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Phenylthiourea as a weak activator of aryl hydrocarbon receptor inhibiting 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced CYP1A1 transcription in zebrafish embryo

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Abstract

The aryl hydrocarbon receptor (AHR) is a ligand-dependent transcription factor that can be activated by a diverse synthetic and naturally-occurring chemicals, such as the halogenated aromatic hydrocarbons (HAHs) and the non-halogenated polycyclic aromatic hydrocarbons (PAHs). The liganded AHR modulates the genetic activity of a variety of xenobiotic-responsive genes, including cytochrome P4501A1 (*CYP1A1*). The tyrosinase inhibitor 1-phenyl-2-thiourea (PTU) is widely used in zebrafish research to suppress pigmentation in developing embryos/fry. Here we showed that 0.2 mM PTU induced a basal level of *CYP1A1* transcription in zebrafish embryonic integument as early as 24 h postfertilization (hpf) stage. Subsequently, PTU induced *CYP1A1* transcription in blood vessels at 36 hpf. During larval stage, the liver and all pharyngeal arch vessels of PTU-treated embryos exhibited *CYP1A1* transcription as well. Comparing to TCDD, PTU induces *CYP1A1* transcription with much lower efficacy in zebrafish embryos. Coincubating the embryos with PTU and TCDD led to repressing TCDD-induced *CYP1A1* transcription. Mechanistic studies indicated that both of PTU- and TCDD-mediated *CYP1A1* transcriptions are modulated by the same AHR–ARNTsignaling pathway.

Keywords: Phenylthiourea (PTU); CYP1A1; 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD); Aryl hydrocarbon receptor 2 (AHR2); Aryl hydrocarbon receptor nuclear translocator (ARNT); Zebrafish

1. Introduction

The aryl hydrocarbon rceptor (AHR) is a ligand-dependent transcription factor that belongs to the basic-helix-loop-helix PAS (bHLH-PAS) family. It plays key roles in the adaptive metabolic response to polycyclic aromatic hydrocarbons and in the toxic mechanism of halogenated dioxins and dibenzofurans. The AHR also plays a role in normal development of liver, heart, vascular, spleen, thymus and kidney development [1–4]. Prior to ligand binding, the AHR associates with heat shock protein 90 (hsp90), XAP2 and p23 in the cytoplasm [5–11]. This protein can be activated by a diverse synthetic and naturally occurring chemicals, such as the halogenated aromatic hydrocarbons (HAHs) and the non-halogenated polycyclic aromatic hydrocarbons (PAHs) [12–14]. The best characterized high affinity ligands for the AHR is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which can elicit a wide variety of toxic and biological effects. Presumably the ligand-bound AHR undergoes a conformation change and translocates into the nucleus to associate with AHR nuclear translocator (ARNT) [13,15,16]. Binding of AHR–ARNTcomplex to the xenobiotic responsive element within the upstream regulatory regions of the *CYP1A1* gene leads to disruption of chromatin and nucleosome structure, increase of promoter accessibility and induction of the *CYP1A1* expression [17].

HAHs (such as the polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans and biphenyls and related chemicals) and the PAHs (such as benzo[*a*]pyrene, 3-methylcholanthrene, benzoflavones, rutacarpine alkaloids, aromatic

Abbreviations: PTU, phenylthiourea; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; CYP1A1, cytochrome P4501A1; AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; hpf, hours post-fertilization

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amines and related chemicals) are the most extensively studied classes of AHR ligands. In general, HAHs have a relatively higher binding affinity for the AHR than PAHs [12–14]. In addition to these classical HAH and PAH ligands, numerous nonclassical AHR ligands or agonists have recently been identified by screening analysis of a combinational chemical library using an AHR-responsive reporter gene system [18]. The structure and physiochemical characteristics of these chemicals are dramatically different than that of HAHs and PAHs. Several activators contain only a single unsaturated ring [19]. Most of these nonclassical AHR ligands/agonists are relatively weak inducers of *CYP1A1* and/or low affinity AHR ligands when compared with TCDD [13]. Interestingly, some chemicals, such as omeprazole, have been identified that can induce AhR-dependent gene expression, yet they reportedly fail to competitively bind to the AhR [13]. It has been proposed that these chemicals are not AhR ligands themselves, but that they can activate AhR dependent gene expression indirectly, either via metabolic conversion into a ligand or by their ability to affect some cellular pathway, such as tyrosine kinase-dependent signal transduction pathway, that results in conformational changes of the AhR similar to those caused by ligand binding [13,20,21].

