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The role of the aryl hydrocarbon receptor pathway in mediating synergistic developmental toxicity of polycyclic aromatic hydrocarbons to zebrafish.

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ABSTRACT

Planar halogenated aromatic hydrocarbons (pHAH), such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin), show strong binding affinity for the aryl hydrocarbon receptor (AHR) and are potent inducers of cytochrome P4501A (CYP1A). It is widely accepted that dioxin toxicity is largely AHR-mediated; however the role of CYP1A activity in causing that toxicity is less clear. Another class of AHR agonists of increasing concern because of their known toxicity and ubiquity in the environment is the polycyclic aromatic hydrocarbons (PAH). Like dioxin, some PAH also cause toxicity to early life stages of vertebrates. Symptoms include increased cardiovascular dysfunction, pericardial and yolk sac edemas, subcutaneous hemorrhages, craniofacial deformities, reduced growth and increased mortality rates. Although developmental effects are comparable between these two types of AHR agonists, the roles of both the AHR and CYP1A activity in PAH toxicity are unknown. As observed in previous studies with killifish (*Fundulus heteroclitus*), we demonstrate here that co-exposure of zebrafish (*Danio rerio*) embryos to the PAH-type AHR agonist β-naphthoflavone (BNF), and the CYP1A inhibitor α-naphthoflavone (ANF), significantly enhanced toxicity above that observed for single compound exposures. In order to elucidate the role of the AHR pathway in mediating synergistic toxicity of PAH mixtures to early life stages, we used a morpholino approach to knock down expression of zebrafish AHR2 and CYP1A proteins during development. We observed that while knock down of AHR2 reduces cardiac toxicity of BNF combined with ANF to zebrafish embryos, CYP1A knock down markedly enhanced toxicity of BNF alone and BNF + ANF co-exposures. These data support earlier chemical inducer/inhibitor studies and also suggest that mechanisms underlying developmental toxicity of PAH-type AHR agonists are different from that of pHAH.

Identifying the pathways involved in PAH toxicity will provide for more robust, mechanistic-based tools for risk assessment of single compounds and complex environmental mixtures.

Key Words: AHR; CYP1A; PAH; developmental toxicity; zebrafish; risk assessment.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) originate from incomplete combustion, such as burning of fossil fuels, and invariably occur in the environment as complex mixtures of non-substituted, alkyl-, and/or N-, S-, and O-ring substituted PAH. Although this class of compounds can enter the environment through natural sources, such as ocean oil seeps and forest fires, the majority of PAH deposition can be attributed to anthropogenic activity, including wood treatment and petroleum refining (Latimer and Zheng, 2003; Douben, 2003), and non-point sources such as urban run-off (Van Metre *et al*., 2000). As such, sediment concentrations of PAH in urban and industrialized centers can be up to two orders of magnitude higher than those in rural areas (Dabestani and Ivanov, 1999).

Some PAH and extracts of PAH-contaminated mixtures are toxic to embryonic stages of fish (Billiard *et al.*, 1999; Hawkins *et al.*, 2002; Wassenberg and Di Giulio, 2004b). Collectively, these effects mimic "blue sac disease" characterized for more potent aryl hydrocarbon receptor (AHR) agonists and cytochrome P4501A (CYP1A) inducers such as certain planar halogenated aromatic hydrocarbons (pHAH), including 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (dioxin) and co-planar PCBs (e.g., 3,3',4,4',5-pentachlorobiphenyl, "PCB126") (Helder, 1981; Walker and Peterson, 1991; Wannemacher *et al.*, 1992; Elonen *et al.*, 1998; Chen and Cooper, 1999; Toomey *et al.*, 2001). In these studies, the most pronounced effect of chronic, sustained exposure of early life stages of fish species to either pHAH or PAH is cardiovascular dysfunction. Recently, dioxin has been shown to adversely affect murine heart development (Thackaberry *et al.*, 2005), consistent with effects previously observed in piscine and avian embryonic models (Ivnitski-Steele *et al.*, 2005).

As evidenced in numerous studies, the general consensus in mammalian models is that dioxin toxicity is largely mediated through binding to the AHR (Fernandez-Salguero *et al.*, 1996), a ligand-activated transcription factor found in vertebrate species from fish to humans (Schmidt and Bradfield, 1996). In general, AHR pathways are similar among mammals and lower vertebrates, including fishes, reptiles, and birds (Hahn, 1998). Activated AHR regulates expression of a number of genes of the AHR battery including the phase I enzymes cytochrome P4501A1 (CYP1A1), CYP1A2, and CYP1B1 (Whitlock, 1999; Nebert *et al.*, 2000). Two divergent AHRs, AHR1 and AHR2 (Hahn *et al.*, 1997), have been identified and characterized in killifish (*Fundulus heteroclitus*) (Karchner *et al.*, 1999) and zebrafish (*Danio rerio*) (Andreasen *et al.*, 2002). In zebrafish, AHR2 binds dioxin with high affinity, is transcriptionally active, and mediates toxic response to dioxin. Conversely, zebrafish AHR1 (AHR1A) lacks the ability to bind pHAH or PAH *in vitro* and is transcriptionally inactive (Andreasen *et al.*, 2002). Since it is non-functional in zebrafish, Karchner *et al.* (2005) suggested AHR1A is a pseudogene in this species. More recently, a novel zebrafish AHR protein belonging to the AHR1 clade, AHR1B, has been discovered (Karchner *et al.*, 2005). Unlike *ahr1a*, the *ahr1b* gene encodes a functional, transcriptionally active AHR protein that is expressed early in zebrafish development. In contrast to AHR2A, AHR1B expression is not inducible by dioxin (Karchner *et al.*, 2005). Thus, AHR1B may play an important physiological role during embryogenesis.

The recent advent of antisense morpholino oligonucleotide (MO) gene technology has shown that dioxin-induced toxicity in zebrafish (*Danio rerio*) early life stages is also AHR-dependent and corroborates mammalian studies. Specifically, knock down of the AHR2 isoform in zebrafish embryos blocks CYP1A expression in the vascular endothelium and protects against dioxin-induced toxicity, including pericardial edema and craniofacial deformities (Teraoka *et al.*,

2003; Carney *et al.*, 2004; Dong *et al.*, 2004). Several studies have shown that inhibition of CYP1A activity or targeted knock down of CYP1A protein expression also prevents or rescues toxic responses to dioxin or PCB126 in zebrafish embryos, thus suggesting that CYP1A also plays a role in triggering dioxin-mediated developmental toxicity in fish embryos (Cantrell *et al.*, 1996; Teraoka *et al.*, 2003; Dong *et al.*, 2004). However, a recent zebrafish study showed that while MO knock down of AHR2 protected embryos from the developmental toxicity of dioxin, MO knock down of CYP1A had no effect (Carney *et al.*, 2004).

Although the suite of cardiovascular defects are very similar between PAH and pHAH-type AHR agonists, the roles of the AHR and CYP1A activity in the toxicity of PAH are less clear. In previous studies we have shown that exposure of killifish (*Fundulus heteroclitus*) embryos to model PAH that are agonists for the AHR, for example β-naphthoflavone (BNF) and benzo(a)pyrene (BaP), or PAH-contaminated mixtures, caused significantly greater-than-additive interactions (in terms of cardiovascular effects) when combined with CYP1A inhibitors (Wassenberg and Di Giulio, 2004b; Wassenberg *et al.*, 2005). This synergy is in marked contrast to responses with dioxin and inhibitors, where CYP1A chemical inhibition or protein knock down either reduces (Cantrell *et al.*, 1996; Dong *et al.*, 2002) or does not alter toxic response to dioxin during zebrafish development (Carney *et al.*, 2004). We have also shown that killifish embryos co-treated with the pHAH PCB126 and the CYP1A inhibitor α-naphthoflavone (ANF) decreased both the frequency and severity of deformities compared with embryos exposed to PCB126 alone (Wassenberg and Di Giulio, 2004b).

The co-occurrence of agonists and inhibitors is typical of environmental PAH mixtures (Willett *et al.*, 2001; Wassenberg *et al.*, 2005). However, current risk assessments for PAH assume an additive model of toxicity for PAH (e.g., Barron *et al.*, 2004). Thus, the synergy we

have observed has important ramifications for adequately assessing risks posed by exposures to PAH, and also raises compelling questions concerning mechanisms underlying these greater-than-additive toxicities.

