

Activation of RXR/PPAR heterodimers by organotin environmental endocrine disruptors

Albane le Maire^{1,2}, Marina Grimaldi³, Dominique Roecklin⁴, Sonia Dagnino³, Valérie Vivat-Hannah⁴, Patrick Balaguer³ & William Bourguet^{1,2}

¹INSERM, U554, 34090 Montpellier, France

²CNRS, UMR5048, Universités Montpellier 1 & 2, 34090 Montpellier, France

³IRCM, Institut de Recherche en Cancérologie de Montpellier, 34298 Montpellier, France ; INSERM, U896, 34298 Montpellier, France ; Université Montpellier1, 34298 Montpellier, France ; CRLC Val d'Aurelle Paul Lamarque, 34298 Montpellier, France

⁴AliX, Boulevard Sébastien Brandt, Bioparc, 67400 Illkirch, France

Corresponding author:

William Bourguet: Phone: +(33) 4 67 41 7702
Fax: +(33) 4 67 41 7913
e-mail: bourguet@cbs.cnrs.fr

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ABSTRACT

The nuclear receptor RXR α /PPAR γ heterodimer was recently reported to play a major role in mediating the deleterious effects of organotin compounds which are ubiquitous environmental contaminants. However, because organotins are unrelated to known RXR α and PPAR γ ligands, the mechanism by which these compounds bind and activate the RXR α /PPAR γ heterodimer at nanomolar concentrations has remained elusive. Here, we show that tributyltin (TBT) activates all three RXR/PPAR α , γ , δ heterodimers primarily through its interaction with RXR. Moreover, the 1.9 Å resolution structure of the RXR α ligand-binding domain in complex with TBT reveals a covalent bond between the tin atom and residue C432 of helix H11. This interaction largely accounts for the high binding affinity of TBT which occupies the RXR α ligand-binding pocket only partially. Our data allow understanding of the binding and activation properties of the various organotins and suggest a mechanism by which these tin compounds could affect other nuclear receptor signaling pathways.

Keywords: nuclear receptor; 3D structure; organotins; environment

INTRODUCTION

Organotin compounds are ubiquitously present throughout the environment due to their widespread use since the 1960's in many industrial and agricultural processes (Appel, 2004; Fent, 1996). In the 1980's, these compounds were found to be responsible for a wide variety of deleterious effects in the marine ecosystem, and tributyltin (TBT) has been designated as "the most toxic chemical ever released into the seas" by the World Wildlife Fund. Despite some restrictions on their use, organotins persist in the environment and are absorbed by higher organisms where they accumulate (Antizar-Ladislao, 2008). TBT and its derivatives are so-called endocrine disruptors, inducing deregulation of vertebrate and invertebrate endocrine systems (Golub & Doherty, 2004). In many marine species, exposure to TBT results in the abnormal induction of male sexual characteristics in female as a consequence of decreased aromatase (the enzyme converting androgens to estrogens) activity (Matthiessen & Gibbs, 1998; McAllister & Kime, 2003). In mammals, exposure to organotins induces immunosuppressive, metabolic, reproductive or developmental effects (Boyer, 1989; Nakanishi, 2008; Ogata et al, 2001). Interestingly, recent studies revealed that members of the nuclear receptor family are important targets of organotins (Gumy et al, 2008; Kanayama et al, 2005; Nakanishi et al, 2005). In particular, it was demonstrated that nanomolar concentrations of TBT can activate the retinoid X receptor alpha (RXR α) - peroxisome proliferator-activated receptor gamma (PPAR γ) heterodimer to promote adipocyte differentiation (Grun & Blumberg, 2006; Grun et al, 2006; Kanayama et al, 2005) and deregulation of the aromatase gene (Nakanishi et al, 2005). Part of the endocrine disruptive action of organotins could therefore be mediated *via* the RXR α /PPAR γ signaling pathway. Intriguingly, organotins (Fig 1) do neither structurally nor chemically resemble known RXR (rexinoids) or PPAR γ (thiazolidinediones) ligands (de Lera et al, 2007; Michalik et al, 2006) and the mechanism of RXR α /PPAR γ activation by organotins has remained enigmatic. In the

following, we report on a study in which we combined functional and biophysical approaches to provide insights as to how TBT activates RXR/PPAR heterodimers.

