

Tributyltin chloride and triphenyltin chloride inhibit steroid hormone secretion by mouse Leydig cells TM3 and human adrenocortical cells H295R

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Objective. To find a possible balance between toxicity and potential anticancer activity of novel triorganotin compounds, retinoid X receptor (RXR) agonists, investigation the relationship between the structure, physicochemical properties, and biological activity of these compounds in non-tumorigenic and tumorigenic cells is required. This study aimed to investigate the effects of selected triorganotin compounds, tributyltin chloride (TBT-Cl) and triphenyltin chloride (TPT-Cl), on viability and steroidogenic function of non-tumorigenic mouse Leydig cell line TM3 and human adrenocortical carcinoma cell line H295R.

Methods. Gene and protein expression of RXR subtypes (RXR α , RXR β , RXR γ) in TM3 Leydig cells was identified by qualitative PCR and immunofluorescence. The potency of TBT-Cl and TPT-Cl to induce mRNA expression of RXR subtypes in TM3 cells was analyzed by semi-quantitative real-time PCR. After exposure of TM3 and H295R cells to different concentration of TBT-Cl and TPT-Cl (0.1 nM–1 μ M) for different time periods (24, 48, 72 h), cell viability was evaluated by detection of mitochondrial activity (MTT assay) and membrane integrity (LDH assay), and steroid hormone (testosterone, cortisol) levels in culture media were measured by radioimmunoassay.

Results. Qualitative PCR and immunofluorescence examination revealed that all RXR subtypes, RXR α , RXR β , RXR γ , are expressed in TM3 Leydig cells. TBT-Cl (500 nM) activated the expression of RXR α and RXR β and at a concentration of 100 nM, RXR γ expression. TPT-Cl (10 and 100 nM) activated the expression of all three RXR subtypes and at a concentration of 500 nM, reduced RXR α expression similar to 9cRA (100 nM). Both compounds, TBT-Cl and TPT-Cl, induced a dose- and time-dependent significant decrease in the viability of TM3 Leydig cells and, in addition, TBT-Cl (500 nM and 1 μ M) significantly inhibited androstenedione-induced testosterone secretion by these cells. Similarly, both tested triorganotin compounds (1 μ M) significantly decreased viability of adrenocortical H295R cells and basal as well as forskolin-stimulated testosterone and cortisol secretions by these cells.

Conclusion. TBT-Cl and TPT-Cl compromised cell viability and induced important functional alteration in Leydig cells probably through RXR activation with potential implications for male reproductive function. Endocrine-disrupting properties of TBT-Cl and TPT-Cl were demonstrated in adrenocortical H295R cells.

Keywords: Leydig cells, adrenocortical cells, triorganotin compounds, retinoid X receptors, viability, steroidogenesis, testosterone, cortisol

The authors would like to dedicate this research article to the Endocrine Regulations 60th anniversary.

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Organotin compounds are organic derivatives of tin (Sn) widely used in the plastics and communication industries, and in several agricultural applications. Tri-substituted organotins such as tributyltin (TBT) and triphenyltin (TPT) were previously used primarily as antifouling agents in ship hull paints until their use was banned due to concerns about their toxicity to marine organisms (ATSDR 2005; EU 2006; RPA 2005; WHO 2006). These compounds might still be in use as active ingredients in biocides and pesticides for certain consumer products (plastics, food packages, paints, wood preservatives, and rodent repellants) (ATSDR 2005; RPA 2005). Such widespread use resulted in the release of increasing amounts of triorganotins into the environment. Despite global restrictions, triorganotin compounds remain prevalent in marine sediments and aquatic organisms due to their persistence and bioaccumulation, with human exposure primarily occurring via the food chain (Boyer et al. 1989; Yi et al. 2012; Sousa et al. 2014; Peivasteh-Roudsari et al. 2023).

TBT and TPT are considered highly toxic to aquatic organisms, they have been shown to produce imposex in female snails and fish, and marine mollusks, presumably through elevated testosterone levels and abnormal modulation of the retinoid X receptor (RXR) (Antizar-Ladislao 2008; Lima et al. 2011; Okoro et al. 2011). Based on these findings and the results of reproductive and developmental toxicity reports in rodent models as well as *in vitro* studies, triorganotins are considered as endocrine-disrupting chemicals (EDCs) and metabolic disruptors (Antizar-Ladislao 2008; WHO 2006; Graceli et al. 2013). Animal studies revealed that triorganotin compounds may induce gender-specific changes in the morphological functioning of reproductive organs, including gonadal cell dysfunction and weight variation in the sex organs coupled with decreased circulating levels of steroid hormones, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and induction of oxidative stress and germ cell apoptosis (de Araujo et al. 2018). The underlying molecular mechanisms of their effects and action have not been clearly elucidated; however, interference with steroidogenic pathways and related enzymes including aromatase (CYP19A1) and β -hydroxysteroid dehydrogenase (β HSD), cell signal transduction (via cAMP) and the transcription of various target proteins, including transcription factors, appears to be associated with endocrine effects (Delgado Filho et al. 2011; Nakanishi 2008). Evidences showed that organotin compounds elicited their endocrine disrupting effects through interactions with nuclear and steroid receptors, such

as estrogen receptor (ER), retinoid X receptors (RXR), and peroxisome proliferator activated receptor (PPAR) (Brtko and Dvorak 2015, 2020; Nakanishi 2008). Epidemiological and biomonitoring studies report that TBT concentrations in human blood range from approximately 16 to 400 nM (Whalen et al. 1999). This exposure levels overlap with concentrations demonstrated to activate nuclear receptors such as RXR and PPAR γ , mechanisms linked to adipogenesis, endocrine disruption, and metabolic dysfunction (Nakanishi 2008).

Rexinoids, RXR agonists, play key roles in proliferation, differentiation, and apoptosis, processes that are highly relevant to cancer, making RXRs attractive targets for cancer therapy (Brtko and Dvorak 2020). As potential RXR ligands, organometallic compounds, including organotin derivatives, have been suggested to be promising candidates for new anticancer drugs. Novel triorganotin compounds have been designed and synthesized to find a possible balance between toxicity and feasible anticancer activity (Stefanizzi et al. 2023). Investigating the relationship between the structure, physicochemical properties, and biological activity of novel organotin compounds in non-tumorigenic and tumorigenic cells is still a subject of great interest.

There is the limited availability of physiologically relevant *in vitro* models or standardized procedure to access effects of chemicals on steroidogenesis in gonadal cells. Leydig cells are used as an *in vitro* model for the majority of investigations on the regulation of testicular steroidogenesis and steroidogenic enzymes, however, the existing models have inherent limitations (Rehurkova et al. 2026). In the testes, Leydig cells play a crucial role in regulating the process of spermatogenesis and synthesizing androgens, mainly testosterone, and other factors that regulate male reproductive development, function, and fertility. Morphology, gene expression and steroid hormone profiles of Leydig cells differ markedly across developmental stages and aging. The synthesis of androgens by adult Leydig cells is under the control of pituitary-derived luteinizing hormone (LH) and a number of factors locally produced by the testis (Chen et al. 2009; Saez 2014; Li et al. 2022). Disorders resulting from subtle deficiencies in this process are common in humans; however, environmental exposure and lifestyle factors could contribute to alteration of Leydig cell function leading to adverse effects on testicular functions (Scott et al. 2009; Yang et al. 2025).

