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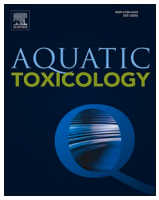
Aquatic Toxicology 279 (2025) 107250

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox



New insights into the regulation of *cyp3a65* expression in transgenic *tg (cyp3a65:GFP)* zebrafish embryos

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ARTICLE INFO

Keywords:
Transgenic zebrafish embryos
CYP3A65
PXR
AHR
GR
Metabolic endocrine disruptors
Intestine

ABSTRACT

Facing the need for alternative models allowing assessment of metabolic-endocrine disrupting chemicals (MDCs), especially in poorly investigated tissues such as the intestine, we recently developed a transgenic zebrafish embryo *in vivo* model, *tg(cyp3a65:GFP),* expressing the Green Fluorescent Protein (GFP) under the control of the zebrafish *cyp3a65* promoter, ortholog of human *cyp3a4*, a gene coding for a key enzyme of intestinal xenobiotic and endobiotic metabolism. In this study, we aimed to better understand the regulation of *cyp3a65* expression by zfPXR, zfAhR2, and zfGR zebrafish orthologs of well-known human xenosensors PXR and AhR, and steroid nuclear receptor GR. For this purpose, we performed zebrafish embryo *tg(cyp3a65:GFP)* (co)exposures to a variety of agonists (clotrimazole, TCDD, fluticasone propionate) and antagonists (econazole nitrate, CH223181, RU486), which were characterized using *in vitro* zebrafish reporter gene assays. We show that zfPXR and zfAhR2 cooperate to positively regulate *cyp3a65* expression, involving different transcription factors and their interaction. Moreover, for the first time, we show that zfGR agonist strongly inhibits the constitutive expression of *cyp3a65*, and we hypothesized the possible involvement of the transcriptional factor zfHNF4α. These results provide a better understanding of the regulation of zebrafish *cyp3a65* expression, highlighting the complex interaction between different transcription factors, which is consistent with the multiple regulatory pathways of *cyp3a4* in humans. Our data support the idea that this gene is a target of multiple contaminants capable of interacting with zfPXR, zfAhR2 and zfGR and highlights the relevance of the *tg(cyp3a65:GFP)* model to screen chemicals potentially acting as MDCs based on their modes of action at the intestinal level, which could be relevant for hazard assessment of chemicals for human and environmental health.

Introduction

Among endocrine disrupting chemicals, some referred to metabolic-endocrine disrupting chemicals (MDCs), can promote metabolic changes responsible for an increased prevalence of metabolic diseases in humans such as obesity, diabetes, and/or non-alcoholic fatty liver disease (NAFLD) (Bernal et al., 2022; Petrakis et al., 2017). The development of biological models and bioassays to evaluate metabolic disruptions induced by MDCs has thus become a major challenge in recent years (Kassotis et al., 2022). This challenge meets the need for alternative models to identify modes of action (MoAs) and investigate possible link with adverse effects (Audouze et al., 2020), especially in poorly investigated organs such as the intestine (Erradhouani et al., 2024).

In this context, we recently established a new *in vivo* transgenic model, *tg(cyp3a65:GFP)* (Erradhouani et al., in submission), developed in pigment-free zebrafish Casper mutant (White et al., 2008). This transgenic line expresses the green fluorescent protein (GFP) under the control of the zebrafish *cyp3a65* gene promoter coding for cytochrome P450 (CYP) CYP3A65, a zebrafish ortholog of human CYP3A4 (Goldstone et al., 2010). The CYP3A4 enzyme plays a central role in intestinal metabolism, as it is involved in the endogenous metabolism such as bile acids, and the protection of the organism against orally

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https://doi.org/10.1016/j.aquatox.2025.107250
Received 31 October 2024; Received in revised form 13 January 2025; Accepted 14 January 2025
Available online 15 January 2025

absorbed xenobiotics, catalyzing one of the first steps of their detoxification processes (Chen et al., 2014; van Herwaarden et al., 2009; van Waterschoot et al., 2009). Previous results showed that *cyp3a65* expression is regulated by two xenobiotic receptors, zfPXR and zfAhR2, orthologs of human PXR (pregnane X receptor) and AhR (aryl hydrocarbon receptor), two well-known ligand-activated transcription factors (Chang et al., 2013; Kubota et al., 2015). Moreover, interactions or crosstalk between zfPXR and zfAhR2 in the regulation of *cyp3a65* have been suggested but are not fully understood. For example, the importance of zfPXR in the regulation of CYP3A65 is subject to questions. It has been shown that depletion of zfPXR does not silence *cyp3a65* expression, suggesting a compensatory mechanism (Salanga et al., 2020). In contrast, two other independent studies showed that *cyp3a65* expression is not induced in zfPXR knock-out zebrafish embryos exposed to zfPXR agonist (Chang et al., 2013; Kubota et al., 2015). For zfAhR2, its role in the expression of cyp3a65 is better defined as repression of zfAhR2 by morpholino oligonucleotides inhibits the *cyp3a65* expression and block the action of prototypical agonist of zfAhR2 such as TCDD, showing the significant role of zfAhR2 in mediating the effect of zfAhR2 agonist to induce *cyp3a65* expression (Chang et al., 2013; Tseng et al., 2005). Moreover, according to Kubota *et al.*, zfPXR and zfAhR2 interact in the regulation of *cyp3a65* expression in a positive and reciprocal crosstalk, *i.e.*, zfAhR2 activation promotes upregulation of *zfpxr* and *cyp3* genes, and zfPXR activation promotes upregulation of *zfahr2* and *cyp1a* genes. Such crosstalk has been suggested for human cells as AhR promotes the upregulation of *cyp3a4* and *pxr* in hepatic cell lines HepaRG and HepG2, and primary hepatocytes (Kumagai et al., 2012; Rasmussen et al., 2017).

In humans, CYP3A4 is also known to be regulated by the glucocorticoid receptor (GR) in the liver and intestine (Khan et al., 2009; Usui et al., 2003). In zebrafish, there is, to our knowledge, no information regarding the role of this steroidal nuclear receptor on the regulation of *cyp3a65*, although our previous data suggest a potential role of the zfGR receptor in the developing intestine (Erradhouani et al., in submission).

In order to further characterize our transgenic model, we have undertaken the exposure of *tg(cyp3a65:GFP)* zebrafish embryos to various substances selected *a priori* from the literature data for their ability to bind and activate (agonist) or block (antagonist) the target receptor and subsequently induce or inhibit the signaling pathways mediated by the zfPXR, zfAhR2 and zfGR. For the zfPXR, agonists were clotrimazole and desogestrel and antagonist was econazole nitrate according to the results obtained *in vitro* with the HG5LN Gal4-zfPXR reporter cell line (Creusot et al., 2021). For the zfAhR2 receptor, 2,3,7,8-tetrachlorodibenzodioxin (TCDD) and 1,2,3,7,8-pentachlorodibenzo-p-dioxin (PeCDD) were selected as agonists, according to their ability to induce *cyp1a* through zfAhR2 in zebrafish embryo (Bugel et al., 2013; Goodale et al., 2012). CH223191 was selected as a zfAHR2 antagonist according to various *in vitro* mammalian models (Zhao et al., 2010), and its ability to reverse TCDD effects in zebrafish embryos (Takeda et al., 2024). In addition to these literature data, we performed an *in vitro* characterization of these agonists and antagonists in HG5LN Gal4-zfPXR and ZXLH reporter cell lines for zfPXR and zfAhR2, respectively, in order to determine whether the selected chemicals were specific for the target receptors.

