Endocrine disrupting organotin compounds are potent inducers of adipogenesis in vertebrates

Running title: Organotins as inducers of adipogenesis

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Abstract

Dietary and xenobiotic compounds can disrupt endocrine signaling, particularly of steroid receptors and sexual differentiation. Evidence is also mounting that implicates environmental agents in the growing epidemic of obesity. Despite a long-standing interest in such compounds, their identity has remained elusive. Here we show that the persistent and ubiquitous environmental contaminant, tributyltin chloride (TBT)**, induces the differentiation of adipocytes in vitro and increases adipose mass in vivo. TBT is a dual, nanomolar affinity ligand for both the retinoid ‘X’ receptor (RXR) and the peroxisome proliferator activated receptor γ (PPARγ). TBT promotes adipogenesis in the murine 3T3-L1 cell model and perturbs key regulators of adipogenesis and lipogenic pathways in vivo. Moreover, in utero exposure to TBT leads to strikingly elevated lipid accumulation in adipose depots, liver, and testis of neonate mice and results in increased epididymal adipose mass in adults. In the amphibian *Xenopus laevis*, ectopic adipocytes form in and around gonadal tissues following organotin, RXR or PPARγ ligand exposure. TBT represents, to our knowledge, the first example of an environmental endocrine disrupter that promotes adipogenesis through RXR and PPARγ activation. Developmental or chronic lifetime exposure to organotins may therefore act as a chemical stressor for obesity and related disorders.

Introduction

Organotins are a diverse group of widely distributed environmental pollutants. Tributyltin chloride (TBT) and bis(triphenyltin) oxide (TPTO), have pleiotropic adverse effects on both invertebrate and vertebrate endocrine systems. Organotins were first used in the 1960s as antifouling agents in marine shipping paints although such use has been restricted in recent
years. Organotins persist as prevalent contaminants in dietary sources, such as fish and shellfish, and through pesticide use on high value food crops (1, 2). Additional human exposure to organotins may occur through their use as antifungal agents in wood treatments, industrial water systems and textiles. Mono- and diorganotins are prevalently used as stabilizers in the manufacture of polyolefin plastics (PVC), which introduces the potential for transfer by contact with drinking water and foods.

Exposure to organotins such as TBT and TPTO results in imposex, the abnormal induction of male sex characteristics in female gastropod mollusks (3, 4). Bioaccumulation of organotins decreases aromatase activity leading to a rise in testosterone levels that promotes development of male characteristics (5). Imposex results in impaired reproductive fitness or sterility in the affected animals and is one of the clearest examples of environmental endocrine disruption. TBT exposure also leads to masculinization of at least two fish species (6, 7), but TBT is only reported to have modest adverse effects on mammalian male and female reproductive tracts and does not alter sex ratios (8, 9). Instead hepatic-, neuro- and immunotoxicity appear to be the predominant effects of organotin exposure (10). Hence, the current mechanistic understanding of the endocrine disrupting potential of organotins is based on their direct actions on the levels or activity of key steroid regulatory enzymes such as aromatase and more general toxicity mediated via damage to mitochondrial functions and subsequent cellular stress responses (11-15).

However, it remains an open question whether in vivo organotins act primarily as protein and enzyme inhibitors, or rather mediate their endocrine disrupting effects at the transcriptional level. Recent work has shown that aromatase mRNA levels can be down regulated in human ovarian granulosa cells by treatment with organotins or ligands for the nuclear hormone receptors, retinoid X receptor (RXRs) or peroxisome proliferator activated receptor gamma (PPARγ) (16-
Furthermore, Nishikawa et al. have demonstrated that the gastropod *T. clavigara* RXR homolog is responsive to 9-cis RA and TBT, and 9-cis RA can also induce imposex (19) suggesting a conserved transcriptional mechanism for TBT action across phyla. These ligand dependent transcription factors belong to the nuclear hormone receptor superfamily – a group of ~150 members (48 human genes) that includes the estrogen receptor (ER), androgen receptor (AR), glucocorticoid receptor (GR), thyroid hormone receptor (TR), vitamin D receptor (VDR), retinoic acid receptors (RARs and RXRs), peroxisome proliferator-activated receptors (PPARs) and numerous orphan receptors. We were therefore intrigued by the similar effects of TBT and RXR/PPAR\(\gamma\) ligands on mammalian aromatase mRNA expression and hypothesized that TBT might be exerting some of its biological effects via transcriptional regulation of gene expression through activation of one or more nuclear hormone receptors.

Our results show that organotins such as TBT, are indeed potent and efficacious agonistic ligands of the vertebrate nuclear receptors, retinoid X receptors (RXRs) and PPAR\(\gamma\). The physiological consequences of receptor activation predict that permissive RXR heterodimer target genes and downstream signaling cascades are sensitive to organotin misregulation. Consistent with this prediction we observe that organotins phenocopy the effects of RXR and PPAR\(\gamma\) ligands using *in vitro* and *in vivo* models of adipogenesis. Therefore, TBT and related organotin compounds are the first of a potentially new class of environmental endocrine disrupters that targets adipogenesis by modulating the activity of key regulatory transcription factors in the adipogenic pathway, RXR\(\alpha\) and PPAR\(\gamma\). The existence of such xenobiotic compounds was previously hypothesized (20, 21). Our results suggest that developmental exposure to TBT and its congeners that activate RXR/PPAR\(\gamma\) might be expected to increase the
incidence of obesity in exposed individuals and that chronic lifetime exposure could act as a potential chemical stressor for obesity and obesity related disorders.

RESULTS

Organotins are agonists of vertebrate RXR and RXR-permissive heterodimers

Many known or suspected environmental endocrine disrupting chemicals (EDCs) mimic natural lipophilic hormones that act through members of the superfamily of nuclear receptor transcription factors (22, 23). In a screen of high priority endocrine disrupting chemicals (EDC) against a bank of vertebrate nuclear receptor ligand binding domains (NR-LBDs), we observed that organotins, specifically tributyltin chloride (TBT) and bis(triphenyltin) oxide (TPTO), could fully activate an RXRα ligand binding domain construct (GAL4-RXRα) in transient transfection assays. Both TBT and TPTO were as potent (EC_{50} ~3-10 nM) as 9-cis retinoic acid, an endogenous RXR ligand and approximately 2-5-fold less potent than the synthetic RXR-specific ligands LG100268 (EC_{50} ~ 2 nM) or AGN195203 (EC_{50} ~ 0.5 nM) (Fig 1A, Table 2). Maximal activation for TBT reached the same levels as LG100268 or AGN195203.