The human adrenocortical carcinoma cell line H295R is widely recognized model for screening the

effects of chemicals on steroidogenesis because of its ability to express the gene, enzymes and hormones involved in adrenal and gonadal steroidogenesis (Hecker *et al.* 2006; Maglich *et al.* 2014). The H295R steroidogenesis assay (Test No. 456: H295R Steroidogenesis Assay) has been developed and validated in comprehensive studies by the Organization for Economic Co-operation and Development (OECD) guideline for identifying steroidogenesis disruptors in a regulatory context (Hollert and Giesy 2007; Hecker *et al.* 2011; OECD 2022).

The aim of the present study was to examine the effects of selected triorganotin compounds, tributyltin chloride (TBT-Cl) and triphenyltin chloride (TPT-Cl), on viability and steroidogenic function of non-tumorigenic mouse Leydig cell line TM3 and human adrenocortical carcinoma cells H295R.

Materials and Methods

Chemicals. Tributyltin chloride (TBT-Cl), triphenyltin chloride (TPT-Cl), 9-*cis*-retinoic acid (9cRA), forskolin (FSK), 4-Androstenedione, dimethyl sulfoxide (DMSO), Triton-X100, and Tween20 were purchased from Merck (Sigma-Aldrich, Germany). Working stock solutions of triorganotin compounds, 9cRA, FSK and androstenedione were prepared by dissolving them in absolute ethanol (99.9%, CentralChem, Slovakia) and stored at -20°C. Before cell treatment, serial dilutions (for TBT-Cl, TPT-Cl) or single dilutions (for 9cRA, FSK, androstenedione) were prepared into the appropriate culture medium to achieve a range of concentrations (0.1 nM – 1µM for TBT-Cl and TPT-Cl) or the tested concentration (100 nM for 9cRA, 1 µM for FSK and androstenedione) so that the final ethanol concentration did not exceed 0.01%. The equal volume of ethanol was added

to the control cells. The chemical structure of 9cRA, TBT-Cl and TPT-Cl is shown in Figure 1.

Cell culture. Non-tumorigenic mouse Leydig cell line TM3 (ECACC 91060526, Merck, Sigma Aldrich, Germany) was purchased from The European Collection of Authenticated Cell Cultures (ECACC) (Salisbury, UK) and cultured in Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F12) (1:1) (Merck, Sigma-Aldrich, Germany) supplemented with penicillin-streptomycin-glutamine (Biosera, France), amphotericin (Biosera, France), 5% horse serum (HS) (Biosera, France), and 2.5% fetal bovine serum (FBS) (Biosera, France) in a humidified incubator at 37°C with 5% CO₂. The medium was changed three times per week and the subculture was performed once a week using 0.25% trypsin-EDTA (Biosera, France). Cells between passages 5–25 were used.

Human adrenocortical carcinoma cell line NCI-H295R [H295R] (ATCC CRL-2128) was purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured in DMEM/F12 supplemented with penicillin-streptomycin, amphotericin, 1% insulin-transferrin-selenium (ITS+Premix, Corning, USA), and 2.5% Nu-Serum I (Corning, USA) in a humidified incubator at 37°C with 5% CO₂. Cells were subculturing one a week using 0.25% trypsin-EDTA, the medium was replaced three times per week. Cells between passages 5–15 were used.

Reverse transcription-semiquantitative real time and qualitative PCR. TM3 cells were seeded in Petri dishes (Ø60 mm, TPP, Switzerland) at a density of 1.2×10⁶ cells/well, left to adhere for 24 h and then exposed to 9cRA (100 nM), TBT-Cl and TPT-Cl (both 10, 100, and 500 nM) for 48 h. After treatment, total RNA was isolated using Monarch® Total RNA Miniprep Kit (New England Biolabs, MA, USA) according to the manufacturer's instructions.

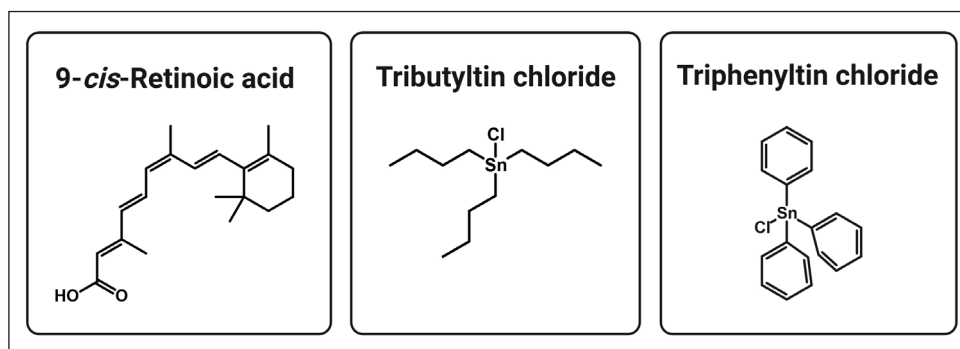


Figure 1. Chemical structure of 9-*cis*-retinoic acid, tributyltin chloride and triphenyltin chloride. Created in BioRender. Scsukova, S. (2026) BioRender.com/9p8iv4s.

The concentration of RNA was determined by spectrophotometry at 260 nm and the purity assessed from the ratio of absorbance, A₂₆₀/A₂₈₀ nm, using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Germany). Reverse transcription (RT) was performed with 2 µg of total RNA and Thermo Scientific™ RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol. Semi-quantitative real-time PCR was performed in a total volume of 20 µl using the SensiFAST™ SYBR Hi-Rox Kit (Bioline, Great Britain) and 0.25 mM of each primer, with RT products at 10 ng (β-actin) and 30 ng (the other genes). Amplification and detection were performed with an ABI Prisma 7900HT detection system (Applied Biosystems, USA) under the following conditions: 95 °C for 2 min, 45 cycles of denaturation (95 °C, 5 s) and annealing (60 °C, 30 s), and a final melting curve analysis. The sequences of the primers employed in this study are as follows: RXRα, F: 5'-ACA TGC AGA TGG ACA AGA CG-3', R: 5'-GGG TTT GAG AGC CCC TTA GA-3'; RXRβ, F: 5'-GTT CTT CCA TGG GGT CTC CT-3', R: 5'-GGA GCG ACA CTG TGG AGT TAA T-3'; and RXRγ, F: 5'-TTG GAA TGG CTC AAA CCA C-3', R: 5'-AGG GCT GGG CAC TAT CTC TT-3'; β-actin, F: 5'-GAC TCA TCG TAC TCC TGC TT-3', R: 5'-AGT GTG ACG TTG ACA TCC GT-3'. The data are expressed using the 2-ΔΔC_q method, with each mRNA level normalized to that of the housekeeping gene β-actin. Qualitative PCR was performed in a 25 µl total volume consisting of 4 µl RT mixture, 1x PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 1 pmol of each specific gene primer set, and 0.3 U of DyNAzyme II DNA polymerase (Finnzymes OY, Finland) in the manufacturer's buffer. After treatment of samples at 94 °C for 3 min to inactivate reverse transcriptase, PCR consisted of 35 cycles of denaturing (95 °C, 45 s), annealing (30 s), extension (72 °C, 90 s), and a final extension at 72 °C for 10 min. The oligonucleotide