For the zfGR, fluticasone propionate (FLU) was selected as a potent agonist and mifepristone (RU486) as an antagonist according to the results obtained *in vitro* with the reporter cell line UMLN-zfGR (Toso et al., 2023). Clobetasol propionate was selected as it has been characterized as a zfGR agonist *in vivo* in adult female zebrafish (Faltermann et al., 2020).

Material & methods

Chemicals

Selected chemicals for zfPXR: clotrimazole (CLO) (CAS 23,593–75–1), desogestrel (DGT) (CAS 54,024–22–5) and econazole nitrate (ECN) (CAS 24,169–02–6). **Selected chemicals for zfAhR2**: 2,3,7,8-tetrachlorodibenzodioxin (TCDD) (CAS 1746–01–06), 1,2,3,7,8-pentachlorodibenzo-p-dioxin (PeCDD) (CAS 40,321–76–4) and CH223191 (CH22) (CAS 301,326–22–7). **Selected chemicals for zfGR**: fluticasone propionate (FLU) (CAS 80,474–14–2), clobetasol propionate (CLOBE) (CAS 25,122–46–7) and mifepristone (RU486) (CAS 84,371–65–3).

All chemicals were powders dissolved in pure dimethyl sulfoxide (DMSO) (CAS 67–68–5). All chemicals were obtained from Sigma-Aldrich (St Quentin Fallavier, France) except for TCDD, which comes from LGC Standards (Molsheim, France), and CLOBE, which comes from USP References Standard (New York City, USA).

Animal facilities

Fish were raised in INERIS (Verneuil-en-Halatte, France) animal facilities, maintained in 3.5 L aquaria with a water recirculation system (Zebtec, Tecniplast, Italy) on a 14:10 light:dark cycle. Water quality was monitored every day: temperature (27.0 ± 2.0 °C), pH (7.5 ± 0.5), conductivity (400 ± 50 µS/cm), dissolved oxygen (>90%), nitrate (not detectable), nitrite (not detectable) and ammonia (not detectable). Zebrafish were fed twice a day with flake food (SDS400, Scientific Fish Food) in the morning and newly hatched brine shrimp (Sep-Art, Ocean Nutrition Europe) in the afternoon. Embryos were obtained from multiple groups of transgenic adult fish (one female and two males) placed together in specific aquaria. Eggs were laid and fertilized at night; in the morning, they were sorted and placed in an incubator at 29 °C and 14 h light/10 h dark cycle.

Exposure of tg(cyp3a65:GFP) embryos

The embryos exposure concentrations were chosen from previous work in which the toxicity of the substances was tested using the fish embryo acute toxicity standardized test (OCDE, 2013). Based on these results, the concentrations of each agonist or antagonist leading to maximal GFP induction or inhibition without any apparent toxicity were selected.

Chemical exposures to zfPXR, zfAhR2 and zfGR agonists and antagonists were performed for 48 h from 72 to 120 hpf in 60 mL capacity glass crystallizers. Each condition of exposure included 3 crystallizers containing 7 embryos each. Tested substances were dissolved in breeding water at different concentrations. The control condition was DMSO 0.01% (v/v) in the case of exposure to a single substance and 0.02% (v/v) in the case of coexposure to two substances. Embryos were then exposed by balneation to the control or experimental medium with 2 mL per embryo (14 mL for 7 embryos per crystallizer) and incubated at 29 °C and 14 h light/10 h dark cycle. Water and dissolved substances were renewed every 24 h.

In vivo fluorescent imaging

To quantify the GFP, living embryos were imaged by using a Zeiss AxioImager.Z1 fluorescence microscope equipped with an AxioCam Mrm camera (Zeiss GmbH, Göttingen, Germany). Embryos were placed on the side to better capture of intestinal fluorescence. Photographs were taken with a 10 ms exposure time using a X10 objective. Data analysis was performed by Zen blue 3.3 imaging software and fluorescence quantification was carried out in the intestine by ImageJ software (Schneider et al., 2012) with a macro-control developed at the laboratory. A detection threshold of 159 ms, determined from a quantification limit in non-transgenic animals, was applied during image analysis to avoid fish autofluorescence and be specific to GFP signal.

Quantification of mRNA expression

In addition to GFP imaging, real-time quantitative PCR (RT-qPCR)

Table 1
In vitro characterization of the tested chemicals in ZXLH and HG5LN Gal4-zfPXR cell lines. EC50 and IC50 were derives from modeled concentration-response curves using Hill regression model.

Agonists	zfAhR2 EC50 (nM)	zfPXR EC50 (μM)
Clotrimazole	not active	0.03
Desogestrel	not active	0.13
TCDD	0.24	not active
PeCDD	0.20	not active
Antagonists	**zfAhR2 IC50 (nM)**	**zfPXR IC50 (μM)**
Econazole nitrate	not active	2.8
CH223191	350	not active

was performed to assess the mRNA expressions of GFP and endogenous genes (*zfahr2, zfpxr, cyp3a65, cyp1a* and *fkbp5*, a zfGR target). Immediately after *in vivo* fluorescence imaging, larvae were placed in RNA stabilization solution (RNAlater™) and stored at -80 °C to preserve the samples, pending future total RNA extraction. RNA extraction, complementary DNAs generation and qPCR were performed as described in (De Oliveira et al., 2020). Primers for *zfahr2, zfpxr, cyp1a* and *fkbp5* were directly purchased from the manufacturer (Thermofisher®, references in Supplementary Material table S1). As *cyp3a65* did not have known commercial primers, we designed them and sent the sequences to the same supplier for custom synthesis (SM table S1).

