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Combination effects of AHR agonists and Wnt/ β -catenin modulators in zebrafish embryos: implications for physiological and toxicological AHR functions

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Abstract

Wnt/ β -catenin signaling regulates essential biological functions and acts in developmental toxicity of some chemicals. The aryl hydrocarbon receptor (AHR) is well-known to mediate developmental toxicity of persistent dioxin-like compounds (DLCs). Recent studies indicate a crosstalk between β -catenin and the AHR in some tissues. However the nature of this crosstalk in embryos is poorly known. We observed that zebrafish embryos exposed to the β -catenin inhibitor XAV939 display effects phenocopying those of the dioxin-like 3,3',4,4',5-pentachlorobiphenyl (PCB126). This led us to investigate AHR interaction with β -catenin during development and ask whether developmental toxicity of DLCs involves antagonism of β -catenin signaling. We examined phenotypes and transcriptional responses in zebrafish embryos exposed to XAV939 or to a β -catenin activator, 1-azakenpauellone, alone or with AHR agonists, either PCB126 or 6-formylindolo[3,2-b]carbazole (FICZ). Alone 1-azakenpauellone and XAV939 both were embryotoxic, and we found that in presence of FICZ, the toxicity of 1-azakenpauellone decreased while the toxicity of XAV939 increased. This rescue of 1-azakenpauellone effects occurred in the time window of Ahr2-mediated toxicity and was reversed by morpholine-oligonucleotide knockdown of Ahr2. Regarding PCB126, addition of either 1-azakenpauellone or XAV939 led to lower mortality than with PCB126 alone but surviving embryos showed severe edemas. 1-Azakenpauellone induced transcription of β -catenin-associated genes, while PCB126 and FICZ blocked this induction. The data indicate a stage-dependent antagonism of β -catenin by Ahr2 in zebrafish embryos. We propose that the AHR has a physiological role in regulating β -catenin during development, and that this is one point of intersection linking toxicological and physiological AHR-governed processes.

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INTRODUCTION

The aryl hydrocarbon receptor (AHR)¹, known for mediating toxicity of dioxin-like compounds (DLCs), is a ligand-activated transcription factor that regulates expression of many genes including those for cytochrome P450 1 (CYP1) enzymes. The AHR plays roles in development, reproduction, immune function, and other processes, and AHR agonist toxicity apparently involves disturbance of these processes (Abel and Haarmann-Stemmann, 2010; Quintana and Sherr, 2013). In zebrafish embryos Ahr2 activation by potent AHR agonists such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3,3',4,4',5-pentachlorobiphenyl (PCB126) causes circulatory failure, skeletal malformations, reduced body growth, and other effects (Carney *et al.*, 2006a; Jönsson *et al.*, 2012; King-Heiden *et al.*, 2012). However, the molecular mechanisms behind the effects are still poorly known. Recently, studies have begun to point to an AHR-dependent dysregulation of Wnt/ β -catenin signaling as a potential mechanism (Schneider *et al.*, 2014a).

The Wnt/ β -catenin pathway plays fundamental roles in stem cell proliferation, development, growth, tissue homeostasis, sex differentiation, and endocrine functions (Nusse, 2008; Schinner *et al.*, 2009; Sreenivasan *et al.*, 2014). The pathway also is implicated in common diseases such as atherosclerosis, type II diabetes, obesity, osteoporosis, and cancer (MacDonald *et al.*, 2009; Schinner *et al.*, 2009; Marinou *et al.*, 2012), and in toxicity of some chemicals, including bisphenol A, dibutyl phthalate, thalidomide, phenanthrene, and benzalkonium chloride (Knobloch *et al.*, 2007; Zhou *et al.*, 2011; Fairbairn *et al.*, 2012; Xu *et al.*, 2013; Tiwari *et al.*, 2014). β -Catenin is a transcriptional activator of the T-cell factor/lymphoid enhancer-binding factor (TCF/LEF1) complex, which controls expression of multiple target genes, many of which are involved in cell proliferation and differentiation (Hödar *et al.*, 2010).

Not surprisingly, the levels of active β -catenin are tightly controlled (Kikuchi *et al.*, 2011). The activators of β -catenin signaling, the Wnts, are secreted lipid-modified glycoproteins that when activated bind to specific transmembrane receptors (Frizzled) and co-receptors (LRP5/6, ROR1/2, etc.) (Niehrs, 2012); the Frizzled receptors are endocytosed and degraded, while β -catenin is released and enters the nucleus to activate transcription (Fig. 1A). Wnt signaling can be enhanced by R-spondins, secreted proteins that block degradation of Wnt receptors (Fig. 1B) (Niehrs, 2012). Regulation also involves proteasomal degradation of β -catenin, which is preceded by phosphorylation of β -catenin by casein kinase I α (CKI α) and glycogen synthase kinase 3 β (GSK-3 β) that together with AXIN and adenomatosis polyposis coli (APC) make up the β -catenin destruction complex (Fig. 1C).

Several studies have observed potential crosstalk between Wnt/ β -catenin and AHR. In male mouse fetuses prostatic budding is antagonized by TCDD apparently through Ahr-mediated down-regulation of R-spondins 2 and 3, associated with suppression of Wnt signaling (Branam *et al.*, 2013). AHR activation by TCDD also triggers neuronal apoptosis in rat brain

¹Zebrafish cytochrome P450 genes/mRNAs and proteins are abbreviated with capital letters (e.g., CYP1A and CYP1A) according to (Nelson *et al.*, 1996). For abbreviations of other genes/mRNAs and proteins in zebrafish we followed ZFIN Nomenclature Guidelines, <https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines>, e.g., ahr2 and Ahr2. The aryl hydrocarbon receptor is denoted AHR when not referring to a particular species.

via down-regulation of Wnt/ β -catenin signaling, and interferes with tissue regeneration in fin-amputated zebrafish via up-regulation of R-spondin 1 (Mathew *et al.*, 2008; Xu *et al.*, 2013). Ligand-activated AHR also might interact more directly with the β -catenin pathway. The ligand/AHR/ARNT complex has been reported to trigger proteasomal degradation of β -catenin in colon cancer cells exposed to 3-methylcholanthrene (3-MC), β -naphthoflavone, or indole-3-acetic acid (Fig. 1D) (Kawajiri *et al.*, 2009). Furthermore β -catenin has been reported to interact with the AHR at *CYP1* gene promoters to enhance transcription and knockdown of β -catenin reduced perivenous *Cyp1a* induction by 3-MC in mouse hepatocytes (Braeuning *et al.*, 2011; Prochazkova *et al.*, 2011; Vaas *et al.*, 2014).

During early development β -catenin is involved in embryo axis determination and in the formation of various organs (Petersen and Reddien, 2009; Yin *et al.*, 2011; Meyers *et al.*, 2012). While there is evidence for interaction between AHR and the Wnt/ β -catenin pathway, many questions remain about such interaction, especially during development. Such questions include: Are interactions between AHR and the Wnt/ β -catenin pathways involved in toxic effects of AHR agonists in early development? Are the AHR/ β -catenin interactions developmental stage-specific? Does the nature of an AHR agonist influence the interaction? Most studies of AHR agonist effects on Wnt signaling in zebrafish have been with TCDD. Do similar effects occur with dioxin-like PCBs? Would transient activation of AHR by rapidly metabolized agonists such as 6-formylindolo[3,2-*b*]carbazole (FICZ) (Rannug *et al.*, 1987; Wincent *et al.*, 2009) differ from persistent AHR activation by slowly metabolized DLCs, in their effects on Wnt signaling?

To address these questions we examined Ahr2 interaction with β -catenin during zebrafish embryo development. To manipulate the activities of Ahr2 and β -catenin we used two AHR agonists with similar AHR affinity but with different resistance to metabolic degradation (PCB126 and FICZ), in combination with chemical modulators of β -catenin activation, *i.e.*, an inhibitor, XAV939 (XAV) and an activator, 1-azakenpaullone (AZP) (Fig. 1E–F). Phenotypic changes and transcriptional responses were examined in zebrafish embryos exposed to XAV or AZP in combination with and as compared to PCB126 or FICZ. Our results suggest that Ahr2 can repress β -catenin activity during zebrafish development in a temporal manner, and that AHR may play a role during embryogenesis by regulating activity of Wnt/ β -catenin signaling.

MATERIALS AND METHODS

Animals

Embryos of the AB zebrafish variant were obtained from the Zebrafish Core Facility at Comparative Medicine, Karolinska Institutet (<http://ki.se/en/research/karolinska-institutet-zebrafish-core-facility>), or the SciLifeLab zebrafish facility at Uppsala University (<http://www.scilifelab.se/facilities/zebrafish/>). Experiments involving morpholino injections were performed at Karolinska Institutet and all other experiments were performed at Uppsala University. Husbandry of the breeding fish stocks was approved by the Swedish Board of Agriculture. Experimental procedures involving zebrafish embryos were according to the directives from the Swedish Board of Agriculture.

