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The tryptophan photoproduct 6-formylindolo[3,2-b]carbazole (FICZ) binds multiple AHRs and induces multiple *CYP1* genes via AHR2 in zebrafish

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Abstract

The tryptophan photooxidation product 6-formylindolo[3,2-b]carbazole (FICZ) has been proposed as a physiological ligand for the mammalian aryl hydrocarbon receptor (AHR), which it binds with high affinity, inducing expression of cytochrome P450 1A1 (CYP1A1). We investigated whether the response to FICZ is evolutionarily conserved in vertebrates by measuring FICZ binding to two zebrafish AHRs (AHR1B and AHR2) and its ability to induce zebrafish *CYP1* genes (*CYP1A*, *CYP1B1*, *CYP1C1*, *CYP1C2*, and *CYP1D1*) *in vivo*. Exposure of zebrafish embryos (48 hours-post-fertilization; hpf) to 10 nM FICZ for 6 hours caused strong induction of *CYP1A* mRNA and a statistically significant but modest induction of *CYP1B1* and *CYP1C1*. Neither *CYP1C2* nor *CYP1D1* expression was induced by FICZ under the conditions of dose, time or developmental stage examined here. *CYP1A* induction was significantly greater after 6 hours than after 12 hours of exposure to FICZ, suggesting a rapid degradation of inducer. The 6-hr EC_{50} values for induction of *CYP1A* and *CYP1B1* by FICZ were 0.6 and 0.5 nM compared to 72-hr EC50 values of 2.3 and 2.7 nM for PCB126, indicating that in zebrafish embryos FICZ is a more potent inducer than PCB126. FICZ at 10 nM was able to completely displace binding of 2,3,7,8-tetrachloro-1,6[^{3}H]-dibenzo-*p*-dioxin to *in vitro*-expressed zebrafish AHR2 and AHR1B. Inhibition of AHR2 translation in zebrafish embryos by an AHR2-specific morpholino antisense oligonucleotide decreased the induction of *CYP1A* and *CYP1B1* by FICZ and by PCB126. Together, these results demonstrate that FICZ is a potent AHR agonist in zebrafish, inducing expression of multiple *CYP1* genes largely through AHR2. Evolutionary conservation of the response to FICZ is consistent with a possible role as an endogenous signaling molecule acting through the AHR.

Introduction

The aryl hydrocarbon receptor (AHR), in addition to its well known role mediating effects of various xenobiotics, has been suggested to participate in a variety of biological processes, including cell cycle control, growth, apoptosis, differentiation, and light-regulated biological

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rhythms [1–3]. Activation of the AHR transcription factor by diverse chemicals alters the expression of genes, most prominently cytochrome P450 1 (CYP1) family genes. AHR agonists include exogenous chemicals such as polynuclear aromatic hydrocarbons, planar halogenated aromatic hydrocarbons and a variety of natural products, as well a diversity of endogenous compounds [1,4,5]. There may be endogenous ligand(s) involved in regulation of physiological pathways, and perhaps environmental sensing, but such ligands have been elusive. 6-Formylindolo[3,2-b]carbazole (FICZ; Fig. 1), a tryptophan oxidation product formed by exposure to ultraviolet (UV) or visible irradiation, binds with high affinity to the AHR in mammalian cells [6], inducing expression of *CYP1A1* [2]. FICZ has been proposed as a physiological ligand for the AHR, possibly involved in light response in animals [2,7–9].

Studies of AHR activation by FICZ have been carried out primarily in mammals, and a consequent question is whether FICZ can activate AHRs in other vertebrate groups. We have begun to address the evolutionary conservation of the response to FICZ by assessing its ability to interact with AHRs and induce *CYP1* gene expression in zebrafish, a teleost model. Zebrafish possess three AHR genes, designated *AHR1A*, *AHR1B* and *AHR2*. AHR1B and AHR2 proteins both are capable of high-affinity binding of TCDD and are transcriptionally active [10,11], whereas AHR1A does not appear to be capable of binding (or becoming activated by) TCDD [11,12]. Zebrafish also have five *CYP1* genes: *CYP1A*, *1B1*, *1C1* and *1C2* are induced by halogenated AHR agonists 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [13–15], while a fifth gene, *CYP1D1*, is not induced by TCDD or PCB [16]. Induction of the four inducible *CYP1* genes by TCDD and PCB126 is regulated primarily by AHR2 [14,15,17].

The objective of the present studies was to determine whether FICZ is an agonist for AHRs and induces *CYP1* genes in zebrafish. Thus, we determined whether FICZ binds avidly to zebrafish AHR2 and AHR1B *in vitro*, how the five *CYP1* genes respond to FICZ *in vivo* in zebrafish embryos, and whether there are differences in response among these multiple *CYP1s*. AHR2 has been found to be most prominent in mediating *CYP1* induction by halogenated AHR ligands in zebrafish [14]. Thus, to determine whether there might be a similar role of AHR2 in FICZ effects, we measured the ability of FICZ to induce *CYP1* genes in embryos in which AHR2 expression had been suppressed using morpholino antisense oligonucleotides.

Materials and methods

Animals

Zebrafish of the Tup/Long fin (TL) type were used in the experiments. Fertilized eggs were obtained by breeding multiple groups of 30 female and 15 male fish as previously described [13]. The day after fertilization unfertilized eggs and dead embryos were removed. Generally, no mortality was observed subsequent to this. Embryos and larvae (up to 7 days post-fertilization; dpf) were held in 0.3× Danieau's solution and juveniles in zebrafish system water at 28°C [13]. Procedures used in these experiments were approved by the Animal Care and Use Committee of the Woods Hole Oceanographic Institution.

Exposure to FICZ

The effect of FICZ on *CYP1* gene expression was studied in developing zebrafish in a series of experiments that were designed to examine *CYP1* inducibility, the dose-dependence of induction, and the developmental time course of inducibility. Depending on time after fertilization (i.e., age and size of the fish), glass petri dishes or small beakers were used for the exposure. FICZ (Biomol International, L.P., Plymouth Meeting, PA) was dissolved in DMSO and mixed into the 0.3× Danieau's solution or system water. Exposure was performed at 28 °

C during the light period of the 14 h light/10 h dark diurnal cycle. The experiments were terminated immediately after the exposure, at which time 3–6 replicates (each of pools of embryos, as specified below) were collected from each treatment group, frozen in liquid nitrogen, and stored at −80 °C until analyzed.

Experiment 1—This experiment was performed to determine if FICZ is able to induce *CYP1* gene expression in zebrafish embryos. Groups of 200 embryos in their chorions were placed in two dishes containing 150 ml 0.3× Danieau's solution. Starting 48 hours post-fertilization (hpf) the embryos were exposed for 6 hours to 10 nM FICZ and carrier (100 ppm DMSO) or carrier only. After exposure, six replicates of 33–34 embryos each were sampled from each dish (6-h DMSO and 6-h FICZ).

