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The unexpected teratogenicity of RXR antagonist UVI3003 *via* activation of PPARγ in *Xenopus tropicalis*

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ABSTRACT

The RXR agonist (triphenyltin, TPT) and the RXR antagonist (UVI3003) both show teratogenicity and, unexpectedly, induce similar malformations in *Xenopus tropicalis* embryos. In the present study, we exposed *X. tropicalis* embryos to UVI3003 in seven specific developmental windows and identified changes in gene expression. We further measured the ability of UVI3003 to activate *Xenopus* RXRα (xRXRα) and PPARγ (xPPARγ) *in vitro* and *in vivo*. We found that UVI3003 activated xPPARγ either in Cos7 cells (*in vitro*) or *Xenopus* embryos (*in vivo*). UVI3003 did not significantly activate human or mouse PPARγ *in vitro*; therefore, the activation of *Xenopus* PPARγ by UVI3003 is novel. The ability of UVI3003 to activate xPPARγ explains why UVI3003 and TPT yield similar phenotypes in *Xenopus* embryos. Our results indicate that activating PPARγ leads to teratogenic effects in *Xenopus* embryos. More generally, we infer that chemicals known to specifically modulate mammalian nuclear hormone receptors cannot be assumed to have the same activity in non-mammalian species, such as *Xenopus*. Rather they must be tested for activity and specificity on receptors of the species in question to avoid making inappropriate conclusions.

1. Introduction

Retinoic acid functions through two classes of receptors: the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (Mangelsdorf et al., 1994). These receptors are members of the nuclear receptor superfamily, modulate ligand-dependent gene expression by interacting as RXR/RAR heterodimers or RXR homodimers on specific target-gene DNA sequences known as hormone response elements (Willhite et al., 1996). In addition to their role in retinoid signaling, RXRs also serve as heterodimeric partners of nuclear receptors for vitamin D (VDR), thyroid hormone (TRs), and peroxisome proliferator activated receptors (PPARs), among others (Mangelsdorf et al., 1994). The RXRα–PPARγ heterodimer regulates transcription of genes involved in glucose and lipid homeostasis, and is considered to be a master regulator of adipocyte differentiation and lipid storage (Rosen and Spiegelman, 2001; Tontonoz and Spiegelman, 2008). Activation of PPARγ by organotins, thiazolidinediones, or lipids promotes the expression of genes that increase fatty acid storage and inhibits expression of genes that induce lipolysis (Ferré, 2004; Grün et al., 2006b; Tontonoz and Spiegelman, 2008).

In addition to natural, endogenous ligands, a few xenobiotic chemicals are known to activate or antagonize RXRs (Alsop et al., 2003; Li et al., 2008; Inoue et al., 2011; Jiang et al., 2012). It is known that RXR activation plays an important role in inducing the development of imposex in gastropods (Nishikawa et al., 2004), and RXR ligands can potentiate some of the teratogenic effects of RAR agonists in mice (*e.g.* spina bifida aperta, micrognathia, anal atresia, and tail defects) (Elmazar et al., 1997; Collins and Mao, 1999). The teratogenicity of RXR antagonists has received much less attention. However, water extracts from six major river systems and three drinking water treatment plants in China have been shown to contain RXR antagonists whereas RXR agonistic activity was not observed (Jiang et al., 2012). The RXR antagonistic activities of source water sample extracts ranged from 15.2% to 57.8% 5 μM 9-*cis*-RA (Jiang et al., 2012). Hexachlorocycolohexane (HCH), *p,p′*-dichlorodiphenyltrichloroethane (*p,p′*-DDT) and 2,4-Dichlorophenol (2,4-DCP) exhibited potent antagonistic activities at very low concentration (1×10^{-6} mol/L, ~10 times environmental level) (Li et al., 2008; Turusov et al., 2002; Li et al., 2014). RAR agonistic activity was found in seven sewage treatment plants and their receiving rivers in Beijing (Zhen et al., 2009). Taken together, these results demonstrate that an understanding of RXR antagonism and its role in teratogenicity in aquatic organisms is important.