Fertilized eggs were generated through group breeding of fish and then used in experiments as described below. All experiments were done while the fish depended on yolk as a nutritional source (before 6 days post-fertilization; dpf). In this paper we refer to zebrafish at all stages before 6 dpf as “embryos”, although fish embryos that have hatched but not started feeding are technically “eleutheroembryos”.

Ahr2 knockdown by morpholinos

Knockdown of Ahr2 was performed using an antisense oligonucleotide morpholino (MO) blocking ahr2 translation. Morpholinos targeting the transcriptional start site of *ahr2* (Ahr2-MO; 5-TGTACCGATACCCGCCGACATGGTT-3) (Prasch et al., 2003) and negative control morpholinos (Ctrl-MO; 5-CCTCTTACCTCAGTTACAATTTATA-3) were obtained from Gene Tools (Philomath, OR, USA). The morpholinos were fluorescein-tagged to allow selection of properly injected embryos. Both morpholinos were diluted in deionized water to a final concentration of 0.15 mM. An Eppendorf FemtoJet with a fine glass needle was used to inject morpholinos into the yolk of 2- to 4-cell stage embryos. Embryos were screened at 6–8 hpf by fluorescence microscopy to verify incorporation of morpholinos. Damaged embryos and those without homogenous fluorescence were removed. Exposure was performed as described below and the experiments were repeated at least twice.

Exposure and experimental design

Chemicals—Groups of embryos were exposed to AZP (97% purity; CAS: 676596-65-9) or XAV (98% purity; CAS: 284028-89-3), both from Sigma-Aldrich Inc. (St. Louis, MO, USA), or to FICZ (CAS: 72922-91-7; Syntastic AB, Stockholm, Sweden) or PCB126 (CAS: 57465-28-8; Larodan Fine Chemicals, Malmö, Sweden), or to combinations of FICZ or PCB126 with AZP or XAV. AZP is an ATP-competitive inhibitor of GSK-3 β (Fig. 1E), a kinase that phosphorylates β -catenin, thus tagging it for proteasomal breakdown (Kunick *et al.*, 2004). Inhibition of GSK-3 β effectively activates or enhances β -catenin signaling. XAV acts by inhibiting the poly-ADP-ribosylating enzymes tankyrase 1 and tankyrase 2 and consequently functions to stabilize Axin (Fig. 1F), with a result of decreasing the amount of active β -catenin (Huang *et al.*, 2009). We refer to this alteration of the levels of active β -catenin as inhibiting or suppressing β -catenin activity. Both FICZ and PCB126 are potent AHR agonists but while FICZ is rapidly metabolized by CYP1 enzymes PCB126 is more resistant to degradation. The four compounds were dissolved in dimethyl sulfoxide (DMSO; CAS: 67-68-5) from Sigma-Aldrich Inc. Nominal final concentrations of the compounds are given below.

Chemical structures of the four compounds and a study overview are shown in Figure 2.

Exposure set up—Groups of embryos were exposed in glass petri dishes containing carbon-filtered Uppsala tap water (28.5 °C). In the study of temporal trends in mRNA expression large dishes were used (described below), while smaller dishes (diameter: 8 cm), each containing 20–30 embryos in 20–25 ml water, were used in all other experiments. Substances were pipetted to the dish bottoms at a space not covered by water (achieved by tilting the dish) and then immediately mixed into the water by thorough swirling of the dish. Unless something else is indicated one dish per exposure group was used. All dishes were

inspected daily and dead embryos were removed when found. Exposure regimens of the different experiments are described in more detailed below and given in the figure legends. Throughout this paper “early” and “late” exposures refers to exposures started at 3 hpf and 1 dpf (25–27 hpf), respectively.

Temporal trends in mRNA expression in zebrafish embryos—Firstly, triplicate samples of 10 unexposed pooled embryos were taken at the 2–4 and 8–16 cell stages. These samples taken before zygotic genome activation (ZGA) were used to determine whether the mRNAs analyzed would be maternally transferred, since if translated they could contribute to the exposure responses. Subsequently, two large dishes (diameter: 14 cm) were prepared with 90 embryos in 90 ml water in each, and with DMSO added to harmonize the conditions with those in control groups. DMSO (100 ppm) was added at 2.5 hpf and shortly after this, sampling began: One sample of 10 pooled embryos were taken from each dish every 2nd hour between 3 and 15 hpf and at 26 hpf ($n=2$). The samples were flash frozen in liquid N₂ and stored at –80 °C until used for quantitative real-time RT-PCR (qPCR). The time course was complemented with the 35, 50 and 74 hpf time points using reanalyzed samples of DMSO controls from another experiment (the one shown in Fig. 9; $n=3$). The results of this experiment are shown in Figure 3.

Phenotypic effects by XAV or AZP after early exposure start—Embryos were exposed to DMSO (200 ppm) XAV or AZP at three concentrations (1, 3, or 10 µM), with exposure starting at 3 hpf. Phenotypic effects were examined and photographed at 14, 34 and 58 hpf (Fig. 4).

Transcriptional effects by XAV or AZP after early exposure start—Embryos were exposed to DMSO (200 ppm), XAV (3 µM), or AZP (3 µM) in triplicate dishes. Exposure started at 3 hpf and at 11, 14 and 36 hpf one sample of 6–9 pooled embryos was taken from each dish ($n=3$). Thus all nine dishes were sampled at three time points. The samples were flash frozen in liquid N₂ and stored at –80 °C until used for qPCR (Fig. 5).

Phenotypic effects by AZP or XAV after late exposure start—Embryos were exposed to DMSO (200 ppm) or PCB126 (10 nM), or to XAV or AZP at three different concentrations (1, 3, or 10 µM) with exposure starting at 1 dpf. The PCB126-exposed embryos were included to demonstrate AHR-mediated toxic effects for comparison with effects of β-catenin effectors. Phenotypic effects were examined and photographed at 3 dpf (Fig. 6).

Mortality rates and phenotypic effects by AZP or XAV combined with PCB126 or FICZ after early exposure start—Embryos were exposed to DMSO (400 ppm), PCB126 (20 nM), FICZ (10 nM), AZP (3 µM), XAV (3 µM), or to combinations of AZP (3 µM) or XAV (3 µM) with PCB126 (20 nM) or FICZ (10 nM). Exposure started at 3 hpf and mortality rates were determined at 1, 2, and 5 dpf (Fig. 7A). Phenotypic effects were photographed at 2 dpf (Fig. 7B).

Mortality rates and phenotypic effects by AZP or XAV combined with PCB126 or FICZ after late exposure start—In a series of experiments zebrafish embryo groups

were co-exposed to the AHR agonists and β -catenin effectors with late exposure start (1 dpf; Fig. 8). Embryos were exposed to DMSO (400 ppm), PCB126 (5–40 nM), or AZP (3 μ M) alone or in combination with PCB126. In another experiment embryos were exposed to DMSO (300 ppm), FICZ (10 nM), or AZP (1, 2, or 3 μ M) alone or in combinations with FICZ. Lastly, embryos were exposed to DMSO (400 ppm) PCB126 (5 or 20 nM), or XAV (3 μ M) alone or in combinations with PCB126, while other embryos were exposed to DMSO (400 ppm), FICZ (5 or 10 nM), or XAV (3 μ M) alone or in combinations with FICZ.

The developing swim-bladder is a sensitive target for toxicity of PCB126 and other chemicals. In zebrafish the swim-bladder normally inflates at 4–5 dpf. Incidences of embryos lacking inflated swim-bladder, embryos with edema, and mortality rates were determined at 5 dpf. Embryos were photographed at 5 dpf.

Transcriptional responses to combinations of AZP or XAV with PCB126 or FICZ after late exposure start—Embryos were exposed to DMSO (400 ppm), AZP (3 μ M), XAV (3 μ M), PCB126 (20 nM), or FICZ (10 nM), or to combinations of AZP (3 μ M) or XAV (3 μ M) with PCB126 (20 nM) or FICZ (10 nM). Each exposure was performed in triplicate dishes. Exposure started at 1 dpf and samples were taken after 8, 24, and 48 hours of exposure. At each time point one sample of 8–10 pooled embryos was taken from each dish ($n=3$). Samples for analysis of mRNA expression by qPCR were flash frozen in liquid N₂ and stored at –80 °C (Fig. 9).

Effect of Ahr2 knockdown on mortality rates and transcriptional responses of embryos exposed to combinations of AZP or XAV with FICZ with late exposure start—The procedure for morpholino injections is described above in “Ahr2 knockdown by morpholinos”. Effects by AZP or XAV alone or in combinations with FICZ were determined in groups of non-injected embryos and Ctrl-MO- and Ahr2-MO-injected embryos. Exposure started at 1 dpf.

