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Structural basis for PPAR γ transactivation by endocrine-disrupting organotin compounds

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Organotin compounds such as triphenyltin (TPT) and tributyltin (TBT) act as endocrine disruptors through the peroxisome proliferator-activated receptor γ (PPAR γ) signaling pathway. We recently found that TPT is a particularly strong agonist of PPAR γ . To elucidate the mechanism underlying organotin-dependent PPAR γ activation, we here analyzed the interactions of PPAR γ ligand-binding domain (LBD) with TPT and TBT by using X-ray crystallography and mass spectroscopy in conjunction with cell-based activity assays. Crystal structures of PPAR γ -LBD/TBT and PPAR γ -LBD/TPT complexes were determined at 1.95 Å and 1.89 Å, respectively. Specific binding of organotins is achieved through non-covalent ionic interactions between the sulfur atom of Cys285 and the tin atom. Comparisons of the determined structures suggest that the strong activity of TPT arises through interactions with helix 12 of LBD primarily via π - π interactions. Our findings elucidate the structural basis of PPAR γ activation by TPT.

The peroxisome proliferator-activated receptors (PPARs), subtypes of which have been identified as α , γ , and δ , belong to the nuclear receptor superfamily and act as transcription factors to control the expression of target genes. PPARs form heterodimers with retinoid X receptor (RXR) and bind to specific regions on various genes¹. It has been well established that PPAR γ regulates the expression of the genes responsible for adipocyte differentiation. Furthermore, PPAR γ has been found in trophoblasts, where it serves as an essential regulator of placental differentiation and has other endocrine functions, including the production of human chorionic gonadotropin (hCG) and in steroidogenesis²⁻⁴.

Organotin compounds have been widely used as antifouling biocides for ships, agricultural fungicides, and so on^{5,6}. However, their widespread use has deleteriously affected marine ecosystems. At very low concentrations, organotin compounds such as triphenyltin (TPT) and tributyltin (TBT) induce “imposex”, which is the masculinization of female gastropod mollusks⁷. Hence these tin compounds came to be known as endocrine-disrupting chemicals. In mammals, organotins also have various undesirable effects on immune mechanisms and metabolic activity^{5,8}. We previously investigated the effects of organotins on PPAR γ and showed that: (1) TPT and TBT at nanomolar concentrations enhance hCG production in human choriocarcinoma cells and stimulate adipocyte differentiation; (2) the endocrine disruptive action of organotins is mediated through the PPAR γ -dependent pathway; (3) TPT has considerably stronger agonistic activity toward PPAR γ than does TBT; and (4) tri-alkyl and aromatic tin compounds have stronger agonist activities than do tetra-, di- and monosubstituted compounds⁹⁻¹⁴.

Recently, a crystal structural analysis of the RXR α /TBT complex was performed that provided insights into how TBT activates the RXR α -PPAR γ signaling pathway¹⁵; transactivation by organotins was attributed to RXR α , not to PPAR γ , because of the weak agonistic activity of TBT toward PPAR γ . In addition, the study¹⁵ reported that organotin compounds employ a covalent interaction between the tin atom and a particular cysteine residue



(Cys432) located in helix 11 (H11) of RXR α . Stabilization of this secondary structural element is believed to be essential to modulate transcriptional activity. Due to the absence of a cysteine residue at the corresponding position in PPAR γ , the authors of the study¹⁵ concluded that the binding of organotin compounds to PPAR γ alone does not allow efficient transactivation. Therefore, our finding that TPT acts as a powerful PPAR γ agonist⁹ suggests the need to clarify the structural basis for PPAR γ transactivation by tin compounds in order to better understand the mechanism of RXR-PPAR γ signaling via these compounds.

Since the first structural determination of the PPAR γ ligand-binding domain (LBD) was reported by using X-ray crystallography¹⁶, multiple structures in both the apo and liganded forms have been determined. From these studies, activation mechanisms induced by various well-known PPAR γ agonists, including thiazolidinediones (TZD) such as rosiglitazone, have been discovered. The binding of the agonist to the ligand-binding pocket of LBD causes its helix 12 (H12) to form an active conformation that promotes the recruitment of a transcriptional coactivator. However, the mechanism of transactivation by tin compounds with various substituted organic groups, which are distinct from well-known PPAR γ agonists with respect to their structural and chemical features (see Supplementary Fig. S1 online), is still poorly understood and cannot be inferred based on previously known structures. Therefore, to elucidate the mechanism, here we determined the three-dimensional structures of PPAR γ -LBD in complexes with TPT and TBT, respectively, and characterized these complexes by using mass spectrometry (MS) and biological activity assays.

Results

Structural determination of the PPAR γ complexes. Crystals of PPAR γ -LBD in complex with TPT and TBT, respectively, were obtained by co-crystallization. They belong to the same space group $P2_1$ with similar cell dimensions. The structures determined at 1.95 Å (PPAR γ -LBD/TBT) and 1.89 Å (PPAR γ -LBD/TPT) resolutions (Table 1) were revealed to have the typical nuclear receptor fold that comprises an α -helical sandwich fold (12 helices) with a four-stranded β -sheet. In an asymmetric unit, two PPAR γ -LBD molecules were found. These structural data have been deposited in the Protein Data Bank database under the accession numbers 3WJ4 and 3WJ5. Both crystals contained two LBDs (chain A and B) in the asymmetric unit, where each LBD was bound to one ligand. The structure of chain A assumed an “active” conformation that was found in the PPAR γ -LBD/agonist/co-activator peptide complex (PDB No. 2PRG)¹⁶, where helix 12 (H12) of LBD exists in a position suitable to interact with the agonist and to form the binding site of the co-activator. By contrast, in the other molecule (chain B), H12 was displaced from the ligand-binding pocket, preventing the co-activator from binding to the LBD, possibly due to extensive interactions with symmetry-related neighboring molecules in the crystal (see Supplementary Fig. S2 online). Because PPAR γ transactivation is induced by the “active” conformation of H12, we hereafter focused on the structure of chain A (Figure 1).