RESULTS AND DISCUSSION

TBT activates RXR α /PPAR γ through RXR α

To unravel the mechanism of RXR α /PPAR γ activation by organotins, we compared the ability of reference ligands and TBT to promote the recruitment of a PGC-1 α coactivator LxxLL motif by RXR α and PPAR γ using fluorescence anisotropy. As expected, the synthetic agonists CD3254 and BRL49653 strongly enhance the recruitment of the fluorescein-labeled PGC-1 α peptide by RXR α and PPAR γ , respectively (Fig 2A, B). In contrast, binding of the antagonists UVI3003 and CD5477 impairs the recruitment of the LxxLL motif by their specific receptors RXR α and PPAR γ . Interestingly, RXR α liganded with TBT recruits the PGC-1 α peptide as efficiently as with CD3254, suggesting that both ligands are equally strong agonists. In contrast, TBT poorly increases the basal interaction of PPAR γ with the LxxLL motif and appears as a weak PPAR γ agonist as compared with BRL49653.

The differential involvement of the two receptors in TBT-induced RXR/PPAR γ signaling was validated by several cell-based assays. Initial transactivation experiments using the stably transfected HGELN Gal4-PPAR γ cell line were carried out in the presence of CD3254, BRL49653 or TBT and confirmed the ability of the organotin to activate the RXR/PPAR γ heterodimer at nanomolar concentrations (Fig 2C). Interestingly, the activation curves obtained with TBT or CD3254 show similar profiles with a maximum activity corresponding to roughly 60-70% of that induced by 1 μ M BRL49653. To assess the specific effect of TBT on RXR and PPAR γ , cells were co-incubated with saturating concentrations of the agonists CD3254 or BRL49653 and increasing concentrations of BRL49653, CD3254 or TBT (Fig 2D). Similarly to CD3254, TBT is able to further activate the BRL49653-saturated

heterodimer. However, in contrast with BRL49653, TBT appears unable to act in conjunction with CD3254 to enhance the activity of RXR/PPAR γ (Fig 2D). Likewise, transient transactivation experiments showed that TBT activates RXR α as efficiently as CD3254 (Fig 2E), whereas it behaves as a very weak PPAR γ agonist (Fig 2F).

TBT binds covalently to RXR α C432

To gain structure-based insight into the binding mode of organotin to RXR, we solved the crystal structure of the RXR α ligand binding domain (LBD) bound to TBT and a TIF-2 coactivator fragment (LxxLL motif) at 1.9 Å resolution (Supplementary Table S1 online). RXR α LBD adopts the canonical agonist-bound conformation (Fig 3A). The TBT tin anomalous difference map allowed to unambiguously identify two positions of the metal in close proximity to two alternative conformations of residue C432 in helix H11 (Fig 3B). Sn-A had a stronger anomalous signal than Sn-B (25σ vs 11σ) and accordingly, the best model refinement was obtained with two TBT molecules with occupations of 0.70 and 0.30 for TBT-A and TBT-B, respectively (Fig 3C, D). Moreover, the distances between the sulfur atoms of C432-A and C432-B and the tin atoms (Fig 3B) are shorter than the sum of their ionic radii (2.54 Å), suggesting the presence of a covalent bond between TBT and C432.

To further characterize the binding mode of TBT, the RXR α -TBT complex was analyzed by electrospray ionization mass spectrometry (ESI-MS) under native conditions. Unexpectedly, the spectrum showed two equally abundant species corresponding to complexes of RXR α with TBT in 1:1 or 1:2 molar ratios, *i.e.* one or two TBT bound to one RXR molecule, respectively (Fig 4A). Addition of the TIF-2 peptide induced strong competition of the TBT bound to the second RXR binding site, and simultaneously the formation of the ternary complex RXR-TBT-TIF2 was observed (Fig 4B). Those results suggested that the second organotin molecule was bound nonspecifically to the hydrophobic coactivator binding-groove. Interestingly, the interaction of TBT with RXR α could not be

disrupted in the mass spectrometer interface when the most stringent parameters were applied (190 V and 3 mbar, generating collisions extremely energetic and disruptive for non covalent interactions), supporting a covalent coupling (Fig 4C). Subsequently, ESI-MS analysis was carried out under denaturing conditions after complexes, either purified or solubilized from crystals, were separated by HPLC and eluted with a gradient of acetonitril. In both cases, the mass calculated from the spectra corresponded to the mass of the unbound RXR α (Fig 4D, E), substantiating the idea that TBT and C432 are connected *via* a fragile Sn-S covalent bond. The weakness of the covalent bond could also be observed in transactivation experiments in which the antagonist UVI3003 at high concentration was able to compete with TBT (Fig 2E).