Mortality—Embryos were exposed to DMSO (300 ppm), FICZ (10 nM), or AZP (3 μ M) alone or in combination with FICZ. Other groups were exposed to DMSO (300 ppm), FICZ (10 nM), or XAV (0.3, 1, or 3 μ M) alone or in combination with FICZ. Mortality rates over the exposure period were determined at 5 dpf (Fig. 10A–B).

Transcriptional responses—Embryos were exposed to DMSO (400 ppm) or FICZ (10 nM), or to AZP (3 μ M) or XAV (3 μ M) alone or in combinations with FICZ. After 24 hours of exposure four samples, each composed of 7–8 pooled embryos, were taken from each exposure group (one dish per exposure). The samples were placed in RNeasy lysis buffer (Qiagen Inc., Austin, TX, USA) and stored at 4 °C until used for qPCR analysis. The experiment was repeated twice and pooled data from both experiments were used for the statistical analyses ($n=7$ –8; Fig. 11).

Mortality and morphological assessment

Embryos were photographed using a Leica MZFLIII dissecting microscope with Leica FireCam software (version 3.1; Leica, Solms, Germany). Generally, mortality rates and morphological effects (edema and failure to inflate the swim-bladder) were assessed at 5

dpf. However, in the early combination exposure experiment mortality rates were assessed at 1, 2, and 5 dpf (Fig. 7A). Mortality rates and incidences of edema and swim-bladder inflation are expressed as the percentage of dead embryos or embryos showing edema and/or non-inflated swim-bladders versus the total number at exposure start. Embryos were classed as having edema if the heart sac and/or the yolk sac were visibly more swollen compared with the controls, or if edema was observed in other body parts. A swim-bladder was considered inflated if an air bubble could be distinguished (even when very small). Mortality and morphological changes were assessed in at least two repeated experiments.

Quantitative real time RT-PCR

RNA extraction, cDNA synthesis, and qPCR analysis were performed as described by Gao *et al.* (2011). Total RNA was isolated and DNase-treated using the AurumTM Total RNA Fatty and Fibrous Tissue kit (Bio-Rad Laboratories Inc., Hercules CA, USA) according to manufacturer's instructions. The concentration of RNA was determined spectrophotometrically using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). Total RNA, 0.5 µg per reaction for the time series (Figs 3 and S1) and 1 µg per reaction in all other experiments, was reverse transcribed using the iScript cDNA Synthesis kit according to instructions (Bio-Rad). Gene-specific qPCR primers were synthesized by Sigma-Aldrich. Primer sequences and GenBank accession number for the cDNAs are given in Table S1. The cDNA was diluted 1:10 in molecular biology water. Reaction solutions (20 µL) were composed by mixing cDNA (corresponding to 12.5 or 25 ng total RNA), forward and reverse primers (5 pmol of each), water, and iQ SYBR Green Supermix (Bio-Rad). Real time PCR was performed with a Rotor Gene 6000 Real-Time PCR Machine (Qiagen, Hilden, Germany). Samples were analyzed in duplicate with the following protocol: 95 °C for 3 min, followed by 35–40 cycles of 95 °C for 15 s and 62 °C for 45 s. To confirm that a single product was amplified, melt curve analysis was performed on the PCR products at the end of each PCR run.

Calculations and statistics

Relative mRNA expression of the target genes was calculated according to Pfaffl (2001). PCR efficiencies (E) for within experiment amplicon groups were determined by the LinRegPCR program (Ruijter *et al.*, 2009) (Table 1). As a reference gene *arn2* was selected since it showed negligible response to the exposures (Jönsson *et al.*, 2007). For all target genes $E^{-CT_{\text{target}}}/E^{-CT_{\text{ref}}}$ was calculated for each sample. Subsequently, this was divided by the mean $E^{-CT_{\text{target}}}/E^{-CT_{\text{ref}}}$ value of the controls. The mRNA levels in the time course study (Fig. 3) were calculated by E^{-CT} (Schmittgen and Livak, 2008), i.e., without normalization to a reference gene. Statistical analysis was performed using Prism 5 by GraphPad Software Inc. (San Diego, CA, USA) with log-transformed data when variance differed. The statistical methods used were Student's *t* test, one-way ANOVA followed by Tukey's or Dunnett's post hoc tests, or Fisher's exact test. With Fisher's exact test the Holm-Bonferroni method was used at multiple comparisons to control family wise error rate (Holm, 1979). Statistical tests used are given in the figure captions.

RESULTS

Temporal trends in basal levels of transcripts associated with Ahr and β -catenin

In order to examine variations in constitutive Ahr and β -catenin signaling during early zebrafish embryo development we determined transcript levels of genes in these pathways over the first three days post-fertilization. Levels of two β -catenin mRNAs (*ctnnb1* and *ctnnb2*), two *ahr* mRNAs (*ahr1b* and *ahr2*), and of mRNAs regulated by β -catenin (*axin2*) and Ahr2 (*CYP1A*) were determined (Fig. 3). Expression of *axin2* is directly induced by active β -catenin and is part of a negative feedback loop controlling the pathway (Leung et al., 2002; Lustig et al., 2002). It is therefore a commonly used marker for active β -catenin signaling. Similarly *CYP1A* expression is a traditional indicator of Ahr2 activities.

As shown in figure 3, all transcripts were detected in the samples taken before 3 hpf, indicating the presence of maternal transcript. The *ctnnb1* and *ctnnb2* transcripts appeared to be present at higher levels than the other transcripts over the entire study period. The expression of *ahr2* and *ctnnb1* showed different time trends; the level of *ahr2* expression dropped at 7 hpf and then stayed low until at some point between 15 and 26 hpf at which it started to increase to peak around 50 hpf, while *ctnnb1* showed a fairly stable expression but with a dramatic peak around 11–13 hpf (late gastrulation – early segmentation). The second *ahr* transcript, *ahr1b*, showed a low expression level before 15 hpf, but after 15 hpf it increased continuously during the remaining experimental period. The expression of *ctnnb2* showed small peaks at 5 hpf and at 35 hpf but other than this the *ctnnb2* level displayed no major changes within the study period. Expression of *axin2* roughly followed the time trend of *ctnnb1* expression but also showed an increase around 26–35 hpf. Similarly, *CYP1A* expression roughly followed the course of *ahr2* expression except for a small peak at 13 hpf. The *CYP1A* level increased markedly between 26 hpf and 36 hpf and stayed at similar expression level during the remaining experimental period.

These results indicate that the activities of the β -catenin and Ahr2 pathways display different time courses during early development.

Effects of β -catenin effectors on zebrafish embryos at “early” and “late” exposure start

We then asked how these temporal variations contribute to the toxic responses to compounds that target the β -catenin and Ahr2 pathways. Consequently, we examined whether there are time dependent differences in the phenotypic and transcriptional effects by such compounds. To manipulate Wnt/ β -catenin signaling we used two common β -catenin effectors, an activator, AZP and an inhibitor, XAV. The Ahr2-mediated toxic effects in zebrafish embryos are well-recognized, and thus PCB126 was included in some of these studies only for comparison. Exposures were started at an early (3 hpf) or a later (1 dpf) developmental stage and effects were recorded several times during the exposure period. The early exposure start made it possible to study effects around the peak in basal *ctnnb1* expression (12 hpf), while the late exposure start enabled study of effects during the peak in *ahr2* expression (about 2 dpf) without influencing early processes. Effects by AHR agonists are known to occur relatively late in embryo development and can be triggered by exposure as late as at 3 dpf (Carney *et al.*, 2006a).

Mortality rates and phenotypic effects by AZP or XAV after early exposure

start—In the first experiment embryos were exposed to DMSO (200 ppm), AZP or XAV (1, 3, or 10 μ M) from 3 hpf. Mortality rates were examined at 14 hpf (not shown) while phenotypes were examined at 14, 34, and 58 hpf (Fig. 4). Embryos exposed to AZP exhibited increasing mortality rates with higher concentrations (60% with 10 μ M), while embryos exposed to XAV (at any concentration) showed little mortality (6% with 10 μ M XAV) compared with the DMSO control (3%).

Embryos exposed to DMSO developed a normal phenotype (Fig. 4A). In contrast, AZP-exposed embryos showed an oval shape at 14 hpf (3 μ M AZP) and malformations of varying degree at later stages (34 and 58 hpf); e.g., some embryos exposed to the lowest AZP concentration (1 μ M) lacked eyes but had a tail, while others showed underdevelopment of both head and tail (Fig. 4B). These effects are characteristic of overactive Wnt/ β -catenin signaling during early embryogenesis (Meyers *et al.*, 2012). Higher AZP concentrations (3 or 10 μ M) produced similar phenotypic effects (or mortality), with the severity and frequency of the effects increasing with higher concentrations (data not shown).