The exact positions of the ligands were determined by using an anomalous difference map derived from the tin anomalous signals (Figure 2). Although the structural analyses showed ligands with

Table 1 | Data collection and structural refinement statistics

	PPAR γ -LBD/TBT	PPAR γ -LBD/TPT
Data Collection		
Beamline	Photon factory BL-6A	Photon factory BL-17A
Wavelength (Å)	1.0000	0.9800
Space group	$P2_1$	$P2_1$
Cell dimensions		
a, b, c (Å)	56.34, 88.29, 57.51	56.52, 88.49, 57.94
α, β, γ (°)	90.00, 90.68, 90.00	90.00, 91.01, 90.00
Resolution range (Å)	50.00-1.95 (1.98-1.95)	50.00-1.89 (1.92-1.89)
R_{merge}^b	0.066 (0.352)	0.064 (0.372)
$\langle I/\sigma \rangle$	43.25 (7.2)	44.12 (6.3)
Completeness (%)	100 (100)	99.8 (99.5)
Redundancy	7.6 (7.5)	7.6 (7.3)
Refinement		
Resolution (Å)	36.438-1.95 (2.00-1.95)	36.515-1.89 (1.94-1.89)
No. reflections	38942	43047
R_{work} (%)	0.194 (0.218)	0.203 (0.233)
R_{free} (%)	0.241 (0.270)	0.247 (0.247)
No. of non-H atoms		
Protein	4164	4116
Ligand	26	38
Water	224	223
Average B-factors		
Overall (Å ²)	25.4	24.0
Protein (Å ²)	25.0	23.7
Ligand (Å ²)	33.2	48.2
Water (Å ²)	26.2	26.2
R.M.S. deviations		
Bond length (Å)	0.017	0.017
Bond angles (°)	1.627	1.516
Ramachandran plot statistics		
Most favored (%)	98.62	98.81
Additional allowed (%)	1.38	0.99
Disallowed (%)	0.00	0.20

^aValues in parentheses are for the highest resolution shell.

^b $R_{\text{merge}} = \sum |I_h - \langle I_h \rangle| / \sum I_h$, where $\langle I_h \rangle$ is the average intensity of reflection h and symmetry-related reflections.

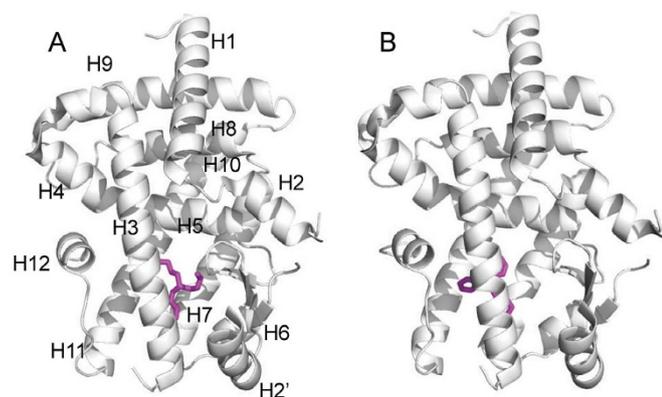


Figure 1 | Structures of the PPAR γ -LBD complex with TBT (A) and TPT (B).

relatively low electron densities (Figure 2) and large B factors (Table 1), clear anomalous signals corresponding to tin atoms were found in close proximity to the sulfur atom of Cys285, which exists in the central region of the ligand-binding pocket. This finding suggests that specific interactions occur between the tin and sulfur atoms. In the TBT complex, only one anomalous peak was identified at the side of the β -strand (B3), at a distance of 2.54 Å from the sulfur atom of Cys285. In contrast, the TPT complex had a major (16σ) and a minor (5σ) anomalous peak around Cys285, which were associated with two alternative binding sites for TPT. Refinements provided the model with the occupancy of TPT molecule at the major site is 80%. Intriguingly, despite the structural similarities of the ligands, the major TPT complex was close to H12, which was on the opposite side of Cys285 from the position of TBT. The distance between the tin atom and the sulfur atom of Cys285 was 2.74 Å (Figure 2). For both complexes, the Sn-S lengths were longer than the sum of the covalent radii (2.42 Å) and equal to, or slightly longer than, the sum of the ionic radii (2.54 Å) of tin and sulfur, suggesting that the Sn-S bond is non-covalent and ionic rather than covalent, as previously observed in the crystal structure of the RXR α /TBT complex.