In this study, we endeavored to further dissect mechanism(s) underlying PAH synergy using the genetic tools available in zebrafish. First, we asked whether synergy observed between PAH-type agonists and CYP1A inhibitors could be repeated in zebrafish, or was this phenomenon specific to killifish embryos. Upon confirming that similar toxic response to inducer/inhibitor mixtures occurred in both species, we then used a morpholino approach in zebrafish to dissect the nature of interaction between PAH that act as AHR agonists and CYP1A inducers and PAH that act as inhibitors of CYP1A. Our findings with zebrafish embryos in the current study lead us to conclude that: 1) PAH synergy appears to be receptor-mediated as AHR2 knock down reduced cardiovascular toxicity characteristic of BNF, ANF co-exposures; and 2) CYP1A plays an adaptive or protective role, as blocking translation of this protein greatly enhanced the toxicities of non-toxic doses of BNF alone and of BNF combined with ANF.

MATERIALS AND METHODS

Animals. Newly fertilized zebrafish embryos were collected at 20-min intervals in 1X Danieau's water (Nasevicius and Ekker, 2000) following mating of AB* strain adults maintained at 28 °C on a 14 hr light, 10 hr dark light cycle. Embryos were maintained in 1X Danieau's water under the same temperature and photoperiod conditions for the duration of experiments.

Morpholinos. Based on cDNA sequences submitted to GenBank, we used morpholinos (Gene Tools, Philomath, OR) that had been designed previously to block initiation of translation of zebrafish aryl hydrocarbon receptor 2 (*zfahr2*-MO) mRNA (AF063446; Teraoka *et al.*, 2003)

and cytochrome P4501A (*zfcyp1a*-MO) mRNA (AB078927; Carney *et al.*, 2004). Sequences used were: *zfahr2*-MO (5'-TGTACCGATACCCGCCGACATGGTT-3') and *zfcyp1a*-MO (5'-TGGATACTTTCCAGTTCTCAGCTCT-3'). Gene Tools' standard control morpholino (5'-CCTCTTACCTCAGTTACAATTTATA-3') was used as control morpholino (control-MO) in experiments. Morpholinos were diluted to working stocks (control-MO and *zfcyp1a*-MO to 0.15 mM; *zfahr2*-MO to 0.1 mM) in 1X Danieau's solution for injection (estimated injection volume 2-4 nl; Nasevicius and Ekker, 2000). Injection working stocks were stored at 4 °C and heated for 5 min at 65 °C prior to use. Control- and *cyp1a*-MOs were tagged with a 3' end carboxyfluorescein modification (Gene Tools) in order to monitor injection success and validate experimental protocol using epifluorescence. The *ahr2*-MO was generously provided by Dr. Elwood Linney (Duke University) and did not have the 3' end fluorescein modification.

Embryo Microinjection. Newly fertilized embryos were screened and healthy embryos at the 1- to 4-cell stage were injected within 2 hours post-fertilization (hpf) with either *zfahr2*-MO, *zfcyp1a*-MO, or control-MO working stocks using a Narishige IM300 Microinjector (Tokyo, Japan). At 24 hpf, dead and damaged embryos were screened and removed. Non-damaged embryos injected with fluoresceinated *cyp1a*- and control-MOs were assessed for fluorescent intensity at this time to evaluate injection success and even distribution of the morpholino throughout the embryo cell mass. Only embryos exhibiting strong, uniform fluorescence at 24 hpf were subsequently used in dosing experiments.

Dosing experiments. Chemicals and dosing concentrations were selected based on our earlier observations of synergistic PAH interactions in killifish (Wassenberg and Di Giulio, 2004b). Model PAH used in this study were BNF (AHR agonist) and ANF (CYP1A inhibitor). Mechanisms of action and chemical properties for compounds used in the current study have

been described in detail elsewhere (Wassenberg and Di Giulio, 2004b).

All chemicals were dissolved in DMSO and stored protected from light at -20 °C. Stock solutions were thawed at room temperature and vortexed prior to dosing. Zebrafish embryos were exposed beginning at 24 hpf to waterborne nominal concentrations of BNF alone and in combination with waterborne nominal concentrations of ANF in DMSO (<0.1% v/v). Embryos were left in original dosing solutions for the duration of the experiment (72-96 hpf).

Because of species differences in sensitivity to dioxin-like toxicity, we first tested a range of BNF and ANF concentrations to characterize dose-response for embryotoxicity to zebrafish embryos. In these experiments, 30 zebrafish embryos were exposed to graded concentrations of BNF or ANF in glass beakers to 25-ml of egg water containing 21 μg/L 7-ethoxyresorufin (7-ER, in DMSO) until 96 hpf when embryos (n=20) were scored blind for pericardial edema; a hallmark symptom of blue sac disease. We then tested whether we could repeat synergy between a PAH-type agonist (BNF) with a CYP1A inhibitor (ANF) previously observed in killifish (Wassenberg and Di Giulio, 2004b). Three replicates of 30 pooled embryos were exposed in glass beakers with 25-ml of egg water containing 7-ER to waterborne BNF with or without ANF (100 μg/L). Embryos were sub-sampled and transferred to clean egg water at 72 hpf for assay of *in vivo* ethoxyresorufin-*O*-deethylase (EROD) activity (n=6-8/treatment) and again at 96 hpf for deformity assessment (n=3-8 embryos/treatment), as described below.

In morpholino experiments, replicate groups (n=2-3) of 5 injected and non-injected control embryos were exposed in glass scintillation vials in 7.5-ml of egg water containing 21 μg/L 7-ER to waterborne concentrations of BNF with or without ANF (100 μg/L). Larvae were transferred to clean egg water at 96 hpf and individual larvae were imaged and scored blind for measurement of *in vivo* EROD activity and deformities. In order to confirm specificity of both

zfahr2- and *zfcyp1a*-MO phenotypes, treatments were repeated using embryos that had been injected with the standard control morpholino (control-MO). While ideally this control would be included in each experiment, the large numbers of embryos required for binary combinations of chemical treatments in our morpholino experimental design was prohibitive. We are confident with this approach given that the AHR2 and CYP1A morpholinos have been extensively characterized in the zebrafish literature (for e.g., Teraoka *et al.*, 2003; Carney *et al.*, 2004; Dong *et al.*, 2004).

In vivo EROD activity. We used a slightly modified *in ovo* EROD method (Nacci *et al.*, 2005) to measure the CYP1A activity of zebrafish larvae. In all experiments, we used *in vivo* measurement of CYP1A catalytic activity as a surrogate for CYP1A protein induction or inhibition. Briefly, on day 3 or 4 (72 or 96 hpf) of development, hatched zebrafish embryos were anesthetized with MS-222 and immobilized in 3% methylcellulose. The accumulated resorufin fluorescent product of CYP1A metabolism of 7-ER in the gastro-intestinal tract was visualized by fluorescent microscopy (50x magnification using a rhodamine red filter set; Axioskop; Zeiss, Thornwood, NY). EROD activity was measured as fluorescent intensity and quantified digitally by IP Lab software (Scanalytics Inc., Fairfax, VA). *In vivo* EROD values were expressed as a percentage of fluorescent intensity in control embryos.

Quantification of Teratogenesis. Day 4 larvae (96 hpf) were anesthesized in MS-222 and immobilized in 3% methyl-cellulose in order to image the lateral left side. Captured digital images were analyzed blind for teratogenic endpoints previously described in developing embryos exposed to dioxin (Carney *et al.*, 2004). The first, truncation of Meckel's cartilage or lower jaw gap length, was measured as the distance from Meckel's cartilage to the anterior edge of the larvae. We also measured total area of the pericardial sac as an index of pericardial sac

edema. Captured images were quantified digitally using IP Lab Software (Scanalytics Inc., Fairfax, VA) and expressed as percent of control values.

Statistical Analysis. Data were analyzed with Statview for Windows (version 5.0.1; SAS Institute, Cary, NC). Dose-response data shown in Figure 1 were analyzed by simple linear regression with Excel (Microsoft 2003). EROD values and deformity measurements (pericardial edema area and lower jaw gap length) were analyzed by factorial analysis of variance (ANOVA). When ANOVA yielded significance ($p < 0.05$), Fisher's protected least-significant differences was used as a post hoc test. We used significance of the interaction term to test our null hypothesis that mixtures tested would yield additive response.

RESULTS

In initial experiments examining dose-response relationships (0-500 μg/L) for model PAH, both BNF-alone (p=0.0076) and ANF-alone (p=0.016) treated larvae showed a dose-dependent increase in the occurrence of pericardial edema (Figure 1). No deformities were observed at doses used in subsequent BNF-alone (1 and 10 μg/L) and ANF-alone (100 μg/L) co-exposure experiments.