Phenylthiourea (PTU) is frequently used in zebrafish research to block melanin synthesis and prevent pigmentation of developing embryos [22]. Although the embryos incubated in a 0.003% (0.2 mM) PTU do not produce any morphological defects except missing pigment [23], recent study revealed that thyroid hormone production is blocked in these PTU-treated embryos [24]. Here, we showed that the PTU acted as a weak AHR activator, which induced *CYP1A1* transcription with low efficacy. When the embryos were co-incubated with both of PTU and TCDD, PTU inhibited TCDD-induced *CYP1A1* gene expression.

2. Materials and methods

2.1. Zebrafish embryos and PTU, TCDD exposure

Wild-type (A B strain) zebrafish embryos were maintained at 28.5 °C and were staged by hours postfertilization (hpf) [25]. For PTU and TCDD treatment, the newly fertilized embryos were continuously incubated at 28.5 °C with 0.2 mM (0.003%, w/v) PTU or/and 0.93 nM waterborne TCDD, respectively.

2.2. CYP1A1 probe and in situ hybridization

Whole-mount in situ hybridization was performed as previously described [23]. For the *CYP1A1* probe, a digoxigenin-labeled cRNA fragment containing 998 bp (nt 217–1214) of the zebrafish *CYP1A1* gene (GenBank AF210727) fragment was synthesized with the DIG RNA Labeling Kit (Sp6/T7) from Roche (USA). The hybridization was detected by anti-digoxigenin (DIG) antibody coupled to alkaline phosphatase. Embryos were sectioned as described previously [23]. The nomenclature and atlas of blood vessels was described elsewhere [26].

2.3. Northern hybridization analysis

Total RNA was isolated from embryos at 48 hpf stage by TRI reagent (Molecular Research Center, USA). RNA at 20 μg was separated by formaldehyde agarose gel electrophoresis and transferred to a zeta-probe membrane (Bio-Rad, USA). For the analysis of *CYP1A1* transcription, the same cDNA fragment of the digoxigenin-labeled probe (nt 217–1214 of *CYP1A1*) is used as template to produce the radiolabeled probe for Northern hybridization analysis [27].

2.4. Morpholino oligonucleotide and blocking AHR2 expression

The *AHR2* antisense morpholino oligonucleotide, obtained from Gene Tools (Corvallis, OR), was designed to target the 5′ UTR region between bases −4 and +21 across the AUG start codon (5′-TGTACCGATACCCGCCGACATGGTT-3′) of zebrafish *AHR2* cDNA (GenBank AAF063446). Prior to injection, morpholinos were diluted to 0.1 mM in 1X Danieau's solution, as described by Nasevicius and Ekker [28]. Twelve nanogram (1.4 pmol) of AHR2-specific morpholino oligonucleotides was injected into the fertilized egg at 1-cell stage.

3. Results

3.1. Phenylthiourea induces CYP1A1 transcription in zebrafish embryo

PTU is frequently used in zebrafish research to block melanin synthesis and prevent pigmentation of developing embryos [22]. By using DIG-labeled antisense *CYP1A1* RNA as a probe, we found that the 0.2 mM PTU induces weak *CYP1A1* transcription in zebrafish embryos (Fig. 1). The *CYP1A1* mRNA was detected in the integument first at 24 h postfertilization (Fig. 1A and B). Subsequently, at 36 hpf, the *CYP1A1* transcription was found in numerous blood vessels (Fig. 1C and D). However, the PTU-mediated *CYP1A1* transcription in the integument was no longer present at hatching stage (48 and 60 hpf) (Fig. 1E-H). At 72 hpf, the PTU-induced *CYP1A1* transcription took place in the blood vessels, eyes and liver (Fig. 1I and J). It is noteworthy that the PTU-induced *CYP1A1* transcription was too weak to be detected by Northern hybridization (Fig. 3).