Experiment 2—In order to study whether the *CYP1A* gene inducibility increases by removal of chorion and to look at sustainability of induction, 48-hpf embryos were dechorionated and exposed to 10 nM FICZ including carrier (100 ppm DMSO) or carrier only for either 6 or 12 hours. Exposure was performed in glass petri dishes (10 cm diameter) containing 25 ml of 0.3× Danieau's solution and 33–34 embryos. Four replicate pools of embryos were analyzed from each exposure group (i.e., 6-h DMSO, 6-h FICZ, 12-h DMSO, and 12-h FICZ).

Experiment 3—The concentration-response relationship for *CYP1* gene expression and FICZ was examined in groups of 48-hpf embryos (100 per concentration) exposed to various concentrations of FICZ (0.1, 0.3, 1, 3, 10, or 30 nM) in carrier (150 ppm DMSO), or carrier only for 6 hours. Triplicate pools of 33–34 embryos were sampled for each concentration.

Experiment 4—Zebrafish at different developmental stages (6, 24, 48, 72, and 96 hpf, and 7, 14, and 28 dpf) were exposed for 6 hours to 10 nM FICZ in carrier (100 ppm DMSO) or carrier only. At the end of exposure the fish were examined for phenotypic changes, and then five replicates (1–30 individuals each, depending on age) were sampled per treatment group, for analysis of *CYP1* expression.

AHR2 knock-down

To examine whether AHR2 is involved in *CYP1* induction by FICZ, we treated zebrafish embryos with a morpholino antisense oligonucleotide (MO) targeting AHR2, as previously described [14,17,18]. MOs targeting the transcriptional start site of *AHR2* (5′-TGTACCGATACCCGCCGACATGGTT-3′) and negative control morpholinos (5′-CCTCTTACCTCAGTTACAATTTATA-3′) were obtained from Gene Tools (Philomath, OR). The morpholinos were fluorescein-tagged to enable selection of successfully injected embryos for the experiments. Two to four-cell stage embryos were injected with morpholinos at approximately 3.3 ng per egg [14]. Embryos were screened at 3 hpf by fluorescence microscopy to verify incorporation of MO. Any damaged embryos or those not displaying homogenous fluorescence were removed. At 48 hpf, groups of embryos injected with the AHR2-MO, the control-MO, or non-injected embryos were placed in glass petri dishes containing 100 ml of 0.3× Danieau's solution and exposed for 6 hours to either carrier (200 ppm of DMSO), 10 nM FICZ in carrier, or 30 nM PCB126 in carrier (*i.e.*, 9 dishes total: control-MO DMSO, control-MO FICZ, control-MO PCB126, AHR2-MO DMSO, AHR2-MO FICZ, AHR2-MO PCB126, no-MO DMSO, no-MO FICZ, and no-MO PCB126). After exposure, 4 replicates of 20 to 25 embryos were sampled from each dish. The samples were frozen in liquid nitrogen and then stored at −80°C until analyzed.

In vitro protein synthesis and ligand-binding assay

The expression construct for zebrafish AHR1B was prepared as described previously [11]. The AHR2 expression construct pBKCMV-zfAHR2 was generously provided by Dr. R. Tanguay

(Oregon State University, Corvallis, OR) and Dr. Richard E. Peterson (University of Wisconsin, Madison, WI). The *in vitro* expression and ligand-binding assays were performed as described previously [11,19]. Briefly, the TNT-Quick Coupled Reticulocyte Lysate System (Promega, Madison, WI) was used to synthesize AHR proteins. In vitro-synthesized AHRs were incubated with [^{3}H]TCDD (2 nM nominal; 1.75–2.02 nM measured) in the presence or absence of FICZ (1 or 10 nM), overnight at 4°C. The amount of [^{3}H]TCDD specific binding was measured by velocity sedimentation on sucrose gradients in a vertical tube rotor [20]. Nonspecific binding was determined by incubating [^{3}H]TCDD with TNT reactions containing an empty vector (unprogrammed lysate (UPL)).

Real-time, quantitative RT-PCR

RNA was isolated using RNA STAT-60™ (Tel-Test Inc. Friendswood, TX, USA) and the isolates were DNase treated by the TURBO DNA-free™ kit (Ambion). The RNA quantity and quality were determined spectrophotometrically (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE, USA). cDNA was synthesized using the Omniscript™ Reverse Transcriptase kit (Qiagen Inc., Valencia, CA, USA), random hexamer primers (Operon Biotechnologies Inc.) and the RNasin® RNase inhibitor (Promega).

Gene specific real time PCR primers for zebrafish *CYP1A*, *CYP1B1*, *CYP1C1*, *CYP1C2*, *CYP1D1*, and *ARNT2* cDNAs were synthesized by Operon Biotechnologies Inc (Table 1). (The *ARNT2* primers were designed to amplify a sequence common to *ARNT2a*, *b*, and *c*.) Real time PCR was performed using the iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) as previously described [13,14]. To ensure that a single product was amplified, melt curve analysis was performed on the PCR products at the end of each PCR run.

Relative mRNA expression of the *CYP1* genes was calculated for each reaction according to the method of Livak and Schmittingen, $E^{\Delta\Delta Ct}$[21] using *ARNT2* as the reference gene [14]. For each target gene $E^{\Delta Ct}$ values of the samples were divided by the mean $E^{\Delta Ct}$ value of the controls ($E^{\Delta Ct[sample]}$/mean $E^{\Delta Ct[control]}$). PCR efficiencies (E) for within-experiment amplicon groups were determined by the new LinRegPCR program [22]. Outliers were excluded based on the Grubbs test [23]. The statistical analyses were performed using Prism 4 by GraphPad Software Inc. (San Diego, CA, USA). Data were log-transformed when the variation differed between groups. In the figures data are shown as mean + standard deviation of the mean (SD).

Results

CYP1 gene expression in 48-hpf zebrafish embryos after exposure to FICZ

Initially we determined whether FICZ added in the water could pass the chorion and induce *CYP1* gene expression in intact zebrafish embryos. Embryos that were exposed to 10 nM FICZ for 6 hours when exposure started at 48 hpf responded with a 34-fold increase in *CYP1A* mRNA expression compared to the DMSO control (Fig. 2A). The same exposure produced significant induction of *CYP1B1* and *CYP1C1*, although the induction was weak for both, only1.6-fold and 1.8-fold, respectively (Fig. 2B and 2C). There was no statistically significant change in expression of *CYP1C2* (1.3-fold; $p = 0.09$ with *t* test) (Fig. 2D) or *CYP1D1* (1.1-fold; $p = 0.38$ with *t* test) (Fig 2E).

To determine whether the presence of the chorion affected the response to FICZ, and to assess the persistence of the *CYP1A* induction, 48-hpf embryos were dechorionated and exposed for 6 or 12 hours to 10 nM FICZ and then analyzed. *CYP1A* was significantly induced after both 6 and 12 hours of exposure to FICZ ($p < 0.001$), but the level of *CYP1A* induction was considerably less after 12 hours than after 6 hours (6-fold versus 26-fold; $p < 0.01$; Fig. 3). The absence of the chorion had no effect on the degree of induction (Fig. 3 versus Fig. 2).