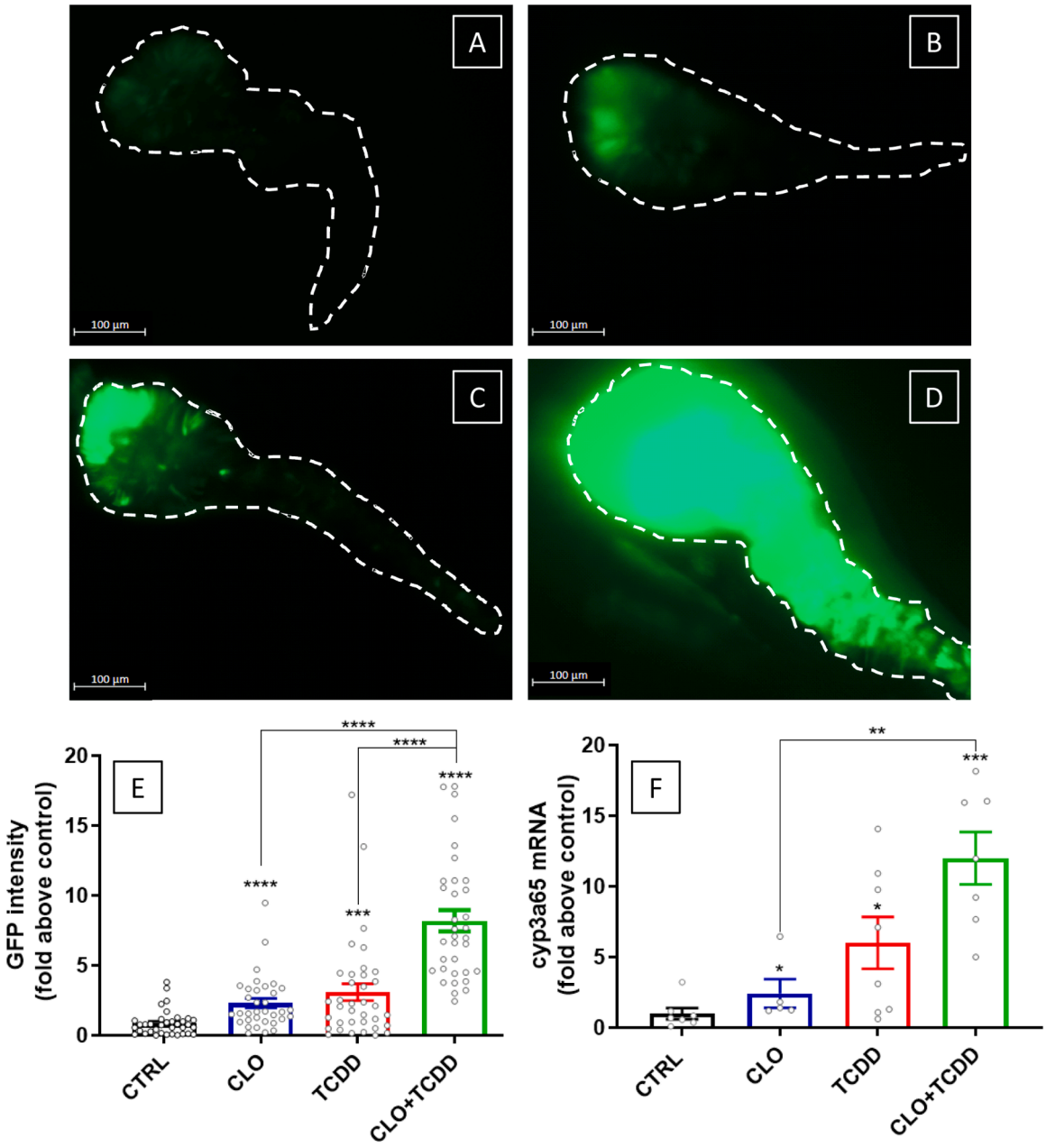


Fig. 1. GFP fluorescence and cyp3a65 mRNA are induced in presence of CLO or TCDD and over-induced when embryos are coexposed to CLO+TCDD. *In vivo* imaging of tg(cyp3a65:GFP) embryos at 120 hpf. The dashed lines delineate the intestine. Expression in control (CTRL) DMSO 0.02% (A) and exposed embryos to CLO 1.5 μM (B); TCDD 0.05 nM (C) and CLO 1.5 μM + TCDD 0.05 nM (D). (E) corresponds to GFP intensity in individuals ($n = 21$ embryos minimum per condition) after exposure to CLO 1.5 μM (in blue); TCDD 0.05 nM (in red) and CLO 1.5 μM + TCDD 0.05 nM (in green), control condition (CTRL) is DMSO 0.02% (in black). (F) cyp3a65 mRNA expression in pooled zebrafish embryos ($n = 5$ embryos/pool) in same condition.

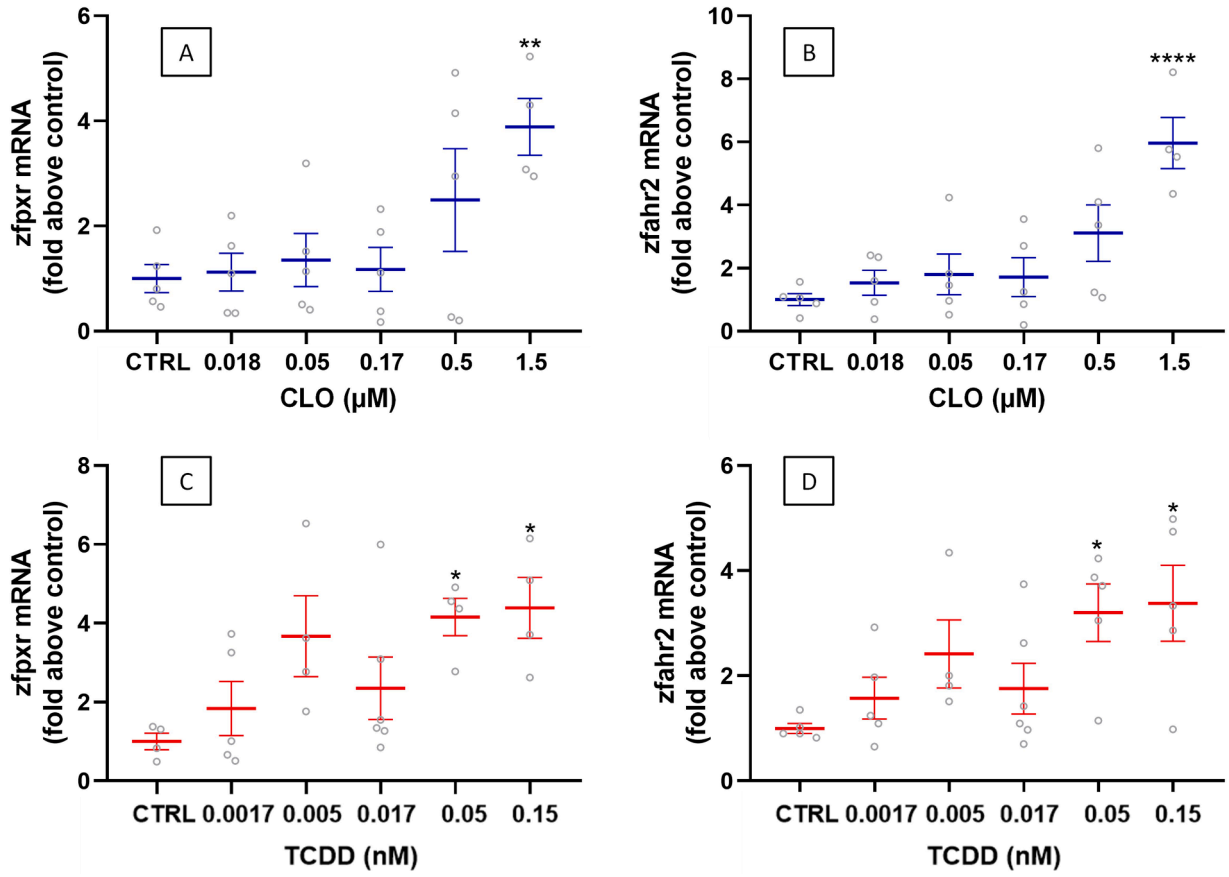


Fig. 2. Clotrimazole-mediated zfPXR activation up-regulates zfahr2 expression and TCDD-mediated zfAhR2 activation up-regulates zfpxr expression. zfpxr mRNA expression in pooled zebrafish embryos ($n = 5$ embryos/pool) when exposed to (A) CLO and (C) TCDD. zfahr2 mRNA expression in pooled zebrafish embryos ($n = 5$ embryos/pool) when exposed to (B) CLO and (D) TCDD. Control condition (CTRL) is DMSO 0.01%.

