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Short Communication

Effects of selected triorganotin compounds on transcriptional activity of vitamin D_3 receptor and peroxisome proliferator-activated receptor gamma

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Abstract. Both, the vitamin D_3 receptor (VDR) and the peroxisome proliferator-activated receptor gamma (PPARγ), are ligand-inducible transcription factors that control expressions of various genes involved in essential biological processes. Structurally diverse chemical substances are capable to bind to VDR and PPARγ, consequently acting in agonistic or antagonistic mode. Ubiquitous triorganotin compounds, key components of antifouling, disinfectant and biocidal agents were found to act as cognate ligands of several nuclear receptors. Triorganotins affect endocrine systems in disruptive manner recruiting proliferative, differentiation and apoptotic pathways. In this study, we have investigated agonistic as well as antagonistic effects of selected triorganotin compounds on VDR and PPARγ in transgenic gene reporter IZ-VDRE and PAZ-PPARγ human cell lines, allowing rapid and sensitive assessment of receptor transcriptional activity. We demonstrated that most of investigated triorganotins at nanomolar concentration exerted significant agonistic effects on VDR with fold activation ranging from 2.0 to 3.0-fold as well as some significant changes ranging from 127 to 199% of the maximal 1,25-dihydroxyvitamin D_3 (calcitriol) induction, in antagonistic mode. In agonistic mode, PPARγ transcriptional activity was not affected by studied triorganotins significantly, but studied tributyltin compounds in antagonistic mode, revealed significant values ranging from 147 to 171% of the maximal 15-deoxy-δ12,14-prostaglandin J2 induction.

Key words: Triorganotin derivatives — Vitamin D_3 receptor — Peroxisome proliferator-activated receptor — Transcriptional activity

The nuclear vitamin D_3 receptor (VDR) was first cloned from chicken in 1987, and its marked expression has been detected in intestine, kidney, parathyroid gland, and skeleton (osteoblasts and chondrocytes) of both, avian and mammalian species. VDR was also found in skin (keratinocytes), pancreas (beta islet cells), placenta, pituitary, ovary, testis, mammary gland, and heart, immune system (monocytes, macrophages, and T-lymphocytes), and germ tissues (Pike and Meyer 2010). On the other hand, the peroxisome proliferator-activated receptor (PPAR) was originally discovered in Xenopus frogs (Dreyer et al. 1992). Alvares et al. (1990) searched for a molecular target for agents that were responsible for increased numbers of peroxisomes in rodent liver tissue. These agents were termed peroxisome proliferators, and became to be first representatives of PPAR ligands. Three tissue specific types of PPARs: α, γ, and δ/β have been identified, whereby, PPARγ was found to be broadly expressed in the cells of adipose tissue, however, lower levels of its expression were observed in the intestine, liver, kidney, brain, heart and skeletal muscle (Abbott 2009).

The human VDR gene is located on chromosome 12 (12q13.11). VDR (NR1I1- nuclear receptor subfamily 1, group I, member 1) is a one of the 48 members of nuclear

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hormone receptor superfamily (Germain et al. 2006a), which binds naturally biologically active form 1,25-dihydroxyvitamin D_3 with high affinity ($K_d = 10^{-10}$ M) (Aranda and Pascual 2001). The human PPAR genes are located on different subunit-specific chromosomes, while PPARγ is transcribed from genes on chromosome 3p25 (Meirhaeghe and Amouyel 2004). PPARγ (NR1C3) belongs also to the superfamily of nuclear receptors and act as a ligand-activated transcription factor (Dreyer et al. 1992). The most important natural activators of PPARγ are eicosanoids, represented by a well-known endogenous ligand 15-deoxy-δ12,14-prostaglandin J2 (15d-PGJ2) that is usually generated in the cells under inflammatory conditions (Forman et al. 1995). On the other hand, thiazolidinedione compounds represent a group of potent synthetic ligands of PPARγ.