Consistent with our previous studies in killifish (Wassenberg and Di Giulio, 2004b), co-exposure of zebrafish embryos to nominal waterborne concentrations of BNF (1 μg/L) and ANF (100 μg/L) inhibited BNF-induced EROD activity by 72 hpf (p=0.0003) and increased cardiovascular toxicity at 96 hpf, specifically pericardial edema (p=0.0002) and craniofacial deformities (p=0.0006), above that observed for single compound exposures (Figure 2).

Based on these results we used a morpholino approach to test the hypothesis that targeted knock down of CYP1A protein would mimic the inhibitory effect of ANF on BNF-induced

EROD activity and deformities previously observed in zebrafish and killifish embryos (Wassenberg and Di Giulio, 2004b). *In vivo* EROD activity was used in all knock down experiments as an index of morpholino function and as a surrogate measure of CYP1A protein induction. In these experiments we used a higher dose of BNF (10 μg/L) because 1 μg/L with CYP1A knock down did not show toxicity. However, the goal of this experiment was to see whether the *cyp1a*-MO would shift BNF toxicity, suggesting that ANF acts to synergize by acting as a CYP1A inhibitor. This dose (10 μg/L) was also effective at inducing EROD activity above controls (significant main effect of BNF, $p=0.0098$) but was non-teratogenic to non-injected zebrafish embryos (Figure 3). As observed in chemical inducer/inhibitor studies, the *zfcyp1a*-MO reduced induction of CYP1A by BNF (significant effect of morpholino and BNF on EROD activity, $p=0.0198$) and enhanced toxicity at 96 hpf in zebrafish morphants (Figure 3). Compared to non-injected control and BNF treated embryos, pericardial edema area was significantly greater (significant effect of morpholino and BNF on edema, $p=0.0485$) and lower jaw gap length significantly enhanced (significant effect of morpholino and BNF on jaw gap length, $p<0.0001$) in BNF-treated *cyp1a* morphants (Figure 3). We did not observe any phenotype associated with the *cyp1a*-MO; injected control morphants also appeared normal.

The next sets of experiments were designed to define the roles of the AHR and CYP1A in mediating synergistic interactions with respect to cardiovascular effects between PAH-type AHR agonists and CYP1A inhibitors by selective knock down of these specific genes during zebrafish embryonic development. First, we examined whether knock down of AHR2 protein would block synergistic toxic response to BNF, ANF co-exposures in zebrafish embryos. As expected, the *zfahr2*-MO effectively inhibited downstream BNF-induced EROD activity by approximately 50% at 96 hpf observed for non-injected control embryos exposed to 1 μg/L BNF alone (Figure

4). Interestingly, lower jaw length appeared to be elongated in non-injected embryos exposed to 100 μg/L ANF alone (Figure 4.). For BNF+ANF co-exposure groups, zebrafish *ahr2* morphants were significantly less deformed than non-injected controls (Figure 5). As shown by the significant interaction p-values, targeted knock down of AHR2 protein clearly protected zebrafish larvae against the severity of pericardial edema ($p=0.0068$) and foreshortened lower jaw ($p=0.0017$) caused by BNF+ANF treatment (Figure 4).

Finally, we examined what role CYP1A activity played in the synergistic embryotoxic effects observed between a PAH-type CYP1A inducer and inhibitor. The deformities caused by the combination of 1 μg/L BNF + 100 μg/L ANF were so severe that while we observed a visual worsening of deformities in CYP1A-morpholino-treated embryos, we were not able to quantitatively capture these as we had effectively saturated our measurement indices at those doses. We thus lowered the dose of ANF from 100 to 50 μg/L in order to achieve a dose that when combined with 1 μg/L BNF would put the embryo at deformity control levels. This way we could quantitatively show a worsening of deformities with CYP1A knock down. This concentration of ANF was sufficient to inhibit BNF-induced EROD activity in zebrafish embryos without causing deformities (Figure 6). In contrast to results observed with *zfahr2*-MO, targeted knock down of CYP1A protein significantly exacerbated PAH embryotoxicity (Figure 7), specifically increased accumulation of pericardial fluid (significant interaction among all factors, $p<0.001$) and retarded growth of the lower jaw (significant interaction among all factors, $p<0.0001$; Figure 6).

DISCUSSION

The early life stages of fish species are among the most sensitive vertebrates to dioxin-like cardiovascular effects (Peterson *et al.*, 1993). For this reason and because of the genetic tools available, we used embryonic stages of zebrafish to elucidate the role of AHR and CYP1A protein in PAH developmental toxicity. To summarize, results from the current study demonstrate a seemingly paradoxical role for the AHR pathway in mediating PAH toxicity during the early life stages of vertebrates. Similar to studies with pHAH-type AHR agonists like dioxin, our data also suggest that PAH synergistic toxicity is, at least in part, AHR2-dependent. Specifically, AHR2 knock down in zebrafish embryos significantly reduced toxicity when a PAH-type AHR agonist and CYP1A inducer (BNF) was combined with ANF, a competitive CYP1A inhibitor. However, the opposite effect was observed in zebrafish CYP1A morphants. That is, knock down of CYP1A protein synergized PAH effects in zebrafish suggesting that CYP1A is an adaptive, protective response and confers protection to the developing vertebrate embryo. This increased PAH teratogenicity is quite different from pHAH studies where CYP1A inhibition or knock down either reduced toxicity (Cantrell *et al.*, 1996; Dong *et al.*, 2002; Wassenberg, 2004a; Wassenberg and Di Giulio, 2004b) or had no effect on dioxin-like symptoms in the developing embryo (Carney *et al.*, 2004). Our data clearly indicate an alternate mechanism by which PAH-type AHR agonists cause developmental toxicity in fish.

Previously we reported that chemical inhibition of CYP1A activity synergized cardiovascular toxicity of PAH-type AHR agonists (i.e. BaP and BNF) to embryonic stages of killifish (Wassenberg and Di Giulio, 2004b). Similar increases in PAH toxicity were observed when agonists were combined with a variety of CYP1A inhibitors (Wassenberg and Di Giulio, 2004a;b). From these data we concluded that increased toxicity of PAH was a function of

CYP1A inhibition, regardless of chemical properties or mechanism (Wassenberg and Di Giulio 2004b). Our first objective in this study was to test whether synergistic toxicity observed in killifish could be repeated using the zebrafish developmental model. As expected, co-exposure of zebrafish embryos to a non-teratogenic dose of ANF inhibited BNF-induced CYP1A activity *in vivo* and synergized BNF cardiovascular toxicity.

Relationships among AHR agonism, CYP1A metabolism and toxicity of PAH are complicated by rapid oxidation of these compounds by CYP1A enzymes. The few studies that have examined these interactions in fish early life stages have yielded somewhat contradictory results including synergism (this study), additivity (Hawkins *et al.,* 2002) and antagonism (Dong *et al*., 2002). At odds with our studies, ANF protected zebrafish embryos from circulation failure in the dorsal midbrain following exposure to BNF (Dong *et al.*, 2002). This could be due to differences between the studies with respect to dose- and time-response. Dong *et al.* (2002) looked at early-onset (50 hpf) mid-brain circulation failure induced by a BNF concentration (272 μg/L) more than 2.5 orders of magnitude higher than the dose used in our studies, which by itself was non-toxic (Wassenberg and Di Giulio, 2004b). In addition, Carney *et al.* (2004) concluded that the earliest onset for dioxin toxicity in zebrafish is around day 3. Particularly at lower doses of dioxin, and we propose for less potent agonists like BNF, teratogenic endpoints are modest in zebrafish embryos prior to 72 hpf (Carney *et al.*, 2004).

With respect to ANF, there is evidence to support that its mechanism of action varies by concentration (Gasiewicz and Rucci, 1991; Santostefano *et al*., 1994; Aluru *et al.*, 2005). Qureshi (2004) observed that at high concentrations (320 μg/L), ANF eliminated symptoms of blue sac disease in rainbow trout larvae caused by exposure to retene and blocked induction of CYP1A protein. This was associated with complete inhibition of Phase I metabolism of parent

PAH suggesting that ANF antagonized activation of AHR by retene (Qureshi, 2004). In contrast, lower concentrations of ANF (32-100 μg/L) caused a concentration-dependent increase in CYP1A protein and enhanced mortality and toxicity of retene to larval trout. Overall, lower ANF concentrations were associated with fewer retene metabolites but selective for less polar hydroxylated intermediates (Qureshi, 2004). This is consistent with our observations that inhibition of CYP1A activity is concomitant with BNF, ANF synergy in zebrafish (Wassenberg and Di Giulio, 2004b). Taken together, these data suggest that at lower concentrations ANF by itself acts to increase CYP1A protein either by acting as a partial AHR agonist and/or by inhibiting CYP1A activity to increase its own half-life to extend the time that it can act as an AHR agonist. However when combined with a strong AHR agonist such as BNF, its role may change to that of a competitive AHR antagonist or it may inhibit CYP1A and extend the half life of the other PAH-type AHR agonist (Hawkins *et al.*, 2002). While it is not entirely clear from our studies whether ANF functions as a receptor agonist or CYP1A inhibitor, either mode of action could lead to an increase in CYP1A protein.