We next tested whether activation by TBT was unique to RXRα only, restricted to RXR heterodimer complexes or a general nuclear receptor transcriptional response (Fig. 1 B-D and Table 1). TBT activated RXRα and RXRγ from the amphibian Xenopus laevis in addition to human RXRs (Table 1). Our results are consistent with recent findings by Nishikawa et al. that organotins promote activation of all 3 human RXR subtypes in a yeast two-hybrid screen (19, 24). We also observed significant activation of receptors typically considered to be permissive heterodimERIC partners of RXR including human PPARγ (Fig. 1B ~30% maximal activation of 10 µM troglitazone, but note that activation is compromised by cellular toxicity above 100 nM),
PPARδ, liver X receptor (LXR) and the orphan receptor NURR1. In contrast, typical non-permissive partners such as retinoic acid receptors (RARs), thyroid hormone receptor (TRβ) and vitamin D receptor (VDR) failed to show activation by organotins (Fig. 1C and Table 1). Murine PPARα was also not activated by TBT although it was fully activated by its specific synthetic agonist WY-14643 (Fig. 1D). The steroid and xenobiotic receptor (SXR) was likewise unresponsive. The orphan receptor NURR1, which has no discernable ligand pocket and is believed to be ligand-independent (25), was nevertheless activated 7 to 10-fold at 100 nM TBT. Similarly, other RXR-specific ligands, e.g. LG100268, activated NURR1 to the same degree suggesting that this response occurred through NURR1’s heterodimeric partner RXR as has been previously described (25, 26). Like other RXR-specific ligands, tributyltin was also able to promote the ligand dependent recruitment of nuclear receptor co-factors such as ACTR, SRC-1 and PBP in mammalian two-hybrid interaction assays (data not shown). We infer from these results that nuclear receptor activation by TBT activation is specific to a small subset of receptors and not a consequence of a general effect on the cellular transcriptional machinery.

We next investigated the relationship between the structure of the tin compounds and RXR activation by testing the response of GAL4-RXRα to mono-, di-, tri- and tetra-substituted butyltin, branched side chains, variations in the alkyl chain length and changes in the halide component (Fig. 1 A and Table 2). Overall, trialkyltin compounds were the most effective with nanomolar EC₅₀ values. Monobutyltin gave no significant activation whereas dibutyltin was moderately active in the µM range (Fig. 1 A and Table 2). Tetrabutyltin was 20-fold less potent than TBT whereas the branched side-chain butyltin tris(2-ethylhexanoate) (BT(2-EHA)₃) was inactive (Table 2). Although activation by dialkyltins is weaker than that of TBT, it is potentially
significant due to their widespread use in the manufacture of polyvinyl chloride (PVC) plastics and greater solubility than TBT.

The effect of the hydrocarbon chain length was very pronounced, suggesting an important structure activity relationship. A reduction in hydrophobicity from butyl to ethyl side chains raised the EC$_{50}$ value by almost a 1000-fold into the micromolar range. Trimethyltin was weakly active only above 100 µM (Table 2). Substitution of the halide component had no significant effect on the EC$_{50}$ values for TBT, probably due to the lability of the halide atom through exchange in aqueous tissue culture media where chloride ions are prevalent.

*TBT is a potent ligand of both RXRα and PPARγ.*

Many, if not most, natural and synthetic nuclear receptor agonists act as ligands that specifically interact with their cognate receptor ligand binding domains. We therefore performed equilibrium competition binding experiments with purified histidine-tagged human RXRα (H$_6$-RXRα) and PPARγ (H$_6$-PPARγ) ligand binding domains to determine if the potent and specific activation of these receptors by TBT was due to direct ligand-receptor interaction (Fig. 1E-F).

The equilibrium binding curves indicate that TBT is a high affinity, competitive ligand for 9-cis RA bound RXRα. The inhibition equilibrium dissociation constant was calculated by the Chang-Prusoff method ($K_i$ equals $K_d$) as 12.5 nM (10-15 nM - 95% confidence interval) (Table 3). By comparison, the value obtained for the synthetic RXR agonist LG100268 was 7.5 nM which compared favorably with its published value of ~ 3 ± 1 nM (27). Therefore, the identification of TBT as an RXR ligand expands the molecular definition of known “rexinoids” (agonists able to activate RXR) to include this structurally unique class of organotin compounds.
Somewhat surprisingly, we also observed potent specific competitive binding by TBT for rosiglitazone bound to human PPARγ LBD (Fig. 2B). The deduced $K_i$ of 20 nM (17-40 nM - 95% confidence interval) was slightly higher than for RXRα but significantly better than the $K_i$ for the PPARγ agonist troglitazone which yielded a $K_i$ of 300 nM, consistent with its published $K_d$ (28). The $K_d$ values for TBT binding to RXRα (12.5 nM) and PPARγ (20 nM) are also in close agreement with EC$_{50}$ values obtained from transient transfection assays using GAL4-RXRα and GAL4-PPARγ constructs (Table 2).

Taken together, these data show that organotins such as TBT, although structurally distinct from previously described natural or synthetic ligands, can interact with RXRα and PPARγ, via direct ligand binding to induce productive receptor:coactivator interactions and promote transcription in a concentration dependent manner. Organotins are therefore potent nanomolar receptor activators on par with synthetic RXR and PPARγ ligands such as LG100268, AGN195203 and thiazolidinediones.