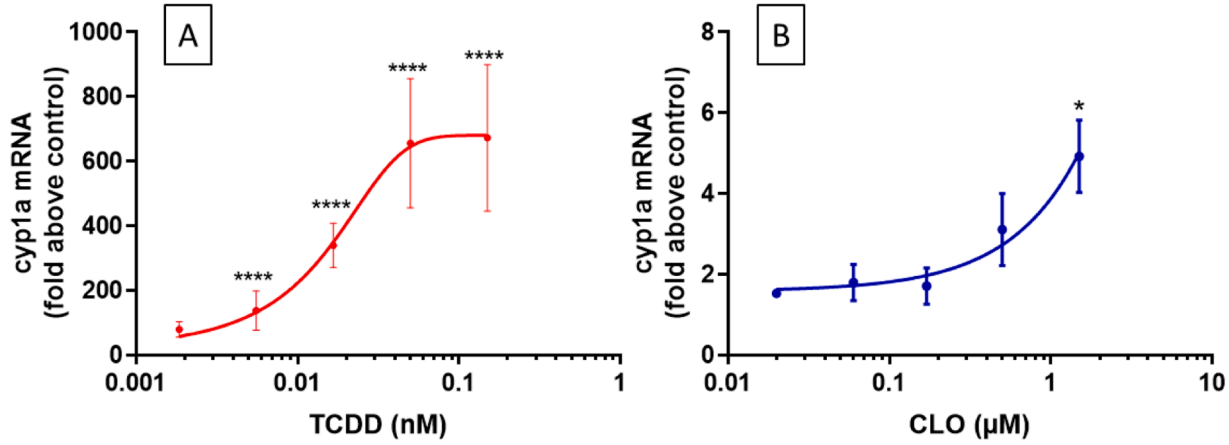


Fig. 3. TCDD promotes strong induction of cyp1a mRNA expression when clotrimazole slightly induces it compared to TCDD. Cyp1a mRNA expression in pooled zebrafish embryos ($n = 5$ embryos/pool) when exposed to (A) TCDD and (B) CLO.

In vitro characterization of zfPXR and zfAhR2 antagonists

***In vitro* reporter gene assay for zfAhR2** was performed in ZXLH (Zebrafish XRE Luciferase Hygromycin) cells, established from ZFL cell line (Ghosh et al., 1994) stably transfected by XRE3-TATA-Luc-Hygro construct to express the luciferase reporter gene under the transcriptional control of zfAhR2. These cells were grown at 28 °C in LDF culture medium consisting of 50% L15 medium, 35% DMEM-HG (Dulbecco's modified Eagle Medium-High Glucose), 15% Ham's F12 medium, 50 U/mL penicillin and streptomycin, 5% fetal calf serum Epidermal Growth Factor (50 ng/mL), insulin (0.01 mg/mL), HEPES (15 mM), sodium bicarbonate (0.15 g/L) and hygromycin B (0.35 µg/mL).

***In vitro* reporter gene assay for zfPXR** was performed in HG5LN Gal4-zfPXR cells, corresponding to HeLa cell line transfected using GAL4RE5-βGlobin-Luc-SVNeo construct to get HG5LN cell line, then HG5LN cell line was transfected using pSG5-GAL4(DBD)-zfPXR(LBD)-puro construct (Creusot et al., 2021). These cells were grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12)

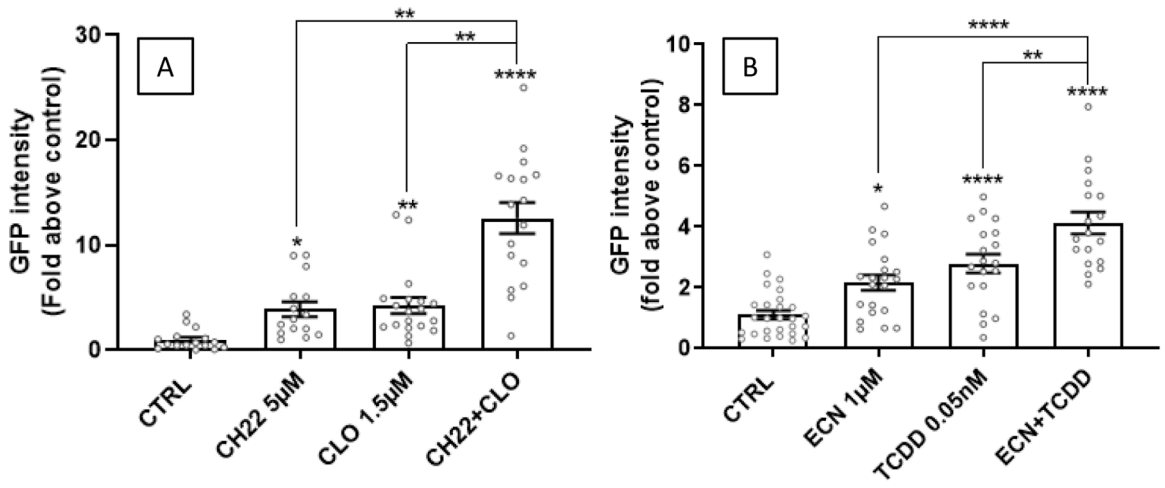


Fig. 4. GFP intensity in tg(cyp3a65:GFP) embryos exposed to zfPXR and zfAhR2 agonist and antagonist ligands alone or in combination ($n = 21$ embryos minimum per condition) at 120 hpf after 48 h. Exposure to CLO 1.5 μM; TCDD 0.05 nM; CH22 5 μM and ECN 1 μM alone or in combination. Control condition (CTRL) is DMSO 0.02%.

containing phenol red and 1 g/L glucose and supplemented with 5% fetal bovine serum, 100 units/mL of penicillin, 100 μg/mL of streptomycin, 1 mg/mL geneticin and 0.5 μg/mL puromycin in a 5% CO_2 humidified atmosphere at 37 °C.

Cells were seeded in white 96-well microplates with a density of 50 000 to 100 000 cells/wells and incubated for 24 h to allow them to adhere to the plastic. Cells were then exposed to a range of concentrations of the tested molecules. Culture medium and DMSO (0.1% vol/vol) were used as negative controls. TCDD (10^{-8} mol/L) was used as a positive control for ZXLH cell line and clotrimazole (10^{-6} mol/L) for HG5LN Gal4-zfPXR cell line.