No obvious difference in phenotype versus the DMSO control was observed in XAV-exposed groups at 14 hpf at any concentration, while minor pericardial edema was observed at 34 hpf in embryos exposed to 3 μ M XAV (the only concentration examined at this stage; Fig. 4C). At 58 hpf pericardial edema in XAV-exposed groups had become more severe and the embryos also exhibited compromised heart development and notably, accumulation of blood below the heart (Fig. 4C), and with 10 μ M XAV edema was observed at multiple locations in addition to the other effects (not shown).

Transcriptional responses to AZP and XAV after early exposure start—We also determined effects of AZP or XAV (both 3 μ M) on the transcription levels of *ahr2*, *ctnnb1*, *CYP1A*, and *axin2*. Exposures were started at 3 hpf and changes in mRNA levels relative the DMSO control were determined at 11, 14, or 36 hpf (Fig. 5). Embryos exposed to XAV showed a slight induction of *ahr2* (1.8-fold) and a strong induction of *CYP1A* at 36 hpf (430-fold), while a suppression of *axin2* (0.7-fold) was observed in the same embryo groups. Exposure to AZP resulted in a slight induction of *ahr2* at 14 hpf (1.6-fold), a strong induction of *CYP1A* at 14 and 36 hpf (93- and 220-fold), and slight induction of *axin2* at 14 and 36 hpf (1.5 and 2.2-fold). Expression of *ctnnb1* was resistant to XAV, but showed a small transient increase in the AZP-exposed group at 14 hpf (1.4-fold). No effects were observed on the transcript levels of *ctnnb2* (data not shown).

Phenotypic changes after late exposure start—In these experiments, embryos were exposed to AZP, XAV, or PCB126 from 1 to 5 dpf. Within this period both AZP and XAV gave rise to concentration-dependent increases in pericardial and yolk sac edemas with the effects beginning to show after 48 h of exposure (3 dpf; Fig. 6), and with the severity of effects increasing over time (not shown). The effects of AZP were mild to begin with; at 3 dpf pronounced edemas and tube-shaped hearts were observed only at the highest concentration (10 μ M; Fig. 6C), but at 4–5 dpf edemas were observed also in groups exposed to 1 or 3 μ M AZP (not shown). In the AZP-exposed groups, lower jaw malformations similar to those induced by PCB126 occurred only in individuals with severe

edema (Fig. 6C and Fig. 8). Commonly, AZP-exposed embryos displayed a curved spine, with the tail bent backwards or to the side.

The phenotype developed by XAV-exposure was strikingly similar to that elicited by PCB126 (Fig. 6B and 6D). Typically XAV-exposed embryos showed pericardial and yolk sac edema, circulatory failure, tube-shaped heart, disrupted swim-bladder inflation, shortened lower jaw, and a reduced body growth, all which are hallmark responses of Ahr2-mediated developmental toxicity in zebrafish (Carney *et al.*, 2006a). However, XAV was considerably less potent than PCB126, and unique for the XAV-induced phenotype was an accumulation of blood below the heart (Fig. 6D). At 5 dpf all DMSO controls showed normal phenotype (with inflated swim-bladders; not shown), while embryos exposed to 1 or 3 μ M XAV or AZP failed to inflate the swim-bladder, and groups exposed to 10 μ M XAV or AZP showed 100% mortality.

In summary, AZP was lethal or induced severe malformations at early stages, while no obvious phenotypic effects by XAV occurred before 1 dpf. At late exposure start both XAV and AZP produced cardiovascular and craniofacial malformations and edema, beginning to show at 2–3 dpf. However, the compounds also had unique effects; typically XAV-exposed embryos showed blood accumulation below the heart while AZP-exposed embryos exhibited bent spine.

At the transcriptional level AZP induced *ctnnb1* and *axin2*, while XAV suppressed *axin2* expression. Both compounds induced *ahr2* and *CYP1A* although with different temporal appearance and magnitude.

Temporal effects of co-exposures to AZP or XAV with PCB126 or FICZ

The observed similarities in toxic phenotype elicited by XAV (β -catenin inhibitor) and PCB126 (Fig. 6B and 6D), raised the question whether AHR-mediated toxicity involves repression of the β -catenin pathway. To address this, we investigated the effects of co-exposures to AZP or XAV with AHR agonists. We used the non-toxic rapidly metabolized AHR agonist FICZ in addition to the slowly metabolized PCB126. Considering the observed time-dependence of the toxic effects with XAV and AZP alone, exposures were again started at early or late time points (3 hpf and 1 dpf).

Mortality rates after co-exposures with early exposure start—Mortality rates were recorded at 1, 2, and 5 dpf. Groups exposed to DMSO (300 ppm) or XAV (3 μ M) showed low mortality rates at 1 dpf (8 and 4 %, respectively) and no or minor further mortality was observed within the study period (Fig. 7A). As previously noted AZP (3 μ M) was severely toxic to early-stage embryos, causing 64% mortality within the first day of exposure and at 5 dpf all embryos in the AZP group had died (Fig. 7A).

Among embryos exposed to PCB126 (20 nM) the mortality rate was low at 1 dpf (8%) but increased towards the end of the experimental period, reaching 44% at 5 dpf (Fig. 7A). The mortality rate in the AZP+PCB group was 79% at 1 dpf and 100% at 5 dpf, while embryos exposed to XAV+PCB showed comparatively low mortality (12% at 5 dpf; Fig. 7A). Thus,

relative to PCB126 alone the mortality rate increased by addition of AZP and decreased by addition of XAV and these changes were statistically significant.

In groups exposed to FICZ (10 nM) or XAV+FICZ the mortality rates were at the control level at 1 dpf, and with FICZ alone no further mortality was observed, but with XAV+FICZ the mortality rate increased considerably towards the end of the exposure period (77% at 5 dpf; Fig. 7A). The combination of FICZ with AZP caused 50% mortality within 1 day and 100% within 5 days of development (Fig. 7A). Consequently, with AZP+FICZ we observed a temporal pattern of mortality roughly similar to those with AZP alone and AZP+PCB.

Phenotypic changes with co-exposures after early exposure start—Phenotypic effects were recorded at 2 dpf (Fig. 7B). Surviving embryos in the DMSO group developed normally, and as previously described (Fig. 4B–C), AZP induced head and tail underdevelopment including lack of eyes, while XAV induced pericardial edema, malformed heart, and blood accumulation below the heart (Fig. 7B, upper row).

The only effect by PCB126 (20 nM) observed at 2 dpf was mild pericardial edema (Fig. 7B, middle row) and FICZ exposed embryos appeared normal (Fig. 7B, lower row). The phenotypes induced by AZP in combinations with PCB126 or FICZ were similar to that of AZP alone, and phenotypes induced by the XAV combinations were similar to that of XAV. Over time, embryos exposed to XAV, PCB126, or the XAV+PCB combination developed more pronounced cardiovascular effects, reduced growth, and large pericardial and yolk sac edemas (not shown). At 5 dpf embryos in the XAV and XAV+PCB groups exhibited generalized edema at multiple locations around the body, including, heart, yolk, eyes, muscle, etc. (not shown). Embryos exposed to AZP or its combinations did often not hatch.

Mortality rates and phenotypic changes with co-exposures after late exposure start—In these experiments groups of embryos were co-exposed to PCB126 or FICZ with AZP (Fig. 8A–B), PCB126 or FICZ with XAV (Fig. 8C), or to the same compounds alone. Exposure started at 1 dpf. Generally, the DMSO control and FICZ exposure groups showed no mortality and almost all embryos developed normally.

PCB126 with AZP: Embryos were exposed to PCB126 at four concentrations (5–40 nM; Fig. 8A, left graph), or to AZP (3 μ M) alone or in combinations with 5–40 nM PCB126 (Fig. 8A, right graph). While there was no mortality in groups exposed to PCB126 at the two lower concentrations, the two higher concentrations resulted in 50 and 80% mortality. Exposure to AZP alone resulted in 90% mortality, while at co-exposure with PCB126 the mortality rate dropped to 60 and 35% at the lower PCB126 concentrations (5 and 10 nM), and a further reduction down to 11% was observed at higher concentrations (20 and 40 nM PCB126). All exposures to AZP or PCB126 alone or in combinations resulted in blocked swim-bladder inflation and most individuals showed pronounced pericardial and yolk sac edemas.

FICZ with AZP: At co-exposure of AZP (1, 2 and 3 μ M) with FICZ (10 nM) the toxicity was lower at all AZP concentrations than with AZP alone (Fig. 8B). While most of the embryos exposed to AZP alone at the lowest concentration exhibited edema and lack of

swim-bladder inflation, embryos exposed to the combination of AZP and FICZ looked perfectly healthy with fully inflated swim-bladders. Exposure to 2 or 3 μ M AZP resulted in 46% and 96% mortality while no mortality was observed in the groups co-exposed to 2 or 3 μ M AZP and FICZ. However, embryos in these groups showed a change in the shape of the lower jaw in mildly affected individuals while severely affected individuals showed a shortening of the lower jaw (Fig. 8B).