To confirm the importance of the Sn-S interaction for RXR α activation by TBT, the residue equivalent to C432 in mouse RXR α (C437) was mutated into alanine. Consistent with the prediction, this mutation abrogated TBT-induced RXR α activity whereas RXR α activation by CD3254 was only modestly affected (Fig 2E).

Structural basis for RXR activation by TBT and congeners

Classically, rexinoids contain an acidic head group and a long aliphatic/aromatic chain (Fig 1) bridging R316 (H5) on one side and helix H11 on the other (Fig 5A, B). Although TBT interacts with only a subset of binding pocket residues in the H11 region, it is engaged in enough essential contacts to stabilize RXR α in its active conformation. Comparison of the RXR α -TBT structure with that of RXR α bound to its natural agonist 9-cis retinoic acid (Egea et al, 2000) reveals that for the most part, residues in contact with the organotin also belong to the 9cis-RA binding pocket (Fig 5A). Moreover, both ligands induce identical residue positioning and side-chain orientations. Notably, TBT preserves the conformation of L436 which has been previously demonstrated to be crucial for full-agonism (Nahoum et al, 2007). This observation demonstrates that interactions involving residues of the TBT binding site are critical for stabilization of the receptor active form whereas interactions made with residues

on the R316 side serve to increase the number of protein/ligand contacts and enhance affinity and specificity of classical rexinoids (Fig 5B).

Organotins form a collection of more than 250 tin compounds containing a variety of mono-, di-, tri- or tetra-substituted organic groups. Our structure reveals that the high affinity of TBT for RXR (12.5 nM; Grun et al, 2006) derives from the covalent interaction linking the tin atom to residue C432 and the direct van der Waals contacts between all TBT atoms and RXR α residues. Accordingly, modeling studies indicate that the most active organotins like triphenyltin exhibit such features (Supplementary Fig S1 and Table S2 online). In contrast, organotins such as triethyltin with fewer and/or shorter alkyl groups do not establish enough contacts with the protein to correctly position the tin atom against residue C432 and ensure high affinity binding. Finally, RXR is unable to accommodate compounds with longer alkyl groups like trioctyltin whose volume, in some directions, exceeds that of the ligand binding pocket (LBP).

Organotins as nuclear receptor modulators?

TBT is able to activate RXR/PPAR γ *via* RXR because this heterodimer poorly interacts with corepressors in cells and belongs to the group of so-called “permissive” heterodimers which can be stimulated by RXR ligands on their own (Germain et al, 2002). Accordingly, significant activation of other permissive heterodimers such as RXR/LXR or RXR/NURR1 has been reported (Grun et al, 2006). Using stably transfected HGELN Gal4-PPAR α and HGELN Gal4-PPAR δ cell lines, we could demonstrate that TBT is able to activate all three RXR/PPAR α , γ , δ heterodimers (Supplementary Fig S2).

Because RXR α C432 plays a key role in the mechanisms of binding and activation by TBT, we looked whether this residue is conserved in other nuclear receptors and found that the presence of a cysteine residue at this particular position is unique to RXR α , β and γ . On the other hand, the PPAR γ LBP contains a cysteine (C285) which couples covalently with

conjugated oxo fatty acids (Itoh et al, 2008) and could serve as an anchoring point for TBT. However, in contrast to C432 which is located in RXR α helix H11, C285 of PPAR γ resides in helix H3. Hence, TBT could bind to PPAR γ in a region of the LBP which does not allow efficient stabilization of the active receptor conformation. Finally, it was recently reported that dibutyltin (DBT) acts as a potent antagonist of the glucocorticoid receptor (GR; Gumy et al, 2008). As other oxo-steroid receptors, GR contains cysteine residues which could help fixing DBT in the hormone binding site. Involvement of cysteine residues in the binding of organotin to receptors other than RXR remains to be established. Nevertheless, our data suggest that tin compounds could use the specific Sn-S interaction to modulate the transcriptional activity of a number of nuclear receptors, the functional outcome being dictated by the structure of the organotin and the position of the anchoring cysteine in the LBP.

METHODS

Ligands and peptides. Tributyltin and BRL49653 were purchased from Sigma-Aldrich and Interchim, respectively. CD5477 and CD3254 were kindly provided by Galderma (Sophia-Antipolis, France). UVI3003 was synthesized by A. R. de Lera (University of Vigo, Spain). GW327647 and GW610742 were gifts of Dr. T. M. Wilson (GlaxoSmithKline, Research Triangle Park, USA). The fluorescein-¹³⁹EEPSLLKKLLLAPA¹⁵² peptide corresponding to the NR box 2-binding motif of PGC-1 α and the NR box 2 peptide (⁶⁸⁶KHKILHRLQLDSS⁶⁹⁸) of TIF-2 were purchased from EZbiolab (Westfield, Indiana, USA).

