Ligand Activity of Group 15 Compounds Possessing Triphenyl Substituent for the RXR and PPARγ Nuclear Receptors

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Abstract

We investigated the ability of group 15 compounds with a triphenyl substituent to bind to and activate human retinoic X receptor (RXR) and peroxisome proliferator-activated receptor (PPAR) γ and their ability to activate the receptor. Triphenylphosphine oxide (TPPO) transcriptionally activated both RXR and PPARγ. Triphenylbismuth (TPBi) transcriptionally activated PPARγ but not RXR. However, TPBi significantly inhibited RXR transcriptional activity induced by 9-cis retinoic acid (9cRA) and PPARγ transcriptional activity induced by rosiglitazone (Rosii). Triphenylarsine (TPAs) also significantly inhibited the 9cRA- and Rosii-induced transcriptional activity of both receptors, whereas TPAs alone had no effect on the transcriptional activity of RXR and PPARγ. Consistent with these results, TPAs and TPBi blocked the binding of [3H]9cRA to RXR and of [3H]Rosii to PPARγ in a competitive manner. However, contrary to the results of the reporter gene assay, TPPO did not compete with [3H]9cRA and [3H]Rosii for binding to RXR and PPARγ, respectively. Our findings indicate that 1) TPPO is a transcriptional activator—but not a ligand—of RXR and PPARγ; 2) TPBi is an antagonist of RXR and a partial agonist of PPARγ; and 3) TPAs is a dual antagonist of RXR and PPARγ. These results suggest that TPPO, TPAs, and TPBi are potential endocrine disrupters of the PPARγ-RXR signaling pathway.

Keywords: peroxisome proliferator-activated receptor; retinoic X receptor; triphenylarsine; triphenylbismuth; triphenylphosphine oxide

Regular Article

Triphenyl compounds can contain various group 15 elements, such as phosphorus, arsenic, and bismuth. For example, triphenylphosphine (TPP) is widely used in the synthesis of organic and organometallic compounds; TPP is oxidized to triphenylphosphine oxide (TPPO, Fig. 1A) in organic synthesis reactions such as the Wittig, Staudinger, and Mitsunobu reactions. Therefore, residual TPP and TPPO are potential impurities in active pharmaceutical ingredients that are synthesized by using this chemistry, and effluents from the pharmaceutical industry typically contain these compounds. TPP exerts low acute aquatic toxicity within a wide range of species, and potential adverse effects after exposure to TPP and TPPO have been documented. For example, exposure to TPP leads to neurotoxicity in rodents, and TPP has immunomodulating effects in mouse and human cells in vitro. Like TPP and TPPO, triphenylarsine (TPAs, Fig. 1B) and triphenylbismuth (TPBi, Fig. 1C) are used as ligands and reagents in coordination chemistry and organic synthesis. Furthermore, TPBi is stable in heat and air, and it has been used as a bactericide, an antioxidant, and a stabilizer. However, toxicity studies of TPAs and TPBi are incomplete.

Nuclear receptors are a family of ligand-activated transcription factors that are important regulators of metabolism, differentiation, apoptosis, and cell cycle progression. The ligands of nuclear receptors are small, lipophilic molecules and include various pharmaceutical agents and environmental chemicals. The nuclear receptor retinoid X receptor (RXR) specifically binds a natural retinoid, 9-cis-retinoic acid (9cRA), and thus may be directly involved in the transcription of retinoid signals. RXR agonists can activate RXR homodimers and heterodimers containing permissive partners, such as peroxisome proliferator-activated receptor (PPAR), liver X receptor and farnesoid X receptor. PPARγ is activated by numerous fatty acids and by a class of synthetic anti-diabetic agents, the thiazolidinediones. PPARγ regulates the transcription of genes by heterodimerizing with RXR and by binding to PPAR response elements in the promoter of the target gene.

We previously reported that some organotin compounds act as nonamor organic agonists of both RXR and PPARγ. Our previous studies demonstrated that the potency of the effects induced by organotin compounds is related to the number of alkyl and aryl groups on the tin atom and that the order of potency is tri- > tetra- > di- > mono-substituted. In addition, the potency of organotins for these nuclear receptors’ transactivation varies according to which functional groups are present. For example, compared with alkyl substitutes, phenyl substitutes on a tin atom substantially enhance the potency of the resulting organotin compounds as PPARγ agonists. We recently used X-ray crystallography and mass spectroscopy to determine the three-dimensional structure of the PPARγ-ligand binding domain (LBD) in complex with triphenyltin (TPT), which is the most powerful agonist among organotins. We found that a π–π interaction between TPT and PPARγ contributed to TPT’s ability to strongly induce the transcriptional activity of PPARγ. These results suggest that the triphenyl substituent is important for the powerful agonistic activity of organotins and may make it possible to bind other analogous...
elements to these nuclear receptors.

