Thyroid in a Jar:
Towards an Integrated In Vitro Testing Strategy for Thyroid-Active Compounds

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Thesis

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Chapter 1

General introduction

Barae Jomaa

Based on Thyroid toxicogenomics: A multi-organ paradigm. In J. Kleinjans [ed.], Toxicogenomics-Based Cellular Models: Alternatives to Animal Testing for Safety Assessment, 159–180 (2014). Academic Press.

1.1 The thyroid scare

Disorders related to the thyroid have long been known to man owing to the visible enlargement of the gland known as goiter. This condition can be due to either an overactive or an underactive thyroid gland, with the former increasing and the latter decreasing the rate at which the body breaks down nutrients and produces cellular components necessary to sustain life (Terris and Gourin, 2008). The successful treatment of goiter with animal thyroid glands was reported in China as early as 643 CE (Temple and Needham, 2007). That environmental contaminants, particularly lead, are associated with goiter had already been proposed by Paracelsus in the sixteenth century. In the nineteenth century, the essential element iodine that is a basic constituent of thyroid hormones (THs) was discovered (Anon, 2013; Kelly, 1961). Thyroid hormones were characterized in the twentieth century, starting with tetraiodothyronine (T4) in 1915, and later triiodothyronine (T3) in 1952 (Figure 1.1) (Edward Calvin Kendall, 1915; Gross and Pitt-Rivers, 1952).

The twentieth century saw a chain of events that raised the level of public concern over compounds that affect the thyroid, and ultimately culminated in the inclusion of thyroid disruption endpoints by the Organisation for Economic Co-operation and Development (OECD) in guidelines for the testing of chemicals (Figure 1.2). The "cranberry scare" of 1959 brought a herbicide, aminotriazole, and its effect on the thyroid into US politics, and toxicology to the forefront of public life. At the very core of the debate was a rat study that showed a high incidence of thyroid cancer upon exposure to high amounts of the pesticide. The idea that chemicals affecting rodents at high doses will affect humans at moderate doses, also known as the "mouse-as-little-man" principle, was so entrenched in people's minds at the time that it formed part of an amendment to the Federal Food, Drug, and Cosmetic Act known as the Delaney clause. To ease the newly created "chemophobia," the then-vice-president Richard Nixon ate cranberry sauce during his presidential election campaign (Lieberman, Adam J., 2004). A decade later, Nixon became president and proposed the establishment of the US Environmental Protection Agency (USEPA), which was approved by congress. Today, the jury is still out on the human relevance of the adverse effect of aminotriazole that was detected in the rat study, even after a retrospective cohort study of exposed Swedish railroad workers found it to be a "suspicious" carcinogen (Axelson et al., 1980). On the one hand, epidemiological data are often mired with confounders such as the presence of a mixture of active compounds in pesticides, and on the other hand, rodents have been shown to be overly sensitive to thyroid hormone disruption (Alison et al., 1994). These uncertainties have led the USEPA to consider aminotriazole as a "probable" human carcinogen (Group B) instead of a definitive human carcinogen (Group A) (USEPA, 1996b).

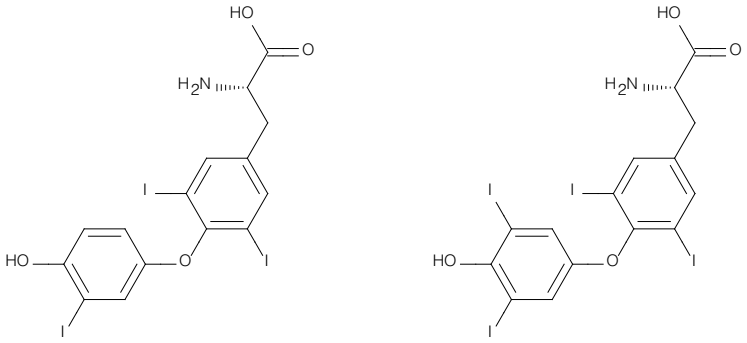

Fig. 1: Chemical structures of T3 and T4, respectively.

The establishment of the European Chemicals Agency (ECHA) in 2007 came on the heels of another public concern, that of endocrine disruption. Again, as one of the three main classes of endocrine disruptors, thyroid disruptors are at the very heart of modern toxicology—the other two classes being estrogen disruptors and androgen disruptors. The main fear today is that a change to thyroid homeostasis could be caused by man-made chemicals and affect the hormone system's well-documented orchestration of sexual and mental development, and this at much smaller doses than predicted from the effects observed at high doses (Vandenberg et al., 2012).

1.2 The thyroid system

The thyroid system is highly responsive to a wide range of external stressors. The hypothalamus responds to conditions requiring increased energy expenditure, such as pregnancy or prolonged periods of cold, by secreting thyrotropin-releasing hormone (TRH), which stimulates the anterior pituitary to produce thyroid-stimulating hormone (TSH), which in turn stimulates the thyroid to secrete thyroid hormones. The hypothalamus also responds to changes in diet by setting off a similar downstream chain of events. A diet that is high in carbohydrates promotes energy expenditure and leads to increased T3 levels, whereas fasting promotes energy conservation and therefore leads to a drop in T3 levels (Palmblad et al., 1977).

In order to keep the rise in T3 production and resulting increase in energy consumption and thermogenesis under control, the body has a negative feedback loop, whereby high levels of T3 downregulate TRH and TSH production. The resultant drop in these tropic hormones leads to decreased stimulation of thyroid hormone production and secretion, and ultimately a drop in serum thyroid hormone levels.

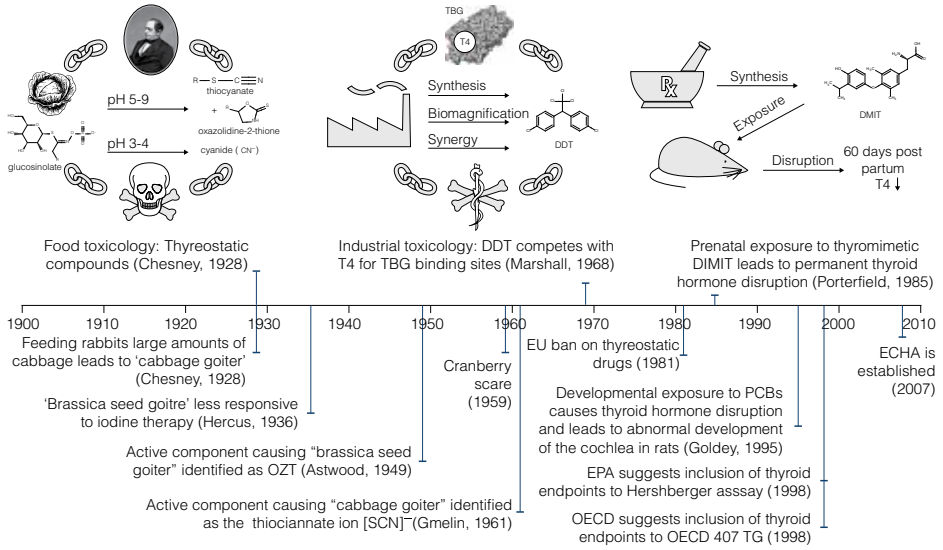

Fig. 2: Timeline of the events that have shaped our understanding of thyroid hormone disruption in the century preceding the establishment of the European Chemicals Agency in 2007. TBG stands for thyroxine binding globulin; thyroxine is another name for T4 [Source: Barae Jomaa/Wikimedia Commons].

The circulating levels of the hormones T3, T4, and TSH are constantly reacting to environmental factors that include temperature, oxygen levels, light, diet, and physical and emotional stress, in addition to chemicals and drugs (Capen and Martin, 1989; Chan et al., 1978; Hackney et al., 1995; Roth et al., 2002; Singh and Turner, 1969; Tingley et al., 1965). TH levels are also influenced by age and sex (Franklyn et al., 1985). Moreover, an individual's range is half the width of the population reference range, which means that TH levels that are normal for one individual can be abnormal for another (Andersen et al., 2002). This also means that there is a genetically determined set-point, whose heritability was estimated in a study on healthy twins to be around 65%, with the rest being attributed to environmental factors (Hansen et al., 2004).

1.3 Rodent toxicity tests with thyroid endpoints

The first laws requiring animal testing were passed in 1938 for marketed pharmaceuticals following the death of 105 users of Elixir Sulfanilamide, which consisted of sulfanilamide dissolved in the sweet but poisonous solvent diethylene glycol (Geiling EK and Cannon PR, 1938; United States Department of Agriculture, 1937). More than half a century later, in 1996, the first legislations requiring the USEPA to address endocrine disruption were put in place (USEPA, 1996a; c). A decade

later, the European Union regulation on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) was passed, which also required an authorization for endocrine disrupting chemicals for which evidence of serious effects on humans or the environment exists (REACH, 2006). In 2009, the USEPA selected 67 chemicals for screening based on exposure potential and in 2013, an additional 109 were selected for endocrine disruptor screening (USEPA, 2009, 2013). Today, the establishment and standardization of animal tests with endocrine endpoints is a work in progress. Table 1.1 presents an overview of rodent test guidelines with thyroid-related endpoints, which include serum thyroid hormone levels, thyrotropin levels, thyroid and pituitary weights as well as thyroid histopathology.

Standardized rodent tests for thyroid-active compounds are inherently unethical, low-throughput and do not always represent effects seen in humans. This means that large-scale chemical screening programs need to move away from animal testing.

1.4 Mode-of-action-based alternative testing strategies for thyroid activity

The development of in vitro testing strategies for thyroid-active compounds must be directed at a variety of endpoints, taking into account all the physiological targets that are known to be sensitive to interference by exogenous thyroid-active chemicals with various modes of action (MOAs). Figure 1.3 illustrates a breakdown and simplification of the potential targets of thyroid-active compounds that are relevant to the thyroid system.

Table 1.2 gives an overview of the currently available in vitro assays that could serve as potential candidates for high-throughput screening. The MOAs that they represent are discussed hereafter, providing a basis for the future development of screens for thyroid-active compounds, including thyroid toxicogenomics-based screens. The number of studies using transcriptomics, let alone other 'omics, when studying thyroid hormone disruption is still very limited. Nonetheless, the aim is still the same—to delineate modes/mechanisms of action, to identify signatures and biomarkers of exposure and effect, and to classify phenotypes of thyroid toxicity (Shirai and Asamoto, 2003).

Primary effects on the thyroid system

Compounds with a primary effect target hormonogenesis at the level of the thyroid and might lead to primary hypothyroidism/hyperthyroidism. The main MOAs involved include inhibition of TSH receptor (TSHR) activation, iodide transport, iodination and coupling, proteolysis, and iodide scavenging (Figure 1.4). The following sections will present these targets for primary effects on the thyroid system in some more detail.