The PPAR γ ligand-binding pocket is surrounded by secondary elements, B3, H3, H5, H7, H11, and H12. Cys285 in H3 serves an anchor for the tin atom to interact with the residues lining the pocket, which are predominantly hydrophobic in character, by using the alkylic or aromatic moieties of the organotin. Both ligands share common interacting residues, namely Ile281 (H3), Phe282 (H3), Ile326 (H5), Tyr327 (H5), Phe360 (H7), Phe363 (H7), Met364 (H7), and His449 (H11). Besides these residues, TPT also interacts

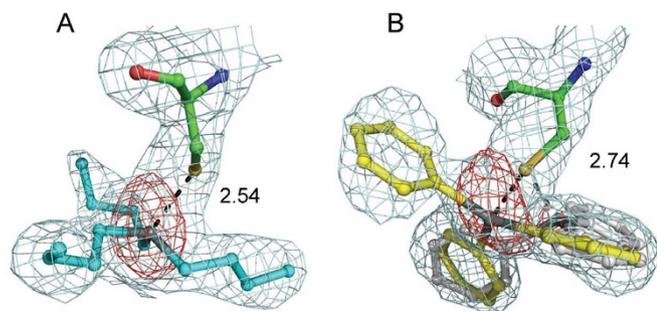


Figure 2 | The organotin atoms ((A): TBT, (B): TPT) and Cys285 in the ligand-binding pocket of PPAR γ . Anomalous difference electron density maps contoured at 3.5σ (red) indicate the position of the tin atom and omit $2F_o - F_c$ electron density maps contoured at 0.5σ (cyan) indicate the geometry of the aliphatic or aromatic chain. Distances between the tin and sulfur atoms are indicated. In panel (B), the major and minor conformations of TPT are shown in yellow and gray, respectively.

with Gln286 (H3), Ser289 (H3), Leu330 (H5), Lys367 (H7), Leu453 (H11), Leu469 (H12), and Tyr473 (H12). In contrast, TBT interacts with Val339 (B3) and Ile341 (B3) (Figure 3).

Activity of tin compounds as PPAR γ agonists. To evaluate the functional potency of tin compounds as PPAR γ agonists, we performed reporter assays using the PPAR γ -specific GAL4-luc system, where wild-type PPAR γ -LBD is fused to the GAL4 DNA-binding domain and a luciferase reporter gene is under the control of GAL4 binding elements (Figure 4). TPT (100 nM) enhanced the transactivation function of PPAR γ by 11-fold, and this level of activation was comparable to that of rosiglitazone, a representative full agonist for PPAR γ . However, although lower concentrations of TPT and TBT provided similar responses to that of rosiglitazone, 100 nM TBT showed only half the level of activation achieved with rosiglitazone and TPT. These results indicate that, despite similar structural and chemical features, TPT and TBT differ in their PPAR γ transactivation and/or binding.

To verify the involvement of the π - π interaction in the full-agonistic activity of TPT, we substituted Phe363 of PPAR γ with alanine

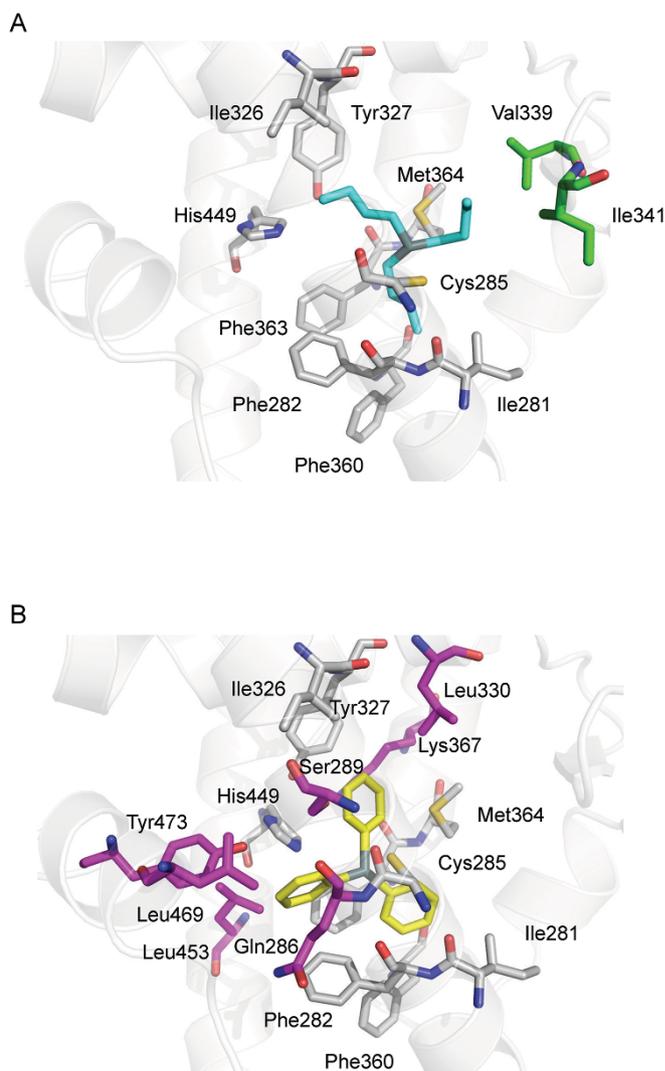


Figure 3 | Interactions of PPAR γ -LBD with TBT (A) or TPT (B). Ligands are shown as cyan and yellow sticks. Ligand-interacting residues, which are close (within 4.2 Å) to the ligands, are also shown. Common interacting residues for both ligands are shown as gray sticks. Interacting residues for TBT or TPT only are shown in green or pink, respectively.