VDR heterodimerizes with the promiscuous partners, nuclear retinoid-X receptors (RXRs). This complex binds to vitamin D_3 responsive element (VDRE) in target gene promoters and modulates gene expression. The VDRE in responsive genes (osteocalcin, osteopontin, 24-hydroxylase) has been well characterized and the consensus sequence consists of two imperfect repeats of AGGTCA separated by three non-specified nucleotides (DR-3 motif) (Aranda and Pascual 2001). PPARγ similarly to VDR, forms heterodimers with RXRs. The PPARγ-mediated regulation of gene expression is provided by direct interaction of PPARγ/RXR heterodimers with peroxisome proliferator-activated receptor responsive elements (PPREs) present in the regulatory regions of target genes. PPRE consists of two AGGTCA consensus sequences that are arranged as a direct repeat separated by a single nucleotide (DR-1 motif) (Aranda and Pascual 2001; Yu and Reddy 2007).

Genes regulated by VDR primarily maintain calcium homeostasis, but they participate in focal actions that control the growth, differentiation and functional activity of numerous cell types including those of the immune system, skin, the pancreas and bone (Makishima et al. 2002; Bouillon et al. 2008). Disruption of VDR function leads to rickets associated with dysregulation of calcium homeostasis, including muscle weakness, growth retardation, and bone deformity, along with secondary hyperparathyroidism and aminoaciduria (Kato et al. 2004). PPARs, on the contrary, play essential roles in the regulation of differentiation, development, and metabolism as well as tumorigenesis of higher organisms. PPARγ plays an essential role in adipogenesis (Tontonoz et al. 1994), but it is also involved in regulation of lipids and glucose metabolism (Rieusset et al. 2002) and in immune response of organism (Ricote et al. 1998). Thus, disruption of PPARγ transcriptional activity can be implicated in numerous physiological disorders, including diabetes, obesity or cardiovascular diseases (Meirhaeghe and Amouyel 2004).

Triorganotin derivatives, especially tributyltin, at pico- or nanomolar concentrations may induce irreversible sexual abnormalities (imposex) in several aquatic organisms. Trialkyl and triaryltins induce metabolic, reproductive and developmental defects in mammals (le Maire et al. 2009). Specifically, triphenyltins inhibit the catalytic activity of human aromatase, trialkyltins in general promote adipocyte differentiation, and both are declared immuno- and neurotoxicants (Brtko and Dvorak 2015). Triorganotins are potent RXR agonists, and despite of their detrimental impact on vertebrates and invertebrates, they may have some therapeutic potential as antitumour drugs, considering their mechanism of action. Inhibition of synthesis of macromolecules, mitochondrial energy metabolism, and reduction of DNA synthesis, as well as direct interaction with the cell membrane leading to increase in cytosolic Ca^{2+} concentration has been implicated in triorganotin-induced cytotoxicity. These effects can be the major factors contributing to triorganotin-induced apoptosis in many cell lines, tumour ones included (Alama et al. 2009; Hunakova et al. 2016).

The aim of this study was to evaluate agonistic and antagonistic effects of tributyltin chloride (TBT-Cl), tributyltin bromide (TBT-Br), tributyltin iodide (TBT-I), tributyltin hydride (TBT-H), tributyltin isothiocyanate (TBT-ITC); and triphenyltin chloride (TPT-Cl), triphenyltin hydride (TPT-H), triphenyltin hydroxide (TPT-OH), triphenyltin acetate (TPT-Ac), triphenyltin isothiocyanate (TPT-ITC), on VDR, using a novel IZ-VDRE transgenic human luciferase reporter cell line, and on PPARγ in PAZ-PPARγ, a stably transfected luciferase reporter cell line. The development and properties of IZ-VDRE cell line were described elsewhere (Bartonkova et al. 2016). The PAZ-PPARγ cell line was prepared by transfection of human bladder carcinoma cells T24/83 with pNL2.1[Nluc/Hygro] reporter plasmid (Promega, USA) containing three copies of the PPARγ response element coupled with the minimal promoter, cloned into the multiple cloning site of the plasmid. Both investigated gene reporter cell lines provide cell-based experimental models allowing selective identification of VDR, PPARγ agonists and assessment of VDR, PPARγ transcriptional activities for toxicological, environmental, and food safety applications.