Similar to chemical inhibition of CYP1A activity (Wassenberg and Di Giulio, 2004b) we observed that CYP1A knock down made a non-toxic dose of BNF (10 μg/L) highly teratogenic and that the combined toxicity of BNF (1 μg/L) + ANF (100 μg/L) was also enhanced in CYP1A morphants even though EROD activity was only maximally reduced by half that of BNF-induced embryos. The transient nature of the morpholino approach could account for EROD activity at 96 hpf in CYP1A (and AHR2) knock downs. Although the same morpholino concentrations were used, it is feasible that incomplete knock down could be explained by differences in injection volume between this and other studies (for e.g., Carney et al. 2004). The fact that we observed such striking effects even without complete knock down speaks to the biological

significance of our results. Collectively, these data show that CYP1A is playing an important role in mediating toxicity of PAH in early life stages of fish. The fact that CYP1A knock down mimics ANF effects on BNF also suggests that in our system ANF is acting more as a CYP1A inhibitor than as an AHR agonist or antagonist.

Several studies have clearly shown that pHAH-induced embryotoxicity is receptor-mediated in fish (Prasch *et al.*, 2003; Teraoka *et al.*, 2003; Carney *et al.*, 2004). Likewise our data suggest that synergistic effects of PAH co-exposures require functional AHR2. We show that AHR2 knock down reduces synergistic cardiovascular toxicity in zebrafish embryos co-treated with BNF and ANF, specifically pericardial edema and retarded growth of the lower jaw. Although not directly tested in the current study, we would expect that AHR2 knock down would block cardiotoxic effects observed in zebrafish exposed to higher embryotoxic doses of BNF alone. Incardona *et al.* (2004) demonstrated that exposure of three-ring PAH abundant in weathered Alaska North Slope (ANS) crude oil induced a specific suite of developmental defects consistent with and secondary to disruption of atrioventricular (AV) conduction block. In a subsequent study, the authors speculated that the AHR pathway plays a protective or adaptive role against cardiovascular defects caused by exposure of zebrafish embryos to high concentrations (viz. above solubility limits) of low molecular weight tricyclic PAH (Incardona *et al.*, 2005). In that study, morpholino knock down of translated zebrafish AHR isoforms and CYP1A protein did not prevent toxicity associated with weathered crude oil and tricyclic PAH leading the authors to conclude that cardiotoxicity was independent of AHR activation or CYP1A induction (Incardona *et al.*, 2005). Rather, cardiovascular dysfunction was exacerbated in zebrafish co-injected with AHR1 and AHR2 morpholinos or CYP1A zebrafish morphants exposed to weathered ANS oil. In contrast, we observed that synergistic response caused by co-

exposure to PAH-type AHR agonists and CYP1A inducers is mediated at least in part by AHR2 since knock down of the receptor protein reduced toxicity. It should be noted that tricyclic PAH tested in Incardona *et al.* (2005) are at best weak AHR agonists and CYP1A inducers compared to more potent 4-5 ring PAH-type AHR agonists that have been used as model compounds in our studies (Wassenberg and Di Giulio, 2004b; Wassenberg *et al.*, 2005). Thus, it is likely that we could be looking at different mechanisms of cardiotoxicity that vary with PAH-AHR binding affinity. Similar to Incardona *et al.* (2005), however, our data do support a protective role of CYP1A activity in PAH toxicity.

Although the exact mechanism(s) by which PAH cause developmental toxicity are unclear, it is evident that CYP1A activity can be beneficial *in vivo*. Thus, part of the difference in the relationships between CYP1A activity and pHAH versus PAH toxicity could be attributed to differential metabolism of these two classes of AHR agonists. Coplanar pHAH, like dioxin, are recalcitrant to oxidative metabolism because they are poor substrates for the CYP1A enzyme (White *et al.*, 1997; Brown *et al.*, 2002). As such, toxicity of pHAH is usually attributed to the parent compound, rather than its metabolites (Hankinson, 2005). By comparison, PAH are excellent substrates for CYP1A metabolism in vertebrates (Niimi and Palazzo, 1986; Brown *et al.*, 2002; Hawkins *et al.*, 2002).

Our data are consistent with the hypothesis that the protective effects of CYP1A1 activity have ensured preservation of this gene in vertebrates for more than 400 million years (Nebert and Dieter, 2000; Uno *et al*. 2004a) and review of the mammalian literature supports our conclusion that CYP1A activity is protective with respect to PAH-type AHR agonists. Consistent with knock down studies in zebrafish, mice lacking a functional *cyp1a1* gene are protected against dioxin-induced toxicity at high doses (Uno *et al.*, 2004b). In contrast, *cyp1a1* knockout mice are

NOT protected from BaP-induced toxicity (Uno *et al.*, 2004a). *Cyp1a1* knockout mice orally dosed with BaP demonstrated significantly higher levels of BaP-DNA adducts than *cyp1a1* wild type mice (Uno *et al.*, 2004a). In that same study, *cyp1a1* wild type mice were also better protected from BaP-induced immunotoxicity, anorexia, bone marrow hypocellularity, and lethality (Uno *et al.*, 2004a). This is in stark contrast to the widely accepted cancer paradigm that CYP1A activity increases PAH toxicity. Important to note however is that the evolutionary pressure that may have selected for preservation of CYP1A activity probably did not apply to the development of cancer.

Certainly caution should be exercised when comparing our studies with fish to the mammalian literature. One key difference is that in contrast to the emphasis on carcinogenic mechanisms of toxicity in mammalian studies of PAH like BaP, cancer does not appear to be the cause of toxicity of PAH to early life stages of fish. Rather, the important message to take home from recent mice knockout studies is that functional CYP1A1 appears to be important in detoxification and protection against PAH toxicity.

Other AHR responsive genes may play a role in embryotoxicity of PAH. CYP1B1 overlaps in function with CYP1A; however, it is generally thought to have a greater tendency to metabolize substrates to more toxic products than CYP1A. A recent study suggests that CYP1B1 in immune tissues governs metabolic activation and immunotoxicity of BaP when CYP1A1 is genetically ablated (Uno *et al.*, 2006). CYP1B1 has been studied in flatfish (*Pleuronectes platessa*), carp (*Cyprinus carpio*), channel catfish (*Ictalurus punctatus*), and brown bullhead (*Ameiurus nebulosus*). In flatfish, CYP1B1 expression is restricted to the gills and, unlike CYP1A, is not induced by BNF (Leaver and George, 2000). CYP1B1 is, however, inducible by the AHR agonist 3-methylcholanthrene (3-MC) in carp tissues (El-kady *et al.*,

2004a). With the exception of gill and blood, CYP1B1 is induced by BaP in channel catfish and brown bullhead in most tissues examined (Willett *et al*., 2003). A second form, CYP1B2, has also been isolated in carp gill and is inducible by 3-MC, although only in this location, and it is not constitutively expressed in any other carp tissues (El-kady *et al*., 2004b). No studies in zebrafish could be found, although the mRNA has been identified and sequenced (Godard *et al*. 2005). Two new P450 genes, CYP1C1 and CYP1C2, have recently been isolated from scup (*Stenotomus chrysops*) (Godard *et al*. 2005). The zebrafish homologues of these genes have also been identified and sequence analysis shows that both contain at least one putative dioxin response element (DRE) in the promoter regions (Godard *et al*., 2005). Homologues to either CYP1C genes were not identified in mammals, suggesting this is a fish specific gene (Godard *et al*., 2005). Although the function of these proteins is currently unknown, sequence similarity suggests a close relationship with CYP1B1. While speculative, these novel AHR-regulated genes could also potentially play a role in PAH synergistic toxicity observed in our study.