FICZ concentration-response relationship in 48-hpf zebrafish embryos

A 6 hour exposure to a range of FICZ concentrations (0.1 nM to 30 nM) caused a concentration-dependent induction of *CYP1A*, *CYP1B1*, and *CYP1C1* (Fig. 4). A significant induction of *CYP1A* mRNA was observed at 0.1 nM, the lowest FICZ concentration tested (Fig. 3A). The induction of *CYP1A* peaked at 10 nM FICZ, although the differences in the degree of induction (expressed as fold increase in FICZ-exposed versus DMSO-exposed fish) were not statistically significant in the concentration range of 0.3–30 nM FICZ. The lowest FICZ concentration that caused a significant induction of *CYP1B1* was 3 nM, and higher concentrations caused no further induction (Fig. 4B). *CYP1C1* was induced at 10 nM FICZ in the first experiment (Fig. 2C); however, in the concentration-response experiment induction was statistically significant only at the 30 nM FICZ dose (Fig. 4C). This is probably because triplicates were used in the concentration-response experiment, while in the first experiment there were six replicates. There was a trend toward higher *CYP1C2* expression in embryos exposed to 10 and 30 nM FICZ, but the levels were not significantly different from that of the control (Fig. 4D). *CYP1D1* expression was not induced in the 48-hpf embryos at any of the FICZ concentrations tested (Fig. 4E). For *CYP1A* and *CYP1B1* the EC_{50} values for induction were almost the same, 0.6 and 0.5 nM FICZ, respectively.

Developmental profile of CYP1A inducibility by FICZ

To determine whether sensitivity to *CYP1* induction by FICZ varies with developmental stage, zebrafish embryos or larvae were exposed to 10 nM FICZ for a 6-hour period starting at various times after fertilization. Exposures began at 6, 24, 48, 72, and 96 hours, and 7, 14, or 28 days post-fertilization. (The times listed in Table 2 and plotted in Figure 5 refer to the times when the experiment was terminated.) The relative induction of *CYP1A* by FICZ was greatest at 12 hpf (100-fold) and least at 7.25 dpf (20-fold) (Fig 5A; Table 2). To compare the levels of FICZ-induced *CYP1A* among developmental stages, we also calculated the change in *CYP1A* expression for the same data, after the data had been normalized to the mean value for 12-hpf control embryos (Fig. 5B and Table 2). Calculated this way, the highest levels of FICZ-induced *CYP1A* expression were observed in 30- and 102-hpf zebrafish (210-fold and 200-fold greater than the 12-hpf DMSO control value, respectively; Fig 5B and Table 2). The lowest levels of FICZ-induced *CYP1A* expression were recorded in groups exposed to FICZ in the period between these time points, that is, at 54 and 78 hpf (47-fold and 60-fold greater than the 12-hpf DMSO control value, respectively).

Assessment of embryo toxicity in FICZ-exposed zebrafish embryos

Planar halogenated aromatic hydrocarbons (*e.g.* TCDD and PCB126) cause a variety of pathological effects in early life stages of fish, *e.g.*, lack of swimbladder inflation, edemas, craniofacial and heart malformations, and hemorrhages [24,25]. Zebrafish embryos exposed to PCB126 show lack of inflation of the swim bladder at 1 nM, and edemas and malformations at 10 nM (or higher concentrations) [14]. Zebrafish are most sensitive in the period before hatching. However, in the present study no phenotypic changes due to FICZ were observed in a group of zebrafish embryos that were exposed to FICZ (10 nM) starting at 48 hpf (with renewal of the FICZ solution after 6 hours), and followed for 48 hours.

Competitive binding of FICZ to AHR1B and AHR2

To determine whether FICZ is capable of binding to both AHR2 and AHR1B of zebrafish, and whether these two AHRs might differ in this regard, we measured the ability of FICZ to displace the specific binding of [^{3}H]TCDD to each AHR, using a sucrose gradient assay. For both AHRs, FICZ at 1 nM had little or no effect on the specific binding of [^{3}H]TCDD. However, at 10 nM, FICZ completely inhibited the binding of [^{3}H]TCDD to both AHR1B and AHR2 (Fig. 6). These results suggest that FICZ is a high-affinity ligand for both zebrafish AHRs.

Effect of AHR2 knock-down on FICZ-induced CYP1A expression

In mammals, the induction of *CYP1A1* by FICZ *in vivo* is dependent on the presence of the single mammalian AHR [9]. The ability of FICZ to interact with zebrafish AHR2 and AHR1b *in vitro* suggests that they may be involved in mediating the response to FICZ in zebrafish, but does not reveal which of these AHRs regulates *CYP1* induction by this compound *in vivo*. Using the morpholino knock-down technique, we and others have shown previously that transcriptional induction of *CYP1A*, *1B1*, *1C1*, and *1C2* by PCB126 and TCDD is regulated primarily by AHR2 in zebrafish embryos [14,15,17]. To determine whether *CYP1* induction by FICZ also involves AHR2, we microinjected zebrafish embryos with a morpholino targeting *AHR2* translation. Non-injected embryos (data not shown) and embryos treated with a standard control morpholino were used as controls. Embryos of each treatment group were exposed to DMSO (200 ppm), FICZ (10 nM), or PCB126 (30 nM). Both the non-injected and the control MO-injected embryos exposed to FICZ or PCB126 responded with a strong induction of *CYP1A*, which was similar for the two compounds (Fig. 7A). In the AHR2 MO-injected FICZ- and PCB126-exposed embryos the induction of *CYP1A* was reduced to 11% and 7%, respectively, of the levels seen in the group injected with control morpholino and exposed to the same compounds ($p < 0.001$; Fig. 7A). The induction of *CYP1B1* by FICZ, although weak, also was similar for FICZ and PCB-126, and was decreased in the AHR2 MO-injected embryos ($p < 0.001$) to 36% and 43% of the corresponding levels in control morpholino-injected embryos (Fig. 7B). Both *CYP1C*s were strongly induced by PCB126 and this induction was suppressed by the AHR2-MO (Fig. 7C and 7D). However, in this experiment neither *CYP1C* was significantly induced by FICZ, precluding detection of an effect of AHR2 knockdown on the expression of these two genes.

Discussion

The tryptophan oxidation product FICZ has been suggested as a physiological ligand for the AHR. Studies have shown that FICZ is formed in mammalian cells upon exposure to ultraviolet (UV) radiation and that it has a high affinity for rodent and human AHR [6,7]. FICZ also induces *CYP1s* in human and rodent, and these *CYP1s* are able to metabolize FICZ very rapidly [8,26]. The results of the present study indicate that the features of FICZ interaction with AHR and induction of *CYP1s* seen in mammals apply to zebrafish as well. At low concentrations, FICZ completely displaced TCDD from AHR2 and AHR1B, and thus apparently is an avid ligand for both. FICZ was a potent inducer of *CYP1* genes in zebrafish embryos, particularly *CYP1A*, and as in mammals this induction of *CYP1A* appeared to be transient, declining in a matter of hours, consistent with rapid metabolism of an inducer. Our results also indicate that FICZ induction at least of *CYP1A* and *CYP1B1* in zebrafish is mediated largely by AHR2.