PCB126 or FICZ with XAV: Embryo groups were also exposed to PCB126 (5 and 20 nM; Fig. 8C left graph) or FICZ (5 and 10 nM; Fig. 8C right graph) in combination with XAV (3 μ M). No mortality was observed at the lower concentration of PCB126, whether alone or in combination with XAV. However, at the higher concentration (20 nM) PCB126 alone resulted in 80% mortality, while embryos co-exposed to PCB126 and XAV showed only 5% mortality. Groups exposed to XAV alone showed no mortality, although all these embryos showed blocked swim-bladder inflation and pronounced pericardial and yolk sac edemas. FICZ alone was not toxic. However, the combinations of 5 and 10 nM FICZ with XAV caused 30 and 60% mortality.

In summary, the results indicate interactions between the β -catenin effectors and AHR agonists in developing zebrafish which differed depending on timing of exposure start and type of AHR agonist (FICZ or PCB126; Table 1). There was no difference in toxicity between AZP alone and its two combinations when exposure began at 3 hpf, but when exposure began at 1 dpf the toxicity was lower with the combinations than with AZP alone. A completely normal phenotype was observed at exposure to 1 μ M AZP with 10 nM FICZ. However, the mortality rate with XAV+FICZ was higher than with XAV alone at both exposure start times. Regarding PCB126 the mortality rate was lower with XAV+PCB than with PCB126 alone regardless of exposure start time, but the surviving embryos showed severe edemas.

Transcriptional responses to combinations of AZP or XAV with PCB126 or FICZ after late exposure start

We also aimed to examine possible transcriptional changes associated with the changes in toxicity with the compounds in dual combinations versus alone that were observed when exposure started at 1 dpf. Thus again embryos were exposed to DMSO (400 ppm), AZP (3 μ M), XAV (3 μ M), PCB126 (20 nM) or FICZ (10 nM) or to combinations of AZP or XAV with PCB126 or FICZ. Samples were taken after 8, 24, and 48 h. For transcripts shown in Fig. 9A and Fig. 9C changes in expression levels in the exposed groups versus the DMSO group were determined at each time point. For transcripts shown in Fig. 9B changes in expression levels were determined among all groups at each time point.

Firstly we determined effects of the exposures on the transcriptional regulators of the two pathways, Ahr2 and β -catenin, by analyzing mRNA levels of their genes (*ahr2* and *ctnnb1*; Fig. 9A). The expression of *ahr2* was induced by all exposures except DMSO and FICZ, with induction levels of 1.5–2-fold versus the control. Groups exposed to combinations showed *ahr2* induction after both 24 and 48 h, while PCB126, XAV, and AZP alone

induced *ahr2* after either 24 or 48 h of exposure. The *ctnnb1* transcript showed no statistically significant change by any of the exposures.

Subsequently the response of genes known to be regulated by the two pathways was examined. Among Ahr2-regulated genes we chose *CYP1A* and *ahr2*, both of which are directly regulated by Ahr2 via Ahr response elements in their respective promoters. Expression of *CYP1A* was strongly induced in all exposure groups at all exposure times (Fig. 9B). Highest levels of *CYP1A* induction were observed with PCB126 and XAV+FICZ at 24 h of exposure (660- and 770-fold the control). As shown in figure 9B, both AZP and XAV induced *CYP1A*, but while the relative level of induction by AZP tended to decrease over time, induction by XAV increased over time (95-, 89-, and 38-fold versus 53-, 150-, and 170-fold the controls at 8, 24, and 48 h of exposure). FICZ combined with AZP or XAV induced considerably stronger *CYP1A* responses than FICZ and AZP alone at all exposure times, while PCB126 combined with AZP or XAV induced a similarly strong (AZP) or weaker (XAV) response than PCB126 by itself. The *ahr2* transcript showed an induction pattern very similar to that of *CYP1A* although the magnitude of induction was lower (Fig. S1A).

As an indicator of β -catenin transcriptional activity we used *axin2*. Expression of *axin2* was induced by AZP (2.6- and 1.7-fold the controls at 24 and 48 h), while it was on the control level in groups exposed to combinations of AZP with PCB126 or FICZ (Fig. 9B). Exposure to XAV suppressed *axin2* expression to about 0.6-fold the control (statistically significant at 48 h). Co-treatment with FICZ or PCB126 did not change the effect of XAV on *axin2* expression.

We also determined effects of the exposures on the expression of other genes related to Wnt signaling, i.e., *rspo1-4*, *pcna*, and *runx2b*. The four R-spondins enhance the effect of Wnts by stabilizing Wnt receptors, thus acting upstream of β -catenin (Fig. 1B). How their transcripts are regulated appears not to be known at present. Proliferating cell nuclear antigen (Pcna) plays a central role in cell proliferation and is regulated via E2F transcription factors; β -catenin interacts with E2F in networks involving p53 (Harris and Levine, 2005). Runt-related transcription factor 2b (Runx2b) is essential for cartilage and bone development and is directly regulated by β -catenin (Gaur *et al.*, 2005). Similar to *axin2*, the *rspo2*, *pcna*, and *runx2b* transcripts were induced by AZP (1.5–2.2-fold the control at 48 h) but not by the combinations of AZP with FICZ or PCB126 (Fig. 9C). Expression of *rspo3* was induced by XAV and XAV+PCB at 48 h (1.7–1.8 fold the control after 48 h), while the *rspo1* and *rspo 4* levels showed tendencies for changes by the exposures, but these changes were not statistically significant (Fig. S1B).

In summary, combinations of AHR agonists with XAV or AZP were able to modify transcription of genes in both pathways. The Ahr2-regulated genes (*CYP1A* and *ahr2*) were induced by AZP and XAV alone and in combination with AHR agonists as well as by AHR agonists alone. The AZP-induced expression of genes associated with β -catenin (*axin2*, *rspo2*, *pcna*, and *runx2b*) was blocked when AZP was administered together with FICZ or PCB126.

Effect of Ahr2 knockdown on the effects by the combinations of AZP or XAV with FICZ

Finally we investigated the role of Ahr2 in mediating the effects by FICZ on AZP- and XAV-mediated toxicity by knockdown of Ahr2 with *ahr2*-specific morpholinos (Ahr2-MO). In these experiments we used FICZ as the only AHR agonist since FICZ alone appeared to be non-toxic in zebrafish embryos, while PCB126 elicited classical AHR-mediated toxic effects. As previously shown, Ahr2-MO treatment rescues toxic effects by PCB126 in zebrafish embryos (Jönsson *et al.*, 2007), and thus effects of Ahr2 knockdown on the toxicities of AZP or XAV in combinations with PCB126 could be difficult to discriminate from its effect on the toxicity by PCB126.

A series of experiments were performed with embryos injected with the Ahr2-MO, or with a control morpholino having no reported effect (Ctrl-MO) (Prasch *et al.*, 2003; Jönsson *et al.*, 2007). Groups of non-injected, Ctrl-MO-injected or Ahr2-MO-injected embryos were exposed to AZP or XAV alone or in combination with FICZ, or to FICZ alone. Exposure started at 1 dpf. As expected, no significant differences in mortality or transcriptional effects were observed between groups of non-injected and Ctrl-MO injected embryos. Results for non-injected groups are therefore not shown.

Mortality rates and phenotypic changes—Mortality and incidence of edema (pericardial and/or yolk sac edema; not shown) were determined at 5 dpf (Fig. 10). Low mortality rates and no edema were observed in Ctrl-MO and Ahr2-MO injected embryos exposed to DMSO or FICZ (10 nM), while exposure to AZP (3 μ M) resulted in 45–55% mortality (Fig. 10A) and all surviving embryos exhibited edema. Treatment with Ahr2-MO had no significant effect on the response to AZP. However, while FICZ abolished the AZP-induced mortality in Ctrl-MO-embryos, treatment with Ahr2-MO blocked this effect by FICZ, resulting in 44% mortality (Fig. 10A) and severe edema in all surviving embryos. XAV had minor effects regardless of morpholino treatment at the lowest concentration (0.3 μ M), while treatment with Ahr2-MO significantly reduced the mortality in embryos exposed to the two higher XAV concentrations (1 and 3 μ M) from 56 to 24% and from 92 to 56%, respectively (Fig. 10B). Tendencies for decreased mortality after Ahr-MO treatment were observed also in the groups exposed to 1 or 3 μ M XAV in combination with FICZ, although this was not statistically significant (from 76% to 62% with 1 μ M XAV+FICZ and from 100% to 92% with 3 μ M XAV+FICZ; Fig. 10B).