There are a number of questions that arise from the conjecture that PAH developmental toxicity is tied to metabolism. First, does inhibiting CYP1A convert PAH to a more developmentally toxic form by extending the half life of metabolically active PAH so that they resemble dioxin-like compounds which are recalcitrant to CYP-mediated metabolism? Or, does PAH toxicity result from a shift in metabolism when CYP1A activity is blocked or inhibited? We are currently addressing these questions. Additionally, for years, CYP1A activity and induction has been utilized as a biomarker of exposure for both PAH and pHAH (Payne *et al.*, 1987). While historically CYP1A activity has been associated with toxicity of pHAH, increased CYP1A activity does not predict chronic PAH effects to vertebrates (Lee and Anderson, 2005).

Finally, these data create uncertainty as to whether models of risk assessment for PAH, which assume additivity, are appropriate. Our studies suggest that a TEQ model based on AHR agonism would underestimate the embryotoxic potential of PAH-dominated mixtures. Considering the complexity of environmental mixtures of PAH, more effective and mechanistic driven models to predict hazard to vertebrate species would appear to be in order.

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FIGURE CAPTIONS

Figure 1. Dose-response of percent pericardial edema area in zebrafish (96 hpf) following exposure as embryos to waterborne concentrations (μg/L) of A. BNF and B. ANF. Both BNF and ANF showed a dose-dependent increase in pericardial edema as percent of control ($p=0.0076$ and $p=0.016$, respectively). $n = 20$ fish for each treatment group.

Figure 2. Effects of BNF (1 μg/L) with or without co-exposure to 100 μg/L ANF on *in vivo* EROD activity (72 hpf) and deformities (96 hpf) in zebrafish larvae. Zebrafish embryos were simultaneously exposed at 24 hpf to waterborne concentrations of 7-ER and either DMSO vehicle, 1 μg/L BNF and/or 100 μg/L ANF. All dosing solutions were prepared in DMSO (<0.1 % v/v). There was a main effect of both BNF ($p=0.0002$) and ANF ($p=0.0011$), as well as a significant interaction between doses ($p=0.0003$) on EROD activity. A. BNF ($p=0.003$), ANF ($p<0.0001$), and their interaction ($p=0.0002$) had a significant effect on percent of control edema area; B. BNF ($p=0.0006$), ANF ($p=0.0007$), and their interaction ($p=0.0006$) had a significant effect on percent of control gap length. $n = 3$ to 9 fish for each treatment group and data shown here are presented as the average percent control ± SEM from three replicate experiments.

Figure 3. Knock down of CYP1A mimics ANF effect on BNF-induced EROD activity and deformities (96 hpf). Zebrafish embryos were injected with the *zfcyp1a* morpholino (MO) within 2 hpf. MO and non-injected (NI) control embryos were simultaneously exposed at 24 hpf to waterborne concentrations of 7-ER and either DMSO vehicle or 10 μg/L BNF. All dosing solutions were prepared in DMSO (<0.1 % v/v). There was a main effect of both morpholino

(p=0.0267) and BNF (p=0.0098) treatments, as well as a significant interaction between these factors (p=0.0198) on EROD activity. A. The main effects of MO and BNF treatments were nearly significant (p=0.05 and 0.06, respectively) , whereas a significant interaction between these factors (p=0.0485) was observed on percent control edema area. Note that symbols overlap for DMSO treatment groups; B. There was a significant main effect of morpholino (p=0.003) and BNF (p<0.0001) treatments as well as their interaction on percent of control lower jaw gap length (p<0.0001). Data shown here are presented as the average percent control ± SEM from a representative experiment that has been repeated at least twice. Each data point is representative of at least 30 fish.

Figure 4. Knock down of AHR2 in zebrafish embryos reduces toxicity of BNF, ANF co-exposures (96 hpf). Zebrafish embryos were injected with the *zfahr2* morpholino (MO) within 2 hpf. MO and non-injected (NI) control embryos were simultaneously exposed at 24 hpf to waterborne concentrations of 7-ER and either DMSO vehicle, 1 µg/L BNF, 100 µg/L ANF, or combination of BNF and ANF. All dosing solutions were prepared in DMSO (<0.1 % v/v). There was a significant main effect of both BNF (p=0.0013) and ANF (p=0.0002) treatments on EROD activity. A. Main effects of BNF (p=0.0053), ANF (p=0.0018) and interaction among BNF, ANF, and morpholino (p=0.0068) treatments on percent control edema were significant; B. Main effects of BNF (p<0.0001), ANF (p=0.0041) and interaction among BNF, ANF, and morpholino treatments (p=0.0017) on percent control lower jaw gap length were significant. Data shown here are presented as the average percent control ± SEM from a representative experiment that has been repeated at least twice. Each data point is representative of at least 30 fish.

Figure 5. Knock down of AHR2 in zebrafish embryos reduces toxicity of BNF, ANF co-exposures (96 hpf). Zebrafish embryos were injected with the *zfcyp1a* morpholino (MO) within 2 hpf. MO and non-injected (NI) control embryos were exposed at 24 hpf to waterborne concentrations of DMSO vehicle (<0.1 % v/v), 1 µg/L BNF, 100 µg/L ANF, or combination of BNF and ANF. Left lateral view images of representative embryos from all treatment groups were captured to measure pericardial sac area (dashed arrow) and truncation of Meckel's cartilage or lower jaw gap length (solid arrow; see Material and Methods).

Figure 6. Knock down of CYP1A in zebrafish embryos increases toxicity of BNF, ANF co-exposures (96 hpf). Zebrafish embryos were injected with the *zfcyp1a* morpholino (MO) within 2 hpf. MO and non-injected (NI) control embryos were simultaneously exposed at 24 hpf to waterborne concentrations of 7-ER and either DMSO vehicle, 1 µg/L BNF, 50 µg/L ANF, or combination of BNF and ANF. All dosing solutions were prepared in DMSO (<0.1 % v/v). There was a significant main effect of BNF ($p=0.0023$), ANF ($p<0.0001$), and morpholino treatments ($p<0.0001$) on percent control EROD. Interaction among the three factors was non-significant. A. Main effects and interaction among all three factors (BNF, ANF, and morpolino treatments) on percent control edema area was significant ($p<0.0001$); B. Main effects and interaction among all three factors (BNF, ANF, and morpolino treatments) on percent control lower jaw gap length were significant ($p<0.0001$). Data shown here are presented as the average percent control ± SEM from a representative experiment that has been repeated at least three times. Each data point is representative of at least 45 fish.

Figure 7. Knock down of CYP1A in zebrafish increases toxicity of BNF, ANF co-exposures (96 hpf). Zebrafish embryos were injected with the *zfcyp1a* morpholino (MO) within 2 hpf. MO and non-injected (NI) control embryos were exposed at 24 hpf to waterborne concentrations of DMSO vehicle (<0.1 % v/v), 1 µg/L BNF, 50 µg/L ANF, or combination of BNF and ANF. Left lateral view images of representative embryos from all treatment groups were captured to measure pericardial sac area (dashed arrow) and truncation of Meckel's cartilage or lower jaw gap length (solid arrow; see Material and Methods).

