Evaluation of a panel of *in vitro* methods for assessing thyroid receptor β and transthyretin transporter disrupting activities

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Highlights

- Developed a novel test panel to assess thyroid hormones and transporter interferences
- Generated a list of reference compounds for thyroid-related bioassays evaluation
- Evaluation of agonistic and antagonistic thyroid receptor β CALUX with pure compounds
- Evaluation of transthyretin-TRβ CALUX bioassay with known T4 competitors
- Analysis of waste water extracts on transthyretin-TRβ CALUX
- Waste water samples showed T4 competitive activity and TTR binding potency

Abstract

We developed a thyroid testing panel to assess endocrine disrupting chemicals (EDCs) capacities to bind either the thyroid receptor β (TRβ) or the thyroid hormones transporter trans-thyretin (TTR). We first stably transfected a human U2OS cell line with TRβ and a luciferase reporter construct to develop the TRβ CALUX® reporter gene assay to assess chemicals' potential to interact with TRβ. Secondly, we combined a TTR-binding assay with the TRβ CALUX (TTR-TRβ CALUX) and optimized the system to evaluate the competitive properties of EDCs towards T4 for TTR binding. Both systems were evaluated with a range of known thyroid-disrupting compounds. The agonistic/antagonistic TRβ CALUX successfully predicted 9/9 and 9/12 test compounds, respectively. The TTR-TRβ CALUX predicted 9/9 compounds and demonstrated competitive activities when analyzing waste water samples. We concluded that the proposed test battery is a promising screening method able to efficiently generate data on thyroid hormone interferences by chemicals.

Keywords: thyroid hormones; TTR binding potency; CALUX; bioassay; water extract; endocrine disruption

Introduction

Over the course of several decades, some evidence has suggested the sensitivity of fetal development and adult physiology towards endocrine disrupting chemicals (EDCs) [1]. It has been reported that a repeated exposure to these EDCs can interfere with endogenous hormones leading to adverse health effects, even at very low concentrations [1]. The importance of the endocrine system in major physiological processes and disease etiology emphasizes the growing need of developing reliable high throughput tools to efficiently predict the disrupting activity of chemicals and their mixtures. In the past, we developed CALUX® (Chemically Activated

LUciferase eXpression) reporter gene assays able to assess androgen receptor (AR) and estrogen receptor (ER) alterations by environmental chemicals [2, 3]. These bioassays cover a variety of endpoints related to EATS (Estrogens, Androgens, Thyroid, and Steroidogenesis) adverse outcomes. In order to further expand our current testing panel, we selected the thyroid system as a next step in developing a complete *in vitro* test battery for assessing developmental and reproductive toxicity (DART) effects.

Thyroid hormones (THs) are important in numerous physiological processes such as regulation of metabolism, bone remodeling, cardiac function and mental status. The two main THs, the prohormone 5′,3′,5,3-tetraiodo-[L]-thyronine (thyroxine, T4) and its bioactive form the 3′,5,3-triiodo-[L]-thyronine (triiodothyronine, T3), play a major role in maintaining body homeostasis [4]. Thyroid hormonal output is regulated by a finely tuned feedback system acting at both hypothalamic and pituitary levels [4, 5]. First, the thyroid-releasing hormone (TRH) is secreted by the hypothalamus into blood, through the hypophyseal portal system. Once in the anterior pituitary, TRH induces the production of thyroid-stimulating hormone (TSH) which is released in the general circulation towards the thyroid gland. Within the thyroid gland, TSH stimulates the synthesis and secretion of THs. Depending on serum levels, THs can modulate their own production and release by exerting a retro-negative control on both TRH and TSH [4, 5]. Once in the bloodstream, T4, the major thyroid hormone produced, binds to transport proteins so as to be distributed to target cells across the body. In humans there are three major serum carrier proteins, thyroxine-binding globulin (TBG), transthyretin (TTR) and human serum albumin (HSA) [6, 7]. Although the major transporter is TBG, specific structural properties allow TTR to pass through the blood-brain barrier and the uterine-placental wall making this protein the main carrier of T4 in the cerebrospinal fluid and the developing fetus [7]. Within the

target tissues, T4 is mainly converted into its bioactive form T3 by deiodinase enzymes [5, 8]. Both T4 and T3 are natural ligands for the thyroid receptors α and β (TRα and TRβ) (TRs), with T3 having a 10-fold higher affinity [9, 10]. TRs primary isoforms are differentially expressed in fetal and adult tissues, TRα being predominant in the brain, heart ventricles, intestine and skeletal muscle while TRβ is more widely distributed (brain, retina, inner ear, heart, kidney, liver and lung) [4, 10]. In 2014, Ortiga-Carvalho *et al.* demonstrated that patients with thyroid hormone β (THβ) resistance presented a severe deregulation of the hypothalamic-pituitary-thyroid (HPT) axis as well as abnormally elevated THs levels [10]. On the contrary, patients with thyroid hormone α resistance showed nearly normal THs and TSH levels, implying a lower impact of the condition on the HPT axis. These results suggest that TRβ isoforms might play a crucial role in maintaining circulating THs levels by participating in the negative feedback of the HPT axis, an important target of EDCs [10]. As a response to THs stimulation, TRs enter the nucleus and bind to thyroid hormone response elements (TREs), triggering the transcription of T3 target genes [9, 12]. TRs can either act as homodimers or form heterodimers with retinoid X receptor (RXR) able to bind to TRE, inducing the stimulation or inhibition of associated genes [13, 14]. The thyroid system plays a central role in proper fetal development as well as preserving homeostasis during life, therefore maintaining a normal thyroid function and hormone levels is essential.

In recent years a link has been found between disturbed thyroid hormones' functions and exposure of man and wildlife to pollutants with endocrine disrupting properties [10]. Although multiple sites of the thyroid system can be disrupted, it is extremely complex to assess them all as there is currently no practical battery that can cover all of these potential targets of EDCs. In

the present paper, we designed a condensed thyroid hormone disruption panel aiming to evaluate both the potential agonistic and antagonistic aspects of chemicals towards TRβ, as well as their possible interferences with T4/TTR-binding. First, we developed the *in vitro* TRβ CALUX reporter gene assay based on the human U2OS cell line stably transfected to endogenously express TRβ. The cells were additionally transfected with a reporter construct containing a luciferase gene linked to two copies of TREs. In this bioassay, a TRβ activation stimulated by ligand-binding consecutively induces both the transcription of THs target genes and of gene coding for the luciferase protein. This method allows assessment of *in vitro* hormonal activity of agonistic chemicals by luciferase production quantification. Moreover, the TRβ CALUX assay can also be used to assess the activity of antagonistic compounds through cells pre-stimulation with the natural ligand T3. The current paper describes the design and operating mode of the new TRβ CALUX bioassay. In addition, this manuscript details the evaluation process used to assess the overall performance of both agonistic and antagonistic methods. As no prior set of recommended chemicals was available at the time of the beginning of the study, we generated our own list of compounds selected on their thyroid-related activities reported in literature.

While a limited number of chemicals are known to directly interfere with TRβ, TTR is a well-known target for phenolic organo-halogen EDCs which may lead to thyroid depletion of the fetal brain and associated disorders [15-18]. In this manner, and as a complement to the TRβ CALUX bioassay, we included a TTR-binding assay to our method in order to assess TTR/THs potential interferences by EDCs. The TTR-binding assay involves the incubation of possible T4 competitor with a fixed amount of T4 and TTR. After separation from free remaining T4/compound, the fraction of T4 which bound to TTR can be measured using TRβ CALUX

bioassay. This publication presents the detailed procedure to perform the optimized TTR-binding assay using TRβ CALUX as read-out system, hence referred as TTR-TRβ CALUX. The overall combined method's performance was evaluated by the analysis of known T4 competitor for TTR binding. In this report, we demonstrate that the newly developed TRβ CALUX method can be used as a readout of the existing TTR-binding assay, so as to assess potency of compounds for competing with T4 for TTR binding sites. Step by step, we detail the development of the TRβ CALUX and the TTR-TRβ CALUX bioassays as well as the intra-laboratory evaluation strategy used for each method (Fig 1.).

We completed this study by investigating the applicability of the TTR-TRβ CALUX bioassay through a short case-study. We analyzed waste- and HPLC-water samples on the combined assay and highlighted net T4 competitive activities for TTR binding. This result showed a potential field application for the TTR-TRβ CALUX bioassay.
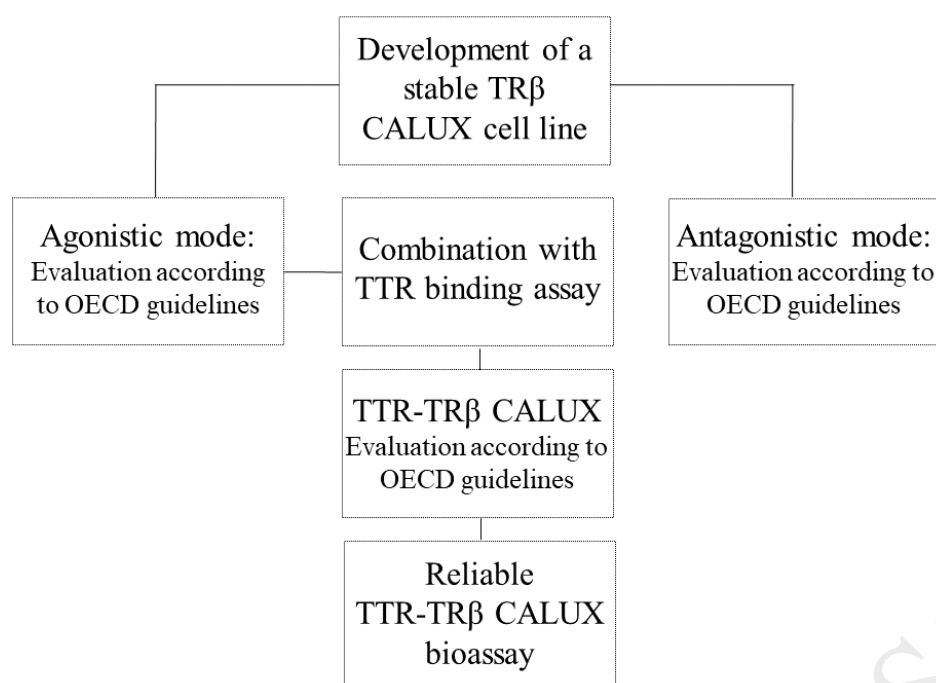
Fig 1. *Study design for the evaluation of TRβ CALUX and TTR-TRβ CALUX bioassays. OECD: Organisation for Economic Co-operation and Development.*

Overall, three different CALUX-based bioassays (TRβ CALUX, anti- TRβ CALUX and TTR-TRβ CALUX) were developed and controlled for variance and reproducibility. This study shows that chemicals presenting both agonistic and antagonistic activities on the TRβ as well as T4 competitive properties for the thyroid transporter TTR can be accurately and efficiently predicted using our thyroid *in vitro* human testing panel.