sequences of primers used in the PCR, as well as the annealing temperatures and sizes of expected PCR products, are summarized in Table 1. The PCR products were separated on 2% agarose gel and stained with ethidium bromide. The band intensities were measured using the STS 6220I Documentation System (Ultralum, USA) and normalized to the GAPDH PCR product band intensity.

Immunofluorescence. TM3 Leydig cells were seeded on plastic tissue culture coverslips (Ø13 mm, Sarstedt, Germany) in 24-well culture plates (TPP, Switzerland) at a density of 5×10⁴ cells/well. After 48 h of incubation without any treatment, cells were washed with cold phosphate-buffered saline (PBS) (Biosera, France), fixed using 4% paraformaldehyde (PFA) for 10 min at room temperature (RT), permeabilized with 0.1% Triton-X100 in PBS for 5 min and blocked with 10% normal goat serum (Abcam, UK) in PBST (0.1% Tween20 in PBS) for 1 h at RT. After blocking, cells were incubated with primary antibodies anti-rabbit anti-retinoid X receptor alpha/RXRα antibody [EPR7106] (1:100) (ab125001, Abcam, UK), anti-mouse RXRβ Antibody (13-17): sc-741 (1:100) (Santa Cruz, Germany), and anti-rabbit anti-RXRγ antibody (prediluted, ab15519, Abcam, UK) overnight at 4 °C. After washing with PBST, cells were incubated with a fluorescence secondary antibody goat anti-rabbit IgG H&L (Alexa Fluor® 555) (1:1000) (ab150078, Abcam, UK) or goat anti-mouse IgG H&L (Alexa Fluor® 555) (1:1000) (ab150114, Abcam, UK) for 1 h at RT, followed by nuclear counterstaining with Hoechst 33342 (1 µg/ml in PBS) (Thermo Fisher Scientific, MA, USA) for 5 min at RT. After washing with DPBS, coverslips were carefully removed from the plates and mounted with glycerol (Merck, Sigma-Aldrich, Germany) on adhesive slides. Zeiss Axio Imager A1 and AxioCam ERc 5s camera (Carl Zeiss, Germany) was used to obtain fluorescence images.

Cell viability. Effects of TBT-Cl and TPT-Cl on

Table 1
List of primers for qualitative PCR

Gene	Oligonucleotide primers	Annealing temperature	Expected size (bp)
RXRα	5'-CTCTACCCAGGTGAACCTCTT-3' 5'-TGCTGCTCACAGGGTTCATG-3'	58.4 °C	293
RXRβ	5'-CCAGACAGCTCCTCCCCAAA-3' 5'-TGCACAGAGCCGTTTGCCAG-3'	57 °C	342
RXRγ	5'-GAGAGCGAGCAGAGAGTGAGG-3' 5'-CACACTCATGTGCGCCGTAGG-3'	57 °C	125
GAPDH	5'-TGAACGGGAAGCTCACTGG-3' 5'-TCCACCACCCTGTTGCTGTA-3'	60 °C	307

Abbreviations: GAPDH – glyceraldehyde 3-phosphate dehydrogenase; RXR – retinoid X receptor.

viability of TM3 and H295R cells were assessed by examining the inhibition of cell metabolic activity performed through a colorimetric method based on the reduction of tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Merck, Sigma-Aldrich, Germany). The MTT reagent was diluted in sterile PBS at a concentration of 5 mg/ml. For TM3 cells, the assay was performed by seeding 2×10^4 cells/well in 96-well culture plates (TPP, Switzerland), cells were left to attach for 24 h and incubated in complete culture medium in the presence or absence of various concentrations of TBT-Cl and TPT-Cl (both 0.1 nM–1 μ M) for 24, 48, and 72 h. H295R cells were seeded at a density of 4×10^4 cells/well in 96-well culture plates, left to attach for 24 h and incubated in complete culture medium in the presence or absence of TBT-Cl and TPT-Cl (both 0.1 nM and 1 μ M) for 24 and 48 h. Each concentration of the tested compounds (added in the volume of 100 μ l) was tested in sextuplicate. After exposure, 10 μ l of MTT solution were added to each well and incubated in the dark at 37°C in a CO₂ incubator for an additional 3 h. Thereafter, the culture media were removed and 100 μ l of DMSO was added to each well to solubilize formed formazan crystals. The absorbance was measured at wavelengths of 570 nm (absorption maximum) and 630 nm (background) using the Synergy MX Microplate Reader (BioTek Instruments, Winooski, VT, USA). The concentrations of TBT-Cl and TPT-Cl that inhibited cell survival to 50% (IC₅₀) was determined by GraphPad Prism version 8 (GraphPad Software, La Jolla, California, USA).

Lactate dehydrogenase assay. Effects of TBT-Cl and TPT-Cl on cell membrane integrity were analyzed by determining the activity of lactate dehydrogenase (LDH) using CytoTox 96° Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA) according to the manufacturer's protocol. TM3 Leydig cells were seeded at a density of 2×10^4 cells/well in 96-well culture plates (TPP, Switzerland), left to attach for 24 h, and subsequently exposed to various concentrations of TBT-Cl and TPT-Cl (both 1 nM–1 μ M) in phenol red-free complete culture medium for 24 h, 48 h and 72 h. Each concentration of the tested compounds (added in the volume of 100 μ l) was tested in sextuplicate. After treatment, 50 μ l aliquots from all test and control wells were transferred to 96-well flat clear bottom plate (Sarstedt, Germany) and 50 μ l of the CytoTox 96° Reagent was added to each well. After 30-min incubation in the dark at RT, 50 μ l of Stop Solution was added to each well. The optical density of the color generated was determined at a wavelength

of 490 nm using the Synergy MX Microplate Reader (BioTek Instruments, Winooski, VT, USA).