Triorganotin derivatives: TBT-Cl, TBT-Br, TBT-I, TBT-H; and TPT-Cl, TPT-H, TPT-OH and TPT-Ac were purchased from Sigma Aldrich (Schnelldorf, Germany). TBT-ITC and TPT-ITC, commercially unavailable compounds, were synthesized and characterized at the Department of Chemical Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic (Bohacova et al. 2018). The IZ-VDRE were seeded on 96-well plates at density 2.5×10^4 cells *per* well in 0.2 ml of Dulbecco's Modified Eagle's Medium (DMEM) culture medium supplemented with 10% charcoal-stripped fetal bovine serum and antibiotics. The PAZ-PPARγ cells were seeded into 96-well plates at a density of 4×10^4 cells in 0.2 ml of McCoy's 5A culture medium supplemented with

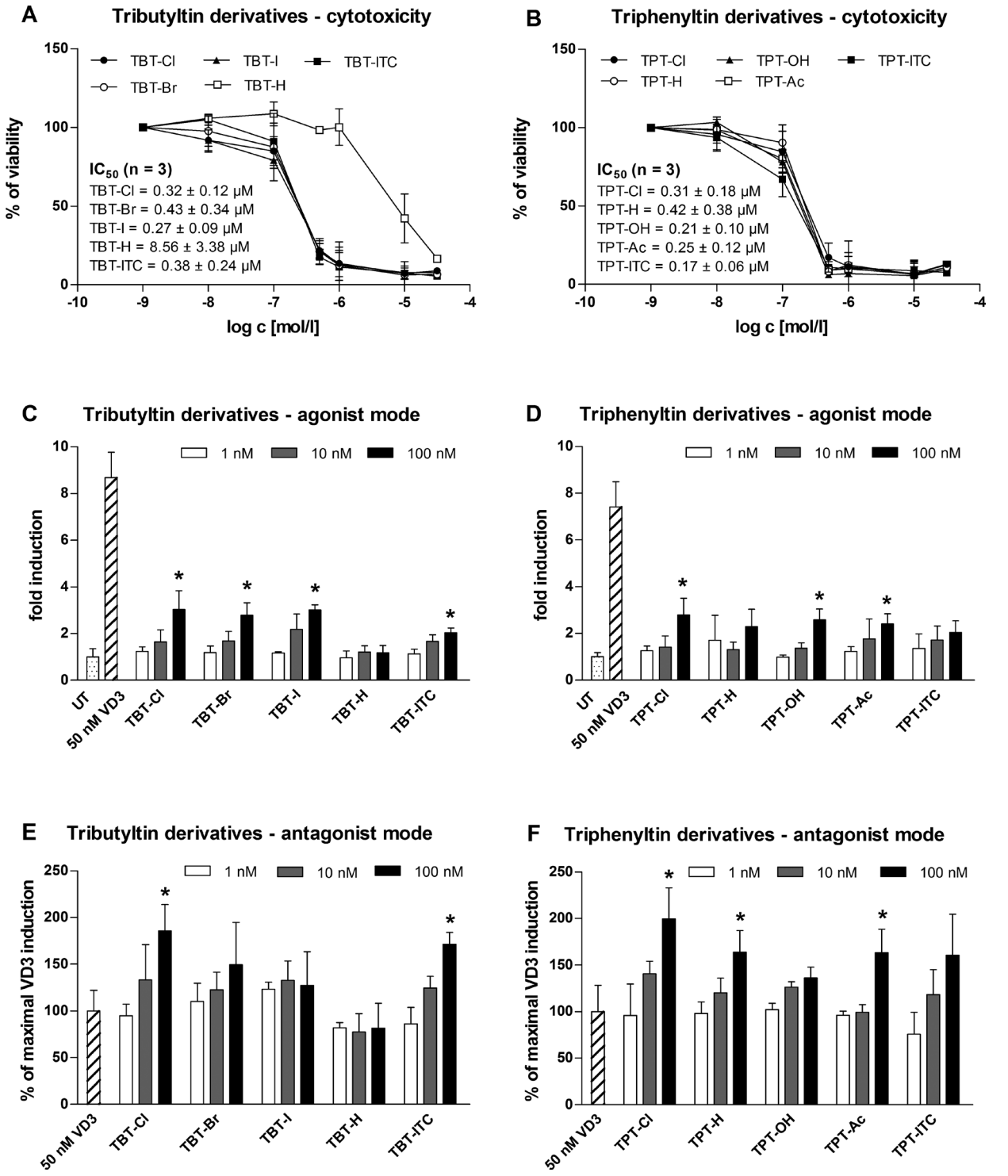

Figure 1. Effects of triorganotin derivatives on the transcriptional activity of VDR in the IZ-VDRE reporter cell line. Cells seeded into 24-well plates were stabilized for 16 h and treated with vehicle (UT; 0.1% DMSO or ethanol v/v), tributyltin derivatives (TBT-Cl, TBT-Br, TBT-I, TBT-OH, TBT-ITC) (**A, C**), or triphenyltin derivatives (TPT-Cl, TPT-H, TPT-OH TPT-Ac, TPT-ITC) (**B, D**) alone (cytotoxicity assay – A, B, agonist mode – C, D), and in combination with 50 nM VD_3 (**E, F**) (antagonist mode). After 24 h, the cells were lysed and the luciferase activity was measured. The data represent the means ± SEM of triplicate measurements from three independent experiments and are expressed as the fold induction over the vehicle-treated cells; * values significantly different from the vehicle-treated cells ($p < 0.05$) as determined by ANOVA followed by Tukey's test.