Fluorescence assays. Fluorescence anisotropy assays were performed using a Safire microplate reader (TECAN) with the excitation wavelength set at 470 nm and emission measured at 530 nm. Experiments were performed as previously described (Pogenberg et al, 2005). The reported data are the average of at least 3 independent experiments and error bars correspond to standard deviations.

Stable transactivation experiments. HGELN human PPAR α , δ , γ cell lines were generated as described in Supplementary Methods online. To measure TBT activity, HGELN Gal4-PPAR cells were seeded at a density of 40,000 cells per well in 96-well white opaque tissue culture plates (Greiner). Compounds were added 8h later and cells were incubated for 16 hours. At the end of incubation, culture medium was replaced by medium containing 0.3 mM luciferin. Luciferase activity was measured for 2s in intact living cells using a microBeta Wallac luminometer (Perkin Elmer). Tests were performed in quadruplicate in at least three independent experiments and data were expressed as mean \pm SD. Dose-response curves were fitted using the sigmoid dose-response function of a graphics and statistics software package (Graph-Pas Prism, version 4., 2003 Graph-Pas Software Inc., San Diego, CA, USA).

Transient transactivation experiments.

PPAR γ and RXR α homodimers activities were monitored on (PPRE)₃-TK- and (RXRE)₆-TK luciferase reporter constructs. pSG5-hPPAR γ and (PPRE)₃-TK-Luc are gifts of Dr L Fajas (IRCM, Montpellier, France). pSG5-hRXR α , mRXR α C437A and (RXRE)₆-TK-Luc were kindly provided by Dr H Gronemeyer (IGMC, Illkirch, France). Transient transfections assays were performed in HeLa cells using Jet-PEI (Ozyme, Saint-Quentin en Yvelines, France) according to manufacturer's instructions. Luciferase assays were performed with the Promega dual-reporter kit, according to the manufacturer's instructions. Renilla luciferase encoded by the normalization vector phRLTK (Promega) was used as internal control for firefly luciferase normalization. Tests were performed in duplicate in at least three independent experiments and data were expressed as mean \pm SD.

Crystallization of the RXR α LBD-TBT complex. Expression and purification of human RXR α LBD have been described previously (Nahoum et al, 2007). Fractions containing the purified receptor were pooled, mixed with a 3-fold molar excess of TBT and a 5-fold molar excess of the TIF-2 NR2 co-activator peptide and concentrated to 10.5 mg/ml. Crystals were obtained by vapor diffusion at 293K. The well buffer contained 28% PEG 4000, 0.1M Tris.HCl pH 7.5, 1.0 M ammonium acetate. Crystals were of space group P43212. A single crystal was mounted from mother liquor onto a cryoloop (Hampton research), soaked in the reservoir solution containing an additional 20 % glycerol and frozen in liquid nitrogen.

Crystallographic data collection, processing and structure refinement. Diffraction data were collected using an ADSC Q315r CCD detector at the BM30A beamline of ESRF (France) at 1.9 Å resolution. Diffraction data were processed using MOSFLM (Leslie, 2006) and scaled with SCALA from the CCP4 program suite (Collaborative Computational Project, 1994). The structure was solved by using the previously reported structure 2P1T (Nahoum et al, 2007) of which the ligand was omitted. Initial *F_o-F_c* difference maps had strong signal for

the ligand which could be fitted accurately into the electron density. The structure was modeled with COOT (Emsley & Cowtan, 2004) and refined with REFMAC (Collaborative Computational Project, 1994) using rigid body refinement, restrained refinement, and individual B-factor refinements.

Mass spectrometry. For LC-MS analysis, 80 µg of purified RXRα LBD-TBT complexes and 15µl of solubilized crystals (eight crystals in 15µl of 5M ammonium acetate, pH 7.0) were injected into C4 reversed-phase HPLC column (Vydac, Grace). Separation was obtained with a linear gradient (10 to 60%) of acetonitril and detection of eluting protein was done at 205nm. Three hundred µl fractions were collected, dried under vacuum, solubilized in 20µl of 50% acetonitril solution acidified with 1% HCOOH, and infused for mass spectrometry analysis using automated Triversa Nanomate (Advion) coupled to LC-TOF mass spectrometer (Waters). Data acquisition was done with the following interface parameters: Vc of 30V, Pi of 2.6mbar. Non-denaturing mass spectrometry analysis requires the final purification buffer of RXRα LBD-TBT complexes to be exchanged for ammonium acetate buffer (50mM, pH 7.5) using Zeba spinning column system (Pierce). Mass spectrometer interface parameters were setup as follow: Vc varied from 190 to 50V, Pi varied from 5 to 3 mbar.