1. Materials and methods

2.1 TRβ CALUX reporter gene assay

The TRβ CALUX cell line is a human osteoblastic osteosarcoma U2OS line (American Type Culture Collection) stably transfected with a pSG5-neo-hTRβ plasmid to express TRβ receptors. The plasmid contains a full-length receptor and is expected to be able to heterodi-merize with RXR expressed endogenously. The cells were additionally transfected with a pGL3 (Promega)-based reporter construct containing a luciferase gene linked to two copies of TREs (pGL3-2xTRE-Luc) (5'agctt/gatatcaggtcatttcaggtcagcatgc/gagctt/gatatcaggtcatttcaggtcag-catgc/g3' x2). The TRβ CALUX bioassay assesses TREs based responses triggered by TRβ activation, or RXR when present in a heterodimer. Either way the thyroid system will be af-fected: a TRβ/RXR activation will induce the transcription of the TRE following the same path-way than a TRβ homo-dimerization. This activation will consecutively induce the transcription of the T3-related genes and genes coding for luciferase protein.

TRβ CALUX were cultured as described before [2]. In brief, TRβ CALUX cells were cultured in Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12) (Gibco, product no.: 31331-028) supplemented with 7.5% charcoal-stripped fetal calf serum (FCS), non-essential amino acids (NEAA) (Gibco, product no.: 11140-03) and 10U/mL peni-cillin and 10μg/mL streptomycin (P/S) (culture medium). Cells were maintained at 37°C and 5% CO_2 at all times and sub-cultured every 3-4 days.

One day prior to the exposure, cultured TRβ CALUX cells were trypsinized and re-sus-pended at a final concentration of $1x10^5$ cells/mL in DMEM/F12 medium without phenol red indicator supplemented with P/S, NEAA and 5% charcoal-stripped fatal calf serum (DCC) (as-say medium). Cell suspension was seeded in clear 96-well plates and incubated for a minimum

8

of 16 hours and maximum 24 hours in a humidified atmosphere at 37°C and 5% CO_2. The next day, the medium was removed and cells were incubated with 200µL of exposure medium consisting of DMEM/F12 supplemented with P/S, NEAA and the compound to be assessed dissolved in DMSO. To avoid any aspecific proteins' interference, the exposure medium does not contain DCC. For each chemical, 8 serial dilutions were tested to a final concentration of 0.1% DMSO, in exposure medium. Each dilution of the test chemicals, including the reference compounds, were tested in triplicate. Plates were placed back in the incubator at 37°C and 5% CO_2 for a period of 24±2 hours. After incubation, the medium was discarded and cells were lysed using 30µL/well of a Triton-lysis buffer. Plates were shaken for 10 minutes after which luciferase activity in cellular lysates was measured for 4 seconds using a luminometer (Tristar, Berthold). The described procedure is used to determine the agonistic potency of chemicals, reflected by light production which can be quantified. For antagonistic assessment, the same procedure was followed with the exception that the exposure medium was supplemented with a non-saturating level of T3 (1.7e-6 M). Therefore, an antagonistic compound is expected to compete with T3 for TRβ binding resulting in a reduction in light emission.

For all measurements, a full dose response dilution series of the reference compound was included on each plate for response quantification. To strengthen quality control, a positive and a negative compound control as well as a solvent control (DMSO) were added to each plate. Regarding the agonistic mode of the TRβ CALUX bioassay, the selected reference compound was the natural ligand T3. 3,3'5-triiodothyroacetic acid (TRIAC), a T4-like compound was chosen as positive control, and bisphenol A (BPA), a TRs antagonist, as negative control. For the antagonist approach, deoxynivalenol was set as the reference, T-2 mycotoxin (T2-toxin) as the positive control and amiodarone as a negative control.

2.2 TTR- TRβ CALUX: the competitive binding assay and analysis

Prior to the TTR-binding assay, all compounds including the reference tetrabromo-bisphenol A (TBBPA) were dissolved in DMSO in dark glass vials. TTR and T4 working solutions were prepared in Tris buffer, made by mixing 12.11g of Tris base (CAS no.: 77-86-1, Sigma Aldrich), 5.84g NaCl (CAS no.: 7647-14-5, Sigma Aldrich) and 0.372g of ethylenedia-minetetraacetic acid (EDTA) (CAS no.: 60-00-4, Sigma Aldrich) in 1L of HPLC water (J.T Baker). The final Tris buffer pH was adjusted to 8.0. Expected T4 and TTR working solution concentrations were 0.08μM and 0.190μM, respectively. The TTR-binding assay is based on an incubation mixture containing 100μL of T4, 50μL TTR and 5μL of tested or model compet-itor compound, prepared in a 1.5mL Eppendorf tube (Fig. 2). In order to prevent TTR denatur-ation, it is important to handle the protein carefully and avoid mixing with a vortex at any point of the procedure. After one hour of incubation at room temperature, tubes were stored at 4°C for at least 20 minutes to overnight. As a next step, self-made Bio-Gel P-6DG columns were prepared to separate T4- or test compound-TTR complexes from unbound compounds. In short, columns were prepared in 1ml disposable Omnifix®-F Solo syringes (B. Braun, product no.: 9161406V). For one 96-well plate, 3g of Bio-Gel P-6DC (Bio-Rad, product no.: 150-0739) was dissolved in 30mL of Tris buffer and incubated at 4°C overnight. From this point, tubes con-taining mixtures were kept on ice at any time to avoid the dissociation of the compound-TTR complexes. Syringes were placed into 15mL Greiner tubes and approximately 1.3mL of Bio-Gel was added, followed by a minute of centrifugation at 120g at room temperature. Bio-Gel P-6DG columns were transferred into clean collector 15mL Greiner tubes placed on ice, where-upon incubate mixtures were immediately loaded. Columns were centrifuged for an additional

10

one minute at 120g. Collected fractions can either be stored up to 8 days at room temperature or directly tested in the TRβ CALUX bioassay.
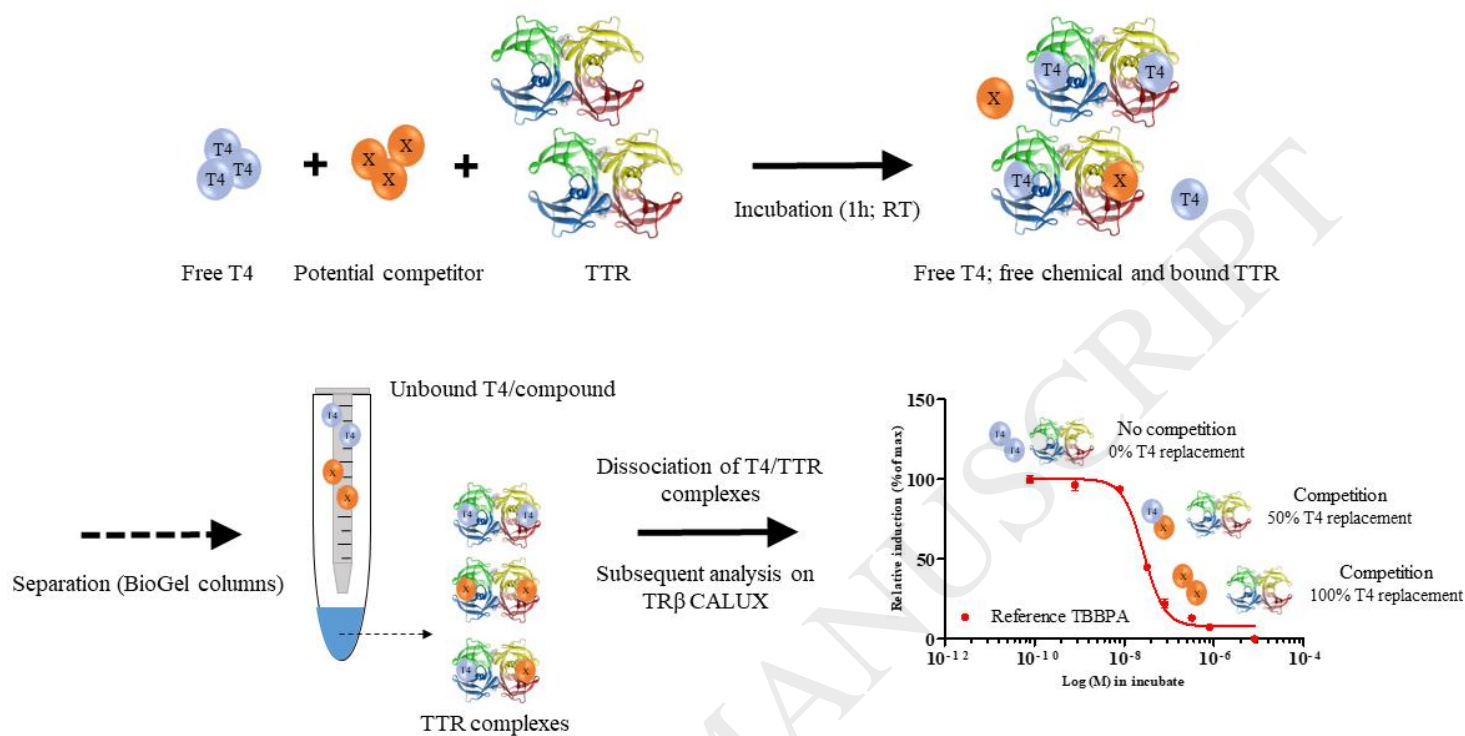