Tab. 1.1: Current standardized rodent toxicity tests with thyroid system endpoints

Screening Assay	Thyroid weight	Pituitary weight	Thyroid Histopathology	Serum TH levels
OECD TG 407	+	+	+	+ (optional)
OECD TG 408	-	-	+	-
OECD TG 416	+	+	-	-
OECD TG 422	-	-	+	-
OECD TG 441	-	-	-	+ (T3 and T4, optional)
OECD TG 443	+	+	+ (optional)	+ (T4 and TSH)
OECD TG 451			+	
OECD TG 452	+		+	
OECD TG 453	+		+	
EPA 15-day intact adult male rat assay	+	-	+	+
EPA Pubertal male	+	+	+	+ (T4 and TSH)
EPA Pubertal female	+	+	+	+ (T4 and TSH)

Inhibition of TSHR activation

TSH, also known as thyrotropin, is a glycoprotein hormone that is produced by thyrotrophic cells in the anterior pituitary and secreted into the circulation where it eventually reaches the thyroid. Once at the basolateral side of thyroid follicular cells, it binds the thyrotropin receptor (TSHR), a G-protein coupled receptor (GPCR), and the ligand–receptor pair gets internalized, thereby initiating a signaling cascade that leads to thyroid hormone production as well as cell proliferation (Werthmann et al., 2012).

There are two pathways that are mostly initiated by TSH-regulated TSHR activation, likely owing to the presence of different active conformations of this receptor (Wonerow et al., 2000). The first involves the adenyl cyclase (AC) pathway. TSHR-mediated G-protein alpha s (Gαs) subtype activity leads to AC catalyzed conversion of ATP to cyclic AMP (cAMP), which then activates cAMP-dependent protein kinase (PKA) and exchange proteins directly activated by cAMP (EPACs), both of which are mediators of the mitogenic action of TSH (Hochbaum et al., 2008). The second involves the phospholipase C (PLC) pathway. TSHR-mediated G-protein alpha q (Gαq) subtype activity leads to PLC-catalyzed hydrolysis of phosphoinositol 4,5 biphosphate (PIP2) and production of diacylglycerol (DAG) and inositol triphosphate (IP3). DAG activates the calcium-dependent protein kinase C (PKC) cascade that results in mitogenic activity, while IP3 signaling leads to the release of the required calcium from the endoplasmic reticulum.

Tab. 1.2: Overview of a selection of assays that are deemed potential candidates for high-throughput screens. They cover all or part of 1) thyroidal hormonogenesis, 2) pituitary control, 3) hypothalamic CNS control, 4) thyroid hormone activity at the target tissue, 5) kinetics, and 6) epigenetics, immune response and other mechanisms.

	MOA	Target(s)	Endpoint	Readout	Cellular model	Origin	Organism	Ref.
1)	TSHR activation	TSHR	Cell proliferation	FL	FRTL-5 (WT)	Thyroid	Rat	(Jomaa et al., 2013)
			[I-125]TSH	GR	Cell-free	Cell-free	Cell-free	(Minich et al., 2004)
			cAMP levels	FL	rHEK293 (R)	Kidney	Human	(Titus et al., 2008)
			cAMP levels	LU	CHO (R)	Ovary	Hamster	(Evans et al., 1999)
	Iodide transport	NIS	FL quenching	FL	FRTL-5 (R)	Thyroid	Rat	(Di Bernardo et al., 2011)
		Na+/K+ ATPase	Molybdenum reduction	OD	FRTL-5 (WT)	Thyroid	Rat	(Pekary et al., 1997)
			Gene expression	-	FRTL-5	Thyroid	Rat	-
		Pendrin	I-125 levels	GR	MDCK (R)	Kidney	Dog	(Gillam et al., 2004)
	Iodination & coupling	TPO	Guaiacol Ox	OD	Cell-free	Cell-free	Cell-free	(Schmutzler et al., 2007)
		DUOX/H_2O_2	CM-H2DCFDA Ox	FL	FRTL-5 (WT)	Thyroid	Rat	(Yoshihara et al., 2012)
	Proteolysis	Cts	Proteolysis	-	-	-	-	-
	Iodide scavenging	IYD	[125I]-DIT, [125I]-MIT	GR	Cell-free	Cell-free	Cell-free	(Gnidehou et al., 2004)
	TH synthesis	T4	T4 levels	FL	Whole embryo	Whole embryo	Zebrafish	(Raldúa and Babin, 2009)

2)	TRHR activation	TRHR	Calcium flux	FL	Chem-1(HR)	Bone marrow	Rat	(Millipore, 2012)
	TSH synthesis	TSH	TSH levels	OD	TαT1 (WT)	Pituitary	Mouse	
	(Zatelli et al., 2010)							
3)	TRH synthesis	TRH	TRH levels	GR	U-373-MG (WT)	Brain	Human	(García et al., 2000)
4)	TR activation	TR	Cell proliferation	FL	GH3 (WT)	Pituitary	Rat	(Gutleb et al., 2005)
			Gene expression	LU	GH3 (R)	Pituitary	Rat	(Freitas et al., 2011)
	Coregulator interaction	TR CoR	TRβ-SRC2-2	FP	Cell-free	Cell-free	Cell-free	(Johnson et al., 2011)
5)	TH blood transport	TTR, TBG	Competitive binding	FL	Cell-free	Cell-free	Cell-free	(Montaño et al., 2012)
	Cellular uptake of TH	OATP1C1	[125I]T4	GR	HEK293 (R)	Kidney	Human	(Westholm et al., 2009)
		MCT8/10	[125I]T3	GR	MDCK1 (R)	Kidney	Dog	(Kinne et al., 2009)
	TH metabolism	DIOs	[125I] levels	GR	Cell-free	Cell-free	Cell-free	(Kuiper et al., 2005)
		hrSULTs	3H-DHEA	GR	Cell-free	Cell-free	Cell-free	(Ekuase et al., 2011)
		hrUGTs	[125I]T4-G	GR	Cell-free	Cell-free	Cell-free	(Martin et al., 2012)
6)	ITG αvβ3 activation	ITG αvβ3	[125I]T4	GR	CV-1 (R)	Kidney	Monkey	(Bergh et al., 2005)

WT stands for wild type, R for recombinant (stable and transient), HR for human recombinant, OD for optical density, FL for fluorescence, FP for fluorescence polarization, LU for luminescence, GR for gamma radiation, Red for reduction, Ox for oxidation, DUOX for dual oxidase, CM-H2DCFDA for 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, Cts for cysteine cathepsin proteases, IYD for iodotyrosine deiodinase, [125I]-DIT for iodide-125 radiolabeled DIT, [125I]-MIT for iodide-125 radiolabeled MIT, OATP for organic anion-transporting polypeptide, CoR for coregulator, ITG for integrin, and a dash is included (-) when an assay either has not been developed or is not suited for the purpose of high throughput analysis.

Inhibition of iodide transport

All dietary iodine is reduced to iodide prior to absorption in the small intestine (Hays, 1984). Once in the circulation, iodide is absorbed by tissues through the sodium iodide symporter (NIS), also known as solute carrier family 5, member 5 (SLC5A5). NIS is a transmembrane glycoprotein that is mainly expressed on the basolateral side of thyroid follicles and which concentrates iodide to a level that is 20 to 50 times that in plasma (Caillou et al., 1998; Eskandari et al., 1997).

NIS is also functional in extrathyroidal tissues, including the small intestine where dietary iodide is taken up into the bloodstream, and lactating mammary glands, through which iodide is delivered to the newborn for proper development. Gastric mucosa, salivary glands, and rectal mucosa also express NIS but its function in these tissues is still unknown. In the thyroid, TSH up-regulates the expression of the NIS gene, whereas iodide inhibits it (Dohán et al., 2003). Anions such as thiocyanate (SCN^-) compete with iodide for transport through NIS.

NIS activity is dependent on the sodium potassium pump (Na/K-ATPase) to provide a sodium gradient. It is through this sodium gradient that iodide is transported along with sodium into the cell through NIS. Accordingly, the Na/K-ATPase inhibitor ouabain inhibits NIS-mediated iodide transport (Josefsson et al., 2006). Na/K-ATPase ATP1A1 and ATP1B1 expression levels are more than threefold and tenfold higher, respectively, in the brain, kidney, and thyroid than in other tissues (Su et al., 2004).

In 1997, a century after Vaughan Pendred described a genetic condition giving rise to hearing loss and goiter, mutations in the pendrin gene (SLC26A4) were associated with the disease (Everett et al., 1997). As can be seen from Figure 1.4, iodide does not only need to be transported in the blood and enter the basolateral side of the thyroid cell, it also needs to cross the apical side in order to reach the follicular lumen where the iodination of thyroglobulin (TG) and the coupling of monoiodotyrosine (MIT) and diiodotyrosine (DIT) take place as penultimate steps in thyroid hormone production. While research on the apical transport of iodide is ongoing, pendrin is, at the very least, one of the key transporters involved in TSH-mediated iodide efflux at the apical side of follicular cells, thus completing the transport into the follicular lumen that was started by NIS at the basolateral side (Pesce et al., 2012; Twyffels et al., 2011).

Inhibition of iodination and coupling

At the apical surface of the thyroid follicle, thyroglobulin is iodinated in the presence of H_2O_2 by thyroid peroxidase (TPO) (Schweizer et al., 2008). Iodinated tyrosyl residues on thyroglobulin form MIT and DIT — the building blocks for T3 and T4 (Figure 1.4). TPO inhibitors can be divided into three classes: drugs, environmental pollutants, and

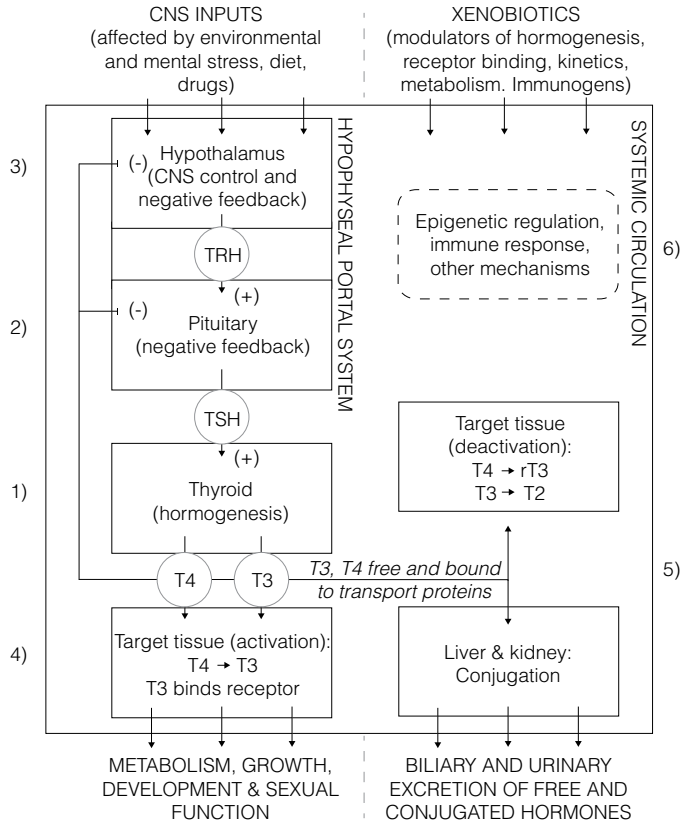

Fig. 3: Overview of the thyroid hormone system from synthesis to clearance. Chemicals can exert their effects through MOAs relating to 1) thyroidal hormonogenesis 2) pituitary control 3) hypothalamic CNS control 4) thyroid activity at the target tissue 5) kinetics and 6) epigenetics, immune response and other mechanisms [Source: Barae Jomaa/Wikimedia Commons].

food products. Antithyroid drugs, which are given in cases of hyperthyroidism, act on TPO, and include methimazole and propylthiouracil. Psychotropic drugs such as tricyclic antidepressants deactivate TPO by binding to its heme (Bou Khalil and Richa, 2011). Environmental contaminants shown to inhibit TPO include benzophenone 2 (BP2) (Schmutzler et al., 2007). Flavonoids contained in food can also inhibit this enzyme both in vitro and in vivo (de Souza Dos Santos et al., 2011).