Steroid hormone measurement. For the steroid assay, TM3 Leydig cells were seeded at a density of 1×10^5 cells/well in 96-well culture plates (TPP, Switzerland). After the 24 h adherence period in complete culture medium, cells were incubated with TBT-Cl and TPT-Cl (both 500 nM and 1 μ M) in phenol red-free culture medium in the absence (control) or presence of androstenedione (1 μ M) for 24 h. Each concentration of the tested compounds (added in the volume of 100 μ l) was tested in sextuplicate. Adrenocortical H295R cells were seeded at a density of 3×10^5 cells/well in 24-well plates (TPP, Switzerland), incubated in complete growth medium for 48 h and subsequently exposed to TBT-Cl and TPT-Cl (both 1 μ M) in phenol red-free culture medium in the absence (control) or presence of FSK (1 μ M) for 48 h. Each concentration of the tested compounds (added in the volume of 500 μ l) was tested in duplicate. After exposure, culture media were collected and stored at –20°C until further analysis. Selected steroid hormone levels were measured using commercial radioimmunoassay kits according to the manufacturer's instructions.

Testosterone levels were measured using a Testosterone [I-125] RIA Kit (RK-61CT; range 0–60 nM; IZOTOP, Institute of Isotopes, Budapest, Hungary) with a sensitivity of 0.12 nM (0.035 ng/ml). The intra-assay and inter-assay coefficients of variation were between 2.8–8.8% and 6.4–12.0%, respectively. Cortisol levels were measured using a Cortisol [125I] RIA Kit (RK-240CT; range 0–1600 nM; IZOTOP, Institute of Isotopes, Budapest, Hungary) with a sensitivity of 2.9 nM (1.05 ng/ml). The intra-assay and inter-assay coefficients of variation were between 2.47–6.81% and 1.65–4.70%, respectively.

Statistical analysis. Data are expressed as mean $\pm$ standard deviation (SD) or as mean $\pm$ standard error of mean (SEM) from two to four independent experiments depending on the analysis performed. Statistical analysis and data visualization were conducted using GraphPad Prism version 8 (GraphPad Software, La Jolla, California, USA). Statistical comparisons between experimental groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A *p*-value <0.05 was considered statistically significant.

Results

Gene and protein expression of RXR subtypes

in TM3 Leydig cells. Since TBT-Cl and TPT-Cl have been shown to act as RXR agonists, we aimed to confirm expression of RXR subtypes in mouse TM3 Leydig cells. Qualitative PCR analysis revealed the gene expression of all three RXR subtypes in TM3 cells. For comparison, we also identified the presence of RXR subtypes in the uterus (Figure 2). The sizes of

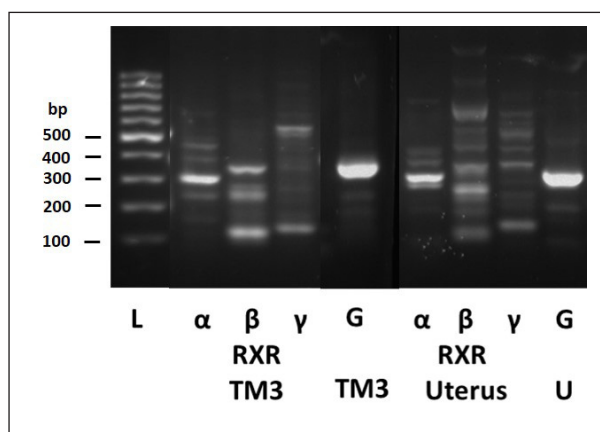


Figure 2. Identification of retinoid X receptor (RXR) subtypes (RXR α , RXR β , RXR γ) in untreated TM3 Leydig cells and uterus (U) by qualitative PCR. Abbreviations: G – GADPH; L – ladder.

the products matched those predicted from the sites of primer annealing (Table 1). The protein expression of RXR subtypes, RXR α , RXR β and RXR γ , was detected in nuclei of TM3 Leydig cells by immunofluorescence (Figure 3).

To examine induction of the transactivation function of RXRs in TM3 Leydig cells by TBT-Cl and TPT-Cl, a semiquantitative real-time PCR was used. After 48-h treatment, the natural RXR ligand 9cRA at 100 nM concentration significantly reduced the mRNA expression of RXR α ($p < 0.01$) compared to untreated cells (control) (Figure 4A); however, had no effect on the gene expression of RXR β and RXR γ subtypes (Figure 4B, C). TBT-Cl significantly increased the RXR α and RXR β mRNA expression at a concentration of 500 nM (both $p < 0.001$) (Figure 4A, B) and the RXR γ mRNA expression at 100 nM concentration ($p < 0.001$) (Figure 4C) compared to corresponding controls and 9cRA. TPT-Cl significantly enhanced the expression of all RXR subtypes at concentrations of 10 and 100 nM ($p < 0.001$) compared to corresponding controls and 9cRA (Figure 4). Comparable to the effect of 9cRA on the RXR α expression, TBT-Cl at 500 nM concentration significantly inhibited the mRNA expression of RXR α ($p < 0.001$) compared to control (Figure 4A), but

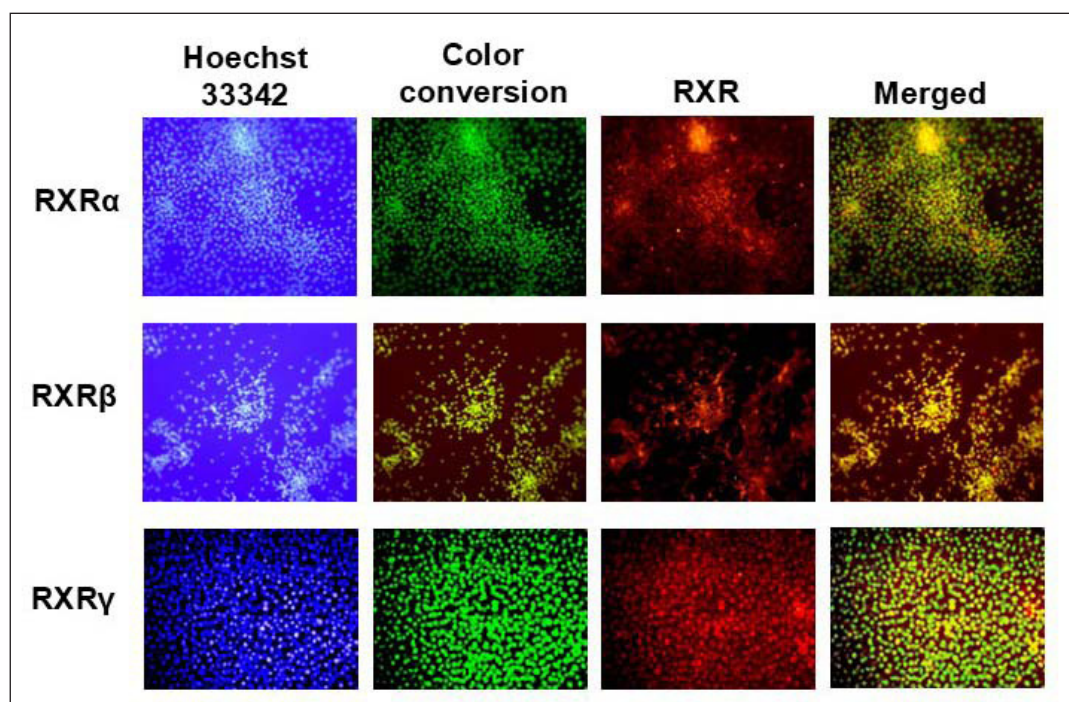


Figure 3. Immunofluorescence detection of nuclear retinoid receptors (RXR) subtypes, RXR α , RXR β , RXR γ , in untreated TM3 Leydig cells. Staining for cell nuclei is visualized in blue, converted into green, and for RXR α , RXR β , RXR γ in red. Co-localized staining appears in yellow in merged images.