10% charcoal-stripped fetal bovine serum. Following 24 h incubation, cells were incubated with triorganotin compounds, vehicle (DMSO; 0.1%, v/v) and Triton X-100 (2%, v/v) to assess the minimal (positive control) and maximal (negative control) cell response, respectively. MTT test, a colorimetric assay for assessing cell viability was used in experiments. After 24 h treatment with tested compounds, samples were measured at λ = 540 nm (TECAN, Schoeller Instruments LLC). The treatments were performed in quadruplicates in three independent cell passages. For a gene reporter assay, as a positive control, a model agonist of VDR, calcitriol, at the concentration 50 nM and as a positive control for PPARγ, 40 μM 15-deoxy-δ12,14-prostaglandin J2 (15d-PGJ2) were used. After treatments, cells were lysed with reporter lysis buffer (Promega, USA) and luciferase activity was measured in 96-well plate format, using the Nano-Glo® Luciferase Assay System (Promega, USA) and a Tecan Infinite M200 plate reader.

In this work, we have examined agonistic and antagonistic effects of selected tributyl/triphenyltin derivatives (TBT-Cl, TBT-Br, TBT-I, TBT-H, TBT-ITC; TPT-Cl, TPT-H, TPT-OH, TPT-Ac and TPT-ITC) on VDR (in human luciferase reporter IZ-VDRE cells) and PPARγ (in human luciferase reporter PAZ-PPARγ cells). Firstly, we have tested effects of triorganotin derivatives on viability of both cell lines.