Since the reactive oxygen species H_2O_2, needed along with TPO for hormonogenesis, can induce oxidative damage, additional safety mechanisms are needed by the thyrocytes. H_2O_2-generating dual oxidases (DUOXs) are localized at the apical membrane bordering the follicular lumen (Senou et al., 2010). As will be detailed later in the text, TPO is capable of degrading T4. Hence, TPO and H_2O_2, needed in the production of T4,

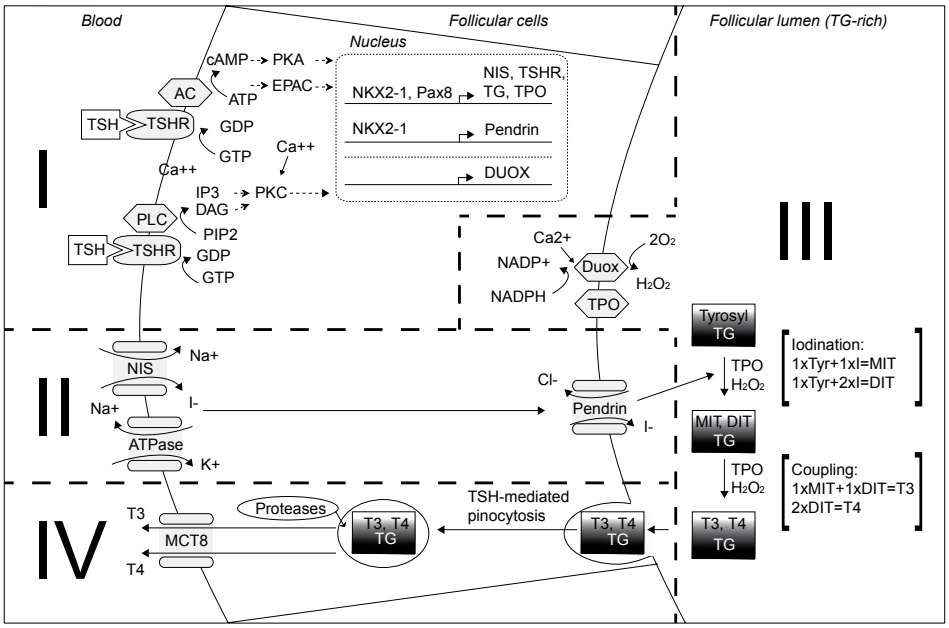

Fig. 4: Primary MOAs that take place at the level of the thyroid and affect thyroid hormone synthesis, including: I) TSH secreted from the pituitary reaches the thyroid and binds to its membrane receptor (TSHR) leading to upregulation of the expression of genes that encode both structural and functional proteins necessary for thyroid hormone production, II) iodide is transported through the cell and into the lumen, III) iodination and coupling reactions take place and form iodinated TG molecules, IV) iodinated thyroglobulin undergoes lysosomal proteolysis to release thyroid hormones as well as V) MIT and DIT whose iodide anions are scavenged [Source: Barae Jomaa/ Wikimedia Commons].

could both antagonize hormonogenesis and must be constantly kept under control. The extracellular surface of the cell membrane, facing the TG-rich lumen, is covered with a thin film rich in H_2O_2, while the intracellular surface of the cell membrane is lined with regions that are high in TPO (Ekholm and Björkman, 1997; Masini-Repiso et al., 2004). The apical surface of the thyroid cell can be thought of as a toxic bioreactor. This functional morphology is critical and might help explain why thyroid hormone production has not yet been achieved in an in vitro model. In 2012, a step in the right direction was taken and involved the in vitro overexpression of transcription factors needed for thyroid cell differentiation followed by in vivo grafting onto mice to finally produce hormones (Antonica et al., 2012). Nonetheless, in vitro results of this breakthrough experiment only indicated the in vitro iodination of TG, suggesting that either the proteolysis of TG to produce thyroid hormones is not taking place or the hormones are being degraded before they can be measured. Follow-up research could soon lead to an in vitro model for thyroid toxicity that encompasses all the complex mechanisms involved in the synthesis of thyroid hormones.

Inhibition of proteolysis

Iodinated thyroglobulin is pinocytosed back into the cell from the thyroglobulin-rich follicular lumen and is subjected to proteolysis by lysosomal proteases, which releases active hormones (Selmi and Rousset, 1988). This process is inhibited by excess iodide either from the diet, the environment, or iodine-rich drugs such as amiodarone (Radvila et al., 1976).

Inhibition of iodide scavenging

In addition to the release of thyroid hormones, proteolysis of iodinated TG also results in the release of MIT and DIT. These molecules carry the valuable iodide anion, which is scavenged by iodotyrosine deiodinase (IYD) and recycled. The importance of this mechanism of intrathyroidal iodide conservation was further elucidated after the cloning and characterization of IYD in 2002, and the implication of its mutation in 2008 in various cases of hypothyroidism (Afink et al., 2008; Moreno et al., 2002, 2008).

Secondary effects on the thyroid system

Compounds with a secondary effect on the thyroid system exert their action at the level of the pituitary, affect the gland's ability to regulate thyroid hormone secretion, and thereby have the potential to lead to secondary hypothyroidism/hyperthyroidism. TSH is secreted by the pituitary gland in response to hypothalamic TRH, which arrives directly through the hypophyseal portal system and is quickly degraded (half-life of 5 minutes) in the systemic circulation by a TRH-degrading enzyme (Marangell et al., 1997; Schmitmeier et al., 2002).

Inhibition of TRHR activitation and TSH secretion

TRH binds to the TRH receptor (TRHR), a GPCR on the cell membrane of TSH-producing thyrotrophic cells of the anterior pituitary, and sets off a signaling cascade whose net effect is the production of more TSH. TRHR has been studied in detail in rodents, where two subtypes were identified and named TRHR1 and TRHR2. As with many GPCRs, the receptors are desensitized by internalization upon agonist binding in order to regulate the length of the signal (Jones and Hinkle, 2009). Signal transduction that leads to TSH production is mediated by the PLC pathway described earlier. It has been postulated that the anticonvulsant and mood stabilizer carbamazepine inhibits TRH-induced TSH secretion in humans (Joffe et al., 1984) and further studies revealed decreased binding of TRH to TRHR (Rosen et al., 1994).

The activation of signal transduction by TRH takes place when TRHR present on the surface of thyrotrophic cells is coupled to Gq proteins (Sun et al., 2003) and is mediated

by transcription factors including cAMP response-element-binding protein (CREB), AP-1 and Elk-1 (Wang and Gershengorn, 1999). The three main genes of interest that are being expressed in response to this TRH-mediated signaling are TSH, DIO2, and THRB (the gene for TRβ).

Factors that suppress the eventual secretion of TSH include somatostatin, thyroid hormones, growth hormone, glucocorticoids, melatonin, and opioid peptides.

Calcium channel blockers have been found to significantly decrease serum levels of T3 and T4 and to elevate serum TSH in rabbits, which is thought to result from impaired thyroid hormone synthesis or release at the level of the thyroid (Mittal et al., 1993). In man, the pituitary is more affected by calcium channel blockers than the thyroid, as studies have revealed that there is a significant decrease in TRH-stimulated TSH release but no significant change in levels of either T3 or T4 (Teba et al., 1985; Yamada et al., 1986).

Thyroid cancer in rats follows continuous secretion of TSH, which leads to the overstimulation of thyroid cells and results in hyperplasia that progresses to adenoma and later to carcinoma (Segev et al., 2003). Iodine-deficient and goiterous patients have elevated levels of TSH but not the thyroid cancer that is observed in rats. This implies that rat thyroids are more responsive to TSH than human thyroids and that the human relevance of TSH has more to do with its role in modulating the secretion of thyroid hormones. While this underlines species differences, excessive and prolonged TSH stimulation of the thyroid can lead to thyrotoxicosis in humans (Ahmed and Steve, 2011). Excessive TSH secretion can be the result of increased TRH activity but can also be due to improper negative feedback, which involves the other two genes of interest that are expressed in the thyroid—DIO2 and THRB.

Tertiary effects on the thyroid system

Compounds with a tertiary effect exert their action at the level of the hypothalamus and compromise its role as a control center that is able to translate various stimuli into messages to the pituitary where the message is relayed to the thyroid.

TRH is present throughout the central nervous system and is highly expressed in the paraventricular nucleus (PVN) region of the hypothalamus. The median eminence at the base of the brain contains both the hypothalamo-hypophyseal portal vessels and axons of a group of neurons that originate in the PVN. These neuroendocrine cells release TRH directly into the portal vessels that vascularize the pituitary. There, the hormone acts as a stimulant of TSH release by binding the TRH receptor (TRHR) on the membrane of thyrotrophic cells (Shibusawa et al., 2008).

This function as a stimulant of TSH release seems to have evolved as animals moved to land, since in poikilotherms, including the zebrafish that is increasingly being used in thyroid research, TRH does not stimulate TSH release. The ancestral role of TRH as a neuroregulator is being increasingly recognized and an analog has been successfully used in vitro as a neuroprotective agent in the glutamate-induced toxicity that is associated with neurodegenerative diseases such as Alzheimer's disease (Veronesi et al., 2007).

The secretion of TRH is down-regulated by T3 through local deiodination of T4 by deiodinase type 2 (DIO2). This action is thought to be TRβ mediated. A rapid fall in serum TSH, T4, and T3 is brought about by fasting and leads to a drop in the appetite suppressant leptin, which has been found to increase DIO2 activity (Coppola et al., 2005). Other regulators of TRH neurons include neuropeptide Y, melanocortin-stimulating hormone, and agouti-related peptide. What is known about the regulation of the TRH neuron has been reviewed in detail by Eduardo Nillni (Nillni, 2010).