Here, by focusing on group-15-element compounds that had a triphenyl substituent, we used reporter gene systems and ligand binding assays to clarify the toxicologic modes of action of TPPO, TPAs, and TPBi on RXR and PPARγ.

**MATERIALS AND METHODS**

**Chemicals** The compounds tested are listed in Table 1. Octanol–water partition coefficients ($\log P_{ow}$) for the tin compounds were calculated by using Chemdred software (PerkinElmer, Inc., Waltham, MA, U.S.A.). Triphenylarsine (TPAs) (amino acids 201–462) were subcloned into pGEX-4T (GE Healthcare Life Sciences, Buckinghamshire, U.K.). Triphenylphosphine oxide (TPPO) was purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Rosiglitazone (Rosi) was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). 9cRA was obtained from Dr. Y. Kamei (Kyoto Prefectural University, Japan). The plasmids we used have been described previously. Briefly, for the RXR transactivation assay, the ligand-binding domain (LBD, encoding amino acids 201–462) of human RXRα was fused to the C-terminal end of the GAL4 DNA-binding domain (amino acids 1–147) in the pBK-CMV expression vector (Agilent Technologies, Santa Clara, CA, U.S.A.) to yield pBK-CMV-GAL4-hRXRα. For the PPARγ transactivation assay, full-length cDNA of human PPARγ (hPPARγ) was fused to the C-terminal end of the GAL4 DNA-binding domain in the pM expression vector (Clontech/Takara, Bio, Katsushika, Japan) to yield pM-hPPARγ. GAL4-hRXRα mutant constructs in which C432 and GAL4-hPPARγ mutant construct in which C285 was mutated to alanine were generated by site-directed mutagenesis of the pBK-CMV-GAL4-hRXRα plasmid and the pM-hPPARγ plasmid, respectively, by using a PrimeSTAR Mutagenesis Basal Kit (TaKaRa Bio). The resulting mutant constructs are designated pBK-CMV-GAL4-hRXRαC432A, and pM-hPPARγC285A, respectively. The LBDs of hRXRα (amino acids 201–462) were subcloned into pGEX-4T (GE Healthcare Life Sciences, Buckinghamshire, U.K.). The DNA sequence encoding hPPARγ was cloned into pGEX-2T (GE Healthcare Life Sciences). These constructs were used to generate glutathione S-transferase (GST)-hRXRα and hPPARγ fusion proteins. The LUC reporter construct containing four copies of the GAL4 DNA-binding site (UAS) followed by the thymidine kinase promoter (p4×UAS-tk-luc) that was used in the chimeric receptor assay was a kind gift from Dr. Y. Kamei (Kyoto Prefectural University, Japan).

**[3H]Thymidine Uptake Assay** To determine the cytotoxicity of TPPO, TPAs, and TPBi, JEG-3 cells (10^4 cells/well) were seeded on to 24-well plates, precultured at 37°C for 24 h, and then treated for 48 h with various concentrations of these compounds. Each culture was pulsed with 20 kBq of [3H] thymidine (GE Healthcare Life Sciences) for 2 h before harvest, and the radioactivity incorporated into the cells was determined by liquid scintillation. A nontoxic concentration of a triphenyl compound was defined as a concentration at which the uptake of [3H] thymidine was >20% that seen with the vehicle alone.

**Transient Transfection Assay** Transfection was performed by using Lipofectamine reagent (Invitrogen/Thermo Fisher Scientific) in accordance with the manufacturer’s instructions. JEG-3 cells (3×10^4 cells/well) were seeded on to 24-well plates, precultured at 37°C for 24 h, and then

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**Table 1. Triphenyl Compounds Tested**

| Triphenyl compound     | Acronym | Purity (%) | Log $P_{ow}$ | CAS No.   | Source                          |
|------------------------|---------|------------|--------------|-----------|---------------------------------|
| Triphenylarsine TPAs   | TPAs    | >97        | 5.97         | 2767-54-6 | Tokyo Kasei Kougyo (Tokyo, Japan) |
| Triphenylbismuth TPBi  | TPBi    | >98        | 4.73         | 2279-76-7 | Merck (Darmstadt, Germany)      |
| Triphenylphosphine oxide TPPO | TPPO | >98        | 2.83         | 1066-45-1 | Kanto Kagaku (Tokyo, Japan)     |