Transcriptional responses of CYP1A and axin2—To examine involvement of Ahr2 in the effects on *CYP1A* and *axin2* mRNA expression we compared effects on the levels of these two transcripts in embryos treated with Ctrl-MO or Ahr2-MO. At 1 dpf the embryos were exposed to DMSO, AZP (3 μ M), XAV (1 μ M), or FICZ (10 nM) or to the combinations of AZP or XAV with FICZ. Relative mRNA expression was determined in samples collected after 24 h of exposure and the results are shown in Figure 11.

Compared to the levels in the corresponding Ctrl-MO-treated groups, treatment with Ahr2-MO reduced *CYP1A* induction in the XAV, FICZ, and XAV+FICZ groups to 58, 57, and 48% and increased *axin2* expression in the AZP, FICZ, or AZP+FICZ groups to 170, 130,

and 160%. Treatment with Ahr2-MO did not have any effect on induction of *CYP1A* expression by AZP or AZP+FICZ.

In summary, treatment with the Ahr2-MO blocked the protective effect of FICZ against AZP toxicity. Furthermore, Ahr2-MO treatment reduced the mortality rate by XAV itself and tended to counteract the increased mortality by the XAV-FICZ combination versus XAV alone (Table 1). At the transcriptional level Ahr2 knockdown led to reduced *CYP1A* expression in embryos exposed to FICZ, XAV, and their combination while it resulted in increased *axin2* expression in embryos exposed to FICZ, AZP and their combination.

The impact on mortality rates of the different treatments is summarized in Table 1.

DISCUSSION

Ahr2 antagonizes β -catenin signaling in developing zebrafish

Evidence is building that AHR and Wnt signaling are connected, but despite progress, the complexities and mechanisms in the interaction are still clouded. The results of this study demonstrate that XAV (which inhibits β -catenin activity by stabilizing axin) and AZP (which enhances β -catenin activity by inhibiting GSK-3 β) are toxic to zebrafish embryos. In this context the transient AHR agonist FICZ repressed the toxicity caused by AZP and increased the toxicity caused by XAV. Moreover, induction of β -catenin-regulated genes by AZP was blocked in presence of FICZ or PCB126 (*axin2*, *runx2b*, *rspo2*, and *pcna*) and enhanced by knockdown of Ahr2 (*axin2*). These results indicate an interaction in which the Ahr2 represses active β -catenin signaling.

Additionally, both XAV and AZP strongly induced *CYP1A* mRNA expression although with different temporal appearance and magnitude. However, while Ahr2 knockdown led to reduced *CYP1A* induction and toxicity by XAV it had no impact on the same effects by AZP. This could mean that XAV is a partial Ahr2 agonist, while AZP activates Ahr signaling by another mechanism, possibly involving β -catenin. Overall, our results point to a direct interaction between the Ahr2 and β -catenin pathways in the normally developing embryo.

The AHR plays roles in cell cycle regulation and is considered to have a developmental function (Nebert *et al.*, 2000; Elferink, 2003; Smith *et al.*, 2013). We hypothesize that the AHR plays a physiological role in antagonizing β -catenin activities in certain developmental processes. It is intriguing to speculate that this could involve a physiological AHR ligand, rapidly degraded via CYP1 enzymes, which would provide a means to control amplitude and duration of AHR activation in a negative feedback loop, and thus modulating the antagonizing effect on β -catenin signaling. Exposure to persistent AHR agonists that cause sustained AHR activation and β -catenin inactivation, would instead lead to disorganized cell proliferation and differentiation, and ultimately malformations and other toxic manifestations.

There appear to be multiple avenues for crosstalk between AHR and the β -catenin pathway. Thus, one interaction between AHR and Wnt signaling has been shown to involve AHR

dependent effects on R-spondin levels (Mathew *et al.*, 2008; Branam *et al.*, 2013; Xu *et al.*, 2013). Branam *et al.* (2013) found that TCDD, like Wnt/ β -catenin signaling inhibitors (including XAV), reduced the number of ventral prostatic buds in mouse embryos. This effect of TCDD was associated with a down-regulation of Wnt/ β -catenin signaling and reduction in *Rspo2* and *Rspo3* mRNA levels, and addition of R-spondin 2 and 3 proteins partially rescued these effects of TCDD (Branam *et al.*, 2013). In a recent study the same group showed that TCDD prevents the onset of β -catenin signaling in the ventral basal epithelium (Schneider *et al.*, 2014b). In zebrafish TCDD blocks tissue regeneration through induction of R-spondin1 and this effect also depend on LRP6, the Wnt co-receptor associated with R-spondin1 (Mathew *et al.*, 2008).

One mechanism by which the AHR could suppress β -catenin signaling more directly is in its role as a substrate recognition subunit of ubiquitin E3 ligase cullin-4B, where the ligand-activated AHR, localized in the nucleus, stimulates ubiquitination of β -catenin and thus increases β -catenin degradation (Fig. 1D) (Wormke *et al.*, 2003; Ohtake *et al.*, 2007; Kawajiri *et al.*, 2009). This was shown to occur with anthropogenic as well as with natural AHR ligands (Kawajiri *et al.*, 2009). Whether this mechanism, which is downstream of Wnt and R-spondin effects, is involved in our results is not known.

The effects of AZP and AZP-AHR agonist interaction are stage dependent

The toxicity of the β -catenin pathway activator AZP was more severe when exposure began earlier (3 hpf) than later (1 dpf), a stage-dependence like that reported also by Meyers *et al.* (2012). In many animals body plan development is guided by a pattern of posterior activation and anterior inhibition of β -catenin (Petersen and Reddien, 2009). In zebrafish β -catenin hyperactivation results in malformations like those we observed with AZP when exposure began early (3 hpf), with effects in the head region ranging from reduced eye size to a complete lack of eyes, and underdevelopment of both embryo poles (Figs. 4B and 7B). Exposure to AZP starting later (1 dpf) resulted in reduced eye growth, bent spine, edema, etc. (Figs. 6C and 8B). Thus, the results imply a stage-dependence in the consequences of β -catenin hyperactivation.

The finding that FICZ was able to completely rescue AZP toxicity and that this effect was Ahr2-dependent suggests a crosstalk whereby the activated Ahr2 somehow down-regulates AZP-induced hyperactivity of β -catenin to normal levels. However, FICZ reduced AZP toxicity only when exposure started at the later time (1 dpf), and had no effect on AZP toxicity at early stages. This suggests that Ahr2 can interact with β -catenin in this way only at later embryo stages in zebrafish. The temporal trend for basal *ahr2* and *CYP1A* mRNA expression showed peaks around hatching (2 dpf), consistent with reports that CYP1A protein level/activity is elevated after hatching (Binder and Stegeman, 1984; Mattingly and Toscano, 2001). Phenotypic effects associated with toxicity of TCDD also begin to appear during this time indicating that processes sensitive to Ahr2 activation occur around hatching (Carney *et al.*, 2006a).

The stage dependence of the effects by AZP and the influence of AHR agonists on these effects could be explained by contrasting temporal variation in Ahr2 and β -catenin activities which is supported by the time course for expression of the genes encoding Ahr2 and β -

catenin and their target genes (*CYP1A* and *axin2*). We found that expression of *ahr2/CYP1A* peaked later than *ctnnb1/axin2* (Fig. 3). If Ahr2 suppresses β -catenin activities, then these results could mean that the Ahr2 to β -catenin ratio is high enough for Ahr2 to suppress β -catenin signaling during the pharyngula and hatching periods (and possibly later stages) but not at earlier embryonic stages.

AZP toxicity was rescued less completely by PCB126 than it was by FICZ

The reason for the lower efficiency of PCB126 than of FICZ in rescuing the toxic effect of AZP could be that both PCB126 and AZP are toxic, and while they affect β -catenin in opposing directions, the extent of rescue could depend on the balance of AHR activation and GSK-3 β inhibition, which could also be influenced by cell specific conditions in target cells. The low toxicity of FICZ, despite its strong activation of Ahr2, could reflect a very rapid metabolism of FICZ (Wincent *et al.*, 2009). This would mean that the FICZ activation of Ahr2 and consequent effects of AZP on active β -catenin levels would be transient while the effects of PCB126 would be persistent. The decline in FICZ-induced *CYP1A* levels observed one to two days after exposure start is consistent with a transient activation of Ahr2 by FICZ.

XAV and PCB126 have similar but not identical effects on the developing heart

The heart is a common developmental target for halogenated AHR agonists, like PCB126, and the effects include a tubular un-looped heart, decreased stroke volume and peripheral blood flow, and halted heart growth (Carney *et al.*, 2006b). We found that XAV elicited heart malformations largely similar to those by PCB126 although the heart effects by XAV occurred earlier and appeared to be more severe than those induced by PCB126, especially at early exposure start (Figs 4, 6, and 7). During normal heart formation Wnt/ β -catenin signaling plays critical role in different phases of cardiomyocyte formation in zebrafish (Dohn and Waxman, 2012). XAV was shown to induce cardiomyocyte differentiation in mouse embryonic stem cell (mESC) cultures (Wang *et al.*, 2011). Like in our study in zebrafish embryos *axin2* expression was down-regulated by XAV in the mESCs, indicating inhibition of β -catenin signaling by XAV (Wang *et al.*, 2011).