We found that FICZ was a more potent *CYP1* inducer than PCB126 in zebrafish, i.e., for induction of *CYP1A* and *1B1* by FICZ the 6 hour EC_{50} values were 0.5–0.6 nM, whereas for PCB126 the 72 hour EC_{50} values are in the range of 2.3–2.7 nM [14]. However, while PCB126 can strongly induce the four *CYP1* genes (*CYP1A*, *1B1*, *1C1*, and *1C2*) [14], FICZ strongly induced only *CYP1A* (>30-fold; Fig. 2,Fig. 4,Fig 7A). *CYP1B1* was induced only about 3-fold by 6 hours of exposure to FICZ or PCB126 (Fig. 7B). Although the levels of induction differed greatly between *CYP1A* and *CYP1B1*, 6 hours of exposure to either of the two compounds (FICZ and PCB126) induced each gene to a similar level. In contrast, responses of *CYP1C1* and *CYP1C2* to FICZ not only were quite weak (at most 2- to 3-fold), the responses to FICZ were much less than that after 6 hours of exposure to PCB126 (13-fold and 8-fold, respectively; Fig 7C-D), or to that after 48 hours of exposure to PCB126 (19- and 34-fold, respectively) [14]. *CYP1D1* was not induced by FICZ, consistent with findings that zebrafish *CYP1D1* is not induced by other AHR agonists [16]. However, the reason for the much weaker induction of *CYP1C*s by FICZ than by PCB126 is not known.

Weak induction of the *CYP1Cs* could be a consequence of rapid degradation of the inducer. In studies performed with recombinant human enzymes, FICZ was shown to be an exceptionally good substrate for CYP1A1 and to be metabolized also by CYP1A2 and CYP1B1 [8,27]. Furthermore, hydroxylated FICZ metabolites were good substrates for sulfotransferases, and human urine was found to contain a variety of FICZ-derived metabolites [8]. FICZ might induce its own metabolism in zebrafish via transcriptional activation of *CYP1* genes. Although the capability of zebrafish CYP1 enzymes to metabolize FICZ has not yet been studied, the decline in *CYP1A* expression after 12 hours compared to 6 hours of exposure is consistent with a rapid degradation of the inducer. The half-lives of the *CYP1C* mRNAs are not known; if they are longer than the half-life of *CYP1A* message, then a rapid metabolism could reduce concentrations of FICZ to ineffective levels before induction of the other *CYP1s* could be detected. However, the relatively strong response of the *CYP1Cs* after a 6-hour exposure to PCB126 is not consistent with that (Fig. 7). CYP1A protein is strongly induced in the endothelium of fish, and if FICZ is a substrate for zebrafish CYP1A that could reduce the amounts of FICZ reaching other parts of the organism. That could in turn affect induction of the other *CYP1s*, if their expression is predominantly extravascular; cell specific expression of all the *CYP1s* is yet to be determined in zebrafish. This situation would be different with exposure to PCB126, which is slowly metabolized and penetrates thoroughly. A possible alternative or additional explanation for the relatively weak induction of *CYP1Cs* by FICZ is that FICZ-AHR complexes may interact differently with the promoters of these genes, in comparison to its interaction with the *CYP1A* promoter. Experiments using chromatin immunoprecipitation will be required to examine the possibility of ligand- and gene-specific AHR-promoter interactions.

Looking at FICZ-induced *CYP1A* expression at different times during development in zebrafish we found a strong induction as early as 12 hpf (49-fold; Fig 4A). In fact, the largest increase relative to the control was recorded at this time point. The high relative induction is partially explained by the low basal level of *CYP1A* expression early in zebrafish embryo development, which results in a high ratio between induced and control. The present study confirms our previous finding that the basal level of *CYP1A* expression is lowest early in zebrafish embryo development and increases after hatching [14].

In order to compare *CYP1A* gene expression over time we normalized all data to the 12-hpf control level. This revealed that the highest FICZ-induced *CYP1A* expression occurred during the first week. However, there was a temporal drop in level of FICZ-induced *CYP1A* expression (Fig 4B) from over 200-fold at day 1 post-fertilization, to about 50- and 60-fold at day 2 and day 3, and then an increase to 200-fold at day 4, all versus the 12-hpf control value. Interestingly, the decrease in FICZ-induced *CYP1A* at day 2–3 coincides with the time for hatching in zebrafish, but at present we do not know what this implies physiologically.

In mammals, induction of *CYP1A* by FICZ occurs in an AHR-dependent fashion [2,9], and FICZ has been shown to be a ligand for the AHR [6]. Unlike mammals, which have a single AHR, fish have multiple AHR paralogs, classified within the AHR1 and AHR2 subfamilies. Zebrafish possess three AHRs, one of which (AHR1A) is unable to bind typical AHR ligands such as TCDD and BNF and lacks a functional transactivation domain [11,12]. In the present study, FICZ was able to bind to both of the other two AHRs, as indicated by its ability to displace the binding of [^{3}H]TCDD to *in vitro*-expressed AHR2 and AHR1B. AHR2 has been shown previously to be the major AHR form involved in *CYP1A* induction and embryo toxicity in response to TCDD, PCB126, and some PAHs [17,28–30], while the function of AHR1B is not yet well understood [11]. Similar to results with xenobiotic ligands, we showed that *CYP1A* and *CYP1B1* induction by FICZ is largely dependent on AHR2. The residual level of induced *CYP1A* or *CYP1B1* in embryos in which AHR2 expression had been knocked down could result from low levels of AHR2 protein remaining in these embryos. It also could

represent a contribution of AHR1B to *CYP1A* or *CYP1B* induction by FICZ, for example in specific tissues or cell types. Studies of organ and cell-specific differences in the expression of the two AHRs, in relation to cell-specific expression of *CYP1s* are underway. Likewise, studies to clarify the role of AHR1B *in vivo* in the response to a variety of AHR ligands (including FICZ as well as TCDD and PAHs) are underway and will be reported separately. The results of such studies could shed light on the possible physiological roles of FICZ and AHRs, taking advantage of the multiple AHR paralogs found in zebrafish and the possibility that they may have partitioned the subfunctions of the single mammalian AHR.

Conclusion

Together, the results here demonstrate that FICZ is a potent AHR agonist in zebrafish, inducing expression of multiple *CYP1* genes. *CYP1A* expression was strongly induced by FICZ, but as in mammals this induction appeared to be transient. Interestingly, FICZ induced the expression of other inducible *CYP1s* very slightly compared to *CYP1A*, or to the effect of PCB126, for reasons not yet understood. We also found that while FICZ has a high affinity for two of the three AHRs in zebrafish, i.e., AHR1B and AHR2, the induction of *CYP1A* and *CYP1B1* (in whole embryos) was primarily through the AHR2. It appears that the high potency of FICZ to bind and activate the AHR is evolutionarily conserved from fish to mammals, consistent with a possible role as an endogenous signaling molecule, acting through the AHR. Our knowledge of the endogenous roles of mammalian and fish AHRs is evolving. If AHRs are involved in physiological sensing of UV and visible light that involves formation of FICZ *in vivo*, this may be important in aquatic vertebrates, including those such as zebrafish with embryos that are transparent. Studies of *in vivo* gene expression responses of zebrafish to UV light are underway.