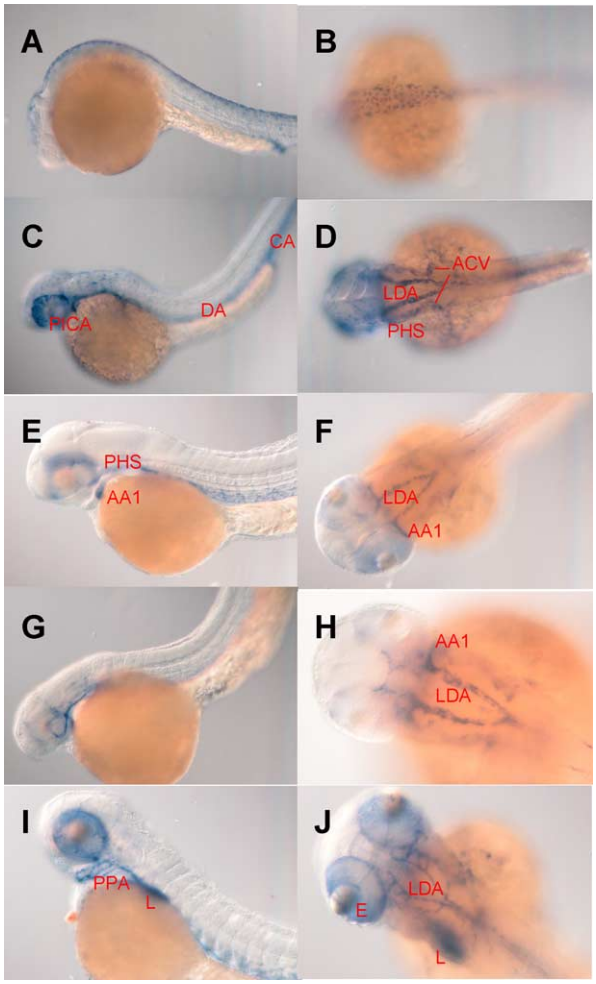

Fig. 1. PTU induces *CYP1A1* transcription in zebrafish embryos. Fertilized embryos were incubated by 0.003% (w/v) PTU. Whole mount in situ hybridization was carried out using a digoxigenin-labeled cRNA probe for zebrafish *CYP1A1* (GeneBank number AF210727), as described in Section 2. (A and B), 24 hpf; (C and D), 36 hpf; (E and F), 48 hpf; (G and H), 60 hpf; (I and J), 72 hpf. (A, C, E, G, I), lateral view; (B, D, F, H, J), dorsal view. *Abbreviations*: AA1, mandibular arch artery; ACV, anterior cardinal vein; CA, caudal artery; DA, dorsal artery; E, eye; L, liver; LDA, lateral dorsal aortas; PHS, primary head sinus; PICA, primitive internal carotid arteries.

3.2. Phenylthiourea blocks TCDD-induced CYP1A1 transcription in zebrafish embryo

Previous studies have shown that fish embryos are highly sensitive to TCDD exposure [29–34]. The major events of TCDD exposure in fish embryos include initiating developmental defects and stimulation of transcription of *CYP1A* family via AHR and its associated factor, ARNT. In waterborne TCDD-treated embryos, *CYP1A1* transcription was markedly induced in blood vessels, pronephros, liver, and skin at 48 hpf stage (Figs. 2G–I and 4K–O)) [35,36]. As revealed by cross-section, it was noted that the melanin expression and the TCDD-induced *CYP1A1* transcription occurs in the hypodermis and epidermis, respectively (Fig. 4M) [37]. The *CYP1A1* was not expressed in the skin of wild type and PTU-treated embryos at 48 hpf stage (Fig. 4C and H). In blood vessels, the strength of *CYP1A1* transcription mediated by 0.2 mM PTU was substantially lower than that induced by 0.93 nM TCDD (Figs. 2G–I versus D–F, 4K–O versus F–J). In addition to the blood vessels, pronephros, and integument tissues, TCDD also induced *CYP1A1* transcription in pectoral fin Figs. 2M and 4M) and lateral lines (Fig. 4N and O).

When compared to TCDD-modulated *CYP1A1* transcription, the induction of *CYP1A1* mRNA was considerably reduced by co-incubation of PTU and TCDD (Figs. 2J–L and O, 4P–T). The inhibition effect of PTU on TCDD-induced *CYP1A1* transcription was further confirmed by Northern hybridization (Fig. 3).

