DEVELOPMENT AND VALIDATION OF *IN VITRO* BIOASSAYS FOR THYROID HORMONE RECEPTOR MEDIATED ENDOCRINE DISRUPTION

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DEVELOPMENT AND VALIDATION OF *IN VITRO* BIOASSAYS FOR THYROID HORMONE RECEPTOR MEDIATED ENDOCRINE DISRUPTION

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For my family and Liliana

Thank you for always believing in me

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CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

In the last decades, endocrine disruption from exposure to environmental contaminants has been driven by the considerable work performed on the estrogenic system. Nonetheless, the endocrine system is not restricted to the female sex hormone axis and several other hormone-driven signaling systems (androgens, progestogens, glucocorticoids, retinoids, thyroid hormones, etc.) play crucial roles in the maintenance of critical processes such as homeostasis (He et al., 2010), sexual development (Li and Kim, 2004), metabolism (Wiegratz and Kuhl, 2006), growth (Wightman et al., 2002) and behavior (Mani and Oyola, 2012) throughout the different life-stages of numerous species. It is now clear that other branches of the endocrine system may also be affected upon exposure to environmental pollutants (Zoeller, 2007; Jugan et al., 2010), although considerably less information is available on the effects of chemicals on these other endocrine systems. This thesis focuses on the disruption of the thyroid hormone system, a key player in the regulation of critical physiological processes such as growth (Cabello and Wrutniak, 1989), differentiation (Tata, 1993), energy metabolism (Cheng et al., 2010), temperature control (Ribeiro, 2008), brain development and function (Bernal, 2007; Horn and Heuer, 2010; Warner and Mittag, 2012), sexual behaviour and fertility (Wagner et al., 2008), heart rate (Danzi and Klein, 2012) and other biochemical communication systems of the overall endocrine system (De Vito et al., 2011). Disruption of the thyroid hormone system by foreign compounds is associated with the increasing incidence of hormone-related cancers and developmental defects, such that new European legislation (REACH) demands extensive testing of newly marketed compounds. The aim of this thesis was to develop and validate functional *in vitro* bioassays for thyroid hormone receptor-mediated toxicity, focusing on thyroid hormone receptor interaction.

THYROID HORMONE SYSTEM

The thyroid hormones (TH), triiodothyronine (T_3) and thyroxine (T_4) (**Figure 1**), are tyrosine-based hormones produced by the thyroid gland, a butterfly-shaped gland which in humans is located in front of the larynx just below the Adams apple.

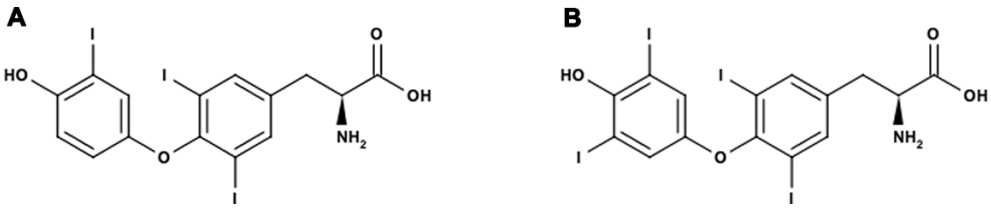

Figure 1. Chemical structures of the thyroid hormones. A) 3,3′,5-triiodo-L-thyronine (triiodothyronine, T_3) and B) 3,3′,5,5′-tetraiodo-L- thyronine (thyroxine, T_4).

Low circulating TH levels are detected by the hypothalamus, that responds by releasing thyrotropin-releasing hormone (TRH). The released TRH, stimulates the pituitary gland to produce thyrotropin (also known as thyroid-stimulating hormone, TSH). In turn, TSH stimulates the thyroid to produce TH until levels in the blood return to normal. Circulating TH exerts a negative feedback control over the hypothalamus and pituitary, eventually controlling the release of TRH from the hypothalamus and TSH from the pituitary gland. This hormone system that involves the hypothalamus, pituitary, and thyroid gland, is known as the hypothalamus-pituitary-thyroid (HPT) axis (Zoeller et al., 2007) (**Figure 2**) and regulates TH synthesis by a negative feedback regulatory system.

Thyroid hormones have low water-solubility, and therefore they are chaperoned by special TH protein carriers; transthyretin (TTR), thyroxin binding globulin (TBG) and albumin, and distributed in this bound form virtually all over the body. Uptake in cells of the various tissues proceeds by a process previously assumed as simple diffusion (Robbins and Rall, 1960), although these hormones may rather be taken up by cells via an energy dependent (adenosine triphosphate, ATP) carrier-mediated transport (Hennemann et al., 2001). Following uptake, TH are translocated to the nucleus or may interact with non-specific cytosolic binding proteins (CTHBPs). Organic ion transport proteins (OATPs) (Hagenbuch, 2007), L-type amino acid

transporters (LAT1 and LAT2) (Taylor and Ritchie, 2007), and members of the monocarboxylate transporter (MCT) family have also been identified as TH membrane transporters (Halestrap, 2012).

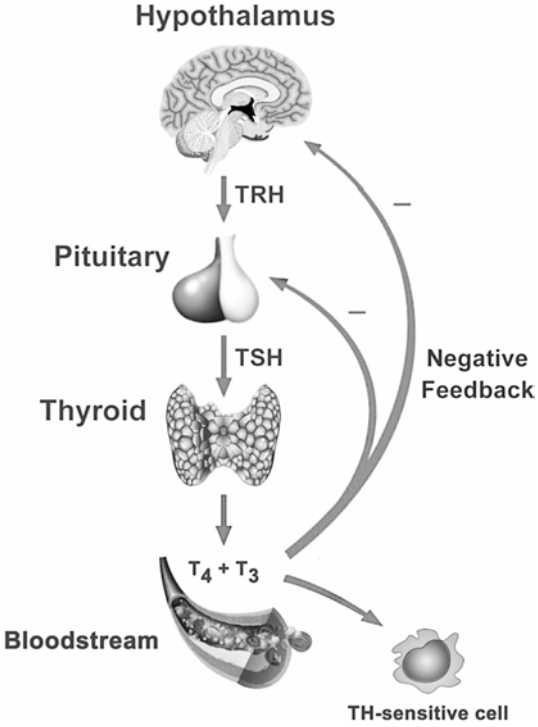

Figure 2. The Hypothalamic-Pituitary-Thyroid axis, including the roles of thyrotropin releasing hormone (TRH), thyroid stimulating hormone (TSH), thyroxine (T_4) and triiodothyronine (T_3). Other forms of thyroid hormones are not included (e.g. T_2 and rT_3). Minus indicates a negative feedback loop. Reproduced with modifications from (Boas et al., 2006).

The thyroid gland secretes mostly T_4 that, in target cells, is converted by the so-called outer ring deiodinases that remove one of thyroxine's four iodine atoms converting the hormone into the highly active T_3. Most of the physiological effects induced by TH are mediated via the interaction of the active form T_3 with specific ligand-dependent transcription factors, the thyroid hormone receptors (TRs). Thyroid hormone receptors are type II receptors that belong to the large super family of nuclear

hormone receptors (Ribeiro et al., 1995) including the steroid, vitamin D and retinoic acid receptors and are virtually expressed in all cells. A cell surface thyroid hormone receptor (integrin alphavbeta3, $\alpha_V\beta_3$) has also been identified and is known for the less studied nongenomic action of the TH (Davis et al., 2011). Integrin binds TH near the Arg-Gly-Asp (RGD) recognition site, which propels conformational changes that transduce the signal into the mitogen-activated protein kinase (MAPK) activity via phospholipase C and PKC (Davis et al., 2000). This enables T_4 to activate complex intracellular signal transduction cascades, independently of the TRs.

Thyroid hormone receptors are encoded by two different genes: TRα (NR1A1) and TRβ (NR1A2), in humans on chromosomes 17 and 3, respectively, and several isoforms are generated through alternative splicing (Lazar, 1993). TRα1 and TRα2 are expressed predominantly in the central nervous system (CNS), but the TRα2 isoform is unable to bind T_3, possibly exerting a dominant negative effect on TRα1 and TRβ (Katz and Lazar, 1993). Instead, TRβ1 is mostly prevalent in the liver and kidney and TRβ2 in the pituitary and hypothalamus (Yen, 2001). Each TR isoform displays tissue and developmental stage differences in the level of its expression, suggesting an important role in mediating tissue specific effects (Bradley et al., 1989; Furlow and Neff, 2006). The receptors can interact with DNA as monomers (TR:DNA), homodimers (TR/TR:DNA) or as heterodimers with the retinoid X receptor (TR/RXR:DNA), the latter being the major functional form of the receptor. In the absence of T_3 the receptors recruit cofactors such as the nuclear receptor corepressor (N-CoR) or the silencing mediator for retinoid and thyroid receptors (SMRT) that modify nearby chromatin to inhibit target gene transcription. Upon binding of T_3, the corepressors are released and the TR/RXR heterodimer complex now recruits coactivators such as the steroid receptor coactivator-1 (SRC-1). This complex forces an open configuration on the adjacent chromatin and facilitates RNA polymerase II recruitment, thereby activating transcription (Oñate et al., 1995; Koenig, 1998), by interacting with DNA sequences usually in the vicinity of target genes known as thyroid hormone response elements (TRE) (Desvergne, 1994). Response elements consist of one or more short copies of a DNA sequence that matches a consensus motif: 5' AGGTCA 3'. Naturally occurring thyroid hormone response elements include at least two copies of this motif frequently separated by 4 base pairs arranged

as direct repeats (DR-4 elements), palindromes (e.g. AGGTCATGACCT) or inverted repeats. The TR DNA-binding domain contains two sets of four cysteine residues forming the so-called zinc fingers, which confer specific binding to the response elements (Aranda and Pascual, 2001).

DISRUPTION OF THE THYROID HORMONE SYSTEM

Exposure to a wide range of natural and manufactured chemicals has shown to disrupt the TH signaling as seen in early studies reporting unusual thyroid gland development in many species of birds and fish (Colborn, 2002). Research with animal models (rodent, amphibian and zebrafish) and studies of hypothyroidism, hyperthyroidism and other genetic diseases related to the HPT axis have also supported concerns about transient and persistent dysregulation of the TH system by compounds present in the environment.

The thyroid hormone system homeostasis is tightly controlled by a complex network of regulatory interactions, rendering it almost impossible to isolate a single mode of disruption, since this may occur through interactions with the several components of the HPT axis. For example, disruption can interfere with TH synthesis, storage and release of TH by the thyroid gland (Capen, 1997; Wolff, 1998), protein-bound TH distribution (Marchesini et al., 2008), cellular uptake (Chalmers et al., 1993), intracellular metabolism (Butt et al., 2011), TH deactivation (Morse et al., 1993), with membrane receptors (Sheng et al., 2012) and with the classical nuclear receptors, the latter being the main focus of this thesis.

THYROID HORMONE RECEPTOR-MEDIATED DISRUPTION

The transcriptional control of target genes by the TRs can be considered an important checkpoint in the complex network of regulatory mechanisms involving the thyroid hormone system, and the endpoint most directly related to altered cellular responses. Ultimately, changes in gene expression mediated by the TRs result in observable cellular phenotypes that are specific for a given cell type. For example, in response to T_3 the rat pituitary tumour GH3 cell line proliferates, T_3-treated primary cultures of cerebellar Purkinje cells show increased dendritic branching (Heuer and Mason, 2003; Ibhazehiebo et al., 2011), cardiomyocytes self-organize into three dimensional heart

muscle-like structures that alter their contractility properties (Khait and Birla, 2008) and primary liver cells show altered expression of metabolic enzymes (Attia et al., 2010). It should however be emphasized that a multitude of mechanisms are possible beyond direct binding to the receptors for disruption of the TH signalling. Compared to the estrogen receptor, interactions with the TRs appear to be restricted to chemicals with high structural resemblance to the natural hormones, due to the steric limitations of the receptor ligand-binding domain (LBD) (DeVito et al., 1999; Zoeller, 2005). As described above, upon ligand binding to the TRs corepressors are released and coactivators recruited, and chemicals have also been described to act via disruption of this interaction (Nguyen et al., 2002). Similarly, disruption of the TR/RXR receptor interaction, or direct interactions with the RXR LBD, may also affect a subset of important TH target genes in specific tissues (Castillo et al., 2004). Given the complexities of the transcriptional control regulated by these receptors, measurement of their activity in their native context is highly relevant for predicting any type of TH disruption. Detection of TH-like activity and the resulting biological effects can be facilitated by *in vitro* bioassays for TR-mediated effects.

IN VITRO ASSAYS FOR THYROID HORMONE RECEPTOR-MEDIATED ACTIVITY

Several *in vitro* bioassays have been developed to determine the ability of compounds or mixtures to interfere with the TR. Ligand binding assays typically involve measuring the displacement efficiency of radiolabeled T_3 bound to TRs overexpressed in bacteria or obtained from nuclear extracts of mammalian cells (Kitamura et al., 2002; Kitamura et al., 2005; You et al., 2006). The relative simplicity of these assays renders them quite attractive to determine direct interaction of chemicals with the TRs and since these are cell-free assays, there are no issues with cytotoxicity of the compound in question. However ligand-binding assays do not predict *in vivo* agonism or antagonism per se and the isolated receptors are not in their native environment where proper folding or stability might play a role. Coregulator assays use the interaction of domain peptides derived from nuclear receptor coactivators or corepressors to the TR LBD, with the recruitment or displacement of these labelled peptides followed by fluorescence (Lévy-Bimbot et al., 2012). Due to their potential to predict agonism or antagonism based on the pattern of peptide recruitment or

displacement these assays have been used for high-throughput screening of thousands of compounds (Johnson et al., 2011). However, they present disadvantages similar to those already outlined for the ligand binding assays. Furthermore, they appear to be relatively insensitive even to T_3 with high concentrations up to 10 μM often needed to induce a response. An alternative to the ligand binding assays can be found in so-called reporter gene assays (**Figure 3**), which can be either transient or stable transfection assays.

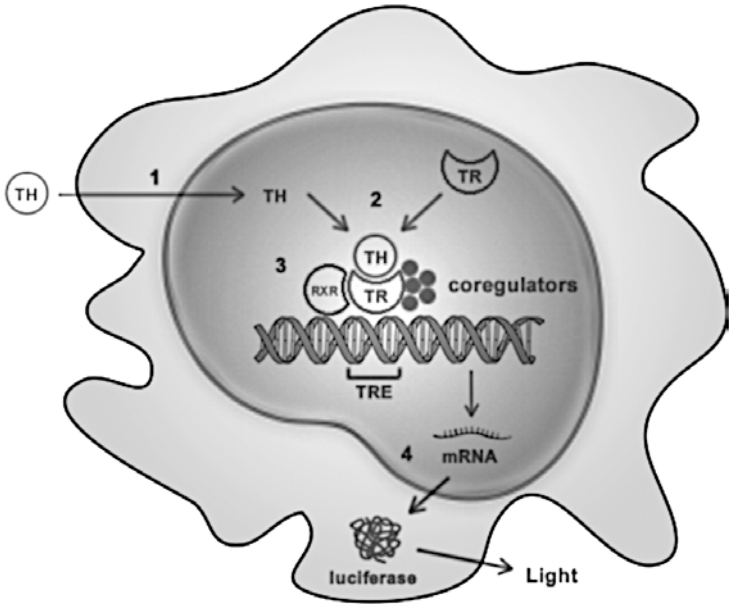

Figure 3. Schematic representation of a thyroid hormone receptor-dependent reporter gene cell line. 1) Thyroid hormones (TH) are taken up 2) and bind to the thyroid hormone receptor (TR) which may form a homodimer with another TR or a heterodimer with a retinoid X receptor (RXR). Corepressors are released, followed by 3) coactivators recruitment to the complex bound to a thyroid hormone responsive element (TRE) and triggering the 4) expression of the reporter protein (luciferase) that ultimately generate a detectable signal (light).

Transient transfection assays usually involve the co-transfection of a TRE-driven reporter gene in parallel with a TR-expressing vector if cells lack endogenous receptors. These assays can be fairly sensitive and reproducible and have been used to demonstrate the effect of compounds like bisphenol A (BPA), its halogenated

derivative tetrabromobisphenol A (TBBPA), polychlorinated biphenyls (PCBs), polybrominated diphenylethers (PBDEs) and their metabolites on the TR transcriptional activity (Zoeller, 2005). Despite their wide acceptance, flexibility, and promise, transient transfection assays are relatively expensive and not readily adaptable for high-throughput screening due to well-to-well variations across the plate, and the fact that the reporter gene is not fully bundled in chromatin like endogenous genes. Stable transfection assays circumvent many of the limitations of their transient counterparts. Furthermore, development of stable transfection assays in cell lines normally responsive to TH, as presented in this thesis, have multiple advantages for screening TR-active compounds. For example, such cells express endogenous, full-length receptors and a full cohort of the cellular machinery required for TH uptake and transcriptional control of target genes. Use of full length receptors also exploits the full range of interactions with a native response element configuration via the receptor's DNA binding domain, and with endogenous co-regulatory proteins via multiple sites in the amino and carboxytermini of the receptors, to allow regulation of a TRE-based reporter embedded in native chromatin.

THESIS SCOPE

This thesis focuses on the development and validation of functional *in vitro* bioassays for TR-mediated toxicity. This aim can be further specified as follows:

1. Development and validation of *in vitro* assays for TH disruption;
2. Identification of new TR agonists and antagonists;
3. Identification of novel TH target genes;
4. Elucidation of potential mechanisms of TR-mediated disruption;
5. Advise on whether predictions can be made based on *in vitro* studies for *in vivo* disruption of TH action.

In Chapter 1, background information on the topic is given, the aims are defined and a short outline of the thesis is presented.
Chapter 2 describes the development of the GH3.TRE-Luc assay using the rat pituitary cell line (GH3), which is also used in the proliferation based T-screen assay

(Hohenwarter et al., 1996; Gutleb et al., 2005; Schriks et al., 2006). The assay is based on endogenous TRs and proven to be highly sensitive and specific. For example, some compounds were identified that were agonists in the T-screen but not in the TR-Luc because they do not directly act via the TR.

The use and validation of this newly developed GH3.TRE-Luc assay in a quantitative high-throughput screening platform is described in Chapter 3. The assay proved to be quite robust, with excellent performance in an automated 1536-well plate format. The LOPAC and NTP 1408 libraries (Library of Pharmacologically Active Compounds (1281 chemicals) and National Toxicology Program library (1408 chemicals)) were screened and a number of potentially novel agonists and antagonists were identified.

In Chapter 4 expression levels of thyroid hormone signalling and responsive genes upon exposure to TH were revealed by microarray analysis. These data further support the validation of the GH3.TRE-Luc cell line as an *in vitro* model for TR-mediated disruption of endogenous target genes, and also contribute to the understanding of some of the molecular mechanisms triggered by selected TR disruptors.

The expression of the TRs is tissue and life-stage dependent, and therefore classification and quantification of TR-isoform specific transactivation allow a better translation of *in vitro* responses to expected *in vivo* effects (tissue specific effects). Chapter 5 describes the development of a stable TRα specific reporter gene cell line designated TRα.HeLa-Luc. The developed TRα.HeLa-Luc stable reporter gene line adds a new level of specificity, representing an improvement of the available tools for *in vitro* high-throughput assessment of TR subtype-specific activity of drugs and environmental pollutants.

In Chapter 6 concluding remarks as well as some future perspectives are presented on the feasibility to predict *in vivo* thyroid hormone system disruption based on a battery of *in vitro* assays.

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CHAPTER 2

DETECTION OF THYROID HORMONE RECEPTOR DISRUPTORS BY A NOVEL STABLE *IN VITRO* REPORTER GENE ASSAY

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ABSTRACT

A stable luciferase reporter gene assay was developed based on the thyroid hormone responsive rat pituitary tumor GH3 cell line that constitutively expresses both thyroid hormone receptor isoforms. Stable transfection of the pGL4CP-SV40-2xtaDR4 construct into the GH3 cells resulted in a highly sensitive cell line (GH3.TRE-Luc), which was further optimized into an assay that allowed the detection of triiodothyronine (T_3) and thyroxine (T_4) concentrations in the picomolar range after only 24 h of exposure. The greater than 20-fold induction of T_3 relative to the solvent control is illustrative of the high responsiveness of the system. The assay was validated by the quantification of the agonistic effect of the natural hormones (T_3 and T_4), the acetic acid derivatives of T_3 (triiodothyroaceticacid, or Triac) and T_4 (tetraiodothyroacetic acid, or Tetrac), hydroxy polybrominated diphenylethers (OH-PBDEs), hydroxy polychlorinated biphenyls (OH-PCBs) and the antagonistic action of sodium arsenite (NaAsO2). The putative antagonist amiodarone, bisphenol A (BPA) and its halogenated derivatives (TCBPA and TBBPA) for which effects reported in the literature are not consistent, showed comparable dose-response curves with a slight agonistic effect (5% of T_3-max) followed by a slight antagonistic effect. The magnitude and reproducibility of the responses to various compounds confirms this assay as a promising tool for the identification and quantification of specific thyroid hormone receptor disrupting potency of compounds.

INTRODUCTION

Certain environmental pollutants have been shown to display adverse effects on the normal function of the endocrine system (Harrison et al., 1997). In particular, adverse effects on the thyroid endocrine axis are very important to assess since the thyroid hormones, triiodothyronine and thyroxine (T_3 and T_4), play a crucial role in growth, development and energy homeostasis (Silva, 2001; Yen, 2001). Thyroid hormones induce their physiological effects through the thyroid hormone receptors (TRs) (Samuels and Tsai, 1974), which belong to the large super family of nuclear hormone receptors including the steroid, vitamin D and retinoic acid receptors (Beato et al., 1995). These receptors are ligand-dependent transcription factors able to interact with DNA sequences known as response elements, usually located in the vicinity of target genes (Desvergne, 1994). Several environmental contaminants and their metabolites, to which humans are routinely and involuntarily exposed, can disturb the thyroid hormone endocrine system (Crofton, 2008) at several different pre-receptor points of action: synthesis, transport, metabolism and cellular uptake. These different targets are the most widely studied for disruption to date (Brouwer et al., 1998; Marchesini et al., 2008; McKinney and Waller, 1994; Schmutzler et al., 2007). For instance, the polychlorinated biphenyls (PCBs) and more specifically the hydroxylated PCB-metabolites (OH-PCBs) that structurally mimic the thyroid hormones have both high affinity for thyroid hormone binding proteins and thyroid hormone metabolizing enzymes (Cheek et al., 1999; Marchesini et al., 2008; Meerts et al., 2002; Miyazaki et al., 2004; Schuur et al., 1998), although some studies have shown that these chemicals are also able to disturb thyroid hormone receptor-mediated gene transcription (Bogazzi et al., 2003; Miyazaki et al., 2008). The hydroxylated metabolites of the flame-retardants polybrominated diphenylethers (PBDEs) have been shown to interfere with the thyroid hormone system as well (Hamers et al., 2006, 2008). If these environmental pollutants act as thyroid hormone analogs (Zoeller, 2007) by binding to thyroid hormone receptors, they may directly disrupt normal thyroid hormone signaling in target cells.

Considering the large number of natural and manufactured chemicals that may

disrupt the thyroid hormone mode of action, EPA's endocrine disruption screening program (EDSP) requires Tier 1 screening information on estrogen-, androgen-, and thyroid-mediated endocrine effects. Currently the EDSP Tier 1 screening battery includes the amphibian metamorphosis assay (using the frog *Xenopus laevis*). A defined testing program as described by EPA does not apply in Europe; testing is carried out on a case-by-case basis. Under the regulatory system for chemicals controlled by the European Chemical Agency (ECHA), Registration, Evaluation and Authorization of Chemicals (REACH), it is stated that when a clear link between serious adverse effects and an endocrine mode of action can be established, the substance may be included in Annex XIV of substances subject to the authorization procedure. The guidance prepared by ECHA contains testing strategies, including evaluation of non-testing data, *in vitro* screening data and *in vivo* screening and testing data. However, *in vitro* screening assays as lower tier tests for identification of a potential thyroid hormone disrupting mode of action do not yet exist therefore this evaluation of chemicals is mainly based on the amphibian metamorphosis assay (Opitz et al., 2005), requiring a large number of test individuals. One key point of the new legislation is the reduction of vertebrate studies, which should only be used if no other suitable assays exist. Great effort has been put into the development of integrated and intelligent testing strategies for evaluation of the potential adverse effects of thyroid disrupting compounds, such as the use of (quantitative) structure–activity (QSAR) relationships as well as *in vitro* assays which are expected to provide useful information on the mechanism of action of many different chemicals. The extraordinarily high number of chemicals to be assessed within a few years (about 30,000 substances are currently marketed at volumes greater than 1 ton/year) places a premium on specific, functional and high-throughput *in vitro* screening systems. Although some promising assays have been developed for interference with thyroid hormone transport (Marchesini et al., 2008), to our knowledge, there are no rapid and specific tests enabling high-throughput detection of compounds directly activating or inhibiting the thyroid hormone receptor.

A number of rat pituitary tumor cell lines (GH1, GH3 and the derivatives GC and GH4C1) cloned from the same rat pituitary tumor (Tashjian, 1968), display an increased level of cell proliferation and growth hormone secretion in response to

physiologic levels of thyroid hormones (Hinkle, 1986; Samuels et al., 1973; Seo et al., 1977). Based on the thyroid hormone-induced proliferation of the rat pituitary tumor cell line GH3, the T-screen has been developed to study the interference of compounds with thyroid hormone action (Gutleb et al., 2005; Hohenwarter et al., 1996; Schriks et al., 2006). Although the T-screen has proven to successfully predict the effects of some thyroid hormone disrupting chemicals, the assay is relatively time consuming and not entirely specific since effects on cell proliferation through non-thyroid receptor-mediated mechanisms cannot be excluded.

This study describes the development and chemical validation of a stable thyroid hormone receptor specific reporter gene assay based on the GH3 cell line. The validation of the assay was performed using the natural hormones T_3 and T_4 and their metabolites Triac and Tetrac which are pharmaceutical structural analogs that have also been used in TH replacement therapy, all shown to be active in the T-screen (Gutleb et al., 2005). Amiodarone (Norman and Lavin, 1989) and sodium arsenite (Davey et al., 2008) were used as model compounds to demonstrate antagonistic behavior on the TR-mediated gene induction mechanism. As model toxic compounds we have chosen to evaluate chemicals that structurally resemble thyroid hormones: T_3 and T_4-like hydroxylated polybrominated diphenylethers (4-OH-BDE 69 and 4-OH-BDE 121, respectively) and the T_3-like hydroxylated polychlorinated biphenyls 4-OH-PCB 69 and 4-OH-PCB 106. In addition, bisphenol A (BPA), tetrabromobisphenol A (TBBPA) and tetrachlorobisphenol A (TCBPA) were also included because of the apparent conflicting reports on their thyroid hormone receptor-mediated activity in different *in vitro* assays (Kitamura et al., 2002, 2005b; Moriyama et al., 2002; Schriks et al., 2006).

Our results demonstrate that the newly developed GH3.TRE-Luc cell line is a promising tool for high-throughput *in vitro* screening, allowing simple, rapid, and specific testing of thyroid hormone receptor agonists and antagonists.

MATERIALS AND METHODS

Chemicals. All chemicals were of ≥98% purity unless stated otherwise. 3,3',5-triiodo-L-thyronine (T_3, CAS no. 6893-02-3), 3,3',5,5'-tetraiodo-L-thyronine (T_4, CAS no. 51-48-9), 3,5,3',5'-tetraiodothyroacetic acid (Tetrac, CAS no. 67-30-1), 3,5,3'-triiodothyroacetic acid (Triac, CAS no. 51-24-1), 2,2-bis(4-hydroxyphenyl)propane (BPA, CAS no. 80-05-7), 2,2-bis(4-hydroxy-3,5-dichlorophenyl)propane (TCBPA, CAS no. 79-95-8), 4,4'-propane-2,2-diylbis(2,6-dibromophenol) (TBBPA, CAS no. 79-94-7), 2-butyl-3-benzofuranyl-4-(2-(diethylamino)ethoxy)-3,5-diiodophenyl ketone hydrochloride (Amiodarone hydrochloride, CAS no. 19774-82-4) and sodium arsenite (CAS no. 7784-46-5) were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, Netherlands). T_3-like 4'-hydroxy-2,3',4,6-tetrabromodiphenylether (4-OH-BDE 69, CAS no. 218303-98-1) and the T_4-like 4'-hydroxy-2,3',4,5',6-pentabromodiphenylether (4-OH-BDE 121, CAS no. 91370-78-4) were kindly provided by Åke Bergman (Stockholm University, Sweden) and synthesized as described (Marsh et al., 1998). T_3-like 4-hydroxy-2',3,4',6'-tetrachlorobiphenyl (4-OH-PCB 69, CAS no. 189578-00-5) and T_3-like 4-hydroxy-2',3,3',4',5'-pentachlorobiphenyl (4-OH-PCB 106, CAS no. 192190-09-3) were obtained from AccuStandard (New Haven, USA). All compounds were dissolved in dimethylsulfoxide (DMSO, 99.9%; Acros Organics, Geel, Belgium) and kept at room temperature in the dark. **Figure 1** illustrates the chemical structure of the compounds used in this study.