Statistics

Results were analyzed with Kruskal-Wallis and Dunn's post-hoc multiple comparisons on GraphPad Prism 8.0.2. In the figures, the number of stars corresponds to the P value summary: $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***), $P \leq 0.0001$ (****). The standard error of the arithmetic mean (SEM) is represented on each graph.

Results

In vitro characterization of the transactivating potential of selected agonists and antagonists

As a first step, we used well-established *in vitro* transactivation models expressing either the ligand binding domain for the zfPXR or the entire zfAHR2 receptor coupled with luciferase as reporter gene to characterize the chemicals towards the two receptors investigated herein. We found that CLO selectively activated zfPXR with no transactivation activity on zfAHR2. Conversely, TCDD activated zfAhR2 with no transactivation activity on zfPXR (Table 1). CH22 and ECN applied alone were unable to induce or inhibit the basal luciferase activity of both receptors, while ECN inhibited the luciferase-induced activity by CLO in zfPXR cell line and CH22 inhibited the TCDD-induced luciferase activity in ZXLH cell line (Table 1). These data show that the selected chemicals elicited specific activation or blocking of the target receptor and signaling pathway of interest (*e.g.*, CLO and zfPXR or TCDD and zfAhR2) without interfering with the other receptor signaling at the cellular level (SM figure S1).

Crosstalk of zfPXR and zfAhR2 pathways in the regulation of cyp3a65 expression

Effect of each zfPXR or zfAhR2 agonist alone and in combination on cyp3a65 expression

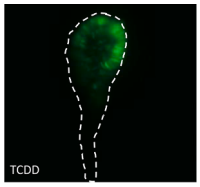
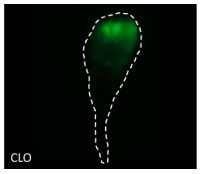
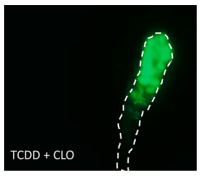
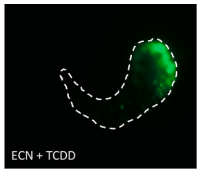
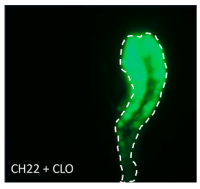
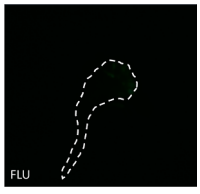
Exposures of *tg(cyp3a65:GFP)* zebrafish embryos from 72 to 120 hpf to zfPXR or zfAhR2 agonists alone promote *cyp3a65* expression as shown by induction of the GFP intensity using *in vivo* fluorescence imaging (Figs. 1B 1C and 1E) as well as induction of the relative mRNA expression using RT-qPCR (Fig. 1F). Exposure to CLO 1.5 μM led to 3-fold induction of GFP intensity and TCDD led to 4-fold induction as compared to control (Fig. 1E). Using mRNA quantification, we show that the inductions measured in CLO or TCDD-exposed zebrafish embryos led to similar induction of the *cyp3a65* expression (Fig. 1E) as compared to GFP intensity, thereby confirming the relevance of using GFP as reported gene of the endogenous *cyp3a65* gene expression while permitting to spatialize the effect of the two compounds on the *cyp3a65* expression in the gastro-intestinal tract.

Coexposure to both CLO 1.5 μM and TCDD 0.05 nM led to a strong induction of the *cyp3a65* expression as compared to exposure to CLO or TCDD alone as seen through *in vivo* imaging (Fig. 1D). Quantification of the GFP intensity by image analysis and *cyp3a65* expression by RT-qPCR further support this observation. The GFP intensity (11-fold induction compared to the control) and the *cyp3a65* mRNA expression (12-fold induction compared to control) in zebrafish embryos exposed to both CLO and TCDD (Figs. 1E and 1F) were also significantly different from those measured in embryos exposed to CLO or TCDD alone. To investigate if similar response profile can be observed in zebrafish embryos exposed to other agonists of zfPXR and zfAhR2, coexposure experiments were performed with DGT 1 μM and PeCDD 0.025 nM, alone or in combination (SM figure S2). Interestingly, a similar response profile was observed, characterized by a strong induction of the GFP fluorescence and *cyp3a65* expression in the coexposure group as compared to DGT or PeCDD alone.

Effect of zfPXR and zfAhR2 agonists on both receptors expression levels

Using the same individuals used to perform *in vivo* imaging and mRNA *cyp3a65* quantification, we also investigated the mRNA expression levels of each receptor in zebrafish embryos exposed to CLO and TCDD alone. We showed that exposure CLO, the agonist of zfPXR led to an up-regulation of *zfpxr* mRNA levels (Fig. 2A) but also an up-regulation of *zfahr2* mRNA levels (Fig. 2B). In case of exposure to TCDD, the agonist of zfAhR2, we also measured an up-regulation of *zfahr2* mRNA levels (Fig. 2D) as well as *zfpxr* mRNA levels (Fig. 2C).

Table 2
Overview of the effects of reference chemicals on cyp3a65 expression in tg (cyp3a65:GFP) zebrafish embryos. Results show cooperation and ambivalent relationship between zfPXR and zfAhR2 signaling pathways as agonist and antagonist promote cyp3a65 expression, and inhibition of its constitutive expression by zfGR.

Chemicals	Effect on *cyp3a65* expression	GFP imaging
zfAhR2 agonist	Induction +	
zfPXR agonist	Induction +	
zfAhR2 agonist + zfPXR agonist	Induction ++	
zfAhR2 agonist + zfPXR antagonist	Induction ++	
zfPXR agonist + zfAhR2 antagonist	Induction ++++	
zfGR agonist	Inhibition ++++	

Effect of (co)exposures to zfPXR and zfAhR2 antagonist with CLO or TCDD

To further explore the potential interaction between zfAhR2 and zfPXR signaling pathways on the regulation of the *cyp3a65* gene, we then exposed zebrafish embryos to a specific and potent antagonist of zfAhR2, *i.e.*, CH22 5 μM, alone or in combination with CLO, the most potent agonist of zfPXR. Similarly, we used an antagonist of zfPXR, *i.e.*, ECN 1 μM, which was tested alone or in the presence of TCDD 0.05 nM, the most potent agonist of zfAhR2. The results showed that CH22 and ECN alone were able to induce GFP intensity as compared to control (Figs. 4A and 4B). For CH22 1 μM, we found that at this concentration, the substance is efficient to block the action of TCDD resulting in a clear reduction of the TCDD-induced 7-ethoxyresorufin-O-deethylase activity and *cyp1a* mRNA in zebrafish embryos (SM figures S3A and S3B). Together with the data obtained in ZXLH cell line and in zebrafish embryos, it shows that CH22 does not act as a partial agonist of zfAhR2 and is efficient in inhibiting the zfAhR2 signaling pathway. For zfPXR, there is, to our knowledge, no other specific zfPXR-regulated gene that could be investigated, and we were therefore unable to verify at the *in vivo* level the efficacity of ECN to block the zfPXR signaling pathway.