Fig. 2. *Diagram of the TTR-TRβ CALUX bioassay. Free T4, potential competitor chemical and TTR are incubated as a mixture for 1h at room temperature. Unbound T4 and free chemical molecules are separated from TTR complexes using BioGel columns. The bound fraction containing T4/TTR complexes is left at room temperature for few minutes in order to allowed T4 to dissociate prior to analysis on the TRβ CALUX bioassay.*

The method of TRβ CALUX testing was carried out essentially as described in 2.1 with only slight variations in the exposure method. In short, exposure medium was prepared by adding 140µL of TTR-T4 mixture to 500µL of phenol red-free DMEM/F12 medium supplemented with non-essential amino acids (NEAA) and P/S. Additionally, quality control was included to each plate by filling one triplicate with 200µL of 8e-6M T4 at a final DMSO concentration of 0.1%. All data points were carried out in triplicate. After 24±2-hour incubation, the exposure medium was removed and cells were lysed using 10µL/well triton-lysis buffer. Afterwards, the luciferase activity in cellular lysates was measured with a luminometer (Berthold) as described above.

Each TTR-binding assay procedure included the solvent DMSO, in which the reference compound, competing chemicals and sample extracts were dissolved, as a solvent control. For normal testing, one dilution series of the reference compound TBBPA was added to each plate as a quality control for the proper functioning of the assay. For each compound, a second negative control including TTR and the assessed competitor, without the addition of T4, was included to the procedure. This step allowed to evaluate potential direct interactions between the tested compound and TRβ.

2.3 Compound selection and evaluation guidelines

The evaluation of the bioassays was conducted following the Organisation for Economic Co-operation and Development (OECD) guidelines [19]. According to OECD requirements intra- and inter-laboratory studies have to be performed. In this paper, only the intra-laboratory phase was conducted. As in the foreseeable future, TRβ CALUX bioassays will be evaluated side by side with other thyroid-relevant endpoints such as thyroperoxydase (TPO), TBG and deiodinase activities as a part of a larger study organized by the European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM). The intra-laboratory evaluation described in this paper required the generation of three lists of compounds chosen for their properties to interact or not with the TRβ and/or TTR. While the selection was mainly built on literature review, we selected few additional chemicals from our own internal database.

For intra-laboratory assessment of the TRβ CALUX bioassay (agonistic mode), an initial set of eighteen chemicals were chosen (See Annex 2, Table 1). There are very few compounds referred in literature for their agonistic interaction with TRβ besides the four well known TRb

agonists (T3, T4, 3,3'5,5'-tetraidothyroacetic acid TETRAC and TRIAC). Hence, along with these chemicals, we decided to also include two compounds from our internal screening that showed a weak response on the TRβ (2-acetamidofluorene 2-AAF and all trans-retinoic acid atRA). To complete this set we incorporated 10 non-active chemicals, 5 were derived from a literature review (1-850, amiodarone, bisphenol A (BPA), endosulfan and TBBPA) and 5 from our own database (aflatoxin B1, dieldrin, methoxyacetic acid, valproic acid and vinclozolin). T2-toxin and hydroquinone were also analyzed on the TRβ CALUX bioassay but not included in the performance calculations as no prior data were reported at the time of the study.

A list of fifteen chemicals were established to test the reliability of the antagonistic TRb CALUX assay (See Annex 2, Table 2). Nine antagonistic compounds were selected from literature (1-850, 4-nonyphenol, BPA, deoxynivalenol, dibutylphthalate, endosulfan, pinoresinol, TBBPA and zearalenone). We also included the mycotoxin T2-toxin due to its antagonistic characteristics found in previous unpublished results obtained in our laboratory. Four chemicals were chosen for their non-active properties (aflatoxin B1, amiodarone, dronedarone and T3). We decided to include the RXR ligand at-RA to our study to assess its potential activity on the TRβ.

To test the TTR-TRβ CALUX bioassay, eleven compounds were assessed for their interference with the T4-TTR binding. Nine compounds were selected based on published information on their capacities to interact with either TRβ or TTR (4-nonyphenol, BPA, diethylstilbestrol (DES), pentabromophenol (PBP), pentachlorophenol (PCP), perfluorooctanoic acid (PFOA), perfluorooctane sulfonic acid (PFOS), TBBPA and tetrachlorobisphenol A (TCBPA)). Alachlor is known to be inactive and was chosen from literature. T2-toxin was included based on results obtained during an internal screening.

13

The evaluation procedure included a pre-screen step followed by at least two independent comprehensive measurements (biological replicate = 3). The general objectives of a pre-screen are to determine the solubility of test chemicals in both DMSO and assay medium and assess their potential cytotoxicity towards U2OS cells. Therefore, during this pilot phase, all concentration tested in the (anti-)TRβ CALUX bioassays were also assessed on the Cytotox CALUX® bioassay that can be used to assess non-specific CALUX assay interferences, e.g. caused by cytotoxicity [2,20]. In short the Cytotox CALUX bioassay uses U2OS cell line which constitutively express luciferase, the addition of cytotoxic compound to the cells would consequently result in a reduction in light production quantifiable with a luminometer. For the pre-screen, 8 serial dilutions of each compound were made from the highest soluble concentration using 10-step dilutions. In order to obtain a proper full dose response curve, the highest concentration tested should not be cytotoxic (cytotoxicity ≤ 20%). If one or several dilutions induced cytotoxicity, the test was repeated using lower concentrations (Annex 1. Table 1-3). Results of the pre-screen study were used to refine the concentration ranges of the test compounds to be used for the comprehensive studies. Unlike the pre-screen phase, comprehensive measurements were based on 3-step dilutions prepared from the highest concentration of each chemical not showing cytotoxicity. In case the results of the comprehensive tests were divergent to each other, a third measurement was added to the study increasing the biological replicates from 3 to 4. Each dilution of the test chemicals including the reference compounds were tested in triplicate on the same 96-well plate.

2.4 Data handling and acceptance criteria

Luciferase activity per well was expressed as Relative Light Units (RLUs) and corrected for background using solvent control measurements (pure DMSO). The maximum signal response elicited by the reference compound was set to 100% (full receptor activation). Subsequently, all registered RLU produced by the test compounds or samples were expressed in % of maximum response of the reference compound. The statistical software package GraphPad Prism 5.0 was used to fit transformed data (non-linear regression, variable slope, 4 parameters and robust fit). Compounds were scored positive if a minimum of two consecutive concentrations showed an increase of at least 10% (PC_{10}, agonist mode) or a decrease in TRβ activation of more than 20% (PC_{80}, antagonist mode) of the maximal effect of the reference compound. Depending on the selected mode of the TRβ CALUX bioassay, either EC_{50} or IC_{50} were evaluated using the same software package. EC_{50} refers to the maximal induced effect of the compound tested. For the antagonist procedure, 50% maximal inhibitory concentration (IC_{50}) was evaluated for each chemical and compared with available data in literature, if available. EC_{50} and IC_{50} can be used to assess and rank the potencies of tested chemicals. For agonistic studies, the limit of detection (LOD; average (solvent blank) +3*SD (solvent blank))) and the limit of quantitation (LOQ; average (solvent blank) +10*SD (solvent blank))) were also determined for all plates based on the standard deviation of the solvent blank DMSO. Z-factor was systematically calculated for the reference compound according to the following formula (1). Z-factor values should be above 0.6 to fit the acceptance criteria.

(1) $$\text{Z-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}.$$

For the evaluation of intra-laboratory assessment results, the coefficient of variance (%CV) and reproducibility (%VC_R) were calculated. The intra-laboratory coefficient of variance was determined based on the average log (EC_{50}) and log (PC_{10}) (agonist), and log (IC_{50}) and log (PC_{80}) (antagonist) derived from both comprehensive test runs. In addition, the intra-laboratory reproducibility variation coefficient was determined based on the log (EC_{50}) and log (PC_{10}) (agonist), and log (IC_{50}) and log (PC_{80}) (antagonist) derived from both comprehensive test runs. The reproducibility is the variation in measurements taken by a single/multiple persons or instruments on the same item at different time-points. Reproducibility was calculated according to formula (2).

$$(2) \qquad \% VC_R = \sqrt{\frac{\sum_{i=1}^{n}\left(\frac{x_{i1}-x_{i2}}{0.5(x_{i1}-x_{i2})}\right)^2}{2n}} \times 100\%$$

%VC_R = reproducibility variation coefficient

x_{i1} = measuring result i^{th} determination first observation

x_{i2} = measuring result i^{th} determination second observation

n = number of determination

The qualitative intra-laboratory concordance of classifications (active/non-active) was determined based on the results of two independent comprehensive tests under reproducibility conditions. From these results, overall accuracy, sensitivity and predictability of the methods were calculated and expressed as a percentage.