Plasmids. The plasmids pBluescript SK- (Genbank Accession# X52324) and pCI-neo (Genbank Accession# U47120) were purchased from Promega (Madison, WI). The vectors pCS2-βgalactosidase and pCS-GFP3 were a kind gift from Dave Turner (University of Michigan, USA) and Enrique Amaya (University of Manchester, UK), respectively. The thyroid hormone receptor-regulated luciferase reporter plasmid pGL4CP-SV40-2xtaDR4 contains two thyroid hormone response elements upstream

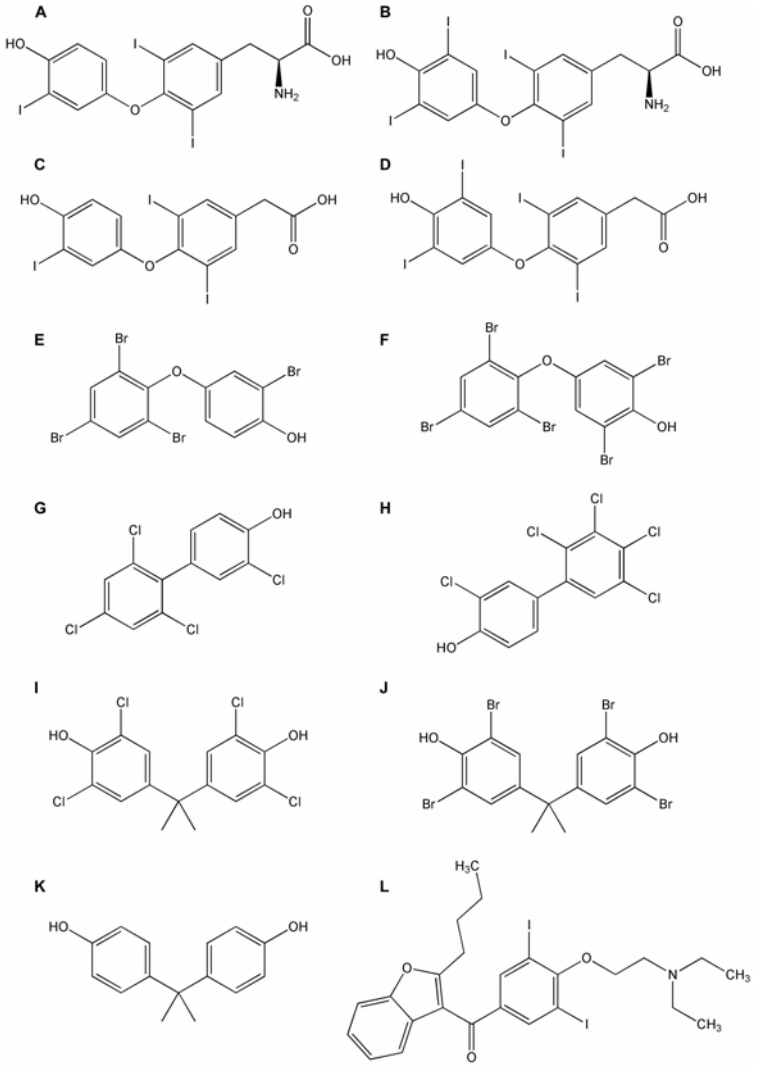

Figure 1. Chemical structures of A) 3,3′,5-triiodo-L-thyronine (T_3), B) 3,3′,5,5′-tetraiodo-L-thyronine (T_4), C) 3,5,3'-triiodothyroacetic acid (Triac), D) 3,5,3',5'-tetraiodothyroacetic acid (Tetrac), E) T_3-like 4′-hydroxy-2,3′,4,6-tetrabromodiphenylether (4-OH-BDE 69), F) T_4-like 4′-hydroxy-2,3′,4,5′,6-pentabromodiphenylether (4-OH-BDE 121), G) T3-like 4-hydroxy-2',3,4',6'-tetrachlorobiphenyl (4-OH-PCB 69), H) T_3-like 4-hydroxy-2',3,3',4',5'-pentachlorobiphenyl (4-OH-PCB 106), I) 2,2-bis(4-hydroxy-3,5-dichlorophenyl)propane (TCBPA), J) 4,4'-propane-2,2-diylbis(2,6-dibromophenol) (TBBPA), K) 2,2-bis(4-hydroxyphenyl)propane (BPA), L) 2-butyl-3-benzofuranyl-4-(2-(diethylamino)ethoxy)-3,5-diiodophenyl ketone hydrochloride (amiodarone hydrochloride).

of an SV40 minimal promoter driving expression of a modified firefly luciferase reporter. The SV40 minimal promoter was amplified by PCR from the pGL3-Promoter plasmid (Genbank Accession# U47298; Promega, Madison, WI) using the SV40-Up (gatcAGATCTCCTAGGAAGCTTtgcatctcaattagtcagcaaccatagtc) and SV40-Dn (aag cttttttgcaaaagcctaggcctc) primers, digested with Bgl II and ligated into the pGL4.12(luc2CP) vector (Genbank Accession# AY738224; Promega, Madison, WI) which was digested with HindIII, filled with DNA polymerase I Klenow fragment and redigested with Bgl II. The pGL4CP-SV40-2xtaDR4 plasmid was created by ligating a 2xtaDR4 adapter (2xtaDR4-Top: tcgagTAAGGTCATTTAAGGTCATTTAA GGTCATTTAAGGTCAa and 2xtaDR4-Btm: gatctTGACCTTAAATGACCTTAAATG ACCTTAAATGACCTTAc) that contains two tandem consensus thyroid response elements (direct repeats of the consensus AGGTCA halfsite sequence, separated by the tetranucleotide sequence TTTA) between the Xho I and Bgl II sites of pGL4CP-SV40. Sequence data for pGL4CP-SV40 and the pGL4CP-SV40-2xtaDR4 can be found at www.mic.ucdavis.edu/privalsky/Sequences/pGL4CPseries/pGL4_series.

Transfections. The rat pituitary tumor GH3 cells were routinely sub-cultured once a week in fresh 75 cm^2 culture flasks (Corning, Schiphol-Rijk, The Netherlands), in a humid atmosphere at 37°C and 95% air/5% CO_2 and in Dulbecco's Modified Eagle's medium/ Ham's F12 (DMEM:F12, Gibco, Paisley, Scotland) supplemented with 10% Fetal Calf Serum (FCS, Gibco, Paisley, Scotland). Twenty-four hours before transfection, GH3 cells were seeded into 12-well plates (Corning, Schiphol-Rijk, The Netherlands), at a density of 1.6×10^5 cells per well in regular growth medium. Transient transfections were performed with Lipofectamine 2000 according to the manufacturer's instruction (Invitrogen, Paisley, Scotland). Transfection optimization was performed with increasing amounts of DNA (100 ng pCS2-βgalactosidase, 100 ng pCS-GFP3 and 200 to 800 ng pBluescript SK-) and 2 to 8 µl of Lipofectamine 2000 (100 ng pCS2 cbgal, 100 ng pCS3 GFP and 600 ng pBluescript SK). Cells were then allowed to recover for 6 h after transfection in DMEM:F12+10% FCS for 48 h. Cells were then visually inspected under a microscope for GFP expression and harvested to measure β-galactosidase activity.

To create the stable cell line, cells were passaged 1:10 into fresh growth medium 24 h after transfection. Forty-eight hours post-transfection, standard growth medium was replaced with selective medium containing 320 μg/ml of Geneticin (Gibco, Paisley, Scotland).

Screening of GH3.TRE-Luc clones. Single Geneticin resistant colonies were harvested using cloning rings and genomic DNA was isolated with GenElute Mammalian Genomic DNA Purification Kit according to the manufacturer's instruction (Sigma-Aldrich Corp, St. Louis, MO, USA). A nested-PCR targeting a 480 bp fragment of the luc4CP reporter gene was performed with the following primers: sense1 5′-CTTCGTGACTTCCCATTTGC-3′; antisense 5′-GCCTCACCTACCTCCTTG CT-3′ and sense2 5′-ACTTGATCTGCGGCTTTCG-3′. The nested-PCR was carried out with a preliminary incubation step at 94°C for 10 min, 35 cycles of denaturation at 94°C for 60 s, annealing at 56.6°C/55.3°C for 30 s, extension at 72°C for 30 s then finally followed by an elongation step at 72°C for 10 min.
PCR positive clones were screened for T_3-induced luciferase activity in triplicate including the vehicle control. Twenty-four hours before plating, standard culture medium was replaced by serum free medium PCM as originally described (Sirbasku et al., 1991). Cells were plated at 100% confluency in white clear bottom 96-well microplate (PerkinElmer, Groningen, The Netherlands), exposed to T_3 for 24 h and luciferase activity was assayed with the Luciferase Assay System (Promega, Madison, WI, USA). Total protein content was assessed in parallel with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and used to normalize luciferase measurements.

Luciferase reporter gene assay using GH3.TRE-Luc cells line. GH3.TRE-Luc cells were seeded at 80% confluency in 75 cm^2 culture flasks (Corning, Schiphol-Rijk, The Netherlands) in regular growth medium. Twenty-four hours later, growth medium was replaced by PCM for an additional period of 24 h. Cells were then collected by scraping and seeded into 96-well plates at a density of 3×10^4 cells per well and incubated for 24 h in the presence (0.25 nM) or absence of T_3, with or without the indicated test chemical in DMSO. The DMSO concentration in 200 μl of exposure

medium was always the same for all exposures within an experiment and always kept ≤0.5% (v/v) to avoid cytotoxicity. A full T_3 standard curve comprising a concentration range from 0 to 1000 nM, was included on each exposure plate.

Cell viability in each well was determined by measuring total cellular metabolic activity using the reduction of resazurine to the fluorescent resorufin as previously described (O'Brien et al., 2000), (Schriks et al., 2006). Following the 24 h exposure described above, 8 µl of a 400 µM resazurine (Sigma-Aldrich Chemie B.V., Zwijndrecht, Netherlands) solution was added to each well. After four hours of incubation in the dark (37°C; 95% air/5% CO_2) fluorescence was measured at λex=530 nm and λem=590 nm (Milli-pore Cytofluor 2350 Fluorescence Measurement System). Luciferase activity was then measured on lysed cells in a microplate luminometer with two injectors (Thermo LabSystems luminoskan Ascent), as previously described (Murk et al., 1998). All exposures were performed in triplicate and every assay was repeated at least three times.

Data and statistical analysis. Results show the representative of three independent experiments. Data correspond to the mean ± standard deviation of triplicate measurements expressed as relative light units, normalized for viable cell number (RLU/RFU) if statistically significant differences were measured via the resazurine assay.

The dose-response curves were characterized by the half maximal effect concentration (EC_{50}) and the EC_{10}, the concentration needed to induce the same effect as 10% of the E_2-maximum induction. The relative potency (RP) of each compound was determined as the ratio between the EC_{50} of T_3 and the EC_{50} of the compound. When the maximum induction was less than 50% of the T_3-maximum, the RP was calculated with the respective EC_{10} value. Compounds are considered to be not active when the response is lower than the vehicle control plus 2× the standard deviation, which was defined as limit of detection (Veld et al, 2006).

Percentages of maximal luciferase induction for each test compound were calculated by setting luciferase response to solvent control (DMSO) as 0% and the maximum luciferase induction by 10 nM T_3 as 100%. The induction factor is calculated as the highest response divided by the response of the solvent control, after subtraction of

the plate background.

Statistical significance was tested by one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls test. Differences were considered significant at $p < 0.05$. Curve fitting, EC_{50}, EC_{10} and IC_{50} values were computed using GraphPad Prism version 5 for Mac OSX (GraphPad Software, San Diego California USA, www.graphpad.com).

RESULTS

Validation of the TRE based luciferase reporter gene assay with natural and synthetic thyroid hormones. All experimental conditions for culturing, transfecting, shifting to serum free media and measuring luciferase activity with the stable GH3 cells were thoroughly optimized before the final method for transfection and screening compounds was decided upon. For instance, transfection efficiency is optimal when transfecting GH3 cells with 2 µl/well of Lipofectamine 2000 and 800 ng of total DNA (**Figure 2**).

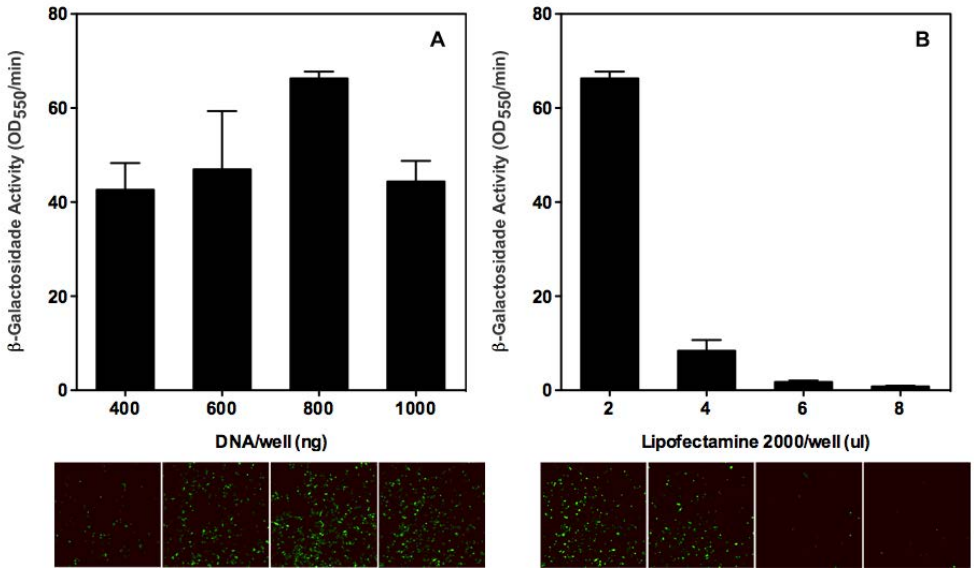

Figure 2. Results of transfection optimization prior to the development of stable clones. Transient transfection efficiency determined through visual inspection under the microscope of GFP-positive cells (representative microscopic fields of cells) and β-galactosidase activity (OD_{550}/min) measured 48 h after transfection with increasing amounts of A) DNA and B) Lipofectamine 2000. For details see materials and methods section.

When testing TR-mediated luciferase induction by compounds it is particularly important to use serum free PCM medium instead of culture medium supplemented

with stripped serum to avoid background induction by low levels of thyroid hormones that might still be in the serum (**Figure 3**).

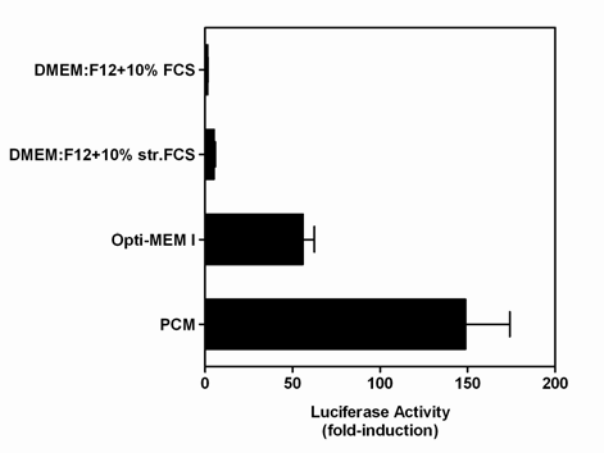

Figure 3. Effect of exposure medium on luciferase induction by 100 nM T_3 relative to solvent control (DMSO) in stably transfected GH3.TRE-Luc cells exposed for 24 h in 12-wells tissue culture plates. Exposure media tested: Dulbecco's Modified Eagle's medium / Ham's F12 (DMEM:F12) supplemented with either regular (FCS) or stripped 10% Fetal Calf Serum (str.FCS), reduced serum medium (Opti-MEM I) or serum-free medium (PCM). Results are mean ± SD of triplicates, corrected for viable cell numbers.

For quantification of cell proliferation the resazurine method was preferred as it proved to be more sensitive than total protein (**Figure 4A**). Also, 24 h of exposure to increasing concentrations of T_3 barely induced cell proliferation as opposed to 96 h exposure in the T-screen (**Figure 4B**).

GH3.TRE-Luc cells stably transfected with the expression vector pCI-neo and the reporter plasmid pGL4CP-SV40-2xtaDR4, were obtained by antibiotic selection and further tested to select lines with the best inducibility. The dose-dependent response of these cells to T_3 and T_4 is shown in **Figure 5A**. T_3 was almost 100 times more potent than T_4 with an EC_{50} of 0.1 ± 0.02 nM (n=3) and 9 nM ± 0.4 nM (n=3) respectively. The maximum T_3-induced effect was reached at 10 nM. T_4 reached its maximum induction at 100 nM, which is 90% of the T_3-maximum response. The average coefficient of variation (CV) of triplicate measurements is 5%.

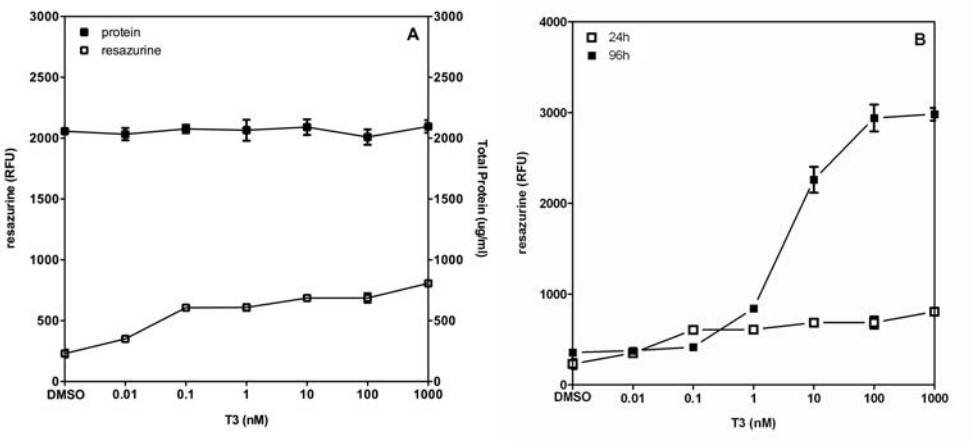

Figure 4. Effect of T_3 on GH3.TRE-Luc cells proliferation in serum free medium (PCM). A) Determined after 24 h by the resazurine method and expressed as relative fluorescence units (□, left axis), and measured by the BCA protein assay expressed as total protein (■, right axis). B) Determined with the resazurine method after 24 h (□) and 96 h (■). Error bars indicate SD of triplicate data points.

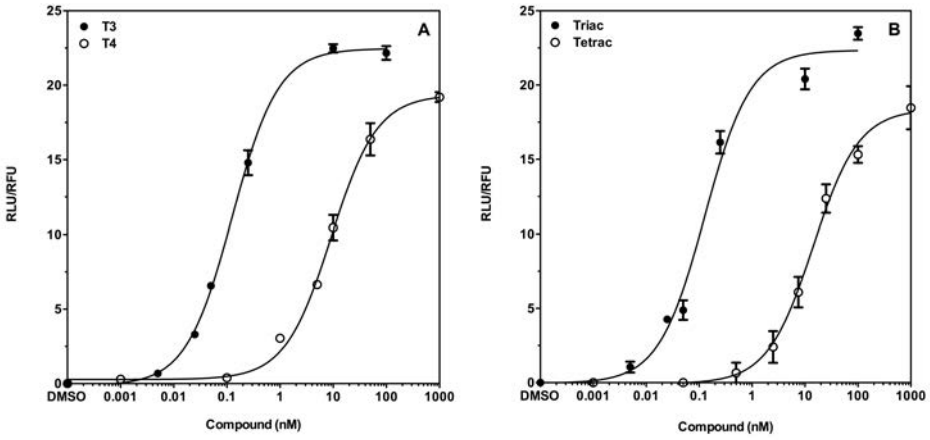

Figure 5. GH3.TRE-Luc induction after 24 h exposure to A) T_3 (●) and T_4 (○), B) Triac (●) and Tetrac (○). Results (mean ± SD of triplicate data points) are expressed as relative light units (RLU) for luciferase activity per relative fluorescence units (RFU) for normalization of viable cell number; absolute values for vehicle control (DMSO) from each plate were subtracted from values obtained from each ligand treated well.

The acetic acid derivative of T_3, Triac, was as effective as T_3 with an EC_{50} of 0.1 ± 0.04 nM (n=3) and inducing the same maximal luciferase activity at 100 nM (**Figure 5B**). Tetrac was approximately as effective as T_4, with an EC_{50} of 14.2 ± 0.8 nM (n=3) and a maximum response of approximately 80% of the T_3-maximum (**Figure 5B**). Taken together, these results demonstrate the sensitivity and robust inducibility of the system for detecting thyroid hormone receptor agonists.

Effects of compounds that antagonize TH action: amiodarone and sodium arsenite. Contrary to previous findings (Schriks et al., 2006), amiodarone did not antagonize the response induced by 0.25 nM of T_3 (**Figure 6A**). Amiodarone alone had no effect (data not shown), but at concentrations up to 1 μM the T_3-induced response is significantly increased. At higher concentrations, as the viability of the cells is reduced, the response decreases. Visible cytotoxicity occurs at 10 μM. However, consistent with other reports (Davey et al., 2008), sodium arsenite clearly inhibits luciferase activity induced by 0.25 nM of T_3 (**Figure 6B**). The IC_{50} was 3 μM and concentrations of sodium arsenite above 100 μM completely antagonize the T_3-induced luciferase activity without any signs of cytotoxicity upon visual inspection and as indicated by the resazurine assay.

Effects of environmental compounds in GH3.TRE-Luc cells. The T_3-like (4-OH-BDE 69) and T_4-like OH-BDEs (4-OH-BDE 121) were able to induce luciferase activity in the GH3.TRE.Luc cells (**Figure 7A**), although with approximately 10^4 times lower potency and 5 times lower maximal induction for the T_3-like OH-BDE when compared to T_3 (**Table 1**). In addition, the T_3-like OH-PCBs (4-OH-PCB 69 and 4-OH-PCB 106) clearly induced luciferase activity in a dose-related manner (**Figure 7B**), and their respective relative potencies and maximal induction are roughly in the same order of magnitude as the two OH-BDEs tested (**Table 1**).

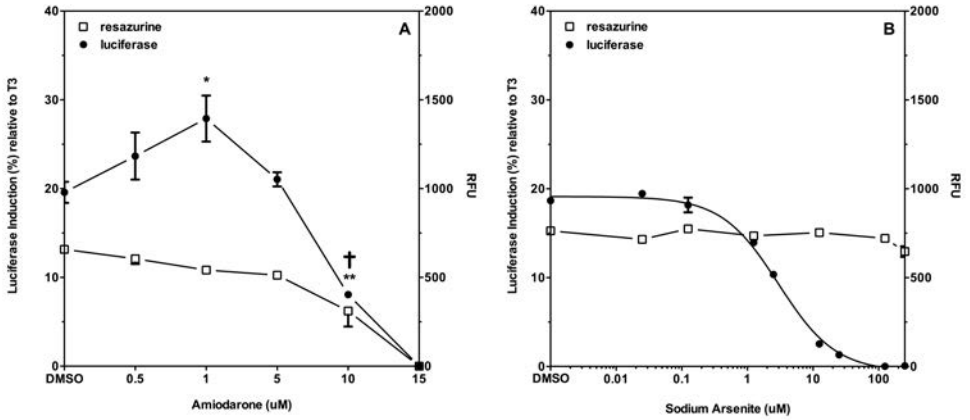

Figure 6. GH3.TRE-Luc induction after 24 h exposure, in the presence of 0.25 nM of T_3, to A) amiodarone and B) sodium arsenite. Luciferase activity (●, left axis) relative to T3 maximal induction (at 10 nM set at 100%, DMSO set at 0%) and the number of viable cells (□, right axis) expressed as relative fluorescence units (RFU). Error bars indicate SD of triplicate data points. *Significantly different from control (*$p < 0.05$, **$p < 0.01$). † = visible cytotoxicity.

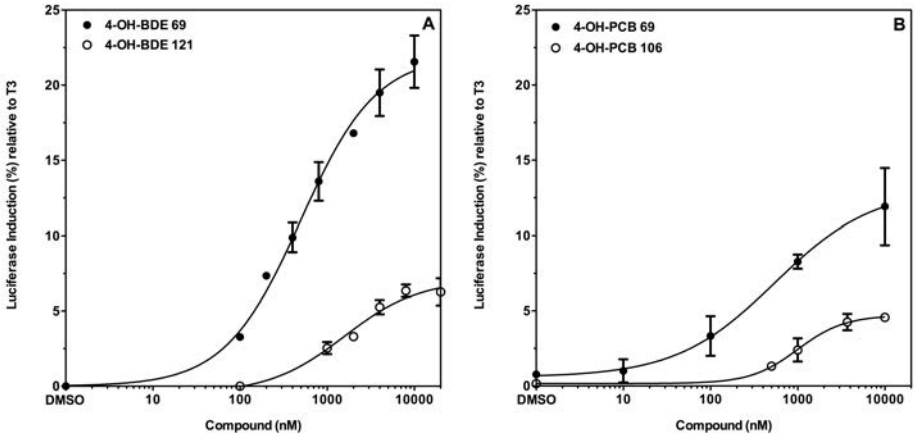

Figure 7. GH3.TRE-Luc induction after 24 h exposure to A) T_3-like 4-OH-BDE 69 (●) and T_4-like 4-OH-BDE 121 (○), B) T_3-like 4-OH-PCB 69 (●) and 4-OH-PCB 106 (○). Luciferase activity relative to T_3 maximal induction (at 10 nM set at 100%, DMSO set at 0%). Error bars indicate SD of triplicate data points.

Table 1. Effects of compounds in the stably transfected GH3.TRE-Luc reporter gene assay. Maximal Induction (%) expressed relative to T_3 (10 nM) set at 100%. For three individual experiments, the Relative Potency (RP) was calculated as the ratio of the half maximal effective concentration (EC_{50}) for T_3 with each chemical. Chemicals not inducing up to 50% of T_3 maximal effective concentration have RP based on EC_{10} levels and given between brackets (n.a. = not active). BDE 69, BDE 121, PCB 69, PCB 106. BPA, TBBPA and TCBPA do not induce a significant effect.

Compound	EC_{10} (nM)	EC_{50} (nM)	RP $EC_{50}/EC_{50\ (10)}$	Max. luciferase Induction (%)
T_3	0.01	0.1 ± 0.02	1	100 (10 nM)
T_4	-	9.1 ± 0.4	1×10^{-02}	94
Triac	-	0.1 ± 0.04	1	100
Tetrac	-	14.2 ± 0.8	7×10^{-03}	82
T_3-like 4-OH-BDE 69	61 ± 11	-	(2×10^{-04})	22
T_4-like 4-OH-BDE 121	460 ± 23	-	(3×10^{-05})	7
T_3-like 4-OH-PCB 69	23 ± 7	-	(6×10^{-04})	13
T_3-like 4-OH-PCB 106	290 ± 15	-	(5×10^{-05})	4

BPA (**Figure 8**), TCBPA and TBBPA (**Figure 9A** and **9B**, respectively) induced a similar and very characteristic effect. After an initial slight increase of the luciferase activity up to 1 μM (approximately 5% of T_3-maximum induction), the luciferase induction then decreases again at higher concentrations. However, at concentrations above 10 μM for TCBPA and TBBPA and 100 μM for BPA visible cytotoxicity occurs.

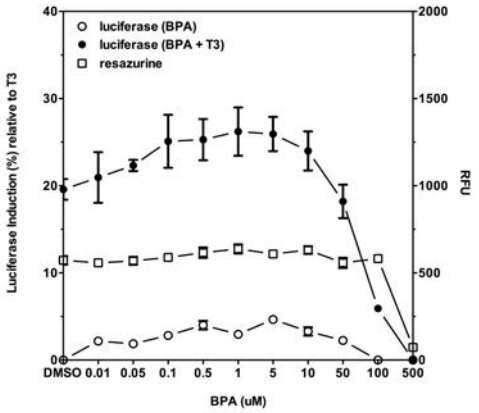

Figure 8. GH3.TRE-Luc induction after 24 h exposure to BPA with (●) and without (○) 0.25 nM T_3. Luciferase activity (left axis) as relative percentage of T_3 maximal induction (at 10 nM set at 100%, DMSO set at 0%) and the number of viable cells (□, right axis) expressed as relative fluorescence units (RFU). Error bars were calculated as SD of triplicate data points.

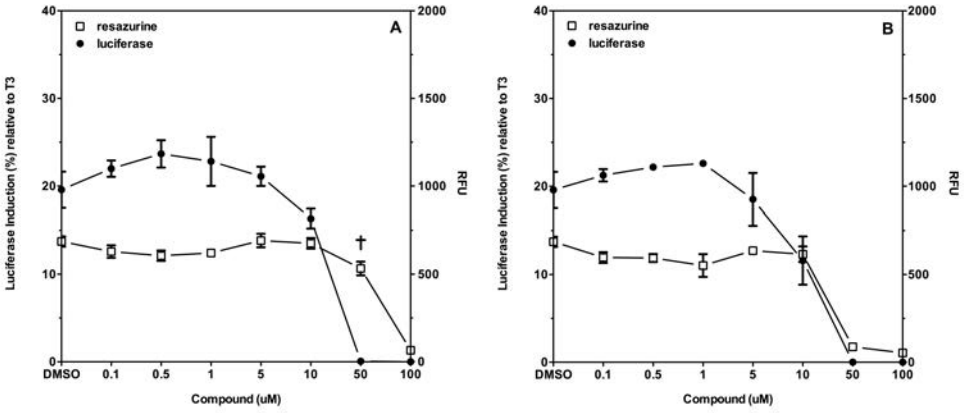

Figure 9. GH3.TRE-Luc induction after 24 h exposure, in the presence of 0.25 nM of T_3, to A) TCBPA and B) TBBPA. Luciferase activity (●, left axis) as relative percentage of T_3 maximal induction (at 10 nM set at 100%, DMSO set at 0%) and the number of viable cells (□, right axis) expressed as relative fluorescence units (RFU). Without T_3 both compounds did not induce a response. Error bars indicate SD of triplicate data points. † = visible cytotoxicity.