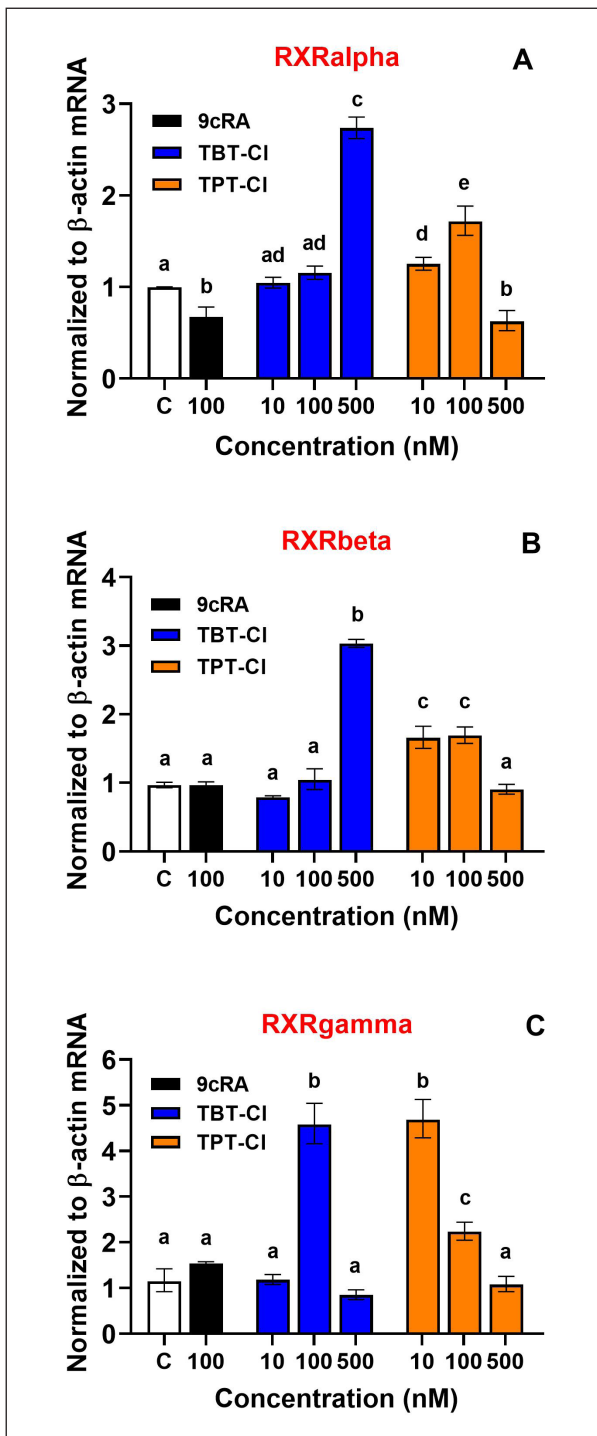


Figure 4. Dose-dependent effects of 9-*cis*-retinoic acid (9cRA) (100 nM), tributyltin chloride (TBT-Cl) and triphenyltin chloride (TPT-Cl) (both 10, 100, 500 nM) on mRNA expression of RXR α (A), RXR β (B) and RXR γ (C) in TM3 Leydig cells after 48-h exposure determined by semiquantitative real-time PCR. Data are expressed as geometrical mean $\pm$ SD of two independent experiments ($n=4$) and were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Different superscripts indicate significant differences between groups.

had no effect on the expression of RXR β and RXR γ (Figure 4B, C).

Effects of TBT-Cl and TPT-Cl on cell viability and testosterone secretion by TM3 Leydig cells. Both TBT-Cl and TPT-Cl induced dose- (0.1 nM–1 μ M) and time-dependent (24, 48, and 72 h) decrease in viability of TM3 cells assessed by MTT test reflecting metabolic activity of cells. TBT-Cl significantly decreased viability of TM3 cells from a concentration of 100 nM by 26–100% ($p<0.05$ –0.001), from a concentration of 10 nM by 16–100% ($p<0.01$ –0.001) and from a concentration of 0.1 nM by 11–100% ($p<0.01$ –0.001)

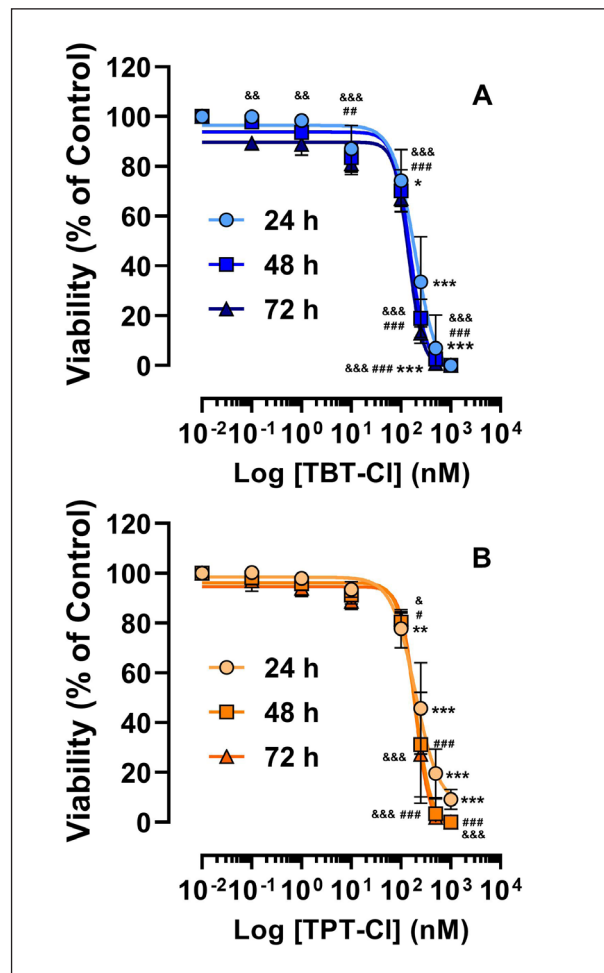


Figure 5. Dose- (0.1–1000 nM) and time-dependent (24, 48, 72 h) effects of tributyltin chloride (TBT-Cl) (A) and triphenyltin chloride (TPT-Cl) (B) on viability of TM3 Leydig cells assessed by MTT assay. Data are presented as mean $\pm$ SD of four independent experiments. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. 24-h untreated cells (control); ## $p<0.01$, ### $p<0.001$ vs. 48-h control; & $p<0.05$, && $p<0.01$, &&& $p<0.001$ vs. 72-h control.

after 24 h, 48 h, and 72 h of treatment, respectively (Figure 5A). TPT-Cl induced a significant decrease in viability of TM3 cells from a concentration of 100 nM by 20–98% ($p < 0.05$ –0.001) for all exposure times (Figure 5B). The concentrations of TBT-Cl and TPT-Cl that inhibited cell viability to 50% (IC₅₀) at 24 h, 48 h and 72 h are shown in Table 2.