*TBT promotes adipogenesis in the murine 3T3-L1 cell model.*

Numerous studies have demonstrated the critical role played by RXRα:PPARγ signaling in regulation of mammalian adipogenesis (29-31). In the murine 3T3-L1 preadipocyte cell model, adipogenic signals induce early key transcriptional regulators such as CCAAT/enhancer binding proteins C/EBPβ and δ that lead to mitotic clonal expansion of growth arrested preadipocytes and induction of the late differentiation factors C/EBPα and PPARγ (32-34). The combination of C/EBPα expression together with PPARγ signaling efficiently drives terminal adipocyte differentiation and lipid accumulation. We therefore tested whether TBT signaling through RXR:PPARγ could promote adipogenesis in the murine 3T3-L1 differentiation assay and
compared its effect to other RXR-specific or PPARγ ligands (Fig. 2). Undifferentiated 3T3-L1 cells were cultured for 1 week in the presence of ligands either with or without a prior 2 day treatment with MDIT (an adipogenic sensitizing cocktail of IBMX, dexamethasone, insulin and T₃) (35). Cells were then scored for lipid accumulation using Oil Red O staining to determine the degree of terminal adipocyte differentiation. TBT was as effective as LG100268 or AGN195203 in promoting differentiation in the absence of MDIT treatment, increasing the number of differentiated adipocytes about 7-fold over solvent only controls (Fig. 2A, C). The PPARγ agonist troglitazone was a weak inducer in the absence of MDIT. Prior treatment with MDIT increased the response to TBT, LG100268 and AGN195203 a further 3-5 fold (Fig. 2B,D). MDIT treatment also boosted the response to troglitazone to equivalent levels as expected from published studies showing that combination treatment with PPARγ ligands promote efficient adipocyte differentiation (36-38). In contrast the RAR-agonist TTNPB inhibited the differentiation of 3T3-L1 cells consistent with previously published data that showed RAR signaling blocks adipogenesis during the early stages of differentiation in vitro and can modulate adiposity and whole body weight in vivo (39-41). The differential response of 3T3-L1 cells to receptor selective retinoids indicates that TBT favors RXR homodimer or permissive RXR-heterodimer rather than RXR:RAR signaling in this cell model.

Adipocyte differentiation by TBT was accompanied by direct transcriptional effects on RXR:PPARγ targets such as adipocyte-specific fatty acid binding protein (aP2) mRNA. The aP2 promoter contains response elements sensitive to CCAAT/enhancer binding protein factors (C/EBPs) and RXRα:PPARγ signaling (42). QRT-PCR analysis showed aP2 levels were elevated by TBT treatment approximately 5-fold at 24 hrs (Fig. 2E) and 45-fold at 72 hrs (data not shown). LG100268, troglitazone and MDIT treatment also increased aP2 expression at these
time points whereas the RAR agonist TTNPB was inhibitory, consistent with the observed cellular responses.

*TBT induces adipogenic regulators and markers of RXRα:PPARγ signaling in vivo.*

The ability of organotins to regulate RXRα:PPARγ target genes and key modulators of adipogenesis and lipid homeostasis *in vivo* has not previously been examined. Therefore, we next asked whether TBT could perturb expression of critical transcriptional mediators of adipogenesis such as RXRα, PPARγ, C/EBPα/β/δ and sterol regulatory element binding factor 1 (Srebf1) as well as known target genes of RXRα:PPARγ signaling from liver, epididymal adipose tissue and testis of six week old male mice dosed for 24 hours with TBT (0.3 mg/kg b.w.), AGN195203 (0.3 mg/kg b.w.), troglitazone (3 mg/kg b.w) or vehicle (corn oil) administered by intraperitoneal injection. TBT either had no effect or weakly repressed RXRα and PPARγ transcription in liver (Fig. 3A, B). A more pronounced decrease was observed for RXRα, PPARγ, C/EBPα and C/EBPδ in adipose tissue and testis (Fig. 3B,C). In contrast, TBT, AGN195203 and troglitazone significantly induced expression of the early adipogenic transcription factor C/EBPβ in liver and testis whereas it was more weakly induced in adipose tissue. Induction was strongest in testis where TBT and troglitazone increased expression greater than 10-fold and AGN195203 increased expression 60-fold compared to vehicle controls (Fig. 3C). In addition to C/EBPβ, the proadipogenic transcription factor Srebf1 was also significantly increased in adipose tissue by all three receptor ligands and weakly induced in liver.

We also observed coordinate changes in several well characterized direct target genes of RXR:PPARγ signaling. Fatty acid transport protein (Fatp) acts as a key control point for regulation of cellular fatty acid content. The Fatp promoter contains a functional PPRE shown to
be sensitive to RXR:PPARγ signaling in 3T3-L1 adipocytes and white fat (43-46). Fatp mRNA levels were up regulated 2-3 fold in liver and epididymal adipose tissue but not testis by TBT, AGN195203 and troglitazone (Fig. 5A,B). Similarly, the PPARγ target phosphoenolpyruvate carboxykinase 1 (PEPCK/Pck1) (47), the rate-limiting step in hepatic gluconeogenesis and adipose glyceroneogenesis, was up regulated in liver and adipose tissues by TBT or troglitazone treatment.

Signaling through RXR:PPARγ, RXR:LXR and ADD1/Srebf1 in hepatocytes has been shown to modulate fatty acid synthesis through transcriptional control of acetyl-CoA carboxylase (Acac), the rate limiting step in long chain fatty acid synthesis (48, 49), as well as fatty acid synthase (Fasn) (50-53). Hepatic expression of both Acac and Fasn was unregulated between 1.5-2.5-fold by TBT, AGN195203 and troglitazone. Therefore, the coordinate increased expression of Fatp, Pck1, Acac and Fasn in liver suggests that TBT stimulates fatty acid uptake and triglyceride synthesis. Similar changes have been reported in the induction of hepatic steatosis by overactive PPARγ signaling (49, 54).

Taken together, these data show that TBT exposure induces lipogenic RXR:PPARγ target gene expression in adipose tissue and liver, and modulates associated early adipocyte differentiation factors such as C/EBPβ and Srebf1. We inferred from these data that organotins are potential adipogenic agents in vivo.

Developmental exposure to TBT disrupts lipid homeostasis and adipogenesis in vertebrates.

Based on its molecular pharmacology, ability to induce 3T3-L1 adipocyte differentiation and in vivo transcriptional responses, we reasoned that TBT would disrupt normal endocrine control over lipid homeostasis and impact adipogenesis particularly when exposure occurred during
sensitive periods of development. We therefore tested this hypothesis in two vertebrate model systems, mouse and *Xenopus laevis*, during embryogenesis.

Pregnant C57BL/6 mice were injected daily from gestational day 12-18 with TBT (0.05 or 0.5 mg/kg body weight i.p.) dissolved in sesame oil or vehicle alone. Pups were then sacrificed at birth and histological sections prepared from liver, testis, mammary gland and inguinal adipose tissue. Sections were stained with Oil Red O to assess changes in total tissue lipid accumulation. TBT exposure caused a disorganization of hepatic (Fig. 4A,B) and gonadal (Fig. 4 C,D) architecture and significantly increased Oil Red O staining in treated animals versus controls. Liver sections exhibited signs of steatosis consistent with the misregulation of fatty acid uptake and synthesis observed using molecular markers. In addition Oil Red O positive staining in mammary, and inguinal adipose (Fig. 4E-H) tissues was dramatically elevated reflecting either an increase in lipid accumulation or an increase in mature adipocytes.