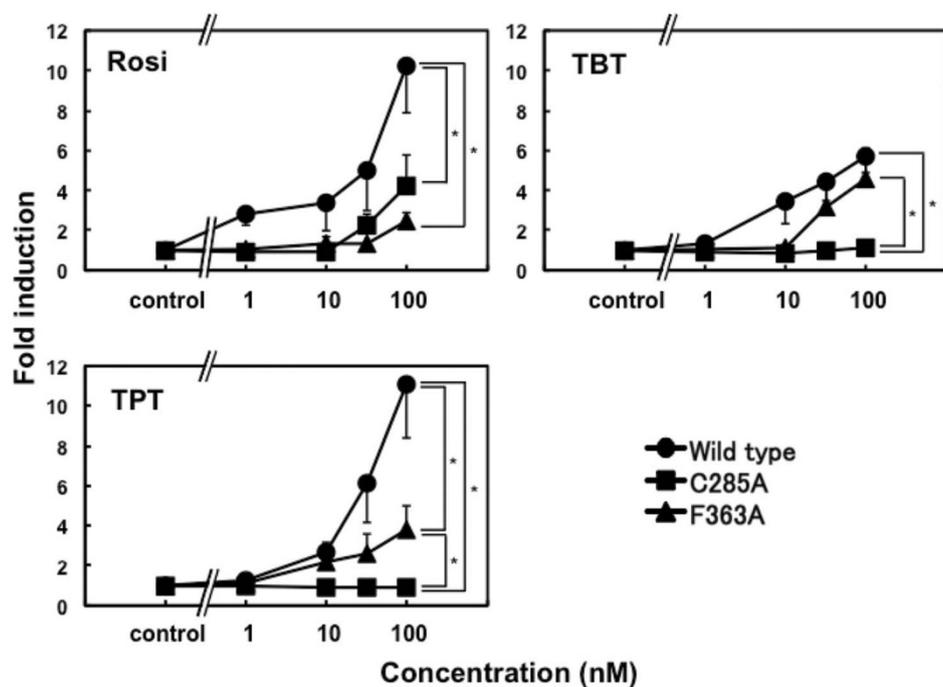


Figure 4 | Cell-based transcriptional activation assay of rosiglitazone, TPT, and TBT on wild-type, C285A, and F363A mutants of PPAR γ . Data are expressed relative to the levels of vehicle-treated cells; these levels were set to 1. Results are expressed as means \pm 1 S.D. of three independent cultures. * P < 0.05 indicates values significantly different between 2 groups analyzed by using 2-way ANOVA.

and carried out the cell-based assay. In the case of TPT, the transcriptional activity of the mutant was about three-fold lower than that of wild-type, whereas no significant difference in activity was observed for TBT upon the introduction of the mutation (Figure 4).

Characterization of organotin binding to PPAR γ Cys285. The PPAR γ mutant with its Cys285 replaced to alanine was not activated by TBT and TPT (Figure 4). Thus, as was previously shown for RXR α ¹⁵, the specific interaction between the cysteine residue and the tin atom is essential for the activation of PPAR γ by organotins. However, our structural data suggested that the Sn-S bonds in PPAR γ complexes are ionic in nature, in contrast to the covalent bond reported for the RXR α -LBD/TBT complex¹⁵. To clarify whether complex formation of PPAR γ -LBD with tin compounds is mediated through covalent or non-covalent bonding, we performed MS analysis under non-denaturing conditions for PPAR γ -LBD complexes with TPT or TBT (Figure 5). The results indicated that the complexes of PPAR γ with these organotins formed in 1 : 1 molar ratios (Figure 5C and 5E). No free PPAR γ -LBD was detected in these spectra and even under highly stringent ionization conditions of up to 190 V of sample cone voltage (V_c), the complex peaks were not disrupted, indicating that the complexes for both cases were highly stable.

On the other hand, the addition of aliquots of formic acid to the mixtures, which should induce PPAR γ -LBD unfolding, resulted in different MS patterns (Figure 5A, B, and D). The newly emerged ion series under the partially (Figure 5B) or fully (Figure 5A and 5D) PPAR γ -LBD unfolded conditions yielded the molecular mass of free PPAR γ -LBD (31,370.6 Da), indicating the dissociation of TPT or TBT from PPAR γ -LBD upon the acid-induced unfolding of PPAR γ -LBD in the mixture. Furthermore, the addition of a sufficient amount of formic acid to the mixture of PPAR γ -LBD and TPT led to the complete disappearance of the peaks for the PPAR γ -LBD/TPT complex, whereas, in addition to the peaks of unfolded PPAR γ -LBD, the peak of dissociated TPT was consistently observed as a singly charged species with a molecular mass of 350.1 Da (see

Supplementary Fig. S3 online). In the previous study¹⁵, they proposed that TBT is connected to RXR α through a “covalent bond” involving the sulfur atom of RXR α Cys432 based on the crystal structure and on MS results that showed no disruption of the RXR α -LBD/TBT complex even when highly stringent parameters (V_c : 190 V) were applied. Similar to their results, in our current study, the PPAR γ -LBD/TPT and PPAR γ -LBD/TBT complexes were retained even under highly stringent conditions (V_c : 190 V). However, our current structural study clearly showed the absence of a covalent bond in the PPAR γ -LBD/TPT and PPAR γ -LBD/TBT complexes. Given that little or no dissociation of protein metal complexes in the gas phase has been observed in the mass spectra of metal complexes^{17,18} and that hydrogen bonding and electrostatic interactions are maintained to a greater extent than are hydrophobic (van der Waals) interactions¹⁹, the retention of the non-covalent interactions of nuclear receptors/organotin complexes under non-denaturing MS conditions with highly stringent parameters is not unexpected. We also performed MS analysis under fully denaturing conditions in which a covalently-bound 15-deoxy- Δ 12,14-prostaglandin J2 (15d-PGJ2)²⁰ did not dissociate from PPAR γ -LBD while a non-covalent ligand, rosiglitazone, did. The results demonstrated that the complexes of PPAR γ with organotins dissociates in the denaturing conditions (see Supplementary Fig. S4 online).

Therefore, we re-investigated the binding mode of the RXR α -LBD/TBT interaction by performing a MS analysis of the RXR α -LBD/TBT mixture. Consistent with the previous report¹⁵, peaks of RXR α -LBD/TBT complex with similar charge states could be observed without disruption of the complex formation. However, the bound TBT was dissociated with ease when RXR α was partially or completely unfolded by the addition of acetonitrile or formic acid to the solution (see Supplementary Fig. S5 online). These results indicate that TBT is bound to RXR α via a non-covalent interaction.