Another effect that was observed with XAV (but not with PCB126 or AZP) in our study was accumulation of blood below the heart. A similar effect was reported in zebrafish embryos after knockdown of survivin 2, a β -catenin-regulated gene which promotes cell cycle progression (Delvaeye *et al.*, 2009; Herbst *et al.*, 2014). Mechanical blockage of blood influx to the heart also can result in accumulation of blood below the heart (Hove *et al.*, 2003). Thus, this effect in XAV-exposed embryos could reflect blockage of blood influx to the heart, perhaps due to heart malformations associated with inhibition of β -catenin signaling in the developing heart cells.

Recent findings indicate that cardiomyocyte development is a major target in embryo toxicity by TCDD in zebrafish (Lanham *et al.*, 2014). Lanham *et al.* (2014) found that constitutive Ahr2 activation in cardiomyocytes results in a phenotype similar to that induced by TCDD. It was suggested that Ahr2 activation in developing cardiomyocytes has systemic effects (e.g. circulatory failure) leading to the various toxicities seen with TCDD in

zebrafish embryos. The molecular target for activated Ahr2 in the cardiomyocytes could be β -catenin, and the mechanism could involve a crosstalk in which Ahr2 represses β -catenin signaling inappropriately.

Both AZP and XAV stimulate induction of Ahr2 regulated genes

One suggested mechanism of AHR- β -catenin crosstalk involves cooperation to enhance induction of *Cyp1* family genes. In mouse liver cells β -catenin appeared to enhance *Cyp1* transcription by direct binding to the ligand-Ahr-Arnt complex at DREs in the *Cyp1* promoters (Braeuning *et al.*, 2011; Prochazkova *et al.*, 2011). This mechanism could explain the stronger *CYP1A* induction by AZP combined with FICZ than with AZP alone in our studies (Fig. 8). It also could explain why *CYP1A* was induced by AZP alone, assuming the presence of an endogenous AHR ligand. This is suggested by the small peak in *CYP1A* basal expression at 13 hpf, which coincided with the peak in *ctnnb1* basal expression (Fig. 3).

Induction of *CYP1A* by XAV requires another explanation model. This could involve inhibition of *CYP1A* catalytic activity by XAV, which we have observed (data not shown). In the presence of a potent endogenous AHR ligand that is degraded by *CYP1A*, a *CYP1A* inhibitor could cause reduced metabolic clearance of the ligand and thereby induction of AHR regulated genes. We showed in cell cultures that co-exposure to the rapidly metabolized agonist FICZ with *CYP1* inhibitors results in potentiation of the *CYP1A* response, compared to the response to FICZ alone (Wincent *et al.*, 2009; Mohammadi-Bardbori *et al.*, 2012; Wincent *et al.*, 2012).

Further, any chemical inhibitor will have off-target effects and in the case of XAV and AZP the possibility that they interact directly with the AHR cannot be excluded. The decrease of XAV-mediated *CYP1A* induction and toxicity after knockdown of Ahr2 (Fig. 10 and Fig. 11) indicates that XAV could be a partial AHR agonist. This has been suggested for the GSK-3 inhibitor SB216763 (Braeuning and Buchmann, 2009). Also by the *CYP1A* induction pattern it is suggested that XAV (and possibly also AZP) could be a partial agonist for the AHR. Recent studies by Soshilov and Denison (2014) indicate that AHR binds and can be activated by a wide range of chemicals and they suggest a molecular explanation for agonist versus antagonist action, depending on amino acid residues in the ligand binding domain. Whether AZP and/or XAV bind the AHR ligand-binding domain, and in which mode, is yet to be determined.

The expression of *ahr2* increased (1.5–2-fold) in all exposure groups except for the FICZ alone group (Fig. 9A); thus both AZP and XAV induced the levels of *ahr2* transcript. How AHR expression is controlled is not fully understood but it seems to be regulated by a variety of factors at both transcriptional and protein levels (including β -catenin) (Hahn *et al.*, 2009; Abel and Haarmann-Stemann, 2010). Whether the increased *ahr2* mRNA expression results in an increase in Ahr2 protein with activation and subsequent induction of *CYP1A* is not known.

In summary, partial agonism of the AHR, direct interaction of β -catenin with the AHR, disrupted feedback regulation of AHR signaling by inhibition of *CYP1A*, and induced levels of Ahr2 protein all could be involved in the AZP and XAV mediated effects on *CYP1A*

expression we observed. Chemical modifiers of Wnt/ β -catenin signaling increasingly are being used in analysis of functions and interactions of these pathways. Awareness that modifiers such as XAV or AZP might affect other pathways that interact with Wnt/ β -catenin is important for inference from studies employing such chemicals. Assessing the transcriptomic responses to AZP and XAV not only would establish the scope of their action as Wnt signaling modifiers, but should reveal the extent of XAV action via the AHR.

Conclusion

We found that both XAV and AZP were toxic to zebrafish embryos, demonstrating the importance of a balance in β -catenin levels to overall development. In dual combinations with potent AHR agonists the toxicity of the β -catenin effectors changed. The transient AHR agonist FICZ increased the toxicity by the β -catenin inhibitor XAV and rescued the toxic effects of the β -catenin enhancer AZP. The rescuing effect was Ahr2-dependent, indicating that activated Ahr2 antagonizes β -catenin in zebrafish embryos. Furthermore, our results confirm that XAV is a β -catenin inhibitor, but also suggest that it is a partial AHR agonist. Together these findings indicate that β -catenin pathway is an important target in the effects of AHR agonists in early development. We propose that the AHR has a physiological function to regulate β -catenin signaling in development, and that AHR activation by persistent ligands may interfere with this function. The picture is far from complete, however, and the precise mechanism by which activated AHR might interact with β -catenin is unknown.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AHR	aryl hydrocarbon receptor
Ahr2	aryl hydrocarbon receptor 2
APC	adenomatosis polyposis coli
AZP	1-azakenpaullone
CKIα	casein kinase I α
CYP1	cytochrome P450 1
DLCs	dioxin-like compounds
DMSO	dimethyl sulfoxide
dpf	days post-fertilization

E	PCR efficiencies
FICZ	6-formylindolo[3,2- <i>b</i>]carbazole
GSK3β	glycogen synthase kinase 3 β
hpf	hours post-fertilization
3-MC	3-methylcholanthrene
MO	morpholino
PCB126	3,3',4,4',5-pentachlorobiphenyl
Pcna	proliferating cell nuclear antigen
qPCR	quantitative real-time RT-PCR
Runx2b	runt-related transcription factor 2b
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TCF/LEF1	T-cell factor/lymphoid enhancer-binding factor
XAV	XAV939
ZGA	zygotic genome activation

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Figure 1. Models for regulation of Wnt/β-catenin signaling

Wnt/β-catenin signaling is stimulated by Wnts activating specific transmembrane receptors (A), and is further enhanced by R-spondins that act by increasing the stability of the Wnt receptors (B) (Niehrs, 2012). Negative regulation is mediated via the “β-catenin destruction complex” which tags β-catenin for proteasomal degradation (C). The ligand-activated AHR associates with ARNT in the nucleus and functions as a substrate recognition subunit of ubiquitin E3 ligase cullin-4B, thus stimulating ubiquitination of β-catenin and its degradation independently of the destruction complex (D) (Kawajiri *et al.*, 2009). Degradation of β-catenin is reduced in presence of a glycogen synthase kinase 3β (GSK3) inhibitor, such as 1-azakenpaullone (AZP), leading to increased levels of active β-catenin (E), while it is increased in presence of XAV939 (XAV) which stabilizes Axin, a limiting component of the destruction complex (F) (Kunick *et al.*, 2004; Huang *et al.*, 2009).

Figure 2. Chemical structures and overview of the experiments

A) Structures of the Wnt regulators XAV939 (XAV) and 1-azakenpauellone (AZP), and the AHR agonists 3,3',4,4',5-pentachlorobiphenyl (PCB126) and 6-formylindolo[3,2-*b*]carbazole (FICZ). B) Schematic overview of the exposure regimens and examined endpoints used in this study.