To determine whether exposure induced long term changes in growth or adipose tissue, we followed mice from birth to adulthood after *in utero* exposure to TBT as indicated above. At birth, mice were cross-fostered to unexposed dams and total body weight recorded until 10 weeks of age (Fig. 5A). Growth curves for male and female pups showed a slight trend for lower total body weight consistent with published observations (9) but were not statistically significant at 10 weeks (Control vs TBT: male 26.00g ± 0.70, n=9 vs 25.53g ± 0.39, n=10, p=0.583; female 21.22g ± 0.41, n =10, vs 20.24g ± 0.24, n=10, p = 0.0529). Males were sacrificed at 10 weeks and epidydimal fat pads weighed (Fig. 5B). Adipose mass in TBT treated males was increased significantly by 20 % over controls (Control vs TBT: 0.30g ± 0.020, n=9 vs 0.36g ± 0.018, n =10, p=0.0374). These data support the conclusion that TBT can increase body adiposity without
overtly increasing total body weight. Similar lipid accumulation and changes in adipose tissue 
mass have also been observed following TZD or rexinoid treatment (55-57).

We had previously observed that TBT activated *Xenopus* RXRs (Table 1) and reasoned that 
the strong conservation in vertebrate nuclear receptor signaling pathways should result in 
consistent responses to organotin and RXR/PPARγ ligands across diverse vertebrate species. We 
therefore tested chronic exposure to environmentally relevant low doses of TBT (1-10 nM), the 
RXR-specific ligands LG100268 and AGN195203 (10-100 nM), troglitazone (0.1-1 µM) and 
estradiol (1-10 nM) on developing *Xenopus laevis* tadpoles from stage 48 to metamorphs. To 
determine the effectiveness of these doses in *Xenopus laevis* tadpoles we used aromatase 
expression as a molecular marker since activity and expression is sensitive to endocrine 
disruption by organotins and RXR/PPARγ ligands in mammals (17, 18). *Xenopus* aromatase 
expression was similarly repressed 2 to 3-fold by 10 nM TBT, AGN195203, LG100268 or 1 µM 
troglitazone at stage 56 tadpoles (Fig. 6A) and at all subsequent stages. Despite significant ligand 
induced aromatase downregulation, neither sex ratios nor the time required to reach 
metamorphosis were altered (data not shown). *Xenopus* liver and kidney also exhibited no gross 
structural abnormalities at the doses given.

However, consistent with the testis and adipose results from mice presented above, we 
observed a dose-dependent increase in ectopic adipocyte formation posterior to the fat-bodies in 
and around the gonads of both sexes following TBT or RXR/PPARγ ligand exposure (Fig. 6B). 
In contrast, estradiol treated animals did not show increased adipocyte formation compared to 
controls. At 10 nM TBT, 10 nM AGN195203 or 1 µM troglitazone, ectopic adipocytes were 
observed in approximately 45-60% of animals. At the highest dose of TBT in males, testicular
tissue was interspersed with, or replaced by adipocytes along the anterior-posterior axis (Fig. 6D,E,G).

The concordant changes observed in *Xenopus* aromatase expression, gonadal adipocyte formation and increased murine adiposity following exposure to TBT, RXR and PPARγ ligands are therefore consistent with a common mechanism of action through RXR:PPARγ activation, supporting the conclusion that endocrine disruption via nuclear receptor transcriptional regulation is a novel and key feature of organotin toxicity.

**DISCUSSION**

We have shown above that TBT is a potent inducer of adipogenesis, *in vitro* and *in vivo*, by acting as a novel, high-affinity xenobiotic ligand for RXRα and PPARγ. The ability of organotins to bind and activate these receptors, in particular the RXRs which exhibit very restricted ligand specificity, is unexpected given the radically different chemical composition and 3D-molecular structure of organotins when compared to known natural and synthetic nuclear receptor ligands. Typically, RXR ligands comprise of a carboxylic acid functional group and a 3D-molecular shape that mimics 9-*cis* RA. Structure-activity profiles indicate distinct structural preferences for organotins but also a relatively broad accommodation for agonist activity that is not easily reconciled with the classical ligand binding model. Organotins may therefore interact somewhat differently than previously described RXR/PPARγ ligands with receptor LBDs to induce productive conformational changes required for co-activator recruitment. However, the binding data indicate that TBT is a potent and efficacious ligand for both RXRs and PPARγ that interacts, at least partially, with the same receptor binding sites of other high-affinity ligands and
promotes the necessary co-factor interactions required for agonist activation. In Kanayama et al., TBT was only effective in co-activator recruitment assays with PPARγ above 10 µM in vitro. However, in accord with our findings, they show that TBT activated PPARγ significantly at nanomolar concentrations in transfection assays. This may reflect a limitation of preference in the co-factor used in vitro. Alternatively, the lower maximal activation observed with TBT on PPARγ in cells (~30% at 100 nM TBT cf troglitazone) is consistent with one of two possibilities: either non-specific cellular toxicity at high levels or activation as a partial agonist.

The ability of TBT to act as a dual ligand for permissive heterodimers such as RXRα:PPARγ, which can be activated by specific ligands for either receptor, also raises the possibility for additive or synergistic effects that might increase the potency of these compounds in vivo at low doses for this specific signaling pathway. Of note is that receptor activation is observed at nanomolar concentrations, whereas other mechanisms of toxicity and endocrine disruption e.g. direct inhibition of aromatase activity, typically occur in the micromolar range. Furthermore, the activation of other permissive RXR heterodimeric partners, e.g. LXR and NURR1, suggests that organotins may act more widely to disrupt multiple nuclear receptor mediated hormonal signaling pathways.

The biological consequences of organotin activation of the RXR:PPARγ signaling pathway are predictable and should follow known aspects of RXR/PPARγ biology. The RXR:PPARγ pathway plays a key role in adipocyte differentiation and energy storage, and is central to the control of whole body metabolism (58). PPARγ activation increases the expression of genes that promote fatty acid storage and represses genes that induce lipolysis in adipocytes in white adipose tissue (59). PPARγ such as the thiazolidinediones can modulate insulin sensitivity due to these effects on the adipocyte, reversing insulin resistance in the whole body by sensitizing the
muscle and liver tissue to insulin (60). However, a consequence of this increase in whole body insulin sensitivity is that fat mass is increased through the promotion of triglyceride storage in adipocytes. Evidence is also mounting that depot-specific remodeling and adipocyte numbers increase following thiazolidinedione treatment (55-57). Therefore, PPARγ agonists comprise a class of pharmaceutical therapies for type 2 diabetes that can also promote obesity by increasing fat storage. Likewise, retinoid X receptor (RXR) ligands also act as insulin-sensitizing agonists in rodents (61) underscoring the permissive nature of the PPARγ:RXR heterodimer and the potential effects on diabetes and obesity of both PPARγ and RXR agonists.