2.5 Demonstration study using water samples

2.5.1 Spiked water sample

16

In order to assess the TTR-TRβ CALUX assay applicability for environmental studies, HPLC-grade water was spiked with three pure compounds: DES, TCBPA and PBP, under controlled conditions. These three chemicals were defined as T4-competitors in the previous TTR-TRβ CALUX evaluation, allowing us to further calculate the relative potency resulting from their mixture. All compounds were dissolved in methanol (MeOH) to a stock concentration of $2.5E^{-3}M$. 120µL of this mixture was added to 250mL HPLC-grade water, resulting in a final concentration of $4.1E^{-7}M$ for each of the spike-compounds. A procedure blank, consisting of 250mL of water with MeOH, was also included into the experiment. Samples were extracted using Solid Phase Extraction (SPE) columns (OASIS HLB SPE cartridges 500mg 6 cc, Waters product no.: 186000115) conditioned with approximately 500ml of water and eluted with 10ml of MeOH followed by 10ml of acetonitrile. Fractions were pooled and evaporated until dryness under a gentle stream of nitrogen at 40°C. The final extracts were dissolved in 100µL of DMSO in order to keep the concentration of the spiking mixture and extract equal. The diluted extracts (dilution series 1-3-10-30-100-300-1000-3000-10000-30000x) as well as the neat spiking mixture were tested on the TTR-TRβ CALUX assay and analyzed following the procedure previously described in section 2.2.

2.5.2 Environmental water samples

Two effluent samples collected from a waste water treatment plant and one HPLC-water blank were extracted and analyzed following the previously described method (2.5.1). In short, 1L of each sample were extracted through SPE columns eluted with 10ml of MeOH and 10ml of acetonitrile. After evaporation, the final extracts were dissolved in 150µl of DMSO and used to prepare serial dilution (1-3-10-30-100x). A first range finding analysis was performed in the

17

TTR-TRβ CALUX bioassay to obtain the appropriate dose-response range of the sample extracts. Based on which the dilution series was adjusted (1-1.5-2-2.5-3-10x) and extracts were analyzed in the TTR-TRβ CALUX bioassay.

3. Results

3.1 Determination of thyroid receptor agonistic activity of compounds using TRβ CALUX bioassay

The sensitivity and responsiveness of the TRβ CALUX assay were assessed by measuring the signal of the reference compound T3 along with eighteen chemicals (see Annex 2. Table 1.). This set contained known TRβ agonists and chemicals that showed no affinity towards the TRβ receptor, also referred to as "non-responders". As a start, the performance and stability of the agonist test were determined by comparing EC_{50} values of the reference compound, T3 from the different measurements (Table 1; Fig. 3). The range in EC_{50} values was small, reflected by a %CV of 0.8%. Additionally, the LOD and LOQ were calculated for each measurements and compared. The variations in LOD and LOQ values were low (%CV≤1%) clearly indicating that the TRβ CALUX bioassay is a stable bioassay producing repeatable results.

Table 1.
Log EC50, PC10, PC50, LOD and LOQ values for the reference agonistic compound T3 on the TRβ CALUX bioassay.

Run	log EC_{50} (M)	log PC_{10} (M)	log PC_{50} (M)	log LOD (M)	log LOQ (M)
Pre-screen	-9.77	-10.28	-9.74	-10.99	-10.51
Comprehensive 1	-9.88	-10.37	-9.85	-10.84	-10.36
Comprehensive 2	-9.92	-10.43	-9.92	-10.80	-10.32
Average	-9.86	-10.36	-9.83	-10.88	-10.40
%CV	0.8%	0.7%	0.9%	0.9%	1.0%

Average represents the average values and %CV corresponds to the calculated coefficient of variation for three measurements.
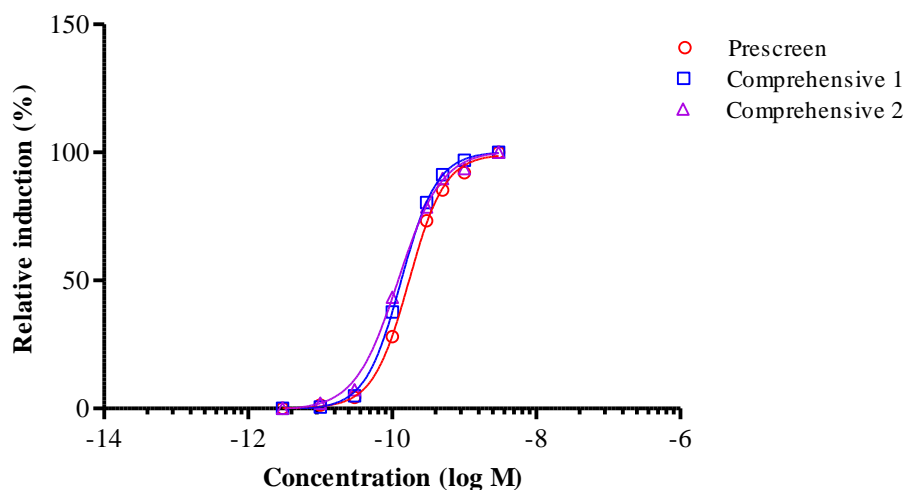
Fig.3. *Dose-response curves obtained with the reference compound T3, analyzed at least twice for the prescreen and each comprehensive. Each point represents the average of these measurements.*

Next, the panel of test compounds was tested on the TRβ CALUX assay by two different technicians. Out of these eighteen chemicals, six scored positive on the agonistic bioassay (Table 2). The TRβ CALUX bioassay gave a general z-factor of 0.89. Whereas T3, T4, TRIAC and TETRAC provided similar full dose-response curves, both at-RA and 2-AAF showed differences in shape and lower activity resulting in an absence of PC_{50} value (Fig. 4). All the natural THs, as well as their analogues TRIAC and TETRAC (T3 and T4-like, respectively), showed agonistic activities. Regarding this valuation, 2-AAF scored positive due to the fact of its low but still noticeable activity (Fig 4). Indeed, this compound showed a slight partial agonistic response on the TRβ CALUX bioassay matching preset OECD scoring criteria of at least two consecutive points above 10% T3's activity to be considered as an active chemical. Following the same criteria, at-RA was also scored positive in the bioassay. Whereas 2-AAF showed no cytotoxicity at high concentration, at-RA was reported as cytotoxic at the highest concentration tested (i.e. 3.e-5M and higher) (data not shown). Although the activity was quite low both 2-AAF and at-RA were defined as activators of the TRβ. From the initial list, twelve compounds

(1-850, aflatoxin B1, amiodarone, methoxyacetic acid, BPA, dieldrin, endosulfan, hydroqui-none, T2-toxin, TBBPA, valproic acid and vinclozolin) scored negative on the agonist TRβ CALUX bioassay (data not shown).

Table 2.
Log EC50, PC10 and PC50 values for positive expected and tested compounds in agonist TRβ CALUX bioassay.

		log EC$_{50}$ (M)	log PC$_{10}$ (M)	log PC$_{50}$ (M)	Relative potency (PC$_{10}$)	Measured activity	Reported activity
T3	avg	-9.44	-10.13	-9.48	1.0	active	active
	%CV	0.80%	1.60%	0.80%			
2-AAF	avg	-6.15	-5.54	n.a.	2.8	active	unknown
	%CV	0.1%	3.6%	n.a.			
at-RA	avg	-6.26	-6.25	n.a.	5.9E-1	active	unknown
	%CV	0.3%	0.4%	n.a.			
T4	avg	-9.01	-9.75	-9.05	5.8	active	active
	%CV	0.4%	0.6%	0.1%			
TRIAC	avg	-10.8	-11.6	-10.9	3.5E-1	active	active
	%CV	0.5%	0.7%	0.3%			
TETRAC	avg	-9.30	-9.73	-9.02	2.2E+1	active	active
	%CV	0.3%	1.2%	3.7%			

Average represents the average values and %CV corresponds to the calculated coefficient of variation for two comprehensive measurements.
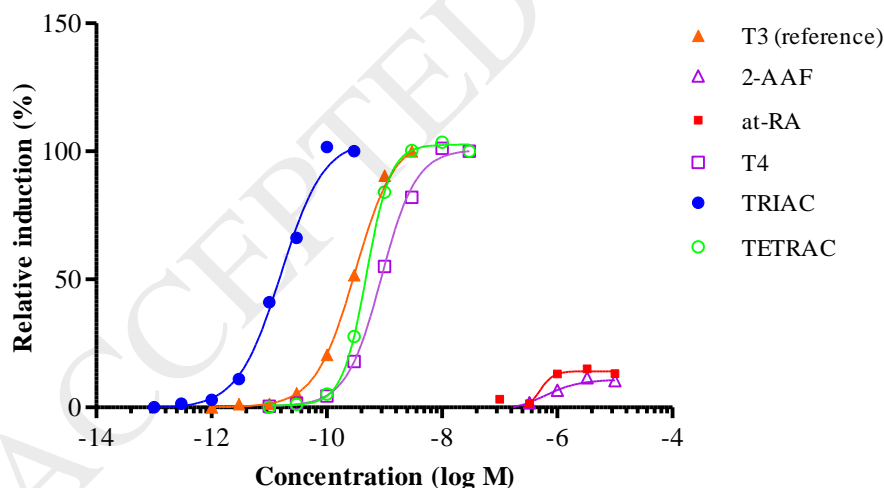

Fig. 4. *Dose-response curves of positive compounds obtained in the agonist mode of the TRβ CALUX bioassay.*

21

3.2 Determination of thyroid receptor antagonistic activity of pure compounds using TRβ CALUX bioassay

The accuracy and reliability of the antagonistic mode of the TRβ CALUX bioassay were investigated using the reference compound deoxynivalenol along with 15 pure chemicals (see Annex 2. Table 2.). Deoxynivalenol was first assessed alone in three separate experiments performed by at least two technicians. Both prescreen and comprehensive tests gave similar dose-response curves, resulting in low variation between the calculated log IC_{50} (%CV = 1.4%) (Table 3. Fig. 5). The repeatability of the method was reflected by an overall %CV $\leq$ 4.1% while the calculated reproducibility %VC_R was even lower (=1.35%). These finding confirm the stability of the antagonistic TRβ CALUX bioassay in generating reproducible results.