Taken together, we conclude that TPT and TBT bind to PPAR γ principally via a non-covalent, ionic bond between the tin atom and Cys285 that requires the correct folding of PPAR γ -LBD, which provides appropriate electrostatic and van der Waals interactions.

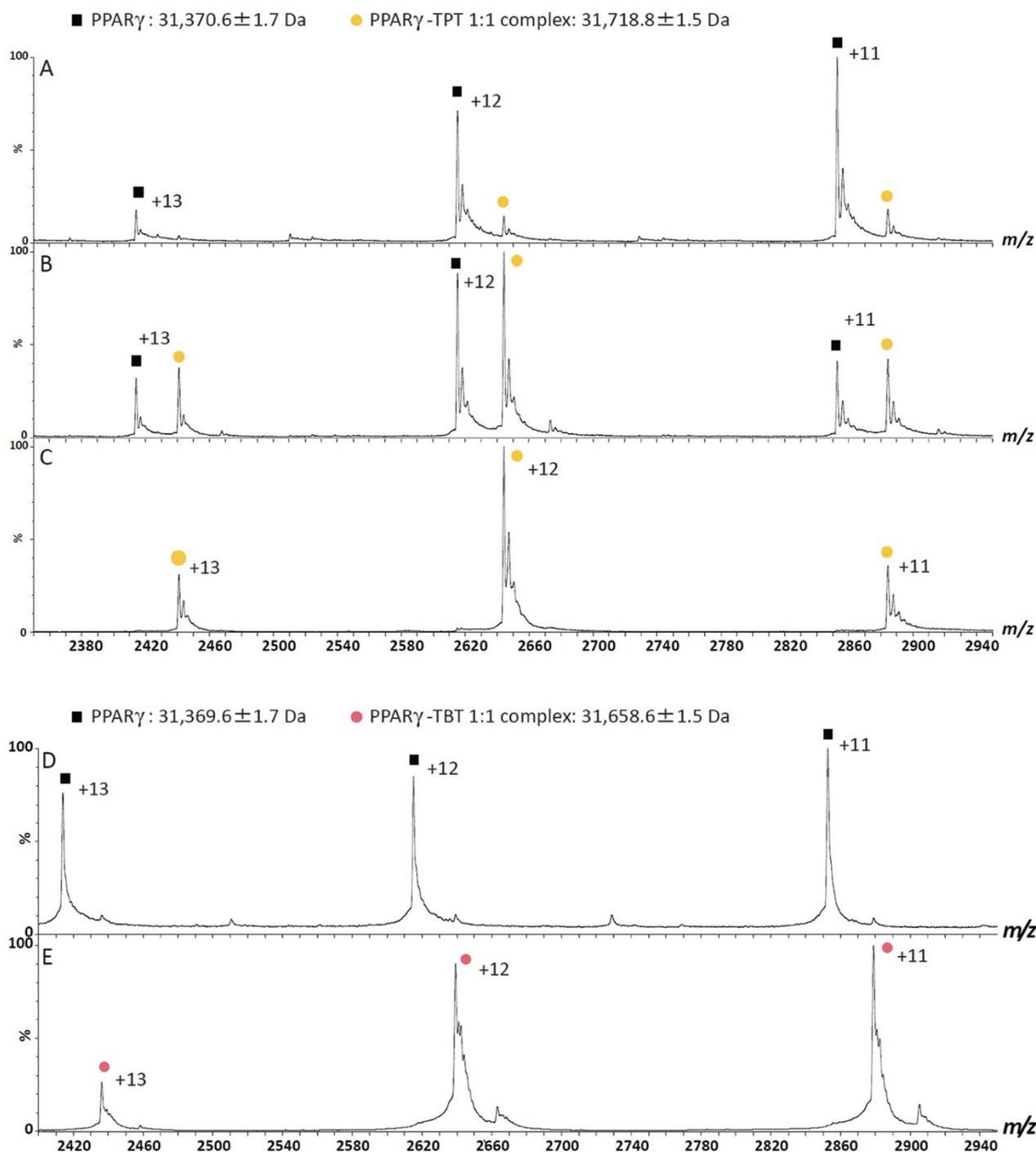


Figure 5 | Mass spectrometry of the PPAR γ -LBD complex with TPT (A–C) or TBT (D, E) under non-denaturing conditions. Mass spectra show that PPAR γ forms a complex with TPT (C) or TBT (E) in a 1 : 1 molar ratio. Mass patterns after the addition of aliquots of formic acid (A, D = 3%, B = 1%) to the complex indicate that the dissociation of the interaction is caused by the unfolding of PPAR γ -LBD.

Discussion

Recent pharmacological studies classify agonists of nuclear receptors as full or partial agonists, depending on their transcriptional activities. The difference between these two types of agonist can be explained in terms of the structural features of PPAR γ -LBD/agonist complexes and whether they use the H12-mediated or non-H12-mediated mechanism. The full agonists directly stabilize H12, allowing it to dock against H3 and H11^{16,21}. The coactivator-binding interface is configured by this conformational change. In contrast, the partial agonists have no direct contact with H12, predominantly interacting with residues on H3 and B3²². This classification also

provides a means to distinguish between the differences in activity between the tin compounds (Figure 4). TPT, a full agonist as shown by cell-based assays (Figure 4), has hydrophobic interactions with Leu469 and Tyr473, stabilizing H12. In contrast, the hydrophobic interactions of TBT with the side chains of Val339 and Ile341, located in B3, displace TBT from H12. This structural feature of the TBT complex suggests some limited agonistic activity for TBT, as observed in the cell-based assay (Figure 4).