DISCUSSION

In the present study, we developed and validated a stable *in vitro* reporter gene assay that enables rapid and specific quantification of thyroid hormone receptor-mediated activity of hormones such as T_3 or T_4, their structural analogs and environmentally relevant contaminants. The bioassay was established using the rat pituitary tumor cell line GH3 that constitutively expresses both thyroid hormone receptor isoforms (Lazar, 1990). The GH3 cells thyroid hormone-induced proliferation is used as basis for the T-screen, a cell-based proliferation assay applied in the investigation of the interference of compounds with thyroid receptor-mediated cellular responses. However, as with other proliferation assays, the relatively time consuming T-screen bears the major drawback of assuming that cell proliferation is an entirely genomic thyroid hormone receptor-mediated mechanism. To be able to measure a thyroid receptor-specific end point in a high-throughput configuration, the GH3 cell line was stably transfected with a thyroid hormone response element regulated luciferase reporter gene enabling the detection of T_3 concentrations in the picomolar range within only 24 h of exposure, rather than the 96 h exposure used in the T-screen. Although 24 h of exposure to even high concentrations of T_3 only minimally induced cell proliferation (**Figure 4A**), the luciferase response was normalized for cell viability determined as the reduction of resazurine to resorufin. The resazurine method, which gives an immediate indication of cytotoxicity, is a more sensitive measure of cell viability than total protein quantification (**Figure 4B**). Moreover, it can be performed in the same wells since it doesn't require cell lysis and there isn't any interference with the luciferase measurement, allowing further reduction of the time required to run the assay. The maximal induction factor obtained with the newly developed GH3.TRE-Luc cells (23-fold at 10 nM, **Figure 5A**) with an average CV of 5% provides a good dynamic range to detect agonists, antagonists and potentiators. By comparison, using the same cell line, the previously reported maximum induction factors upon T_3-exposure were only 3.5-fold (Gauger et al., 2007; You et al., 2006) and 4-fold (Davey et al., 2008) in a transient transfection assay format. The use of the destabilized luciferase in our assay may contribute to the

high inducibility, as this strongly reduces the background activity after 24 h (Almond et al., 2004). The relative potency of natural thyroid hormones and the acetic acid derivatives of T_3 and T_4 (Triac and Tetrac) for inducing luciferase activity is highly consistent with previous reports; i.e. T_3 > Triac > T_4 > Tetrac (Cheek et al., 1999; Gutleb et al., 2005; Halpern and Hinkle, 1984), (Samuels et al., 1979).

We also examined the ability of the assay to detect compounds that antagonize TR-mediated transcriptional activity. The presumed antagonist amiodarone (Hamers et al., 2006; Norman and Lavin, 1989; Schriks et al., 2006) significantly increased the T_3-induced thyroid receptor-mediated luciferase activity at concentrations below 1 μM, accompanied by a statistically significant decrease in cell viability (**Figure 6A**). At higher concentrations the luciferase activity was reduced in parallel with the number of viable cells. Above 10 μM cytotoxicity became visible. This finding demonstrates the importance of a receptor-specific assay, as inhibition of T_3-dependent cell proliferation can be due to cytotoxicity as well. Other reports, however, show that amiodarone's antagonistic behavior is actually mediated through its major metabolite, desethylamiodarone (Bakker et al., 1994; van Beeren et al., 2003). The ambiguous effect of amiodarone in the GH3.TRE-Luc assay compared with previously reported effects, suggests a different mechanism of action for this chemical other than a standard thyroid receptor agonism or antagonism. Recently we have demonstrated the ability of amiodarone to interfere with cellular efflux pumps (in prep.), which could account for the initial increase in luciferase response in the presence of T_3, until at higher concentrations the compound becomes cytotoxic. Taking all into account amiodarone can hardly be used as a suitable antagonist, and unfortunately desethylamiodarone cannot easily be obtained. On the other hand, sodium arsenite was able to inhibit the thyroid receptor-mediated luciferase expression at non-cytotoxic concentrations (**Figure 6B**). This effect is in accordance with previous studies reporting that sodium arsenite acts as a strong endocrine disruptor by altering gene regulation by several nuclear receptors, including the thyroid hormone receptor (Davey et al., 2007; Davey et al., 2008; Kaltreider et al., 2001; Rosenblatt and Burnstein, 2009) suggesting a common inhibitory mechanism of action for this family of receptors.

We also examined the thyroid hormone receptor disrupting activity of hydroxyl

metabolites of PCBs and BDEs, which are important environmental contaminants. The relative potencies of the synthetic T_3- and T_4-like OH-BDEs tested in the GH3.TRE-Luc cells were in the same order of magnitude of those reported for the T-screen (Schriks et al., 2006) and in good agreement with a previously reported thyroid receptor binding assay (Marsh et al., 1998). Like the OH-BDEs, both the OH-PCB 69 and OH-PCB 106 showed a dose-dependent agonistic response, which is consistent with previously reported results (Ghisari and Bonefeld-Jorgensen, 2005; Kitamura et al., 2005a; You et al., 2006). Interestingly, the same two chemicals have shown to antagonize thyroid receptor-mediated luciferase induction in different cell-based assays (Iwasaki et al., 2002; Miyazaki et al., 2004). The apparent discrepancy found, for this chemical family and others, may be a reflection of their tissue and receptor subtype specific mode of action. The GH3 cells used here are well characterized and are known to express both thyroid receptor isoforms as well as their heterodimer partners and respective cofactors; these and other unknown transcription factors may be absent in other cell-based systems yielding completely different transcriptional outcomes. It would not be surprising if the cell type selective actions of these environmental compounds are reminiscent of the actions of the so-called selective estrogen receptor modulators (SERMs), whose agonist or antagonistic activities depend at least in part on qualitative and quantitative differences in receptor coactivators and corepressors in different cell types (Smith and O'Malley, 2004).

As was to be expected, the parent (non-hydroxylated) BDEs and PCBs were not able to induce any luciferase activity in our cells (data not shown) as they did not induce cell proliferation in the T-screen (Schriks et al., 2006) nor displace thyroid hormones from their transport proteins with high affinity (Lans et al., 1993; Marchesini et al., 2008; Meerts et al., 2000). The presence of a hydroxyl group preferably adjacent to one halogen seems to be required to confer the thyroid hormone receptor binding affinity. One study has reported PCB inducible P4501A1 activity for their clone of GH3 cells (Gauger et al, 2007). This would be very relevant as the metabolism of PCBs and other compounds to hydroxylated metabolites occurs *in vivo*. However, the wild type GH3 cells used to clone the new stably transfected GH3.TRE-Luc cell line do not show cytochrome P4501A activity in our hands and before (Gutleb et al., 2005; Schriks et al., 2006). P4501A1 transcripts were also not detected by microarray analysis (data not

shown). An option to test non-hydroxylated compounds of interest is by pre-incubation with microsomes to allow metabolism before exposure. It needs to be studied, however, to what extent the OH-metabolites can reach the same intracellular levels as the more lipophilic parent compounds (Schriks et al., 2006). Either reduced uptake or phase II metabolism may play a role. Also the relatively low induction of the reporter gene with the very T_3-like OH-metabolites in our study suggest that the availability of the OH-metabolites might be the limiting factor in an *in vitro* cell system in contrast to *in vitro* binding assays. Therefore, we are currently performing subsequent experiments, which will allow these chemicals to be intracellularly hydroxylated.

Our results also indicate that BPA (**Figure 8**), and its halogenated derivatives TBBPA and TCBPA may have the same mechanism of action as amiodarone in the GH3 cells. Up to a concentration of 1 μM they act as weak agonists (max. 5% effect), but above 5 μM an antagonistic effect was observed (respectively **Figure 9A** and **9B**). These very weak results suggest that the low dose response is not receptor-mediated, and with increasing concentration towards the receptor binding constant (K_i), binding may occur resulting in a tissue specific antagonist effect overriding the weak agonist effect. Previous studies have also reported that estrogenic chemicals may induce pituitary cell growth, however no estrogen-responsive proliferation was observed (data not shown) with this particular strain. A closer look into previous reports reveals that BPA induces cell proliferation alone and in combination with T_3 in the T-screen performed with 10% dextran-charcoal treated FCS (Ghisari and Bonefeld-Jorgensen, 2005). In a similar study with serum free medium only potentiation occurs (Schriks et al., 2006). It cannot be excluded that in the 10% DCC treated serum some hormones were still present as opposed to our serum free system. For BPA and its relatives, a multitude of contradicting effects in different reporter gene cells lines with different exposure conditions have also been reported (Moriyama et al., 2002; Kitamura et al., 2005b; Yamada-Okabe et al., 2004), of which some are in agreement with recent *in vivo* studies (Heimeier et al., 2009). Therefore experimental dissimilarities, such as exposure concentrations and conditions as well as cellular contexts should be taken into account when trying to explain different effects of the same compound in different assays.

In conclusion, the results of this study present the GH3.TRE-Luc assay as a promising tool to identify and quantify the thyroid receptor-mediated mechanism of action of compounds to which humans, animals and environment are regularly exposed. The assay is sensitive and versatile enough to screen for chemicals that are able to promote agonistic, antagonistic and potentiating thyroid receptor-mediated effects, very much like the T-screen. However, coupling the viable cell number end-point with the thyroid receptor-mediated luciferase activity, a distinction can be made between TR-mediated and other mechanisms of action that induce cell proliferation. Also putative antagonists, in the T-screen, revealed to be merely reducing cell viability. In addition, indications for compounds with other possible modes of action were found for a variety of different chemical classes of environmentally relevant compounds. The modes of action of these compounds should be further studied.

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CHAPTER 3

IDENTIFICATION OF THYROID HORMONE RECEPTOR ACTIVE COMPOUNDS USING A QUANTITATIVE HIGH-THROUGHPUT SCREENING PLATFORM

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ABSTRACT

To validate the use of the recently developed GH3.TRE-Luc cells for quantitative high-throughput screening, the GH3.TRE-luc assay was miniaturized and validated in a 1536-well plate format. The Library of Pharmacologically Active Compounds (LOPAC) and the National Toxicology Program (NTP) collection were screened for thyroid hormone receptor agonists and antagonists. Of the 2688 compounds tested 8 or 1 were agonists depending on the positive hit cut-off defined at ≥10% or ≥20% efficacy, respectively and 5 were identified as antagonists. None of the inactive compounds were structurally related to thyroid hormones, nor reported to be thyroid hormone receptor disruptors, so false negatives were not detected. None of the low potency agonists structurally resemble thyroid hormones, thus these may not be active directly through the ligand-binding domain of the receptor and may not represent effective agonists. Defining agonists in the qHTS as requiring a hit cut off of ≥20% efficacy at 100 μM may avoid identification of positives without physiological relevance. It is concluded that the miniaturized GH3.TRE-Luc assay offers a promising addition to the *in vitro* test battery for endocrine disruption, and that, given the low percentage of compounds testing positive, its high-throughput nature is an important advantage for future toxicological screening.

INTRODUCTION

The thyroid hormone (TH) system of vertebrates consists of an elaborate signaling network controlling critical processes throughout different life-stages such as regulation of energy metabolism (Cheng et al., 2010), growth and differentiation, development and maintenance of brain function (Horn and Heuer, 2010; Warner and Mittag, 2012), thermo-regulation (Ribeiro, 2008), osmo-regulation and renal function (Vargas et al., 2006), regulating onset and proper function of other endocrine systems, sexual behaviour and fertility, cardiovascular functioning (Danzi and Klein, 2012). The possibility of alterations to the TH system by natural or synthetic compounds present in our food or the environment could therefore have substantial implications. In this context, the demonstration that certain manufactured chemicals exhibit thyroid-like activity (Gray et al., 1993; Porterfield and Hendry, 1998) generated an interest in the TH system due to its crucial role in growth, development and energy homeostasis (Silva, 2001; Yen, 2001). The TH signaling network relies on efficient and accurate interpretation of chemical signals by the thyroid receptors (TRs). Thyroid active compounds may interact at the level of these TRs but may also generate effects at several other targets in normal thyroid functioning. These include TH transport by transthyretin (TTR) or thyroxine-binding globulin (TBG) (Lans et al., 1993); hormone production which essentially depends on iodine uptake (Hornung et al., 2010) and the enzyme thyroid peroxidase or thyroperoxidase (TPO) that incorporates iodine onto tyrosines from thyroglobulin for the production of thyroid hormones (Biegel et al., 1995); hormone activation or deactivation by iodothyronine deiodinases types I, II, and III (D1, D2, and D3, respectively) which regulate the activity of thyroid hormones via removal of specific iodine substituents, UDP glucuronosyltransferases (UGTs) or sulfotransferases (SULTs) which conjugate the thyroid hormones and facilitate their excretion from the body (Visser et al., 1979; Schuur et al., 1998); or transport of thyroid hormones over the membrane into the cell (Guo et al., 2002). Although for most of these targets an *in vitro* assay has already been developed (Murk et al., 2012), current risk assessment strategies still rely heavily on chemical safety data obtained in animal models. This low-throughput approach is relatively expensive and sometimes provides an unreliable representation of human

toxicity. Furthermore, the use of large number of animals for toxicity testing raises legal and ethical considerations. The development of integrated and intelligent testing strategies for toxicity evaluation, such as innovative *in vitro* and *in silico* approaches have paved the way for the reduction of these vertebrate studies. The regulatory system for chemicals controlled by the European Chemical Agency (ECHA), called Registration, Evaluation and Authorization of Chemicals (REACH) placed a premium on functional quantitative high-throughput *in vitro* screening assays (qHTS) for the toxicological evaluation of the extraordinarily high number of natural and synthetic chemicals to be assessed within a few years (about 30,000 substances are currently marketed at volumes greater than 1 ton/year). A collaboration known as Tox21, comprised of the United States Environmental Protection Agency (US EPA), the US National Institutes of Health (NIH), and the US Food and Drug Regulatory Agency (FDA), has initiated a program of screening a large chemical library composed of environmental chemicals and pharmaceuticals through different qHTS assays developed based on specific biological mechanisms relevant to toxicity (Kavlock et al., 2009; Shukla et al., 2010; Huang et al., 2011). These screening assays directly study the effects of thousands of potentially harmful chemicals on particular cellular systems or molecular targets. However, for TR-mediated disruption a functional qHTS assay based on endogenous full-length receptors is still lacking. Recently, we have developed and validated a stably transfected reporter gene cellular model based on the TH responsive rat pituitary tumor GH3 cell line that constitutively expresses both TR isoforms with TRβ being predominant. Here, we present the development and application of the GH3.TRE-luc cell line using a qHTS system to test chemicals for TR activity with potential for endocrine disruption. This includes the miniaturization and optimization of the GH3.TRE-Luc assay into a 1536-wells plate format for agonistic, antagonistic and cytotoxic activities of the compounds tested. The qHTS system obtained was subsequently used to test the 1280 compounds of the LOPAC library (Library of Pharmacologically Active Compounds) (Xia et al., 2011a) and the 1408 chemicals from the National Toxicology Program collection (NTP) (Xia et al., 2008). Intracellular ATP content was measured to ascertain that effects observed were not due to cytotoxicity. The chemical collections are used for validation of the high-throughput screen (qHTS) because of their diverse chemical families, some proven to

be pharmacologically active, with almost all compounds previously tested in one or more standard toxicological assays. The outcomes of this preliminary screen were further examined to identify potential false positives and false negatives using the publically available PubChem Bioassay database.

MATERIALS AND METHODS

Cell line and culture conditions. The GH3.TRE-Luc cell line developed as described (Freitas et al., 2011), stably expresses a modified firefly luciferase reporter gene under the regulation of a thyroid hormone response element (TRE). Cells were routinely sub-cultured once a week in fresh 75 cm^2 culture flasks (Corning, Acton, MA), in a humid atmosphere at 37°C and 95% air/5% CO_2 in Dulbecco's Modified Eagle's medium/Ham's F12 (DMEM:F12, Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (FBS, Hyclone, Logan, UT).

qHTS TRE luciferase reporter gene assay. For the TRE luciferase reporter gene assay GH3.TRE-Luc cells were seeded at 80% confluency in 225 cm^2 culture flasks (Corning, Acton, MA) in regular growth medium and cultured overnight. Growth medium was then replaced by assay medium (Sirbasku et al., 1991) (DMEM:F12 supplemented with 10 μg/ml insulin, 10 μM ethanolamine, 10 ng/ml sodium selenite, 10 μg/ml human apotransferrin and 500 μg/ml bovine serum albumin) followed by a 20 hours incubation. Subsequently, cells were detached, re-suspended in assay medium and seeded at 1500 cells/well in 1536-well assay plates. For agonist mode screening cells were plated in 5 μl of assay medium. After culturing for 4-5 hours, 23 nl of required concentrations of test compounds or DMSO control were transferred using a Pintool (Wako, San Diego, CA) into each well resulting in a final DMSO concentration of 0.46%. For antagonist mode screening, cells were plated in 4 μl, test compounds were added as indicated above and 1 μl of 1 nM final concentration T_3 or assay media control was dispensed to each well. Plates were incubated with compound treatment for 24 hours. After this incubation, 5 μl of One-Glo luciferase reagent (Promega, Madison, WI) was added and plates were incubated at room temperature for 30 minutes before reading on a Viewlux plate reader (PerkinElmer, Waltham, MA).
The 1280 compounds from the Library of Pharmacologically Active Compounds (LOPAC, Sigma, St. Louis, Missouri, USA) (Xia et al., 2011a) and the 1408 chemicals from the National Toxicology Program collection (NTP) (Xia et al., 2008) were tested in series of 7 to 15 dilutions with final concentrations ranging from 0.6 nM to 92 μM

and 3 nM to 46 μM for the NTP and LOPAC collections respectively. The highest concentrations tested were judged to be at the very upper range of potential environmental exposures. The four left columns in each plate were reserved for controls. The control format for the agonist mode plate was column-1 T_3 from 0.3 pM to 4.6 μM, column-2 T_3 100 nM and column-3 to 4 DMSO only. The control format for the antagonist mode plate was column-1 to 2 DMSO only, column-3 to 48 T_3 1 nM. In order to exclude the compounds that inhibit TR-induced luciferase reporter gene expression due to cytotoxicity, the LOPAC and NTP libraries were also tested for cell viability by measuring intracellular ATP content using a luciferase-coupled ATP quantitation assay (CellTiter-Glo viability assay, Promega, Madison, Wisconsin, USA). The cells were dispensed in 5 μl at 1500 cells/well in 1536-well white/solid bottom assay plates (Greiner, Monroe, NC) and the assay was run identically to the antagonist screen method mentioned above, with addition of 5 μl/well of CellTiter-Glo reagent in place of One-Glo. After 30 minutes incubation at room temperature, the luminescence intensity was measured using a ViewLux plate reader (PerkinElmer).

Data analysis. The primary data analysis was performed as previously described (Xia et al., 2011b). Briefly, raw plate reads for each titration point were first normalized relative to the T_3 control (100 nM T_3, set at 100% for agonist mode; 1 nM T_3, set at 100% for antagonist mode) and DMSO only wells (basal, set at 0%), and then corrected by applying a pattern correction algorithm using compound-free control plates (DMSO plates) (Xia et al., 2011b).

Concentration-response titration points for each compound were fitted to the Hill equation yielding concentrations for half-maximal activity for agonists (EC_{50}) or half-maximal inhibition for antagonists (IC_{50}) and maximal response (efficacy) values. The concentration response curves of the compounds were classified into four major classes (1-4) based on the completeness of curve, goodness of fit, and efficacy (Xia et al., 2011b). Antagonists were identified using the selection criterion that their IC_{50} values should be at least three times lower than the IC50 in the viability assay to exclude cases of cytotoxicity.

RESULTS

Assay optimization and miniaturization in the 1536-well plate format. The GH3.TRE-luc assay was initially miniaturized in a 1536-well plate with a 5 μl final assay volume. To find optimal cell density in the well, three different cell numbers were tested after treatment with various concentrations of the known agonist T_3 ranging from 0.3 pM to 4.6 μM for 24 hours. The EC_{50} values of T_3 obtained amounted to 0.33, 0.55, and 0.39 nM at cell densities of 1000, 1500, and 2000 cells/well respectively (**Table 1**). The signal-to-background ratio (S/B) for these three cell densities was 7 to 9.5 fold. Z′ factor value from the density of 1500 cells/well was 0.88, which was the highest compared to other cell densities (1000 or 2000 cells/well). Therefore 1500 cells/well was chosen for use in the subsequent studies.

Table 1. Assay optimization in 1536-well format. Cells were plated 4 hours prior to assay and stimulated with T_3. Signal-to-background ratio (S/B) was calculated comparing the response of 4.6 μM T_3 to DMSO controls. EC_{50} values were calculated from full dose response curves (n=3). Z′ factor was calculated using T_3 EC_{100} (n=3).

Cells/well (5μl)	S/B	EC_{50} (nM)	Z′ factor
1,000	9.18	0.33 ± 0.032	0.76
1,500	7.13	0.55 ± 0.036	0.88
2,000	9.49	0.39 ± 0.038	0.76

Further studies were conducted to optimize conditions to screen for thyroid receptor antagonist activity. Antagonist action can be identified based on the ability of the compound tested to block the effect of a sub-maximal concentration of the agonist T_3. In order to determine the concentration of T_3 to be used for qHTS determination of antagonist activity, two concentrations slightly above the EC_{50} of T_3 (**Table 2**) were tested. At both T_3 concentrations the assay shows a similar S/B ratio (3.6 and 3.7 fold) but the 1 nM T_3 group gave a minimal CV (coefficient of variation, 7%) compared to the 0.5 nM T_3 group (CV, 11%). Z′ factors (Zhang et al., 1999) were 0.44 and 0.65 for the 0.5 nM and 1 nM T_3 exposure groups, indicating that 1 nM was a better concentration for screening for antagonist activity. This concentration represents one

that optimizes a large screening window without significant loss of sensitivity to detecting antagonists due to receptor binding competition with the T_3 used in this assay mode. All further inhibition (antagonism) assays were run using 1 nM of T_3 as the agonist.

Table 2. T_3 concentration optimization for antagonist mode screening in 1536-well format. Cells were plated 4 hours prior to assay and stimulated with T_3. Signal-to-background ratio (S/B) was calculated comparing the response of the respective T_3 concentrations to DMSO controls. Coefficient of variation (CV) and Z′ factor were calculated using the indicated T_3 concentrations (n=2).

T_3 (nM)	S/B	CV (%)	Z′ factor
0.5	3.6	11.1	0.44
1	3.7	7.0	0.65

Identification of TR agonists by qHTS. The qHTS GH3.TRE-luc assay was used to screen the LOPAC and NTP libraries for TR agonists and antagonists to provide a proof of principle for use of the newly developed biomolecular screening tool. For agonist screening, the concentration titration of T_3, used as a positive control, was performed in each plate. The control dose-response curves of T_3 were well reproduced in all 27 plates used for the screening of the two libraries, including 6 DMSO plates, with an EC_{50} average of 0.66 ± 0.13 nM (**Figure 1**). T_3 controls averaged a signal-to-background ratio of 6.88 and 7.42 and the average Z′ factor was 0.82 and 0.77 in the LOPAC and NTP libraries screen, respectively (**Table 3**).
Of the 1280 compounds from the LOPAC library, 6 (0.5%) were identified as potential TR agonists (**Figure 2**), with the positive hit cut off being compounds that gave a ≥10% efficacy. The potency and efficacy of these compounds are listed in **Table 4**.

Table 3. Screening statistics for LOPAC and NTP libraries. Coefficient of variation (CV) calculated from control and low concentration plates. Signal-to-background ratio (S/B) and Z′ factor for agonist and antagonist mode determined for 100 nM T_3 and 1 nM T_3, respectively.

	Agonist Mode			Antagonist Mode		
Library	**CV (%)**	**S/B**	**Z′ factor**	**CV (%)**	**S/B**	**Z′ factor**
NTP	9.21 ± 1.81	7.42 ± 0.51	0.77 ± 0.05	7.25 ± 3.77	4.71 ± 0.29	0.65 ± 0.08
LOPAC	11.04 ± 1.99	6.88 ± 0.54	0.82 ± 0.05	7.83 ± 1.27	3.37 ± 0.18	0.76 ± 0.04

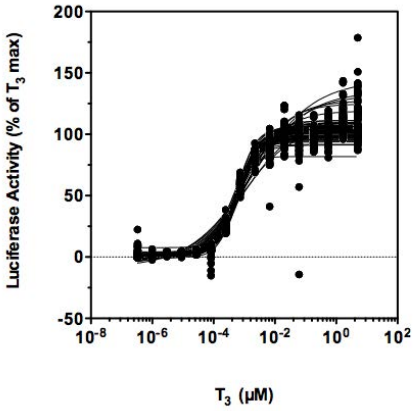

Figure 1. The intra-plate T_3 dose-response curves for 27 different plates. In each plate T_3 was used as positive control. After plated at 1500 cells/well in 1536-well plates and incubated for 4 hours, cells were stimulated with the indicated concentration of T_3 for 24 hours.

Table 4. Potency and efficacy for compounds identified in the LOPAC library screening. Potency (EC_{50} and IC_{50}) values were calculated from full dose response curves in the absence and presence of T_3 (1 nM), respectively. Efficacy was defined as percentage of maximal induction (agonist mode) or inhibition (antagonist mode) relative to T_3 set at 100% (100 nM for testing of agonist and 1 nM for antagonist activity). Re-screen values listed in parenthesis.

Agonists	**Potency, μM (re-screen)**	**Efficacy, % (re-screen)**
retinoic acid	0.8	22.2
CGP-7930	2.7 (2.6)	18.2 (18.0)
CGP-13501	2.8 (2.3)	16.8 (15.0)
13-cis-retinoic acid	6.4 (2.9)	18.8 (15.5)
SKF-89145 hydrobromide	21.9 (20.6)	10.9 (16.5)
4-hydroxybenzhydrazide	24.6 (26.0)	11.5 (17.5)
Antagonists		
Tranilast	0.7 (0.8)	-86 (81.8)
SB 205384	2.6 (2.5)	-75 (73.0)
NS-1619	5.2 (13.4)	-58 (75.7)
5-fluorouracil	5.2 (3.1)	-72 (71.1)

Of the 1408 compounds from the NTP library, 2 (0.1%) were identified as potential TR agonists, with the positive hit cut off being compounds that gave a ≥10% efficacy. The potency and affinity of these compounds are listed in **Table 5**.

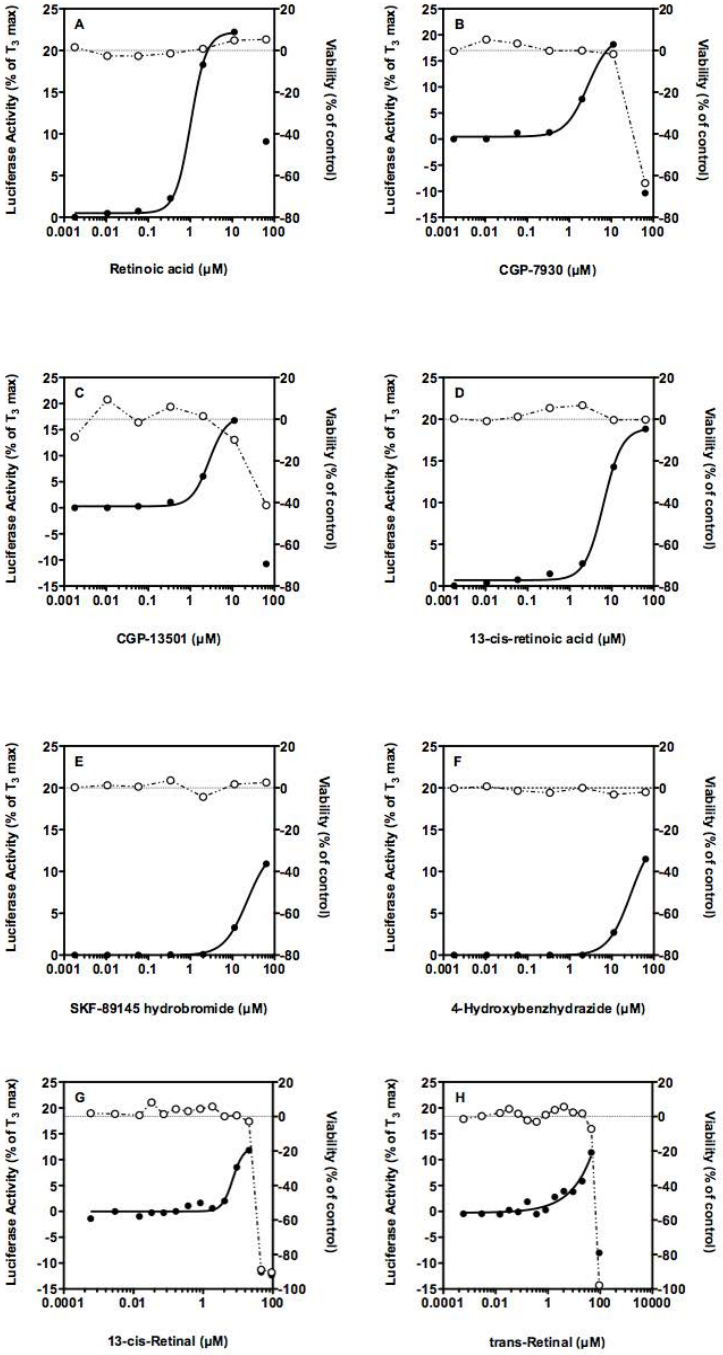
A
Luciferase Activity (% of T3 max)
Viability (% of control)
Retinoic acid (µM)
B
CGP-7930 (µM)
C
CGP-13501 (µM)
D
13-cis-retinoic acid (µM)
E
SKF-89145 hydrobromide (µM)
F
4-Hydroxybenzhydrazide (µM)
G
13-cis-Retinal (µM)
H
trans-Retinal (µM)

Figure 2. Examples of dose response curves in the qHTS for agonists identified in the LOPAC and NTP libraries screening. GH3.TRE-Luc induction after exposure to A) retinoic acid, B) CGP-7930, C) CGP-13501, D) 13-cis-retinoic acid, E) SKF-89145 hydrobromide, F) 4-hydroxybenzhydrazide, G) 13-cis-retinal, H) trans-retinal. Luciferase activity (l, left axis) as relative percentage of T_3 control (at 100 nM set at 100%, DMSO set at 0%) and the number of viable cells (○, right axis) expressed as relative percentage of DMSO control. Chemicals were tested in series of 7 to 15 dilutions with final concentrations ranging from 0.6 nM to 92 μM and 3 nM to 46 μM for the NTP and LOPAC collections respectively.

In order to evaluate the reproducibility of the hits and validate the screen in qHTS format, the LOPAC library was re-screened three times. All 6 compounds identified from the primary screen showed similar activity in the re-screen (**Table 4**). When, for defining TR agonist activity, the positive hit cut off was set at ≥20% efficacy, only one TR agonist was identified in the LOPAC library and none in the NTP library. Close evaluation of the dose response curves obtained for the compounds that did not induce ≥20% efficacy, revealed that at the highest dose levels tested, 46 or 92 μM, maximum activity of some of the possible agonists may not yet have been reached. However, given that the highest concentrations tested were judged to be at the upper range of what would be relevant possible exposure levels, this supports the conclusion that they may not represent effective TR agonists. Furthermore, none of these compounds has structural characteristics that resemble the thyroid hormones T_3 and T_4 (**Figure 3**), providing further support for the conclusion that they may not represent effective TR agonists.