Interference by chemicals at the level TRH signalling might lead to tertiary hypothyroidism and tertiary hyperthyroidism; the latter condition, although theoretically possible, is very rare (Gavras and Thomson, 1972).

Peripheral effects

Compounds with a peripheral effect exert their action at the level of the target organ and could result in either hypothyroidism or hyperthyroidism. The effects of thyroid hormones are mediated in a large part by their binding to thyroid hormone receptors (TRs), which act as transcription factors that mediate specific patterns of gene expression. There are four different isoforms: A1, A2, and B1 that are widely expressed, with B1 having its highest levels in the liver, and B2 that is expressed mainly in the hypothalamus and pituitary, where it regulates TH negative feedback, as well as in the inner ear and retina during development (Abel et al., 2001; Zandieh Doulabi et al., 2002). TRα2 does not bind thyroid hormones but as an apoprotein can still play a role in the regulation of TR-mediated gene expression, especially in its dephosphorylated state (Katz et al., 1995).

Inhibition of TR activity

DNA-binding domains on TRs have a high affinity for certain sequences of DNA called thyroid hormone response elements (TREs) and the resulting binding of the receptors to the DNA dictates the expression of linked genes. TRs can be found as monomers, homodimers, and heterodimers with retinoid X receptor (RXR), with the last having the highest affinity for TREs. Positive regulation of gene activity by thyroid hormones involves three general states of expression of genes that are linked to TREs. First, there is basal

activity, which can be exemplified in experimental knockout models of TRs. Second, there is repression of gene expression when TRs are bound to DNA in the absence of T3, since this state favors the formation of a co-repressor complex with histone deacetylase (HDA) activity. Third and last, there is activation of gene expression when TRs are bound to DNA in the presence of T3, since this state favors the formation of a co-activator complex with histone acetyltransferase (HAT) activity (Yen, 2001). This is relevant when considering that acetylated histones pack DNA less tightly and allow for greater gene expression. Negative regulation of gene activity is exemplified by T3-dependent repression of TRH expression in the hypothalamus and repression of TSH expression in the pituitary, a process that involves TRβ2 binding to negative TREs (nTREs) and interaction of TRβ2 with the co-activator SRC-1 (Abel et al., 2001; Carr and Wong, 1994; Weiss et al., 1999).

Co-regulator activity

Resistance to thyroid hormones is a condition in which the majority of tissues are not responsive even when the level of thyroid hormones is high. A mutation in the TRβ gene has been implicated and is thought to cause a disruption of the charge clusters (acidic, basic, or a mixture of both) in the tertiary structure of the protein that allows for co-activator binding (Lee et al., 2011). It is therefore evident that co-regulators play a physiologically significant role in the regulation of TR-mediated gene expression. As was mentioned above, thyroid-hormone-mediated transcriptional regulation involves either co-activator or co-repressor recruitment. While canonical steroid hormone receptors are inactive in the unliganded state, TR and retinoic acid receptor (RAR) form heterodimers with RXR that bind to DNA and recruit nuclear receptor co-repressor (NCoR) and silencing mediator for retinoid or thyroid-hormone receptors (SMRT), as well as Sin3 and HDAC that actively repress gene expression (Safi et al., 2009; Yen, 2001).

Hormone-kinetics-based effects

Thyroid hormone kinetics involves the distribution of T3 and T4 to the body's tissues, their metabolism, and their eventual clearance. Though these are mainly peripheral effects, they are classified separately due to the different MOAs of chemical disruptors acting on this level and their potential for kinetic modeling.

Circulation

Due to the lipophilic nature of the thyroid hormones T3 and T4, their transport in the body's circulatory system must be assisted by transport proteins. Thyroxine-binding globulin (TBG), transthyretin (TTR), and albumin (ALB) are, in order, the three proteins with the highest affinity for T4 and T3. While most of the thyroid hormones in humans

are transported by TBG, it is thought that albumin alone is sufficient for carrying these hormones (Schussler, 2000). "Free" T3 and T4 that are unbound to blood transport proteins are called fT3 and fT4 respectively. These free hormones are thought to be good indicators of the amount that is available at the cellular level.

Unlike humans, rodents only express thyroxine-binding globulin (TBG), which acts as a reservoir for thyroid hormones, when they need it the most—during development and again later on during senescence (Savu et al., 1991). TTR is the main T4 carrier in the circulation of rodents but TTR null mice did not respond any differently than wild-type mice when challenged with exposure to cold, which is known to result in thyroid-hormone-mediated thermogenesis. Additional experiments including thyroidectomy and gene-expression analysis led the authors to conclude that TTR has no role to play in thyroid hormone homeostasis (Sousa et al., 2005). In humans, decreased thyroid hormone binding to transport proteins raises the ratio of fT4 to T4, which results in greater clearance of T4 and a drop in total T4 (Schussler, 2000). Patients with undetectable levels of TBG in their serum due to a mutation in the gene had low total T4, which is associated with fatigue (Carvalho et al., 1998). Another study examined patients with sepsis and found a clear association between a drop in TBG, TTR, and ALB, a rise in fT4/T4, and a drop in total T4 (Afandi et al., 2000). It can be concluded that using rodents as models in animal experiments does not reflect the human situation and that in vitro experiments should take into consideration all of the main three thyroid-hormone-binding proteins.

Transport into cells

While it was previously thought that the lipophilic nature of thyroid hormones is sufficient for them to passively diffuse across the cell membrane, it is now clear that their transport is facilitated by plasma membrane transporters, including monocarboxylate (MCT) MCT8, MCT10, and organic anion transporting polypeptide (OATP) OATP1C1 (Pizzagalli et al., 2002; Friesema et al., 2003, 2008). A mutation in the MCT8 gene causes Allan–Herndon–Dudley syndrome, which includes high serum T3 levels coupled with severe psychomotor retardation. This transporter has been found to be expressed in a variety of tissues, including the heart, brain, liver, kidney, intestine, and placenta, while MCT10 is expressed in the skeletal muscles, liver, kidney, intestine, and placenta (Nishimura and Naito, 2008). To date, there have been no clinical cases of thyroid disease linked to either MCT10 or OATP1C1. While MCT8 is a specific thyroid hormone transporter, OATPs are also known to transport xenobiotics (Deure et al., 2010). However, OATP1C1 is specific to the brain and has high affinity for T4, and its lack in an OATP1C1 knockout mouse has been shown to lead to central nervous system (CNS)-specific hypothyroidism (Mayerl et al., 2012).

In addition to the previously mentioned role of calcium channels in thyroid hormone production, it appears that they are also involved in the transport of these hormones into peripheral organs.

Calcium channels have been found to be involved in T4 uptake into cardiomyocytes. In fact, the calcium channel blocker dilitazem reduced T4 uptake by 45%, whereas T3 uptake was unaffected (Verhoeven et al., 2001). Moreover, calcium has been found to be a first messenger in thyroid-hormone-mediated increases in sugar uptake, and calcium blockers inhibited this physiologically relevant action of thyroid hormones (Segal, 1990).

Metabolism and clearance

The metabolism and clearance of thyroid hormones is an essential process in the regulation of thyroid hormone activity and serum levels (Figure 1.5) with deiodination being the main mechanism and accounting for 85% of T4 and around 50% of T3 breakdown (Benedetti et al., 2005). The rest is accounted for by sulfonation and glucuronidation.

Thyroid-hormone-regulated transcriptional activity is largely dependent on DIO2 for the removal of an iodine atom from the outer ring of T4, turning it into T3, in order to boost the hormone's affinity for the TRs. Deiodinase type 3 (DIO3) removes an iodine from the inner ring of T4, turning it into rT3, an inactive congener (Figure 1.5). Deiodinase type 1 (DIO1) is capable of both inner and outer ring deiodination. rT3, containing two outer ring iodine atoms, can now be deiodinated into T2 by DIO2, while T3, which has two inner ring iodine atoms, can be deiodinated by DIO3 to form T2.

DIO3 is highly expressed in the placenta and embryonic tissue but has only been detected in a few adult tissues. Nonetheless, DIO3 has been shown to be responsible for the degradation of up to 80% of thyroid hormone production, which might indicate that the anatomical location of the bulk of this enzyme has yet to be defined. This enzyme has been found to be re-expressed during neoplasia and critical illness, leading to the hypothesis that it is a contributing factor in the low T3 levels that are observed in these situations (Huang and Bianco, 2008).

In the liver and to a lesser extent the kidney, sulfotransferases (SULTs) add a sulfate or, alternatively, UDP-glucuronosyltransferases (UGTs) add a glucuronic acid moiety to the phenolic hydroxyl group of T4, T3, rT3, and T2, rendering them more water soluble thereby facilitating excretion. Among the studied SULTs, 1A1, which is also involved in the inactivation of estrogens, has the lowest Km value and thus the highest affinity for thyroid hormones; in order, T2 > > rT3 > T3 > T4 (Wu et al., 2005). Glucuronidation takes place mainly in the liver, with the result being an increase in the water solubility of thyroid hormones, which is followed by biliary excretion. While drug-metabolizing enzymes are

not very abundant under normal circumstances, they are up-regulated upon xenobiotic challenge (Xu et al., 2005). The importance of glucuronidation for the thyroid system became apparent when compounds such as anti-epileptic drugs (AEDs) were tested on rats and were found to cause thyroid hyperplasia and tumors. These drugs, by acting as microsomal enzyme inducers (MEIs), increase the breakdown of thyroid hormones, which in turn leads to compensatory TSH production and its associated thyroid cell hyperplasia and tumor formation in rats. The only problem with these results is that AEDs are well studied in man and do not result in an increase in TSH production, nor have they ever been correlated with thyroid cancer. This is an important species difference between humans and rodents with regards to thyroid-active compounds, both in terms of sensitivity and outcome.

Clearly, there are differences between humans and rodents in the way they metabolize thyroid hormones. Indeed, it was found that in humans, unlike rats, T3 is not significantly glucuronidated. Nonetheless, in both species, T4 and rT3 are conjugated by UGT1A1 and UGT1A9. What is missing in humans is UGT1A9-mediated glucuronidation of T3 (Findlay et al., 2000; Visser et al., 1993). This means that T3 is first converted to rT3 by deiodinases before being glucuronidated, and rT3 is by far the preferred iodothyronine substrate for both UGT1A1 and UGT1A9.

Phenobarbital, an anticonvulsant, has been shown to increase thyroid hormone clearance by inducing the expression of OATP and UGT genes (Wieneke et al., 2009). This increased expression of hepatic transport and conjugation genes is mediated by the constitutive androstane receptor (CAR), which in addition to OATP and UGT, has also been shown to regulate SULT, leading to a drop in circulating levels of thyroid hormone. The same is thought to happen with other anticonvulsants, including phenytoin and carbamazepine, as well as certain antibiotics such as rifampicin (Zavacki and Larsen, 2005).