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transfected with optimal doses of each DNA construct. At 24 h after transfection, the test compounds were added to the transfected cells, which were then cultured in regular culture medium supplemented with 1% charcoal-stripped FCS instead of 10% normal FCS. The cells were harvested 24 h later, and extracts were prepared and assayed for firefly LUC activity by using Dual luciferase reporter system (Promega, Madison, WI, U.S.A.) in accordance with the manufacturer’s instructions. To normalize firefly LUC activity for transfection and harvesting efficiency, the Renilla LUC control reporter construct pGL 4.74 (Promega) was cotransfected as an internal standard in all reporter experiments. The results are expressed as the average relative firefly LUC activity of at least quadruplicate samples.

Ligand Binding Assay The GST-hRXRα and GST-hPPARγ fusion proteins were expressed in Escherichia coli BL21 (DE3) cells and purified by using glutathione–Sepharose 4B (GE Healthcare Life Sciences). The purified proteins (30 µg/mL) were incubated with 10 nM of [3H]9cRA and 50 nM of [3H]Rosi or 200 nM of [14C]TPTOH without or with the above-described unlabeled ligand or test compounds. After incubation at 4°C for 1 h, specific binding was determined by using the hydroxyapatite binding assay.20,21) Binding in the presence of a 100-fold molar excess of unlabeled ligand was defined as nonspecific binding; specific binding was defined as total binding minus nonspecific binding. The upper concentration of the test compounds was limited to 60 µM, because denaturation of the receptor protein due to greater concentrations of the test compounds markedly increased the non-specific binding of radioisotope-labeled ligands.

Statistics All data from the control and treatment groups were obtained from the same numbers of replicate samples per

Fig. 2. Effects of TPPO, TPAs, and TPBi on DNA Synthesis in JEG-3 Cells

Cells were treated with various concentrations of TPPO, TPAs, and TPBi for 48 h. Results are expressed as the mean±1 S.D. of triplicate cultures. The compounds used and their acronyms are listed in Table 1.

Fig. 3. Ability of TPPO, TPAs, and TPBi to Activate or Suppress GAL–RXRα

JEG-3 cells were cotransfected with p4×UAS-tk-luc and pBK-CMV-GAL4-hRXRα and then treated with various concentrations of TPPO, TPAs, and TPBi with 0.1% DMSO (vehicle) (A–C) or with 100 nM 9-cis retinoic acid (9cRA) (D–F). pGL 4.74 was cotransfected as the control for normalization of Renilla LUC activity. Results are expressed as fold activation compared with vehicle (A–C) or 100 nM 9cRA alone (D–F) (mean±1 S.D.) after normalization to Renilla LUC activity. *Value is significantly (p<0.05) different from that for the vehicle only (A–C) or 100 nM 9cRA alone (D–F).
**RESULTS**

Effects of TPPO, TPAs, and TPBi on DNA Synthesis in JEG-3 Cells  To confirm the nontoxic concentration ranges of TPPO, TPAs, and TPBi and to determine whether treatment with triphenyl compounds was associated with cytotoxic effects, we performed DNA synthesis assays. JEG-3 human choriocarcinoma cells were treated for 48 h with the three tin compounds at various concentrations, and DNA synthesis was evaluated by [3H]thymidine incorporation (Fig. 2). TPPO at 0.1 to 100 µM had no significant effect on [3H]thymidine incorporation, whereas 100 µM TPAs and 100 µM TPBi both markedly reduced [3H]thymidine incorporation. By using these results as a guide, we established the maximal nontoxic concentration of each compound for use in investigating possible effects in cell-based assays.

Ligand Activity of TPPO, TPAs, and TPBi on RXRα in Cell-Based Assays  We then defined the functional potency of TPPO, TPAs, and TPBi as RXR agonists. To this end, we exposed JEG-3 cells to various concentrations of TPPO, TPAs, and TPBi at which the uptake of [3H]thymidine was ≈20% of that for the vehicle and measured the responsiveness of the RXRα receptor by using a chimeric receptor consisting of the DNA-binding domain of the yeast transcription factor GAL4 and RXRα (GAL–RXRα) and a LUC reporter system. Although TPAs and TPBi lacked agonistic activity in this system, 100 µM TPPO modestly but significantly (p<0.05) induced the transactivation function of hRXRα (Figs. 3A–C).