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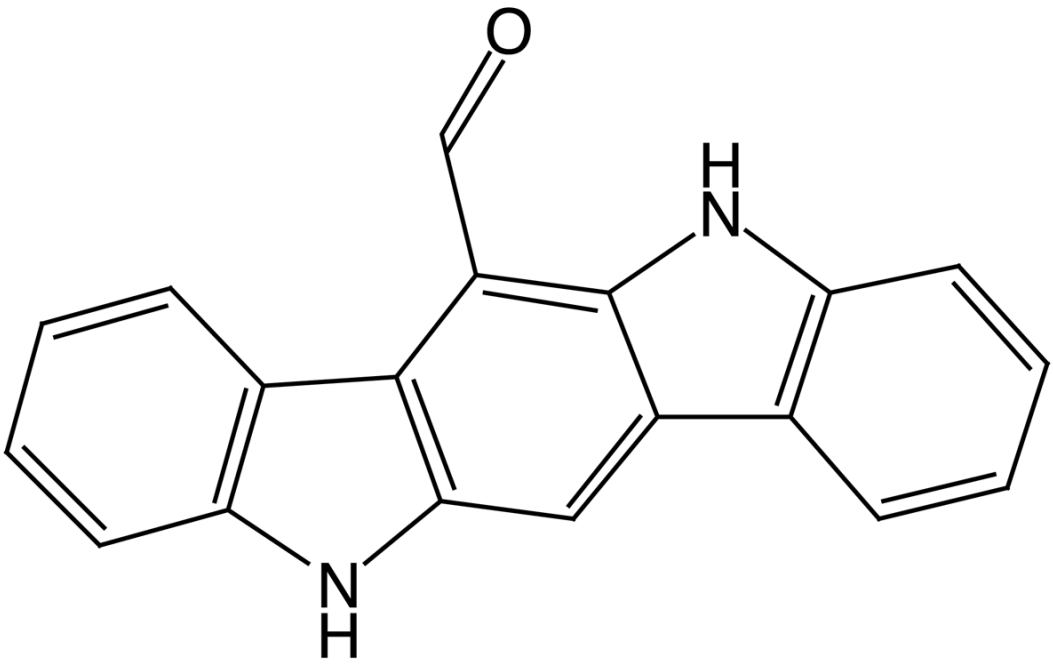

Fig 1.
Structure of 6-Formylindolo[3,2-b]carbazole

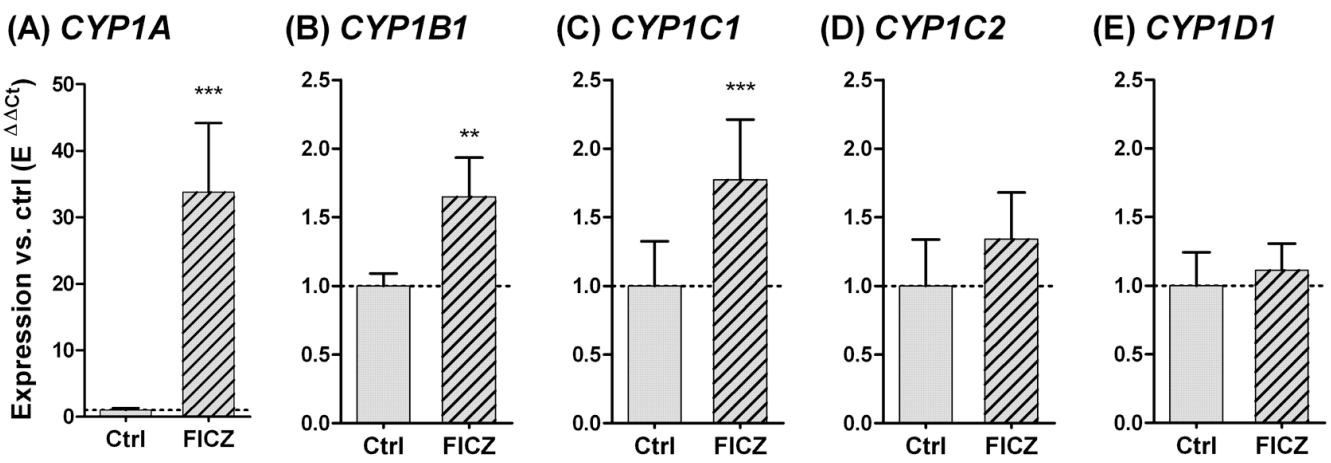

Fig 2.

Relative *CYP1A*, *1B1*, *1C1*, *1C2*, and *1D1* gene expression (A-E) in 48-hpf zebrafish embryos after exposure to 10 nM 6-formylindolo[3,2-b]carbazole (FICZ; hatched bars) or 100 ppm DMSO (Ctrl; filled bars) for 6 hours. Relative expression was calculated by $E^{\Delta Ct[sample]}$/mean $E^{\Delta Ct[control]}$. A significant statistical difference compared with the control (n=6) was determined with student's t test and is shown by stars (*** = p<0.001 and ** = p<0.01).