Minor degradation pathways for thyroid hormones are much less known and studied but are arousing interest simply because the resulting metabolites, while in small quantities, may have a significant biological role. Tetrac and triac, the deaminated forms of T4 and T3 respectively, are potent metabolites that act on the thyroid receptor in vitro and suppress TSH secretion in vivo (Everts et al., 1994; Juge-Aubry et al., 1995; Lameloise et al., 2001).

The deamination reactions are catalyzed by L-amino acid oxidase (LAO) and thyroid hormone aminotransferase (AT). Triac, which is present in human serum at a picomolar range, represents 14% of T3 degradation, and has a higher clearance rate than T3 owing to its rapid conjugation in the liver (Gavin et al., 1980). T4, sold as Synthroid, has long been used illicitly for weight loss, and now triac is being sold under various names

as a dietary supplement for weight loss, leading the US Food and Drug Administration (FDA) to issue repeated safety alerts and go to court in the case United States v. Syntrax Innovations, Inc., et al. (FDA, 2004). According to the FDA, the health consequences of consuming products containing triac include heart attacks and strokes (FDA, 2009).

Decarboxylation of T4 and T3 by a still unknown decarboxylase can lead to the formation of 3,3,5,5-tetraiodothyronamine (tetram) and 3,3,5-triiodothyronamine (triam), respectively. Though these were not found in vivo, other decarboxylation metabolites of thyroid hormones were. A 2004 Nature Medicine article by Scanlan et al. surprised the thyroid community with results suggesting that the observed non-genomic effects of thyroid hormones are in fact mediated by even less recognized metabolites of thyroid hormones. The authors found that 3-iodothyronamine (T1AM) and thyronamine (T0AM) are in vitro agonists of G-protein-coupled trace amine receptor TAR1 and in vivo induce hypothermia and bradycardia within minutes (Scanlan et al., 2004). It is increasingly becoming evident that the action of these minor metabolites within new signaling pathways should be of major concern for toxicologists; however, in vitro models of the disruption of thyroid hormone metabolism and clearance still look solely at inhibition of SULTs, UGTs, and DIOs (Murk et al., 2013).

Ether link cleavage of T4 by peroxidases in the presence of H_2O_2 leads to DIT. It is not surprising then that, in the thyroid, TPO and its substrate H_2O_2 can also act on newly synthesized T4 and degrade it. This means that the thyroid follicles have mechanisms in place to safeguard T4 from TPO-mediated degradation. Glutathione peroxidase, which is found intracellularly, has been found to inhibit TPO activity by disposing of the substrate H_2O_2 (Ekholm and Björkman, 1997).

Other MOAs affecting the thyroid hormone system

Epigenetics

Abnormal thyroid gland development, also called thyroid dysgenesis (TD), accounts for 80% of reported cases of congenital hypothyroidism (CH), with the remaining 20% of patients having a normal thyroid but a dysfunction in the synthesis of thyroid hormones (Gruters, 2004). A study on monozygotic twins led its authors to conclude that a classic genetic hypothesis for thyroid dysgenesis is unlikely (Kuehnen et al., 2009). In addition, mutations in genes commonly associated with thyroid development, such as PAX8, FOXE1, NKX2.1, and NKX2.5, were identified in only 3% of patients with congenital hypothyroidism from thyroid dysgenesis (Castanet et al., 2005). The sporadic nature of thyroid dysgenesis suggests that there could be epigenetic factors involved (Felice and Lauro, 2004; Vassart and Dumont, 2005). Xenobiotic exposure during critical time-

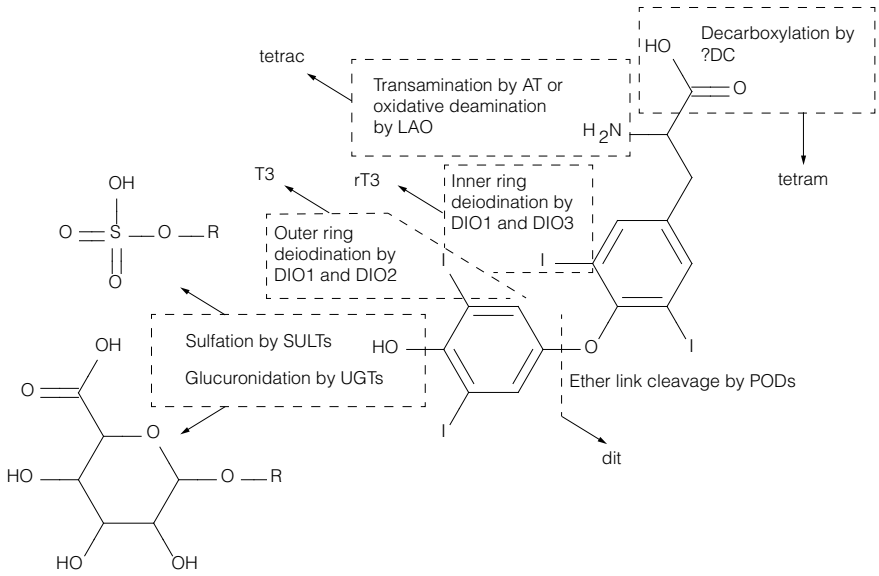

Fig. 5: Enzymes involved in the metabolism of T4, sulfotransferases (SULTs), UDP-glucuronosyltransferases (UGTs), deiodinases (DIOs), L-amino acid oxidase (LAO), thyroid hormone aminotransferase (AT), peroxidases (PODs) and a yet to be discovered decarboxylase (?DC) [Source: Barae Jomaa/Wikimedia Commons].

windows of development could affect both biochemical and epigenetic mechanisms and help explain the lack of a clear genetic underpinning for this disease.

While it is a challenge to gather epidemiological data showing the contribution of xenobiotics to thyroid dysgenesis, it is well established that hyperthyroid mothers receiving treatment with antithyroid drugs such as methimazole and propylthiouracil or who have been given radioactive iodine (RAI) are at higher risk of having a child with hypothyroidism (Atkins et al., 2000; Gorman, 1999). CH is associated with an increased risk of other congenital malformations and of these, most are cardiac malformations (Olivieri et al., 2002).

A recent study correlating pituitary cell proliferation upon exposure to thyroid-active compounds to pituitary gland weight ended up finding high correlation with the weight of another organ—the heart (Jomaa et al., 2013). This underlines the strong interplay between the heart and the thyroid system and also emphasizes the potential of in vitro systems to unravel physiological effects.

Autoimmunity

Autoimmune thyroid disease (AITD) can be divided into diseases that lead to hypothyroidism, such as Hashimoto's (chronic autoimmune thyroditis) and postpartum thyroditis, and diseases that lead to hyperthyroidism, such as Grave's disease. Autoimmune hypothyroidism results mainly from autoantibodies against TPO and TG, whereas autoimmune hyperthyroidism results from autoantibodies that bind the TSHR and activate it in a similar way to TSH. Even though the symptoms of autoimmune hypothyroidism and autoimmune hyperthyroidism are different, they both have a high degree of heritability and share susceptibility genes, including those for HLA-DR3, HLA-C*07, PTPN22, and CTLA4—all linked to the deregulation of T-cell activation (Panicker, 2011). Nonetheless, a Danish twin cohort revealed that 21% of Grave's disease could not be explained by heredity alone and must be due to the environment (Brix et al., 2001). On the other hand, a steady increase in the incidence of autoimmune thyroditis could be due to increased diagnosis, but increasing evidence is linking iodine to this disease (McLeod and Cooper, 2012; Rose et al., 1999). Some have even suggested the involvement of soy isoflavones in the formation of TPO neoantigens during their covalent binding and inactivation of the enzyme (Doerge and Sheehan, 2002).

Novel pathways

Integrin alpha v beta 3 (ITG αvβ3) has been found to be a cell surface receptor for T4 inducing a downstream signaling cascade that might help elucidate some of the in vivo effects that cannot be accounted for fully by other mechanisms (Bergh et al., 2005).

Multiple effects

The widespread use of lithium that followed its approval in the 1970s by the FDA for the treatment of bipolar disorder quickly led to its association with goiter (Bocchetta and Loviselli, 2006; Maletzky and Shore, 1978). However, a single MOA cannot explain this drug's antithyroidal effects. The multiple effects of lithium include abnormal iodine kinetics, altered thyroglobulin structure, inhibition of iodotyrosine coupling and thyroid hormone secretion, DIO2 inhibition, immunostimulation, and altered TR gene expression (Chakrabarti, 2011). These effects may be exacerbated by other factors such as iodine exposure, dietary goitrogens, and immunogenic background (Lazarus, 1998).

1.5 Objective and outline of the thesis

The objective of the studies described in this thesis is to identify and develop in vitro and toxicogenomics-based alternatives to in vivo thyroid hormone disruption tests. Given that thyroid-active compounds could exert their effect through numerous MOAs occurring

across multiple organs, a battery of in vitro tests has to be developed, validated and implemented. The research work presented in the following chapters begins with one-to-one replacements of in vivo endpoints with in vitro assays and proceeds in exploring additional tests and strategies that aim to tackle the pitfalls of these assays, such as the lack of the feedback mechanisms involved in thyroid homeostasis.

In the first part of Chapter 1, some of the key events that have led to the current interest in testing for thyroid active compounds have been outlined. These events have fuelled public interest and have led to regulations that have reverberated across continents. This was followed by a list of the available rodent in vivo test guidelines with thyroid endpoints. The latter part of this chapter presented known mechanisms of action that can serve as the basis for alternative in vitro tests.

The following two chapters address the current in vivo endpoints and explore potential alternatives as well as their relevance. While rodent tests for thyroid activity have three main parameters, namely, thyroid weight, serum thyroid hormone levels, and thyroid histopathology, the latter cannot be replaced with current in vitro tests. Hence, Chapter 2 compares chemically-induced in vivo changes in thyroid and pituitary organ weights with the in vitro proliferation of representative cell lines. Chapter 3 follows with a comparison between the effect of model compounds on serum thyroid hormone level changes in vivo and their effect on a potential in vitro counterpart. In this regard, the thyroid peroxidase enzyme, which is involved in the synthesis of thyroid hormones, is exposed to known inhibitors and known non-inhibitors and the results were compared to in vivo changes in thyroid hormone levels as reported in literature. Chapter 4 introduces the up and coming 'omics technology of Microarray Assay for Real-time Coregulator-Nuclear Receptor Interaction (MARCoNI) and details its implementation to the thyroid system in tests for thyroid hormone receptor-corregulator interaction upon exposure to known thyroid hormone disrupting compounds. In Chapter 5, zebrafish embryos are proposed as an in vitro test organism due to their intact thyroid system and potential for high throughput. The dose-dependent effect of model thyroid-active compounds on embryo development was assessed from 0 – 120 hours post fertilization and an adjusted developmental scoring system was developed. Chapter 6 provides an overview of the results obtained in this thesis and proposes an integrated testing strategy for the testing of thyroid active compounds. This includes the systematic and tiered integration of both highly sensitive and highly specific in vitro endpoints for thyroid activity.