3.3. The PTU- and TCDD-induced CYP1A1 transcription is mediated by the same AHR-related signaling pathway

Recently it was shown that the developmental toxicity of TCDD in zebrafish is mediated by an AHR2-related signaling pathway [38]. To investigate the mechanism of PTU-mediated *CYP1A1* transcription and its inhibitory activity on AHR ligands, we employed an antisense approach using morpholino oligonucleotides against *AHR2* (*AHR2*-MO) to repress the level of endogenous AHR2 protein translation. Injection of 12 ng (1.4 pmol) *AHR2*-MO into fertilized embryos at 1 cell stage led to the repression of *CYP1A1* transcription in both of PTU-treated and PTU plus TCDD-co-incubated embryos (Fig. 5). Only a low level of *CYP1A1* transcription was retained in the eyes (Fig. 5G, H, J and K). It suggests that both PTU- and TCDD-induced *CYP1A1* transcriptions are mediated by the same AHR/ARNT signaling pathway. Reducing the AHR translation blocks both compounds-modulated *CYP1A1* transcription. However, the mechanism of PTU-mediated AHR activation still remains to be elucidated.

4. Discussion

The majority of the high affinity AHR ligands that have been identified and characterized to date are planar, hydrophobic HAHs and PAHs and their related compounds [13]. In addition to these classical HAH/PAH compounds, a large number of non-classical HAH/PAH chemicals have been identified as AHR ligands/agonists that can induce weak *CYP1A1* transcription [19,39]. For instance, 3′methoxy-4′-nitroflavone (3′M4′NF) competes with TCDD to bind to the AHR and induces basal level of *CYP1A1* transcription [40–42]. On the other hand, some other chemicals, such as omeprazole, were proposed to activate AhR dependent gene expression indirectly, either via metabolic conversion into a ligand or by their ability to affect some cellular pathway that results in conformational