Human luciferase reporter IZ-VDRE cells

All examined tributyltin derivatives with exception of TBT-H exhibited strong cytotoxic effects already at concentration of 100 nM (Fig. 1A). IC_{50} values were ranging from 0.3 ± 0.1 μM (TBT-I) to 8.4 ± 3.4 μM (TBT-H). This enabled us to order tributyltin compounds toxicity as follows: TBT-I > TBT-Cl > TBT-ITC > TBT-Br >> TBT-H. Triphenyltin derivatives showed similar cytotoxic effects with IC_{50} values ranging from 0.2 ± 0.1 μM (TPT-ITC) to 0.4 ± 0.4 μM (TPT-H) (Fig. 1B). Cytotoxic potency declined in the order: TPT-ITC > TPT-OH > TPT-Ac > TPT-Cl >> TPT-H. Cells were incubated with individual triorganotin compounds in the absence (agonist mode) or in the presence of natural ligand (antagonist mode) for 24 h. Concentration scale of investigated derivatives was derived according to cytotoxicity in nanomolar range (1 nM – 100 nM). The vehicle for tested compounds was DMSO (0.1%, v/v). Luciferase activity was measured in relative light units (RLU) and expressed as a fold induction over the DMSO-treated cells. As a positive control, we settled the natural ligand of VDR, calcitriol (50 nM) (7.4-fold induction). Luciferase activity in agonistic mode was dose-dependently induced and reached highest statistically significant values at the 100 nM concentration with the following compounds: TBT-Cl (3.0 ± 0.8-fold), TBT-Br (2.8 ± 0.5-fold), TBT-I (3.0 ± 0.2-fold) and TBT-ITC (2.0 ± 0.2-fold) (Fig. 1C). TPT derivatives revealed signification with the following compounds: TPT-Cl (2.8 ± 0.7-fold), TPT-OH (2.6 ± 0.5-fold) and TPT-Ac (2.4 ± 0.4-fold) (Fig. 1D). Neither TPT-H (2.3 ± 0.8-fold) nor TPT-ITC (2.0 ± 0.5-fold) showed any significance; moreover TBT-H exerts just weak agonistic effects as its fold induction was 1.2 ± 0.3. In parallel, we examined the antagonistic effects of the tributyltin compounds on the transcriptional activity of VDR in the presence of calcitriol (50 nM). We observed significant changes of luciferase activity with the following compounds: TBT-Cl, TBT-ITC, TPT-Cl, TPT-H and TPT-ITC. The fold inductions at 100 nM corresponded to: 186 ± 28% (TBT-Cl), 172 ± 13% (TBT-ITC), 200 ± 34% (TPT-Cl), 164 ± 24% (TPT-H), and 160 ± 44% (TPT-ITC) of the maximal calcitriol induction (Fig. 1E, F).

Human luciferase reporter PAZ-PPARγ cells

Similarly to previous experiments, test of viability revealed that all examined tributyltin compounds with exception of TBT-H had significant cytotoxic effect on the PAZ-PPARγ cells at concentration of 100 nM (Fig. 2A). The estimated cytotoxicity with the corresponding values of IC_{50} was in following order: TBT-ITC (0.2 ± 0.1 μM) > TBT-I (0.3 ± 0.1 μM) > TBT-Cl (0.4 ± 0.2 μM) > TBT-Br (0.6 ± 0.5 μM) >> TBT-H (5.2 ± 3.1 μM). Triphenyltin compounds resulted in even stronger drop of cell viability of the PAZ-PPARγ cells (Fig. 2B). Cytotoxic effect of examined compounds decreased in the order: TPT-ITC (0.09 ± 0.02 μM) > TPT-OH (0.14 ± 0.02 μM) > TPT-Cl (0.19 ± 0.05 μM) > TPT-Ac (0.20 ± 0.06 μM) > TPT-H (0.21 ± 0.05 μM). After the treatment of the PAZ-PPARγ cells with increasing concentrations (1–100 nM) of both tributyltin and triphenyltin derivatives, we did not observe any significant changes in luciferase activity (Fig. 2C, D). The values of luciferase induction obtained by 40 μM 15-deoxy-δ12,14-prostaglandin J2 (positive control) ranged between 39.9 ± 9.1 and 40.3 ± 7.1-fold over the DMSO-treated cells. In parallel experiments, the PAZ-PPARγ cells were exposed to tributyltin or triphenyltin compounds at concentrations of 1–100 nM in the presence of 40 μM 15-deoxy-δ12,14-prostaglandin J2 (Fig. 2E, F). Combined treatment did not induce any significant changes in luciferase activity in the PAZ-PPARγ cells treated with triphenyltin compounds, but application of TBT-Cl, TBT-Br, TBT-I and TBT-ITC resulted in substantial increase in luciferase activity at concentration of 100 nM (Fig. 2E). The levels of luciferase induction corresponded to 156 ± 20% (TBT-Cl), 147 ± 14% (TBT-Br), 168 ± 19% (TBT-I) and 171 ± 31% (TBT-ITC) of maximal luciferase activity attained by 40 μM 15-deoxy-δ12,14-prostaglandin J2, only.