The activity of LDH released into the culture

Table 2
Cytotoxicity of tributyltin chloride (TBT-Cl) and triphenyltin chloride (TPT-Cl) in TM3 Leydig cells

Time of exposure	IC ₅₀ [nM]	
	TBT-Cl	TPT-Cl
24 h	191±68	222±83
48 h	152±21	191±61
72 h	142±17	187±27

Data are presented as mean ± standard error of mean (SEM) of four independent experiments.

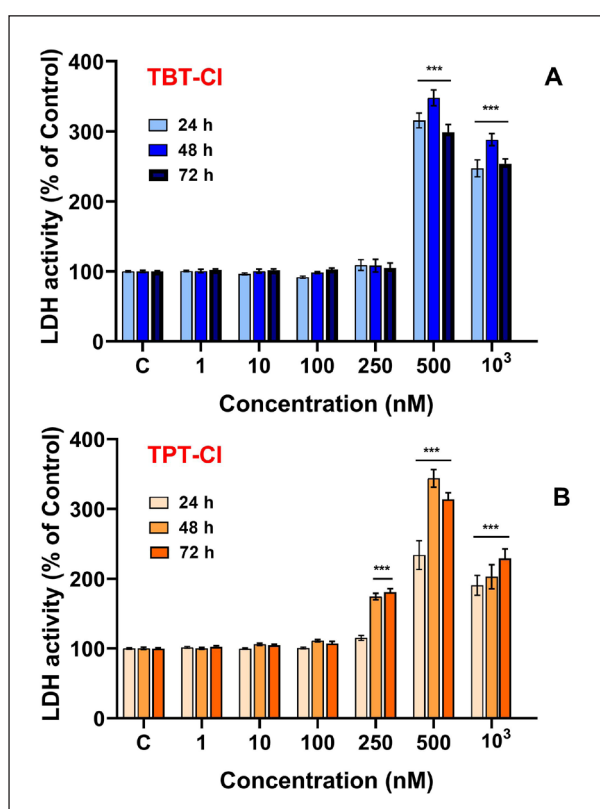


Figure 6. Dose- (1–1000 nM) and time-dependent (24, 48, 72 h) effects of tributyltin chloride (TBT-Cl) (A) and triphenyltin chloride (TPT-Cl) (B) on activity of lactate dehydrogenase (LDH) released from TM3 Leydig cells to culture medium. Data are presented as mean ± SEM of three independent experiments. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. *** $p < 0.001$ vs. corresponding controls.

medium was examined following exposure to various concentrations (1 nM–1 μ M) of TBT-Cl and TPT-Cl for 24, 48 and 72 h. After all exposure times, TBT-Cl induced a significant increase in LDH activity at concentrations of 500 nM and 1 μ M ($p < 0.001$) compared to the respective controls (Figure 6A). TPT-Cl significantly increased LDH activity ($p < 0.001$) at 250 nM concentration after 48 h and 72 h of treatment and at concentrations of 500 nM and 1 μ M after all tested exposure times compared to corresponding controls (Figure 6B).

To evaluate the endocrine-disrupting potential of TBT-Cl and TPT-Cl, we examined effects of these compounds on testosterone secretion by TM3 Leydig cells to culture medium. Since the basal testosterone levels (control) were very low (0.034 ± 0.001 ng/ml), TM3 cells were treated in the presence of testosterone precursor, androstenedione (1 μ M), resulting in 120-fold increase of testosterone levels ($p < 0.001$) compared to control (Figure 7). Exposure of TM3 cells to TBT-Cl for 24 h significantly decreased androstenedione-induced testosterone levels by about 50% ($p < 0.001$) at both tested concentrations (500 nM and 1 μ M) (Figure 7). Treatment of TM3 cells with TPT-Cl (500 nM and 1 μ M) for 24 h decreased

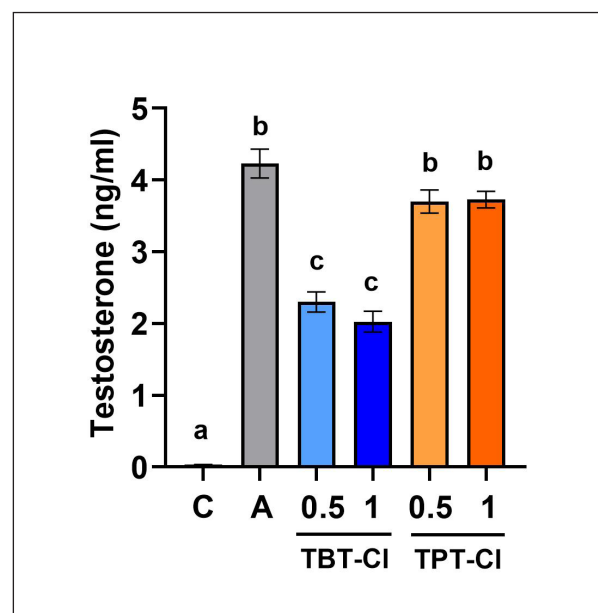


Figure 7. Effects of tributyltin chloride (TBT-Cl) and triphenyltin chloride (TPT-Cl) (both 0.5 and 1 μ M) on testosterone production by TM3 Leydig cells after 24-h exposure in the absence (C-control) and presence of androstenedione (A) (1 μ M). Data are presented as mean ± SEM of three independent experiments. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Different superscripts indicate significant differences between groups.

androstenedione-induced testosterone levels only by 12% ($p>0.05$) (Figure 7).

Effects of TBT-Cl and TPT-Cl on cell viability and steroid hormone secretion by H295R adrenocortical cells. To confirm the inhibitory effect of TBT-Cl and TPT-Cl on steroid hormone secretion in other steroid hormone synthesizing cells, the human adrenocortical cell line H295R was used as a validated OECD standard assay for identifying steroidogenesis disruptors. Both TBT-Cl and TPT-Cl at a concentration of 1 μM induced a significant decrease in viability of H295R cells by 40% and 25%, respectively ($p<0.001$) after 24-h exposure, and by 92% and 85%, respectively ($p<0.001$) after 48 h of treatment compared to corresponding controls (Figure 8A). Treatment of H295R cells with FSK (1 μM) for 48 h induced a significant increase of testosterone (3-fold) ($p<0.001$) (Figure 8B) and cortisol (2-fold) ($p<0.001$) (Figure 8C) secretions compared to controls. TBT-Cl and TPT-Cl at a concentration (1 μM) significantly inhibited basal as well as FSK-stimulated testosterone (by 86–93%) ($p<0.001$) (Figure 8B) and cortisol (by 86–94%) ($p<0.001$) (Figure 8C) secretions after 48 h of treatment compared to controls and FSK treatment alone.