Table 3.
Log IC50, PC50, PC80 values for deoxynivalenol in the antagonist mode of the TRβ CALUX bioassay.

Run	log IC_{50} (M)	log PC_{50} (M)	log PC_{80} (M)
Pre-screen	-6.28	-6.29	-6.68
Comprehensive 1	-6.14	-6.15	-6.58
Comprehensive 2	-6.14	-6.18	-6.18
Average	-6.18	-6.21	-6.48
%CV	1.3%	1.2%	4.1%

Average represents the average values and %CV corresponds to the calculated coefficient of variation for three measurements.
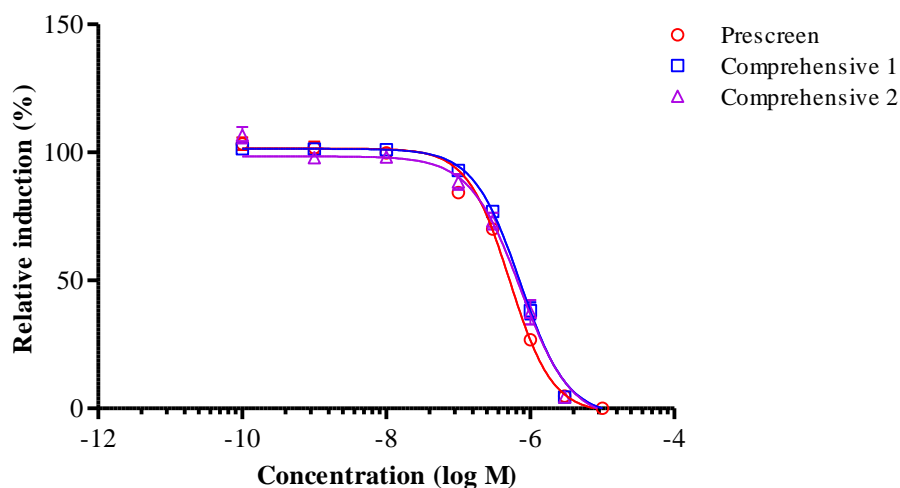
Fig. 5. *Dose-response curves of deoxynivalenol obtained in the antagonist mode of the TRβ CALUX bioassay. The reference compound was analyzed at least twice for the prescreen and each comprehensive. Each point represents the average of these measurements.*

Fifteen pre-selected chemicals were tested in the TRβ CALUX bioassay in order to determine their thyroid receptor antagonistic potential. Nine chemicals showed antagonistic activity with different potency and shape of dose-response curves (Table 4, Fig. 6.a.b.). None of the tested compounds were found to be cytotoxic in the assessed concentration range indicating that the decrease in activity represents antagonistic activity. 1-850, at-RA, amiodarone, BPA and zearalenone all scored positive but did not give a full-dose response curve as their higher concentration did not induce full antagonism. Alongside with 4-nonyphenol and deoxynivalenol, TBBPA showed a full dose-response curve indicating the complete inhibition of the TRβ receptor activation. The mycotoxin T2 showed activity in the anti-TRβ CALUX bioassay which was higher in $logIC_{50}$ than any in the compound set. Six compounds (aflatoxin B1, dibutylphthalate, dronedarone, endosulfan, pinoresinol and T3) did not show activity nor fit the pre-set criteria of the bioassay.

As a quality criteria, %CV was calculated for each compound and an average z-factor was given (0.72). All chemicals showed good repeatability in their results and thereby showed a good stability of the antagonistic TRβ CALUX bioassay ($0 \leq \%CV \leq 3.5\%$).

Table 4.
Log IC50, PC80 and PC50 values for positive expected and tested compounds in the antagonist mode of the TRβ CALUX bioassay.

		Log IC$_{50}$ (M)	Log PC$_{80}$ (M)	Log PC$_{50}$ (M)	Relative potency (PC$_{80}$)	Measured activity	Reported activity
deoxynivalenol	avg	-6.22	-6.54	-6.21	1	active	active
	%CV	0.60%	0.70%	3.50%			
1-850	avg	-5.02	-5.19	-4.93	4.50E-02	active	active
	%CV	1.00%	0.20%	0.30%			
4-nonylphenol	avg	-5.45	-5.63	-5.45	1.20E-01	active	active
	%CV	1.00%	2.70%	1.60%			
at-RA	avg	-4.75	-4.96	-4.75	2.60E-02	active	unknown
	%CV	1.10%	0.20%	3.50%			
amiodarone	avg	-5.59	-5.95	-5.59	2.60E-01	active	active
	%CV	2.30%	0.70%	3.50%			
BPA	avg	-4.96	-4.73	-4.45	1.50E-02	active	active
	%CV	0.50%	1.70%	0.00%			
TBBPA	avg	-6.07	-6.62	-6.15	1.2	active	active
	%CV	1.00%	0.70%	3.50%			
zearalenone	avg	-4.51	-4.8	-4.51	1.80E-02	active	active
	%CV	0.30%	1.10%	0.40%			
T2-toxin	avg	-8.47	-10.38	-10.5	6.90E+03	active	unknown
	%CV	0.00%	0.10%	0.00%			

Avg represents the average and %CV corresponds to coefficient of variation for two comprehensives.
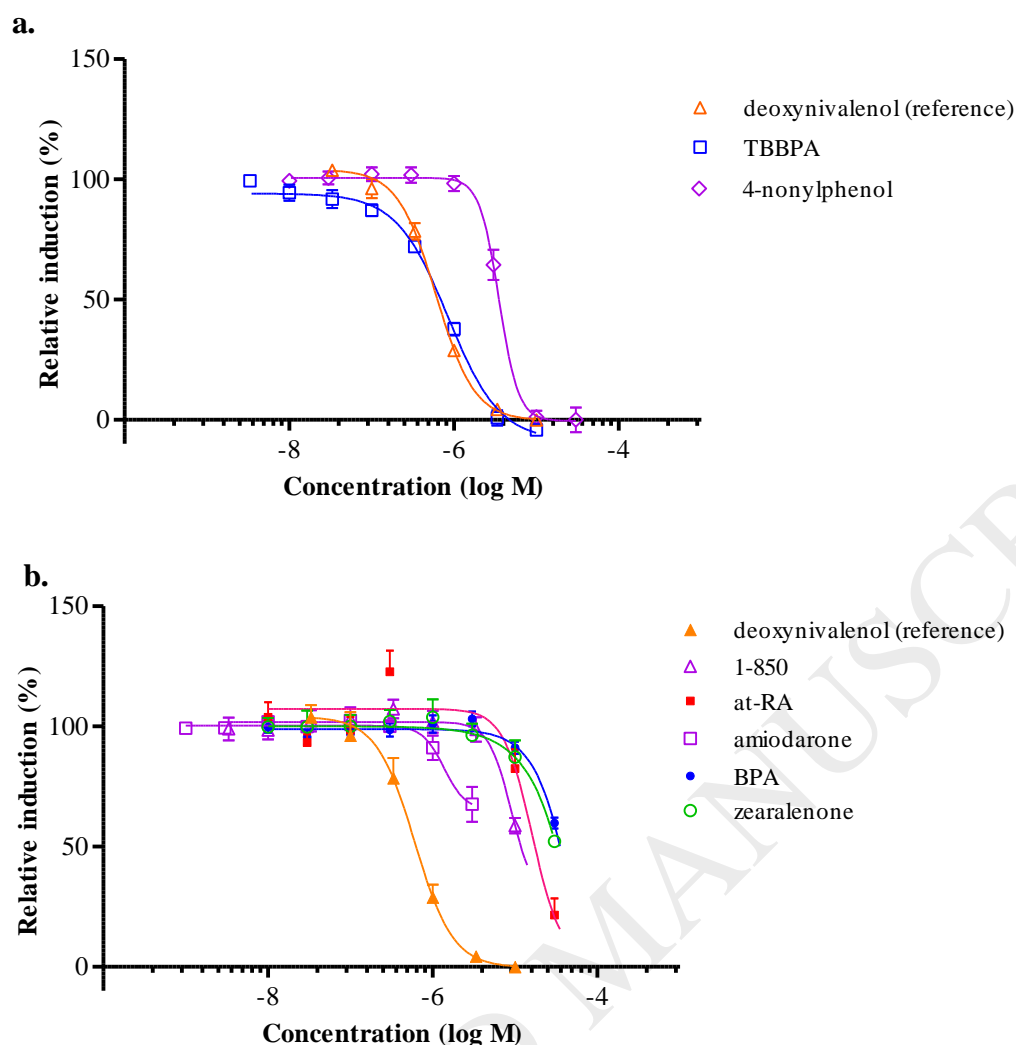
Fig. 6.a.b. *Representative dose-responsive curves of tested chemicals obtained using the antagonist mode of the TRβ CALUX bioassay. Each data point is the mean of at least two measurements.*

3.3 Evaluation of the TTR-TRβ CALUX bioassay

The test design of the evaluation of the TTR-TRβ CALUX bioassay was based on eleven pure compounds selected on their abilities to interact with TTR according to the literature (see Annex 2. Table 3.). As a start, the performance of the reference compound TBBPA was investigated in three independent experiments performed by at least two different technicians. The shape of the dose-response curves were similar between the individuals experiments, giving comparable log IC_{50} and PC_{80} (-7.60M±0.10 and -8.01M±0.9, respectively) (Table 5; Fig. 7.).

25

These results indicate the stability of the TTR bioassay when used in combination with the TRβ

CALUX.