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Chapter 2

In vitro pituitary and thyroid cell proliferation assays and their relevance as alternatives to animal testing

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Summary

This study investigates the in vitro effect of eleven thyroid-active compounds known to affect pituitary and/or thyroid weights in vivo, using the proliferation of GH3 rat pituitary cells in the so-called "T-screen," and of FRTL-5 rat thyroid cells in a newly developed test denoted "TSH-screen" to gain insight into the relative value of these in vitro proliferation tests for an integrated testing strategy (ITS) for thyroid activity. Pituitary cell proliferation in the T-screen was stimulated by three out of eleven tested compounds, namely thyrotropin releasing hormone (TRH), triiodothyronine (T3) and thyroxine (T4). Of these three compounds, only T4 causes an increase in relative pituitary weight, and thus T4 was the only compound for which the effect in the in vitro assay correlated with a reported in vivo effect. As to the newly developed TSH-screen, two compounds had an effect, namely, thyroid-stimulating hormone (TSH) induced and T4 antagonized FRTL-5 cell proliferation. These effects correlated with in vivo changes induced by these compounds on thyroid weight. Altogether, the results indicate that most of the selected compounds affect pituitary and thyroid weights by modes of action different from a direct thyroid hormone receptor (THR) or TSH receptor (TSHR)-mediated effect, and point to the need for additional in vitro tests for an ITS. Additional analysis of the T-screen revealed a positive correlation between the THR-mediated effects of the tested compounds in vitro and their effects on relative heart weight in vivo, suggesting that the T-screen may directly predict this THR-mediated in vivo adverse effect.

1 Introduction

Endocrine disruption has been the source of considerable debate since 1991 when Ana Soto reported that man-made compounds could act as estrogen mimics (Soto et al., 1991), an observation that was popularized with the publication of Our Stolen Future by Theo Colborne in 1996. Although some environmental chemicals have been shown to affect endocrine activity in in vitro and in vivo test systems, there is still debate as to whether this activity can truly result in adverse effects to humans at realistic levels of exposure (Boas et al., 2006; Brucker-Davis, 1998; Leghait et al., 2010; Brouwer et al., 1998). So far, attention on the endocrine activity of chemicals has largely focused on estrogen disruption. An analysis of literature search results in Scopus™ reveals that, in the last decade, the majority of the papers published on endocrine disruption have focused on estrogen rather than on androgen or thyroid hormone disruption. This is in contrast to the number of chemicals listed on the Toxnet hazardous substance data bank (HSDB) as of 25 January 2013 which lists 159 chemicals for androgen, 352 chemicals for estrogen, and 924 chemicals for thyroid activity. This is a cause for concern as altered thyroid hormone (TH) levels can cause adverse effects such as decreased fertility and retarded development, especially of the bones and the brain (Poppe and Velkeniers, 2004; Wajner et al., 2009; Göthe et al., 1999; Aronson et al., 1990; Zoeller and Crofton, 2000).

Changes in thyroid hormone levels are also directly related to changes in cardiac output, heart rate, and systemic vascular resistance (Zamoner et al., 2011). The clinical outcomes of such disturbances include arrhythmias, reduced exercise performance, and an increased risk of cardiovascular mortality (Klein and Ojamaa, 2001; Fazio et al., 2004).

Considering that thyroid hormones play a crucial role in the development of the human brain, bones, and gonads, in pregnancy as well as in cardiac pathogenesis, the possibility of alterations to the normal function of the thyroid system by xenobiotics could have substantial societal implications (Oppenheimer and Schwartz, 1997; Zoeller et al., 2002; Göthe et al., 1999; Aronson et al., 1990; Wagner et al., 2008; Gardiner-Hill, 1929; Mestman et al., 1974; Sugrue and Drury, 1980; Fazio et al., 2004; Char, 1996). For this reason, the Organization of Economic Cooperation and Development (OECD) has amended and validated its test guideline number 407 (TG 407) for repeated-dose 28-day oral toxicity studies in rodents to include endpoints relevant to the thyroid system, including histopathology of the pituitary and thyroid weight. Thyroid hormone levels in plasma or serum are included as an optional endpoint for the confirmation of toxicants with a mode of action related to the thyroid system (OECD, 2007). However, testing on animals raises issues related to ethics, high costs, long duration, and difficulties in the interpretation of inter-species data. This has prompted research into suitable in vitro

assays that, once validated, could serve as alternatives.

Wang et al. (2012) recently published an in-depth comparison of the proliferative response of different estrogen-responsive human cell lines with data on the in vivo change in uterine weight in the rat uterotrophic assay upon exposure to a series of model compounds. Proliferation of the MCF-7 human breast cancer cell line subclone MCF-7/BOS upon exposure to the test compounds appeared to be predictive for the in vivo effect on rat uterine weight resulting in a squared sample correlation coefficient of 0.85 (Wang et al., 2012). Hence, as in the case of the estrogen-responsive MCF-7 cell proliferation assay, called "E-screen" (Soto et al., 1992), in vitro proliferation correlates well with the in vivo increase in uterine wet weight. For thyroid hormone activity, the so-called "T-screen" has been developed, detecting proliferation of rat pituitary adenoma cells upon exposure to thyroid hormone receptor (THR)-active compounds, whereas in vivo pituitary weight is an endpoint for the disruption of thyroid activity (Gutleb et al., 2005; Umano et al., 2009; Sellers and Schänbaum, 1965). Given the correlation of the E-screen with uterine weight, the objective of the present study was to investigate the correlation between the effects of a series of model thyroid-active compounds on cell proliferation in the T-screen and in vivo data for their effects on pituitary weight. Compounds that tested positive in the T-screen were further tested in the GH3-TRE-Luc reporter gene assay using the same cell line in order to find out if the observed proliferation was directly caused by activation of the thyroid hormone receptor (Freitas et al., 2011). GH3 cells express all thyroid nuclear receptor isoforms, with THRβ2 and THRα1 being, in order, the most abundant, which is in line with pituitary expression patterns (Hahn et al., 1999; Freitas, 2012; Yen et al., 1992). Since these receptors shuttle between the nucleus and the cytoplasm, THR-active compounds must be able to cross the cell membrane to have an effect (Mavinakere et al., 2012). The cellular uptake of TH-like compounds is mediated by specific plasma membrane transporters showing preferential transport of T4 or T3 or their metabolites (Hennemann et al., 2001). Very lipophilic compounds also can cross the cell membranes by passive diffusion. Moreover, as thyroid weight is another endpoint in in vivo studies for thyroid hormone activity, an additional objective was to develop an in vitro counterpart (OECD, 2007). To this end, a physiologically-relevant assay based on thyroid stimulating hormone (TSH)-induced proliferation of cells from the FRTL-5 rat thyroid cell line was developed (denoted TSH-screen). This screen was subsequently used to explore parallels between the effects of the selected model thyroid-active compounds on in vitro FRTL-5 rat thyroid cell proliferation and their effects on in vivo rat thyroid weight. Changes in thyroid weight are associated mainly with hyperplasia and hypervascularization and, to a lesser extent, hypertrophy, thereby suggesting that proliferation assays may be able to reflect the in vivo effects (Martin et al., 1973; Kero et al., 2007). Given the complexity of the in

Tab. 1: List of compounds used in this study with their main mode of action on the thyroid hormone system and concentration ranges used in the in vitro experiments.

Compound	Abbreviation	CAS No.	Description	Mode of Action	Concentration Range (nM)
Thyrotropin-Releasing Hormone	TRH	24305-27-9	Endogenous ligand	Binds thyrotropin-releasing hormone receptor	0.001 - 160,000
bovine Thyroid Stimulating Hormone	bTSH	9002-71-5	Endogenous ligand	Binds thyroid stimulating hormone receptor	0.001 x 10^{-3} - 143
Triiodothyronine	T3	6893-02-3	Endogenous ligand	Binds thyroid hormone receptor	0.001 - 1,000
Thyroxine	T4	51-48-9	Endogenous ligand	Binds thyroid hormone receptor	0.001 - 1,000
Propylthiouracil	PTU	51-52-5	Antithyroid drug	Inhibits enzyme thyroid peroxidase	1 - 100,000
Methimazole	MMI	60-56-0	Antithyroid drug	Inhibits enzyme thyroid peroxidase	1 - 100,000
Ethylene thiourea	ETU	96-45-7	Environmental contaminant	Inhibits enzyme thyroid peroxidase	0.01 - 100,000
Aminotriazole	3-AT	61-82-5	Environmental contaminant	Inhibits enzyme thyroid peroxidase	0.01 - 100,000
Sodium perchlorate monohydrate	$NaClO_4 \bullet H_2O$	7791-07-3	Environmental contaminant	Inhibition of iodide uptake	0.01 - 200,000
Bisphenol F	BPF	620-92-8	Environmental contaminant	Binds to estrogen receptor	0.01 - 1,000
4,4'-Butylidenebis	BBBC	85-60-9	Environmental contaminant	Unknown	0.001 - 100

vivo hypothalamic-pituitarythyroid (HPT) axis, it may be expected that the T-screen and the newly developed TSH-screen may prove less predictive for effects on pituitary and thyroid weight in vivo than the E-screen is for effects on uterine weight in the uterotrophic assay. Thus, the ultimate aim of this study was to provide insight into the predictive potential and relative value of the two in vitro cell proliferation screens within an integrated testing strategy (ITS) for thyroid activity.

Tab. 2: List of compounds used in this study and overview of the in vivo data. Selection was based on significant in vivo change in either thyroid weight (Δ thyroid wt) or pituitary weight (Δ pituitary wt) in rats. The direction of the changes and their magnitude are presented in Tables 3 and 4. Two studies are included for the effect of TRH on thyroid weight so that both sexes are represented and two studies using different exposure periods are included for the effect of T4 on pituitary weight. M, male; F, female; OM, Osborne-Mendel; SD, Sprague-Dawley; DM, Dutch-Miranda; CR, Charles River; LEW, Lewis; WSTR, Wistar; IV, intravenous; IP, intraperitoneal; NA, not available.