Table 5. Potency and efficacy for compounds identified in the NTP library screening. Potency (EC_{50} and IC_{50}) values were calculated from full dose response curves in the absence and presence of T_3 (1 nM), respectively. Efficacy was defined as percentage of maximal induction (agonist mode) or inhibition (antagonist mode) relative to T_3 set at 100% (100 nM for testing of agonist and 1 nM for antagonist activity).

Agonists	**Potency, μM**	**Efficacy, %**
13-cis-retinal	7.1	11.8
trans-retinal	14.9	11.4
Antagonists		
5-fluorouracil	6.7	-86.7
1-acetyl-2-phenylhydrazine	10.6	-87.5

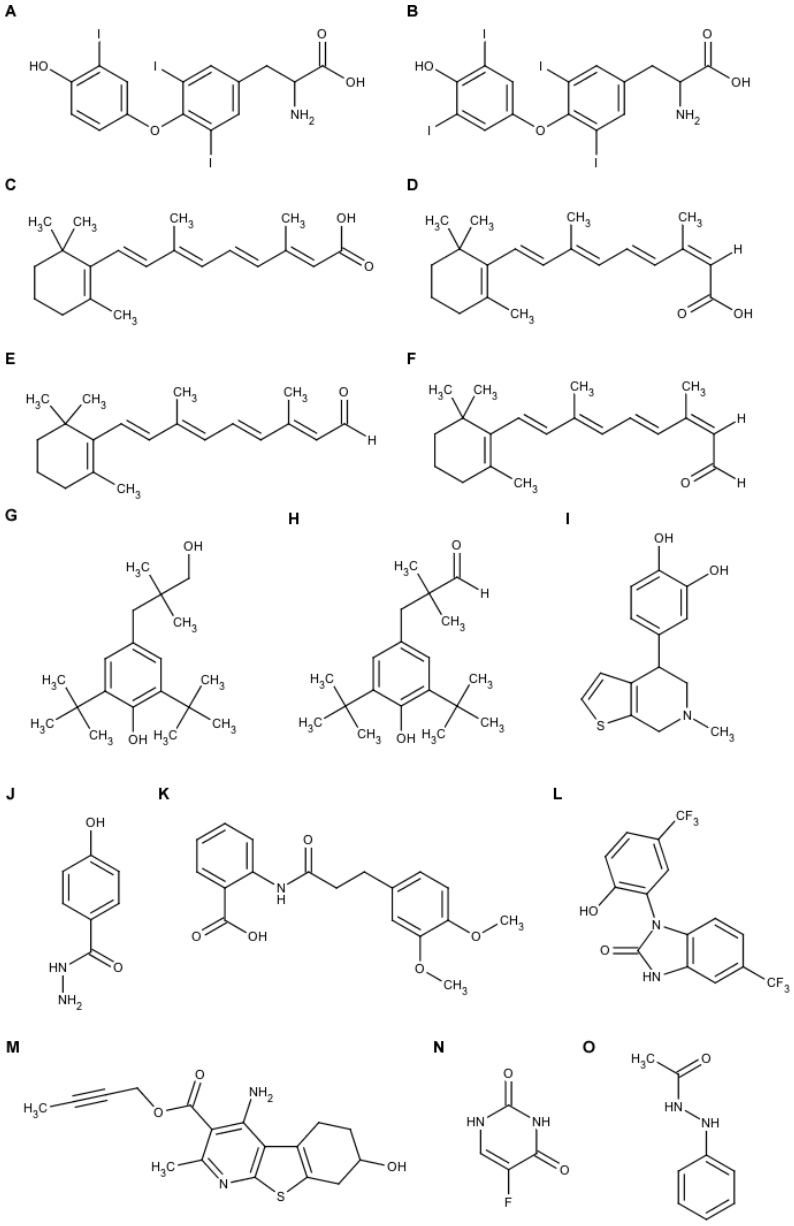

Figure 3. Chemical structures of compounds tested positive for TR agonist activity. A) triiodothyronine (T_3), B) thyroxine (T_4), C) retinoic acid, D) 13-cis-retinoic acid, E) trans-retinal, F) 13-cis-retinal, G) CGP-7930, H) CGP-13501, I) SKF-89145 hydrobromide, J) 4-hydroxybenzhydrazide, K) Tranilast, L) NS-1619, M) SB 205384, N) 5-fluorouracil and O) 1-acetyl-2-phenylhydrazine.

Identification of TR antagonists by qHTS. To identify the potential TR antagonists by using this GH3.TRE-luc assay, the LOPAC and NTP libraries were screened. In the antagonist mode screening, cells were exposed to the test compounds in the presence of 1 nM T_3 and the ability of the compounds to inhibit T_3-mediated TR activation was measured. The average signal-to-background ratio was 3.37 and 4.71, and the average Z' factor was 0.76 and 0.65 for the LOPAC and NTP libraries, respectively. To ascertain that the inhibitory effect of the potential hits was not due to cytotoxic effects, a cell viability screen was performed in parallel. From the LOPAC screen, 4 compounds (0.3%) were identified with an IC_{50} that was 3-fold lower than their viability IC_{50}. The potency and efficacy of these compounds are listed in **Table 4** and graphs given in **Figure 4**. From the NTP library, 2 compounds (0.1%) were identified with an IC_{50} that was 3-fold lower than their corresponding viability IC_{50}. The potency and efficacy of these compounds are listed in **Table 5** and graphs given in **Figure 4**. In order to evaluate the reproducibility of the hits and to validate the screen in qHTS format, the LOPAC library was also screened three times. All 4 compounds were confirmed in the re-screening.

Altogether the data presented indicate reproducibility and provide a first proof of principle that the novel and robust GH3.TRE-luc assay can be utilized to screen large compound libraries in 1536-well plate format for TR agonist as well as antagonist activity.

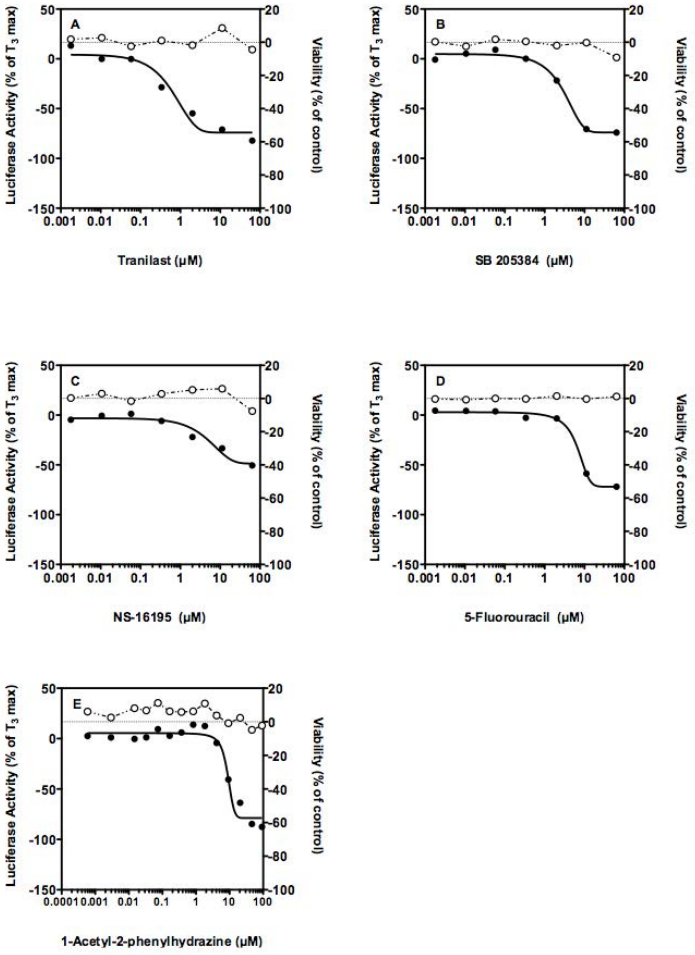

Figure 4. Examples of dose response curves in the qHTS for antagonists identified in the LOPAC and NTP libraries screening. GH3.TRE-Luc induction after exposure, in the presence of 1 nM of T_3, to A) Tranilast, B) SB 205384, C) NS-1619, D) 5-fluorouracil, E) 1-acetyl-2-phenylhydrazine. Luciferase activity (l, left axis) as relative percentage of T_3 control (at 1 nM set at 100%, DMSO set at 0%) and the number of viable cells (○, right axis) expressed as relative percentage of DMSO control. Chemicals were tested in series of 7 to 15 dilutions with final concentrations ranging from 0.6 nM to 92 μM and 3 nM to 46 μM for the NTP and LOPAC collections respectively.

DISCUSSION

In the present study a quantitative high-throughput screen (qHTS) for TR agonist and antagonist activity was developed based on the recently developed *in vitro* reporter gene assay using the stably transfected GH3.TRE-Luc cell line (Freitas et al., 2011). The assay was miniaturized and validated in a 1536-well plate format. Subsequently the 1280 compounds of the LOPAC library (Library of Pharmacologically Active Compounds) and the 1408 compounds of the NTP collection were tested for their TR agonist or antagonist activity. Of the 2688 compounds tested in the qHTS 8 (0.3%) or 1 (0.04%) were found to be TR agonists depending on whether the positive hit cut off was defined at ≥10% or ≥20% efficacy. None of the inactive compounds were structurally related to T_3, nor reported elsewhere to be thyroid hormone disruptors, so false negatives were not obvious in the screen. Furthermore, none of the low potency TR agonists has structural characteristics that resemble the thyroid hormones (**Figure 3**), providing further support for the conclusion that they may not represent effective TR agonists and may not activate the assay through binding to the TR ligand-binding domain. Defining TR agonists in the qHTS with a hit cut off of ≥20% efficacy at 100 μM may avoid identification of positives that are only very weak agonists and/or not acting through the ligand-binding domain. The GH3.TRE-Luc cells performed very well in an automated 1536-well plate format. The CV varied between 7-11% depending on the potency of the compounds tested, which is well within the performance standards for comparable assays in 1536-well plate format.

The two chemical collections were used for validation of the high-throughput screen (HTS) because of their diverse chemical space, some proven to be pharmacologically active, with almost all compounds previously tested in one or more standard toxicological assays. They do, however, not specifically contain compounds known for their *in vivo* or *in vitro* thyroid hormone disrupting potency. For example, the known T_3- and T_4-like compounds such as Tetrac (3,5,3',5'-tetraiodothyroacetic acid), Triac (3,5,3'-triiodothyroacetic acid) and OH-PHAHs (hydroxylated polyhalogenated aromatic hydrocarbons) such as TBBPA (4,4'-propane-2,2-diylbis(2,6-dibromophenol)) or OH-PCBs (hydroxylated polychlorinated biphenyls), PBDEs (polybrominated

diphenylethers) shown before to be active in the GH3.TRE-Luc assay (Freitas et al., 2011) were not included. The LOPAC library contains pharmacologically active compounds, including many cytostatic compounds that could give growth inhibition hence reducing the luciferase activity. For compounds that do not directly antagonize the TR activity, the cell viability and the TRE-Luc inhibition IC_{50}'s will be similar. Therefore it is important to quantify the cell viability and calculate the ratio between the cell viability and the TRE-Luc inhibition IC_{50}'s. When this ratio is less than 3, the compound is assumed to be merely cytotoxic instead of antagonistic. This approach has been used before for other reporter gene screens (Miller et al., 2010). One of the most potent TRE-Luc antagonists identified in the present screen was the anti-cancer drug 5-fluorouracil inducing a full dose-response curve without apparent cytotoxicity although it would be worthwhile to evaluate for cytotoxicity by alternative methods to provide higher confidence in interpretation of these results (**Figure 4**). It is of interest to note that one of the side effects of 5-fluorouracil is cardiotoxicity, the mechanism of which is still poorly understood (McGlinchey et al., 2001). Given however, that T_3 is an important signaling molecules for cardiac function it seems worthwhile to study the possible role of the TR antagonism of 5-fluorouracil detected in the present study in the mode of action underlying this side effect.

The most active agonists with EC_{50} values lower than 10 μM were the retinoids (13-cis-retinoic acid and 13-cis-retinal) and the positive allosteric modulators of the GABA receptors GCP-7930 and GCP-13501 (**Figure 2**). The 13-cis-retinoids are known ligands of the TR heterodimer partner retinoid X receptor RXR (Li et al., 2002). RXR has been described both as a permissive heterodimer partner, i.e. activity at a thyroid receptor response element can be driven by RXR ligands, and as a non-permissive (Li et al., 2002). It may be that the status of permissive versus non-permissive is defined by the specific cell system used for evaluation. In these rat pituitary GH3 cells, TR:RXR acts consistent with a permissive heterodimer. Interestingly, in another study 9-cis-retinoic acid significantly induced luciferase activity up to 20% of the T_3-maximum response in the GH3.TRE-Luc cells but not in the TRα.HeLa-Luc cells (Freitas et al., 2012). The TRα.HeLa-Luc cells, with low expression levels of endogenous TRs, are stably transfected with TRα and the same TRE-Luc construct as the GH3.TRE-Luc cells. The absence of luciferase induction in the TRα.HeLa-Luc cells by the retinoic acids suggest

that they also lack a functional RXR and this would corroborate the conclusion that the agonist activity detected in the GH3.TRE-luc cells may reflect RXR rather than direct TR agonist activity. This would also explain the retinoids antagonistic like behavior in the GH3.TRE-Luc assay without any structural resemblance to T_3 or T_4. It should be further studied whether a comparison between the TRE-Luc activation in the GH3.TRE-Luc and the TRα.Hela-Luc cells could be used to filter out RXR-active compounds, thereby reducing the chances on false positives in the GH3.TRE-Luc assay.

The TRE-Luc potency of compounds that do not structurally resemble T_3, is less than that of true TR-agonists such as the T_3-like OH-BDE's (Freitas et al., 2011). Therefore the cut-off of a maximum efficacy of ≥20% is also of use to filter out these compounds that are non-true TR-agonists. These less effective activators could be of interest as they may interact with pathways indirectly modulating TR activation, such as interactions with co-regulators or epigenetic modifications like effects on DNA methylation, chromatin structure or miRNA expression patterns.

Taken together, the results obtained in the present study demonstrate the potential of the qHTS for the identification of novel thyromimetic compounds acting via a TR mediated mode of action. It is concluded that the miniaturized GH3.TRE-Luc assay offers a promising addition to the *in vitro* test battery for endocrine disruption, and that, given the low percentage of compounds testing positive, its high-throughput nature is an important advantage for future toxicological screening.

ACKNOWLEDGMENTS

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CHAPTER 4

GENOMIC RESPONSES TO THYROID HORMONE RECEPTOR ISOTYPE SELECTIVE MODULATORS AND THYROID HORMONE DISRUPTING CHEMICALS IN GH3 RAT PITUITARY TUMOR CELLS

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ABSTRACT

GH3 cells are highly responsive to thyroid hormone (TH) and are increasingly used in *in vitro* bioassays for TH disrupting chemical detection. These assays often rely on measurements of cell proliferation that may be subject to interference by compounds independently of their effects on TH signaling per se. Recently, we developed an integrated thyroid hormone response element (TRE) based luciferase reporter gene system using stably transfected GH3 cells (GH3.TRE-LUC) that offers a more specific means to detect compounds affecting thyroid hormone receptor (TR) activity. While these cells possess properties desirable for high-throughput screening approaches, altered reporter gene activity has not been compared to *bona fide* endogenous TH target gene expression changes. In this study, we treated GH3 cells with 3,3',5-triiodothyronine (T_3) as well as two TR isotype selective thyromimetics for either TRα or TRβ (CO23 and GC-1, respectively). Response profiles in the TRE-based reporter gene assay showed excellent concordance with endogenous growth hormone (GH) gene expression, a known direct TH target gene. Further, we demonstrate that sodium arsenite, bisphenol A and the phthalate DEHP inhibit T_3-mediated induction of the reporter gene as well as the endogenous GH gene. We then performed a microarray experiment to identify a fuller spectrum of T_3 responsive genes in these cells, since promoter selective effects of synthetic nuclear receptor ligands may occur. In addition to identifying several important components of the TH signaling pathway, the microarray allowed us to identify a battery of both known and novel TH target genes, including genes related to angiogenesis such as HIF2α (EPAS1). Identification of these genes is important for validation of assays designed to identify new synthetic TR modulators and TR disrupting compounds, and their potential genomic versus nongenomic modes of action.

INTRODUCTION

Thyroid hormones (TH), including the major product of the thyroid gland, thyroxine (T_4), and the more active 3,3',5-triiodothyronine (T_3), are critical regulators of vertebrate development and metabolism. In humans, inadequate TH levels lead to moderate to severe mental retardation, ataxia, and sensory deficits along with delayed skeletal development and growth arrest (Williams, 2008). Even marginally low maternal thyroid function during pregnancy leads to significant decreases in mental capacity of the offspring. In adults, low TH levels lead to slowed basal metabolic rate and heart rate, as well as neurological issues such as attention deficit and depression-like symptoms. There is growing evidence that several environmental chemicals can negatively impact various aspects of the TH endocrine system, which has been the subject of several recent reviews (Diamanti-Kandarakis et al. 2009; Miller et al. 2009). Given the major role of TH in development, metabolic homeostasis, and reproduction, exposure to these chemicals could have adverse effects on human and animal health (Zoeller, 2007). In response to these concerns, the EPA incorporated animal based assays to detect potential thyroid hormone disrupting chemicals as part of the Tier 1 battery of the Endocrine Disruptor Screening Program (http://www.epa.gov/endo). While the prevailing notion to date has been that most TH disrupting chemicals act primarily to disturb TH synthesis or serum protein binding, recent evidence points to additional direct influences on the TRs themselves (Zoeller, 2005; Diamanti-Kandarakis et al. 2009). These effects may occur by directly binding to the receptors as agonists or antagonists, or indirectly by altering corepressor/coactivator recruitment and activity, for example. Furthermore, observed effects on the HPT axis could be mediated via the TRs, particularly by influencing negative feedback at the hypothalamus or pituitary via the TRβ isotype. Therefore, it is of paramount importance to develop sensitive, reliable, and specific screening methods to detect TR mediated actions of chemicals, to include in a battery of *in vitro* methods with predictive value for the *in vivo* situation. Several cell-based methods for this purpose have been published (e.g. (Gauger et al. 2007; Hofmann et al. 2009; Shiizaki et al. 2010)), but suffer from various limitations such as low fold inductions,

reliance on transient transfection methods, and/or the use of cells that are not normally TH responsive and with introduced TR over-expression may still lack important endogenous factors.

The best understood actions of TH are mediated through a pair of thyroid hormone receptors, TRα or TRβ, and their splicing isoforms (Cheng et al. 2010). These highly conserved proteins are ligand-regulated transcription factors that bind near target genes via well-defined thyroid hormone response elements, and, on classically up-regulated genes, repress or activate transcription in the absence and presence of hormone, respectively (Shi, 2009). TRα and TRβ are expressed in tissue-specific manner and are believed to play both distinct and overlapping roles during development and in the control of metabolism (Flamant and Gauthier 2012). GH3 cells proliferate in response to T_3 and T_4, as well as environmental compounds such as hydroxylated metabolites of polybrominated diphenyl ethers (OH-PBDEs) (Schriks et al. 2006). However, the relative role of TR isotypes in GH3 cell proliferation is not known. Non-genomic actions of TH have also been proposed, acting via TRs outside the nucleus or via membrane receptors for TH or TH metabolites (Cheng et al. 2010). In particular, the nongenomic response has been linked to T_4 induction of angiogenesis (Bergh et al. 2005). While the response to TH might be initiated at the membrane or in the cytoplasm, ultimately, downstream changes in gene expression could also occur. However, far less is known about the relative contribution of non-genomic pathways in the myriad roles for TH in developing or adult organisms.

A major unmet need in the study of TR disrupting chemicals has been the development of a sensitive and specific cell based assay, potentially amenable to high-throughput screening. A rat somatotroph tumor-derived cell line GH3 that expresses all endogenous TR isoforms (Ball et al. 1997) and proliferates upon exposure to T_3 or T_3-like compounds (Schriks et al. 2006; Gutleb et al. 2005) was used to develop the stably transfected GH3.TRE-Luc reporter line (Freitas et al. 2011). The GH3.TRE-Luc line contains a stably integrated reporter construct containing two DR4 thyroid hormone response elements in tandem upstream of the SV40 small T antigen minimal promoter, driving a destabilized firefly luciferase cDNA (luc2CP). This luciferase reporter was chosen to reduce baseline expression yet retain high sensitivity. The T_3 responsiveness of the most inducible clone was optimized to twenty-four hour

exposure (prior to noticeable induction of proliferation) in serum free media. A cell viability assay conveniently quantifies metabolic activity of these cells prior to cell lysis for luciferase measurements, by monitoring conversion of resazurine to the fluorescent resorufin. Luciferase reporter activity was highly inducible and sensitive to low levels of T_3, and there was a low coefficient of variation between assays (<5%) (Freitas et al. 2011). Furthermore, candidate environmental toxicants such as OH-PBDEs and hydroxylated polychlorinated biphenyls (OH-PCBs) showed partial agonist activity and arsenic trioxide and BPA antagonized T_3 induced reporter gene activity (Freitas et al. 2011), all consistent with previous findings in the literature. Despite these promising results, it is not yet known how well the integrated luciferase reporter gene serves as a surrogate for actual TH responsive target genes, a critical consideration should these cells be adopted by government, industry and academic scientists for TR disrupting compound screening. Furthermore, only a handful of endogenous TH responsive genes has been reported in GH3 cells despite the cell line's long history in the field, including the discovery of the first TRE in the GH gene (Koenig et al. 1987). Therefore, determination of the genomic response to TH exposure in these cells will help reveal the mechanism of the proliferation response as well as a broader spectrum of TH target genes for post-screening validation.

MATERIALS AND METHODS

Chemicals. All chemicals were of ≥98% purity unless stated otherwise. 3,3',5-Triiodo-L-thyronine (T_3, CAS no. 6893-02-3), 2,2-Bis(4-hydroxyphenyl)propane (BPA, CAS no. 80-05-7), Bis(2-ethylhexyl) phthalate (DEHP, CAS no. 117-81-7), and sodium arsenite (CAS no. 7784-46-5) were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, Netherlands). 5-({4-[4-Hydroxy-3-(propan-2-yl)phenoxy]-3,5-diiodophenyl}methyl) imidazolidine-2,4-dione (CO23, CAS no. 911689-00-4) and 2-(4-{[4-hydroxy-3-(propan-2-yl)phenyl]methyl}-3,5-dimethylphenoxy) acetic acid (GC-1, CAS no. 211110-63-3) were a gift from Thomas Scanlan (Oregon Health Sciences University, Portland, OR) and synthesized as described elsewhere (Chiellini et al. 2000; Ocasio and Scanlan 2006). All compounds were dissolved in dimethylsulfoxide (DMSO, 99.9%; Acros Organics, Geel, Belgium) and kept in the dark. Stocks were stored at −20°C until used.

T-screen. The T-screen was carried out as previously described (Schriks et al. 2006). Briefly, GH3 cells were cultured in Dulbecco's modified eagle's medium/Ham's F12 (DMEM:F12) with 15mM HEPES (Gibco) supplemented with 10% fetal calf serum (Gibco-Invitrogen). Cells were sub-cultured once a week in new 75cm^2 culture flasks (Greiner). Forty-eight hours before T-screen, cells were incubated in a serum free medium (PCM medium (Sirbasku et al. 1991; Gutleb et al. 2005)) to deplete cells of thyroid hormones. Cells were then collected by scraping and seeded into transparent 96-well plates (Greiner) at a density of 2500 cells/well. GH3 cells were exposed in triplicate and incubated for 96 h in the presence or absence of 0.25 nM T_3, with or without the indicated test chemical (ranging from 0.1 μM to 10 μM) in DMSO (0.2%). A full T_3 standard curve comprising a concentration range from 0 to 100 nM was included on each plate. Following the 96 h incubation, 8 μL of a 400 μM resazurine solution in PBS (Gibco) was added to each well. After 4 h incubation in the dark (37°C; 5% CO_2) fluorescence was measured at λ_{ex}=530 nm and λ_{em}=590 nm on a Millipore Cytofluor 2350 Fluorescence Measurement System.

Reporter gene (luciferase) assay. The reporter gene (luciferase) assay was performed as described (Freitas et al. 2011). Briefly, GH3.TRE-Luc cells were seeded at 80% confluency in 75 cm^2 culture flasks (Greiner) in DMEM:F12 with 15 mM HEPES (Gibco) supplemented with 10% fetal bovine serum (Gibco-Invitrogen) and 320 μg / ml of Geneticin (Gibco). Twenty-four hours later, growth medium was replaced by PCM medium for an additional period of 24 h. Cells were then collected by scraping and seeded into white clear bottom 96-well plates (Greiner) at a density of $3x10^4$ cells / well and incubated for 24 h in the presence or absence of 0.25 nM T_3, with or without the indicated test chemical in 0.2% DMSO. A full T_3 standard curve comprising a concentration range from 0 to 100 nM, was included on each plate. Cell viability in each well was determined as described above and luciferase activity was measured in lysed cells in a microplate luminometer with two injectors (Thermo LabSystems luminoskan Ascent), as previously described (Murk et al. 1998).

Microarray analysis. GH3 cells (ATCC CCL-82.1) were seeded in six well plates (Falcon) and grown to approximately 80% confluency in DMEM:F12 media with 10% fetal bovine serum (Invitrogen). Cells were then shifted to PCM medium for 48 hours, then treated in duplicate with 0.2% DMSO (vehicle), or 1 nM T_3 (in an equivalent amount of DMSO) for 48 hours. Cells were harvested and total RNA prepared using an RNeasy kit (Qiagen). Integrity of the RNA was checked with an Agilent Bioanalyzer, and quantified with a Nanodrop ND-1000 spectrophotometer. 0.5 μg total RNA from each well was labeled with TotalPrep RNA Amplification Kit (Ambion), cRNA quality was verified with the BioAnalyzer, and found to have an average size of 1.2 kb. The biotinylated cRNA was used to probe an IIllumina *RatRef-12* Expression BeadChips (containing 22,523 probes representing 21,912 unique rat genes) at the UC Davis Genome Center gene expression core. Samples from two separate wells from each treatment group were used on the microarray. Illumina Beadstudio software was used to normalize the data. Genes with an average signal three times over background in at least one condition, and with an array detection p value < 0.01 were selected for further analysis.

Quantitative reverse transcriptase real time PCR (qPCR). GH3 cells were grown in six well plates in triplicate and treated as described for the microarray analysis. RNA was extracted as described in the previous section. cDNA synthesis of 1 μg total RNA was performed using the Quantitect Reverse Transcription kit (Qiagen). Quantitative real time PCR was performed using the SYBR green master mix (Applied Biosystems) in an Applied Biosystems 7900 HT FAST instrument at the Real-time PCR Research and Diagnostics Core Facility, School of Veterinary Medicine, UC Davis. Primers used were: Growth hormone forward 5′-CTCCCTGGCTCCTGACCTTC-3′, growth hormone reverse 5′-ATGTAGGCACGCTCGAACTCTT-3′; hairless forward 5′-CCTCCAG AGCGTTCGTATGAC-3′, hairless reverse 5′-GCTAAGCCCAACATCCCAGACT-3′; IGFBP3 forward 5′-GCGTGCAGAGCCAGTAGATACC-3′, IGFBP3 reverse 5′-TGTT GGCAGTCTTTTGTGCAA-3′; Aurora B Kinase forward 5′-AGAGCGGAACCTTCG ATGAG-3′, Aurora B kinase reverse 5′-CATCAGCGCGTCTGACAGTT-3′. Expression was normalized with the ribosomal L8 gene (rpl8), rpl8 forward 5′-TGCTAACCGAG CTGTTGTTGGT-3′, rpl8 reverse 5′- CAGCAGTTCCTCTTTGCCTTGT-3′.

RESULTS

Effects of T_3, GC-1 and CO23 on GH3 cell proliferation. As shown in **Figure 1**, T_3 was the most potent in inducing proliferation, with an EC_{50} of 0.27 nM. Proliferation concentration-response curves for the relatively TRβ selective ligand GC-1 and the relatively TR*α* selective ligand CO23, resulted in EC_{50}'s of 1.5 nM and 18 nM for GC-1 and CO23, indicating they were approximately 5-fold and 67 times less potent than the T_3 EC_{50}, respectively.

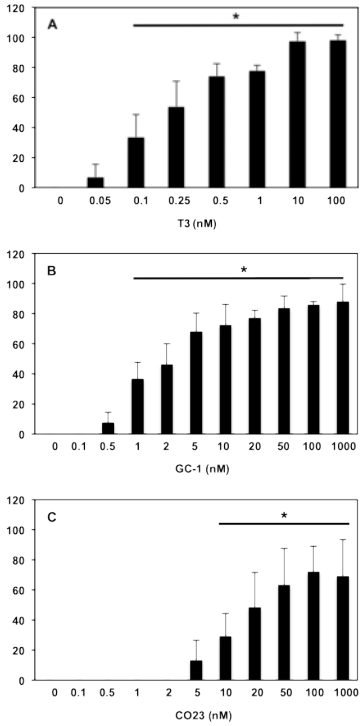

Figure 1. Effect of T_3 and the isotype selective thyromimetics GC-1 and CO23 on GH3 cell proliferation. GH3 cells were treated for four days in serum free medium with the indicated concentrations of T_3 (A), GC-1 (B) or CO23 (C), and cell proliferation was measured using the conversion of resazurine to the fluorescent resorufin (RFU). 100% on y-axis represents the maximum T_3 induced value. Bars represent the average value for each dose from 3-5 separate experiments, error bars represent SD. Representative graphs for each compound are shown here; a summary of the EC_{50} results averaged from these experiments is found in **Table 1**.