To evaluate the antagonist activity of TPPO, TPAs, and TPBi, we exposed JEG-3 cells to these compounds in the presence of 100 nM 9cRA and measured the resulting LUC activity. Whereas TPAs and TPBi at 10 µM inhibited RXRα-associated transcription, TPPO lacked antagonist activity in this context (Figs. 3D–F).

We previously reported that C432 of hRXRα was critical for transactivation of hRXRα by organotin compounds, respectively.21) Accordingly, to investigate whether C432 of hRXRα is involved in the ligand activity of triphenyl compounds to RXRα or not, we investigated the ability of triphenyl compounds to activate or inhibit C432A mutant-associated transcription. The agonist activity of the mutant to TPPO was equivalent to the response of the wild type (Fig. 4A). In addition, we evaluated the antagonist activity of TPAs, and TPBi to the C432A mutant. Although the response of the mutant to 100 nM 9cRA was markedly reduced [3H]thymidine incorporation, whereas 100 µM TPAs and 100 µM TPBi both exhibited significant antagonist activity (p<0.05) toward blocking Rosi-induced LUC activity (Fig. 4A). As previously described in, TPAs at 1 and 10 µM inhibited RXRα-associated transcription, TPPO lacked antagonist activity in this context (Figs. 3D–F).

We also reported that C285 of hPPARγ was critical for transactivation of hPPARγ by organotin compounds, respectively.19) Accordingly, to investigate whether C285 of hPPARγ is involved in the ligand activity of triphenyl compounds to PPARγ or not, we investigated the ability of triphenyl compounds to activate or inhibit C285A mutant-associated transcription. The agonist activity of the mutant to TPPO was equivalent to the response of the wild type (Figs. 5A–C). In these experiments, TPAs had no agonistic activity, whereas 100 µM TPPO and 10 µM TPBi both induced the transactivation function of hPPARγ (Figs. 5A–C). In the presence of 100 nM Rosi, a well-known typical PPARγ agonist, both 1 and 10 µM TPAs, as well as 10 µM TPBi, inhibited (p<0.05) PPARγ transactivation activity (Figs. 5E, F). In addition, TPPO demonstrated a statistical trend (p=0.092) toward blocking Rosi-induced PPARγ activity (Fig. 5D).

We also reported that C285 of hPPARγ was critical for...
transactivation of hPPARγ by organotin compounds. Accordingly, to investigate whether C285 of hPPARγ is involved in the ligand activity of triphenyl compounds to hPPARγ or not, we investigated the ability of triphenyl compounds to activate or inhibit mutant hPPARγ C285A. TPPO and TPBi also induced the transactivation function of C285A mutant equivalent to the wild type (Figs. 6A, B). Furthermore, we evaluated the antagonist activity of TPAs and TPBi to the C285A mutant. Although the fold-activation value of 100 nM Rosi was markedly lower for the mutant than for the wild-type (Figs. 6A, B), the antagonistic activity of TPAs and TPBi also remained for the mutant comparable to for the wild-type (Figs. 6C, D).

**Competition of TPPO, TPAs, and TPBi with Radio-ligands for Binding to RXR and PPARγ**

We performed competitive ligand-binding assays to characterize the binding affinities of TPPO, TPAs, and TPBi for RXR in greater detail. Accordingly, we measured the ability of [3H]9cRA or [14C]TPT to compete with TPPO, TPAs, and TPBi for binding to GST-RXRα. TPAs and TPBi competed with [3H]9cRA for binding to GST-RXRα in a concentration-dependent manner (Fig. 7A). However, these compounds failed to compete with [14C]TPT (Fig. 7B). TPPO failed to compete with both [3H]Rosi and [14C]TPT for binding to PPARγ (Figs. 8A, B).

**DISCUSSION**

We investigated the ligand activity of TPAs, TPBi, and TPPO for RXRα and PPARγ by using reporter gene systems and ligand binding assays. The results of the reporter assays suggested that TPAs is a partial antagonist of both RXR and PPARγ (Figs. 3, 5). In addition, TPBi is a potential partial antagonist of RXR but a partial agonist of PPARγ (Figs. 3, 5). Consistent with the results of reporter gene assays, TPAs and TPBi blocked the binding of [3H]9cRA to RXR and of [3H]Rosi to PPARγ in a competitive manner (Figs. 7A, 8A). These results suggest that TPAs and TPBi might modulate the transactivation of RXR and PPARγ by directly binding to each receptor as a ligand.