Treatment of the cells (IZ-VDRE as well as the PAZ-PPARγ) with nanomolar concentrations of triorganotin compounds results in strong cytotoxicity and the cytotoxic effect of these compounds on various cell lines were fully en-

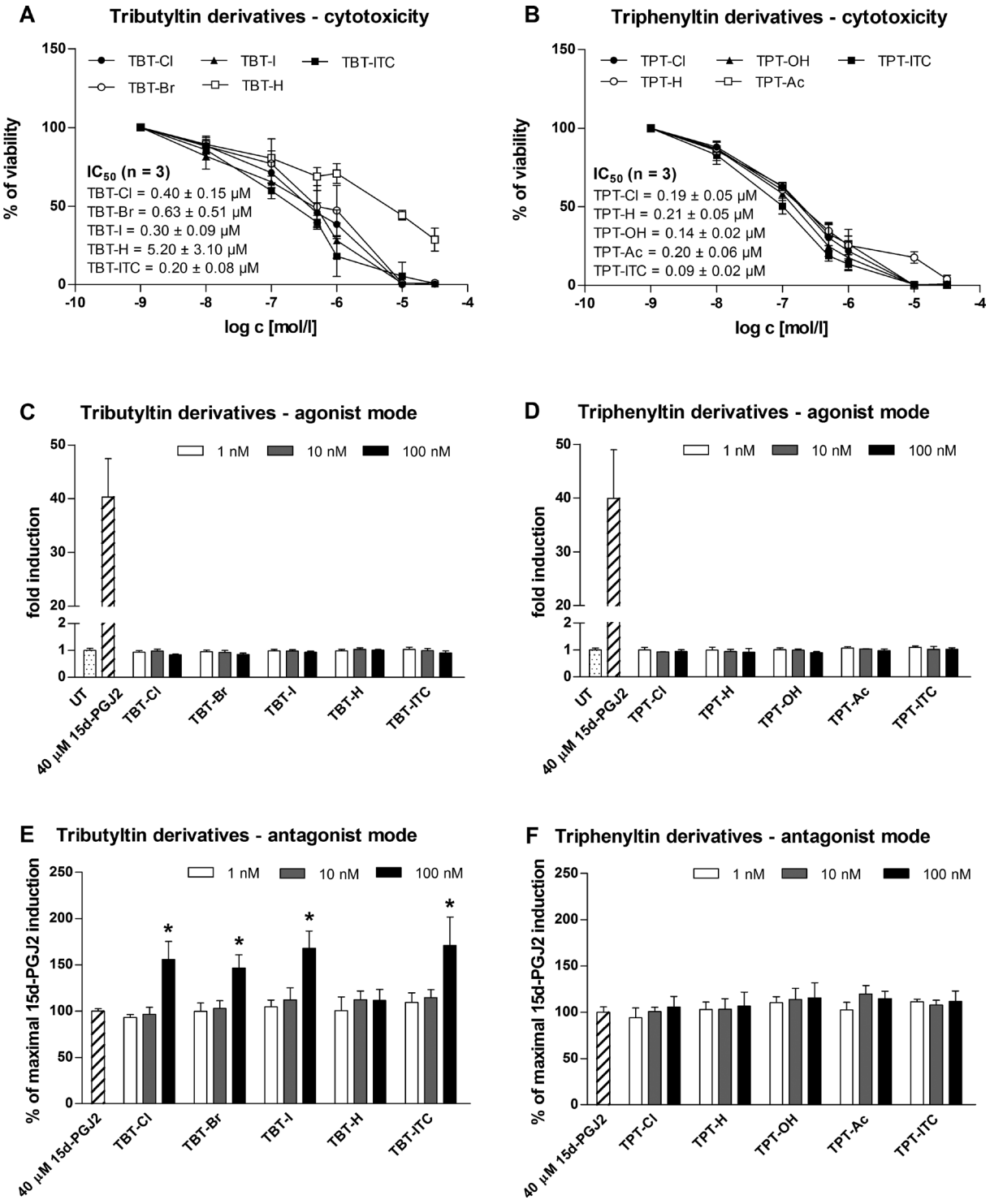

Figure 2. Effects of triorganotin derivatives on the transcriptional activity of PPARγ in the PAZ-PPARg reporter cell line. Cells seeded into 24-well plates were stabilized for 16 h and treated with vehicle (UT; 0.1% DMSO or ethanol v/v), tributyltin derivatives (TBT-Cl, TBT-Br, TBT-I, TBT-OH, TBT-ITC) (**A, C**), or triphenyltin derivatives (TPT-Cl, TPT-H, TPT-OH TPT-Ac, TPT-ITC) (**B, D**) alone (cytotoxicity assay – A, B, agonist mode – C, D), and in combination with 50 nM VD_3 (**E, F**) (antagonist mode). After 24 h, the cells were lysed and the luciferase activity was measured. The data represent the means ± SEM of triplicate measurements from three independent experiments and are expressed as the fold induction over the vehicle-treated cells; * values significantly different from the vehicle-treated cells ($p < 0.05$) as determined by ANOVA followed by Tukey's test.

dorsed by other studies (Hoth et al. 2005; Nakanishi 2008). Triorganotin toxicity is strongly related to interactions with nuclear hormone receptor pathways, as TBTs exert high affinity values to both the RXR and PPARγ (Germain et al. 2006b). Cytotoxicity of that compounds seems to be influenced by size and chemical character of the anionic part of triorganotin skeleton, so modification of this part led to milder cytotoxicity in case of both hydrides, TBT-H and TPT-H. Modelling studies indicate the high affinity of TBTs and TPTs for RXR molecule derived from the covalent interaction linking the tin atom to residue Cys432 and the direct van der Waals contacts between triorganotins and RXR residues (le Maire et al. 2009). On the other hand, alkyl-aryl interchange reflected the severe cytotoxicity of aryl derivatives. This effect could be caused by process of triorganotin molecule degradation, as tributyltin was found to metabolize more readily than triphenyltin in all the species (Ohhira et al. 2003). Potential of TBTs to activate heterodimeric partner – RXR engages the multiple nuclear receptors involved in a tangle of signalling pathways (Szeles et al. 2010).

