunted ISV sprout length was detectable in zebrafish exposed to 1.76–5.60 μM AG1478 during development (Fig. 4D and E). Mortality was observed in 80–100% of embryos exposed to 8.0 μM AG1478 from 24 to 72 hpf, precluding ISV quantification (Figs. 4B). Time-lapse microscopy of tg(kdrl:EGFP);mitfa/b692/b692 embryos exposed to AG1478 produced concentration-dependent deficits in ISV sprout growth rates relative to vehicle controls (Supplemental Videos 5–8 and Fig. 4F). Supplemental Videos 5–8 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2014.05.014.

3.4. Sustained defects observed in zebrafish developmentally exposed to AG1478

Following exposure to 0–8 μM AG1478 from 24 to 72 hpf, embryos were reared in chemical-free medium until 120 hpf (Fig. 1A). At 120 hpf, a suite of gross malformations was observed in 60–100% of embryos exposed to 3.14–5.60 μM AG1478, respectively (Fig. 5A and C). Observed malformations included shortened trunk and pericardial, eye, and yolk sac edema (Fig. 5A and B). Interestingly, pectoral fin defects were noted in 120 hpf embryos developmentally exposed to ≥0.55 μM AG1478 (Fig. 5A and B). Representative fluorescent images also showed evidence of sustained effects on ISV sprout length at 120 hpf (Fig. 5B).

3.5. Developmental exposure to PTK787 or AG1478 increases mortality in juvenile fish

To determine the effects of embryonic developmental ISV toxicity on growth and development endpoints, a 30 days study
was performed. Embryos were exposed to 0.07–0.7 μM PTK787, 0.17–3.14 μM AG1478, or 0.4% DMSO from 24 to 72 hpf and then reared in chemical-free medium through 30 dpf (Fig. 6A). Statistically significant effects on survival (Fig. 6B and C) were observed in cohorts exposed to 0.7 μM PTK787 or 3.14 μM AG1478 during development relative to vehicle control. The primary window of mortality occurred at 8–10 dpf for both compounds. No effect on weight or length at 30 dpf or on length at 15 dpf and 30 dpf was noted in juveniles exposed to 0.07–0.22 μM PTK787 or 0.17–3.14 μM AG1478 during early development (Supplementary Figs. S3 and S4). A significant decrease in body length was observed in the 0.7 μM PTK787 group at 15 dpf but not 30 dpf (Supplementary Fig. S3).

Supplementary Figs. S3 and S4 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2014.05.014.

3.6. Normal vascular morphology in PTK787 and AG1478 survivors at 30 dpf

Vascular morphology was evaluated in a subset of 30 dpf fish developmentally exposed to PTK787 or AG1478. Structures evaluated included ISVs, cardinal veins, dorsal aorta, and branchial and retinal vessels. Across all groups, trunk and tail somitic myotomes were regularly spaced and separated by discrete ISVs branching from the dorsal aorta (Fig. 7A and B). Vascular structures were limited to mild acute hemorrhage within trunk myotomes and mild vascular attenuation of the caudal dorsal aorta, both in a single animal in the PTK787 0.22 μM group. Otherwise, irregular ISV tracts and other vascular or perivascular changes were not observed. Features of ISVs in all groups included well-spaced endothelial cell nuclei and scattered intraluminal erythrocytes, confirming vessel patency. Across the subset of control and high-dose PTK787 fish examined by immunohistochemistry, tomato lectin staining of endothelial cells was present within ISV tracts from the dorsal aorta to the dorsal longitudinal anastomotic vessel, indicating continuous endothelium; no differences were seen in intensity or other lectin staining patterns between groups (Fig. 7C and D). The caudal and/or common cardinal veins, dorsal aorta, branchial vessels, and other prominent vascular structures contained abundant erythrocytes and showed no evidence of surrounding edema or inflammation (Fig. 7). These results demonstrate normal vascular morphology in surviving fish at 30 dpf.