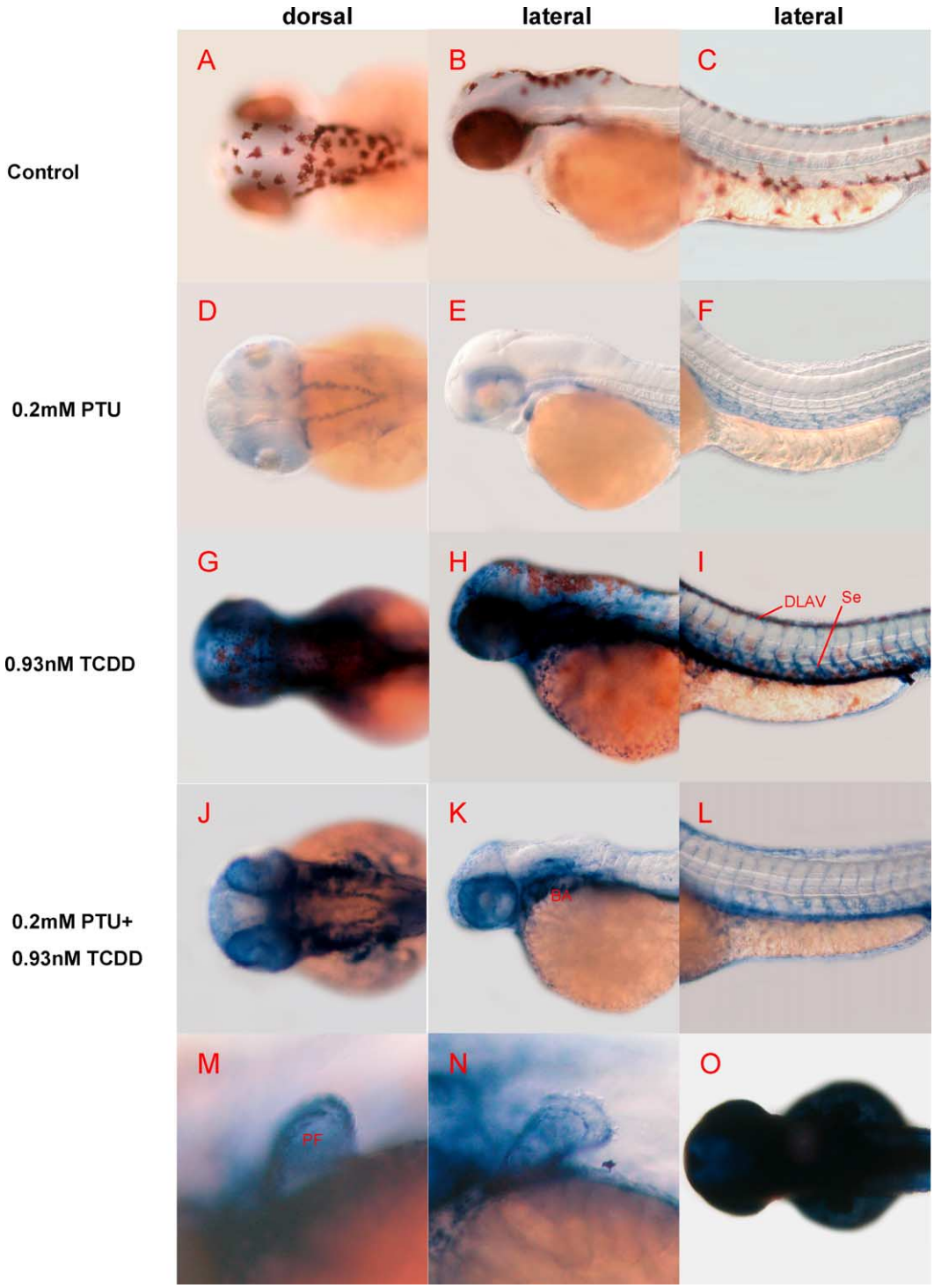

Fig. 2. PTU represses TCDD-mediated *CYP1A1* transcription. Whole mount in situ hybridization was carried out at 48 hpf with the process as described in Fig. 1, except the process of NBT/BCIP staining in TCDD-treated embryos (G–I and M) was taken in half (3.5 h) to prevent overstaining due to the abundance of *CYP1A1* induction by TCDD (O). (A–C), control embryos without any chemical exposure; (D–F), incubated with 0.2 mM PTU; (G–I, M, O), incubated with 0.93 nM TCDD; (J–L, N), incubated with 0.2 mM PTU plus 0.93 nM TCDD. (A, D, G, J), dorsal view of the anterior region; (B, E, H, K), lateral view of the anterior region; (C, F, I, L), lateral view of the trunk region; (M and N) lateral view of the pectoral fin. The pigmentation is repressed by PTU treatment (D–F and J–L). *Abbreviations*: DLAV, dorsal longitudinal anastomotic vessel; PAA, pharyngeal arch arteries; PF, pectoral fin; Se, intersegmental vessel.

changes of the AhR similar to those caused by ligand binding [13,20,21]. The current study we show that PTU induces a weak *CYP1A1* transcription in zebrafish embryos. As with TCDD [35,36], PTU first induces *CYP1A1* transcription in the embryonic integument and later on in blood vessel, liver and eyes. However, the PTU-induced *CYP1A1* transcription in the skin does not last throughout hatching stage. In contrast, a significant amount of *CYP1A1*

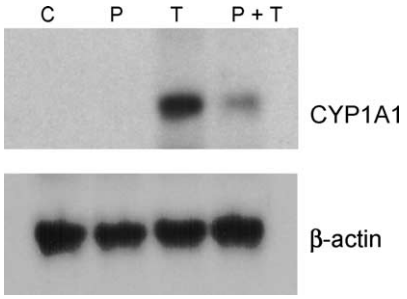

Fig. 3. Northern blot analysis of *CYP1A1* transcription in the developing embryo. C, control embryos; P, incubated with 0.2 mM PTU; T, incubated with 0.93 nM TCDD; P + T, co-incubated with 0.2 mM PTU plus 0.93 nM TCDD. The process of Northern hybridization is described in Section 2. β-Actin is used as a control to evaluate the quality and quantity of the loaded RNA.

transcription is detected steadily in the epidermis of TCDD-treated or TCDD plus PTU co-treated embryos at 48 hpf. It suggests that the steady state of *CYP1A1* transcription in the epidermis is TCDD-dependent and it is not related to the melanonin synthesis in the hypodermis.

When the embryos are treated by PTU and TCDD together, the TCDD-mediated *CYP1A1* transcription is significantly reduced. Since repressing the AHR2-related pathway leads to blocking both of PTU and TCDD-mediated *CYP1A1* transcription, it suggests that the inhibition effect of PTU in TCDD-induced *CYP1A1* transcription is mediated via the AHR–ARNTsignaling pathway. However, the exact biochemical events of PTU-mediated *CYP1A1* regulation still remain to be elucidated. The endogenous AHR can be activated either by the direct binding of ligands or via an indirect signaling pathway that results in conformational changes of the AhR.

Previous studies have shown that both of skin and vascular endothelium cells are sensitive to TCDD and produce high level of *CYP1A1* transcription [43]. The vascular system is a major initial site affected by TCDD in lake trout early life stages [31]. It implies that a functional AHR–ARNTpathway is probably first established in the skin and vascular cells during development. However, the physiological function of the AHR–ARNTpathway in the embryonic skin and vascular cells remains to be characterized.