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Accession code. The atomic coordinates have been deposited in the Protein Data Bank under the accession code 3E94.

The authors declare that they have no conflict of interest.

Supplementary information is available at EMBO reports online.

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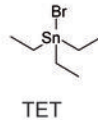
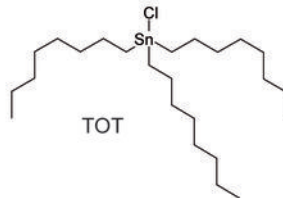
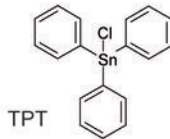
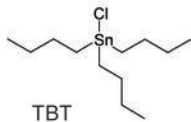
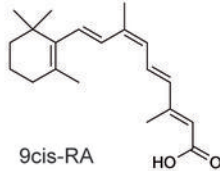
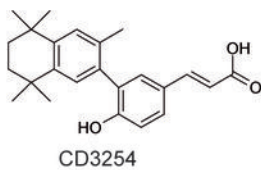
Fig 1 Chemical structures of some ligands used in this study. CD3254 and 9cis-RA are RXR α agonists whereas BRL49653 is a PPAR γ agonist. Tributyltin (TBT), triphenyltin (TPT), trioctyltin (TOT) and triethyltin (TET).

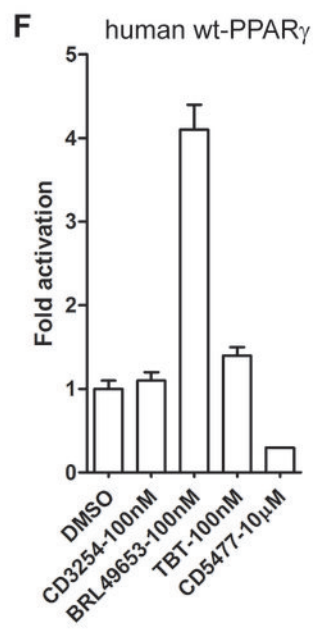
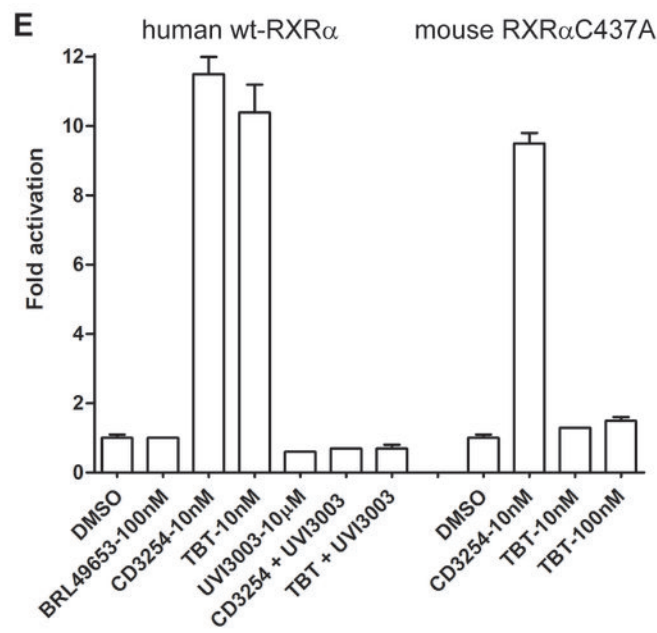
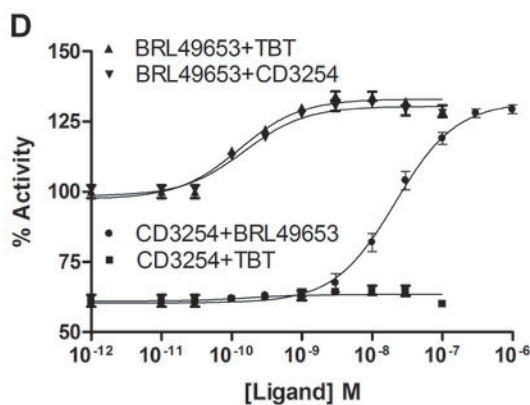
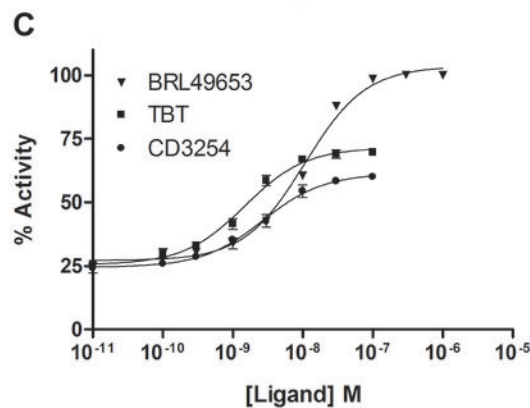
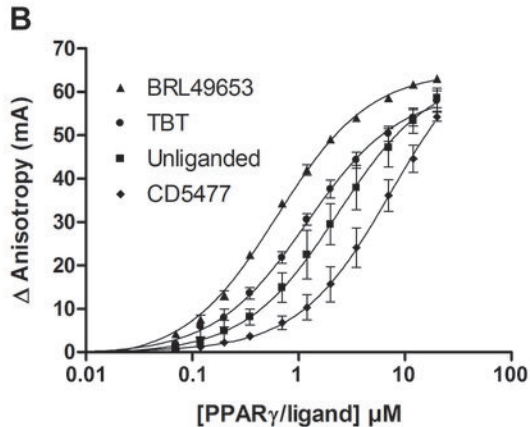
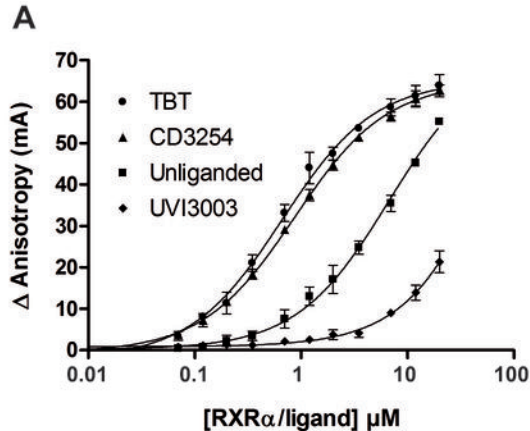
