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

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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 RXRs (sRXRs) and PPARγ (PPARγ) in vitro and in vivo. We found that UVI3003 activated PPARγ 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 PPARγ 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.

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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 (Wllhite 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 RXRs–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% to 57.8% (more than 10 times). 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 UV3003 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 UV3003 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 UV3003 and sought to distinguish phenotypic malformations and gene expression changes characteristic of seven different chemical exposure windows. We further assessed the activity of UV3003 on XR/Rxs 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 UV3003 in Xenopus embryos. The data show that although UV3003 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 UV3003 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). UV3003 (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 EC50 of UV3003 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 UV3003 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 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 (Kliever et al., 1994) were previously described (Chamorro-Garcia 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 1. Two 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 (Sanbrook and Russell, 2005). UV3003 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^{-5} M for RXRs and PPARγ activation assays. The control compounds HX531 (RXR antagonist), I RX4204 (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; Vuglingda 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 Nieuwkoop and 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): UV3003 (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). EC50 (half effective concentration) and IC50 (half inhibitory concentration) of nuclear receptor activation or antagonism assays were calculated by nonlinear regression.
(variable slope) of log (concentration of agonist or antagonist) versus response using GraphPad Prism 5.

3. Results

3.1. Multiple malformations are induced by UVI3003 in Xenopus tropicalis embryos

Exposure to UVI3003 during different developmental windows induced obviously developmental delay and multiple malformations (Fig. 1). The most common phenotypes were reduced forehead, turbid eye lens and narrow fin in UVI3003 treatment groups. Proctodaeum elongation was observed in all NF10–19 treatment groups and in the NF19–25 high dose group, while enlarged proctodaeum phenotype occurred in late exposure windows. The teratogenic ability of UVI3003 was weak in NF 10–25 stages, while it was significantly increased in NF 25–39 and then decreased in NF 39–43. The body length of embryos decreased significantly in all NF 31–36 NF36–39 treatment groups and in the NF39–41 middle and high dose groups (Supplementary Fig. 3).

3.2. PPARγ was down-regulated by UVI3003 in Xenopus tropicalis embryos

The expression of mRNAs encoding RXRs and their heterodimeric partners RARs, PPARs and TRs were evaluated after 7 different

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Fig. 1. Multiple malformations induced by UVI3003 in X. tropicalis embryos during different stages. Embryos were collected for morphological observations when the control embryos were cultured to stage 43. Each dish of 10 embryos was considered to be one replicate, and there were 4 dishes per group (n = 4). The percent of embryos observed with the malformation is provided in the top right of each photo. Abbreviations: af, absence of fin; bn, bent notochord; cg, cement gland; dcg, displaced cement gland; eh, edema in heart; ep, enlarged proctodaeum; f, fin; h, head; hp, hypopigmentation; n, notochord; nf, narrow fin; p, proctodaeum; pe, proctodaeum elongation; rf, reduced forehead; sp, skin pigmentation; tel, turbid eye lens; Scale bar = 0.5 mm.
UVI3003-exposure windows. We found RARβ was down-regulated in early exposure periods (Fig. 2B2), whereas RXRs, TRα and TRβ were affected after late embryogenesis treatment (Fig. 2A, D). The expression of PPARγ was clearly decreased during all the treatment periods (Fig. 2C3). We tested the effects of TPT treatment during the most sensitive exposure window and found that PPARγ was also down-regulated at high dose (Fig. 3). Thus, our results showed that PPARγ was down-regulated by UVI3003 and TPT in X. tropicalis embryos.

3.3. Inhibition of xRXRα and hRXRα by UVI3003

In order to better understand the phenotypes elicited by UVI3003 in Xenopus embryos, we sought to test the specificity of UVI3003 on Xenopus nuclear receptors. Transient transfection assays in Cos7 cells showed that UVI3003 inhibited the activity of Xenopus and human RXRα (Fig. 4A, B), yielding a half inhibitory concentration (IC50) of approximately 0.2 μM (Table 1). UVI3003 was approximately 10- and 5-fold more potent on Xenopus and human RXRα than was another RXR antagonist HX531 (Table 1). TPT strongly activated Xenopus and human RXRα, and the half effective concentration (EC50) was 1.3 × 10^{-4} μM and 2.2 × 10^{-4} μM (Fig. 4C, D). TPT produced noticeable cytotoxicity at 0.1 μM judged by approximately 10-fold reduced β-galactosidase activity.

3.4. Activation of xPPARγ by UVI3003

In transient transfection assays, UVI3003 fully activated xPPARγ (EC50 = 12.6 μM) (Table 1). The maximal activation reached the same level as a well-known agonist of PPARγ, rosiglitazone (ROSİ), although UVI3003 was not as efficacious as ROSI (Fig. 5A). In contrast, UVI3003 was almost completely inactive on hPPARγ and mPPARγ (Fig. 5B–C). Next, we tested the ability of UVI3003 to activate xPPARγ, in vivo. xPPARγ mRNA and luciferase reporter DNA were microinjected into Xenopus embryos. The results showed that UVI3003 could activate xPPARγ (Fig. 6). 1 μM UVI3003 significantly activated xPPARγ (~2-fold), similar to the activation observed with either 0.05 μM TPT or...
0.05 μM TBT (Fig. 6). Increasing the dose to 5 μM or 10 μM, UVI3003 enhanced activation of xPPARγ to 3–3.5 fold over DMSO controls. TPT activated xPPARγ in an appropriate, concentration-dependent manner.