The VDR is a member of the steroid hormone receptor superfamily that exerts transcriptional activation and repression of target genes in a ligand-dependent manner. Its natural ligand, calcitriol, has prominent antiproliferative, anti-angiogenic and pro-differentiative effects in a broad range of cancers: breast, colorectal and prostate cancers, squamous cell carcinoma, head and neck cancer included. These effects are mediated through perturbation of several important signalling pathways mediated through genomic and non-genomic mechanisms (Deeb et al. 2007). Transcriptional activity is modulated through dimerization with RXR, while formed heterodimer has non-permissive character, referred to as „silecing phenomenon" (Germain et al. 2006b). Our IZ-VDRE data did not support the theory of RXR/VDR heterodimer strict non-permissivity, which implies that RXR/VDR heterodimer is considered to be non-permissive and cannot be activated by rexinoid, either in the presence of VDR ligand. On the contrary, we have detected luciferase activity in antagonistic mode and these suggest that the RXR does not act as a silent partner to the VDR. Moreover, Sanchez-Martinez (2006) found that RXR/VDR heterodimer can recruit coactivators in response to either vitamin D or 9*c*RA, and that both cooperate to stimulate the activity of VDRE reporters to increase transcription of the *cyp24* gene or to promote differentiation of colon carcinoma cells. So, it is not clear, if binding of the RXR agonist to VDR heterodimer causes association with coactivators and transcriptional stimulation by a VDR ligand (Sanchez-Martinez et al. 2006). Nevertheless, there are no reports available dealing with triorganotin derivatives *vs*. VDR, and thus, our findings unambiguously suggest that triorganotins as RXR agonists have important role in VDR functioning and signalling with important physiological and potential pharmacological implications.

In contrast to VDR, PPARγ is a dimerization partner forming permissive class of RXR heterodimers (Germain et al. 2006a). Very interesting aspect of PPAR/RXR physiology is the potential for synergistic transactivation in the presence of both PPARγ and RXR ligands (Gampe et al. 2000). Treatment with both agonists resulted in an enhanced PPARγ-dependent transactivation of reporter genes; adipocyte differentiation in cultured cells (Sato et al. 2001), lipid metabolism in skeletal muscle (Cha et al. 2001), differentiation of liposarcomas *in vivo* (Tontonoz et al. 1994), etc. TBT, declared as dual PPAR/RXR ligand, should bind and activate both PPARγ and RXR. Later, it has been specified that TBT exerts its physiological effects predominantly through activation of RXR, because of higher efficacy for RXRs activation (Grun et al. 2006; le Maire et al. 2009). We surprisingly did not observe any significant changes in agonistic mode after the treatment of the PAZ-PPARγ cells with triorganotin derivatives. In parallel, antagonistic mode (cotreatment with 40 μM 15-deoxy-δ12,14-prostaglandin J2) revealed significant increase of luciferase activity in the PAZ-PPARγ cells treated only with tributyltin compounds. This supports the data, where TBT was highly potent at inducing the expression and activation of PPARγ, resulting in lipid accumulation and terminal adipocyte differentiation. Therefore, the efficacy of TBT was higher than that of commercially used RXR ligand bexarotene, suggesting the contribution of a mechanism in addition to RXR activation to TBT-induced effects. TBT-induced differentiation requires PPARγ, but TBT likely engages multiple nuclear receptor pathways, as it were declared by the increased expression of RXR homodimer and LXR gene targets (Yanik et al. 2011). There is growing evidence about exposure of organisms to environmental contaminants that are ligands for some nuclear receptors (Antizar-Ladislao 2008). Importantly, humans are exposed to multiple organotins, and the data suggest that organotins structurally related to TBT or TPT are PPAR/RXR dual agonists (Kanayama et al. 2005; Hiromori et al. 2009). Selected triorganotin compounds used in this study interact with VDR and PPARγ heterodimers, preferentially with their dimerization partner – retinoid X receptor, allowing direct effect on the expression of VDR and PPARγ regulated genes. However, triorganotins may also interact with other nuclear receptors known to form heterodimers with RXR, and thus they may induce competitive reaction with PPAR and VDR in heterodimerization with RXR, which may induce effects on protein expression.

Finally, triorganotin – RXR – nuclear receptor complex may also affect other protein components of different regulatory pathways, and thus induce multimodal effects on protein expression. More detailed and extensive analyzes

are required to determine the nature of molecular mechanisms of triorganotin compounds through their cognate nuclear receptors.

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