However, neither TPAs nor TPBi altered the binding of [14C]TPT, which is a nanomolar dual agonist of RXR and PPARγ, to either receptor (Figs. 7B, 8B), suggesting that the protein–ligand interaction between TPT and RXR or PPARγ differs markedly from those between TPAs or TPBi and these receptors. We previously demonstrated that specific binding of organotin compounds is achieved through non-covalent
ionic interactions between the tin atom and the sulfur atom of C432 of hRXRα or C285 of hPPARγ. However, the either C432 mutation of hRXRα or C285 mutation of hPPARγ did not result in significant effect of the ligand activity of TPAs and TPBi, for each receptor (Figs. 4, 6). Our current results suggest that these non-covalent ionic interactions are not involved in the binding of TPAs and TPBi to RXRα and PPARγ. Furthermore, in the case of PPARγ, we previously demonstrated that TPT has considerably stronger agonistic activity than does tributyltin (TBT) and that the phenyl rings of TPT enable it to form a network of π–π interactions with Phe363 of PPARγ, thus contributing to TPT’s increased ability to activate the transcriptional activity of this receptor.23) Hence, the protein–ligand interaction between PPARγ and TPAs or TPBi might similarly involve π–π interaction. Further studies are necessary to clarify which amino acids of RXRα and PPARγ are important for the binding of TPAs and TPBi to the ligand-binding pocket.

In contrast to TPAs and TPBi, TPPO slightly but significantly transactivated both RXR and PPARγ (Figs. 3, 5) but failed to compete with either [3H]9cRA for binding to RXR or [3H]Rosi for binding to PPARγ (Figs. 7A, 8A). These ap-

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Fig. 6. Ability of TPPO, TPAs, and TPBi to Activate orSuppress Mutant hPPARγ

JEG-3 cells were cotransfected with p4×UAS-tk-luc and pM-hPPARγ C285A and then treated with TPPO and TPBi with 0.1% DMSO (A, B) and with TPAs and TPBi with 100 nM Rosi (C, D). pGL 4.74 was cotransfected as the control for normalization of Renilla LUC activity. Results are expressed as fold activation compared with vehicle (A, B) or 100 nM Rosi alone (C, D) (mean±1 S.D.) after normalization to Renilla LUC activity. *Value is significantly (p<0.05) different from that for the vehicle only (A, B) or 100 nM Rosi alone (C, D).

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Fig. 7. Competition by TPPO, TPAs, and TPBi with Radioligands for Binding to RXRα

A GST–hRXRα fusion protein was incubated with increasing concentrations of unlabeled TPPO, TPAs, and TPBi as competitors in the presence of 10 nM [3H]9cRA (A) or 200 nM [3H]TPTOH (B) as ligands. Each experiment was performed at least twice, and representative curves are shown.
parently contradictory results suggest that TPPO induces the transactivation of RXR and PPARγ without directly binding to either receptor (that is, through a mechanism other than ligand binding).

RXR and PPARγ are involved in control of the immune system. The anti-inflammatory properties of these nuclear receptors suppress the immunogenicity of antigen-presenting cells35 and reduce cytokine production.22,28 In one study, TPPO significantly suppressed antigen-specific lymphocyte proliferation by interfering with monocyte antigen presentation.30 By combining these previous findings with our current results, we consider that TPPO might suppress lymphocyte proliferation by acting through the RXR or PPAR signaling pathway, or both. However, TPBi induces apoptosis in rat thymocytes, but its toxic effect is less than that of TPT.29 Given that PPARγ activation appears to promote age-related thymic involution through reductions in thymic size, thymic weight, and thymocyte number,30 TPBi and TPT might induce thymic involution by agonistically transactivating PPARγ.

In summary, we here determined that RXR and PPARγ are potential target molecules of TPPO, TPAs, and TPBi. These triphenyl compounds of group 15 elements might act as endocrine disrupters by interfering with the PPAR–RXR signaling pathway. Our findings may facilitate the design of new types of RXR and PPARγ ligands with binding modes that differ from those of the well-known ligands for each receptor.

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Conflict of Interest The authors declare no conflict of interest.

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