In contrast to the fairly large collection of RXR agonists known to date (Lehmann et al., 1992; Vuligonda et al., 1996; Dawson, 2004), only a few RXR antagonists have been identified (Hashimoto and Miyachi, 2005). UVI3003 was reported to be a highly selective antagonist of RXRs and has been suggested to be a valid tool to study the function of RXRs (Nahoum et al., 2007). In our previous study, we found that UVI3003 induced multiple malformations in *X. tropicalis* embryos (Zhu et al., 2014). Unexpectedly, the phenotypes induced by UVI3003 are

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very similar to those induced by the organotin, triphenyltin (Supplementary Fig. 1) (Zhu et al., 2014), which is a well-known RXR and PPARγ agonist (Kanayama et al., 2005 and Grün and Blumberg, 2006a). Triphenyltin has been widely used as a biocide in antifouling paints and agriculture since the 1960s (Alzieu, 1996). Therefore, we hypothesized that UVI3003 was exerting its teratogenic effects through a mechanism similar to TPT (*e.g.*, through RXR-PPARγ), but perhaps not through RXR to induce malformations in *X. tropicalis* embryos.

RXRs have varied and complex expression and functions during the development of vertebrate embryos (Kastner et al., 1994). Long-term exposure experiments make it difficult to link the exact function of RXRs and the teratogenic characteristics of chemicals. For example, the RXR antagonists UVI3003 and HX531 induced divergent malformations in *X. tropicalis* embryos following 12 h of exposure (Hu et al., 2015b). Therefore, identifying stage-specific gene expression changes in response to RXR antagonists should be useful in shedding light on teratogenic mechanisms in *X. tropicalis*.

To test our hypothesis that antagonizing RXR could produce teratogenic effects, we treated *X. tropicalis* embryos with UVI3003 and sought to distinguish phenotypic malformations and gene expression changes characteristic of seven different chemical exposure windows. We further assessed the activity of UVI3003 on xRXRα and xPPARγ by transient transfection in Cos7 cells (*in vitro*) and by microinjection of *Xenopus* embryos (*in vivo*). Our aim was to determine the molecular mechanism of teratogenicity induced by UVI3003 in *Xenopus* embryos. The data show that although UVI3003 is a *bona fide* antagonist of *Xenopus* RXR, it has the novel and unexpected ability to activate *Xenopus* PPARγ (but not mouse or human PPARγ). This can explain the similar phenotypes induced by both UVI3003 and TPT, and suggests that these occur *via* activation of PPARγ.

2. Materials and methods

2.1. Exposure experiments using Xenopus tropicalis embryos

Xenopus tropicalis adults were obtained from Nasco (Fort Atkinson, WI, USA) and maintained according to previous methods (Yu et al., 2011). Breeding was induced by subcutaneous injection of human chorionic gonadotrophin (hCG) (Zhejiang, China) as described (Yu et al., 2011; Hu et al., 2015a). The exposure experiments were conducted following the Frog Embryo Teratogenesis Assay (FETAX) protocol (Fort and Paul, 2002) with some modifications. Briefly, approximately 12 h after the second injection of hCG, adults were removed from their tanks, and embryos were harvested without removing the jelly coats (Supplementary Fig. 2). UVI3003 (Cat# 847239-17-2, Tocris Bioscience, Bristol, UK) was dissolved in DMSO and then diluted into FETAX medium. Four replicate dishes ($n = 4$) were used in each control or treatment group of 20 embryos for morphological observations and real-time quantitative PCR analysis.

The EC_{50} of UVI3003 is 0.5 μM after 48 h treatment from NF10 in *X. tropicalis* embryos (Zhu et al., 2014). In this study, we chose 1, 1.5, 2 μM of UVI3003 to treat embryos in short exposure windows (6–8.5 h) from gastrulation (Nieuwkoop and Faber stage 10) to larval stage (NF43). 10 embryos were collected immediately after the exposure windows ended for real-time quantitative PCR analysis; the other 10 embryos were rinsed with FETAX medium three times and maintained at 26 ± 0.5 °C in the dark for later morphological analysis. All exposure experiments ended when the control embryos reached NF43. To minimize biological variation, embryos for each exposure window were chosen from one pair of frogs.