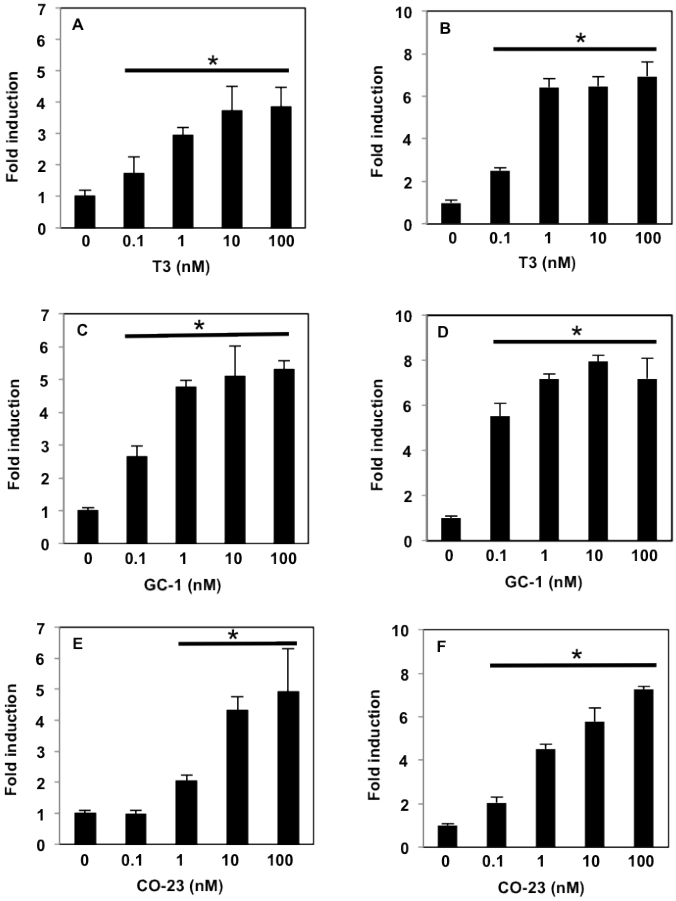

Figure 2. Concentration-dependent effect of T_3 and the isotype selective thyromimetics GC-1 and CO23 on TRE based reporter gene and endogenous growth hormone gene expression in GH3 cells. GH3.TRE-LUC cells were treated with the indicated concentrations of T_3 (A), GC-1 (C) or CO23 (E) and luciferase activity measured in cell extracts, normalized to resorufin formation. Parental GH3 cells were treated with the same concentrations of T_3 (B), GC-1 (D), or CO23 (F) and analyzed for GH RNA expression by quantitative PCR, normalized to rpL8 expression. Error bars represent SD. *, $p < 0.05$ vs. untreated controls.

Effects of T_3, GC-1 and CO23 on a TRE-regulated reporter gene and endogenous growth hormone gene expression. Next, we used T_3, GC-1 and CO23 in gene expression studies to provide a direct comparison to the T-screen results shown in **Figure 1**, and to uncover any potential isotype selectivity in target gene regulation in

GH3 cells. The response curves of the luciferase reporter in GH3.TRE-Luc cells were remarkably similar to the T-screen based proliferation results. GH expression in the parental GH3 cells in response to T_3, GC-1 and CO23 (**Figure 2**) is somewhat more sensitive than the T-screen and reporter gene assays, but overall, the same pattern of relative potencies exists (T_3>GC-1>>CO23). Thus, the proliferation and transcriptional responses to GC-1 (as measured by reporter gene activity and GH expression) are much closer to the natural ligand T_3 than they are for CO23. The EC_{50} values for proliferation, reporter gene, and GH gene expression assays in response to T_3, GC-1 and CO23 are summarized in **Table 1**.

Table 1. EC_{50} values of T_3 and the thyromimetic compounds GC-1 and CO23 for proliferation, reporter gene, and endogenous gene regulation in GH3 cells.

Compound	T-screen EC_{50} (nM)	T screen: % T_3 max	TRE-LUC EC_{50} (nM)	TRE-LUC % T_3 max	GH qPCR EC_{50} (nM)	GH qPCR % T_3 max
T_3	0.27	100	0.3	100	0.2	100
GC-1	1.5	82	1.1	108	0.5	117
CO23	18	86	22	94	7.0	104

The effect of putative environmental TR antagonists on reporter gene and GH gene expression in GH3 cells. In order to assess the reliability of the reporter gene assay to detect compounds that inhibit TR activity, we chose three environmentally relevant compounds previously linked to TH disruption: sodium arsenite (SA), bisphenol A (BPA), and di(2-ethylhexyl)phthalate (DEHP). SA and BPA have previously been shown to alter responses to TH in *Xenopus laevis* tadpoles and mammalian cells, but less is known about DEHP and TR activity. SA, BPA, and DEHP inhibited T_3 induced luciferase activity as well as endogenous GH expression in a concentration-dependent manner (**Figure 3**). The effect of SA is biphasic on the GH gene, with significant induction at low doses and strong inhibition at the highest doses. On caveat of these findings is that the non T_3 responsive gene used to normalize GH expression, rpl8, was also repressed at doses of SA>10 μM but to a lesser extent than GH expression was repressed, leading to the observed strong inhibition even upon normalization. SA did not affect viability as judged by the resazurine/resorufin assays, however.

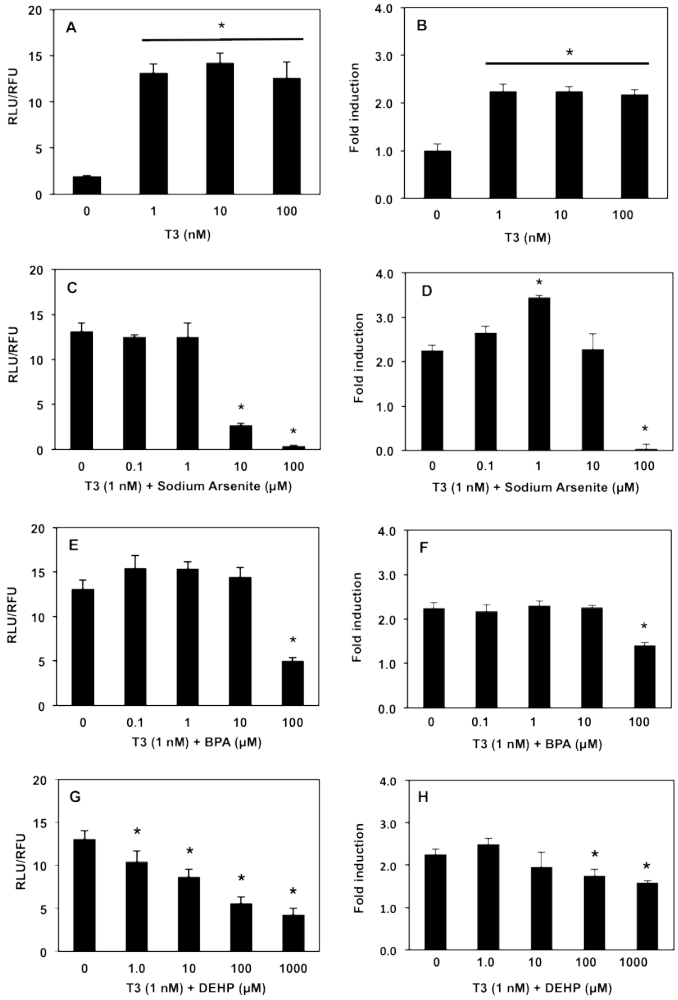

Figure 3. Effect of selected endocrine disrupting chemicals on TRE based reporter gene and endogenous gene expression in GH3 cells. GH3.TRE-LUC cells were treated with DMSO (control), increasing concentrations of T_3 (1, 10 and 100 nM), 1 nM T3 + increasing concentrations of sodium arsenite (C,D) (SA, 0.1, 1, 10 and 100 μM), bisphenol A (E, F) (BPA, 0.1, 1, 10 and 100 μM) or Bis(2-ethylhexyl)phthalate (G,H) (DEHP, 1, 10, 100 and 1000 μM). In one set of cells (A, C, E, G), luciferase activity was measured in cell extracts, and normalized to resorufin formation. In a parallel set of cells, RNA was prepared for analysis of GH expression (B, D, F, H) by quantitative RT-PCR, normalized to rpL8 expression. Experiments were performed in triplicate; error bars represent SD. *, $p < 0.05$ vs. untreated controls (A, B), or versus 1 nM T_3 treated but compound untreated controls (C-H).

Identification of the T_3 regulated transcriptome in GH3 cells. An important secondary screen of positive compounds following proliferation based assays in parental GH3 cells, or reporter gene based assays in GH3.TRE-Luc cells, will be to test positive "hits" for effects on endogenous TH responsive genes. We exploited an important advantage of using this normally TH responsive cell line to identify a signature of T_3 genomic signaling for use in validation. Toward this end, we performed microarray analysis on the parental GH3 cells treated with 1 nM of T_3 for 48 hours. First, as shown in **Table 2**, the array results allowed us to examine the relative expression levels of multiple components of TR signaling in control and ligand treated GH3 cells. The array detected both TR isotypes, and both were modestly down-regulated by T_3. The location of the probe in the TRβ transcript, however, did not allow discrimination between TRβ1 and TRβ2. The TR heterodimer partners RXRβ and γ, but not α, were also detected. In addition, expression of the coactivator SRC-1 and the co-repressors NCoR and SMRT genes were detected, and SRC-1 is induced roughly two-fold by T_3. The most abundant deiodinase detected in GH3 cells by the array was the Type I deiodinase (Dio1), and T_3 induced the expression of this gene nearly 8 fold. Type II deiodinase expression was very low, but significantly expressed above background, whereas Type III deiodinase expression was not detected. Of several putative TH transporters included on the array, only the L-type amino acid transporter LAT1 and its heterodimer partner 4F2hc were significantly expressed, whereas other known transporters such as MCT8 and OATP1c1 were not detected.

To determine a full set of genes differentially regulated by T_3, we expanded our analysis of the array to focus on genes that were significantly expressed and changed at least 2-fold up or down compared to the vehicle treated control cells. We identified 207 genes two-fold up-regulated and 312 genes two-fold down-regulated by T_3, including several previously known TH response genes from other cell types and tissues including Dio1. Genes induced over four-fold are shown in **Table 3**.

qPCR was used to validate expression of selected genes from the array, confirming the pattern and magnitude of up-regulation of the hairless and IGFBP3 genes, and down-regulation of the Aurora B kinase (AURKB) gene (**Figure 4**).

Table 2. Expression of selected thyroid hormone signaling pathway components in GH3 cells detected by Illumina microarray.

Gene	Definition	Average signal, Control	Average signal, T_3
	Nuclear receptors		
Thra	Thyroid hormone receptor alpha	1356.8	1164.7
Thrb	Thyroid hormone receptor beta	2448.1	1562.9
Rxra	Retinoid-X receptor alpha	ND	ND
Rxrb	Retinoid-X receptor beta	1465.0	2028.9
Rxrg	Retinoid-X receptor gamma	245.2	203.7
	Nuclear receptor coregulators		
Ncoa1	Nuclear receptor coactivator 1 (SRC-1)	2021.3	4243.0
Ncoa2	Nuclear receptor coactivator 2 (SRC-2, Tif2)	ND	ND
Ncoa3	Nuclear receptor coactivator 3 (SRC-3, ACTR)	ND	ND
Ncor1	Nuclear receptor corepressor 1 (NCoR)	602.2	723.1
Ncor2	Nuclear receptor corepressor 2 (SMRT)	527.0	655.8
	Deiodinases		
Dio1	Deiodinase, Type I	238.2	1872.7
Dio2	Deiodinase, Type II	56.3	65.4
Dio3	Deiodinase, Type III	ND	ND
	TH transporters		
SLC16A2	Solute carrier family 16, member 2 (MCT8)	ND	ND
SLCO1C1	Solute carrier organic anion transporter family, member 1C1 (OATP1c1)	ND	ND
SLC3A2	Solute carrier family 3, member 2 (4F2hc)	8997.8	7666.7
SLC7A5	Solute carrier family 7, member 5 (LAT1)	2737.5	1606.2
	Housekeeping genes		
Rpl8	Ribosomal protein L8	32692.9	34535.4
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase	3580.6	3878.3

Table 3. List of genes up regulated over 4 fold by T_3 in GH3 cells.

Gene	Definition	T_3 Fold change
RGD1562107	PREDICTED: similar to class-alpha glutathione S-transferase	37.5
Es1	esterase 1	13.2
LOC686871	PREDICTED: similar to B-cell leukemia/lymphoma 3 (Bcl3)	11.8
Slc14a1	solute carrier family 14 (urea transporter), member 1	10.2
LOC681126	PREDICTED: similar to keratin complex 2, basic, gene 25, transcript variant 1	9.2
RGD1566155	PREDICTED: similar to 2610030H06Rik protein	8.9
Dio1	deiodinase, iodothyronine, type I	7.9
Prm2	protamine 2	7.6
LOC363618	PREDICTED: similar to heparan sulfate (glucosamine) 3-O-sulfotransferase 3A1	7.6
RGD1562954	PREDICTED: similar to aldo-keto reductase family 1, member C12	6.3
Actn2	PREDICTED: actinin alpha 2	5.8
Shh	sonic hedgehog	5.4
Abcb1b	ATP-binding cassette, sub-family B (MDR/TAP), member 1B	5.3
LOC683720	PREDICTED: similar to keratin 6L	5.3
Epas1	endothelial PAS domain protein 1	5.2
Pgf	placental growth factor	5.1
RGD1565373	PREDICTED: similar to CD69 antigen (p60, early T-cell activation antigen)	4.8
Hr	hairless homolog	4.8
Sfxn5	sideroflexin 5	4.6
Irak2	interleukin-1 receptor-associated kinase 2	4.5
Sstr2	somatostatin receptor 2	4.5
Rims1	regulating synaptic membrane exocytosis 1	4.4
Tnnc2	troponin C type 2 (fast)	4.3
RGD1564287	PREDICTED: similar to mKIAA0704 protein	4.3
Bhlhb2	basic helix-loop-helix domain containing, class B2	4.2
Igfbp3	insulin-like growth factor binding protein 3	4.1
Lgals3	lectin, galactose binding, soluble 3	4.1
Acta1	actin, alpha 1, skeletal muscle	4.1
Epn3	epsin 3	4.0
Bcar3	PREDICTED: breast cancer anti-estrogen resistance 3	4.0
Vdr	vitamin D (1,25- dihydroxyvitamin D3) receptor	4.0

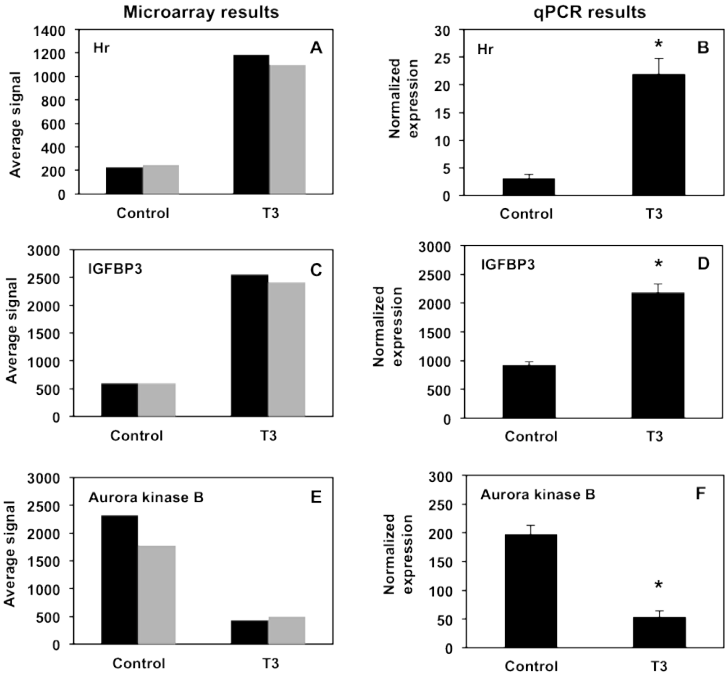

Figure 4. Validation of selected response gene expression changes from the microarray analysis by quantitative RT-PCR. Comparison of microarray derived gene expression changes (A, C, and E; black bars, value of one replicate; gray bars, value of second replicate) versus quantitative PCR derived gene expression changes (B, D, and F). Selected genes were hairless (Hr, A and B), insulin-like growth factor binding protein 3 (IGFBP3, C and D), and Aurora kinase B (E and F). Control and 1 nM T_3 treated cells were used for qPCR (B, D, and F) and for microarray experiment (A, C, E). *, $p < 0.05$ vs. untreated controls.

We then investigated whether there were enriched T_3 regulated biological pathways in GH3 cells, using the web based DAVID program (**Table 4**). The most over-represented pathways for T_3 up-regulated genes cluster into two basic categories: 1) responses to extracellular (i.e. hormonal) signals, and 2) blood vessel development, or angiogenesis. The response to extracellular signals cluster includes several nuclear receptors, such as the estrogen receptor α, retinoic acid receptor α, and Vitamin D receptor genes, as well as the previously noted SRC-1 nuclear receptor coactivator genes. The blood vessel development cluster includes regulators of angiogenesis such as Endothelial PAS domain-containing protein 1 (Epas1), or HIF2α, placental growth

factor (Pgf), a member of the VEGF family, and angiopoietin 1 (Angpt1).
Unexpectedly, the most over-represented pathways for T_3 down-regulated genes were related to cell cycle control, including multiple cell division cycle genes (cdca1, cdca3, cdca7, cdc2, cdc6, cdc20, and cdc23) that are active at various stages of the cell cycle, multiple minichromosome complex maintenance genes (MCM2-7, MCM10) involved in origin of replication licensing at S phase, and Aurora kinase B (AURKB) involved in chromatin condensation at M phase. IGF-1 and associated IGFBPs have been previously shown to be up-regulated by T_3 in other pituitary tumor cell lines, and are proposed to be mediators of the proliferative response of these cells to the hormone (Gilchrist et al. 1995). Indeed, IGFBP3 was up-regulated by 1 nM T_3 (**Table 3**, **Figure 4C**, **D**). To investigate the apparent paradox of induced proliferation in the face of repressed expression of cell cycle genes, we examined expression of IGF-1 (not represented on the array) and a marker of proliferation (AURKB) both at 24 hours prior to the time point chosen for the array, and at 96 hours, just prior to when proliferation was measured by the resazurine/resorufin assay.
As shown in **Figure 5**, we found that IGF-1 is modestly induced 24 and 96 hours after exposure, consistent with previous reports, whereas AURKB is repressed 24 hours (and at 48 hours, see **Figure 4**) after exposure, but not 96 hours after exposure. This demonstrates that the early repressive effect of T_3 on cell cycle gene transcription might be overcome by induced growth factor expression and return of cell cycle gene expression to baseline.

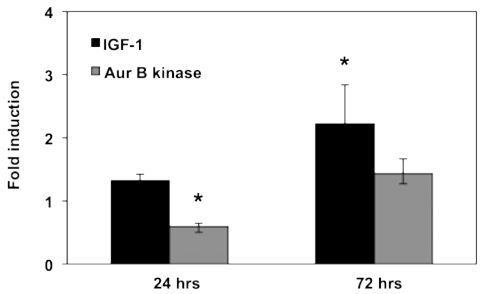

Figure 5. T_3 induces delayed IGF-1 expression while transiently suppressing Aurora B kinase expression in GH3 cells. GH3 cells were cultured in PCM media, then treated with DMSO or T_3 for 24 or 72 hours. RNA was isolated for quantitative RT-PCR for the IGF-1 (black bars) or Aurora kinase B (gray bars) genes, normalized to rpL8 expression. Error bars represent SD. *, $p < 0.05$ vs. untreated controls.

Table 4. Top ranking Gene Ontology terms for T_3 up- and down-regulated genes.

Biological Process Term	Count	%	P Value
Up-regulated genes			
GO:0048545~response to steroid hormone stimulus	13	11.11	3.86E-06
GO:0010033~response to organic substance	22	18.80	1.32E-05
GO:0009719~response to endogenous stimulus	17	14.53	1.34E-05
GO:0001944~vasculature development	11	9.40	2.14E-05
GO:0009725~response to hormone stimulus	15	12.82	5.91E-05
GO:0001525~angiogenesis	8	6.84	6.11E-05
GO:0001568~blood vessel development	10	8.55	1.01E-04
GO:0048514~blood vessel morphogenesis	9	7.69	1.30E-04
GO:0007242~intracellular signaling cascade	20	17.09	1.35E-04
GO:0001666~response to hypoxia	9	7.69	1.57E-04
GO:0009991~response to extracellular stimulus	11	9.40	1.63E-04
Down-regulated genes			
GO:0007049~cell cycle	28	18.42	3.36E-15
GO:0022403~cell cycle phase	20	13.16	2.13E-13
GO:0000279~M phase	18	11.84	2.29E-13
GO:0000087~M phase of mitotic cell cycle	14	9.21	8.75E-12
GO:0022402~cell cycle process	21	13.82	4.64E-11
GO:0006260~DNA replication	14	9.21	8.27E-11
GO:0007067~mitosis	13	8.55	9.49E-11
GO:0000280~nuclear division	13	8.55	9.49E-11
GO:0000278~mitotic cell cycle	17	11.18	1.56E-10
GO:0048285~organelle fission	13	8.55	1.89E-10
GO:0006259~DNA metabolic process	20	13.16	2.97E-10

DISCUSSION

In this study, we demonstrate that the reporter gene assay developed in GH3.TRE-LUC cells is an excellent surrogate for several specific endogenous gene responses to T_3, isotype thyromimetic drugs, and environmental endocrine disrupting chemicals. The reporter assay provides greater speed and more specificity than the previously developed T-screen that relies on an indirect detection of cell proliferation. Since induction or inhibition of cell proliferation could occur via multiple pathways unrelated to the activity of the TRs, development of more specific assays for TR function, such as the reporter system described here that closely mimics endogenous gene behaviors, is highly desirable. In comparing the T-screen proliferation results to the reporter and endogenous gene (GH) assays, we found close concordance among the assays, including the same rank order of potency of T_3 vs. two thyromimetic isotype selective compounds, GC-1 and CO23 (T_3>GC-1>>CO-23). Thus, for these compounds, the reporter gene assay is highly predictive of both a phenotypic response (proliferation) and an endogenous gene response, but in a much more convenient format that is adaptable for high-throughput assay development. Additionally, our results with the isotype selective ligands suggest that the GH3 cellular responses induced by T_3 are predominantly TRβ mediated, due to the strong selectivity of GC-1 for TRβ over TRα. Additional experiments using both isotype and isoform selective siRNA knockdown approaches, for example, will be necessary to more fully address this issue.

In addition to T_3 and the selective thyromimetic agonists, reporter gene and endogenous gene responses were also similar for environmental TR antagonists, such as arsenic trioxide, BPA, and DEHP. In all three cases, T_3 induced reporter gene and GH gene expression was inhibited in a concentration-dependent manner. Another direct target gene, hairless, also showed a similar pattern of responses (data not shown). The inhibition of T_3 induced genomic responses in *Xenopus laevis* tadpole tissues reported previously for both arsenic and BPA (Heimeier and Shi 2010; Davey et al. 2008) is consistent with our results. One difference between the reporter gene and endogenous gene assays was that GH gene expression was induced at low

concentrations of arsenic but repressed at higher concentrations. Modest induction of the Dio1 gene in GH3 cells by arsenic was previously reported (Davey et al. 2008). DEHP also inhibits T_3 induced transcription in GH3 cells, an important observation given the ubiquitous presence of phthalates in the environment (Schettler, 2006) and consistent with other reports of links to potential TR disruption, including reporter gene assays (Shen et al. 2009; Sugiyama et al. 2005; Shi et al. 2012). Work on the endocrine disrupting activity of BPA and phthalates like DEHP have generally focused on ER rather than TR activity. Our results, as well as other *in vitro* and *in vivo* results, support further investigation into effects of phthalates on additional nuclear receptors, including the TRs. While the exact mechanisms underlying repression of T_3 induced transcription by compounds like arsenic, BPA, and DEHP is unknown at present, they may not necessarily involve direct interaction of the compounds with the TRs themselves. Rather, these and related compounds may affect TR mediated transcriptional control via interference with associated coactivator or corepressor interactions, post-translational modifications and/or activity, or indirectly via membrane TH transport or deiodinase activity. For example, arsenic has been shown to affect corepressor SMRT phosphorylation and nuclear translocation (Hong et al. 2001). Since GH3 cells naturally respond to T_3, these cells present a good *in vitro* model system to investigate the mode of action of these and other environmentally relevant chemicals affecting TH signaling.

Given the importance of GH3 cells as an *in vitro* model for TR action, and their use as a screening system for TH disruption, a fuller understanding of the T_3 induced gene expression responses in these cells is important. Further, while the GH gene has proven to be a useful marker of T_3 action in these cells, other hormones and signaling cascades may influence GH expression as well; for example glucocorticoids, cAMP, and retinoids among others (Brent et al. 1988; Bedo et al. 1989). Therefore, we conducted a microarray analysis to delineate the T_3 induced transcriptome in these cells. Our microarray analysis revealed a number of important characteristics regarding the responsiveness of these cells to T_3. First, the array detected expression of both TR isotypes, and GH3 cells have been shown to express TRα1 and the non-ligand binding TRα2 splicing isoform, and two TRβ splicing isoforms (TRβ1 and TRβ2) (Ball et al. 1997; Hahn et al. 1999; Lazar, 1990). In addition to the TRs, the array indicated

that these cells express two RXR isotypes (β and γ), at least one nuclear receptor co-activator which is T_3 inducible (SRC-1), and two nuclear receptor co-repressors (NCoR and SMRT), again in agreement with previous published findings in GH3 cells (Misiti et al. 1998; Haugen et al. 1997; Davis and Lazar 1992). Type I deiodinase (Dio1) expression was both detected in controls and strongly induced by T_3, with lower expression of the Type II deiodinase (Dio2). The T_3 and T_4-inactivating Type III deiodinase (Dio3) was not detected in these experiments, although a probe was included on the array. Expression of the TRs, RXRs, and associated coregulators, the low level of Dio3 expression, and abundant heterodimeric LAT1/4F2hc TH transporter expression, is consistent with the high sensitivity of these cells to T_3.

In terms of the T_3 induced gene expression program in these cells, in general, up-regulated genes mostly fell into two general categories: genes often induced by various extracellular signals, including hormones, and genes involved in angiogenesis or as a response to hypoxia. In the former group, there were several genes identified that have been identified as TH responsive genes in several different contexts; these genes may be considered a common "core" that when taken together, reflect the presence of T_3 and a functional TR. For example, the aforementioned Dio1 gene (Zhang et al. 1998), the B-cell leukemia/lymphoma 3 (Bcl3) (Choi et al. 2008), sonic hedgehog (Shh) (Desouza et al. 2011), multidrug resistance (Abcb1b) (Kurose et al. 2008), the corepressor hairless (hr) (Thompson and Bottcher 1997), and Klf9/BTEB (Denver and Williamson 2009) genes (**Table 3**, supplemental **Table 1** can be found at https://dl.dropbox.com/u/1184226/GH3_array_1.5_fold_regulated_genes.xlsx) have all been identified as TH inducible in other cell types and contain functional TREs, adding additional confidence that the array detected bona fide T_3 responsive genes. Interestingly, a large group of T_3 up-regulated genes were linked to blood vessel formation and response to hypoxia. TH is a known inducer of angiogenesis in the mammalian heart and brain (Zhang et al. 2010) and the chick chorioallantoic membrane model (Mousa et al. 2005). Data from a recent series of papers support a non-genomic role for T_4 (and, to a certain extent, T_3) in the induction of angiogenesis and associated genes (reviewed in (Cheng et al. 2010; Moeller and Broecker-Preuss 2011)). Tumor growth has also been linked to non-genomic actions of T_4 (Mousa et al. 2005). Essentially, two non-genomic pathways mediating TH signaling have been

proposed: first, integrin αvβ3 binds to and is activated by T_4 at the plasma membrane, and blockade of T_4 binding by various means prevents downstream T_4 induced angiogenesis. GC-1 is also able to induce angiogenesis via integrin αvβ3 (Bergh et al. 2005). Second, cytoplasmic, liganded TRβ1 has been shown to interact with and activate PI3 kinase, inducing downstream pathways leading to HIF1α induction and subsequent activation of HIF1α targets, such as various glycolytic enzyme genes and angiogenic factors. With regards to TH signaling and gene expression in GH3 cells, as investigated here, there are several considerations relevant to understanding the underlying direct genomic versus nongenomic mechanisms at play. For example, tetraiodothyroacetic acid (Tetrac), another thyromimetic analog, acts as an antagonist at integrin αvβ3 and inhibits T_4 induced angiogenesis (Davis et al. 2009). However, Tetrac, like its related natural ligand T_4, is a weak but full agonist in both the GH3 cell proliferation and GH3.TRE-LUC reporter gene assays (Schriks et al. 2006; Freitas et al. 2011), indicating that these responses are mediated by classical genomic mechanisms via nuclear TR actions. Further, classical genomic pathways have been reported for T_3 induction of HIF1α in HepG2 cells, via prior induction of hepatic leukemia factor (HLF), a direct target gene of the TR (Otto and Fandrey 2008), although an HLF probe was not included on this array. In GH3 cells, HIF1α and several of its downstream targets (e.g. VEGFA, PFKP) are induced by T_3 (~1.5 fold), but only modestly (supplemental **Table 1**), whereas HIF2α (EPAS1) is over five-fold induced (**Table 3**) and shares some overlapping gene targets with HIF1α. To our knowledge, this is the first report of HIF2α as a TH responsive gene, and it will be interesting to examine its mechanism of up-regulation. Future experiments will be important to clarify the mechanism of induced angiogenesis/hypoxia related gene expression in this system, including the relative role of classical genomic versus non-classical but receptor mediated (TRβ/PI3K pathway) or integrin αvβ3 mediated pathways.