4. Discussion

This study was designed to evaluate the functional consequences of vascular toxicity during development in embryonic zebrafish. To do so, we used a quantitative model of vascular toxicity in transgenic zebrafish. Here, two chemical inhibitors
that target distinct signaling pathways were leveraged to disrupt angiogenic signaling during development. While previous reports examining the effects of these reference compounds have been published, they relied on qualitative metrics like the percent of normal vessels [25,26]. The current assay was tested over a broad range of inhibitor concentrations and used to detect overt toxicity and quantitative vascular endpoints at early developmental stages in addition to the functional consequences of embryonic vascular toxicity at advanced life stages.

The adverse outcome pathway (AOP) framework provides a useful construct for evaluating data generated from our model. AOPs are used to describe hypothetical linkages between a series of events that follow from molecular perturbation to an observable phenotype [35–37]. AOPs are predicated on a proximal perturbation of a molecular target by chemical exposure, termed a molecular initiating event [37]. Multiple intermediate steps at the cellular, tissue and organ levels lead to an adverse outcome at the level of an individual or population [37]. A key consideration in the AOP concept is that perturbations at higher levels of biological organization should occur at similar or higher chemical doses. Because AOPs are typically constructed based on concentration response data, the framework simply describes hypothetical linkages between different levels of biological organization that can be used for hypothesis generation. In contrast, knockout
or knockdown studies are necessary to demonstrate causality between molecular initiating events and adverse outcomes. Our detailed concentration response curves allowed us to observe that increasing concentrations of PTK787 were associated with toxicity at higher levels of biological complexity (i.e. ISV malformations vs. gross structural defects) (Table 1). These data raise the possibility that VEGFR2 inhibition by PTK787 exposure may be sufficient, as a molecular initiating event, to trigger vessel toxicity and subsequent morbidity and mortality. In support of this AOP concept, functional inactivation of the VEGFR2 gene is embryo lethal in VEGFR2−/− nullizygous mice [38]. Functional inactivation of the so-called ‘decoy’ receptor VEGFR1, which functions to prevent inappropriate VEGFR2-dependent signaling [39], is also embryo lethal in mice as a result of unchecked endothelial growth and vascular disorganization [40]. This implies the role of VEGFR1 in regulating VEGFR2-dependent signaling in the mammalian embryo.

In contrast to PTK787, exposure to the EGFR inhibitor AG1478 presents a more complex molecular paradigm. In agreement with previous reports of AG1478-induced ISV defects in zebrafish [20,26], we observed partial ISV sprouts in embryos and larvae developmentally exposed to AG1478. In comparison to PTK787 exposure, the ISV phenotype induced by AG1478 exposure occurred at concentrations exceeding those that produced overt toxicity including multiple fin malformations. Therefore, according to the concentration response profiles, PTK787 treatment appears to induce a vascular-specific AOP, while AG1478 exposure likely triggers multiple different AOPs (Table 1).

In the current study, we sought to understand the functional consequence of ISV malformations during development. Developmentally exposed embryos were reared in chemical-free medium through 5 or 30 dpf in a stop-exposure experimental design. At 5 dpf, abnormalities in ISV structure appeared to persist (Fig. 3B and C). Larvae developmentally exposed to 0.12–0.22 μM PTK787 presented with partially stunted ISV sprouts in the absence of visible malformations (Fig. 3A and B). Despite detectable vessel malformations, no effects on survival were noted out to 30 dpf (Fig. 6B). In contrast, severe malformations and ISV toxicity were detected in embryos exposed to ≥0.39 μM (Figs. 1B, 2A, B, and 3A–C), while exposure to 0.7 μM PTK787 resulted in pronounced mortality beginning at 8 dpf (Fig. 6B). No clear treatment-related effects on ISVs were observed on histology among survivor fish at 30 dpf (Fig. 7). Taken together, these data support the concept that severe malformations during development predict reduced survivability which coincides with the transition to independent feeding. Of note, several studies report that the cardiovascular system is not required to supply oxygen until 7–14 dpf [41,42]. Thus, the observed delayed mortality may be related to improper formation...
Fig. 6. Developmental exposure to PTK787 is associated with increased mortality in juvenile zebrafish. Embryos were exposed to 0.07, 0.12, 0.22 or 0.7 μM PTK787; 0.17, 0.98, 1.76 or 3.14 μM AG1478 or 0.4% DMSO from 24 to 72 hpf and subsequently reared in chemical-free medium from 3 to 30 dpf. Animals were assessed daily. (A) Study design. Daily survival data for (B) PTK787 and (C) AG1478 (n = 3, 15 animals per biological replicate, *P < 0.05).

of the vascular system. Further studies manipulating oxygen levels would be required to establish a causal link between VEGFR2 inhibition, reduced oxygen and decreased survivability at 8 dpf.