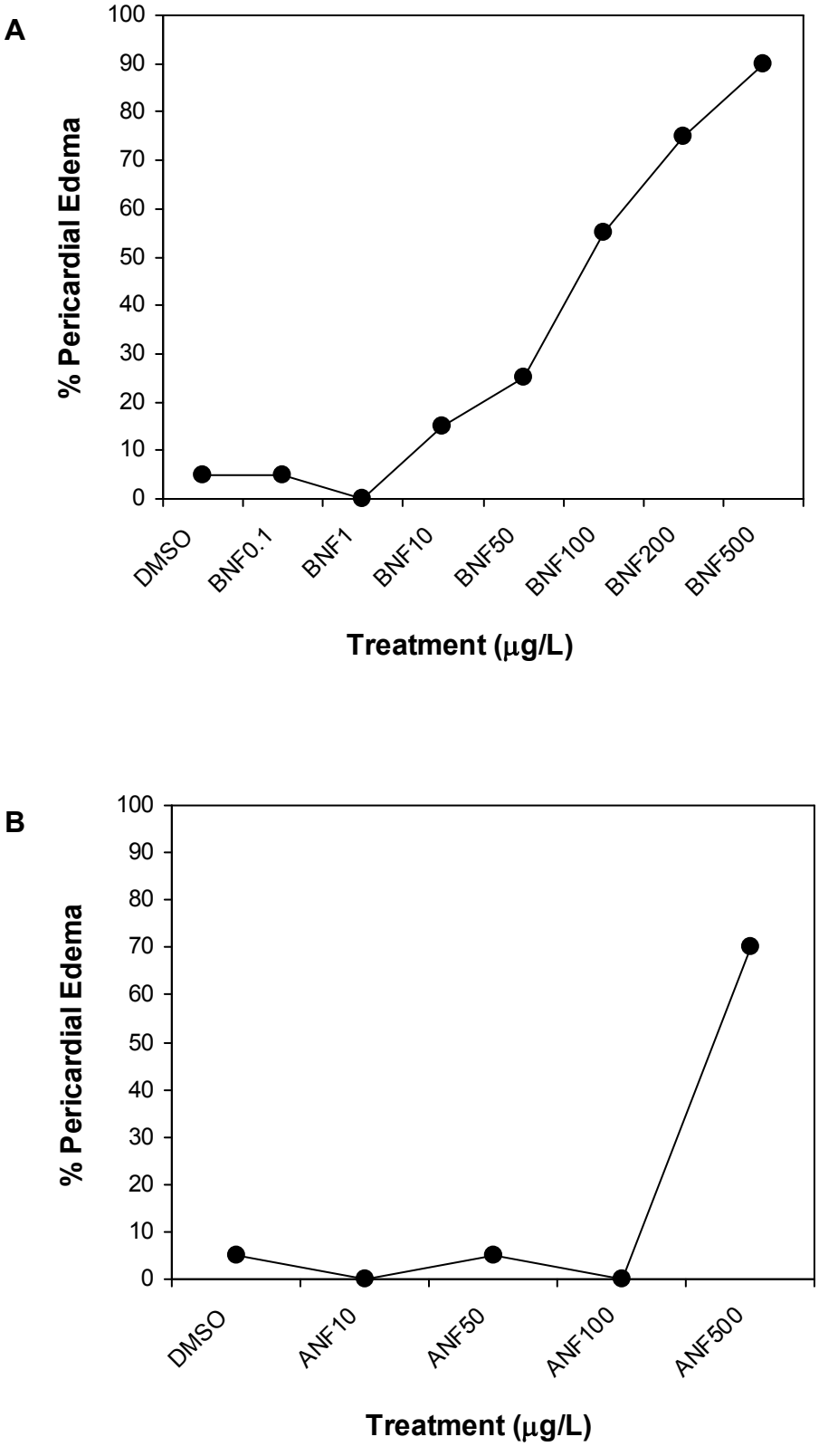

Figure 1.

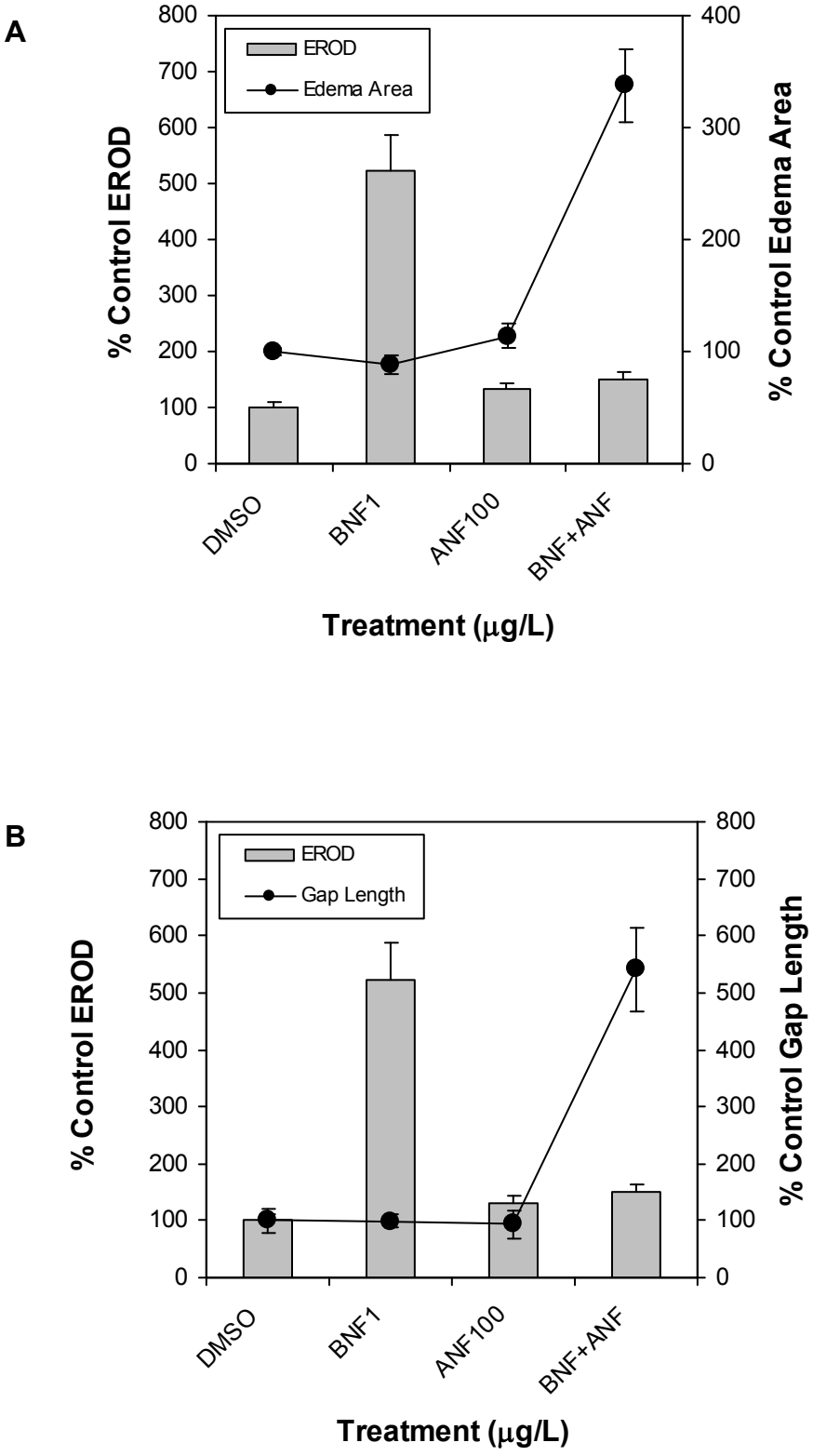

Figure 2.