Compound	Ref. for △ pituitary wt	Type of study	Ref. for △ thyroid wt	Type of study
TRH	Iglesias et al., 1985	34-day repeated oral dose with SD rats (F, n=6)	D'Angelo et al., 1975	14-day repeated oral dose with CR rats (F, n=4)
	-	-	Connors et al., 1988	6-day chronic IV dose with SD rats (M, n=4)
bTSH	NA	NA	Connors et al., 1988	6-day chronic IV dose with SD rats (M, n=4)
T3	Nedvidkova et al., 1996	20-day repeated oral dose with WSTR rats (M, n=6)	Soukup et al., 2001	6-month repeated IP dose with LEW rats (F, n=10)
T4	OECD, 2006b	OECD TG 407 (M, F, n=10)	OECD, 2006b	OECD TG 407 (M, F, n=10)
	Seller and Schonbaum, 1965	64-week repeated oral dose with WSTR rats (M, n=6)	-	-
PTU	OECD, 2006b	OECD TG 407 (M, F, n=10)	OECD, 2006b	OECD TG 407 (M, F, n=10)
MMI	Moreira et al., 2005	21-day repeated oral dose with DM rats (M, F, n=10)	Hood et al., 1999	21-day repeated oral dose with SD rats (M, n=4)
ETU	Lu and Staples, 1978	9-day repeated oral dose with CR rats (F, n=6-12)	Graham et al., 1972	30-day repeated oral dose with OM rats (M, n=10)
3-AT	Umano et al., 2009	OECD TG 407 (M, F, n=10)	Umano et al., 2009	OECD TG 407 (M, F, n=10)
$NaClO_4$	Stoker et al., 2006	31-day repeated oral dose with CR rats (M, n=25)	Siglin et al., 2000	90-day repeated oral dose with SD rats (M, F, n=10)
BPF	Higashihara et al., 2007	OECD TG 407 (M, F, n=10)	Higashihara et al., 2007	OECD TG 407 (M, F, n=10)
BBBC	Yamasaki et al., 2008	OECD TG 407 (M, F, n=10)	Yamasaki et al., 2008	OECD TG 407 (M, F, n=10)

2 Materials and methods

Compound selection

Table 1 presents the list of compounds selected and the concentration range used in the in vitro experiments. BBBC was obtained from Wako Pure Chemical Industries (Tokyo, Japan), while all other compounds were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). All chemicals were of high purity (≥95%). With the exception of bTSH, which was diluted in phosphate buffered saline (pH 7.2) (PBS), all test chemicals were added from 500-fold concentrated stock solutions in dimethylsulfoxide (DMSO, Acros Organics, Geel, Belgium), resulting in a final concentration of DMSO of 0.2%.

Compound selection was based on whether in vivo data for effects of the compounds on pituitary and/or thyroid weight were available (Tab. 2). The mode of action underlying the increase in organ weights was not used as a selection criterion, since the objective was to test whether the proliferation assays could replace in vivo assays detecting organ weight changes, as was shown feasible for the E-screen and in vivo effects on uterus weight (Wang et al., 2012). Changes in pituitary weight induced by the selected model compounds are presented in Table 3, and changes in thyroid weight induced by these compounds are presented in Table 4.

Cell culture

GH3-TRE-Luc cells were cultured at 37 °C in a humid atmosphere containing 5% (v/v) CO_2 and passaged twice a week in Dulbecco's Modified Eagle's medium: Ham's F12 (1:1) with 15 mM HEPES (DMEM:F12, Gibco, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS, Gibco, Paisley, Scotland). The construction and validation of the GH3-TRELuc cells was previously described (Freitas et al., 2011).

FRTL-5, a rat thyroid cell line (obtained from CLS, Germany), was cultured as a monolayer in tissue-culture flasks (obtained from Corning, Badhoevedorp, The Netherlands) at 37 °C in a humid atmosphere containing 5% (v/v) CO_2 and passaged once a week with an interim refresh of the medium, which consisted of Coon's Modified F-12 medium (obtained from Biochrom, Berlin, Germany), supplemented with 5% FCS, and a mixture of six hormones (referred to as 6H5, reflecting the presence of 6 hormones and 5% FCS). The six hormones included bTSH (1 mIU/ml), insulin (10 µg/ml), hydrocortisone (10 nM), apo-transferrin (5 µg/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 ng/ml). All these hormones were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). This 6H5 cell culture medium was supplemented with 1 mM non-essential amino acids, 2 mM L-glutamine, 100

Tab. 3: Effects of the selected model compounds on relative pituitary organ weight (mg/100g body weight) in male rats compared to in vitro pituitary cell proliferation (T-screen). Arrows indicate an increase (↑), decrease (↓), or (↔) no change in relative thyroid weight. If available the fold-change in mean response to the highest tested dose (HTD) compared to the unexposed controls also was presented. * indicates significance ($p < 0.05$) and ** indicates high significance ($p < 0.01$) for fold change calculations. Benchmark doses associated with a 10% change in organ weight (BMD10) were calculated and presented as the lower 95% confidence limit (BMDL10). For the effect on cell proliferation in vitro, besides fold change and when applicable, EC50 values are shown. The highest tested concentrations (HTCs) also are represented and were chosen in such a way that they were not cytotoxic based on results from the resazurin assay. NA, not applicable. In vitro-in vivo correlation (IVIVC) was evaluated qualitatively and represented by +ve when the direction of change was similar in both situations, by 0 when there was no correlation, by -ve when the direction of change was opposite and by X when there was no significant change in both situations leading to an invalid correlation.

	Pituitary organ weight (literature)					Pituitary cell proliferation (T-screen)			
	Male		Female						
Compound	Fold change	BMDL10 (mg/kg bw/day)	Fold change	BMDL10 (mg/kg bw/day)	HTD (mg/kg bw/day)	Fold change	EC50 (nM)	HTC (µM)	IVIVC
TRH	NA	NA	↔	X	50	1.4x ↑**	0.90	160	0
bTSH	NA	NA	NA	NA	NA	↔	NA	0.143	NA
T3	1.1x ↑	X	NA	NA	0.0005	5x ↑**	0.09	1	0
T4	↔	X	↔	X	1	4.3x ↑**	1.73	1	0
	1.3x ↑**	NA	NA	NA	0.012	4.3x ↑**	1.73	1	+ve
PTU	1.4x ↑**	NA	1.4x ↑**	NA	10	↔	NA	100	0
MMI	↔	X	↔	X	75	↔	NA	100	X
ETU	NA	NA	1x ↔	X	40	↔	NA	100	0
3-AT	1.3x ↑**	38.97	1.2x ↑*	51.31	125	↔	NA	100	0
$NaClO_4$	1x ↔	X	NA	NA	500	↔	NA	200	X
BPF	1.1x ↑	X	1.1x ↑	X	500	↔	NA	1	X
BBBC	1x ↔	X	1x ↔	X	125	↔	X	0.1	X

U/ml penicillin and 100 µg/ml streptomycin (Difco, Amsterdam, The Netherlands). Enzymatic passaging was performed using a cell detachment and disaggregation solution containing 20 units/ml collagenase, 0.075% trypsin, and 2% chicken serum (CTC) in PBS. Collagenase and chicken serum were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands) while trypsin was obtained from Difco (Amsterdam, The Netherlands).

T-screen

The T-screen was performed as previously described (Schriks et al., 2006) and using the rat GH3-TRE-Luc cell line. Compared to wild type GH3 cells, this cell line is stably transfected with a thyroid-hormone response element (TRE) driven luciferase expression construct, allowing for the comparison between cell proliferation and TRE-driven gene expression with the same cell line (Freitas et al., 2011). Briefly, GH3-TRE-Luc cells at 80% confluence were incubated for 48 h in serum-free PCM-0 medium, as originally described by Sirbasku (1991). PCM-0 consists of phenol red-free DMEM:F12 with 15 mM HEPES, 10 µg/ml bovine insulin, 10 µM ethanolamine, 10 ng/ml sodium selenite, 10 µg/ml apo-transferrin, and 500 µg/ml bovine serum albumin (BSA, obtained from SigmaAldrich Chemie, Zwijndrecht, The Netherlands). The cells were then harvested and plated at a density of 2,500 cells/ well on a 96-well plate. Following an attachment period of 2 to 3 h, the cells were exposed in triplicate and for 96 h to a concentration range of the chemicals to be tested alone or in combination with 0.25 nM T3. Subsequently, and following a 4-h incubation period with 10 µl/well of 0.1 mg/ml resazurin (obtained from Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) in PBS, cell proliferation was measured as relative fluorescence units (RFUs) resulting from the reduction of non-fluorescent resazurin to the fluorescent product resorufin. Fluorescence was measured at λex = 530 nm and λem = 590 nm on a SpectraMax M2 microplate reader (Molecular Devices, Menlo Park, CA, USA).

TSH-screen

FRTL-5 cells at 70% confluence and below passage 30 were incubated in serum-free, phenol red-free DMEM containing 0.2% BSA for a period of 96 h. The cells were then harvested and plated at a density of 5,000 cells/well on a 96-well plate in phenol red-free DMEM with insulin (10 µg/ml) and, following an attachment period of 2 to 3 h, exposed to a concentration range of the chemicals to be tested alone or in combination with 1 mIU/ml bTSH, in triplicate, for 72 h. Following this incubation, cell proliferation was measured 4 h after the addition of 10 µl/well of 0.1 mg/ml resazurin as described above.

GH3-TRE-Luc Reporter Gene Assay

GH3-TRE-Luc cells at 80% confluence were incubated in serum-free PCM-0 medium for 24 h. The cells were then harvested and plated at a density of 30,000 cells/well on a 96-well plate and, following an attachment period of 2 to 3 h, exposed in triplicate for 24 h to a concentration range of the chemicals to be tested alone or in combination with 0.25 nM T3. To measure luciferase activity, cell culture medium was thoroughly aspirated, and the cells were lysed by addition of low salt buffer, which consisted

Tab. 4: Effects of the selected model compounds on relative thyroid organ weight (mg/100g body weight) in male rats compared to in vitro thyroid cell proliferation (TSH-screen). Arrows indicate an increase (↑), decrease (↓), or (↔) no change in relative thyroid weight. If available the fold-change in mean response to the highest tested dose (HTD) compared to the unexposed controls also was presented. * indicates significance ($p < 0.05$) and ** indicates high significance ($p < 0.01$) for fold change calculations. † TRH and bTSH were chronically administered intravenously to male rats at 240 µg/day and 800 mIU/day, respectively. Benchmark doses associated with a 10% change in organ weight (BMD10) were calculated and presented as the lower 95% confidence limit (BMDL10). For the effect on cell proliferation in vitro, besides fold change and when applicable, EC50 values are shown. The highest tested concentrations (HTCs) are also represented and were chosen in such a way that they were not cytotoxic based on results from the resazurin assay. NA, not applicable. In vitro-in vivo correlation (IVIVC) was evaluated qualitatively and represented by +ve when the direction of change was similar in both situations, by 0 when there was no correlation, by -ve when the direction of change was opposite and by X when there was no significant change in both situations leading to an invalid correlation.