There could be several mechanisms accounting for the differential efficacy of *CYP1A1* induction between PTU and TCDD, such as the differential conformation or post-translational modification of the AHR or differential coactivator recruitment. Previous structure-activity analysis suggests that the native AHR may exhibit different conformations after ligand binding. For instance, low efficacy flavonoids, such as 3′M4′NF, bind to the AHR ligand-binding site but fail to elicit conformational changes similar to those found with the high efficacy agonist TCDD [41,44]. Consequently, the nuclear translocation, DNA binding and *CYP1A1* induction of 3′M4′NF-bound AHR is much lower than with TCDD-liganded AHR.

In addition to the differential conformation of AHR, the efficacy of *CYP1A1* induction could also be regulated by the status of AHR phosphorylation. It was shown that the DNA binding ability of AHR/ARNT heterodimer is determined by the phosphorylation status of tyrosine residues in the heterodimer complex [45,46], whereas the phosphorylation status of serine/threonine residues of AHR and ARNT might regulate the events of transcriptional activation [47]. Activation of the protein kinase C pathway enhances the transcription of AHR/ARNT-modulated genes [48–51]. Therefore, protein kinase C (PKC) might exert its effects by increasing the transactivation potential of the AHR/ARNT heterodimer [48]. In this manner, PKC is thought to increase the ability of coactivator proteins to interact with AHR/ARNT complex [52,53].

A number of nuclear receptor coactivators were shown to interact with AHR, such as ERAP140 [54], RIP140 [55], p300, CBP [56], BRG-1 [57] and the three members of the p160 family of coactivators: NcoA1 (SRC1), NcoA2 (GRIP-1 and TIF-2) and NcoA3 (AIB-1, p/CIP, and ACTR) [58,59]. AHR interacts with these factors via its C-terminal transcativation domain, and ARNT may also be involved in recruiting cofactors to the promoter [59]. The coactivators are involved in recruiting of additional proteins, ATP-dependent chromatin remodeling and acetylation of promoter histones to relax chromatin, reposition nucleosomes and facilitate recruitment of RNA polymerase II. Recently it was shown that 3,3′-diindolylmethane (DIM), a weak partial agonist of AHR [60,61], recruited a subset of coactivators but failed to cause histone acetylation or effective polymerase recruitment [62]. In contrast, the full AHR agonist causes cycles of receptor and coactivator recruitment leading to gene transcription. It reflects that the efficacy of AHR activation can be modulated by differential recruitment of transcriptional coactivators.

TCDD and other AHR agonists suppress 17beta-estradiol (E)-induced responses in the rodent uterus and mammary tumors and in human breast cancer cells by blocking the cross-talk between AHR and estrogen receptor [63,64]. TCDD also induces degradation of ERα via a proteasome-dependent degradation pathway [65]. Consequently, it was proposed that AHR ligands could be used in treatment of breast cancer [63,66]. Accordingly, a number of selective AHR modulators (SahRMs) were developed as chemopreventative agents. These compounds, such as 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF) and 3,3′-diinodolylmethane (DIM), bind the AHR and suppress the estrogenic induced responses in a manner similar to TCDD, but they did not induce AHR-mediated toxic responses in rodent models [64,67]. Since we have shown that PTU is a weak AHR activator that can suppress TCDD-modulated *CYP1A1* transcription, it will be interesting to investigate the efficacy of PTU in antitumorigenic activity.