In both complexes, the protein backbones have almost the same conformation, and the ligand volumes (TBT, 233 Å³; TPT, 245 Å³) are very similar¹⁵. Thus, it is likely that both organotin compounds

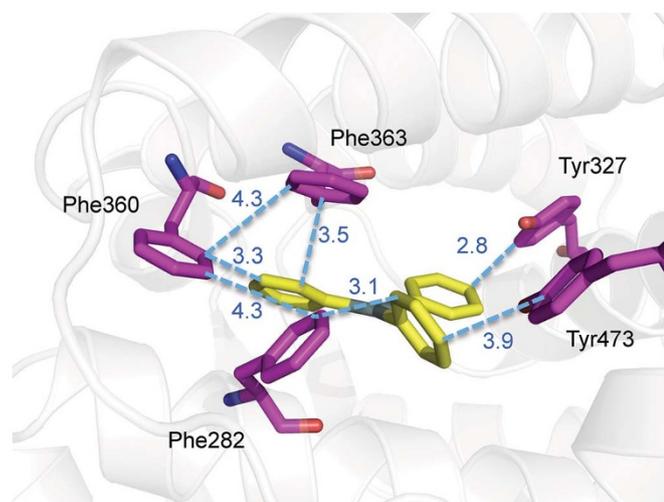


Figure 6 | The π - π interactions in the PPAR γ -LBD/TPT complex. The distances between the nearest neighbor carbon atoms of the aromatic rings are indicated.

would interact with PPAR γ -LBD in the same manner and show similar transactivation activity. However, we found that they occupy different spatial positions within the ligand-binding pocket that result in different levels of activation. Of note, organotin compounds are anchored to the Cys285 of PPAR γ by an ionic bond, which does not impose strict angular or length constraints, unlike covalent bonds. Moreover, PPAR γ -LBD has a relatively large binding pocket, which can accommodate a diverse array of ligands¹⁶. Thus, TPT and TBT can optimally adapt their positions depending on their specific interactions with surrounding residues in the ligand-binding pocket, even though their ligand volumes are almost the same. The origins of positional preference are explained based on high-resolution structural analyses of the ligand-binding pocket, in which the side chains of Phe and Tyr form a cluster on the side of H12. The phenyl rings of TPT make it possible to form a network of π - π interactions, as shown in Figure 6, which stabilizes the active conformation of H12 and results in full agonistic activity. It is well known that π - π interactions can have a significant influence on protein–ligand interactions²³. In a previous report¹⁰, we showed that TPT is powerful agonist for PPAR γ but not for the other PPAR subtypes, α and δ , which have an isoleucine at the position corresponding to Phe363. The mechanism of subtype selectivity responsible for the presence of the π - π interaction at Phe363 has been proposed for benzenesulfonamide derivatives, which are selective PPAR γ agonists²⁴. In fact, although the transactivation of the F363A mutant of PPAR γ by TPT was significant lower than that of wild-type, no difference in activity was observed for TBT (Figure 4). These results indicate that the π - π interactions contribute to the high transcriptional activity of TPT for PPAR γ .

In contrast to TPT, TBT had no direct contact with H12 as shown in Figure 3A. This observation is consistent with previous findings where TBT behaved as a weak agonist against PPAR γ and acted mainly on RXR α ¹⁵. Although no direct interaction between the organotin and H12 was observed in the case of the RXR α /TBT complex, a specific interaction between the tin atom and Cys432 in H11 of RXR α might stabilize the helix 12. However, Cys285 of PPAR γ , which is located at H3, offers an anchoring point to the organotin but is not sufficient to position the ligand such that it can support the active conformation of H12 (see Supplementary Fig. S6 online).

In conclusion, here we show the structural basis for the strong activation of PPAR γ by TPT. We previously showed that hCG secretion in human choriocarcinoma cells, which is upregulated by RXR-PPAR γ signaling pathways, is powerfully induced by phenyltin compounds, relative to butyltin compounds^{9,12,13} and concluded that

the differences in toxicological response caused by these organotins depended on their potencies as agonists for PPAR γ and RXR α . Our current observations show that the mode of action of organotin compounds, via RXR-PPAR γ signaling pathways, is strongly influenced by their chemical structures.

Methods

Cell culture. Cells of the human choriocarcinoma cell line JEG-3 (ATCC No. HTB-36) were obtained from ATCC (Manassas, VA). JEG-3 cells were cultured in MEM with 2 mM L-glutamine, 0.1 mM MEM nonessential amino acid solution (Invitrogen, Carlsbad, CA), and 10% fetal calf serum (FCS). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Plasmid construction. Full-length cDNA of human PPAR γ was amplified by RT-PCR using mRNA from JEG-3 cells. For the PPAR γ transactivation assay, the amplified hPPAR γ fragment was cloned into the pM vector (Clontech, Mountain View, CA). The resulting GAL4 DNA-binding domain- (DBD) fused hPPAR γ expression vector was termed pM-hPPAR γ . pM-hPPAR γ mutant constructs, carrying a Cys285 or Phe363 to Ala mutation, were generated by site-directed mutagenesis of the pM-hPPAR γ plasmid using the PrimeSTAR mutagenesis basal kit (Takara Bio, Shiga, Japan). The sequences of the mutagenic primers are shown in Supplementary Table S1, online.