The analysis of the down-regulated gene program in GH3 cells presents somewhat of a paradox, certainly in explaining the proliferative response to T_3 in these cells. Under our experimental conditions, a large number of cell cycle related genes are repressed, rather than induced, compared to vehicle control after 48 hours of exposure. In contrast, other studies using related pituitary tumor cell lines (GC cells, for example), reported increases in cyclin and cdk gene expression upon T_3 treatment (Barrera-

Hernandez et al. 1999). One explanation for our findings is that repression of cell cycle genes may be transient, and eventually overcome by secondary induction of various growth factors, including IGF-1. Consistent with other studies in related cells, we observed a T_3 induced increase in both IGF-1 and IGFBP (IGFPB3) expression (Gilchrist et al. 1995) (**Figure 4** and **5**), although IGFBP3 has been reported to show both growth stimulatory and inhibitory properties (Jogie-Brahim et al. 2009). It is interesting to note that pituitary (thyrotrope) tumorigenesis is promoted *in vivo* by the expression of a dominant negative TRβ protein (TR^{PV}) that does not bind ligand, and that liganded wild type TRβ1 represses cyclin D2 expression (Furumoto et al. 2005). Thus, in GH3 cells, the wild-type TR mediated response may be inhibition of proliferation-associated genes that is offset by autocrine growth factor expression, allowing a net stimulation of proliferation. Responses of cell cycle related genes might also be sensitive to other variables that differ between our conditions and those used in other studies, such as plating density and media formulations (Gilchrist et al. 1995; Barrera-Hernandez et al. 1999).

In summary, we demonstrate good agreement between the T_3 inducible reporter gene in GH3.TRE-LUC cells and endogenous gene responses, in response to thyromimetic agonists and environmental chemicals that antagonize TR activity. Further, our data support the conclusion that the proliferative response and the T_3 induced transcriptional responses in GH3 cells appear to be largely TRβ mediated. These findings help to validate the use of these cells for high-throughput screening for clinically relevant agonist and antagonists or environmental toxicants affecting TH signaling, particularly mediated by the TRβ isotype. Recently a TRα-responsive reporter gene assay has been developed in HeLa cells, to be able to distinguish TRα and TRβ-specific effects of compounds (Freitas et al., 2012). Lastly, GH3 cells may provide a good experimental system to investigate the relative importance of genomic and nongenomic pathways leading to transcriptional responses to TH, of relevance not only for understanding the role of TH in angiogenesis and tumor progression, but also for modes of endocrine disrupting chemicals affecting TH activity.

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CHAPTER 5

HUMAN THYROID HORMONE RECEPTOR ALPHA REPORTER GENE CELL LINE FOR CHARACTERIZATION OF ENDOCRINE DISRUPTING COMPOUNDS

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ABSTRACT

Thyroid hormone (TH) disrupting compounds can interfere with critical processes such as brain development, growth and energy homeostasis. THs act via thyroid receptors (TR) TRα and TRβ, which have distinct physiological functions, therefore it is important to study the TR-specific potency of TH active compounds. This study describes the development of TRα and TRβ transient luciferase reporter gene bioassays using human cervical HeLa cells, in conjunction with the development of a stable TRα specific reporter gene cell line designated TRα.HeLa-Luc. Responses in this TRα cell line were compared to responses in the GH3.TRE-Luc cell line, which appears to contain TRβ as the major TR isoform. The TR isoform-selective chemicals CO23 and GC-1 induced responses according to the anticipated rank order T_3>CO23>GC-1 in the TRα.HeLa-Luc cells and T_3>GC-1>CO23 in GH3.TRE-Luc cells. In addition to 7 known TR-active standards, 10 chemicals suspected to have TR disrupting activity were tested. Of these, only sodium arsenite and T_3-like 4-OH-BDE69 induced a response in the TRα.HeLa-Luc assay. Together, the developed and validated TRα.HeLa-Luc and GH3.TRE-Luc stable reporter gene lines add a new level of specificity, representing an improvement of the available tools for *in vitro* high-throughput assessment of TR subtype-specific activity of drugs and environmental pollutants.

INTRODUCTION

The thyroid endocrine system is involved in a number of critical physiological processes that regulate brain development, growth and energy homeostasis (Bernal, 2007; Alkemade, 2010; Combs et al., 2011). The regulation of these processes is mediated by the thyroid hormones (THs) triiodothyronine (T_3) and to a lesser extent thyroxine (T_4), both small, lipophilic molecules built from tyrosine and iodine. The thyroid gland secretes mostly T_4; T_3 is produced from enzymatic deiodination of T_4 in peripheral tissues and is considered to be the active hormone form due to its high binding affinity to the thyroid hormone receptors (TRs). It is generally accepted that deiodination of T_4 is the major pathway through which T_3 bioavailability is regulated in mammalian tissues (Köhrle, 2000). THs induce their physiological effects via the TRs (Samuels and Tsai, 1974), which belong to the large super family of nuclear hormone receptors including the steroid hormone, vitamin D and retinoic acid receptors (Beato et al., 1995). These receptors are ligand-dependent transcription factors able to interact with DNA sequences known as response elements, usually located in the vicinity of target genes (Desvergne, 1994). TRs are expressed in virtually all cells, and encoded by two different genes: TRα (NR1A1) and TRβ (NR1A2), in humans on chromosomes 17 and 3 respectively. These two receptors differ in their relative and absolute tissue distribution, also depending on developmental stages (Bradley et al., 1989; Furlow and Neff, 2006), suggesting an important role for TR subtypes in mediating tissue specific responses to the hormone. Indeed, mouse knockout experiments have demonstrated both overlapping and distinct effects for each receptor isotype (Flamant and Samarut, 2003). In addition several isoforms of these receptors are generated through alternative splicing (Lazar, 1993). TRα1 is predominant in the central nervous system (CNS) and heart, and TRα2 is also expressed in the CNS but is unable to bind T_3 and may exert a weak dominant negative effect on TRα1 and TRβ isoforms (Katz and Lazar, 1993). TRβ1 is most prevalent in the liver and kidney and TRβ2 in the pituitary and hypothalamus (Yen, 2001). Since T_3 binding is nonselective towards any of the isoforms, an excess or shortage of THs may cause very different tissue-specific effects. For instance, the

administration of T_3 has a lowering effect on plasma cholesterol levels through TRβ1 activation (Pelletier et al., 2008), however cardiac side effects via the TRα1 such as tachycardia are sometimes difficult to avoid during TH replacement therapy. A TRβ1 selective agonist could be useful to treat chronic diseases such as obesity and hypercholesterolemia, avoiding therapeutic complications with cardiovascular or other predominantly TRα-regulated systems. As selectivity towards the α or β subtypes remains a paramount criterium for the development of such chemicals (i.e. Selective thyroid hormone receptor modulators or STORMS) (Flamant and Samarut, 2003), the possibility of a TR ligand with clinically useful properties makes these receptors very attractive targets for new drug development (Hirano and Kagechika, 2010; Scanlan, 2010).

In addition to clinical implications, understanding receptor isotype selectivity could also be important in determining and predicting the actions of endocrine disrupting chemicals. Xenobiotics defined as thyroid disrupting chemicals (TDCs) (Crofton, 2008) target the TH system by hampering the normal function of the thyroid gland (Hornung et al., 2010), interfering with enzymes linked with TH homeostasis (Barter and Klaassen, 1992), inhibiting plasma TH transport (Brouwer and van den Berg, 1986), altering TH tissue concentrations (Gereben et al., 2008) and ultimately modulating TR-regulated genes (Bogazzi et al., 2003; Amano et al., 2010). Some of these chemicals may act as TH analogs (Zoeller, 2005), binding the TR and disrupting the normal TR-mediated signaling in target cells. By analogy with the estrogen receptors (ER) (Veld et al., 2006), and given the successful development of isotype selective TR ligands, these same chemicals could also transactivate each TR subtype in a selective manner (Schriks et al., 2007) contributing to tissue and developmental stage specific disorders.

Assays attempting to discriminate between receptor subtype transcriptional effects have been previously reported, but these were restricted to green monkey fibroblasts (CV-1 cells) only transiently transfected with *Xenopus* TRs (Schriks et al., 2007), yeast reporter assay systems (Shiizaki et al., 2010) or the rat PC12 cell line expressing the avian TRα1 isoform (Jugan et al., 2007). Recently, we developed a stable transfected reporter gene assay based on the human pituitary adenoma GH3 cell line (Freitas et al., 2011) that is also used as basis for the T-screen (Hohenwarter et al., 1996; Gutleb et

al., 2005). The stably transfected GH3.TRE-Luc cells allow sensitive and rapid screening of TR agonists and antagonists (Freitas et al., 2011). Given that TRβ appears to be the more abundant TR form present in GH3 cells (Hahn et al., 1999) the stably transfected GH3.TRE-Luc cells is likely to detect especially TRβ mediated responses. This implies that a stable TRα specific reporter gene assay in a human cell line is missing. To allow future high-throughput identification of potential TRα1 specific compounds, in the present study we developed a new stably transfected bioassay based on the human uterine cervical cancer cells (HeLa). The cells were stably co-transfected with the receptor subtype TRα1 and the same TR-regulated reporter gene vector used for the development of the GH3.TRE-Luc cell line. For validation of the TR-specificity of the stably transfected TRα-HeLa.Luc and GH3.TRE-Luc cell lines, their responses to model isoform-selective ligands (CO23 and GC-1) (Chiellini et al., 1998; Ocasio and Scanlan, 2006) were compared with the rank order of potency in transient TR isoform-specific reporter gene assays we established in the HeLa cell line. In addition to 7 known TR-active standards, 10 chemicals suspected to have TR disrupting activity, were also tested for their potential TR subtype specificity.

MATERIALS AND METHODS

Chemicals. All chemicals were of ≥98% purity unless stated otherwise. 3,3′,5-triiodo-L-thyronine (T_3, CAS no. 6893-02-3), 3,3′,5,5′-tetraiodo-L-thyronine (T_4, CAS no. 51-48-9), 3,5,3',5'-tetraiodothyroacetic acid (Tetrac, CAS no. 67-30-1), 3,5,3'-triiodothyroacetic acid (Triac, CAS no. 51-24-1), 9-cis-retinoic acid (CAS no. 5300-03-8), 2-butyl-3-benzofuranyl-4-(2-(diethylamino)ethoxy)-3,5-diiodophenyl ketone hydrochloride (Amiodarone, CAS no. 19774-82-4) and sodium arsenite (CAS no. 7784-46-5) were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, Netherlands) and 4,4′-diiodobiphenyl (DIB, CAS no. 3001-15-8) from ABCR GmbH & Co (Karlsruhe, Germany). 2,4,4'-Tribromodiphenyl ether (BDE 28, CAS no. 41318-75-6), 2,2′,3,3′,4,4′,5,5′,6-nonabromodiphenyl ether (BDE 206, CAS no. 63387-28-0), T_3-like 4′-hydroxy-2,3′,4,6-tetrabromodiphenylether (4-OH-BDE 69, CAS no. 218303-98-1) were kindly provided by Åke Bergman (Stockholm University, Sweden). (2-Butyl-1-benzofuran-3-yl)-[4-(2-ethylaminoethoxy)-3,5-diiodophenyl]methanone (Desethyl-amiodarone, CAS no. 83409-32-9) and 2-butyl-3-[4-[3-(butylamino)propoxy]benzoyl]-5-benzofuranyl]-methanesulfonamide (Debutyldronedarone, CAS no. 141626-35-9) were a kind gift from Anita Boelen (Amsterdam University, The Netherlands). 2-[4-({3-[2-(4-aminophenyl)ethynyl]-4-hydroxy-5-(propan-2-yl)phenyl}methyl)-3,5-dimethylphenoxy] acetic acid (NH-3, CAS no. 447415-26-1), 5-({4-[4-Hydroxy-3-(propan-2-yl)phenoxy]-3,5-diiodophenyl}methyl)imidazolidine-2,4-dione (CO23, CAS no. 9116 89-00-4) and 2-(4-{[4-hydroxy-3-(propan-2-yl)phenyl]methyl}-3,5-dimethylphenoxy) acetic acid (GC-1, CAS no. 211110-63-3) were kindly provided by Thomas S. Scanlan (Oregon Health Sciences University, USA) and synthesized as previously described (Chiellini et al., 1998; Nguyen et al., 2002; Ocasio and Scanlan, 2006). All compounds were dissolved in dimethylsulfoxide (DMSO, 99.9%; Acros Organics, Geel, Belgium) and kept in the dark. Stocks were stored at −20°C until used. **Figure 1** illustrates the chemical structure of the compounds used in this study.

Plasmids. The carrier DNA pBluescript SK- (Genbank Accession# X52324) was purchased from Promega (Madison, WI) and the green fluorescent protein (GFP)

vector pCS-GFP3 was a kind gift from Enrique Amaya (University of Manchester, UK). The TR-regulated luciferase reporter plasmids pGL4CP-SV40-2xtaDR4 and pGL4CP-TK-2xtaDR4 (courtesy of Michael L. Goodson and Martin Privalsky, University of California, Davis, USA) and the TRα1 and TRβ1 expression vectors pCI-neo-TRα1 and pCI-neo-TRβ1 (provided by Ivan H. Chan and Martin Privalsky, University of California, Davis, USA) were designed as previously described (Chan and Privalsky, 2009; Freitas et al., 2011).

Transfections. In transiently transfected cells, the maximal T_3-induced luciferase activity using the pGL4CP-SV40-2xtaDR4 reporter gene vector was approximately 6× higher than cells transfected with pGL4CP-TK-2xtaDR4 (data not shown). Without the pCI-neo-TRα1 vector, the luciferase induction in the HeLa cells transiently transfected with pGL4CP-SV40-2xtaDR4 displayed a more than 30× lower fold-induction to T_3 compared to cells transfected with both plasmids (data not shown). Given the low endogenous TR activity and the superior induction factor of the SV40 based reporter gene, HeLa cells were stably transfected with the expression vector pCI-neo-TRα1 and the reporter plasmid pGL4CP-SV40-2xtaDR4 to create the TRα.HeLa-Luc cell line. The stable cell line GH3.TRE-Luc was prepared as previously described (Freitas et al., 2011). The human uterine cervix cancer cell line (HeLa) was routinely sub-cultured once a week in fresh Minimum Essential Medium (MEM, Gibco, Paisley, Scotland) supplemented with 10% Fetal Calf Serum (FCS, Gibco, Paisley, Scotland) and 2 mM L-glutamine (Gibco, Paisley, Scotland) in 75 cm^2 culture flasks (Corning, Schiphol-Rijk, The Netherlands), in a humid atmosphere at 37°C and 95% air / 5% CO_2. Twenty-four hours before transfection, cells were seeded in regular growth medium into new 75 cm^2 culture flasks at a density of 3×10^6 cells per flask. Transfections were performed with Lipofectamine 2000 according to the manufacturer's instruction (Invitrogen, Paisley, Scotland). The responsiveness of the reporter gene vectors pGL4CP-SV40-2xtaDR4 and pGL4CP-TK-2xtaDR4, containing different minimal promoters driving the TR-dependent luciferase expression, were compared. Cells were transfected with 3 μg of each desired vector (pCS-GFP3; pGL4CP-SV40-2xtaDR4 or pGL4CP-TK-2xtaDR4; and pCI-neo-TRα1 or pCI-neo-TRβ1) and pBluescript SK- as filler DNA, up to a total of 15 μg per flask. Six hours after transfection, cells were allowed to recover

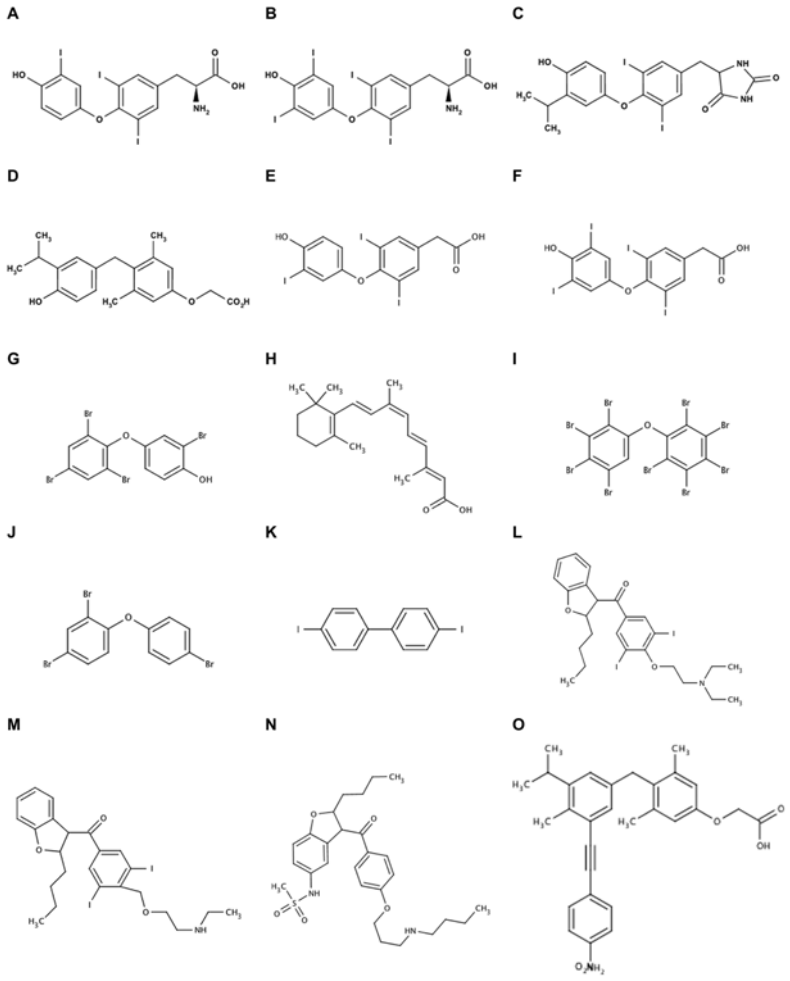

Figure 1. Chemical structures of A) 3,3′,5-triiodo-L-thyronine (T_3), B) 3,3′,5,5′-tetraiodo-L-thyronine (T_4), C) 5-({4-[4-hydroxy-3-(propan-2-yl)phenoxy]-3,5-diiodophenyl}methyl)imidazolidine-2,4-dione (CO23), D) 2-(4-{[4-hydroxy-3-(propan-2-yl)phenyl]methyl}-3,5-dimethylphenoxy) acetic acid (GC-1), E) 3,5,3'-triiodothyroacetic acid (Triac), F) 3,5,3',5'-tetraiodothyroacetic acid (Tetrac), G) T3-like 4′-hydroxy-2,3′,4,6-tetrabromodiphenylether (4-OH-BDE 69), H) 9-cis-retinoic acid, I) 2,2′,3,3′,4,4′,5,5′,6-nonabromodiphenyl ether (BDE 206), J) 2,4,4'-tribromodiphenyl ether (BDE 28), K) 4,4′-diiodobiphenyl (DIB), L) 2-butyl-3-benzofuranyl-4-(2-(diethylamino)ethoxy)-3,5-diiodophenyl ketone hydrochloride (Amiodarone), M) 2-butyl-1-benzofuran-3-yl)-[4-(2-ethylaminoethoxy)-3,5-diiodophenyl]methanone (Desethylamiodarone), N) 2-butyl-3-[4-[3-(butylamino)propoxy]benzoyl]-5-benzofuranyl]-methanesulfonamide (Debutyldronedarone), O) 2-[4-({3-[2-(4-aminophenyl)ethynyl]-4-hydroxy-5-(propan-2-yl)phenyl}methyl)-3,5-dimethylphenoxy] acetic acid (NH-3).

in phenol red-free Dulbecco's Modified Eagle's medium/Ham's F12 (DMEM:F12, Gibco, Paisley, Scotland) supplemented with 5% dextran-coated charcoal-treated Fetal Calf Serum (DCC-FCS, HyClone), for 24 hours before exposure.
For development of stable transfectants, transient transfected cells were transferred 1:10 into fresh growth medium (MEM with 10% FCS and 2 mM L-glutamine) 24 hours after transfection. Forty-eight hours post-transfection, standard growth medium was replaced with selective medium containing 400 μg/ml of geneticin (Gibco, Paisley, Scotland). Single geneticin resistant colonies were harvested using cloning rings and allowed to proliferate until 100% confluency in 12-well plates. The clone with the highest inducible luciferase activity was selected by plating cells at 100% confluency in white clear bottom 96-well microplates (Packard View-Plate, PerkinElmer, Groningen, The Netherlands) and exposing to 0, 1, 10 and 100 nM of T_3 for 24 hours. Luciferase activity was determined as described below.

Stable or transient luciferase reporter gene assays. Stably transfected TRα.HeLa-Luc cells were seeded at 80% confluency in 75 cm^2 culture flasks in MEM supplemented with 10% FCS and 400 μg/ml of Geneticin. Twenty-four hours later, medium was replaced with phenol red-free DMEM:F12 containing 5% DCC-FCS for an additional period of 24 hours. Cells were then harvested by trypsinization and seeded into white clear bottom 96-well plates at a density of 3×10^4 cells per well and incubated for 24 hours with or without the indicated test chemical added from a 2× concentrated stock solution in DMSO.
The GH3.TRE-Luc assay was performed as described (Freitas et al., 2011) with modifications. Cells were seeded at 80% confluency in 75 cm^2 culture flasks in DMEM:F12 supplemented with 10% FCS and 320 μg/ml of geneticin. Twenty-four hours later, medium was replaced with phenol red-free DMEM:F12 containing 5% DCC-FCS for an additional period of 24 hours. Cells were then harvested by trypsinization, instead of scraping. Subsequently, cells were seeded into white clear bottom 96-well plates at a density of 3×10^4 cells per well and exposed for 24 hours to dilutions of the compounds added from a 2× concentrated stock solution in DMSO to culture medium.
Transient transfected HeLa cells were harvested by trypsinization, 24 hours after

recovery of the transfection. Cells were then seeded in phenol red-free DMEM:F12 supplemented with 5% DCC-FCS into white clear bottom 96-well plates at a density of 3×10^4 cells per well and exposed for 24 hours.
Both stably and transiently transfected cells were exposed in the presence (1 nM) or absence of T_3. The DMSO concentration in exposure medium was kept the same for all exposures within an experiment, in all cases ≤0.2% (v/v). A T_3 standard curve ranging from 0 to 100 nM was included in each experiment, in addition to 3 calibration points (0, 1 nM and 100 nM T_3) included on all exposure plates.

Cell viability and luciferase activity measurements. Cell toxicity and proliferation were determined per well in each exposure microplate by measuring total cellular metabolic activity using the reduction of resazurine to the fluorescent resorufin as previously reported (O'Brien et al., 2000; Schriks et al., 2006). For the GH3.TRE-Luc cells serum free medium (PCM) yielded the highest induction factor because of the very low baseline levels of luciferase expression. The stable TRα.HeLa-Luc cells, however, did not perform well in serum free medium (data not shown), with a high degree of well-to-well variation. Therefore, in order to make valid comparisons, both cell lines were exposed in medium (DMEM:F12) without phenol red and supplemented with DCC-FCS to reduce background induction by low levels of thyroid hormones present in FCS (Cao et al., 2012). To the exposure medium of each well 10 μl of a 400 μM resazurine (Sigma-Aldrich Chemie B.V., Zwijndrecht, Netherlands) solution was added and after 2 hours of incubation in the dark (37 °C; 95% air/5% CO2) fluorescence was measured at λex = 530 nm and λem = 590 nm (Millipore Cytofluor 2350 Fluorescence Measurement System). Subsequently, the same plates were used for luciferase determination. Cells were lysed in low salt buffer (10 mM Tris, 2 mM DTT and 2 mM CDTA; pH 7.8) or lysis buffer (25 mM Tris, 2 mM DTT, 2 mM CDTA, 10 glycerol and 1% Triton x-100; pH 7.8) and luciferase activity was measured using the glow methodology, in a microplate luminometer with two injectors (Thermo LabSystems luminoskan Ascent) (Murk et al., 1998). For measurement of luciferase activity a glow instead of a flash luminescence type assays was used since in the GH3.TRE-Luc line T_3-like 4-OH-BDE 69 was able to induce a 76% T_3-maximum response in the glow assay against a maximum of only 22% in the

flash assay, with similar EC_{50} of 431±33 nM and 391±27 nM respectively (data not shown). Flash luminescence is based on a short-lived reaction that generates a rapid peak of measurable light within seconds, while glow luminescence is supported by a slower rate reaction producing a stable signal for up to several minutes. Since no other differences were found, the glow methodology was used in further measurements. Furthermore, since GH3.TRE-Luc cells display a slight T_3-dependent cell proliferation during the 24 h exposure (Freitas et al., 2011), a phenomenon not observed for the TRα.HeLa-Luc cells, all luciferase activities were normalized to the number of cells present at the end of the 24 hours incubation.

Data and statistical analysis. Data shown correspond to the mean ± standard deviation (SD) of three independent experiments expressed as relative light units. Absolute values for the vehicle control (DMSO) were subtracted for all data on the same plate.

The dose-response curves were characterized by the half maximal effect concentration (EC_{50}). Triiodothyronine equivalence factors (T_3EF_{50}) for each compound were determined as the ratio between the EC_{50} of T_3 and the EC_{50} of the compound. Compounds are considered to be not active when the response is lower than the vehicle control plus 2× the standard deviation, which was defined as limit of detection (Veld et al., 2006).

Percentages of maximal luciferase induction for each test compound were calculated by setting luciferase response to solvent control (DMSO) as 0% and the maximum luciferase induction by T_3 as 100% in the same experiment. The induction factor is calculated as the highest response divided by the response of the solvent control, after subtraction of the plate background.

Statistical significance was tested by one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls test. Differences were considered significant at $p<0.05$. Curve fitting and EC_{50} values were computed using GraphPad Prism version 5 for Mac OSX (GraphPad Software, San Diego California USA, www.graphpad.com).

RESULTS

HeLa cells were stably transfected with the expression vector pCI-neo-TRα1 and the reporter plasmid pGL4CP-SV40-2xtaDR4 to create the TRα.HeLa-Luc cell line. Clones were obtained via geneticin selection. The experimental conditions for chemical exposures and luciferase activity measurements were thoroughly optimized. Both stably transfected HeLa-based TRα.HeLa-Luc and GH3-based GH3.TRE-Luc cell lines responded to T_3 in a dose-dependent manner (**Figure 2A**), with the maximum induction reached at 100 nM of T_3 in each line.

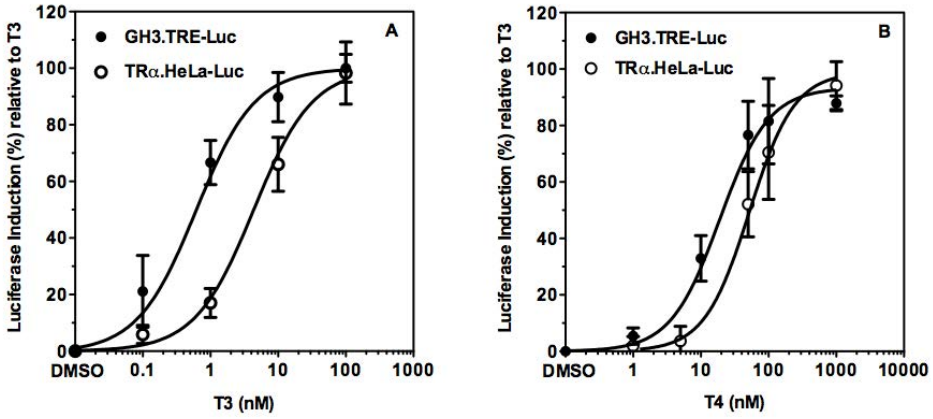

Figure 2. TRα.HeLa-Luc (o) and GH3.TRE-Luc (●) induction after 24 h exposure to A) T_3 and B) T_4. Luciferase activity expressed relative to T_3 maximal induction (at 100 nM set at 100%, DMSO set at 0%). Data represent mean ± SD of triplicate experiments.

Based on the EC_{50} level T_3 was approximately 7× more potent in the GH3.TRE-Luc compared to the TRα.HeLa-Luc cells (**table 1**) and comparable to the EC_{50}'s of the transiently transfected cells (t)TRα.HeLa-Luc and (t)TRβ.HeLa-Luc respectively (**Table 1**, **Figures 3B** and **4B**).

The potency of T_4 was 2.7 fold less in the TRα.HeLa-Luc compared to the GH3.TRE-Luc cells (**Table 1**, **Figure 2B**), reaching a maximum induction of approximately 90% of T_3-maximum response in both assays. The average coefficient of variation (CV) of

triplicate measurements is 5% for all compounds in both cell lines.

To examine whether the TRα.HeLa-Luc and GH3.TRE-Luc lines can discriminate between known subtype selective ligands, we used the α and β-selective agonists, CO23 and GC-1 respectively (Chiellini et al., 1998; Ocasio and Scanlan, 2006). Both compounds were able to induce a full dose-response curve up to 80% induction of T_3-maximum response in both stable cell lines (**Figures 3A** and **4A**), but the TRα-selective agonist CO23 had a 2.8× lower EC_{50} in the TRα.HeLa-Luc than in the GH3.TRE-Luc cells. In the TRα.HeLa-Luc cells the β-selective agonist GC-1 had a 10× higher EC_{50} than in the GH3.TRE-Luc cells (**Table 1**).

Table 1. EC_{50} (half maximal effective concentration), T_3EF_{50} (triiodothyronine equivalence factors) and maximal induction (expressed relative to T_3 set at 100%) values determined in the stably transfected cell lines TRa.HeLa-Luc and GH3.TRE-Luc. Each experiment was performed at least three times in triplicate.