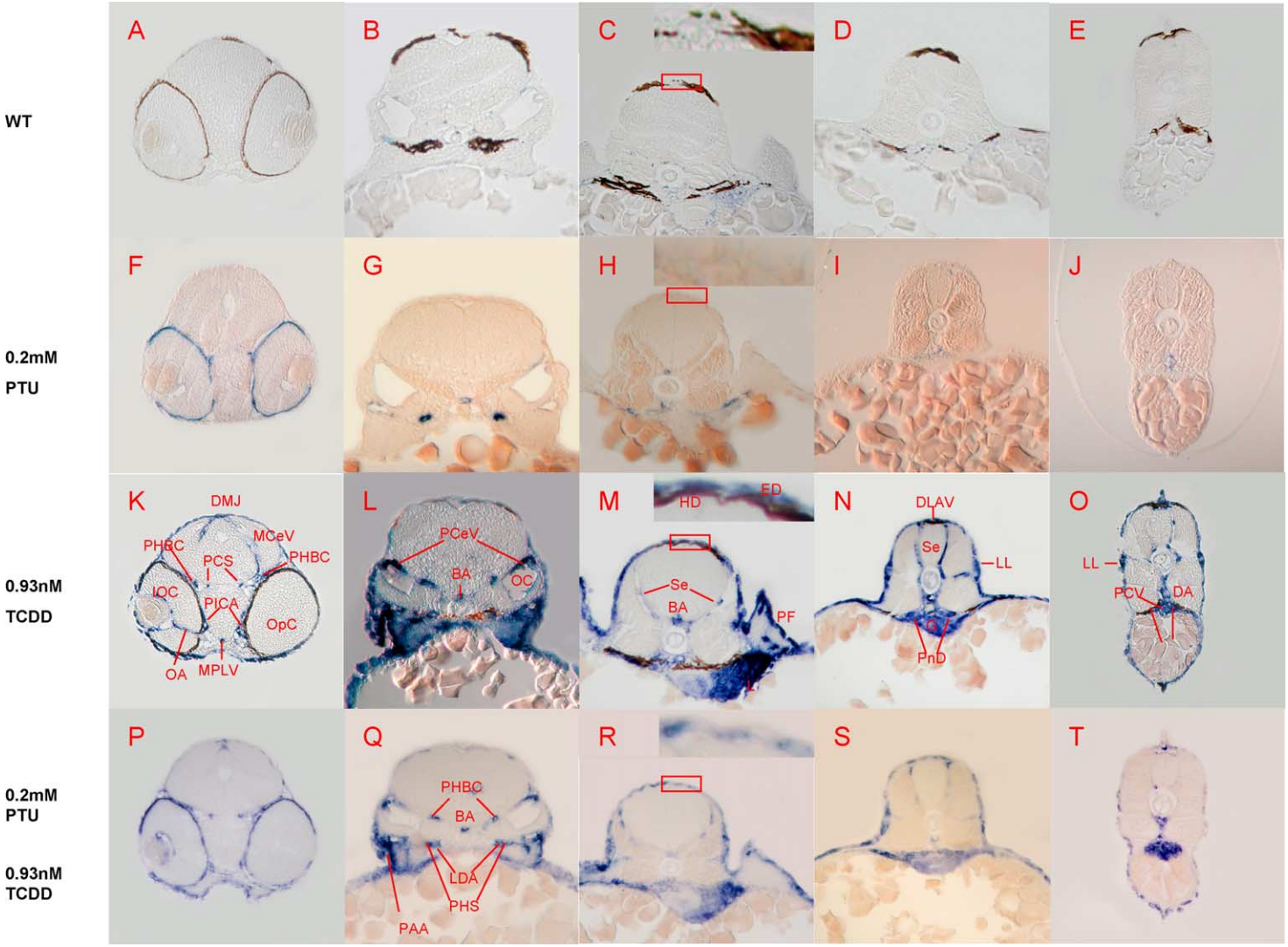

Fig. 4. Expression pattern of *CYP1A1* in 48 hpf-stage embryo. (A–E), control embryos without chemical treatment; (F–J), incubated with 0.2 mM PTU; (K–O), incubated with 0.93 nM TCDD; (P–T), co-incubated with 0.2 mM PTU plus 0.93 nM TCDD. Boxes in (C, H, M, R) show that melanonin (brown) and *CYP1A1* (blue) were expressed separately in hypodermis and epidermis, respectively. After whole mount in situ hybridization at 48 hpf, the fixed embryos were sectioned to reveal the tissue distribution of *CYP1A1* mRNA. *Abbreviations*: BA, basilar artery; DA, dorsal aorta; DMJ, dorsal midline junction; ED, epidermis; G, gut; HD, hypodermis; IOC, inner optic circle; L, liver; LDA, lateral dorsal aorta; LL, lateral line; MCeV, middle cerebral vein; MPLV, median palatocerebral vein; OA, optic artery; OC, otic capsule; OpC, optic capsule; PAA, pharyngeal arch arteries; PCeV, posterior cerebral vein; PCS, posterior communicating segment; PCV, posterior cardinal vein; PF, pectoral fin; PHBC, primordial hindbrain channel; PHS, primary head sinus; PICA, primitive internal carotid artery; PnD, pronephric duct; Se, intersegmental vessel.