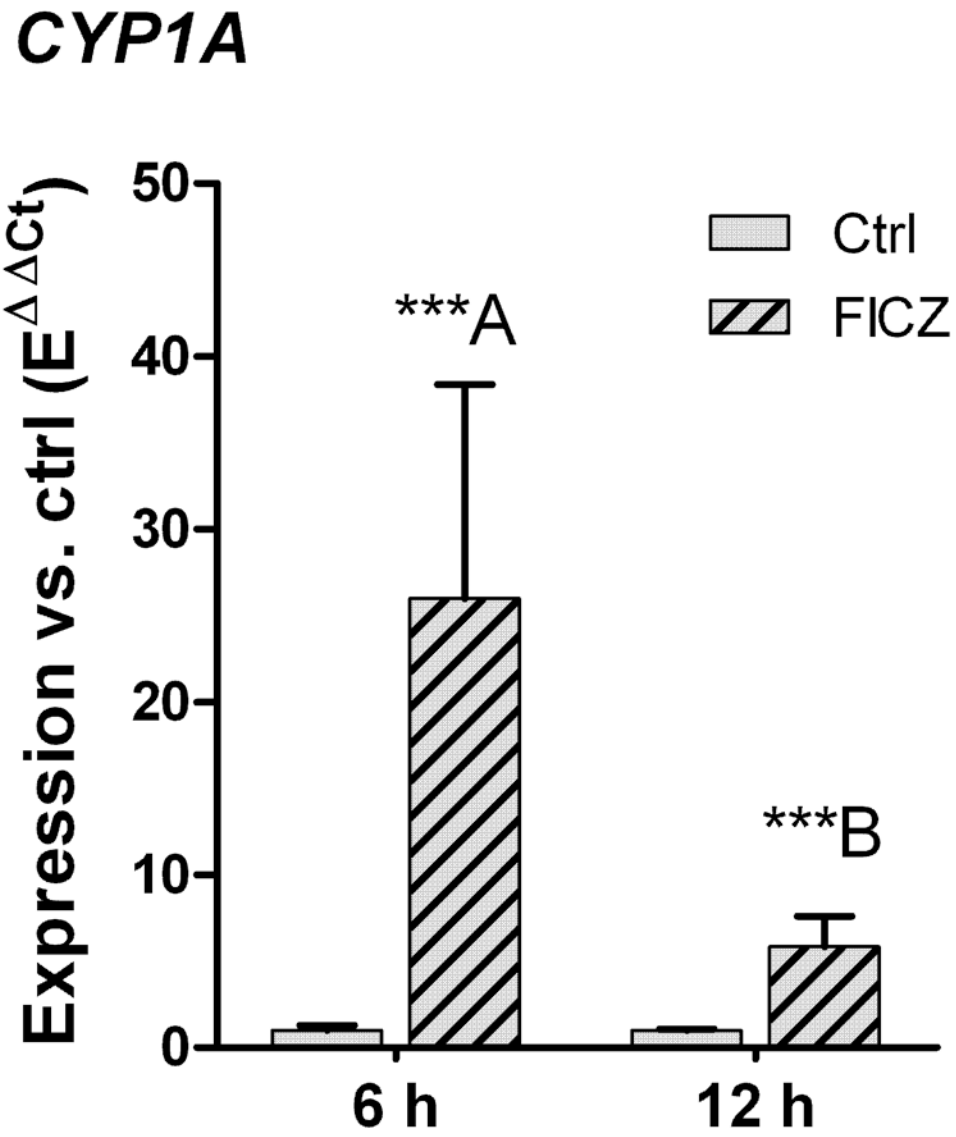

Fig 3.

Effect of 6 or 12 hours of exposure to 6-formylindolo[3,2-b]carbazole (FICZ) on *CYP1A* expression in dechorinated 48-hpf zebrafish embryos. Embryos were exposed to 10 nM FICZ (hatched bars) and 100 ppm DMSO, or 100 ppm DMSO only (Ctrl; filled bars) for 6 or 12 hours. Relative level of *CYP1A* gene expression was calculated by $E^{\Delta Ct[sample]}$/mean $E^{\Delta Ct[control]}$. Significant statistical differences between groups (n=4) were determined using one-way ANOVA followed by Bonferroni's Multiple Comparison Test of selected pairs (post hoc test) with log transformed data. Differences between the FICZ-exposed groups and the corresponding controls and between the groups exposed to FICZ for 6 or 12 hours are shown by stars (*** = $p < 0.001$) and different letters ($p < 0.01$), respectively.

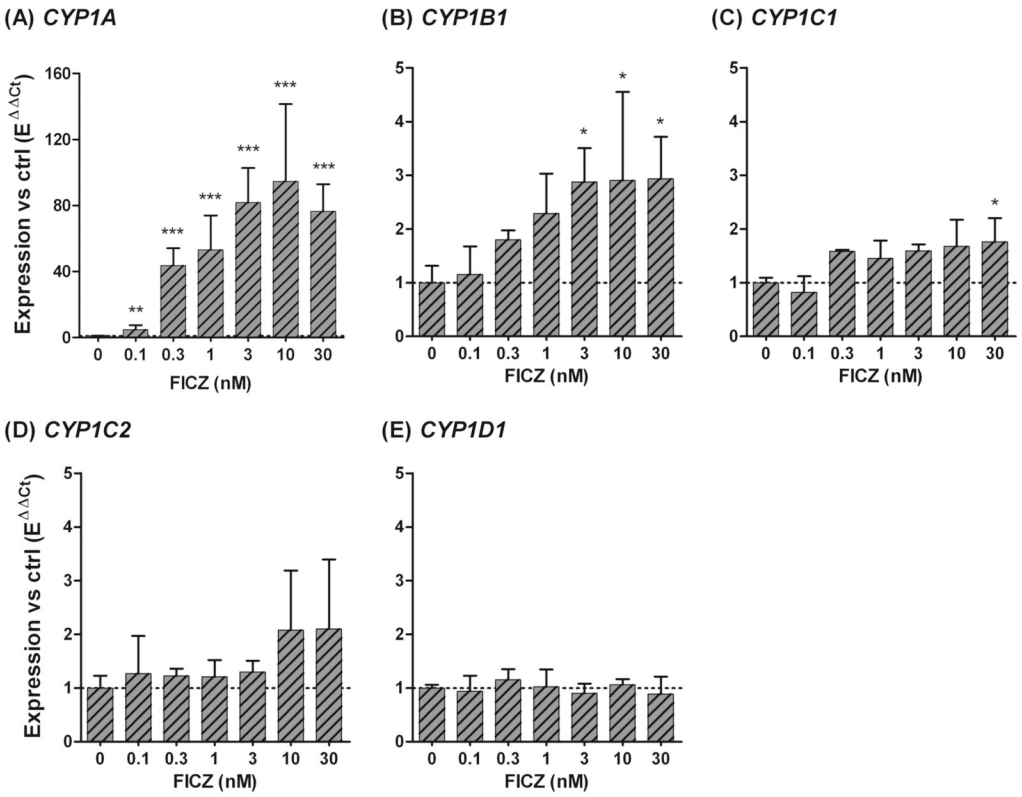

Fig 4.

6-Formylindolo[3,2-b]carbazole (FICZ) concentration response relationships for expression of *CYP1A*, *1B1*, *1C1*, *1C2*, and *1D1* (A-E) in zebrafish embryos. At 48 hpf embryos were exposed for 6 hours to 0.1, 0.3, 1, 3, 10, or 30 nM FICZ, or 150 ppm DMSO ("0"). Relative expression was calculated by $E^{\Delta Ct[sample]}$/mean $E^{\Delta Ct[control]}$. Statistically significant differences between the control and exposed groups (n=3) were determined with one-way ANOVA followed by Dunnett's post hoc test (* = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$).