Compound	TRα.HeLa-Luc			GH3.TRE-Luc			$T_3EF_{50}\alpha$/ $T_3EF_{50}\beta$
	EC_{50} (nM)	T_3EF_{50}	Max. induction (%)	EC_{50} (nM)	T_3EF_{50}	Max. induction (%)	
T_3	4.4±0.6	1	100 (100nM)	0.6±0.08	1	100 (100nM)	1
T_4	52±11	0.08	94	19±3	0.03	88	2.7
Triac	0.4±0.09	11	93	0.2±0.08	3.8	92	3.0
Tetrac	109±14	0.04	87	8.8±0.6	0.07	91	0.6
4-OH-BDE69	155±23	0.03	26	431±33	0.001	78	20
CO23	12±2	0.4	80	33±5	0.02	77	20
GC-1	14±3	0.3	49	1.4±0.2	0.4	91	0.7
9-cis-retinoic acid	-	-	3.8	-	-	19	-
BDE206	-	-	-	-	-	-	-
BDE28	-	-	-	-	-	-	-
DIB	-	-	-	-	-	-	-
Antagonists IC_{50} in the presence of 1 nM T_3							
NH-3	300±34		-31	112±19		-80	-
Sodium Arsenite	94.4±11		-98	2.4±0.8		-99	-
Amiodarone	-	-	-	-	-	-	-
Desethylamiodarone	-	-	-	-	-	-	-
Debutyldronedarone	-	-	-	-	-	-	-

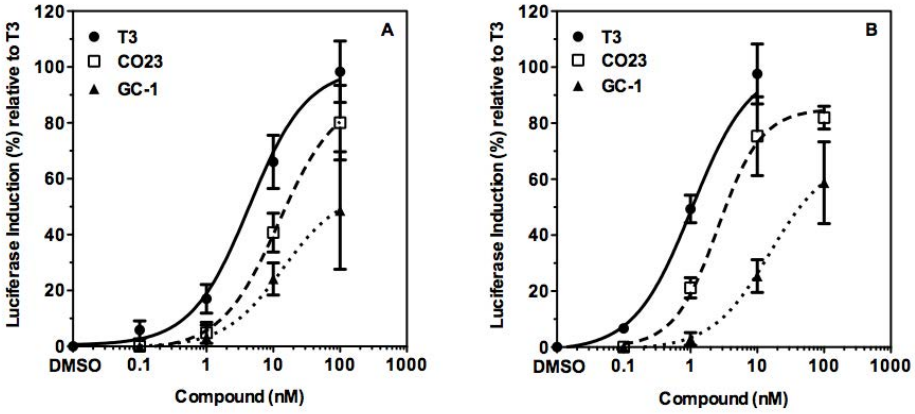

Figure 3. Luciferase induction in A) TRα.HeLa-Luc and B) transiently transfected (t)TRα.HeLa-Luc cells after 24 h exposure to T_3 (●), CO23 (□) and GC-1 (▲). Luciferase activity expressed relative to T_3 maximal induction (at 100 nM set at 100%, DMSO set at 0%). Data represent mean ± SD of triplicate experiments.

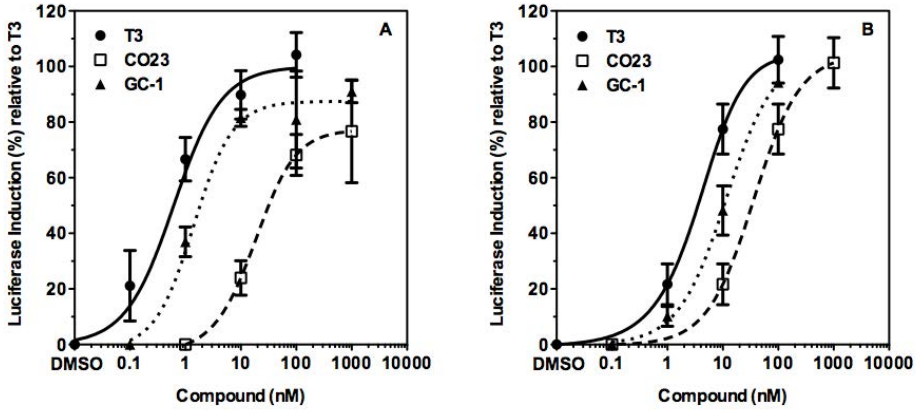

Figure 4. Luciferase induction in A) GH3.TRE-Luc and B) transiently transfected (t)TRβ.HeLa-Luc cells after 24 h exposure to T_3 (●), CO23 (□) and GC-1 (▲). Luciferase activity expressed relative to T_3 maximal induction (at 100 nM set at 100%, DMSO set at 0%). Data represent mean ± SD of triplicate experiments.

In GH3.TRE-Luc cells, GC-1 essentially induced the T_3-maximum response (91%) against a maximum of only 49% in the TRα.HeLa-Luc cell line. Therefore, the potency rank for the selective agonists compared to T_3 was as expected, with T_3>CO23>GC-1 for TRα.HeLa-Luc and T_3>GC-1>>CO23 for the GH3.TRE-Luc cells. These findings are also supported by examining dose response curves in transiently (t) transfected HeLa cells exposed to the same α and β-selective agonists displayed the same order of relative potencies, T_3>CO23>GC-1 for (t)TRα.HeLa-Luc and T_3>GC-1>CO23 for (t)TRβ.HeLa-Luc (**Table 2**, **Figure 3B** and **4B**).

Table 2. EC_{50} (half maximal effective concentration), T_3EF_{50} (triiodothyronine equivalence factors) and maximal induction (expressed relative to T3 set at 100%) values determined in the transiently (t) transfected cell lines TRa.HeLa-Luc and TRb.HeLa-Luc. Each experiment was performed at least three times in triplicate.

Compound	(t)TRα.HeLa-Luc			(t)TRb.HeLa-Luc			$T_3EF_{50}\alpha$/ $T_3EF_{50}\beta$
	EC_{50} (nM)	T_3EF_{50}	Max. induction (%)	EC_{50} (nM)	T_3EF_{50}	Max. induction (%)	
T_3	1.1±2	1	100 (10nM)	3.5±3	1	100 (100nM)	1
CO23	2.6±3	0.4	82	24±6	0.1	101	2.8
GC-1	15±4	0.07	59	11±4	0.3	94	0.2

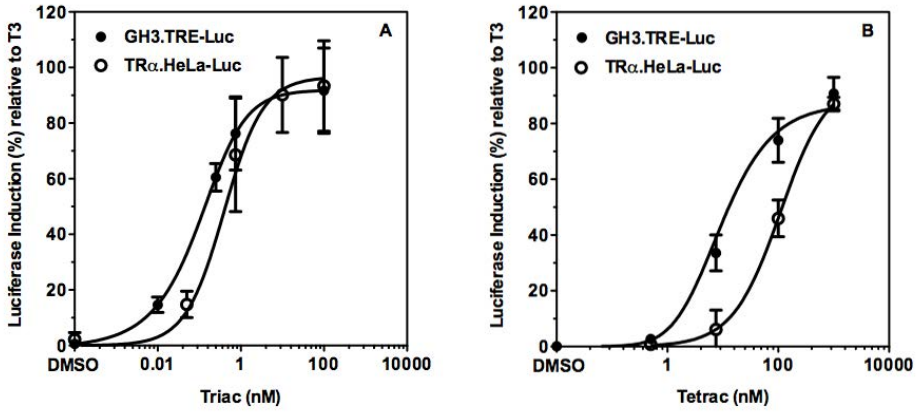

Figure 5. TRα.HeLa-Luc (o) and GH3.TRE-Luc (●) induction after 24 h exposure to A) Triac and B) Tetrac. Luciferase activity expressed relative to T_3 maximal induction (at 100 nM set at 100%, DMSO set at 0%). Data represent mean ± SD of triplicate experiments.

Having established the relative subtype selective responsiveness of the two stable reporter lines, we next examined a series of other chemicals known to induce TR-mediated gene expression. The acetic acid derivatives of T_3 and T_4, Triac and Tetrac are as potent as their natural parent compounds (**Table 1**, **Figure 5**), and both compounds are most potent in GH3.TRE-Luc line. Interestingly, Tetrac was less potent than T_4 in the TRα.HeLa-Luc cell line, but more potent than T_4 in the in GH3.TRE-Luc cells. 9-cis-retinoic acid significantly induced luciferase activity up to 19% of the T_3-maximum response in the GH3.TRE-Luc cells but poorly if at all in the TRα.HeLa-Luc cells (**Figure 6**), whether or not T_3 was also present in the media.

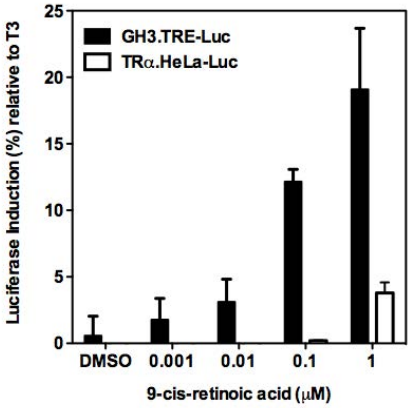

Figure 6. Luciferase induction in GH3.TRE-Luc (black bars) and TRα.HeLa-Luc (white bars) cells after 24 h exposure to 9-cis-retinoic acid. Luciferase activity expressed relative to T_3 maximal induction (at 100 nM set at 100%, DMSO set at 0%). Data represent mean ± SD of triplicate experiments.

The T_3-like 4-OH-BDE 69, a model hydroxylated BDE synthesized for high binding affinity for the receptor (Marsh et al., 1998), was able to induce luciferase activity in both cell lines (**Figure 7**), inducing 78% of T_3-maximum response in the GH3.TRE-Luc cells against a maximum of only 26% in the TRα.HeLa-Luc cell line, contrasting with the almost 3× lower potency in the GH3.TRE-Luc cells (**Table 1**).

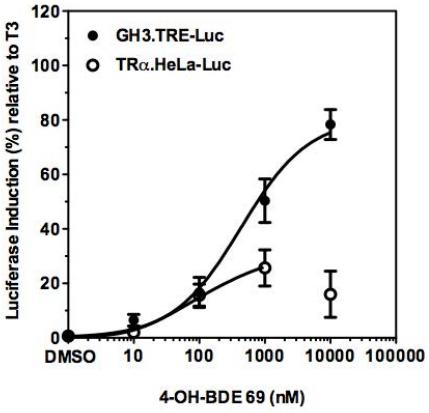

Figure 7. TRα.HeLa-Luc (o) and GH3.TRE-Luc (●) induction after 24 h exposure to 4-OH-BDE 69. Luciferase activity expressed relative to T_3 maximal induction (at 100 nM set at 100%, DMSO set at 0%). Data represent mean ± SD of triplicate experiments.

We next compared the responsiveness of compounds reported to have antagonistic activity against TR mediated gene expression; NH-3, a designed antagonist that binds directly to the receptor (Lim et al., 2002; Nguyen et al., 2002) and sodium arsenite, an environmentally relevant compound with a less understood mode of action (Davey et al., 2007). In the TRα.HeLa-Luc cell line, the antagonist NH-3 slightly but significantly decreased (31%) luciferase activity induced by 1 nM of T_3 after an initial induction of 13% at 10 nM. By contrast, NH-3 strongly antagonized T_3-induced luciferase activity (80%) in the GH3.TRE-Luc cells (**Table 1**, **Figure 8A**). The inhibitor sodium arsenite completely inhibited T_3-induced luciferase activity at 100 μM in both cell lines without any signs of cytotoxicity, but with a much lower IC_{50} in the GH3.TRE-Luc cells (**Table 1**, **Figure 8B**).

By contrast, amiodarone and its metabolite desethylamiodarone, and debutyldronedarone, compounds previously reported to antagonize TR activity (Latham et al., 1987; Van Beeren et al., 2003; Schriks et al., 2006), did not induce any effect on luciferase activity in either cell line, both in the presence or absence of T_3. Likewise, DIB, an organic photo-conductor reported to induce luciferase activity in a reporter cell line endogenously expressing the human TRα (Yamada-Okabe et al., 2005), did

not have an effect in either of the cell lines here. Lastly, the brominated flame-retardants BDE 28 and BDE 206 (Schriks et al., 2007) also did not induce any effect on luciferase activity in either cell line, whereas these compounds have been previously reported to have variable agonist or antagonist activity in the T-screen and/or transient and stable transfection based assays (**Table 1**).

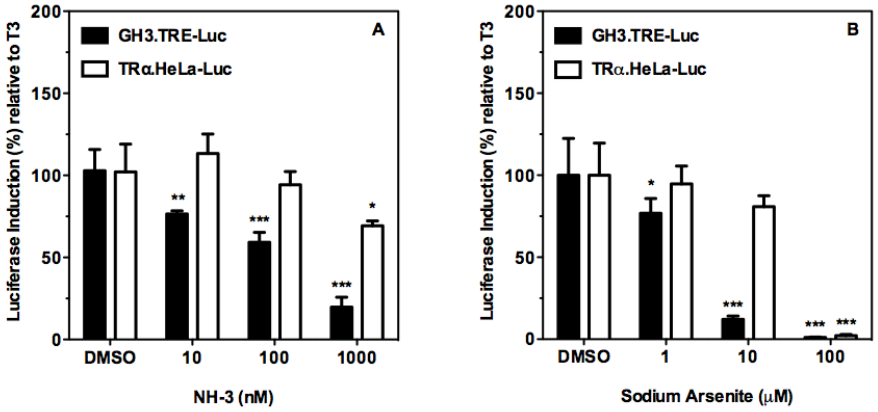

Figure 8. TRα.HeLa-Luc (white bars) and GH3.TRE-Luc (black bars) induction after 24 h exposure to a) NH3 and B) Sodium Arsenite. Luciferase activity expressed relative to T_3 maximal induction (at 100 nM set at 100%, DMSO set at 0%). Data represent mean ± SD of triplicate experiments. *Significantly different from control (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

DISCUSSION

In this study we developed and validated a stable *in vitro* human reporter gene assay to elucidate the receptor-ligand interactions of the α and β TR subtypes. This was achieved through the development of a human uterine cervical HeLa cell line stably co-transfected with the human TR subtype alpha 1 (TRα1) and a TRE-regulated luciferase reporter gene designated TRα.HeLa-Luc. The TR-mediated responses of this cell line were compared with the previously reported GH3.TRE-Luc cells (Freitas et al., 2011) with TRβ appearing to be the dominant endogenous TR isoform (Hahn et al., 1999). Although at low levels, TRs are also expressed in wild-type HeLa cells, such that transient transfection of the TR-dependent reporter gene vector resulted in a low but quantifiable induction of luciferase activity (data not shown); however, co-transfecting the TRα1 expression vector resulted in a 30-fold greater magnitude of response, increasing the TRα specificity of the assay. Previous to the development of the stable cell line, the degree of each TR-isotype specific nature was determined via transiently transfection assays expressing human TRα or TRβ in HeLa cells, with high quality dose-response curves (**Figures 3B** and **4B**). Although great improvements have been made to standardize transient transfections for detecting TR modulating chemicals (Hofmann et al., 2009), we focused on the use of a stably transfected cell lines to avoid the many steps required in the preparation of a transient assay. Although the preparation of a stable reporter gene assay is time consuming and does not always yield well performing cell lines, the experimental variability and cost associated with transient reporter genes assays make the stable assays the most suitable option for high-throughput screening applications. Multiplexing in the same cell culture plate cellular viability and luciferase activity measurements enables a further reduction of total assay time.

Assays attempting to discriminate between receptor subtype transcriptional effects have been previously reported, but these were restricted to green monkey fibroblasts (CV-1 cells) only transiently transfected with *Xenopus* TRs (Schriks et al., 2007), yeast reporter assay systems (Shiizaki et al., 2010) or the rat PC12 cell line expressing the avian TRα1 isoform (Jugan et al., 2007). Recently, we developed a stable transfected

reporter gene assay based on the TRβ dominant human pituitary adenoma GH3 cell line (Freitas et al., 2011) which allows sensitive and rapid screening of TR agonists and antagonists. Given that TRβ is apparently more abundant TR form present in GH3 cells (Hahn et al., 1999) the stably transfected GH3.TRE-Luc cells is likely to detect especially TRβ mediated responses. This implies that the stable TRα.HeLa-Luc cells developed in the present study complete a set of in *in vitro* TR specific reporter gene assays in human cell lines. The established *in vitro* bioassay based on TRα.HeLa-Luc cells enabled the detection of T_3 in the picomolar range within only 24 hours of exposure, with a detection limit of 100pM. Compared to a TR-mediated reporter gene assay in yeast, this is an almost 8× lower EC_{50} (Shiizaki et al., 2010), and this difference between the human cell line based and the yeast based assay is comparable to differences between mammalian cell- and yeast-based reporter gene assays for estrogen-receptor mediated responses (Legler et al., 2002). Another stably transfected reporter gene assay based on the rat PC12 cell line that constitutively expresses the avian TRα1 isoform responds to T_3 with approximately the same sensitivity (Jugan et al., 2007). Although no striking mechanistic differences have been reported between avian and mammalian TRα1-mediated gene expression, one cannot exclude potential discrepancies due to qualitative and quantitative differences in receptor co-activators and co-repressors in different cellular contexts, or potential species specific compound interactions outside the well conserved ligand binding pocket (Wagner et al., 2001). Hence we chose to stably express a human TR isoform in a human cell line with typically low TR expression levels for these studies.

The TRβ dominant GH3.TRE-Luc cell line was stably transfected with the same TR-regulated reporter gene vector used in the present study for the development of the TRα.HeLa-Luc cell line (pGL4CP-SV40-2xtaDR4). Both stable cell lines share the same experimental conditions, with the exception of their respective routine sub-culture media formulations. In order to keep the exposure conditions the same between cell lines, for direct comparison of their response profiles, a common medium with 5% DCC-FCS was used for all exposures. This allowed a similar T_3-induced dose-dependent response (**Figure 2A** and **3A**), with the GH3.TRE-Luc cells slightly more sensitive than the TRα.HeLa-Luc cells. In agreement with other studies, while the binding affinity of TRα or TRβ is virtually identical, TRα-mediated responses induced

by T_3 are consistently more sensitive (at the EC_{50} level) than TRβ-mediated responses in our transient transfection assays. Thus, the higher sensitivity of the TRβ dominant GH3.TRE-Luc line is most likely due to differences in cellular context, such as coregulator expression, deiodinase activity or TH transport.

Differences in potency or maximal luciferase induction relative to T_3 were not found, except for the T_3-like 4-OH-BDE 69 in the GH3.TRE-Luc line. Further validation of the stable receptor subtype dominant reporter gene cell lines was performed with the model isotype-selective ligands CO23 and GC-1. The two stable cell lines as well as HeLa cells transiently transfected with either TR isoforms (TRα1 or TRβ1), were exposed in parallel with these receptor subtype selective chemicals. The transient transfections and exposures to CO23 and GC-1 allow a functional assessment and validation of the receptor variant discriminatory differences between in each stable cell line. As anticipated, the TRα-selective CO23 was more potent in the transient and stable TRα. HeLa-Luc cells contrasting with the higher potency of the TRβ-selective GC-1 in the transient and stable GH3.TRE-Luc cells. This is also reflected by the $T_3EF_{50}\alpha / T_3EF_{50}\beta$ ratios (2.8 and 20 for CO23, 0.2 and 0.7 for GC-1) indicating their respective relative affinity towards TRα and TRβ. In accordance with previous reports (Wagner et al., 2001), these differences are mainly due to the way each ligand interacts with a single different amino acid in the ligand binding domains of the otherwise homologous TRα and TRβ receptor ligand binding domains. Likewise, the T_3-like 4-OH-BDE 69 revealed a relatively increased potency towards the TRα cell line ($T_3EF_{50}\alpha / T_3EF_{50}\beta$ ratio of 20), making a good candidate for further *in vivo* testing due to possible different physiological effects in TRα versus TRβ dominant tissues.

Other chemicals suspected to be TR ligands were tested for their TR-isoform specific responses. The selected chemicals had all previously shown to mediate TR-dependent effects as agonists or antagonists. However, their ligand potencies and specificities to TR subtypes were slightly different. For instance, the potencies of the natural thyroid hormone T_4 and the acetic acid derivatives of T_3 and T_4 (Triac and Tetrac, respectively) were consistent with previous reports (Cheek et al., 1999; Gutleb et al., 2005). Interestingly, Tetrac was more potent than T_4 in the TRβ-dominant GH3.TRE-Luc cells as previously reported using a biochemical assay with either TRα or TRβ in the presence of SRC1-2 co-activator peptide (Koury et al., 2009). The type I and II 5'-

deiodinases are both expressed in the GH3 cells (Baur et al., 2000; Mori et al., 2007), playing an important role in the regulation of hormone production. These enzymes are capable of deiodinating the outer ring of both T_4 and Tetrac, which converts them to their active form T_3 and Triac. Specifically for these chemicals, different levels of expression of these enzymes in the TRα.HeLa-Luc and the GH3.TRE-Luc cells could account for the differences in potency. A similar reasoning applies for 9-cis-retinoic acid, which is a known ligand of the TR normally silent heterodimer partner RXR (Li et al., 2002). A permissive TR-RXR partnership may occur in the GH3 cells enabling the activation of the stably transfected TRE-Luc system by this retinoid, even in the absence of T_3, as has been proposed for the prolactin gene TRE (Castillo et al., 2004). In the HeLa cells, luciferase expression may be triggered by a ligand activated TRα1 monomer, homodimer, or as a TR/RXR heterodimer where RXR is less permissive, possibly due to the lack or presence of particular cofactors that enable the non-silent behavior in certain cell types, or potentially lower RXR expression than GH3 cells. Therefore differences in cellular contexts should be taken into account when interpreting the different TR-related effects of the same compound in TRα.HeLa-Luc vs. GH3.TRE-Luc cells.

We also found differential sensitivity of these two lines to two mechanistically different antagonistic compounds. The designed antagonist NH-3 that competitively inhibits TH binding to the receptor was most potent in the GH3.TRE-Luc cells. This suggests isotype selectivity even though NH-3 binds to TRα and TRβ equivalently (similar EC_{50}). Interestingly, data from our laboratory suggests that NH-3 is a less effective antagonist in TRα dominated tissues than TRβ dominated tissues in *Xenopus laevis* tadpoles (Neff et al. unpublished results). In accordance with previous studies (Davey et al., 2007) the inhibitor sodium arsenite also disrupted the TR-mediated luciferase expression at non-cytotoxic concentrations, implying a common inhibitory mechanism of action for both cell lines. As with NH-3, a clear selectivity towards inhibition of T_3 induced reporter gene activity in the TRβ dominant GH3.TRE-Luc cells was also observed for sodium arsenite but much less is known about its mode of action.

Together, the stably transfected GH3.TRE-Luc and TRα.HeLa-Luc reporter gene cell lines described here complement each other by enabling simple, rapid and specific

identification of chemicals that modulate TR activity in an isotype-selective manner. Further comparisons of the GH3.TRE-Luc and the TRα.HeLa-Luc cell lines in terms of deiodinases, TR coregulators, heterodimer partners and transporters will be important to allow additional differentiation between truly TR-mediated effects and molecules indirectly affecting the receptors in an isotype selective manner, and their further development as front-line rapid screens for TR disrupting compounds.

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CHAPTER 6

SUMMARY

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

SUMMARY

The aim of the present thesis was to develop functional *in vitro* bioassays for the detection of compounds affecting thyroid hormone signaling at the level of the thyroid hormone receptor. The assays to be developed should be appropriate for quantitative high-throughput screening (qHTS) and inclusion in an *in vitro* test battery, replacing the use of animal experiments in the identification of thyroid hormone disrupting compounds and quantification of their potency.

In **Chapter 1**, background information and objectives of the thesis are presented. First it is explained that the thyroid hormone (TH) system is responsible for several important physiological processes, including regulation of energy metabolism, growth and differentiation, development and maintenance of brain function, thermo-regulation, osmo-regulation, the onset and proper function of other endocrine systems, sexual behavior and fertility, and cardiovascular functioning. Given the relevance of the TH system, the concern about TH disruption (THD) has resulted in the development of *in vitro* strategies to identify THD compounds (THDCs). This information is generally derived from animal studies and, for the majority of chemicals present in the environment, quite limited or not available. For ethical, financial and practical reasons, *in vitro* and *in silico* alternatives are crucial for the identification of THDCs and quantification of their effects. THDCs may act by several different mechanisms of action, including altered hormone production, transport, metabolism, receptor activation and disruption of feedback mechanisms. For several, but not all of these endpoints, *in vitro* assays are already available. However, at the time of the start of this thesis no functional *in vitro* bioassay was available for detection of TR agonists and antagonists that also would be appropriate for quantitative high-throughput screening (qHTS) of potential THDCs. Thus the aim of the present study was to develop and validate functional *in vitro* bioassays for thyroid hormone receptor-mediated toxicity, focusing on thyroid hormone receptor interaction.

Chapter 2 describes the development of a stable luciferase reporter gene assay based on the thyroid hormone responsive rat pituitary tumor GH3 cell line that

constitutively expresses both thyroid hormone receptor isoforms, with TRβ appearing to be the major TR isoform. The assay was established by stably transfecting the pGL4CP-SV40-2xtaDR4 construct into the GH3 cells resulting in a highly thyroid hormone sensitive cell line (GH3.TRE-Luc), which was further optimized into an assay that allowed the detection of triiodothyronine (T_3) and thyroxine (T_4) concentrations in the picomolar range after only 24 h of exposure. The greater than 20-fold induction by T_3 relative to the solvent control is illustrative of the high responsiveness of the system. The assay was validated by the quantification of the agonistic effect of the natural hormones (T_3 and T_4), the acetic acid derivatives of T_3 (triiodothyroaceticacid, Triac) and T_4 (tetraiodothyroacetic acid, Tetrac), hydroxypoly-brominated diphenylethers (OH-PBDEs), hydroxypolychlorinated biphenyls (OH-PCBs) and the antagonistic action of sodium arsenite ($NaAsO_2$). The putative antagonist amiodarone, and bisphenol A (BPA) and its halogenated derivatives (TCBPA and TBBPA) for which effects reported in the literature are not consistent, showed comparable dose-response curves with a slight agonistic effect (5% of T_3-max) followed by a slight antagonistic effect. The magnitude and reproducibility of the responses to various compounds confirmed the assay to be a promising tool for the identification and quantification of specific thyroid hormone receptor active compounds.

In **Chapter 3** the use of the GH3.TRE-Luc reporter gene cell line for a quantitative high-throughput screening (qHTS) platform is described. The GH3.TRE-Luc assay was miniaturized and validated in a 1536-well plate format. Using this miniaturized format, the Library of Pharmacologically Active Compounds (LOPAC) and the National Toxicology Program (NTP) collection were screened for TR agonists and antagonists. Of the 2688 compounds tested 8 or 1 were identified as TR agonists depending on whether the positive hit cut off was defined at ≥10% or ≥20% of efficacy relative to T_3, respectively, and 5 were identified as TR antagonists. None of the inactive compounds were structurally related to T_3, nor reported elsewhere to be thyroid hormone disruptors, so false negatives were not detected. The low potency agonists also did not resemble the thyroid hormones, which is an indication that these may not be active directly through the ligand-binding domain of the receptor and

may not represent effective agonists. Defining TR agonists in the qHTS using the criteria of a hit cut off of ≥20% efficacy at 100 μM may avoid identification of positives without physiological relevance. It is concluded that the miniaturized GH3.TRE-Luc assay is an important addition to the *in vitro* test battery for TR-mediated endocrine disruption, and that, given the low percentage of compounds testing positive, its high-throughput nature is an important advantage for future toxicological screening. After the GH3.TRE-Luc cells had shown to be highly responsive and suitable for the application in a qHTS test battery for detecting TH disrupting chemicals, a comparison was performed with the responses of bona fide endogenous TH target genes (**Chapter 4**). In addition to the TRE-driven luciferase induction in the GH3.TRE-Luc cells, TH-dependent cell proliferation was also analyzed since the effect of some THDCs could be independent of the canonical TH signaling per se. In this study, we treated GH3 cells with T_3 as well as two TR isotype (TRα and TRβ) selective thyromimetics, CO23 and GC-1 respectively. Response profiles in the reporter gene assay showed excellent concordance with endogenous growth hormone (GH) gene expression, a known direct TH target gene and the basis of the use of these cells in the proliferation based T-screen. Further, it was demonstrated that sodium arsenite, bisphenol A and the phthalate DEHP inhibit the T_3-mediated induction of the reporter gene as well as the endogenous expression of the GH gene. A subsequently performed microarray experiment identified a fuller spectrum of T_3 responsive genes in these cells, since promoter selective effects of synthetic nuclear receptor ligands may occur. In addition to identifying several important components of the TH signaling pathway, the microarray allowed identification of a battery of both known and novel TH target genes, including genes related to angiogenesis such as HIF2α (EPAS1). Identification of these genes is important for validation of assays designed to identify new synthetic TR modulators and their potential genomic versus non-genomic modes of action.

Chapter 5 deals with the fact that the critical role of TH in processes such as brain development, growth and energy homeostasis is performed via different isoforms of the thyroid hormone receptor. As the main isoforms, TRα and TRβ, present distinct physiological functions, it is important to study the TR-specific potency of TH active

compounds. This chapter firstly describes the development of TRα and TRβ transient luciferase reporter gene bioassays using human cervical HeLa cells enabling quantification of the responses of the TR isoform-selective chemicals CO23 and GC-1. In conjunction with this, a stable TRα specific reporter gene cell line was developed, denominated TRα.HeLa-Luc. Responses in this TRα.HeLa-Luc cell line were compared to responses in the GH3.TRE-Luc cell line, which appears to contain TRβ as the major isoform. The TR isoform-selective chemicals CO23 and GC-1 induced responses according to the anticipated potency rank order T_3>CO23>GC-1 in the TRα.HeLa-Luc cells and T_3>GC-1>CO23 in the GH3.TRE-Luc cell line. In addition to 7 known TR-active standards, 10 chemicals suspected to have TR disrupting activity were tested. Of these, only sodium arsenite and the T_3-like 4-OH-BDE69 induced a response in the TRα.HeLa-Luc assay. Together, the developed TRα.HeLa-Luc and GH3.TRE-Luc stable reporter gene cell lines add a new level of specificity, representing an improvement of the available tools for *in vitro* high-throughput assessment of TR isoform-specific activity of drugs and environmental pollutants.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

As summarized above, this thesis presents the development of functional *in vitro* bioassays for the detection of compounds disrupting the thyroid hormone signaling at the level of the thyroid hormone receptor. These assays could be used in a battery of selected mechanism-specific high-throughput *in vitro* bioassays that could at least partially replace the use of animal experiments for the identification and quantification of specific thyroid hormone receptor disrupting activities of compounds. In this section, the advantages and limitations of the use of *in vitro* assays for replacing animal experiments are discussed in more detail with emphasis on the relevance of the developed TR-dependent reporter gene assays.