2.2. Real-time quantitative PCR analysis of gene expression in Xenopus tropicalis embryos

Total RNA was isolated from treated *X. tropicalis* embryos preserved in RNA*later* using RNeasy® Mini Kit (QIAGEN, GmBH, Germany). RNA concentrations were measured with a SMA4000 UV–vis Spectrophotometer (Merinton, Beijing, China). Reverse transcription of 1 μg of total RNA samples was carried out using PrimeScript™ RT reagent Kit with genomic DNA Eraser (Takara, Dalian, China). Primers were designed using Primer 3 and NCBI Primer-BLAST (Supplementary Table 1). Real-time quantitative PCR was performed according to our previous method (Yu et al., 2011). For each target mRNA, melting curves and gel electrophoresis verified the specificity of the amplified products and absence of primer dimers.

2.3. Luciferase reporter assay using in vitro model (Cos 7 cells)

pCMX-GAL4 plasmid fusion constructs of nuclear receptor ligand binding domains GAL4-human RXRα (Perlmann et al., 1996), - *Xenopus laevis* RXRα (Blumberg et al., 1992), - human PPARγ (Greene et al., 1994), - mouse PPARγ (Kliewer et al., 1994) were previously described (Chamorro-García et al., 2012). We isolated *Xenopus laevis* PPARγ from a cDNA library by PCR and cloned it into pCMX-GAL4 expression vector, its cloning primers are listed in Supplementary Table 2. One microgram pCMX-GAL4 effector plasmid was co-transfected with 5 μg pCMX-β-galactosidase transfection control, 5 μg tk-$(MH100)_4$-luciferase reporter and 14 μg pUC19 carrier plasmid (per 96-well plate) into Cos7 cells using calcium phosphate-mediated transient transfection (Sambrook and Russell, 2005). UVI3003 was added in 3-fold serial dilutions from 10^{-5} and 10^{-4} M for RXRα antagonism and PPARγ activation assays, respectively. TPT was serially diluted 10-fold or 3-fold from 10^{-6} M for RXRα and PPARγ activation assays. The control compounds HX531 (RXR antagonist), IRX4204 (formerly designated AGN194204 and NRX194204, RXR agonist) and ROSI (rosiglitazone, PPARγ agonist) were tested from 10^{-5} M in 10-fold serial dilutions (Kanayasu-Toyoda et al., 2005; Vuligonda et al., 1996). All transfections were performed in triplicate and reproduced in multiple experiments.

2.4. Luciferase reporter assay using in vivo model (Xenopus laevis embryos)

Xenopus laevis eggs were fertilized *in vitro* as described previously (Janesick et al., 2012), and embryos were staged according to Nieuwkoopand Faber (Nieuwkoop and Faber, 1956). Embryos were microinjected at the 2- or 4-cell stage with 50 pg/embryo pCMX-GAL4-xPPARγ mRNA or β-galactosidase (control) mRNA together with 50 pg/embryo tk-$(MH100)_4$-luciferase reporter DNA. Microinjected embryos were treated at stage 8 with the following chemicals (in 0.1× MBS): UVI3003 (1, 5, 10 μM), TPT (0.01, 0.05, 0.1 μM), TBT (RXR and PPARγ agonist, 0.05 μM) or vehicle (0.1% DMSO). For each treatment, 25 embryos were treated in glass 60-mm Petri dishes containing 10 mL of MBS + chemical, and two replicate dishes were used for each concentration. Treated embryos were separated into five-embryo aliquots at neural stage for luciferase assays (Janesick et al., 2012, 2014). Each group of five embryos was considered one biological replicate. All animal experiments were approved and performed in accordance with Institutional Animal Care and Use Committee protocols.

2.5. Statistical analysis

Data were analyzed using SPSS16.0 software. All data were tested for homogeneity of variance with Levene's statistic, if the homogeneity value is >0.05, the variances are equal and the homogeneity of variance assumption has been met. Mean differences among control and treatments were assessed by one-way analysis of variance (ANOVA) followed by Dunnett post-hoc test. Independent samples *t*-test was used for two group comparison. The luciferase data were reported as fold change over vehicle control (0.1% DMSO) ± SEM using standard propagation of error (Bevington and Robinson, 2003). EC_{50} (half effective concentration) and IC_{50} (half inhibitory concentration) of nuclear receptor activation or antagonism assays were calculated by nonlinear regression