Discussion

Using various concentrations of tested triorganotin compounds, TBT-Cl and TPT-Cl, and incubation times, we performed an analysis of their effects on viability and functionality of non-tumorigenic TM3 Leydig cells and tumorigenic adrenocortical H295R cells. In addition, quantitative PCR analysis and immunofluorescence detection revealed the gene and protein expression of all RXR subtypes, RXR α , RXR β , and RXR γ , in mouse TM3 Leydig cells.

In normal rodent testes, mRNA expression of all RXR subtypes has been previously demonstrated using Northern blot, *in situ* hybridization, or RNase protection assays (Nagata et al. 1994; Kastner et al. 1996; Gaemers et al. 1997, 1998). Immunohistochemical and Western blot analyses have demonstrated different temporal and cellular expression patterns of RXR subtypes in all cell types of mice and rodent testis (Gaemers et al. 1998; Boulogne et al. 1999; Dufour and Kim 1999). Unexpectedly, RXR proteins, with the exception of RXR γ , were frequently detected in the cytoplasmic compartment of the cells of fetal and neonatal rat testis, whereas in immature and adult testis, they were found almost exclusively in cell nuclei (Boulogne et al. 1999). Dufour and Kim (1999) studied the developmental changes in cellular

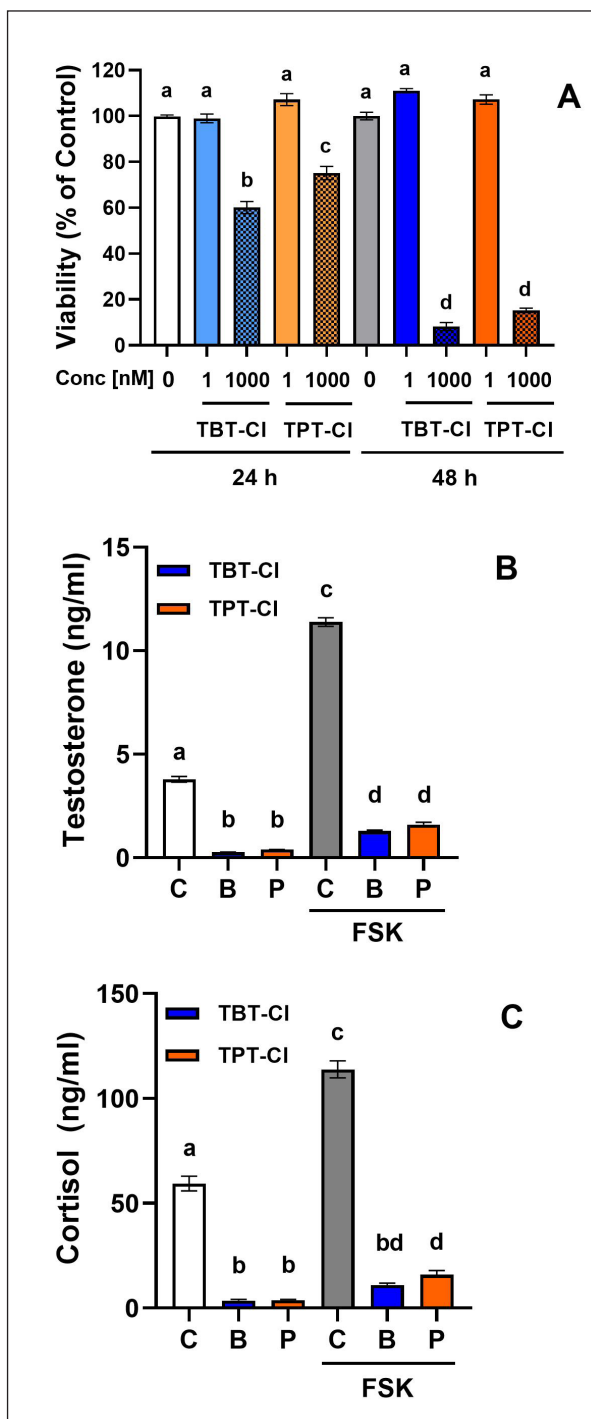


Figure 8. Dose- (1 and 1000 nM) and time-dependent (24 and 48 h) effects of tributyltin chloride (TBT-Cl) and triphenyltin chloride (TPT-Cl) on viability of H295R adrenocortical cells assessed by MTT assay (A). Effects of TBT-Cl and TPT-Cl (both 1000 nM) on basal and forskolin (FSK)-stimulated (1 μM) testosterone (B) and cortisol (C) production by H295 cells after 48-h exposure. Data are presented as mean $\pm$ SD of two independent experiments. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Different superscripts indicate significant differences between groups.

and subcellular localization for RXRs in the rat testis. RXR α and RXR γ were detected in both somatic cells and germ cells, and expressed in Sertoli cells throughout postnatal development, whereas RXR β were present only in Sertoli cells from 30–35 days of age. In Sertoli cells, RXR β and RXR γ were predominantly found in the nucleus, but RXR α was primarily localized in cell cytoplasm in the young animals and later partially in the nucleus in the mature animals. In fertile human testes, immunohistochemical staining revealed RXR β expression mainly in Sertoli cells and strong nuclear expression of RXR γ in most testicular cell types. No specific staining signal was detected in human testis by antibodies against RXR α (Wang et al. 2020).

In the present study, both TBT-Cl and TPT-Cl inhibited TM3 Leydig cell viability in a dose- and time-dependent manner. TBT-Cl induced significant transactivation of the RXR α and RXR β subtypes at a highly cytotoxic concentration of 500 nM, and the RXR γ subtype at a concentration of 100 nM. At the high concentrations tested, significant inhibition of androstenedione-stimulated testosterone secretion by TM3 cell was observed. In contrast, TPT-Cl increased transactivation of all RXR subtypes at a non-cytotoxic concentration of 10 nM and at a cytotoxic concentration of 100 nM. In addition, TPT-Cl at 500 nM concentration inhibited RXR α transactivation in a manner similar to the natural RXR ligand 9cRA (100 nM). The effects of the tested organotin compounds on the expression or activity of enzymes involved in steroidogenesis were not investigated in this study, therefore we cannot confirm the involvement of this system in the mechanism of action of the compounds.