OVERRATED VALUE OF ANIMAL EXPERIMENTS

Information on the potency of THDCs is largely derived from animal studies. For the majority of chemicals, however, this information is limited or not available and performing animal experiments for all relevant chemicals is unlikely to happen in the near future for ethical, financial and practical reasons. Although *in vivo* bioassays are generally perceived as being the most reliable tests for assessing the risk of chemicals, including THDCs, to human health, this approach faces several limitations. For instance, healthy animals may cope with an exposure to THDCs by compensation via HPT feedback mechanisms or other means and effects may be masked, while humans with specific health conditions, such as hypo- or hyperthyroidism or malnutrition, may be more vulnerable. Also, since most *in vivo* studies are hardly ever carried until animals are fully mature, life-stage specific differences may be overlooked when assessing the risk for humans. Furthermore, it is of importance to note that although rodents and human share a common physiology for the HPT axis, several differences between rodents and human have been defined that make the rat more sensitive toward thyroid proliferative effects than human (Berthelsen et al., 2002; EFSA, 2011). The high affinity binding protein thyroxine-binding globulin (TBG) which binds T_4 and to a lesser degree T_3, is present in humans but lacking in rodents, birds, amphibians and fish (Larsson et al., 1985). This implies that rodents have a higher

percentage of unbound active T_4 and T_3 than humans, as well as higher degradation rates (lower half-life) for plasma T_4. This difference in T_4 half-life results in a 10-fold greater requirement for endogenous T_4 in the rat thyroid than in the adult human thyroid and an accelerated production of thyroid hormone in the rat which is driven by serum TSH levels that are about 6- to 60-fold higher than in humans (EFSA, 2011). As a result, the rodent thyroid gland is chronically stimulated by TSH levels to compensate for the increased TH turnover. This may result in increased sensitivity of the thyroid gland toward increased growth and potential neoplastic change in rats than in humans, reflecting an important species difference. This makes the rat and rodent-based models sensitive models for hazard assessment of thyroid disruption although the species difference between rodents and human should be taken into account in subsequent risk assessment.

Therefore, an appropriate *in vitro* test battery for hazard assessment on thyroid disruption should cover mechanisms relevant to different life stages and under different exposure conditions. They should enable fast assessment of a broad range of compounds for potential perturbations of the TH signaling pathways relevant for humans, with virtually no animals sacrificed and at much lower cost. Results based on *in vitro* testing also may offer the possibility to predict interspecies THD effects, provided that species-specific *in vitro* assays will be available taking into account species-specific physiological characteristics.

In addition it is of importance to note that most current rodent-based assays were originally developed for the detection of the disrupting effects on the sex-hormone signaling system, and not optimized for the identification of THDCs. Relevant effects may not be detected in animal studies when the outcomes are delayed, life stage specific, not assessed (e.g. behavior) or only occur when an organism has to adapt to environmental factors by modulating TH levels. Therefore, in *in vivo* studies it is often difficult to determine whether the effect of a compound is a result of THD or another toxic mechanism. This may have critical consequences for policy decisions regarding a compound, as there is a tendency for a stricter regulation on endocrine disrupting compounds than toxic compounds affecting other mechanisms.

TR-MEDIATED THYROID HORMONE DISRUPTION

The TRs control transcription of specific genes directly through interaction with thyroid hormone response elements (TREs). Typically, TRs form a complex with the retinoid X receptor (RXR), a heterodimer where the RXR is usually a "silent partner". Nonetheless, in particular cell types and/or TRE configurations RXR may be ligand activated by specific retinoids (Castillo et al., 2004). In the GH3.TRE-Luc cell line (**Chapter 2**), the response to retinoids was consistent with a permissive heterodimer. In contrast, the TRα.HeLa-Luc cells did not show this effect of RXR activation and crosstalk with the RXR (**Chapter 5**). The results also imply that the agonist activity detected in the GH3.TRE-Luc cells may in some cases reflect RXR rather than TR agonist activity. It should be further studied whether a comparison between both cell lines could be used to filter out RXR-active compounds, reducing the chances of detecting false positives in the GH3.TRE-Luc assay.

Recently, THs have also shown to directly act at the cell membrane, cytoplasm (Cheng et al., 2010) and mitochondria (Chocron et al., 2012; Pessemesse et al., 2012), which is of interest given the important influence of THs on mitochondrial function in warm-blooded vertebrates. Often, these effects are referred to as non-genomic versus the "classical" nuclear receptor-mediated genomic effects described above. However, this may be a misnomer since actions initiated at the membrane or in the cytoplasm may ultimately influence the transcription of specific genes, and even interface with the action of the nuclear TRs. The actions of TH at the cell membrane, cytoplasm or mitochondria, may be mediated by a bona fide TR isoform acting outside the nucleus, a different form of the TR or a distinct receptor for TH signaling altogether. For example, T_4 (and to some extent T_3) can stimulate blood vessel formation (angiogenesis) in various models via an integrin-mediated signaling pathway (Cheng et al., 2010).

While most TH disruption has shown to occur at pre-receptor stages such as TH production, regulation, plasma transport and metabolism, TRs are in the signaling pathway closest to the endpoints that result in altered cellular responses: transcriptional control of target genes. The relatively tight fit of the TR ligand-binding domain only allows compounds with a closely resembling structure to the hormone

itself to bind with reasonable affinity (DeVito et al., 1999; Zoeller, 2005) (**Chapters 2 and 3**). As a result only few of the known THDCs have shown to directly bind to the receptor ligand-binding domain and interfere with the TR action.

On the other hand, although chemical libraries such as the Library of Pharmacologically Active Compounds (LOPAC) and the National Toxicology Program (NTP) collection applied in the research described in this thesis (**Chapter 3**), or the recently screened Tox21 library (>10.000 compounds), comprise a diverse chemical space with chemicals proven to be pharmacologically active and almost all previously tested in one or more standard toxicological assays, they do not include chemicals known for their *in vivo* or *in vitro* THD potency. For example, OH-PHAHs (hydroxylated polyhalogenated aromatic hydrocarbons) such as TBBPA (4,4'-propane-2,2-diylbis(2,6-dibromophenol)) or OH-PCBs (hydroxylated polychlorinated biphenyls), and PBDEs (polybrominated diphenylethers) shown before to be active in the GH3.TRE-Luc assay (**Chapter 2**) are not included in these libraries.

It should also be emphasized, that the complexities of the transcriptional control by these receptors go beyond direct binding to the receptors or altering coactivator/corepressor interactions and a multitude of other mechanisms for disruption of the receptor action are also possible. Interactions with RXRs may affect a subset of important TH target tissues or specific genes in specific cells. Furthermore, the TRs themselves and various coactivator and corepressor proteins are subject to post-translational modifications that may be a target for endocrine disruption. TR and coactivator stability, nuclear localization, and interactions with other cofactors may be additional targets of THDCs. As an example, exposure to arsenic trioxide (**Chapter 4**) activates a MAP kinase cascade that leads to phosphorylation of the corepressor SMRT and its dissociation from the nuclear receptor complex may lead to derepression of target genes (Hong et al., 2001). This complexity is well embodied in the GH3 cells, which are used throughout the research described in this thesis, due to their extensive characterization, expression of both TR isoforms (mainly TRβ), their heterodimer partners and respective cofactors, as well as deiodinase enzymes (Ball et al., 1997; Misiti et al., 1998; Baur et al., 2000). These and other possibly unknown transcription factors and enzymes may be absent in other cell-based systems resulting

in completely different responses to THDCs. Moreover, by coupling the increased level of cell proliferation and growth hormone secretion in response to physiological levels of THs with the TR-mediated luciferase activity, a better distinction can be made between TR-mediated and other mechanisms of action that induce cell proliferation, ultimately reducing the complexity that characterizes the multitude of mechanisms involved in the THD.

COMPOUND BIO-ACTIVATION AND AVAILABILITY *IN VITRO*

In addition to compounds directly interfering with different endpoints of the TH system, other THDCs such as PCBs and PBDEs require metabolic activation to become able to interfere with the TH signaling. This bio-activation into hydroxylated metabolites (OH-metabolites) typically occurs *in vivo* by phase I enzymes (Morse and Brouwer, 1995; Marsh et al., 2006). These OH-metabolites can show remarkable structural similarities to THs and interfere with the TH homeostasis by mimicking T_4 and to a lesser extent T_3 (Meerts et al., 2002; Freitas et al., 2011). These OH-metabolites can interfere with TH transport, action, feedback mechanisms and metabolism. In mammals including humans, they bind with high affinity to specific transport proteins, such as TTR and TBG (Lans et al., 1994; Meerts et al., 2000; Marchesini et al., 2008; Gutleb et al., 2010) and can accumulate in blood plasma reaching levels up to 30 times higher as compared to their respective parent compounds (Sjodin et al., 2000; Gebbink et al., 2008). Furthermore, the TH-like OH-metabolites can be selectively transported over the placenta and blood brain barrier (Morse et al., 1993; Meerts et al., 2002). Given the high plasma levels and the toxicological relevance of these metabolites, it is crucial to include the quantification of their toxic potencies for toxicological risk and hazard assessment. However, since most cell-based *in vitro* test systems lack phase I metabolism, the bio-activation of such chemicals is performed prior to testing by typically using *in vitro* biotransformation techniques with microsomal fractions. Unfortunately this approach is susceptible to low biotransformation efficiencies, the remaining parent compound at toxic concentrations in the extracts and the presence of interfering matrix components co-extracted from the metabolizing system (Meerts et al., 2000; Schriks et al., 2006; Hamers et al., 2008). Recently, a new method has been developed to separate the OH-metabolites from their

parent compounds followed by a non-destructive cleanup which removes potentially interfering matrix compounds such as microsomal fatty acids from the extract (Montano et al., 2012). Alternatively, an *in vitro* system can be exposed directly to the metabolite of interest, if known and available from other sources.

In a recent study (Gutleb et al., 2012) the cellular localization of halogenated compounds and their OH-metabolites was visualized and their concentration estimated via secondary ion mass spectrometry. Relative intracellular concentrations of 4-OH-BDE69 and 4-OH-BDE121 in GH3.TRE.Luc cells were 61% and 18%, respectively, compared to the parent compounds. These differences could be due to phase II metabolism followed by excretion from the cells, and may partly explain the usually higher observed effect concentrations of the 4-OH-BDEs in *in vitro* cell models than what would be expected based on receptor binding studies. Therefore, it is crucial to characterize the phase II and cellular efflux mechanisms in these cell-based systems, as hydroxylated compounds may undergo rapid phase II metabolism and excretion from the cells resulting in false negative results for the testing of their implicit TH activities but possibly adequately defining their *in vivo* activity.

APPLICATION OF *IN VITRO* TESTS IN A TESTING STRATEGY

The TR mediated reporter gene assays developed in this thesis are suitable to be applied in a battery of non-animal tests for THD hazard assessment. While minimizing the risk of false negatives this *in vitro* approach should enable the prioritization of chemicals that need further studies. With a pathway-based chemical hazard assessment, both laboratory testing facilities and regulatory bodies should be able to focus on those chemicals of highest concern. Thus, the first function of a comprehensive battery of *in vitro* tests is to rank the thousands of poorly characterized chemicals. Recently an international workgroup of experts has advised a set of endpoints covering the most important mechanisms of the TH system regulation that are currently known to be sensitive for THDCs and are currently or in the near future applicable in an *in vitro* HTS test battery (Murk et al., 2012). In this Tier 1 testing proposal, the GH3.TRE-Luc assay is included as a promising *in vitro* functional assay suitable for this HTS platform. It is important that this, and any other *in vitro*

approaches developed, include phase I metabolism to detect possible bio-activation of compounds. The GH3 cells used to create the stably transfected GH3.TRE-Luc cell line did not show any cytochrome P4501A activity (Gutleb et al., 2005; Schriks et al., 2006), although one study has reported a PCB inducible P4501A1 activity for their GH3 variant (Gauger et al., 2007). Alternatively, compounds of interest could also be pre-incubated with microsomes to allow activation before exposure. However, it is still not completely clear to what extent the metabolites can reach the same intracellular levels as the parent compounds, since reduced uptake or phase II metabolism and subsequent cellular elimination could also play a role (Gutleb et al., 2010). As described in **Chapter 2** the cellular uptake of the active metabolites when produced outside the TH responsive cell could be the limiting factor in an *in vitro* cell system in contrast to *in vitro* binding assays. Therefore, intracellular bio-activation of potential THDCs seems to be a prerequisite that still needs to be further developed.

COMPUTATIONAL MODELING TO INTERPRET *IN VITRO* ASSAYS FOR *IN VIVO EFFECTS*

Although it was beyond the scope of this thesis to develop an assay to be able to predict *in vivo* effects, in a later stage this could be further developed for the assays here created in combination with other selected assays. Despite the many advantages of an *in vitro* testing battery, estimating *in vivo* toxicity from *in vitro* results can be a daunting task as it is not possible to fully reproduce *in vitro* the complexity of a complete *in vivo* system. The absence of whole-body pharmacokinetic processes (absorption, distribution, metabolism and excretion) in the *in vitro* assay system is one of the main challenges in implementing *in vitro* based risk assessments for chemicals. Integration of data on the toxic mode of action of a chemical with data on its pharmacokinetic behavior is essential for the interpretation of *in vitro* toxicity studies of a compound for the whole animal. With physiologically based kinetic (PBK) models, a better interpretation of the *in vivo* relevance of *in vitro* results can be obtained by converting an *in vitro* concentration-response curve to an *in vivo* dose-response curve. These approaches are also known as *in vitro* to *in vivo* extrapolation (IVIVE) or reverse dosimetry (Verwei et al., 2006; Forsby and Blaauboer, 2007; Louisse et al., 2010; Punt et al. 2012). IVIVE is the process of estimating the environmental

exposures to a chemical that could produce tissue exposures at the site of toxicity in humans equivalent to those associated with effects in an *in vitro* toxicity test (e.g., an EC_{50}, a Benchmark concentration, etc.). IVIVE can provide an estimate of the likelihood of harmful effects from expected environmental exposures to chemicals by integrating diverse information from targeted *in vitro* toxicity and kinetic assays using a computational modeling approach. Pharmacokinetic modeling plays a pivotal role in this quantitative extrapolation, by incorporating the *in vivo* absorption, distribution, metabolism and excretion of the chemical into the process.

In conclusion the results of the present thesis contribute to the further establishment of a battery of *in vitro* tests for the hazard identification of thyroid active compounds. Further implementation of the *in vitro* assays in risk assessment practice requires several future perspectives to be considered, including the following items:

1. To be able to make the step from hazard to risk assessment based on *in vitro* test batteries more fundamental knowledge is needed to reveal the relationship between critical pathway perturbations, timing, physiological effects and diseases. With insight in new mechanisms, *in vitro* test battery results offer more possibilities to predict what compounds are relevant for specifically sensitive groups or conditions. This approach may include development of libraries of model compounds defining their behaviour in series of *in vitro* tests, enabling the identification of the mode of action of unknown compounds based on their patterns of results in this test battery and comparison to the data base in the library.
2. Many environmental pollutants have shown to disrupt the thyroid hormone homeostasis. A few, as also demonstrated by the results of the present thesis, have shown to directly interfere with the TR. This indicates that *in vitro* assays for several other endpoints of the thyroid hormone signalling should be developed and included in the battery of tests. Several endpoints have so far been difficult to be modelled in *in vitro* assays, such as for example thyroid hormone synthesis and excretion. These endpoints do require further attention and may need more complex *in vitro* systems than just one-dimensional cell models.
3. Comparing *in vitro* and *in vivo* responses can help to more specifically determine mechanisms of THD of compounds and assist the further development of models that

can be used in a more predictive way for regulatory toxicology. Defining relationships between *in vitro* and *in vivo* data will also support further validation of the integrated test battery and the library based on its results.

4. When making *in vitro-in vivo* comparisons, it is important to consider not only external doses but also consider the blood plasma levels (internal concentrations) of the compounds and/or its bioactive metabolites in the *in vivo* experiments. PBK modeling may be a way to provide this information and make the link between *in vitro* concentration response curves and *in vivo* dose response data.

5. Since the development of PBK models for a single compound requires a significant amount of research, efforts should be undertaken to further develop general PBK model approaches for series of (related) compounds, possibly using (quantitative) structure activity ((Q)SAR) approaches to define the required parameters and/or the use of simple general PBK models as used in ToxCast.

6. Elucidation of the relative importance of the so-called non-genomic TH action and whether these are relevant targets for TH disrupting chemicals and hence assay development is an underinvestigated topic that deserves further attention.

7. Further development of these future perspectives would provide important further contributions to toxicity testing in the 21st century (Testing and Assessment of Environmental Agents, 2007) and the development of an integrated testing strategy for thyroid active compounds adding additional value to the assays aiming at replacement of animal studies developed in the framework of the present thesis.

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CHAPTER 7

NEDERLANDSE SAMENVATTING

ABOUT THE AUTHOR

ACKNOWLEDGEMENTS

NEDERLANDSE SAMENVATTING

Het doel van de voorliggende thesis was het ontwikkelen van functionele *in vitro* bioassays voor het opsporen van stoffen die invloed hebben op het schildklierhormoonsysteem op het niveau van de schildklierhormoonreceptor.
De te ontwikkelen assays moesten geschikt zijn voor zogenoemde "quantitative high-troughput screening" (qHTS) en moesten in te passen zijn in een *in vitro*-testreeks die gebruikt kan worden als vervanging voor dierproeven bij de identificatie van schildklierhormoonverstorende stoffen en kwantificering van hun sterkte.
In Hoofdstuk 1 wordt achtergrondinformatie gegeven en worden de doelen van dit proefschrift gepresenteerd. Om te beginnen wordt uitgelegd dat het schildklier-hormoonsysteem verantwoordelijk is voor meerdere belangrijke fysiologische processen, zoals regulering van de energiehuishouding, groei en differentiatie, ontwikkeling en onderhoud van de hersenfunctie, warmteregeling, osmoseregulering, het in werking stellen en goed functioneren van andere endocriene systemen, seksueel gedrag en vruchtbaarheid en cardiovasculaire functies. Het belang van het schildklierhormoonsysteem en de zorgen over verstoring ervan hebben geresulteerd in de ontwikkeling van *in vitro*-strategieën om schildklierhormoon-verstorende stoffen te identificeren. Deze informatie wordt doorgaans aan de hand van dierstudies verkregen en is, voor wat betreft de meeste chemische stoffen die in het milieu aanwezig zijn, nogal beperkt of niet beschikbaar. Om ethische, financiële en praktische redenen zijn *in vitro*- en *in silico*-alternatieven cruciaal voor de herkenning van schildklierhormoonverstorende stoffen en voor de kwantificering van hun effecten. Verstoring van de schildklierhormoonfunctie kan optreden via verschillende mechanismen, zoals veranderingen in hormoonproductie, transport, stofwisseling, receptoractivering en verstoring van terugkoppelingsmechanismen. Voor verschillende –zij het niet voor alle- van deze eindpunten zijn al *in vitro*-assays beschikbaar. Op het moment van het begin van dit onderzoek was er geen functionele *in vitro* bioassay beschikbaar voor het opsporen van schildklierhormoonreceptor-agonisten en -antagonisten die ook geschikt zouden zijn voor qHTS van potentiële schildklierhormoonverstorende stoffen. Daarom was het doel van het huidige onderzoek

functionele *in vitro* bioassays te ontwikkelen en te valideren voor schildklierhormoon receptor-gemedieerde toxiciteit, toegespitst op de receptorinteractie van het schildklierhormoon.

Hoofdstuk 2 beschrijft de ontwikkeling van een stabiele luciferase reporter gen assay, gebaseerd op de schildklierhormoon responsieve rat hypofyse tumor GH3 cellijn die structureel beide schildklierhormoonreceptoren laat zien, waarbij TRβ de belangrijkste schildklierhormoonreceptor blijkt te zijn. De assay werd ontwikkeld door stabiele transfectie van het pGL4CP-SV40-2xtaDR4 construct in de GH3-cellen, resulterend in een zeer gevoelige schildklierhormoon cellijn (GH3, TRE-Luc), die verder werd geoptimaliseerd in een assay waarmee de detectie werd bewerkstelligd van triidothyronine- (T_3) en thyroxine- (T_4) concentraties in het picomolaire gebied na slechts 24 uur blootstelling. De meer dan 20-voudige inductie door T_3 ten opzichte van de controle met het oplosmiddel, is illustratief voor de hoge gevoeligheid van het systeem. De assay werd gevalideerd door de kwantificering van het agonistisch effect van de natuurlijke hormonen (T_3 en T_4), de azijnzuurderivaten van T_3 (triiodothyroacetic acid, Triac) en T_4 (tetraidodothyroacetic acid, Tetrac), hydroxypolybrominated diphenylethers (OH-PBDEs), hydroxypolychlorinated biphenyls (OH-PCBs) en de antagonistische actie van natriumarseniet ($NaAsO_2$). De mogelijke antagonisten amiodarone en bisphenol A (BPA) en de gehalogeneerde derivaten daarvan (TCBPA en TBBPA), waarvan de in de literatuur beschreven effecten niet consistent zijn, vertoonden vergelijkbare dosis-respons curves met een gering agonistisch effect (5% van T_3-max). De omvang en reproduceerbaarheid van de responses op diverse stoffen bevestigden dat de assay een veelbelovende techniek is voor de identificatie en het kwantificeren van specifieke schildklierhormoonreceptor actieve stoffen.

In hoofdstuk 3 wordt het gebruik beschreven van de GH3.TRE-Luc reporter gen cellijn in een qHTS platform. De GH3.TRE-Luc assay werd gemodificeerd en gevalideerd voor gebruik van de kleinere volumes, geschikt voor het formaat van een 1536-wells plaat. Door dit sterk verkleinde formaat te gebruiken, werd het mogelijk de collecties van de Library of Pharmacologically Active Compounds (LOPAC) en de National Toxicology Program (NTP) collectie te screenen op schildklierhormoon-receptoragonisten en -antagonisten. Van de 2688 stoffen die werden getest, werden er

8 of 1 als TR-agonisten geïdentificeerd -afhankelijk van de definitie van de positieve cut off- hetzij op ≥10%, hetzij op ≥20% van werkzaamheid in verhouding tot T_3, en 5 stoffen werden geïdentificeerd als antagonisten. Geen van de niet-actieve stoffen was structureel verwant aan T_3, en ook bij verdere structurele analyse van de geteste stoffen werd geen schildklierhormoonverstorende stof ontdekt. De lage-potentie agonisten leken ook niet op de schildklierhormonen, wat een aanwijzing is dat deze niet direct actief zijn via het ligand-binding gebied van de receptor en dat ze mogelijk geen effectieve agonisten zijn. Het bepalen van schildklierhormoonreceptor agonisten in de qHTS, waarbij gebruik wordt gemaakt van de criteria van een hit cut off van ≥20% werkzaamheid bij 100 μM, zou identificatie kunnen voorkómen van positieven zonder fysiologische relevantie. Geconcludeerd werd dat de verkleinde GH3.TRE-Luc assay een belangrijke aanvulling is op de testreeks voor schildklierhormoonreceptor-gemedieerde endocriene verstoring en dat, gezien het lage percentage van positief geteste stoffen, zijn high-throughput karakter een groot voordeel is voor toekomstige toxicologische screening.

Nadat de GH3.TRE-Luc cellen hoog-responsief bleken te zijn en geschikt voor de toepassing in een qHTS testreeks voor het opsporen van schildklierhormoon-verstorende chemicaliën, werd een vergelijking gemaakt met de respons van betrouwbare endogene schildklierhormoon doelgenen (Hoofdstuk 4). In aanvulling op de TRE-gestuurde luciferase inductie in de GH3.TRE-Luc cellen, werd ook schildklierhormoonafhankelijke celproliferatie geanalyseerd, omdat het effect van sommige schildklierhormoonverstorende stoffen onafhankelijk kon zijn van de geldende schildklierhormoon gemedieerde effecten op genexpressie. In deze studie werden GH3 cellen behandeld zowel met T_3 als met twee schildklierhormoonreceptor vormen (TRα and TRβ) selectieve thyromimetics, respectievelijk CO23 en GC-1. Responsprofielen in de reportergen assay vertoonden uitstekende overeenkomst met endogene groeihormoon (GH) genexpressie, een bekend direct schildklierhormoon doelgen en de basis van het gebruik van deze cellen in de op celproliferatie gebaseerde T-screen. Voorts werd duidelijk dat natriumarseniet, bisphenol A en de phtalate DEHP de T_3 gemedieerde inductie van het reportergen onderdrukken evenals de endogene expressie van het GH-gen. Een daaropvolgend uitgevoerd microarray

experiment bracht een vollediger spectrum aan het licht van de T_3 respons-genen in deze cellen. Naast het identificeren van verscheidene belangrijke componenten van de schildklierhormoon-signaling pathway, maakte de microarray de identificatie mogelijk van een reeks van zowel reeds bekende als van nieuwe schildklierhormoon doelgenen, inclusief genen die gerelateerd zijn aan angiogenese zoals HIF2α (ERPAS1). Identificatie van deze genen is belangrijk voor de validatie van assays die ontworpen zijn om nieuwe synthetische schildklierhormoonreceptor modulatoren en hun potentiële genomische, versus non-genomische modes of action te identificeren. Hoofdstuk 5 gaat over de kritische rol van het schildklierhormoon in processen zoals hersenontwikkeling, groei en energie-homeostase die plaats vinden via verschillende vormen van de schildklierhormoonreceptor. Omdat de belangrijkste vormen, TRα and TRβ, onderscheiden fysiologische functies representeren, is het belangrijk de schilklierhormoonreceptor specifieke potentie van schildklierhormoon actieve stoffen te bestuderen. Dit hoofdstuk beschrijft de ontwikkeling van TRα- and TRβ-gebaseerde reporter gen bioassays die humane cervicale HeLa-cellen gebruiken en zo kwantificering mogelijk maken van de respons van de schildklierhormoonreceptor vorm-selectieve chemicaliën CO23 en GC-1. In combinatie hiermee werd een stabiele TRα-specifieke gen cellijn ontwikkeld, die de naam TRα.HeLa-Luc kreeg. Reacties in deze TRα.HeLa-Luc cellijn werden vergeleken met reacties in de GH3.TRE-Luc-cellijn, die TRβ bleek te bevatten als belangrijkste schildklierhormoon receptorvorm. De schildklierhormoonreceptor-selectieve chemicaliën CO23 en GC-1 veroorzaakten reacties overeenkomend met de verwachte potentierangorde T_3>GC-1>CO23 in de TRα.HeLa-Luc-cellijn en T_3>CO23>GC-1 in de GH3.TRE-Luc-cellijn. In aanvulling op de 7 bekende schildklierhormoonreceptor actieve standaarden werden 10 chemicaliën getest die ervan verdacht werden dat zij een schildklierhormoonreceptor verstorende werking zouden hebben. Van deze chemicaliën veroorzaakten alleen natriumarseniet en de T3-achtige 4-OH-BDE69 een reactie in de TRα.HeLa-Luc assay. Samen voegen de ontwikkelde TRα.HeLa-Luc en de GH3.TRE-Luc stabiele reportergen cellijnen een nieuw niveau van specificiteit toe aan de bestaande testreeks en bewerkstelligen daarmee een verbetering van de beschikbare tools voor *in vitro* high throughput

assessment van schildklierhormoonreceptor specifieke activiteit van geneesmiddelen en milieuverontreinigende stoffen.

ABOUT THE AUTHOR

CURRICULUM VITAE

Jaime Freitas was born on the 9th of March 1980, in Maputo (Mozambique). At an early age he moved with his family first to Berlin (Germany), then to Lisbon (Portugal), to finally settle in the north of Portugal in a city called Espinho, where in 1999 he obtained his high school degree in Natural Sciences. In this same year, he enrolled in a 5-year study program in Applied Chemistry at the New University of Lisbon (Portugal).

In 2004 he was granted an Erasmus scholarship to conduce part of his specialization thesis in Biotechnology at the Department of Experimental Immunology in the Academic Medical Center of the University of Amsterdam (Netherlands). Working on a project entitled: "Controlling inflammatory responses in the airways" under the supervision of Dr. Rene Lutter.

While still finishing his University degree, in 2005 he was awarded a scientific initiation grant by the Portuguese Science Foundation to work under the supervision of Prof. Gertrude Thompson on a project entitled: "Deciphering the mechanisms of rabbit genetic resistance to myxomatosis and rabbit haemorrhagic viral disease" at the Laboratory of Infectious Diseases in the Institute of Biomedical Sciences Abel Salazar of the University of Porto (Portugal).

In 2006 he was appointed the PhD candidate position at the Division of Toxicology in collaboration with the Department of Neurology, Physiology and Behavior from the University of California-Davis (USA) to work under supervision of Prof. Tinka Murk, Prof. David Furlow and Prof. Ivonne Rietjens, on the project discussed in the present thesis.

Since 2012, he is working as a post-doctoral researcher on the European Large-Scale Project "Nanofol" at the Cell Activation and Gene Expression Group at the Institute for Molecular and Cell Biology (IBMC, Porto).

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Gutleb AC, **Freitas J**, Murk AJ, Verhaegen S, Ropstad E, Udelhoven T, Hoffmann L, Audinot JN (2012) NanoSIMS50 - a powerful tool to elucidate cellular localization of halogenated organic compounds. Analytical and Bioanalytical Chemistry, 1618-2642.

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Freitas J, Cano P, Craig-Veit C, Furlow JD, Murk AJ. Detection of thyroid hormone receptor disruptors by a novel *in vitro* assay. AiO/OiO days of the Dutch Society for Toxicology, 18-19 June 2009, Veldhoven, Netherlands. (Oral Presentation).

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Freitas J, Craig-Veit C, Murk AJ, Furlow JD. Development and validation of a highly sensitive bioassay to screen TH receptor-specific endocrine disruptors. SETAC Europe 18th Annual Meeting, 25-29 May 2008, Warsaw, Poland. (Oral & Poster Presentation).

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

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Research Context Activity: Writing of postdoc research proposal on the basis of PhD research outcomes: Thyroid Hormone Disruption - Predicting *in vivo* effects based on *in vitro* testing by incorporating in *silico* fate modeling.

Other PhD and MSc Courses

Environmental Toxicology, Wageningen University, Netherlands

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Didactic Skills Training

Supervision of four MSc theses and two MSc internships

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Development of a stable thyroid hormone receptor reporter gene cell line, University of California, Davis, Davis, CA, USA, 2007

Oral Presentations

Development and validation of a highly sensitive bioassay to screen TH receptor-specific endocrine disruptors. SETAC Europe 18th Annual Meeting, 25□29 May 2008, Warsaw, Poland

Detection of thyroid hormone receptor disruptors by a novel in vitro assay. AiO/OiO days of the Dutch Society for Toxicology, 18-19 June 2009, Veldhoven, Netherlands

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