Table 5.
Log IC50, PC50 and PC80 values for TBBPA reference compound measured in the TTR-TRβ CALUX bioassay.

Run	log IC$_{50}$ (M)	log PC$_{50}$ (M)	log PC$_{80}$ (M)
Pre-screen	-7.66	-7.66	-8.10
Comprehensive 1	-7.77	-7.64	-7.92
Comprehensive 2	-7.57	-7.59	-8.0
Average	-7.67	-7.63	-8.01
%CV	1.3%	0.5%	1.1%

Average represents the average values and %CV corresponds to the calculated coefficient of variation for three measurements.
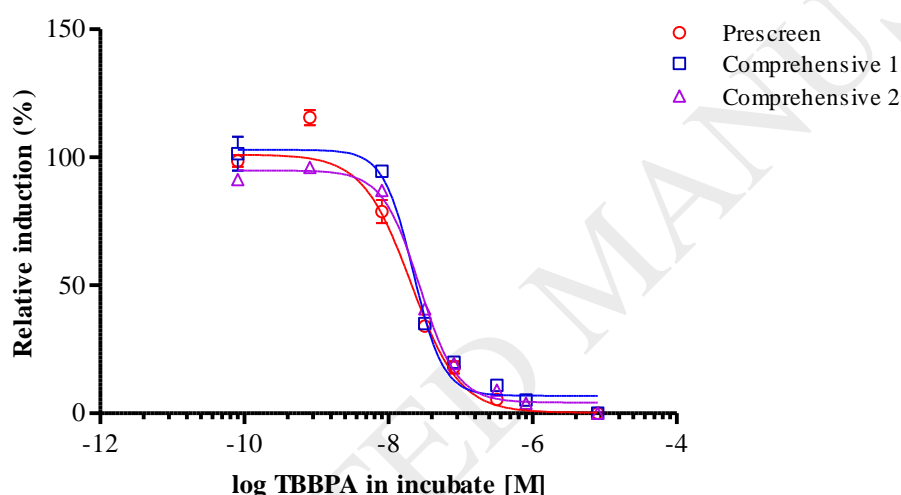

Fig. 7. *Dose-response curves obtained with the reference compound TBBPA in TTR-TRβ CALUX. TBBPA was analyzed at least twice for the prescreen and each comprehensive. Each point represents the average of these measurements.*

In addition, a panel of potential T4-TTR binding competition positive chemicals was as-

sessed following the same procedure. Associated TTR-binding potencies were expressed as log

concentration at 50% inhibition for T4 (Table 6). A general z-factor value of 0.74 was calcu-

lated. For each compound, %CV were calculated for logIC$_{50}$, logPC$_{80}$ and logPC$_{50}$. All test

chemicals (4-nonyphenol, BPA, DES, PBP, PCP, PFOA, PFOS, T2-toxin, TBBPA and

TCBPA) with the exception of alachlor, showed binding inhibition responses and were reported

26

as competitors for T4-TTR binding (Fig. 8). BPA demonstrated capacity to compete with T4 for binding TTR, however no IC_{50} could be calculated due to the fact that this compound did not give a full dose-response curve (data not shown). Out of eleven compounds tested, ten showed T4-competing properties with a stronger effect from perfluorinated chemicals PFOA and PFOS and the reference chemical TBBPA.

Table 6.
Log IC50, PC80 and PC50 values for positive expected and tested compounds in TTR-TRβ CALUX bioassay.

	Run	IC50	PC80	PC50	Relative potency (PC80)	Measured activity	Reported activity
TBBPA	avg	-7.81	-8.06	-7.79	1	active	active
	%CV	0.50%	0.30%	0.10%			
4-nonylphenol	avg	-4.12	-4.54	-4.23	3.00E-04	active	active
	%CV	3.70%	17.30%	9.90%			
BPA	avg		-4.27	-3.32	1.60E-04	active	active
	%CV		5.60%	5.10%			
DES	avg	-6.68	-7.03	-6.67	9.30E-02	active	active
	%CV	1.50%	2.00%	0.70%			
PBP	avg	-6.59	-6.9	-6.61	6.90E-02	active	active
	%CV	0.50%	3.30%	1.50%			
PCP	avg	-4.47	-4.79	-4.47	5.40E-04	active	active
	%CV	0.30%	1.30%	0.20%			
PFOA	avg	-7.5	-7.74	-7.41	4.80E-01	active	active
	%CV	1.30%	1.10%	2.20%			
PFOS	avg	-7.22	-7.45	-7.15	2.50E-01	active	active
	%CV	0.80%	0.50%	0.40%			
T2-toxin	avg	-6.0	-6.08	-5.91	1.00E-02	active	unknown
	%CV	0.50%	1.20%	0.50%			
TCBPA	avg	-6.77	-7.03	-6.73	9.30E-02	active	active
	%CV	1.80%	1.10%	1.40%			

Avg represents the average and %CV corresponds to coefficient of variation for two comprehensive measurements. For abbreviations refer to 2.4.
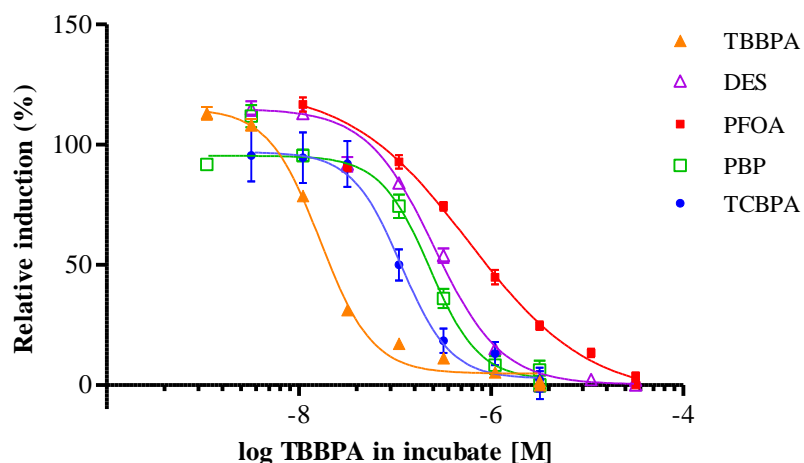
Fig. 8. *Dose-response curves of DES, PFOA, PBP, TBBPA and TBCPA in the TTR-TRβ CALUX. Each data point is the mean of at least two measurements.*

3.4 Demonstration study: water samples

3.4.1 Spiked water sample

Water (HPLC-grade) was spiked with a mixture of three compounds (DES, TCBPA and PBP) with dilution steps of 1, 3, 10, 30, 100, 300, 1000, 3000, 10000 and 30000. After the extraction procedure, extract obtained from spiked water was tested in the TTR-TRβ bioassay alongside the original spiking mixture (Fig. 9). So as to evaluate the quality of the SPE extraction, results obtained from the mixture itself and the spiked water sample were compared revealing a recovery value of 101.3% based on PC80 values. The global potency of the mixture was calculated and expressed as TBBPA equivalent, giving a value of 3.17E-05M. Overall, this experiment suggested that the TTR-TRβ bioassay used in combination with SPE-based extraction might be suitable for water sample analysis.
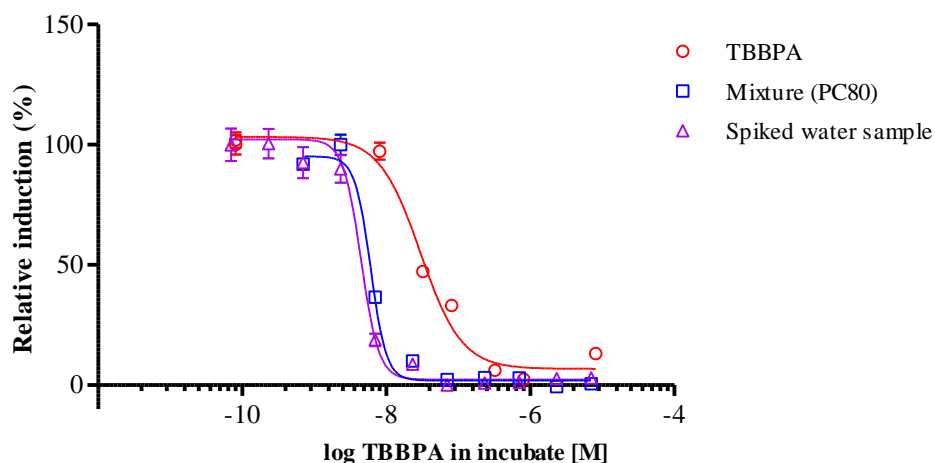
Fig. 9. *Dose-response curves from reference compound TBBPA, mixture and extract from spiked water sample tested in TTR-TRβ-CALUX. The mixture consisted of DES, PBP and TCBPA at a final individual concentration of 4.1e-7M.*

3.4.2 Environmental water samples

To determine the applicability of the TTR-TRβ bioassay for analysis of potential thyroid disrupting chemicals in environmental waters, two waste water treatment plant samples were analyzed. Along with these samples, HPLC-water was used as a procedure blank control and did not show any binding activity towards TTR. Both samples from the waste water treatment plant demonstrated a significant dose-dependent T4-TTR binding competition (Fig. 10).
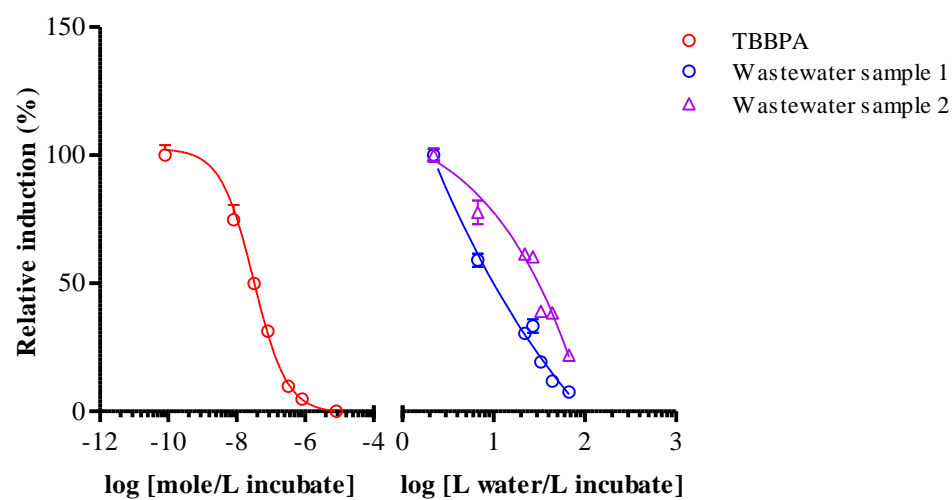
Fig. 10. *Dose-response relationships of wastewater and HPLC-water samples analyzed in TTR-TRβ CALUX bioassay.*