Our data are consistent with recent studies that organotins can mediate some of their endocrine disruption effects by transcriptional regulation through nuclear receptors, in particular RXR:PPARγ signaling (17-19, 24). Consequently, TBT exposure can promote adipocyte differentiation in the same manner as other RXR or PPARγ ligands in vitro using the standard murine 3T3-L1 cell model and in vivo through increased adiposity following intra-uterine organotin exposure in newborn mice. It is currently unknown whether the increased adiposity in vivo results from an increase in adipocyte precursor cell number, enhanced adipocyte differentiation from the same number of precursors, an increase in adipocyte size without an increase in number or some combination of these.

The prevailing epidemiological data ascribes high density caloric and/or fatty diets coupled with decreased physical activity as the root causes for the rise in obesity rates in the general population (62). The contribution of genetic components is less clear. While genetic variation contributes to an individual’s propensity to develop obesity, the rapid worldwide increase in obesity suggests that interaction with the modern environment exposes inherent genetic differences. The Barker hypothesis postulates that in utero fetal nutritional status is a potential
risk factor for metabolic syndrome diseases (63-67). In this view, developmental metabolic programming of a thrifty phenotype limits the range in adaptive responses to the environment, e.g. diet and exercise, in later life (68). Experimental evidence from animal models lends support to this hypothesis (69). Plausible mechanisms include imprinting of obesity sensitive hormonal pathways or changes in cell type and number e.g. adipocytes, established during development.

Others, however, argue that the environment plays another role in obesity. Since the increase in obesity rates parallels the rapid growth in the use of industrial chemicals over the past 40 years, it is plausible and provocative to associate in utero or chronic lifetime exposure to chemical triggers present in the modern environment with this epidemic. Hence, an “obesogen” model predicts the existence of xenobiotic chemicals that inappropriately regulate lipid metabolism and adipogenesis to promote obesity. Several recent studies serve as “proof-of-principle” for such a hypothesis. Environmental estrogens such as bisphenol A and nonylphenol, for instance, can promote adipocyte differentiation or proliferation in murine cell lines (70, 71). Furthermore, epidemiological studies link maternal smoking during pregnancy to an elevated risk of childhood obesity (72-76).

Seen in this context, we propose that organotins such as TBT and its congeners are chemical stressors or “obesogens” that activate RXR:PPARγ signaling to promote long term changes in adipocyte number and/or lipid homeostasis following developmental or chronic lifetime exposure.
MATERIALS AND METHODS

Plasmids and transfections

pCMX-GAL4 and pCMX-VP16 plasmid fusion constructs to nuclear receptors ligand binding domains and coactivators (GAL4-hRARα, hRXRα, -xRXRα/γ, -hPPARγ, -mPPARα, -hSXR, -NURR1, -VDR, -LXR, -hACTR, -hPBP, -hSrc1, hTIF2) have been previously described (77-82). Transfections were performed in Cos7 cells essentially as described in (83) using MH200-x4-TK-Luc as reporter and normalized to pCMX-β-galactosidase controls. Briefly, Cos7 cells were seeded at 5000 cells/well in 96-well tissue culture plates in 10% FBS/DMEM and transfected for 8 hrs with 11 µg/plate of DNA/calcium phosphate precipitate mix (MH200x4-TK-Luc: CMX-β-galactosidase: nuclear receptor/coactivator effector(s) at a ratio of 5:5:1). Cells were washed free of precipitate with phosphate buffered saline (PBS) and media replaced with serum free ITLB/DMEM medium (84) plus ligands for an additional 24 hours prior to assays for luciferase and β-galactosidase activity. All transfection data points were performed in triplicate and all experiments were repeated at least three times.

Quantitative real-time PCR analyses

Total cellular RNA from C57BL/6 mouse and Xenopus laevis tissues was isolated with Trizol reagent and reversed transcribed with oligo dT and Superscript II (Invitrogen, CA) according to the manufacturer’s instructions. Triplicate cDNA samples (50 ng/reaction) were analyzed by QRT-PCR on a DNA Engine Opticon thermal cycler (MJ Research/Biorad) using SYBR Green chemistry (PerkinElmer Life Sciences, MA). Fold changes in expression levels were calculated after normalization to histone Hist2h4 using the delta-delta cycle threshold
method (85). Gene specific primers were as follows - Hist2h4 F
5’CCCGTGTTGGTGTGCTGAAGGTGT3’, R 5’GA ATTGAAGCGCGGCGGCTCTA3’; RXRα F
5’CGGCTGTCAGGGTACTTGTGTT3’; R 5’ CCGCTGTCAGGGGACTTTGTGTTT3’;
PPARγ F 5’TGGGTGAAACTCTGGGAGATTC3’, R 5’
AATTCTTGTGAAGTGCTCATAGGC3’; C/EBPα F 5’CCAAGAAGTCGGTGAGACAAGA
3’, R 5’CGGTCAATTGTCACCTGCTCAACT3’; C/EBPβ F 5’GCCGCGCCCTTTTAGACC 3’, R
5’CGCTCGTGCTCGCCAATG3’; C/EBPδ F 5’ACCCCGCCGCTTTCTACGAG3’, R
5’ACGGCGGCAATGGAGATGTGAC3’; aP2 F 5’GAATTCGATGAAATCACCGCA 3’, R
5’CTCTTTATTGTGCTGACTTTCTCCA3’; FATP F 5’AGCCGCTTCTGGGATGACTGTG3’, R
5’ACCGAAGCGCTGCTGAACT3’; ACS F 5’CCCAGCCAGTCCACCAG3’, R
5’CACACCACACTAGCTAGCTCAGTGT3’, FASN F
5’TCGCGGTTGTTGTTGTTAGAAT3’, R 5’ACTTGGGCGGTGAGATGTGTC3’;
ACAC F 5’GGATGGCAGCTCTGGAGGTGTATG3’, R 5’TGTCCTTAAGCTGGCGGTGT3’;
Pck1 F 5’CTGGCACAGCAGCTGGAGGTGTTG3’, R 5’TGCCGAAGTTAGAGCGGTGAG3’;
Srebfi F 5’GCCCGGCTTCAACCTCAG3’, R 5’ACTGGCAGGCGGCTTCTCC3’;
Xenopus EF1α F 5’GATCCCAGGAAAGCGCAATG3’, R 5’CCGGATCTGCTGCTTCTTTTCT3’;
Xenopus CYP19 (aromatase) – F 5’GTCTGGATTTATGGCGGAGAACA3’, R
5’CTGATGAAGTATGGCCGAATGACC3’.