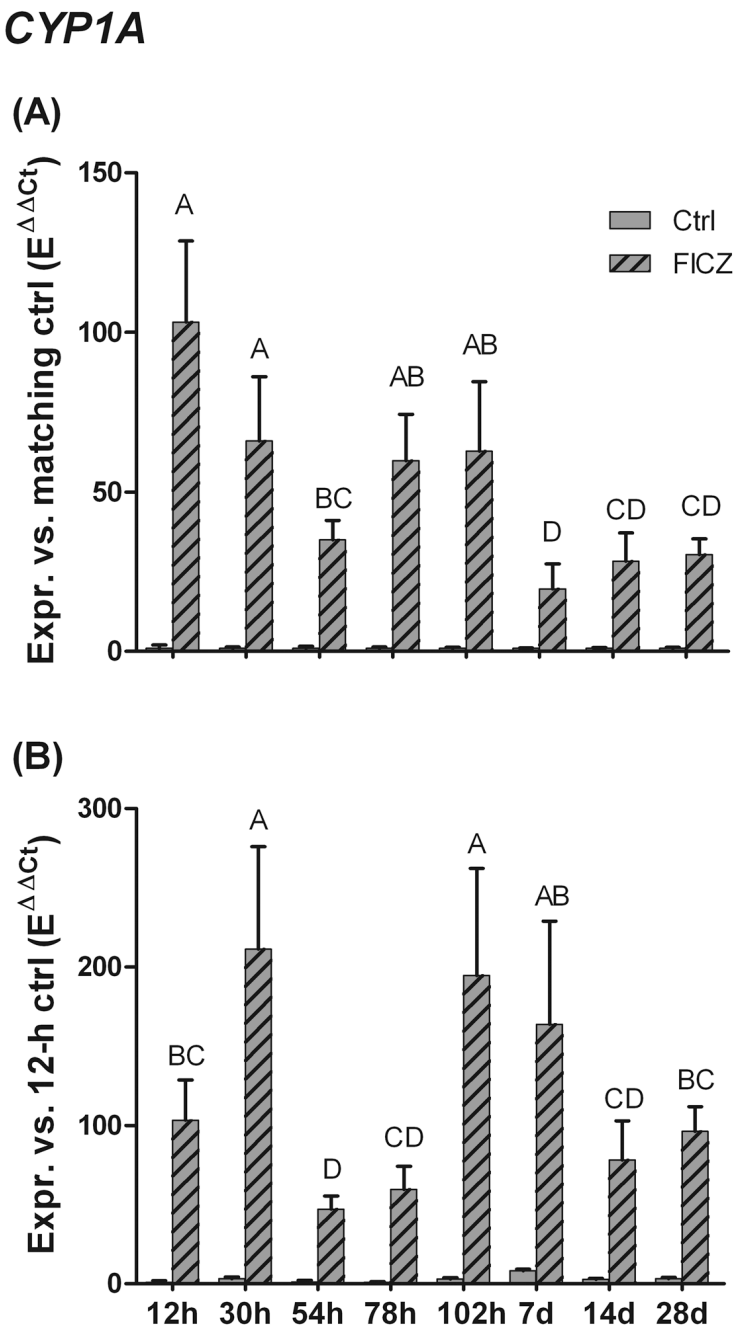

Fig 5.

Developmental profile of *CYP1A* inducibility by 6-formylindolo[3,2-b]carbazole (FICZ) in zebrafish embryos, larvae, and juveniles. Zebrafish at different developmental stages (6 hpf, or 1, 2, 3, 4, 7, 14 or 28 dpf) were exposed to FICZ (10 nM) or DMSO for 6 hours. *CYP1A* induction A) versus the matching control ($E^{\Delta Ct[sample]}$/mean $E^{\Delta Ct[control]}$) and B) versus the 12-hour control ($E^{\Delta Ct[sample]}$/mean $E^{\Delta Ct[12\text{-h control}]}$). Statistically significant differences between groups (n=5) were determined by one-way ANOVA followed by Tukey's post hoc test and are shown by different letters ($p < 0.05$).

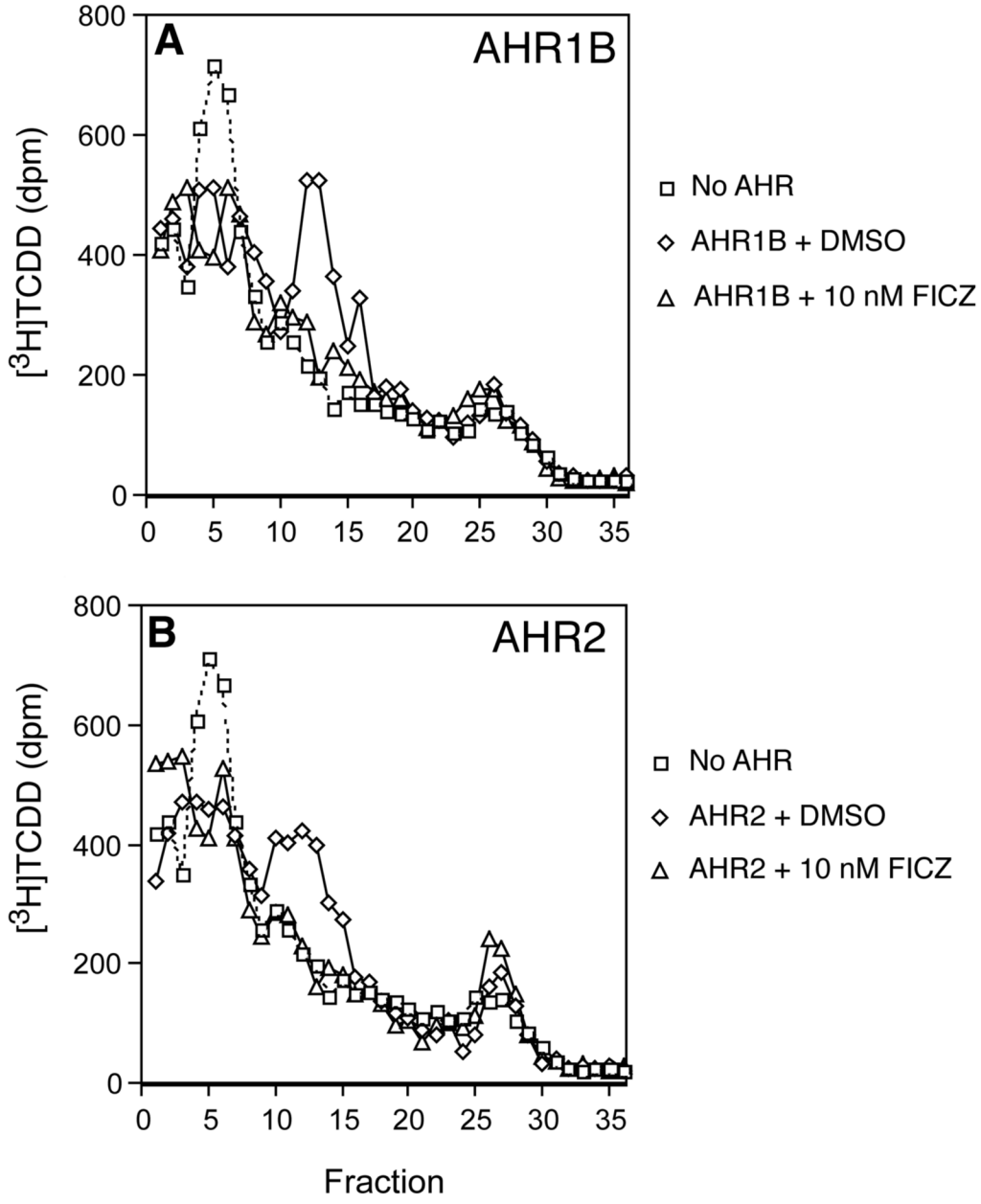

Fig 6.

Competitive binding assay using *in vitro*-expressed zebrafish AHR1B or AHR2 proteins. AHR1B (A) and AHR2 (B) proteins were expressed by *in vitro* transcription and translation, incubated with [^{3}H]TCDD (2 nM) ± 10 nM FICZ or DMSO, and analyzed by velocity sedimentation on sucrose gradients, as described in *Materials and Methods*. No AHR: Unprogrammed lysate incubated with [^{3}H]TCDD (2 nM), showing nonspecific binding; AHR1B or AHR2 protein incubated with [^{3}H]TCDD (2 nM) + DMSO, showing total binding in the absence of FICZ; AHR1B or AHR2 protein incubated with 2 nM [^{3}H]TCDD + 10 nM FICZ, showing displacement of [^{3}H]TCDD binding by FICZ.