Coexposure to CLO and CH22 resulted in a strong induction of GFP as compared to CH22 or CLO alone (Fig. 4A). Coexposure to ECN and TCDD shows a similar trend, *i.e.*, a strong and significantly different induction of GFP as compared to ECN or TCDD alone although the magnitude of effect was less pronounced as compared to CH22 and CLO (Fig. 4B).

Involvement of zfGR pathway in the regulation of cyp3a65 expression

Embryos exposure to zfGR agonist FLU promotes the mRNA expression of *fkbp5,* a well-known GR-regulated gene in zebrafish (Benato et al., 2014). This data clearly indicates that the GR-signaling pathway was activated in zebrafish exposed under this experimental condition. Interestingly, we measured a dramatic down-regulation of the *cyp3a65* mRNA expression (Figs. 5A and 5B). GFP intensity was also strongly reduced in a concentration-dependent fashion (Fig. 5C). To determine whether this reduction was restricted to FLU or can be extended to other zfGR agonist, we performed an exposure to another GR agonist, CLOBE, with similar results (Fig. 5D).

Considering the effects of two zfGR agonists on the basal expression of *cyp3a65*, we also tested zfGR antagonist RU486. In this experiment, TCDD was used as a positive control. As expected TCDD 0.05 nM led to a strong GFP induction, meaning that the zebrafish embryos responded in a correct manner to this treatment. RU486 alone had no effect on GFP intensity as compared to control but was significantly different as compared to FLU suggesting that zfGR activation is key to inhibit *cyp3a65* expression. Moreover, we observed that RU486 blocks the FLU effect on GFP intensity (Figs. 6A and 6B).

Discussion

New insights on the zfPXR/zfAhR2 crosstalk in cyp3a65 expression regulation

In this study, we showed that GFP expression in *tg(cyp3a65:GFP)* is induced by different agonists of two transcription factors, *i.e.*, zfPXR and zfAhR2, both being implicated in *cyp3a65* expression regulation. Combined exposure to zfPXR and zfAhR2 agonists promotes *cyp3a65* over-induction as compared to exposure to zfPXR or zfAhR2 agonists alone (Fig. 2). This indicates that both zfPXR and zfAhR2 signaling pathways cooperate in the regulation of *cyp3a65* expression. In addition, activation of zfPXR by its agonist CLO induces *zfahr2* expression, and activation of zfAhR2 by its agonist TCDD induces *zfpxr* expression (Fig. 3). It is important to remind here that we showed that TCDD is unable to act on zfPXR and similarly, CLO has no activity on zfAhR2 (Table 1). Conversely, at the *in vivo* level, exposure to zfPXR or zfAhR2 agonists led to the induction of the other signaling pathway target genes. In addition to *zfahr2* expression, we also measured *cyp1a* mRNA expression, a cytochrome known to be strongly regulated by zfAhR2 (Petrulis et al., 2001). In TCDD-exposed embryos the induction was strong. In contrast, in CLO-exposed zebrafish, the induction of *cyp1a* was weak but significant (Fig. 3). Altogether, these data are in line with Kubota *et al.* who showed the same crossed up-regulation between the two transcription factors in zebrafish embryos when exposed to candidate zfPXR agonist PCN and zfAhR2 agonist PCB126 (Kubota et al., 2015). This is also in tune with Chang *et al.* who assume that the interaction between the two receptors is necessary for *cyp3a65* expression (Chang et al., 2013). Such positive interaction may account for the strong induction of the *cyp3a65* gene in case of coexposure to TCDD and CLO (Fig. 1).

Surprisingly, the antagonism of zfAhR2 or zfPXR also promotes *cyp3a65* expression as compared to control. One hypothesis could rely on the fact that CH22 or ECN could act as partial agonists of the target