Figure 3. Temporal trends in basal expression of genes in the β -catenin and Ahr pathways in zebrafish embryos

Levels of *cttnb1*, *cttnb2*, *ahr1b*, *ahr2*, *axin2*, and *CYP1A* mRNA were determined from 1 to 74 hours post-fertilization (hpf). The first two samples were taken at the 2–4 and 8–16 cell stages corresponding to 1 and 1.5 hpf, respectively. The shaded area indicates the period before zygotic genome activation where most mRNA is of maternal origin. Expression was calculated by $E^{-CT} (\times 10^6)$. Data are shown as mean $\pm$ SD ($n=2-3$).

Figure 4. Typical zebrafish embryo phenotypes induced by AZP or XAV after early exposure start

Groups of embryos (n=30) were exposed to 200 ppm DMSO (A), 1 or 3 μM AZP (B), or 3 μM XAV (C) with exposure starting at 3 hpf. Phenotypes were observed at 14, 34, and 58 hpf. At the 58-hpf time point embryos exposed to 1 μM AZP are shown because the majority of embryos exposed to 3 μM AZP exhibited severe malformations or had died. Detailed description of the phenotypes is given in the text.

Figure 5. Transcriptional responses to AZP and XAV of *ahr2*, *cttnb1*, *axin2*, and *CYP1A* during early zebrafish development

Zebrafish embryos were exposed to 3 μ M AZP, 3 μ M XAV, or 200 ppm DMSO. Exposure started at 3 hpf and samples for qPCR analysis were taken at 11, 14, and 36 hpf. The exposures are represented by triplicates ($n=3$) except for the exposure to XAV at 14 hpf which is represented by two replicates (due to accidental sample loss). Data are shown as mean + SD. Statistically significant changes compared with the control were determined by one-way ANOVA followed by Dunnett's post-hoc test and are indicated by * $p<0.05$, ** $p<0.01$, or *** $p<0.001$.

Figure 6. Typical zebrafish embryo phenotypes induced by AZP and XAV after late exposure start

Groups of embryos (n=20) were exposed to 200 ppm DMSO (A), 10 nM PCB126 (B), or to 1, 3, or 10 μ M AZP (C) or XAV (D). Exposure started at 1 dpf and phenotypes were observed at 3 dpf. Note the similar phenotype of embryos exposed to PCB126 and 3 μ M XAV. Detailed description of the phenotypes is given in the text.

A) Cumulative mortality

B) Phenotypes

Figure 7. Cumulative mortality and phenotypic effects of AZP or XAV combined with AHR agonists after early exposure start

A) Cumulative mortality and B) phenotypes in zebrafish embryos exposed to 400 ppm DMSO (D), 20 nM PCB126 (P), 10 nM FICZ (F), 3 μ M AZP (A), combinations of 3 μ M AZP with PCB126 (AP) or FICZ (AF), 3 μ M XAV (X), or combinations of 3 μ M XAV with PCB126 (XP) or FICZ (XF). Exposure started at 3 hpf and embryos were monitored until 5 dpf ($n=24-50$). At 2 dpf embryos were photographed to document characteristic phenotypes. For cumulative mortality at 5 dpf statistically significant differences between the D versus A or X groups, or between the P versus AP or XP groups, or between the F versus AF or XF groups were determined by Fisher's exact test and are indicated by ** $p < 0.01$, or *** $p < 0.001$.

Figure 8. Mortality and phenotypic effects of PCB126 or FICZ in combination with AZP or XAV after late exposure start

A) Effects of PCB126 combined with AZP were examined in embryos exposed to 400 ppm DMSO (D), 5–40 nM PCB126 (P5, P10, P20, and P40), or to 3 μ M AZP (A3) alone or in combination with PCB126 (P5+A3, P10+A3, P20+A3, and P40+A3). B) Effects of FICZ combined with AZP were examined after exposure to 300 ppm DMSO, 1–3 μ M AZP (A1, A2, and A3), or to 10 nM FICZ (F10) alone or in combinations with AZP (F10+A1, F10+A2, and F10+A3). C) Effects of PCB126 or FICZ combined with XAV were examined after exposure to DMSO (400 ppm) 5 or 20 nM PCB126 (P5 and P20), or to 3 μ M XAV alone (X3) or in combinations with PCB126 (P5+X3 and P20+X3). C) Other groups were

exposed to DMSO (400 ppm), 5 or 10 nM FICZ (F5 and F10), or to 3 μ M XAV alone (X3) or in combination with FICZ (F5+X3 and F10+X3). Exposure started at 1 dpf. Percentages of embryos with normal appearance (Normal), embryos lacking inflated swim-bladder (–SB), embryos with edema lacking inflated swim-bladder (–SB +Edema), and cumulative mortality (Dead) were determined at 5 dpf (n=19–26). Embryos showing edema generally lacked inflated swim-bladder. Note in B) how the shape of the lower jaw changes with higher AZP concentration in embryos co-exposed to FICZ and AZP. Statistically significant differences in mortality between groups were determined by Fisher's exact test with Holm-Bonferroni's method for compensation at multiple comparisons. Differences between D versus P20 or P40 are indicated by *** ($p<0.001$). Differences between A3 vs P10–40+A3 in A), A2 vs F10+A2 and A3 vs F10+A3 in B), and F5 vs F5+X3 and F10 vs F10+X3 in C) are indicated by †, ††, or ††† ($p<0.05$, $p<0.01$, and $p<0.001$). Finally ### indicates differences ($p<0.001$) between P20 vs P20+A3 and P40 vs P40+A3 in A) and between P20 vs P20+X3 in C).

Figure 9. Effects on mRNA expression after late exposure to AZP or XAV combined with FICZ or PCB126

Levels of A) *ahr2* and *cttnb1* (β -catenin), B) *CYP1A* and *axin 2*, and C) *rspo2*, *pcna*, and *runx2b* in zebrafish embryo groups exposed to 400 ppm of DMSO (D), 3 μ M AZP (A), 3 μ M XAV (X), 20 nM PCB126 (P), 10 nM FICZ (F), or combinations of 20 nM PCB126 or 10 nM FICZ with 3 μ M AZP (AP; AF) or 3 μ M XAV (XP; XF). Exposure started at 1 dpf and samples for qPCR analysis were taken after 8, 24, or 48 h of exposure. In A) and C) statistically significant differences versus the control were determined by one-way ANOVA followed by Dunnett's test and are indicated by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$. In B) statistically significant differences among groups were determined by one-way ANOVA followed by Tukey's test and are indicated by different letters ($p < 0.05$), i.e., two bars having

at least one similar letter are not statistically different from each other. Data are shown as mean + SD ($n=3$).

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Figure 10. Effects of Ahr2 knockdown and FICZ on mortality elicited by AZP and XAV at late exposure start

Groups of embryos injected with Ahr2-MO or Ctrl-MO were exposed to 300 ppm DMSO (D), 10 nM FICZ (F10), or either to A) 3 μ M AZP (A3) alone or in combination with 10 nM FICZ (F10+A3), or to B) 0.3, 1, or 3 μ M XAV alone (X0.3, X1, or X3) or in combination with 10 nM FICZ (F10+X0.3, F10+X1, or F10+X3). Exposure started at 1 dpf and the mortality rate was determined at 5 dpf. Data for mortality are shown as percentages of the total number of embryos in a group at the exposure start. In A) $n=19-25$ and in B) $n=24-25$. A line indicates that there was no mortality. Statistically significant differences between the DMSO and FICZ groups, or between the AZP or XAV alone and combination groups were determined by Fisher's exact test and are indicated by * $p<0.05$ or ** $p<0.01$.

Figure 11. Effects of Ahr2 knockdown on *CYP1A* and *axin2* transcriptional response to combinations of AZP or XAV with FICZ at late exposure start

Groups of zebrafish embryos were exposed to 400 ppm DMSO (D), 3 μ M AZP (A), 1 μ M XAV (X) or 10 nM FICZ (F), or to combinations of AZP or XAV with FICZ (AF and XF). Statistically significant changes between pairs of groups injected with Ctrl-MO or Ahr2-MO but with the same exposure were determined with Student's *t* test using log-transformed data and are indicated by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$. One outlier was removed based on Grubb's test (Grubbs, 1969). Data are shown as mean $\pm$ SD ($n = 7-8$), and represent results from two experiments.

Table 1

Changes in AZP- or XAV-induced mortality rates in zebrafish embryos after addition of PCB 126 or FICZ, or after Ahr2-MO treatment $\pm$ FICZ with exposure starting early (3 hpf) or late (1 dpf) and with assessment at 5 dpf.

Compound	Start	Change in mortality rate			
		+ PCB	+ FICZ	+ Ahr2-MO	+ Ahr2-MO + FICZ
AZP	3 hpf	no ¹⁾	no	NS ²⁾	NS
	1 dpf	↕	↕↕	no	no
XAV	3 hpf	↕	↑↑	NS	NS
	1 dpf	↕	↑↑	↕	(↓) ³⁾

¹⁾ no = no change,

²⁾ NS = not studied, and

³⁾ (↓) = not statistically significant