Ligand binding

Histidine-tagged RXRα ligand binding domain (H6-RXRα LBDs) was expressed and
purified from pET15b(+) vector in BL21(DE3) pLysS bacteria cultures after induction with 1
20 mM IPTG for 3hrs at 37 °C (86). Purified H₆-PPARγ was purchased from Invitrogen. Proteins were bound to 96-well Nickel Chelate Flashplates (PerkinElmer Life Sciences, MA) at 100 µg/ml overnight at 4 °C and washed 5 times with 200 µl/well Flashplate Assay Buffer (20 mM HEPES pH 7.9, 100 mM KCl, 0.1 % CHAPS, 0.1 mM DTT). Competition assays typically used 1-5 nM[^3H]-9-cis-RA (Perkin Elmer Life Sciences, MA) or 10-50 nM[^3H]-rosiglitazone (American Radiochemicals Inc., MO) plus cold competitor ligands in Flashplate Assay Buffer at concentrations indicated in the figures. Plates were incubated at room temperature, protected from light and read after 4 hours on a Packard Topcount scintillation counter. Specific bound cpm were determined by subtraction of cpm from uncoated wells at each ligand concentration. Data were analyzed with GraphPad Prism 4.0 (GraphPad, CA) using a one-site competition binding equation to determine Kᵢ values for competitor ligands; Kᵅ values of 1.4 and 41 nM for 9-cis-RA and rosiglitazone for their respective receptors were used in the calculations (87, 88).

3T3-L1 Cell Assays

3T3-L1 (ATCC, VA) cells were maintained as sub-confluent cultures by passage every 3 days from cultures seeded at 5000 cells/cm² in 8 % calf serum/DMEM medium. For differentiation assays, cells were seeded at 15×10⁶ cells/well into 24-well tissue culture plates in 8% fetal bovine serum/DMEM medium, cultures grown for 2 days and then treated with the indicated RXR, RAR and PPAR ligands either with or without MDIT (100 µM IBMX, 100 nM dexamethasone, 0.1 ng/ml insulin and 2 nM T₃ thyroid hormone) induction cocktail. Media and ligand treatments were renewed every 2 days. After 1 week, cells were scored for adipocyte differentiation by Oil Red staining for lipid droplet accumulation. Cultures were washed with phosphate buffered saline (PBS), fixed with 10 % formaldehyde for 15 minutes, washed with
distilled water and stained with filtered Oil Red solution (4g/L, 60 % isopropanol) for 15 mins. Excess stain was removed by washing three times with distilled water. Three random fields from each well were photographed under phase contrast and analyzed in ImageJ. Images were converted into high contrast black and white images to visualize lipid droplets and scored as the percentage area/field. Data are shown as the mean ± S.E.M from three wells per treatment. The method was validated by extraction of Oil Red O from stained cells into 100 % isopropanol and quantitated by absorbance at 540nm on a spectrophotometer.

In vivo animal exposure experiments

C57BL/6J mice were housed under a 12-hour light/dark cycle. Pregnant mice were dosed by intraperitoneal injection with TBT (0.05 or 0.5 mg/kg body weight (b.w.) or vehicle (sesame oil) from E12 every 24 hours until the day before delivery. Neonates were sacrificed at the day of delivery and analyzed. The samples were embedded in OCT and sectioned (12 mm) using a cryostat. Sections were fixed on slides with 4% paraformaldehyde for 10 min and rinsed in phosphate-buffered saline (PBS). The slides were then sequentially washed with distilled water and 60 % of isopropanol and stained with Oil Red O (4g/L, 60 % isopropanol). After washing with 60 % isopropanol and distilled water, the slides were counterstained with hematoxylin. Sections were evaluated and photographed using a Zeiss microscope.

For long-term growth studies, pups were cross-fostered to unexposed C57BL/6 dams after birth and litter sizes kept constant at 8 pups/dam (control, 2 male + 2 female; TBT treated, 2 male + 2 female). Animals were weaned at 3 weeks of age and maintained on standard rodent chow. Total body weight was followed until 10 weeks of age. Males were then sacrificed, epidydimal fat pads dissected and weighed.
Xenopus laevis tadpoles were sorted at stage 48 (89) and maintained in 1 L glass tanks in 20%
Holtfreter’s buffered salt solution (90) at a density of 10 tadpoles/tank on a diet of ground
Tetramin Fish Flakes and spirulina. Compounds prepared in dimethylsulfoxide (DMSO) as 10^5-
fold stock solutions were tested on triplicate tanks and dosed by static renewal after weekly
water changes. Metamorphs at stage 64 were transferred to individual containers and fed frozen
brine shrimp for 2 weeks until stage 66. Froglets were euthanized with 250 mg/L MS222 in 20%
Holtfreter’s solution and then scored for gonadal abnormalities and inter-renal/gonadal adipocyte
formation under a dissecting microscope. Kidneys with attached gonads and livers were fixed in
10 % formalin-phosphate buffered saline, embedded in paraffin and sectioned at 15 μm
thickness. Sections were developed with Mallory’s trichrome stain.

All animal experiments were approved and performed in accordance with IACUC protocols.
FOOTNOTES

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**Abbreviations used are: TBT, tributyltin chloride; TPTO, triphenyltin oxide; RXR, retinoid x receptor; PPAR, peroxisome proliferator-activated receptor; DMEM, Dulbecco's modified Eagle's medium, FBS, fetal bovine serum; 9-cis RA, 9-cis retinoic acid; T3, 3,5,3'-triiodo-L-thyronine; MDIT, 3-isobutyl-1-methylxanthine, dexamethasone, insulin and 3,5,3'-triiodo-L-thyronine adipocyte differentiation mix; Cos7, transformed green monkey kidney fibroblast cell line; 3T3-L1, embryonic murine preadipocyte fibroblast cell line; OCT, optimal cutting temperature embedding compound.
REFERENCES

7. McAllister BG, Kime DE 2003 Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and irreversible sperm damage in zebrafish (Danio rerio). Aquat Toxicol 65:309-16
10. Boyer IJ 1989 Toxicity of dibutyltin, tributyltin and other organotin compounds to humans and to experimental animals. Toxicology 55:253-98