AG1478 was also used to evaluate the quantitative vascular assay in zebrafish. AG1478 inhibits EGFR, a receptor TK that is indispensable for normal embryonic development. Depending on genetic background, mice lacking EGFR die during embryonic development or shortly after birth [43–45]. The most sensitive phenotypes observed in the present study following exposure to AG1478 were caudal and pectoral fin defects. Interestingly, fin malformations have not been previously reported in zebrafish embryos exposed to this inhibitor [26]. Zebrafish embryos possess caudal and pectoral fin folds. The pectoral fold emerges from a fin bud that is homologous to the apical ectodermal ridge (AER) of tetrapods [46], an epidermal structure that functions to promote cell proliferation and mesenchymal outgrowth [47]. Following its discovery in embryonic wing development in drosophila [48], EGFR has been linked to vertebrate AER formation and limb outgrowth and patterning [49]. In chick embryos, overexpression of EGFR produced multiple AERs and polydactyly and disrupted normal patterning of molecules critical to limb development [49]. Thus, evidence exists across multiple species to support a role for EGFR activity in pectoral fin development by acting on the AER to disrupt expression of key molecular regulators of fin patterning and outgrowth.

The teleost AER transitions into an extended apical fold that is vascularized by the circumferential fin blood vessel [22]. Given that our group and others have observed vascular disruption in larvae exposed to EGFR inhibitors ([26] and Fig. 6D and E), EGFR-dependent effects on pectoral fin formation via a mechanism involving vascular disruption should not be ruled out. The vascular hypothesis is best demonstrated by thalidomide, an antiemetic compound used in the 1960s by pregnant women [50,51]. In addition to increasing rates of miscarriages and still births, over 80% of exposed children were born with severe limb malformations [50–52]. In chick embryos exposed to thalidomide analogs, loss of immature limb blood vessels was determined to be the primary cause of limb defects [53]. In zebrafish, exposure to thalidomide blocked pectoral fin bud development [54]. It remains to be determined whether AG1478 initiates pectoral fin malformations through disruption of the AER and underlying molecular signaling pathways that drive fin formation or by vascular disruption of the circumferential blood vessel.

In summary, we showed that that concentration-dependent disruption of VEGFR2 or EGFR-dependent signaling impacted vascular development and caused overt toxicity that led to increased mortality at the transition to independent feeding. Further studies are necessary to fully elucidate the functional consequences at advance life stages of structural defects in early vessel development that occur without accompanying overt toxicity. Looking forward, assessment of vascular toxicity in zebrafish meets a critical need for an integrative biological system in which predictions by high throughput in vitro and computational in silico models can be rapidly qualified and further extended into mechanistic biological models.
**Conflict of interest**

The authors declare that there are no conflicts of interest.

**Acknowledgements**

We are grateful to Alisha Palekar for measuring blinded images. We greatly appreciate Earl Puckett for fabricating the lateral orientation tool and stage adaptor. The authors thank Gabriele Daniels for her technical assistance with the juvenile studies and Alan Tennant for microscopy and imaging assistance. We thank Kim Howard, Ned Collins and Leslie Martin for fish husbandry and Jeffrey Wolf at Experimental Pathology Laboratories, INC.
for histopathology peer review. We thank the Zebrafish International Resource Center (RR12546) for providing Tg(kdrl:EGFP)P843/+ zebrafish embryos, CWM and MW were supported by the EPA-Texas-Indiana University Virtual STAR Grant (RB34289). We are grateful to William Mundy and E. Sidney Hunter for their critical review of the manuscript. This manuscript has been reviewed by the U.S. Environmental Protection Agency and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

References