4. Discussion

Regarding the fact that no set of recommended chemicals for thyroid disruption was available at the time the study began (2015), a preliminary review was performed so as to generate a list of reference compounds suitable for the evaluation of each aspect of the thyroid panel. From literature, nine, twelve and ten compounds were chosen for assessing the agonistic, antagonistic TRβ CALUX and TTR-TRβ CALUX assays. Along with these reference chemicals, nine, three and one other compounds were respectively added to the sets. These compounds were considered of interest based on results obtained during an internal screening. While jointly evaluated on the panel, none of these additional chemicals were used in predictability nor accuracy calculations, which were strictly based on the compounds properly reported in literature. The evaluation of each thyroid-related bioassay relies on this preliminary work.

In 2016, Wegner *et al.* reviewed Tier 1 endocrine screening assays and generated a list of 34 reference chemicals to be used to assess thyroid system's alterations, however it is unclear which mechanism were involved in this disruption. In this paper, we focused on assessing the (ant)agonistic potency of compounds on one TH receptor using a reporter gene assay (TRβ CALUX) and one non-receptor based TH- response (TTR assay) the later one based on previous research of our group on competitive binding interference of compounds to TTR. Hence, it was crucial for our study to select compounds with available *in vitro* information regarding their potential receptor or transporter disrupting activities. Nevertheless, it would be of interest to analyze the set of chemicals provided by Wegner *et al.* in the future [21].

Within this study, we have developed a robust CALUX-based test method to assess the agonistic and antagonistic potency of chemicals towards the TRβ, as well as T4-TTR binding

competition potencies of chemicals. The evaluation of both agonistic and antagonistic TRβ

CALUX assays showed low variations in EC50 (≤0.8%) and IC50 (≤4.1%) measurements and

high z-factor values (0.89 and 0.72, respectively), indicating a good reproducibility of the methods.

The TRβ CALUX assay generated consistent results in comparison with existing data. The rank order of agonistic potency of active THs (T3 and T4) and chemicals with similar structure (TETRAC and TRIAC) was established as TRIAC>T3>TETRAC>T4, matching the results found in literature [15, 22]. Unlike these very active compounds (logEC50≥9.00), at-RA and 2-AAF showed a weak activity in the TRβ CALUX bioassay (logEC50=6.26 and 6.15, respectively). at-RA is a well-established ligand for retinoic X receptors (RXR), receptor able to act as heterodimer with TRβ [13, 14]. In our case, although a higher concentration of at-RA led to cytotoxicity (Annex 1. Table 1), we observed that this compound elicits an aberrant transactivation of the TRβ which may be the result of RXR binding rather than a direct interaction with TRβ itself. 2-AAF also showed two points above 10% in the TRβ bioassay suggesting at least a partial agonistic activity towards the TRβ. In a general way, even though at-RA and 2-AAF were slightly active on the TRβ CALUX bioassay, we were not able to classify them with certainty due to the lack of available data at the time of the study. Overall, besides these six compounds (2-AAF, at-RA, T3, T4, TETRAC and TRIAC), all chemicals tested scored negative when assessed for TRβ agonistic activity. Although only few reports have related TRβ agonists, most of these negatively scored compounds were clearly reported antagonists and thus, were expected to be inactive in the agonist TRβ bioassay. As examples, amiodarone, BPA and TBBPA were all reported TRβ antagonists in literature, as well as the well-established antagonist reference compound 1-850 [23-26]. No data were available regarding agonistic activity

towards TRβ of the aflatoxin B1, T2-toxin, hydroquinone or valproic acid preventing us from comparing our results with existing data. Whereas these compounds require a closer examination to be part of the evaluation, we still classified them as inactive for the agonistic TRβ bioassay.

Overall out of eighteen compounds tested, half (2-AAF, at-RA, aflatoxin B1, dieldrin, hydroquinone, methoxyacetic acid, T2-toxin, valproic acid and vinclozolin) were not used in predictivity and accuracy calculations due to the lack of reference data. For the compounds for which reference values were available, the agonist mode of the TRβ-CALUX bioassay showed a predictivity and accuracy of 100% (9/9). This study allowed the classification of yet unknown compounds, dieldrin, hydroquinone, methoxyacetic acid, T2-toxin, valproic acid and vinclozolin as non-responders and 2-AAF and at-RA as a weak TRβ agonists. It is not excluded that 2-AAF might act using a RXR-like dependent pathway, since it showed a similar dose-response curve as at-RA. Although we used a limit set of compounds, the results obtained from this intra-laboratory appraisal were promising and consistent with currently available data.

Outcomes acquired through the anti-TRβ CALUX evaluation showed a good correlation with existing data. In line with previously reported results, compound 1-850, BPA and TBBPA all scored positive in the antagonistic TRβ CALUX bioassay [23-25]. Alongside with the known antagonist deoxynivalenol, 4-nonylphenol and TBBPA showed a complete inhibition of the TRβ receptor activation resulting in a full dose response curve [27]. Surprisingly, at-RA was active on both agonist and antagonist modes of the TRβ CALUX bioassay. Amiodarone and zearalenone also showed TR antagonistic activity in the TRβ CALUX bioassay. Previous studies reported these two compounds as antagonist of the TRβ which matched our results [26, 28]. Interestingly, whereas mycotoxins like deoxynivalenol and zearalenone were already reported

33

as TRβ antagonist, no prior data about were found T2-toxin at the time of writing [27,28]. According to our results, T2-toxin is the most potent compound from the tested set (log IC50=8.47). Along with deoxynivalenol and zearalenone, mycotoxins seem to present strong endocrine disrupting activities targeting TRβ. In this way, mycotoxins including T2-toxin will be of particular interest for further studies. Contrary, dibutylphthalate, endosulfan, pinoresinol and T3 were all found not to be antagonistic in the TRβ-CALUX bioassay. Whereas dibutylphthalate, endosulfan and pinoresinol were reported as antagonists in former studies no activity was measured in our bioassay [28-30]. No data allowed us to confirm the antagonistic potential of these two compounds neither of at-RA, aflatoxin B1 nor T2-toxin. In this respect, these three compounds were excluded from predictivity calculations. Overall, the intra-laboratory evaluation demonstrated a good consistency with a global accuracy of 75% (9/12).

Used in combination with the TRβ CALUX, the TTR-binding assay reported consistent results based on comparison with existing data. In 1999, Meerts *et al.* classified TBBPA and TCBPA as T4 competitors in a radioactivity-based study, which entirely matched with our results [17]. In 2011, Cao *et al* assessed BPA using fluorescence probes and reported it as a weak competitor for TTR. In the present study, this compound also demonstrated capacity to compete with T4 at high concentration (1e-4M and higher) in the TTR-TRβ CALUX bioassay. As a whole, although we were not able to define an IC_{50} value, BPA matched our acceptance criteria of at least 20% diminution of TRβ activation and thus, was classified as a T4-TTR competitor [31]. 4-nonyphenol, DES, PBP and PCP also showed T4-TTR binding competition activity with a calculated IC50 in the same range as data found in prior TTR-related studies [17, 32-34]. Perfluorinated chemicals PFOA and PFOS showed a high potency to bind TTR, confirming

what has already been reported in prior study using a combination of TTR-binding and radio-labeled assays [35]. T2-toxin based measurements gave an IC50 of 1.00E-06M suggesting competitive properties towards T4, which has never been reported in literature at the time of the study. In contrast, alachlor is known not to compete for TTR-binding which was coherent with our results generated using the TTR-TRβ CALUX [15]. TTR seems to be an important target for compounds who interfere with the thyroid system, emphasizing the importance of developing test tool for assessing potential T4 competitors. Results obtained from our study demonstrated the reproducibility and stability of the TTR-TRβ CALUX while showing a good correlation with existing data.

The newly developed panel allows the assessement of yet unknown chemicals towards TRβ, as well as their possible interferences with T4/TTR binding. Using TRβ CALUX bioassay, a compound can be assessed for both agonistic and antagonistic activities. With the natural thyroid hormone T3 as a reference, an increase of at least 10% in TRβ activation allows to score the chemical as a TRβ agonist. Based on PC_{10} or EC_{50} values, it is possible to calculate the compound's relative potency expressed as a percentage of the reference's activity. A relative potency value above 1 suggests that the compound is more potent than the natural ligand while if below 1, a higher concentration is required to affect TRβ the same way as T3. This calculation provides an easy way to determine the potency of a new chemical, and to compare it with other compounds or existing data. In a similar way, antagonistic potential can be assessed in the TRβ CALUX bioassay. A decrease in TRβ activiation of more than 20% of the maximal effect of the reference compound deoxynivalenol suggests that the test compound presents antagonistic properties towads the receptor. Relative potency can be calculated based on either PC_{80} or IC_{50}

35

values, allowing to compare the unknown chemical pontecy to others. A new chemical can be also assessed for T4 competition regarding TTR binding using the TTR-TRβ CALUX bioassay. Two points below 80% of the reference TBBPA's activity implies a competitive activity from the unknown compound. Its potency can be calculated and expressed as a TBBPA's potency percentage using PC_{80} or IC_{50} values. Overall the present panel is able to generate data regarding direct activity towards TRβ, while giving informations about their potential capacity to bind TTR thus, to pass through the blood brain barrier and the uterine-placental wall.