23. Watanabe H, Iiguchi T, Morohashi K 2002 [Endocrine disruptors and nuclear receptors]. Nippon Rinsho 60:397-403


32. Lane MD, Tang QQ, Jiang MS 1999 Role of the CCAAT enhancer binding proteins (C/EBPs) in adipocyte differentiation. Biochem Biophys Res Commun 266:677-83

34. **Tang QQ, Lane MD** 1999 Activation and centromeric localization of CCAAT/enhancer-binding proteins during the mitotic clonal expansion of adipocyte differentiation. Genes Dev 13:2231-41


36. **Kletzien RF, Clarke SD, Ulrich RG** 1992 Enhancement of adipocyte differentiation by an insulin-sensitizing agent. Mol Pharmacol 41:393-8


38. **Tafuri SR** 1996 Troglitazone enhances differentiation, basal glucose uptake, and Glut1 protein levels in 3T3-L1 adipocytes. Endocrinology 137:4706-12


49. Schadinger SE, Bucher NL, Schreiber BM, Farmer SR 2005 PPAR{gamma}2 regulates lipogenesis and lipid accumulation in steatotic hepatocytes. Am J Physiol Endocrinol Metab
56. de Souza CJ, Eckhardt M, Gagen K, Dong M, Chen W, Laurent D, Burkey BF 2001 Effects of pioglitazone on adipose tissue remodeling within the setting of obesity and insulin resistance. Diabetes 50:1863-71
58. Auwerx J 1999 PPARgamma, the ultimate thrifty gene. Diabetologia 42:1033-49
67. Barker DJ, Martyn CN, Osmond C, Hales CN, Fall CH 1993 Growth in utero and serum cholesterol concentrations in adult life. BMJ 307:1524-7
70. Masuno H, Kidani T, Sekiya K, Sakayama K, Shiosaka T, Yamamoto H, Honda K 2002 Bisphenol A in combination with insulin can accelerate the conversion of 3T3-L1 fibroblasts to adipocytes. J Lipid Res 43:676-84
homeostasis is mediated by the steroid and xenobiotic receptor SXR. J Biol Chem 278:43919-27


FIGURE LEGENDS

**Figure 1 Organotins are agonist ligands of RXRα and PPARγ.** Organotins are high affinity ligand agonists of RXRα and PPARγ. (A-D) Activation of GAL4-hRXRα, -hPPARγ, -hRARα or -hPPARα in transiently transfected Cos7 cells by organotins and receptor specific ligands. Data represent reporter luciferase activity normalized to β-galactosidase and plotted as the average fold activation ± S.E.M. (n=3) relative to solvent only controls from representative experiments. (E-F) Competition binding curves of histidine-tagged RXRα or PPARγ LBDs with tributyltin chloride (TBT). Data shown are from a representative experiment analyzed in GraphPad Prism 4.0 (GraphPad, CA) and Kᵢ values deduced (Table 3).

**Figure 2 Tributyltin induces adipogenesis in 3T3-L1 cells.** Uninduced (A) and MDIT induced (B) 3T3-L1 cultures grown for 1 week in the presence of vehicle (DMSO) or ligands were analyzed for mature adipocyte differentiation by Oil Red O staining. Scale bar represents 100 µm. (C and D) The percentage area stained was determined by automated analysis of random fields (n=9) from high contrast dissecting scope photographs of monolayers analyzed in ImageJ; 1-100 nM of TBT, AGN195203 and TTNPB or 1-10 µM troglitazone. (E) Quantitative real-time PCR (QRT-PCR) of adipocyte specific fatty acid binding protein aP2 (aP2/Fabp4) expression levels in post-confluent 3T3-L1 cells treated with the indicated ligands for 24 hours. Data were normalized to GAPDH controls and plotted as average fold induction ± S.E.M. (n=3).

**Figure 3 In vivo induction of adipogenic modulators and RXR:PPARγ target genes.** C57BL/6 male mice (3 animals/treatment) were dosed with TBT (0.3 mg/kg b.w.), AGN195203 (0.3 mg/kg), troglitazone (3 mg/kg b.w.) or vehicle (corn oil) only by intraperitoneal injection.
Animals were sacrificed after 24 hours, dissected and cDNA prepared from liver, epidydimal fat pad or testis for QRT-PCR analysis. Expression levels were normalized to histone Hist2h4 and shown as the average fold change ± S.E.M. (n=3) compared to vehicle (corn oil) controls. Control v ligand treatments were analyzed by the unpaired Student’s t-test; * p<0.1, ** p<0.05.

Figure 4 In utero exposure to TBT increases adiposity in mouse liver, testis and adipose depots. Histological sections (12 µm) of newborn mouse liver (A,B), testis (C,D), inguinal adipose (E,F) and mammary adipose (G,H) stained with Oil Red O and counterstained with hematoxylin following in utero exposure to vehicle only (sesame oil) (A,C,E,G) or 0.5 mg/kg b.w. TBT (B,D,F,H) given s.c. daily from E12-18. Scale bar represents 100 µm.

Figure 5 In utero exposure to TBT increases adipose mass but not body weight in adult mice. (A) Growth curves of C57BL/6 male and female pups exposed to control (sesame oil) or TBT in utero (E-12-18). Data are mean ± SEM (n= 10). (B) Epidydimal fat pad weights from control or TBT treated males at 10 weeks. *Epidydimal adipose mass from exposed males was ~20 % greater (Control vs TBT: 0.30g ± 0.020, n=9 vs 0.36g ± 0.018, n =10, * p=0.0374). Data represent mean ± SEM n=9-10.