However, there are several *in vitro* studies investigating the effects of various triorganotin compounds and their possible mechanisms of action on testicular Leydig cells. Chen et al. (2023) demonstrated that environmental-relevant concentration of TBT-Cl (100–400 nM) induced cell injury in TM3 Leydig cells with the IC₅₀ of around 200 nM, similar to our results (IC₅₀ for TBT-Cl: 142–191 nM; IC₅₀ for TPT-Cl: 187–222 nM). TBT-Cl-induced cell apoptosis and cycle arrest were suggested to be depended on endoplasmic reticulum (ER) stress and autophagy flux inhibition. In primary rat Leydig cells, TBT-Cl (300 nM–3 μ M) reduced cell viability and testosterone release after 6 h of exposure (Mitra et al. 2014). TBT-Cl altered calcium homeostasis and triggered redox imbalance within 60 min, followed by cell death at 6 h, involving mitochondrial membrane depolarization, Bax/Bcl2 increase,

and activation of the tested MAPKs (ERK1/2, JNK1/2, p38). In addition, TBT-Cl modulated the expression levels of enzymes involved in steroidogenesis such as 3 β -hydroxysteroid dehydrogenase (3 β -HSD), steroidogenic acute regulatory protein (StAR) and translocator protein (TSPO) (Mitra et al. 2014). *In vitro* exposure (3 h) to dibutyltin (DBT) (0.1, 0.5 and 1 μ M) inhibited androgen biosynthesis under basal or stimulated conditions (LH; 8-bromo-adenosine 3',5'-cyclic monophosphate, 8-Br-cAMP; 22R-hydroxycholesterol; and pregnenolone) in immature rat Leydig cells by selectively interfering with the mRNA expressions and enzyme activities of CYP11A1 and 3 β -HSD1 (Li et al. 2021). Hu et al. (2020) demonstrated that trimethyltin (TMT) (1–10 μ M) decreased androgen production under basal and stimulated condition (LH, 8-Br-cAMP, 22R-hydroxycholesterol, androstenedione) in rat immature Leydig cells via exerting inhibitory effects on the mRNA/protein expression levels or enzyme activities of STAR, CYP11A1, 17 β -HSD3, or NR5A1. Wang et al. (2006) demonstrated that exposure of rat tumorigenic Leydig cells LC-540 to TBT-Cl and TPT-Cl (0–80 nM) for 24 to 96 h suppressed cell viability in a dose- and time-dependent manner and induced apoptosis determined by DNA fragmentation ladder formation. The apoptotic effect of TBT-Cl was likely mediated by the rise in intracellular Ca²⁺ and mitochondrial disorders. Similar to our study, TBT-Cl reduced the basal as well as hCG-stimulated testosterone production by LC-540 cells (Wang et al. 2006).

In Leydig cells isolated from neonatal pigs, TBT-Cl, dibutyltin dichloride (DBT-Cl) and TPT-Cl strongly suppressed human chorionic gonadotropin- (hCG) (10 IU/ml) and 8-Br-cAMP-stimulated (0.1 mM) testosterone production at the concentration range of 10–300 nM without apparent cytotoxicity (Nakajima et al. 2003). TBT-Cl (100 nM) exposure to pig Leydig cells significantly decreased hCG- (10 IU/ml) and FSK-stimulated (25 μ M) testosterone secretion and intracellular cAMP levels. In the same way, TBT-Cl exposure for 6 and 24 h significantly decreased the 17 α -hydroxylase/17,20-lyase (P450c17) mRNA level stimulated by hCG; however, did not affect the mRNA levels of other steroidogenic enzymes (Nakajima et al. 2005). In another study, the authors investigated the effects of TBT-Cl and TPT-Cl on the activity of enzymes involved in testosterone biosynthesis using pig testicular microsomes and mitochondria. TPT-Cl at relatively low concentrations (1–100 nM) inhibited activities of 17 β -hydroxysteroid dehydrogenase (17 β -HSD) (IC₅₀=2.6 μ M) and cytochrome P450

17 α -hydroxylase/C17–20 lyase (IC₅₀=117 μ M). In porcine Leydig cells, TBT-Cl significantly inhibited 17 β -HSD activity beyond 100 nM, while TPT-Cl showed significant inhibition at over 30 nM; the IC₅₀ values of TPT-Cl and TBT-Cl for 17 β -HSD activity were estimated to be 48 and 114 nM, respectively (Ohno *et al.* 2005).

To assess the risk of endocrine disruption in mammals as a result of exposure to organotin compounds, TBT-Cl and TPT-Cl, the human adrenocortical cell line H295R was used as a validated OECD standard assay in this study. Both TBT-Cl and TPT-Cl (1 μ M) induced a significant decrease in cell viability and basal as well as FSK-stimulated testosterone and cortisol secretions by H295R cells. The possible molecular mechanisms of organotin action in H295 cells were not explored in this study. Yan *et al.* (2018a) used H295R cell model to investigate effects of several commonly used organotins, including TPT, TBT, DBT and monobutyltin (MBT) (10, 50, and 100 nM) on steroidogenesis pathway. TPT and TBT decreased the levels of 17 β -estradiol, aldosterone, and cortisol, but increased testosterone levels in culture media after 48 h of exposure. Intracellular levels of ATP and cAMP and the activity of adenylate cyclase decreased in the H295R cells treated with TPT and TBT. In addition, up-regulation of CYP11B2 and down-regulation of StAR, 3 β HSD2, CYP19A1, CYP21 and CYP11B1 by TPT and TBT were observed. No obvious changes were found in H295R cells treated with DBT and MBT (Yan *et al.* 2018a). TBT at up to 100 nM significantly increased the expression of liver X receptor (LXR α) and farnesoid X receptor (FXR) mRNA in H295 cells after 48 h of exposure, indicating that TBT acts as a potential agonist for LXR α /RXR and FXR/RXR.

Proteomic and bioinformatic analysis identified altered expression of 69 proteins mainly involved in the regulation of hormone homeostasis, lipid metabolism, cell death, and energy production in H295 cells after TBT treatment (Yan *et al.* 2018b). In another study, DBT-Cl, TBT-Cl, and TPT-Cl (1–300 nM) decreased aromatase and ethoxyresorufin-O-deethylation (EROD), a measure of the catalytic activity of the CYP1A subfamily of cytochrome P450, activities only at cytotoxic concentrations after 24-h exposure (Sanderson *et al.* 2002). Further studies are needed to elucidate the detailed molecular mechanisms of the complex effects of triorganotin compounds in mammals.

In conclusion, the results of this study show that *in vitro* exposure to the triorganotin compounds, TBT-Cl and TPT-Cl, induced a decrease in cell viability and steroid hormone secretion by TM3 Leydig cells and H295R adrenocortical cells, suggesting potential endocrine disrupting properties of these compounds. In TM3 Leydig cells, the cytotoxic effects of TBT-Cl and TPT-Cl may be mediated by RXR activation. However, further studies are needed to elucidate the precise molecular mechanisms of the TBT-Cl and TPT-Cl action on steroidogenesis.

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