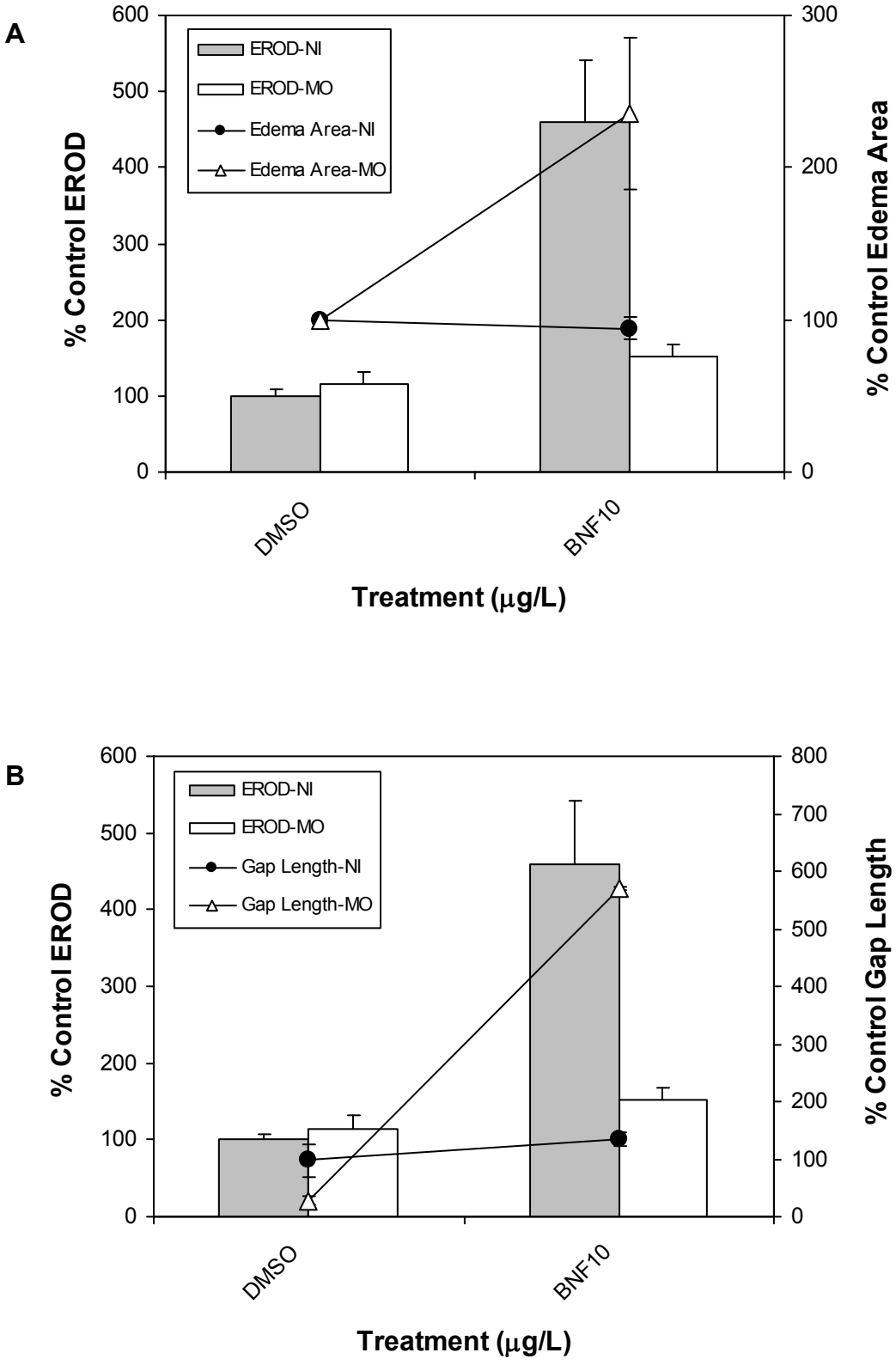

Figure 3.

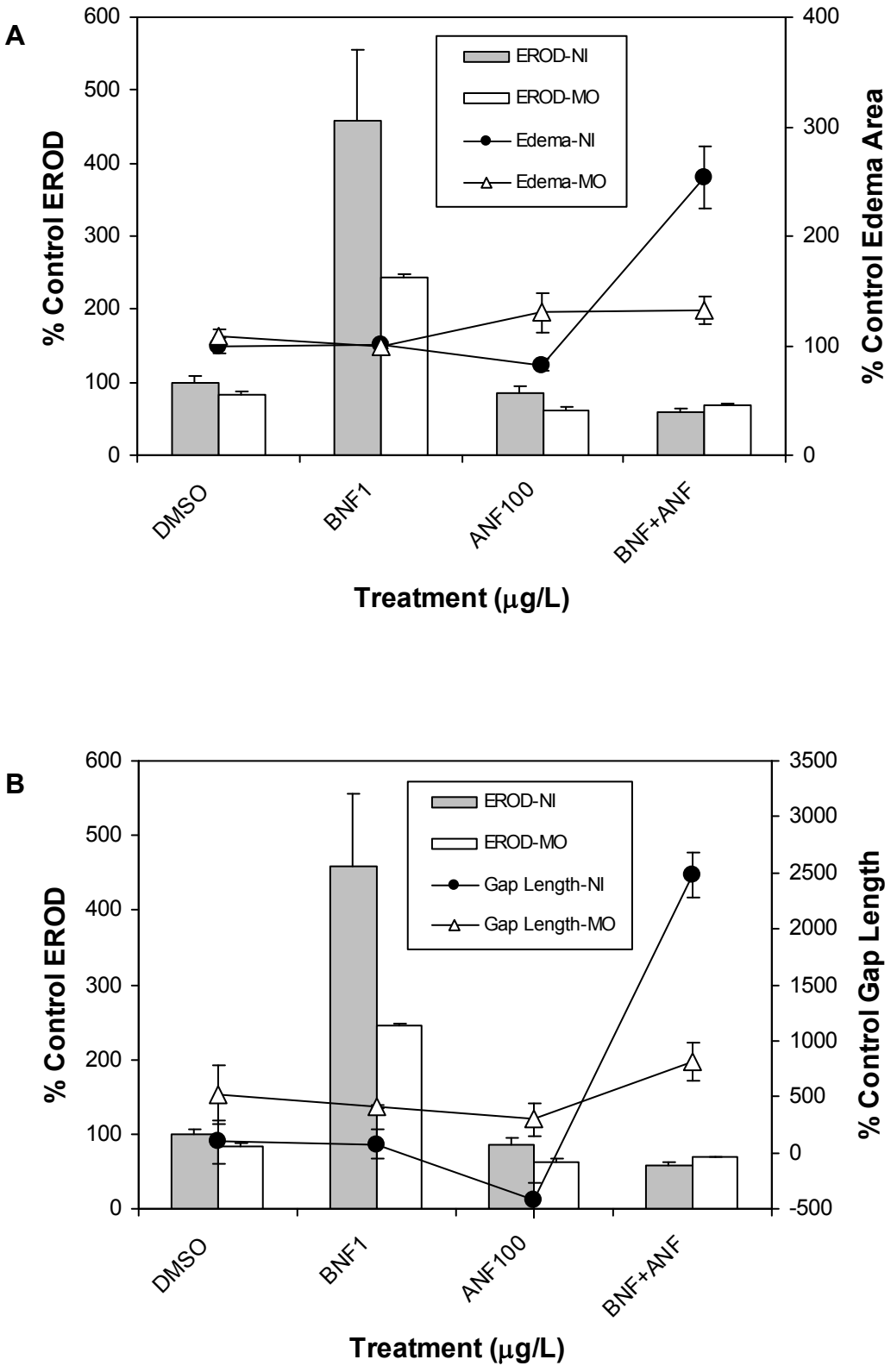

Figure 4.

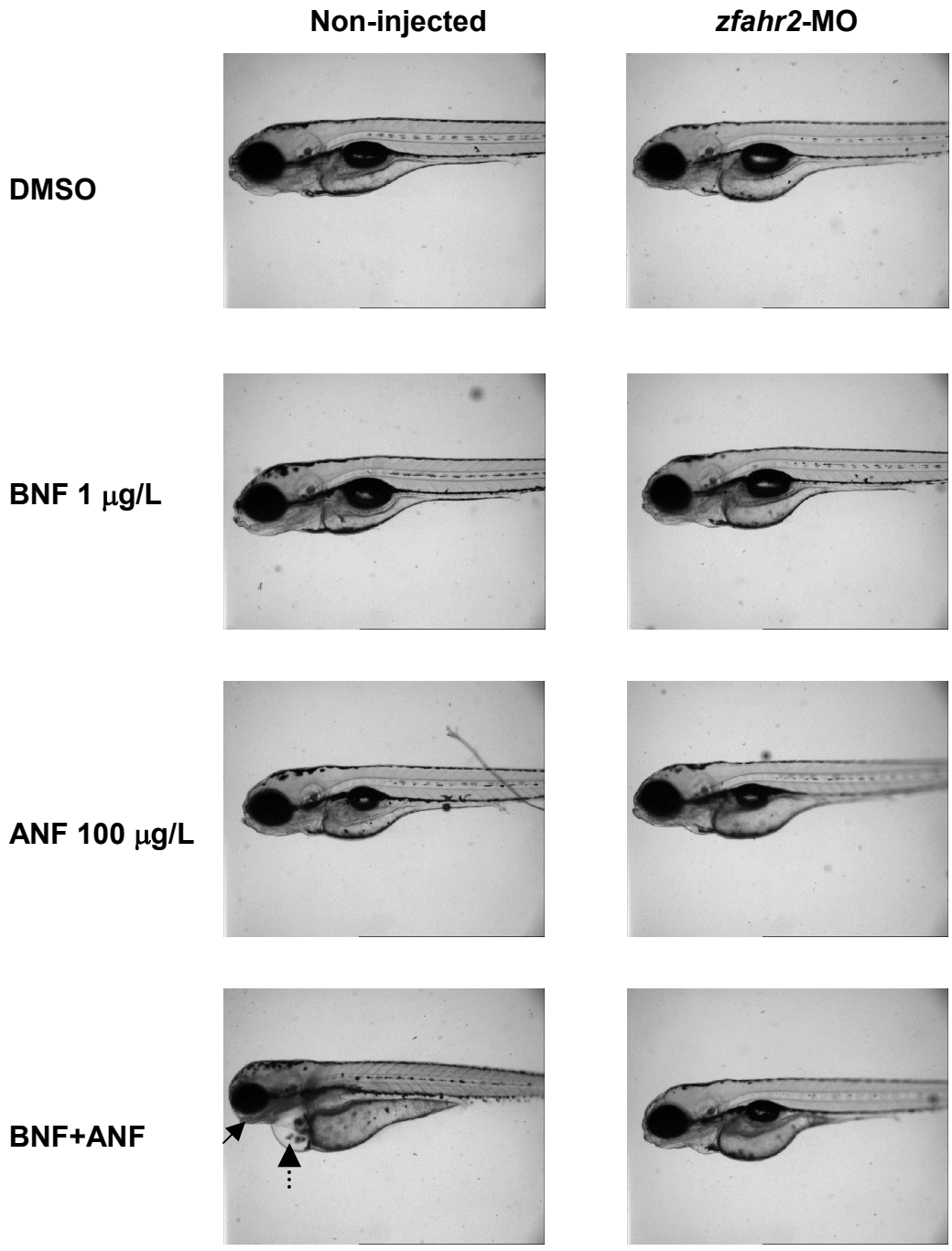

Figure 5.