The demonstration study using water samples as an example, showed a positive case regarding potential thyroid hormones disrupting compounds activity in waste water. No sign of TTR-binding potency were registered in HPLC water. An interesting follow-up of this case-study would be to collect waste water samples at different stages of treatment in a waste water treatment plant and analyze them in all variants of the panel in order to first determine the agonistic or antagonistic potential of these samples and secondly, evaluate the efficiency of various treatment procedures.

Overall, the *in vitro* TTR-TRβ CALUX assay appears to be a simple efficient method for assessing TTR binding disruption, giving a cheaper and safer alternative to other currently available bioassays mostly using radioactivity. This system was added to the (anti-)TRβ CALUX set so as to develop a mini test panel to assess the thyroid disruptive potential of various compounds at multiple levels. Also, the proposed battery can be performed alongside with the AR- and ER- CALUX bioassays in order to cover a variety of endpoints related to EATS (Estrogens Androgens Thyroid and Steroidogenesis) based-endocrine disruption.

Conflict of interest

The authors declare no conflict of interest.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Annex 1

Table 1.
Cytotoxicity results of test compounds for TRβ CALUX evaluation, agonistic mode.

Compound	Minimum (M)	Maximum (M)	Cytotoxicity (M)
1-850	1.0E-12	1.0E-05	≥1.0E-05
2-AAF	1.0E-12	1.0E-05	-
aflatoxin b1	3.0E-12	3.0E-05	-
amiodarone	3.0E-13	3.0E-06	-
at-RA	3.0E-12	3.0E-05	≥3.0E-05
BPA	3.0E-12	3.0E-05	-
dieldrin	3.0E-12	3.0E-05	-
endosulfan	3.0E-12	3.0E-05	-
hydroquinone	3.0E-12	3.0E-05	-
methoxyacetic acid	3.0E-12	3.0E-05	-
T2-toxin	1.0E-01	1.0E-04	≥1E-7
T3	1.0E-12	1.0E-05	-
T4	1.0E-12	1.0E-05	-
TBBPA	1.0E-12	1.0E-05	≥1.0E-05
TETRAC	1.0E-12	1.0E-05	-
TRIAC	1.0E-11	1.0E-04	≥1.0E-04
valproic acid	1.0E-12	1.0E-05	-
vinclozolin	1.0E-12	1.0E-05	-

Concentration range and cytotoxicity results for all test compounds analyzed in the TRβ CALUX. Cytoxicity was determined by Cytotox CALUX assay, background control and/or visual observation.

Table 2.
Cytotoxicity results of test compounds for TRβ CALUX evaluation, antagonistic mode.

Compound	Minimum (M)	Maximum (M)	Cytotoxicity (M)
1-850	1.0E-12	1.0E-05	-
4-nonylphenol	3.0E-12	3.0E-05	≥3.0E-05
aflatoxin b1	3.0E-12	3.0E-05	-
amiodarone	3.0E-12	3.0E-06	-
at-RA	3.0E-12	3.0E-05	-
bisphenol A	3.0E-12	3.0E-05	-
deoxynivalenol	1.0E-09	3.0E-06	-
dibutylphthalate	3.0E-12	3.0E-05	-
dronedarone	1.0E-12	1.0E-05	≥1.0E-05
endosulfan	3.0E-12	3.0E-05	≥3e-6
pinoresinol	3.0E-12	3.0E-05	-
T2-toxin	1.0E-11	1.0E-04	≥1e-5
T3	1.0E-12	1.0E-05	-
TBBPA	1.0E-12	1.0E-05	-
zearalenone	1.0E-11	1.0E-04	≥1e-5

Concentration range and cytotoxicity results for all test compounds analyzed in the anti-TRβ CALUX. Cytoxicitiy was determined by Cytotox CALUX assay, background control and/or visual observation.

Table 3.
Cytotoxicity results of test compounds for TTR-TRβ CALUX evaluation.

Compound	Minimum (M)	Maximum (M)	Cytotoxicity (M)
4-nonyphenol	1.1E-07	3.2E-04	-
alachlor	1.1E-07	3.2E-04	-
BPA	1.1E-06	3.2E-03	-
DES	1.1E-08	3.2E-05	-
PBP	1.1E-09	3.2E-06	-
PCP	1.1E-07	3.2E-04	-
PFOA	1.1E-08	3.2E-05	-
PFOS	1.1E-10	3.2E-06	-
TCBPA	1.1E-09	3.2E-06	-
TBBPA	1.1E-09	3.2E-06	-
t2-toxin	1.1E-09	3.2E-06	-

Concentration range and cytotoxicity results for all test compounds analyzed in the TTRTRβ CALUX. Cytoxicity was determined by background control and visual observation.

Annex 2

Table 1.
Selection of active and non-active compounds for TRβ CALUX evaluation, agonistic mode.

Compound	CAS number	Reported activity	Reference
1-850	251310-57-3	Non-active	Schapira *et al.,* 2003 [1]
2-AAF	53-96-3	Active	BDS internal database
aflatoxin B1	1162-65-8	Non-active	BDS internal database
amiodarone	96027-74-6	Non-active	Drvota *et al.,* 1995 [2]
at-RA	302-79-4	Active	BDS internal database
BPA	80-05-7	Non-active	Sun *et al.,* 2009 [3]
dieldrin	60-57-1	Non-active	BDS internal database
endosulfan	959-98-8	Non-active	ToxCast [4]
hydroquinone	123-31-9	Unknown	BDS internal database
methoxyacetic acid	625-45-6	Non-active	BDS internal database
T2-toxin	21259-20-1	Unknown	BDS internal database
T3	5714-08-9	Active	Cheek *et al.,* 1999 [5]
T4	51-48-9	Active	Cheek *et al.,* 1999
TBBPA	79-94-7	Non-active	Sun *et al.,* 2009
TETRAC	67-30-1	Active	Cheek *et al.,* 1999
TRIAC	51-24-1	Active	Cheek *et al.,* 1999
valproic acid	99-66-1	Non-active	BDS internal database
vinclozolin	50471-44-8	Non-active	BDS internal database

All compounds were selected based on either available data, prior intra-laboratory studies or chemical properties. In italics, the reference compound T3. Compounds referred as "BDS internal database" will not be used for predictivity calculations. Report to 2.3 for chemicals abbreviation. All compounds were purchased from Sigma Aldrich with the exception of 2-AAF and 1-850 which were obtained from Ultra Scientific and Cayman, respectively.

Table 2.
Selection of active and non-active compounds for TRβ CALUX evaluation, antagonistic mode.

Compound	CAS number	Reported activity	Reference
1-850	251310-57-3	Active	Schapira *et al.,* 2003
4-nonylphenol	104-40-5	Active	ToxCast [5]
aflatoxin B1	1162-65-8	Non-active	BDS internal database
amiodarone	96027-74-6	Non-active	Drvota *et al.,* 1995
At-RA	302-79-4	Unknown	BDS internal database
BPA	80-05-7	Active	Sun *et al.,* 2009
deoxynivalenol	4330-21-6	Active	Demaegdt *et al.,* 2016 [5]
dibutylphthalate	84-74-2	Active	Shen *et al.,* 2009 2016 [7]
dronedarone	141626-36-0	Non-active	Droggrell & Hancox, 2004 2016 [8]
endosulfan	959-98-8	Active	ToxCast
pinoresinol	487-36-5	Active	Ogungbe *et al.,* 2014 2016 [9]
T2-toxin	21259-20-1	Active	BDS internal database
T3	5714-08-9	Non-active	Cheek *et al.,* 1999
TBBPA	79-94-7	Active	Sun *et al.,* 2009
zearalenone	17924-92-4	Active	Kiss *et al.,* 2018 [10]

All compounds were selected based on either available data, prior intra-laboratory studies or chemical properties. In italics, the reference compound deoxynivalenol. Compounds referred as "BDS internal database" will not be used for predictivity calculations. Report to 2.3 for chemicals abbreviation. All compounds were purchased from Sigma Aldrich with the exception of 1-850 which was obtained from Cayman.

Table 3.
Selection of active and non-active compounds for TTR-TRβ CALUX evaluation.

Compound	CAS number	Reported activity	Reference
4-nonyphenol	25154-52-3	Active	Simon *et al.,* 2013 [11]
alachlor	15972-60-8	Non-active	Cheek *et al.,* 1999
BPA	80-05-7	Active	Cao *et al.,* 2011 [12]
DES	56-53-1	Active	Ishihara *et al.,* 2003 [13]
PBP	608-71-9	Active	Meerts *et al.,* 2000 [14]
PCP	87-86-5	Active	Van den Berg, 1990 [15]
PFOA	335-67-1	Active	Weiss *et al.,* 2009 [16]
PFOS	1763-23-1	Active	Weiss *et al.,* 2009
T2-toxin	21259-20-1	Unknown	BDS internal database
TBBPA	79-94-7	Active	Meerts *et al.,* 2000
TCBPA	79-95-8	Active	Meerts *et al.,* 2000

All compounds were selected based on either available data, prior intra-laboratory studies or chemical properties. In italics, the reference compound TBBPA. Compounds referred as "BDS internal database" will not be used for predictivity calculations. Report to 2.3 for chemicals abbreviation. All compounds were purchased from Sigma Aldrich with the exception of PFOS which was obtained from Chemika.

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