PPAR γ transactivation assay. The responsiveness of PPAR γ to organotin compounds was measured by using a chimeric receptor consisting of the GAL4-DBD and PPAR γ , pM-hPPAR γ , with a luciferase (LUC) reporter system, p4 \times UAS-tk-luc, which is a LUC reporter construct containing four copies of the GAL4 binding site [upstream activating sequence (UAS) of GAL4] followed by the thymidine kinase promoter^{9,10}. Transient transfection assays were performed in JEG-3 cells with Lipofectamine reagent (Invitrogen). The cells (3×10^4) were seeded in 24-well plates 24 h before transfection with pM-hPPAR γ and p4 \times UAS-tk-luc. At 24 h after transfection, compounds in 0.1% DMSO were added to the cells, which were then cultured in medium supplemented with 1% charcoal-stripped FCS. The cells were harvested 24 h later, and extracts were assayed for firefly LUC activity. To normalize firefly LUC activity, the Renilla LUC control reporter construct pGL 4.74-TK (Promega, Madison, WI) was co-transfected as an internal standard. The results are expressed as the average of measurements of at least quadruplicate samples. Data from the cell-based transcriptional activation assay were analyzed by using two-way ANOVA, with multiple comparisons obtained with the Tukey-HSD test. A *P* value of <0.05 was used to indicate statistical significance. All statistical analyses were performed with SPSS software (Chicago, IL).

Protein expression and purification. The human PPAR γ -LBD (residues 202–477) was cloned into the pGEX-6P-3 vector. *E. coli* Rosetta (DE3) cells (Novagen) transformed by the plasmid were grown at 37°C in LB medium containing 20 μ g/ml chloramphenicol and 50 μ g/ml ampicillin to A₆₀₀ = 0.6, and were induced by the addition of IPTG to a final concentration of 0.1 mM. Then, the cells were grown for 12–14 h at 10°C. Harvested cells were lysed by sonication. After the supernatant was applied to a GSTrap HP column (GE Healthcare), 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 1 mM DTT with 100 U PreScission protease (GE Healthcare) was applied to same column, to remove the GST tag. PPAR γ -LBD was eluted with the same buffer and dialyzed against 20 mM Tris-HCl (pH 8.0). Then PPAR γ -LBD was applied to a HiTrap Q HP column (GE Healthcare) and eluted with an NaCl gradient (0.01–0.5 M). The fractions containing PPAR γ -LBD were pooled and dialyzed against 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, and 2 mM DTT. PPAR γ -LBD was concentrated to 5 mg/mL by using Amicon Ultra 15 concentrator (Millipore).

Crystallization, data collection, and structure determination. We performed co-crystallization with organotin compounds and PPAR γ -LBD by using the sitting-drop vapor-diffusion method at 277 K. Because the organotin compounds have poor aqueous solubility, an excess of each compound was added as powder to the protein solution and incubated for several hours to obtain the PPAR γ -LBD/organotin complex. Insoluble compound was removed before crystallization by centrifugation followed by filtration through a 0.2- μ m membrane filter. Crystals were obtained from drops derived from 1 μ L of protein solution (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 5 mM DTT) mixed with an equal volume of crystallization buffer (100 mM Tris-HCl, pH 8.5, 160 mM CH₃COONH₄, 19%–23% PEG4000). Diffraction data were collected at 100 K on beamline 6A or 17A of Photon Factory, KEK (Tsukuba, Japan), and beamline BL38B1 of Spring-8 (Hyogo, Japan), and were indexed, integrated, and scaled by using HKL2000. All structures were solved by use of the molecular replacement method using MOLREP from the CCP4 suite with the previously reported PPAR γ -LBD structure (PDB 1PRG) as an initial search model. Structural refinement and the addition of water molecules were performed by using Coot and REFMAC5. The final structures were checked and validated by MolProbity. The atomic coordinates were deposited in the Protein Data Bank (PDB) as entry code 3WJ4 and 3WJ5 for PPAR γ -LBD/TBT and PPAR γ -LBD/TPT, respectively. The statistics for the diffraction data collection and structural refinement are shown in Table 1.

Mass spectrometry. The concentration of PPAR γ -LBD was fixed at 1 mM, and an excess amount of TPT or TBT was added. After dilution with buffer (20 mM Tris and



150 mM NaCl pH 7.5) to 10 μ M, sample solutions of PPAR γ -LBD, PPAR γ -LBD/TPT, and PPAR γ -LBD/TBT were subjected to a buffer exchange with 150 mM ammonium acetate, pH 7.5, by passing them through mini gel filtration columns (BioRad) prior to analysis. All samples were analyzed by use of nanoflow electrospray using in-house capillaries prepared as described previously²⁵. Samples were loaded into capillaries, and spectra were recorded on a modified Synapt HDMS mass spectrometer (Waters), which provides the molecular mass of a protein complex formed through a non-covalent interaction²⁶. All mass spectra were calibrated against cesium iodide and analyzed by Mass Lynx software (Waters). Typical conditions included 2–3 μ L of aqueous protein solution, capillary voltage of 1.1–1.7 kV, cone voltage of 190 V, and trap and transfer collision energy voltages of 30 and 10 V, respectively. The source pressure was maintained at 3×10^{-2} mbar.