Fig 2 Effect of TBT on coactivator recruitment and activation of RXR α and PPAR γ . Titration of fluorescein-labeled PGC-1 α peptide by RXR α (**A**) and PPAR γ (**B**), in the absence of ligand or in the presence of CD3254 (RXR agonist), UVI3003 (RXR antagonist), BRL49653 (PPAR γ agonist), CD5477 (PPAR γ antagonist) or TBT. (**C**) Stably transfected HELN PPAR γ cells were incubated with BRL49653, CD3254 or TBT to measure their effect on the transcriptional activity of the heterodimer formed by Gal4-PPAR γ and endogenous RXR. (**D**) To measure the specific effect of the organotin on PPAR γ and RXR, BRL49653, CD3254 or TBT were also tested in the presence of saturating concentrations of RXR (CD3254) or PPAR γ (BRL49653) specific ligands. 100% activity corresponds to the activity obtained with 1 μ M BRL49653. (**E**) Hela cells transiently transfected with the reporter recombinant (RXRE)₆-TK-Luc and pSG5-RXR α (human wild-type or mouse RXR α C437A) were incubated with BRL49653, CD3254 or TBT to assess their agonist potential on RXR α homodimer. The antagonist UVI3003 (10 μ M) was also used to check the reversibility of TBT (10nM) binding. (**F**) Same experiment with (PPRE)₃-TK-Luc and pSG5-hPPAR γ . The antagonist CD5477 was used as a control.