4. Discussion

In the present paper, we employed a modified frog embryo teratogenesis assay Xenopus (FETAX) protocol (Fort and Paul, 2002; Yu et al., 2011) to assess UV13003-induced malformations in X. tropicalis embryos specific to certain developmental windows of chemical exposure. In a previous study, we found that the well-known agonist of RXR, triphenyltin (TPT), induced stage-specific malformations and phenotypic changes in Xenopus embryos (Yuan et al., 2011). Both UV13003 and TPT showed higher toxicity during late embryogenesis and shared very similar characteristics such as reduced forehead, turbid eye lens, and TPT showed higher toxicity during late embryogenesis and shared very similar characteristics such as reduced forehead, turbid eye lens, and TPT (Barak et al., 1999; Kubota et al., 1999; Rosen et al., 1999). Grün et al. (2006b) found that in mouse liver and testis organs, PPARγ expression was decreased after treatment with TBT (tributyltin) and troglitazone (PPARγ agonist). The exposure of zebrafish to 10 ng Sn/L TBT from pre-hatching to 9 months of age alters the bodyweight, hepatosomatic index and up-regulated the transcription of PPARγ in liver (Lysimachou et al., 2015). Thus, the expression of PPARγ was regulated by PPARγ activating ligands but its change is different among species and organs.

Our reporter assays showed that although UV13003 is a bona fide antagonist of RXRs in Xenopus, it has an unexpected ability to activate Xenopus PPARγ but not mouse or human PPARγ. Therefore, chemicals that are stated to activate or antagonize mammalian nuclear hormone receptors cannot be assumed to possess the same activity, or the same selectivity for receptors of non-mammalian species, such as Xenopus. The differences in the activation of PPARγ by UV13003 in different species might be due to the different sequence of PPARγ in specific species. For example, certain phthalates, perfluorinated compounds and halo-nated derivatives of BPA are common activators of human PPARγ and p450, GAL4-xRXRα, GAL4-hRXRα against 10 nM 4204. C–D. TPT activates GAL4-xRXRα, GAL4-hRXRα with EC50 1.3 × 10−4 μM and 2.2 × 10−4 μM. Data represent reporter luciferase activity normalized to β-galactosidase and plotted as the average fold change ± SEM (n = 3) relative to DMSO (0.05%) controls.

Table 1

<table>
<thead>
<tr>
<th>Ligand IC50 and EC50 values for nuclear receptor LBDs.</th>
<th>Gal4-NR LBD</th>
<th>IC50 values, μM</th>
<th>EC50 values, μM</th>
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<tbody>
<tr>
<td></td>
<td>UVI3003</td>
<td>HX531</td>
<td>TPT</td>
</tr>
<tr>
<td>xRXRα</td>
<td>0.22</td>
<td>2.8</td>
<td>1.3 × 10−4</td>
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<tr>
<td>hRXRα</td>
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<td>1.0</td>
<td>2.2 × 10−4</td>
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<tr>
<td>xPPARγ</td>
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<td>ND</td>
<td>0.01</td>
</tr>
<tr>
<td>hPPARγ</td>
<td>12.6</td>
<td>ND</td>
<td>0.02</td>
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</tbody>
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na, not active; ND, not determined. IC50 and EC50 values were calculated from nonlinear regression analysis of dose-response curves of GAL4-NR LBD activation or inhibition in transiently transfected Cos7 cells after 24 h ligand exposure.
zebrafish PPARγ, whereas the potent pharmaceutical iPpARγ agonists thiazolidiones are not recognized by zebrafish PPARγ (Grimaldi et al., 2015; Riu et al., 2014). Comparison of human and zebrafish PPARγ sequences reveals that several residue differences could explain the differential ligand specificity of the various species (Grimaldi et al., 2015). Thus, conclusions based on assumptions about the specificity of mammalian receptor-selective ligands toward non-mammalian species may not be accurate. Our work highlights the need to test the activity of such chemicals against receptors of the target species before making inferences about the mechanisms through which they act.

In brief, we documented for the first time that a well-known RXRs antagonist (UV30003) could also activate iPpARγ, but this unique characterist is only observed in Xenopus rather than in mouse or human in vitro. Further in vivo work is needed to clarify the difference among species using UV3003. Our results indicate that PPARγ is likely to play a critical role in inducing malformations in Xenopus embryos after exposure to UV3003.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2016.11.014.

Conflict of interest statement

None of the authors have competing interests. Every author has seen and contributed to the final draft and also agrees the manuscript is ready to submit.

Transparency document

The Transparency document associated with this article can be found in the online version.

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