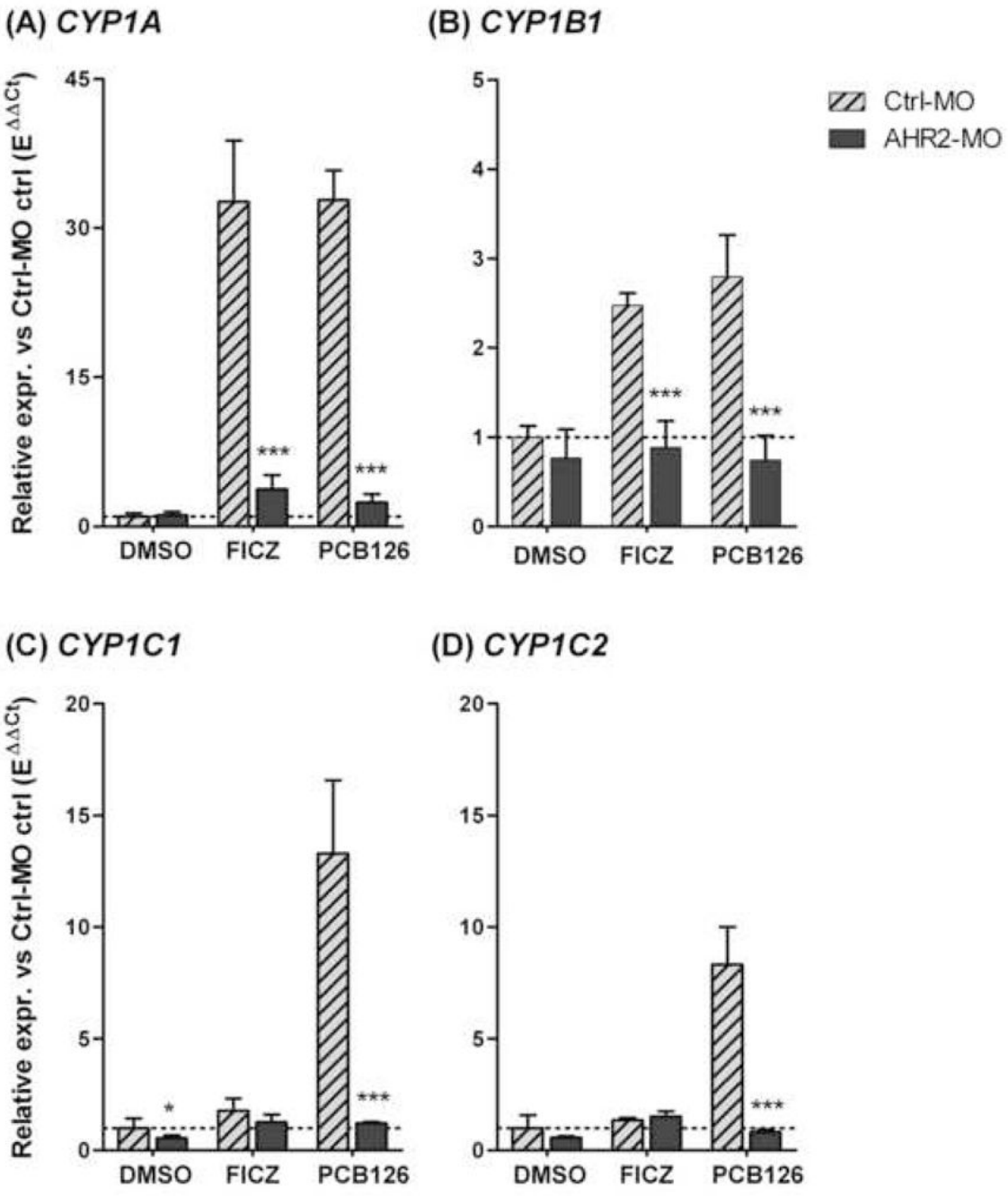

Fig 7.

Effect of AHR2 knock-down on *CYP1* induction by FICZ or PCB126 in zebrafish embryos. Embryos (2–4 cell stage) were injected with an AHR2 morpholino (AHR2-MO; dark bars), a negative control morpholino (Ctrl-MO; hatched bars), or not injected by a morpholino (Not shown). At 48 hpf these embryos were exposed to 200 ppm of DMSO, 10 nM FICZ, or 30 nM PCB126 for 6 hours (n=3–4). Relative expression was calculated by $E^{\Delta Ct[sample]}$/mean $E^{\Delta Ct[control\ MO\ control]}$. A) *CYP1A*. B) *CYP1B1*. C) *CYP1C1*. D) *CYP1C2*. Differences among groups treated with Ctrl-MO or AHR2-MO and with the same exposure (DMSO, FICZ, or PCB126) were examined by one-way ANOVA followed by Bonferroni's Multiple Comparison Test of selected pairs (* = $p < 0.05$ and *** = $p < 0.001$).

Table 1

Real time PCR primer sequences

Primer	Sequences
ZFCYP1A F[a]	GCATTACGATACGTTCGATAAGGAC
ZFCYP1A R[a]	GCTCCGAATAGGTCATTGACGAT
ZFCYP1B1 F[b]	CTGCATTGATTTCCGAGACGTG
ZFCYP1B1 R[b]	CACACTCCGTGTTGACAGC
ZFCYP1C1 F	AGTGGCACAGTCTACTTTGAGAG
ZFCYP1C1 R	TCGTCCATCAGCACTCAG
ZFCYP1C2 F[b]	GTGGTGGAGCACAGACTAAG
ZFCYP1C2 R[b]	TTCAGTATGAGCCTCAGTCAAAC
ARNT2 F[b]	CACCTTTGGATCACATCTCATTG
ARNT2 R[b]	TCACCCTCCTTAGACGGACC
CYP1D1 F[c]	TCAACTTCGACACGAACTGTATC
CYP1D1 R[c]	TGTGAACGATCTGGGAGTTG

[a] [31]

[b] [13,14]

[c] [16]

Table 2

CYP1A expression in zebrafish exposed to 10 nM FICZ or 100 ppm of DMSO for 6 hours at various times in development

The time post-fertilization (pf) is the age at termination of the experiment, FICZ exposure started 6 hours prior to this. The first set of data are normalized to the mean values of the time-matched controls, the second set are normalized to the mean value of the control at 12 hpf.

Time pf	*N*	Expression vs control		Expression vs 12-hpf control	
		Ctrl	FICZ	Ctrl	FICZ
12 h	5	1.0 ± 1.0	100 ± 25	1.0 ± 1.0	100 ± 25
30 h	5	1.0 ± 0.4	66 ± 20	3.2 ± 1.2	210 ± 65
54 h	5	1.0 ± 0.6	35 ± 6	1.4 ± 0.9	47 ± 8
78 h	5	1.0 ± 0.5	60 ± 15	1.0 ± 0.5	60 ± 15
102 h	5	1.0 ± 0.3	63 ± 22	3.1 ± 0.9	200 ± 68
7.25 d	4	1.0 ± 0.1	20 ± 8	8.4 ± 1.0	160 ± 65
14.25 d	5	1.0 ± 0.2	28 ± 9	2.8 ± 0.7	78 ± 25
28.25 d	5	1.0 ± 0.3	30 ± 5	3.2 ± 1.1	100 ± 16