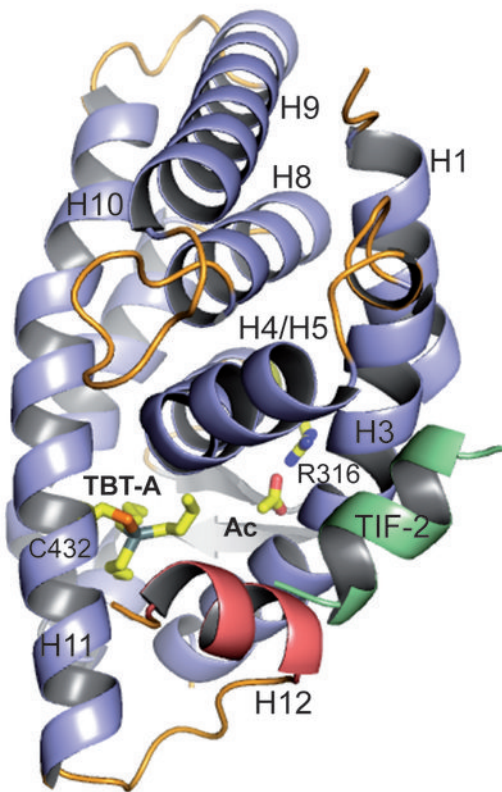
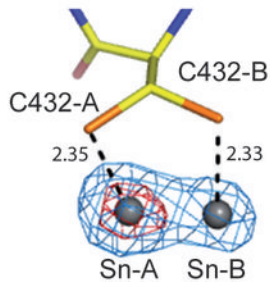
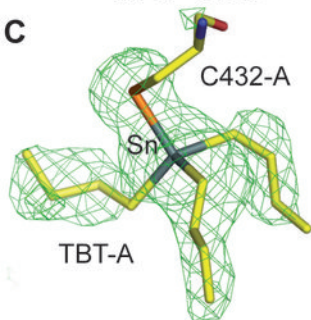
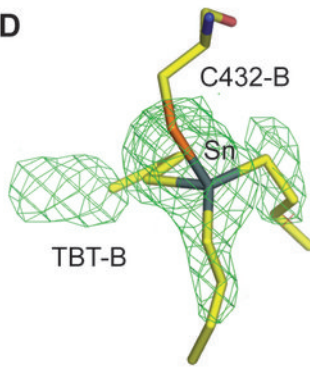
Fig 3 Structure of RXR α LBD in complex with TBT and TIF-2. **(A)** Overall structure of the complex in cartoon representation. The TBT bound to C432 *via* a covalent interaction and an acetate molecule (Ac) involved in a salt bridge with R316 are depicted. This acetate, not essential for RXR activity, derives from the crystallization condition. **(B)** Anomalous difference electron density map contoured at 15.0 σ (red) and 5.0 σ (blue) showing two tin sites facing the two alternative positions of C432 (A and B). **(C)** and **(D)** The two positions of TBT (TBT-A and TBT-B) bound to C432-A and C432-B are shown in the F_O-F_C omit map contoured at 3.0 σ .

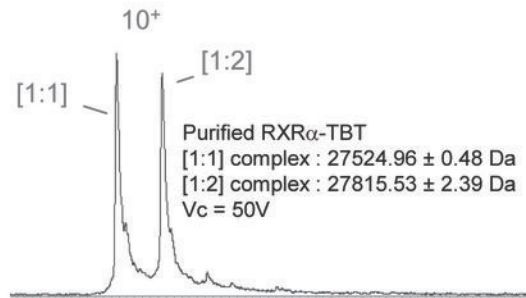
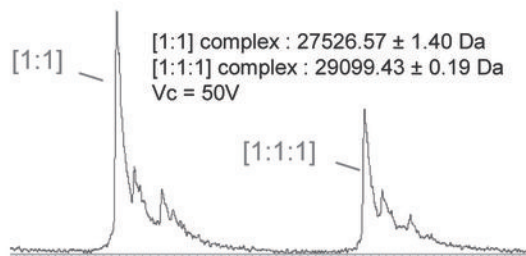
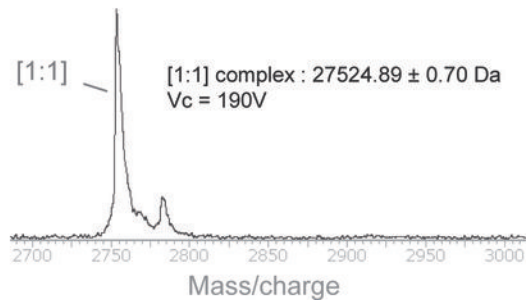
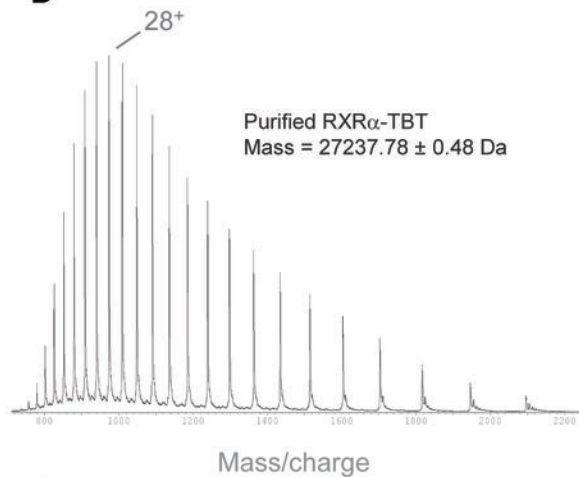
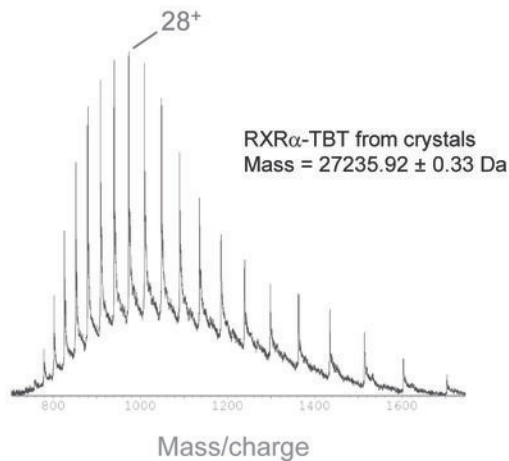
Fig 4 Mass spectrometry analysis. Non-denaturing ESI-MS was used to characterize RXR α -TBT complexes in the absence **(A)** or in the presence of four-fold molar excess of TIF-2 peptide **(B and C)**. Increasing ion acceleration voltage (V_c) from 50V **(A and B)** to 190V **(C)** had no effect on TBT interaction with RXR. LC-MS analysis of RXR α -TBT complexes either purified **(D)** or solubilized from crystals **(E)** was done to assess the reversibility of TBT binding. Measured mass corresponds to unbound RXR α LBD.