- Kliwer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A. & Evans, R. M. Convergence of 9-*cis* retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. *Nature* **358**, 771–774 (1992).
- Fournier, T. *et al.* The role of PPAR-gamma/RXR-alpha heterodimers in the regulation of human trophoblast invasion. *Ann. N. Y. Acad. Sci.* **973**, 26–30 (2002).
- Schaiff, W. T. *et al.* Peroxisome proliferator-activated receptor-gamma modulates differentiation of human trophoblast in a ligand-specific manner. *J. Clin. Endocrinol. Metab.* **85**, 3874–3881 (2000).
- Tarrade, A. *et al.* PPARgamma/RXRalpha heterodimers control human trophoblast invasion. *J. Clin. Endocrinol. Metab.* **86**, 5017–5024 (2001).
- Boyer, I. J. Toxicity of dibutyltin, tributyltin and other organotin compounds to humans and to experimental animals. *Toxicology* **55**, 253–298 (1989).
- Fent, K. Ecotoxicology of organotin compounds. *Crit. Rev. Toxicol.* **26**, 1–117 (1996).
- Horiguchi, T., Shiraishi, H., Shimizu, M. & Morita, M. Effects of triphenyltin chloride and five other organotin compounds on the development of imposex in the rock shell, *Thais clavigera*. *Environ. Pollut.* **95**, 85–91 (1997).
- Ogata, R. *et al.* Two-generation reproductive toxicity study of tributyltin chloride in female rats. *J. Toxicol. Environ. Health A.* **63**, 127–144 (2001).
- Hiromori, Y., Nishikawa, J., Yoshida, I., Nagase, H. & Nakanishi, T. Structure-dependent activation of peroxisome proliferator-activated receptor (PPAR) gamma by organotin compounds. *Chem. Biol. Interact.* **180**, 238–244 (2009).
- Kanayama, T., Kobayashi, N., Mamiya, S., Nakanishi, T. & Nishikawa, J. Organotin compounds promote adipocyte differentiation as agonists of the peroxisome proliferator-activated receptor gamma/retinoid X receptor pathway. *Mol. Pharmacol.* **67**, 766–774 (2005).
- Nakanishi, T. *et al.* Organotin compounds enhance 17beta-hydroxysteroid dehydrogenase type I activity in human choriocarcinoma JAr cells: potential promotion of 17beta-estradiol biosynthesis in human placenta. *Biochem. Pharmacol.* **71**, 1349–1357 (2006).
- Nakanishi, T. *et al.* Trialkyltin compounds enhance human CG secretion and aromatase activity in human placental choriocarcinoma cells. *J. Clin. Endocrinol. Metab.* **87**, 2830–2837 (2002).
- Nakanishi, T. *et al.* Trialkyltin compounds bind retinoid X receptor to alter human placental endocrine functions. *Mol. Endocrinol.* **19**, 2502–2516 (2005).
- Nakanishi, T. Endocrine disruption induced by organotin compounds; organotins function as a powerful agonist for nuclear receptors rather than an aromatase inhibitor. *J. Toxicol. Sci.* **33**, 269–276 (2008).
- le Maire, A. *et al.* Activation of RXR-PPAR heterodimers by organotin environmental endocrine disruptors. *EMBO Rep.* **10**, 367–373 (2009).
- Nolte, R. T. *et al.* Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma. *Nature* **395**, 137–143 (1998).
- Lafitte, D., Capony, J. P., Grassy, G., Haiech, J. & Calas, B. Analysis of the ion binding sites of calmodulin by electrospray ionization mass spectrometry. *Biochemistry* **34**, 13825–13832 (1995).
- Witkowska, H. E., Green, B. N., Morris, M. & Shackleton, C. H. Intact protein electrospray ionization tandem mass spectrometry can be the sole technique used for confirming the structure of a variant hemoglobin. *Rapid communications in mass spectrometry: RCM Spec No.* S111–115 (1995).
- Robinson, C. V. Protein secondary structure investigated by electrospray ionization. *Meth. Mol. Biol.* **61**, 129–139 (1996).
- Waku, T. *et al.* Structural insight into PPARgamma activation through covalent modification with endogenous fatty acids. *J. Mol. Biol.* **385**, 188–199 (2009).
- Kallenberger, B. C., Love, J. D., Chatterjee, V. K. & Schwabe, J. W. A dynamic mechanism of nuclear receptor activation and its perturbation in a human disease. *Nat. Struct. Biol.* **10**, 136–140 (2003).
- Bruning, J. B. *et al.* Partial agonists activate PPARgamma using a helix 12 independent mechanism. *Structure* **15**, 1258–1271 (2007).
- Salonen, L. M., Ellermann, M. & Diederich, F. Aromatic rings in chemical and biological recognition: energetics and structures. *Ang. Chem.* **50**, 4808–4842 (2011).
- Lu, I. L. *et al.* Structure-based drug design of a novel family of PPARgamma partial agonists: virtual screening, X-ray crystallography, and *in vitro/in vivo* biological activities. *J. Med. Chem.* **49**, 2703–2712 (2006).
- Nettleton, E. J. *et al.* Protein subunit interactions and structural integrity of amyloidogenic transthyretins: evidence from electrospray mass spectrometry. *J. Mol. Biol.* **281**, 553–564 (1998).
- Sobott, F. & Robinson, C. V. Protein complexes gain momentum. *Curr. Opin. Struct. Biol.* **12**, 729–734 (2002).

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Author contributions

S.H. prepared crystal samples and performed the X-ray analysis. Y.H. made constructs and performed the PPAR γ transactivation assay. M.N., T.M., S.U. and K.F. performed the mass analysis. S.N., K.K. and S.F. provided technical assistance with crystallography. J.N. and H.N. provided technical assistance with cell biology. S.H., T.Y. and T.N. wrote the manuscript. Y.K. and T.O. edited the manuscript. T.N. and T.Y. conceived and designed the study.

Additional information

Accession Codes: Structural data are available in the Protein Data Bank database under the accession numbers 3WJ4 (PPAR γ -LBD/TBT complex) and 3WJ5 (PPAR γ -LBD/TPT complex).

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