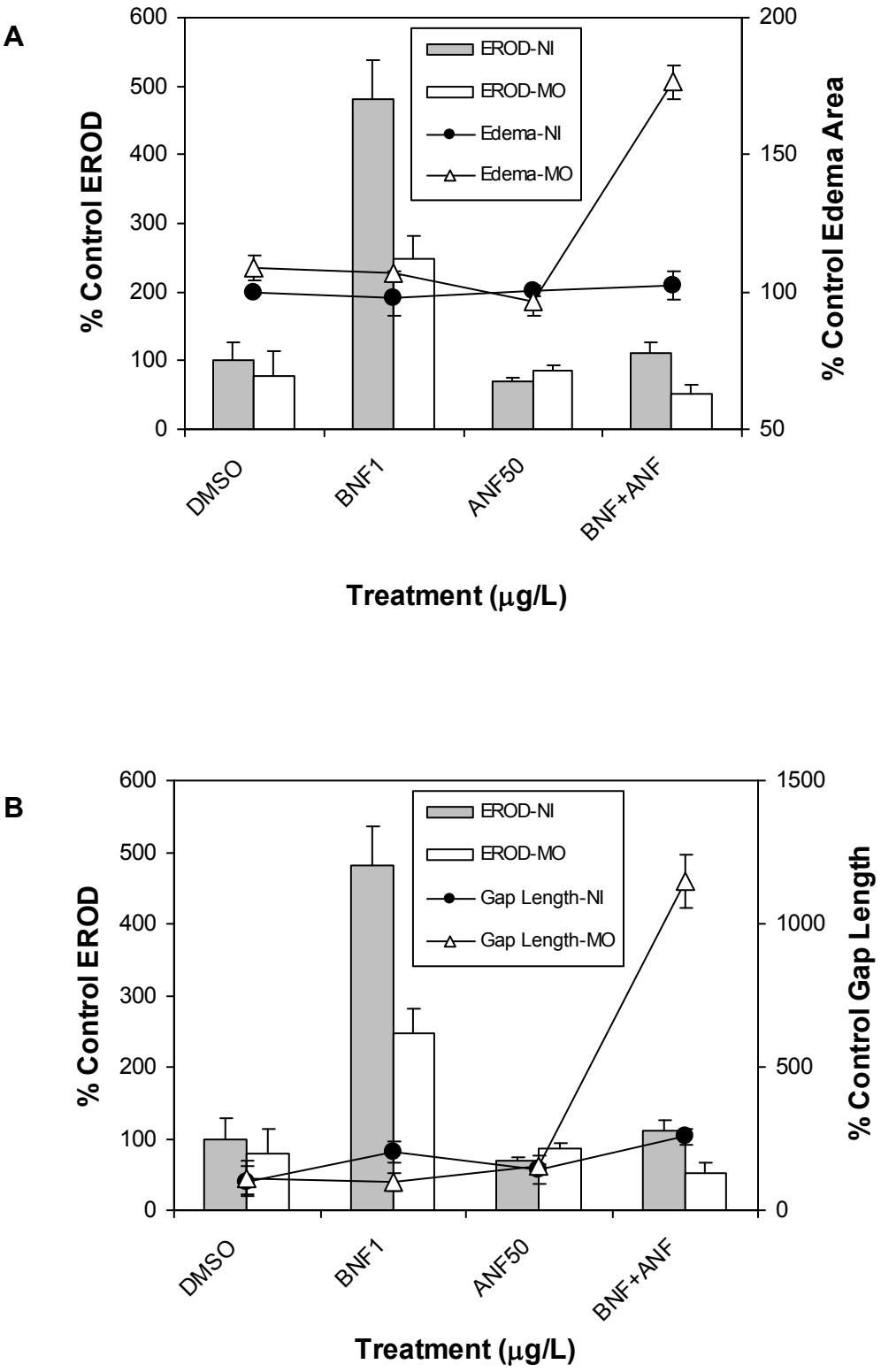

Figure 6.

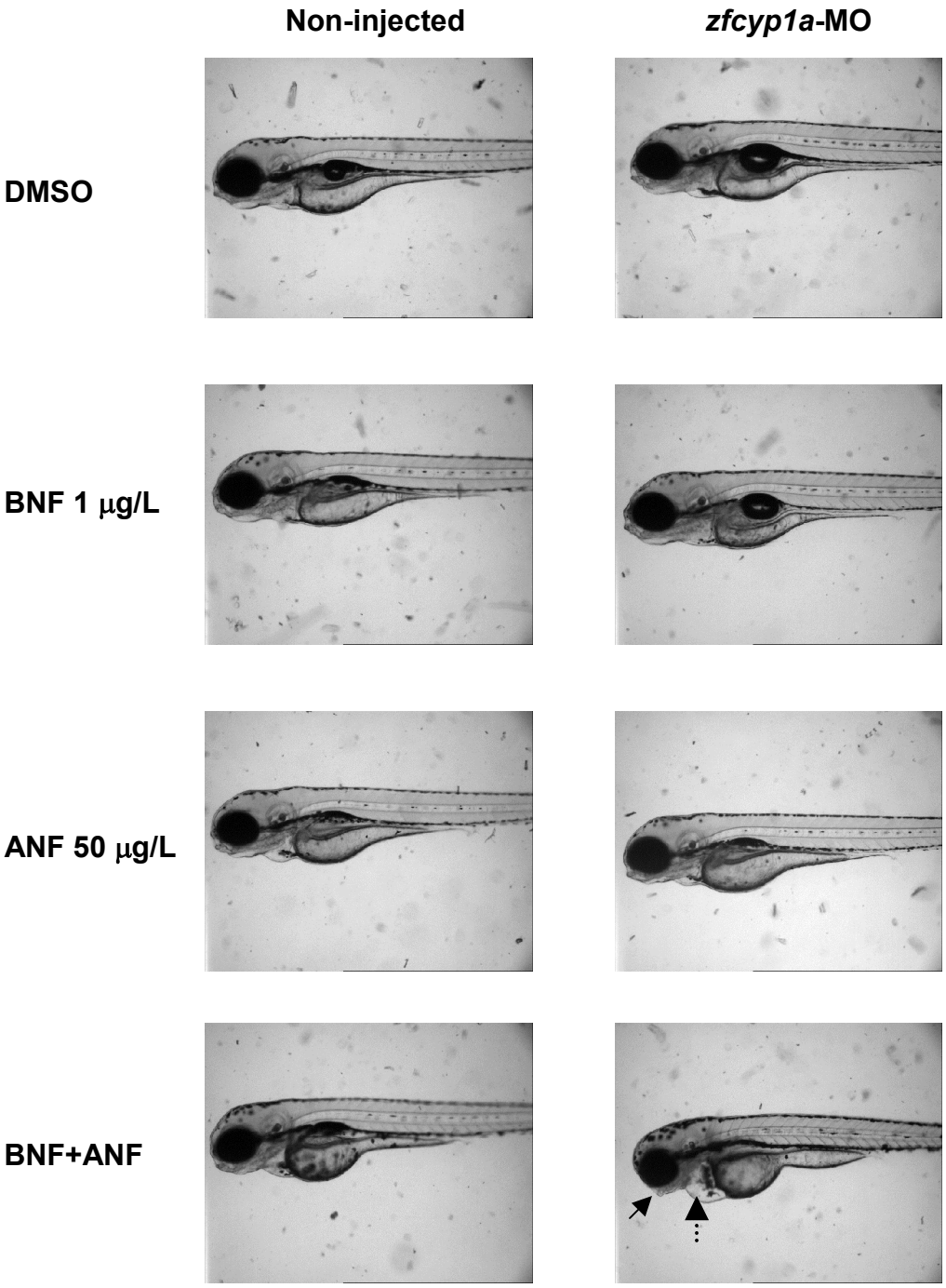

Figure 7.