Fig 5 Structural determinants of TBT recognition by RXR α . **(A)** Stereo view of the superposition of RXR α LBD in complex with TBT (orange) or 9cis-RA (pink, PDB code 1FBY). Residues involved in TBT and 9cis-RA binding are colored in yellow and purple, respectively. **(B)** Schematic representation of the different RXR α LBP parts occupied by TBT and classical rexinoids.

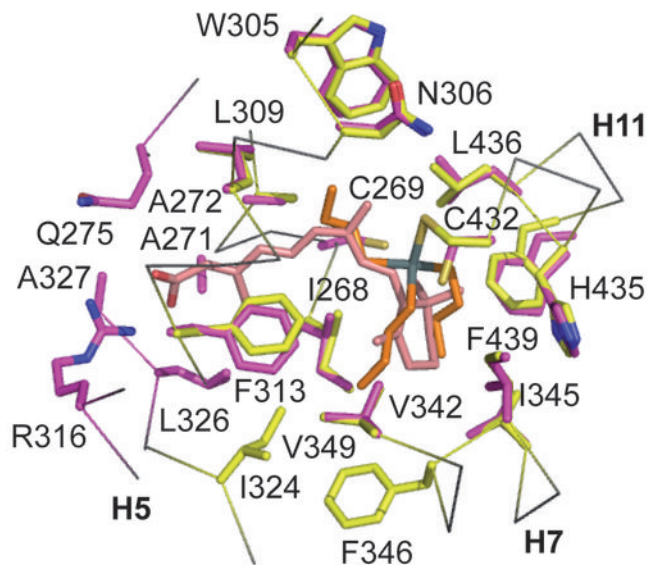
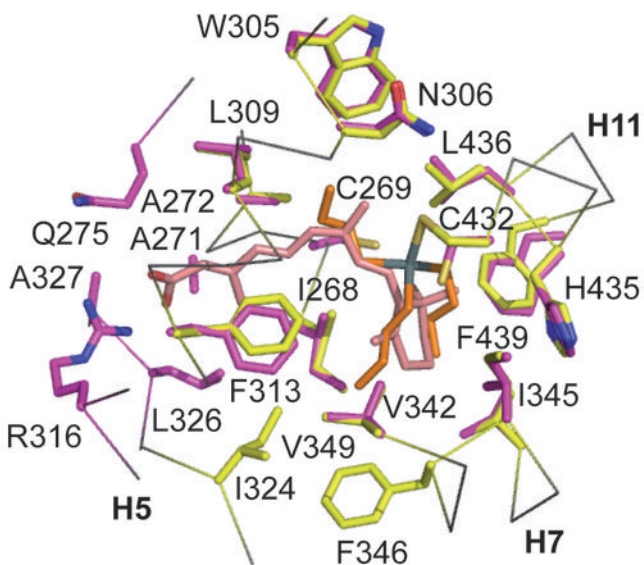




A**B****C****D**

A**B****C****D****E**

A



B