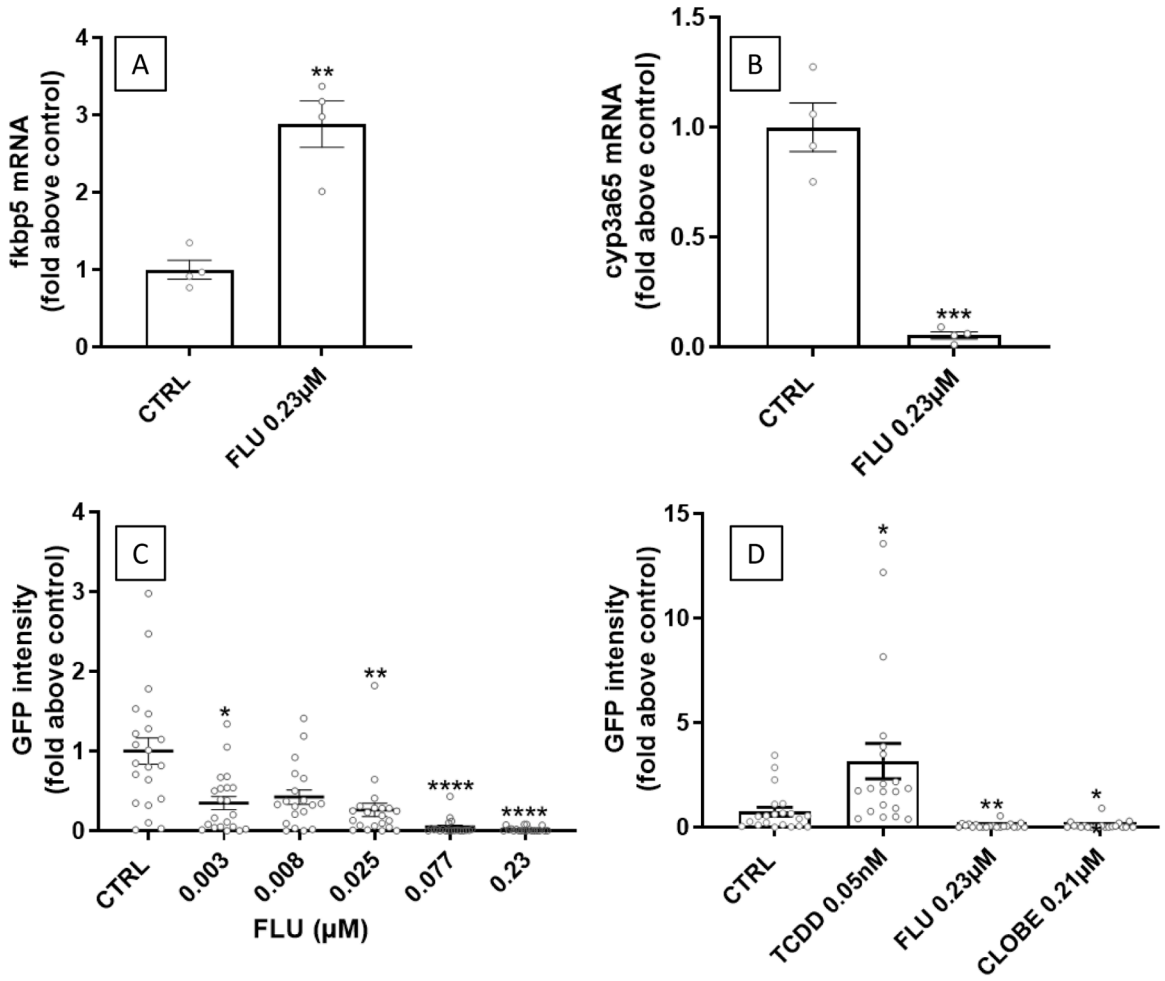


Fig. 5. Fluticasone propionate promotes fkbp5 expression and inhibits cyp3a65 expression in both GFP intensity (as clobetasol propionate) and mRNA expression. mRNA expression of (A) fkbp5 and (B) cyp3a65 in pooled zebrafish embryos ($n = 5$ embryos/pool) when exposed to FLU 0.23 µM. GFP intensity in individuals ($n =$ 21 embryos minimum per condition) after exposure to (C) FLU and (D) CLOBE. In (D) FLU is for comparison and TCDD as positive control. Control condition (CTRL) is DMSO 0.01%.

receptors. However, these two compounds were unable to activate the zfPXR or the zfAhR2 receptors *in vitro* (Table 1). Moreover, we observed a strong induction of the GFP and *cyp3a65* mRNA in case of coexposure to the antagonist of one receptor (*e.g.*, CH22 for zfAhR2) and the agonist of the other signaling pathway (*e.g.*, CLO for zfPXR). In such a situation, coexposure to CH22 and CLO promotes a *cyp3a65* over-induction as compared to CH22 or CLO alone (Fig. 4A). ECN and TCDD coexposure promotes *cyp3a65* over-induction as compared to ECN and TCDD alone (Fig. 4B). These results add complexity to the hypothesis of positive and reciprocal crosstalk as presented above and in literature. The interactions between the two receptors indicate that these two signaling pathways have an ambivalent relationship as they cooperate positively while in the presence of the antagonist of one receptor, the effect of the agonist of the other receptor is maximized. This ambivalent relationship could explain at least partly why in zfPXR knock-out zebrafish, *cyp3a65* appears to be induced as compared to control (Salanga et al., 2020), as in our case, the presence of CH22 or ECN led to an increased *cyp3a65* expression as compared to control. It is clear that the intime mechanism of action between zfAhR2 and zfPXR will require additional experiments. At this stage, we can only report the complex and original interaction between zfAhR2 and zfPXR, which is highly dependent on the state of activation of each receptor to regulate the expression of *cyp3a65* in the developing intestine (Fig. 7 and Table 2).

New insights on zfGR involvement in cyp3a65 expression regulation

While the zfGR agonists FLU and CLOBE promote the expression of the known zfGR-regulated gene *fkbp5*, they also inhibit *cyp3a65* expression, suggesting that zfGR-mediated pathway down-regulates the constitutive expression of *cyp3a65* (Fig. 5). This hypothesis is confirmed when embryos are exposed to both FLU and zfGR antagonist RU486; the coexposure blocks the FLU-mediated inhibition of *cyp3a65* expression (Fig. 6).

We previously performed an *in silico* analysis of the *cyp3a65* promoter which identified response elements specific to zfPXR and zfAhR2 (SM figure S4, (Erradhouani et al., in submission)), in line with the literature (Chang et al., 2013). The search for consensus motifs for zfGR did not allow us to identify specific response elements for this receptor, suggesting that zfGR modulates *cyp3a65* in another way than direct DNA binding. For example, in humans, GR can bind to the transcription factor HNF4α (hepatocyte nuclear factor 4α), which binds the promoter of *cyp3a4* (Pavek et al., 2007). As GR and HNF4α are well-conserved across evolution (Dinarello et al., 2020; Heppert et al., 2022); we could, therefore, pose a similar hypothesis for *cyp3a65* in zebrafish. Moreover, zfHNF4α (zebrafish HNF4α) is present in the zebrafish foregut (Heppert et al., 2022), where *cyp3a65* is expressed at embryonic stage according to the *tg(cyp3a65:GFP)* model. Finally, the search for a classical direct repeat HNF4α motif (DR1, 5′-AGGTCAxAGGTCA-3′) led to its identification downstream of the *cyp3a65* promoter (SM figure S4). The binding