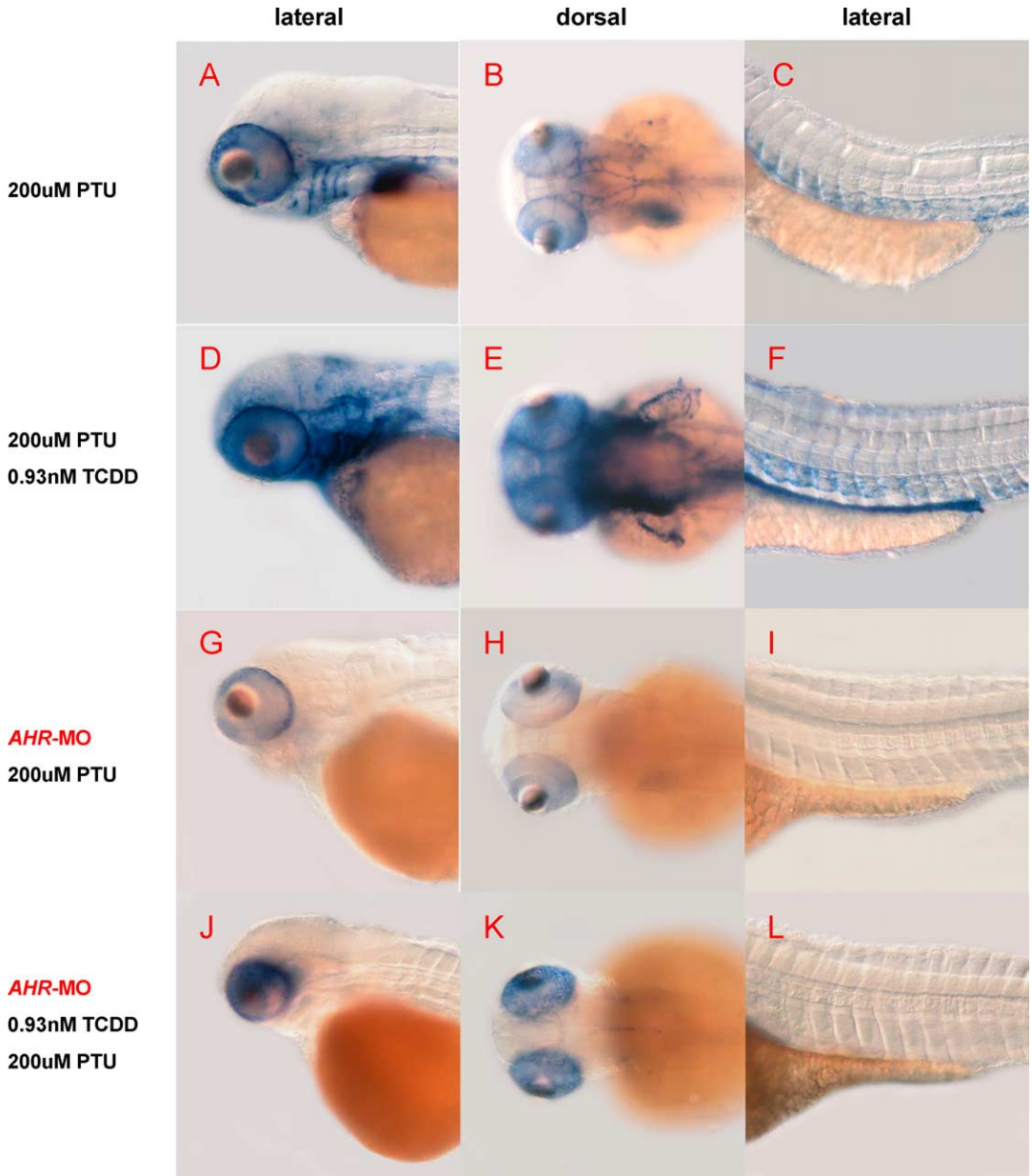

Fig. 5. *AHR* antisense morpholino oligonucleotide represses PTU- and TCDD-mediated *CYP1A1* transcription. To block the translation of AHR protein, 12 ng (1.4 pmol) of *AHR*-specific antisense morpholino oligonucleotide was injected into fertilized embryo at 1-cell stage (G–L). (A–C and G–I), incubated with 0.2 mM PTU; (D–F and J–L), incubated with 0.2 mM PTU plus 0.93 nM TCDD.

Acknowledgments

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