Figure 6 Endocrine disruption of RXR:PPARγ signaling and ectopic induction of adipocytes in Xenopus laevis by TBT. (A) Expression levels of Xenopus aromatase (XCYP19) were determined in tadpoles (stage 56) by QRT-PCR after 24 hours exposure to vehicle only (DMSO) or the indicated ligands. Expression was normalized to Xenopus EF1α and expressed as average fold change in expression ± S.E.M. (n= 9) relative to vehicle controls. (B) Xenopus laevis tadpoles were dosed weekly under static renewal conditions with indicated ligands from...
stage 48 (prior to gonadogenesis) until stage 64 (metamorphic climax). Metamorphs (stage 66) were scored for ectopic adipocyte patches on gonads and urogenital ducts. Data are shown as the percentage of metamorphs exhibiting ectopic adipocyte patches posterior to the fat bodies; mean ± S.D. from triplicate tanks. (C-E) Dissecting microscope photographs of kidneys (k), testis (t) and fat bodies (fb) from DMSO control, 10 nM TBT and 1 µM troglitazone treated male metamorphs. Multiple ectopic adipocyte patches (red arrows) are present posterior to the fat bodies along the anterior-posterior axis of gonads in TBT (D) and troglitazone (E) treated animals but not controls (C). Histological sections of kidneys and gonads from the same control (F) and 10 nM TBT (G) treated males at the level indicated by the white line in C and D. Gonadal and connective tissue was either completely replaced by or interspersed with adipocytes (red arrows) in TBT treated animals. Sections were developed with Mallory’s trichrome stain. Scale bars represent 100 µm.
### TABLES

#### Table 1. TBT activates RXRs and RXR-permissive heterodimers

<table>
<thead>
<tr>
<th>GAL4-NR LBD</th>
<th>Fold Activation at 60 nM TBT</th>
<th>Permissive RXR heterodimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>RXRα (H. sapiens)</td>
<td>60</td>
<td>Yes</td>
</tr>
<tr>
<td>RXRα (X. laevis)</td>
<td>25</td>
<td>Yes</td>
</tr>
<tr>
<td>RXRγ (X. laevis)</td>
<td>7.0</td>
<td>Yes</td>
</tr>
<tr>
<td>NURR1 (H. sapiens)</td>
<td>7.0</td>
<td>Yes</td>
</tr>
<tr>
<td>LXR (H. sapiens)</td>
<td>2.1</td>
<td>Yes</td>
</tr>
<tr>
<td>PPARα (M. musculus)</td>
<td>0.7</td>
<td>Yes</td>
</tr>
<tr>
<td>PPARγ (H. sapiens)</td>
<td>5.3</td>
<td>Yes</td>
</tr>
<tr>
<td>PPARδ (H. sapiens)</td>
<td>1.7</td>
<td>Yes</td>
</tr>
<tr>
<td>RARα (H. sapiens)</td>
<td>0.7</td>
<td>No</td>
</tr>
<tr>
<td>TRβ (H. sapiens)</td>
<td>0.4</td>
<td>No</td>
</tr>
<tr>
<td>VDR (H. sapiens)</td>
<td>0.5</td>
<td>No</td>
</tr>
<tr>
<td>SXR (H. sapiens)</td>
<td>1.0</td>
<td>No</td>
</tr>
</tbody>
</table>

Data are fold activation at 60 nM TBT relative to solvent only controls of transiently transfected Cos7 cells after 24 hour ligand treatment.
Table 2. Organotin EC50 values for nuclear receptor LBDs

<table>
<thead>
<tr>
<th>Ligand</th>
<th>GAL4-NR LBD Transactivation EC50s (nM)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>hRXRα</td>
</tr>
<tr>
<td>LGD268</td>
<td>2-5</td>
</tr>
<tr>
<td>AGN195203</td>
<td>0.5-2</td>
</tr>
<tr>
<td>9-cis RA</td>
<td>15</td>
</tr>
<tr>
<td>all-trans RA</td>
<td>na</td>
</tr>
<tr>
<td>Butyltin chloride</td>
<td>na</td>
</tr>
<tr>
<td>Dibutyltin chloride</td>
<td>3000</td>
</tr>
<tr>
<td>Tributyltin chloride</td>
<td>3-8</td>
</tr>
<tr>
<td>Tetrabutyltin</td>
<td>150</td>
</tr>
<tr>
<td>Di(triphenyltin)oxide</td>
<td>2-10</td>
</tr>
<tr>
<td>Butyltin tris(2-ethylhexanoate)</td>
<td>na</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>na</td>
</tr>
<tr>
<td>Tributyltin fluoride</td>
<td>3</td>
</tr>
<tr>
<td>Tributyltin bromide</td>
<td>4</td>
</tr>
<tr>
<td>Tributyltin iodide</td>
<td>4</td>
</tr>
<tr>
<td>Triethyltin bromide</td>
<td>2800</td>
</tr>
<tr>
<td>Trimethyltin chloride</td>
<td>&gt;10000</td>
</tr>
</tbody>
</table>

na- not active; ND- not determined. EC50 values were determined from dose-response curves of GAL4-NR LBD construct activation in transiently transfected Cos7 cells after 24 hour ligand exposure.
Table 3 Tributyltin chloride binding constants (K\textsubscript{d}) for hRXR\textalpha and hPPAR\textgamma LBDs

<table>
<thead>
<tr>
<th>Ligand</th>
<th>H\textsubscript{6}-RXR\textalpha</th>
<th>H\textsubscript{6}-PPAR\textgamma</th>
<th>Published</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBT</td>
<td>12.5 (10-15)</td>
<td>20 (17-40)</td>
<td>-</td>
</tr>
<tr>
<td>LG100268</td>
<td>7.5 (6-10)</td>
<td>ND</td>
<td>3±1\textsuperscript{a}</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>ND</td>
<td>300 (270-335)</td>
<td>300 ± 30\textsuperscript{b}</td>
</tr>
</tbody>
</table>

Competition binding curves were determined at constant [\textsuperscript{3}H]-specific ligand concentrations (20 nM 9cis-RA, K\textsubscript{d} = 1.4 nM (87) or rosiglitazone, K\textsubscript{d} = 41 nM (88) with increasing cold competitor ligands over the range indicated in Fig. 1 E,F. Data were anlayzed in GraphPad Prism by non-linear regression of a competitive one-site binding equation (Chang-Prusoff method) to determine K\textsubscript{i} values ± 95% confidence interval (n=3). \textsuperscript{a} RXR\textalpha:LG100268 K\textsubscript{d} = 3±1 nM (27); \textsuperscript{b} PPAR\textgamma:troglitazone K\textsubscript{d} = 300 ± 30 (28).
A bar graph showing the fold expression of genes with different treatments. The x-axis represents the treatment concentration in nM, with treatments including DMSO, TBT, AGN203, LG268, TROG, and E₂. The y-axis represents the fold expression.

A pictograph showing the percentage of metamorphs with ectopic adipocytes. The x-axis represents the treatment concentration in nM, with treatments including DMSO, TBT, AGN203, TROG, and E₂. The y-axis represents the percentage of metamorphs with ectopic adipocytes.

Images C, D, and E show histological sections with arrows indicating ectopic adipocytes. Image F shows a higher magnification of the section with arrows indicating ectopic adipocytes. Image G shows a histological section with arrows indicating ectopic adipocytes.