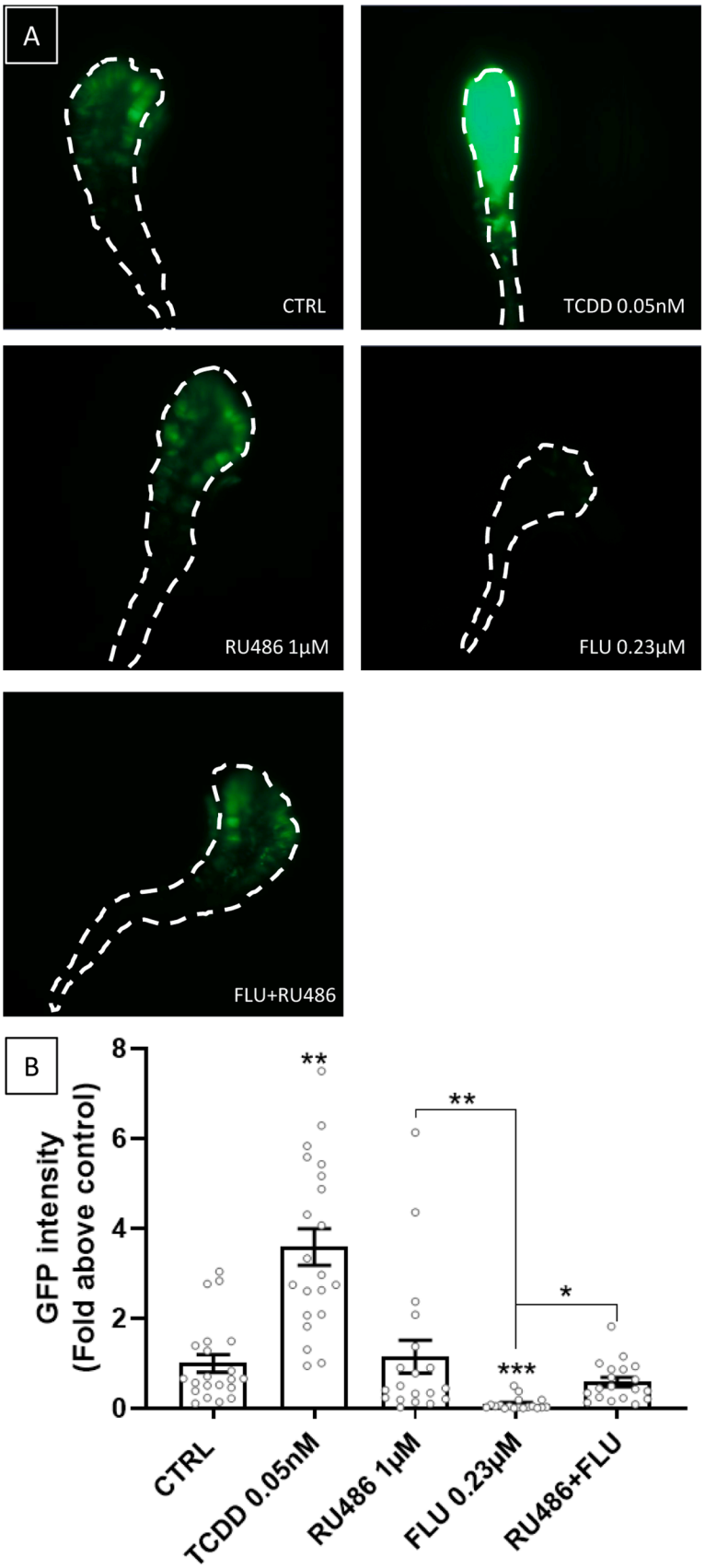


Fig. 6. ***RU486 reverses inhibited expression of cyp3a65 by fluticasone propionate.*** *(A) In vivo imaging of tg(cyp3a65:GFP) embryos at 120 hpf after 48 h exposure to TCDD 0.05 nM; RU486 1 μM; FLU 0.23 μM and RU486+FLU. TCDD as positive control. Control condition (CTRL) is DMSO 0.02%. The dashed lines delineate the intestine. (B) GFP intensity in individuals (n = 21 embryos minimum per condition) in the same conditions.*

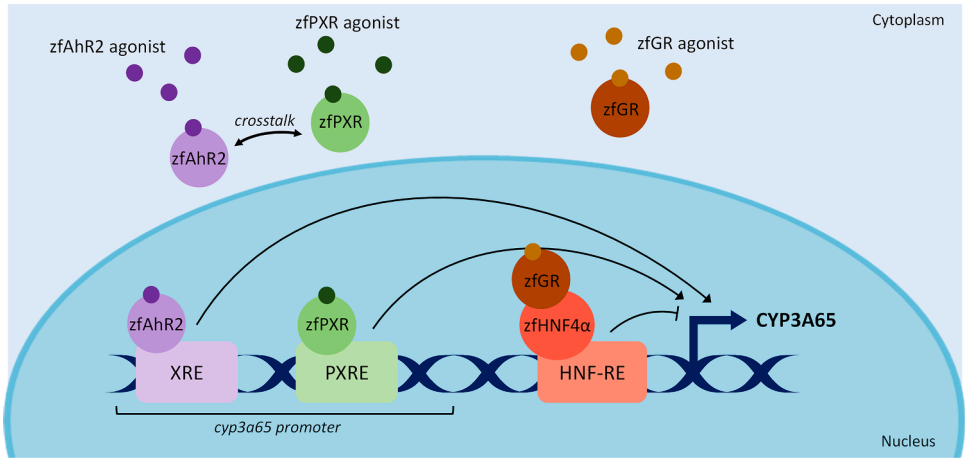


Fig. 7. Hypothesis for the regulation of cyp3a65 by zfAhR2, zfPXR and zfGR. While zfAhR2 and zfPXR link to their own response elements in the core of the cyp3a65 promoter, the binding of zfGR to zfHNF4α, itself linked to specific response elements downstream of the cyp3a65 promoter, modulates its expression. zfAhR2 and zfPXR agonist ligands promote the expression of cyp3a65 and zfGR inhibits its constitutive expression. XRE, PXRE and HNF-RE: response elements for zfAhR2, zfPXR and zfHNF4α respectively.

of zfGR to zfHNF4α, itself linked to its specific response elements (HNF-RE), could therefore modulate *cyp3a65* expression (Fig. 7). HNF4α is a cofactor of PXR, allowing the expression of *cyp3a4* in humans (Tirona et al., 2003; Dvorak, 2011), it could be hypothesized that in zebrafish, the binding of zfGR to HNF4α limits its role as a cofactor of zfPXR and is responsible for the observed decrease in the constitutive expression of *cyp3a65*. Overall, regulation of *cyp3a65* expression appears complex, mediated by both the ambivalent relationship existing between zfPXR and zfAhR2, and the zfGR receptor, probably through the intermediary of zfHNF4α (Fig. 7 and Table 2).

If human CAR (constitutive androstane receptor) is considered "lost" in teleostean fish, human transcription factors PXR, GR and HNF4α are part of the regulation of *cyp3a4* (Erradhouani et al., 2024), as zfPXR, zfGR and zfHNF4α are part of the regulation of *cyp3a65*. In addition, if human AhR does not seem to be directly involved in the regulation of *cyp3a4* expression, as it is the case for zfAhR2 and *cyp3a65* in zebrafish, it could be indirectly involved by promoting PXR expression, as shown in a human hepatocyte cell line (Rasmussen et al., 2017). These similarities between human *cyp3a4* and zebrafish *cyp3a65* regulation are encouraging, in the perspective to promote our assay for MDCs assessment.

Conclusion

In this study, we have shown that zfPXR and zfAhR2 cooperate to regulate *cyp3a65* expression. Their interactions are more complex than a simple reciprocal positive crosstalk but in contrast appear ambivalent. It is clear that results of such interactions on intestinal expression of the *cyp3a65* gene are highly dependent on the nature of the coexposure conditions.

Another major outcome of the study relies on the role of the zfGR in the constitutive expression of the *cyp3a65* gene. To our knowledge, it is the first report showing that agonists of the zfGR strongly inhibit the expression of *cyp3a65* in the developing intestine, an effect blocked in the presence of RU486, demonstrating that a functional zfGR is required in mediating the effect of zfGR agonists. We hypothesized that the mechanism through which zfGR regulates the expression of *cyp3a65* implies zfHNF4α (whose responsive elements are present downstream the *cyp3a65* promoter).

By enhancing our understanding of *cyp3a65* expression regulation, we improve the *tg(cyp3a65:GFP)* model in its ability to provide information on the mode of actions of MDCs in the intestine.

CRediT authorship contribution statement

Chedi Erradhouani: Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Benjamin Piccini:** Data curation. **Emmanuelle Maillot-Marechal:** Data curation. **Selim Aït-Aïssa:** Writing – review & editing. **Patrick Balaguer:** Data curation. **Xavier Coumoul:** Writing – review & editing, Conceptualization. **François Brion:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors deeply thank the anonymous reviewers for their thorough reading of the manuscript and their valuable suggestions. This study was funded by the H2020 OBERON project (grant agreement no 825712). The transgenic model was first developed within the project PestR from French National Research Program for Endocrine Disruptors (PNRPE). C.E. received a doctoral fellowship from INERIS (P190 – Ecotoxicologie). The authors acknowledge INERIS, INSERM and Université Paris-Cité for their financial and scientific support in this project, as well as the members of C.E.'s thesis committee, Dr. Etienne Blanc (Université Paris Cité) and Dr. Nicolas Diotel (Université de la Réunion).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2025.107250.

Data availability

Data will be made available on request.

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