	Thyroid organ weight (literature)					Thyroid cell proliferation (TSH-screen)			
	Male		Female						
Compound	Fold change	BMDL10 (mg/kg bw/day)	Fold change	BMDL10 (mg/kg bw/day)	HTD (mg/kg bw/day)	Fold change	EC50 (nM)	HTC (µM)	IVIVC
TRH	NA	NA	1.1x↑	X	12.5	↔	NA	160	X
	1.7x↑*	NA	NA	NA	***	↔	NA	160	0
bTSH	1.8x↑*	NA	NA	NA	***	1.6x ↑**	4.6	0.143	+ve
T3	NA	NA	1.9x↓*	NA	0.15	↔	NA	1	0
T4	↔	X	1.3x ↓*	0.26	1	1.3x ↓*	0.11	1	+ve
PTU	7.2x ↑**	0.02	7.7x ↑**	0.14	10	↔	NA	100	0
MMI	3.3x ↑**	0.11	NA	NA	50	↔	NA	100	0
ETU	2.7x ↑**	1.52	NA	NA	37.5	↔	NA	100	0
3-AT	3x ↑**	3.86	3.7x ↑**	2.10	125	↔	NA	100	0
$NaClO_4$	1.3x ↑**	0.36	1.2x ↑*	6.44	10	↔	NA	200	0
BPF	1.3x ↑**	30.28	1.2x ↑	X	500	↔	NA	1	0
BBBC	1.2x ↑	X	1.3x ↑**	5.02	125	↔	NA	0.1	0

of 10 mM Tris (Invitrogen, Carlsbad, CA), 2 mM dithiothreitol (DTT) and 2 mM trans-1,2-diaminocyclohexane-N,N,N′,N′-tetra-acetic acid monohydrate (obtained from Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) (pH 7.8). Cell lysis was completed by one subsequent freeze-thaw cycle. Luciferase reagent, which consisted of 20 mM tricine,

1.07 mM $(MgCO_3)4 \cdot Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$ (all obtained from Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands), 0.1 mM EDTA, 2 mM dithiothreitol (obtained from Merck, Darmstadt, Germany), 470 µM D-luciferin and 5 mM ATP (both obtained from Duchefa Biochemie, Haarlem, The Netherlands) (pH 7.8), was adde d to the wells, and luciferase flash-kinetics activity was measured as relative luminescence units (RLUs) using a Luminoskan Ascent luminometer from Labsystems (Helsinki, Finland).

Data analysis

In the T-screen, GH3-TRE-Luc reporter gene assay and TSHscreen, data points are representative of at least two independent experiments and three replicate wells per data point in each experiment. Raw data from triplicate wells were averaged, converted to fold change over solvent control and represented graphically as the means of independent experiments with bars representing the standard error of that mean (SEM). The luminescent signal in the GH3-TRE-Luc reporter gene assay was normalized to cell viability as determined by a resazurin cell viability test with an incubation period of 2 h. Non-linear curve fitting was done using the Hill equation with the help of GraphPad Prism software version 5.04 (GraphPad Software, San Diego, CA, USA). In vitro, EC50 values were calculated from the fitted models as the concentration of the tested compound that gave 50% of the maximal response.

For the in vivo data collected from literature, with included standard deviations and more than one dose level used, the benchmark dose (BMD) approach was used and BMDL (95% lower confidence limit of the BMD) values with a relative deviation from the controls of 10% (BMDL10) were calculated using Benchmark Dose Software (BMDS) version 2.1.2 (US EPA, Washington, DC, USA). For organ weight changes that were significant ($p<0.05$) among dose levels, the curve fitting models used for in vivo BMDL calculations were based on either polynomial, power, or Hill equations, and the choice of the best model to fit the data was based on the highest p-value for three tests, namely, homogenous variance, adequate modeling of the variance and model fit and when these p-values were similar across models, then a lower Akaike Information Criterion (AIC) value was used for selection. Models with a goodness of fit for the means with a p-value<1 were rejected. BMDL10 calculations were based on data from male rats (except for TRH for which data from virgin females were used and ETU for which data from gestating females were used).

Doses of 0, 50, 100, 500, and 750 ppm ETU in feed were converted to 0, 2.5, 5, 25, and 37.5 mg/kg bw/day, a T3 dose of 0.01 mg/kg was converted to 0.0005 mg/kg bw/day and a T4 dose of 0.25 mg/kg diet was converted to 0.012 mg/kg bw/day, based on the assumption that rats consume 5% of their body weight of food each day. 0.03% (0.3 mg/ml) MMI, 0.2 mg/ml, and 0.05 mg/ml TRH in drinking water were converted to

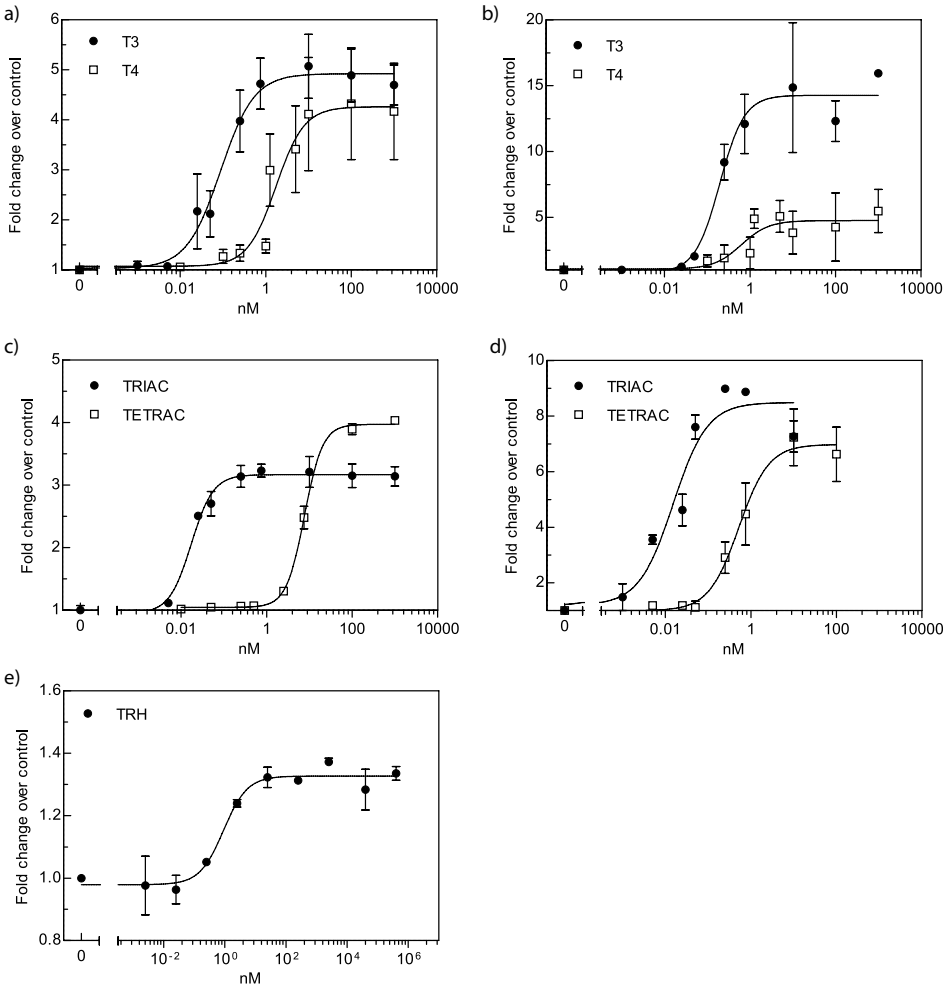

Fig. 1: Concentration-dependent GH3 cell proliferation (T-screen) upon exposure to a) T3 (n=5) and T4 (n=3), c) triac (n=1) and tetrac (n=1) or e) TRH (n=2) and luciferase response in the GH3-TRE-Luc thyroid hormone receptor reporter gene assay for b) T3 (n=3) and T4 (n=2) or d) triac (n=2) and tetrac (n=2). The response is expressed as fold change over control, error bars represent the standard error of the mean (SEM) of the indicated number of independent experiments.

75 mg/kg bw, 50 mg/kg bw and 12.5 mg/kg bw, respectively, based on a previous study reporting an average water intake of similarly aged rats of 250 ml/kg bw (McGivern et al., 1996). To calculate the molar concentrations of bTSH, it was considered that TSH has a molar mass of 28,000 g/mol and conversion to mIU/ml was based on the manufacturer's stated activity of 2 IU/mg (Pierce et al., 1971).

3 Results

3.1 Activity of selected compounds in the T-screen

Figures 1A and 1C show that the endogenous agonists T3 and T4, along with their respective acetic acid analogues triac and tetrac resulted in a concentration-dependent increase in cell proliferation in the T-screen. Figures 1B and 1D show that a concentration-dependent response also was obtained in the GH3- TRE-Luc reporter gene assay, which is an indication that the observed cell proliferation is likely to be THR-mediated. The other model compounds tested with known in vivo effects on pituitary weight, propylthiouracil (PTU) and 3-amino-1,2,4-triazole (3-AT) did not induce a proliferative response in the assay for in vitro GH3 pituitary cell proliferation. On the other hand, TRH, which does not lead to an increase in pituitary weight in vivo, was found to be an agonist in the T-screen with an EC50 of 0.9 nM (95% confidence interval 0.3-2.4 nM, Fig. 1E).

3.2 Correlation of in vitro pituitary cell proliferation with in vivo pituitary weight

Table 3 presents an overview of the effects of the selected model compounds on pituitary weight in in vivo studies derived from literature, as well as the results obtained in the present study on the effects of the compounds in the T-screen assay. A correlation between the in vitro T-screen and effects on pituitary weight reported in the in vivo studies was observed only for T4.

3.3 Development of the TSH-screen based on FRTL-5 cell proliferation

FRTL-5 cells are a differentiated, continuously growing, nontransformed subclone of FRTL rat thyroid cells derived from primary cultures of Fischer rat thyroid glands. Differentiated features such as the cellular uptake of iodide and the secretion of thyroglobulin in FRTL cells are dependent on a low concentration of FCS (0.5-1%) which results in slow growth (AmbesiImpiombato et al., 1980). FRTL-5 is a fast growing subclone of the FRTL cell line that grows in 5% FCS while maintaining iodide uptake and thyroglobulin secretion. In addition, FRTL-5 cells exhibit enhanced TSH sensitivity, especially after TSH starvation (Ambesi-Impiombato, 1986).

To optimize the proliferation of FRTL-5 cells, a series of compounds was tested for its ability to improve TSH-induced cell proliferation, subsequently defining cell culture conditions in the TSH-screen. As can be seen in Figure 2, FRTL-5 cells exhibited little response to bTSH (1 mIU/ml) alone and a limited response to insulin (10 µg/ml), while the combination of both TSH and insulin resulted in a synergistic effect. Forskolin, a compound that directly activates cAMP, the downstream target of TSH's proliferative action, also was tested alone or in combination with TSH (Kimura et al., 2001). The

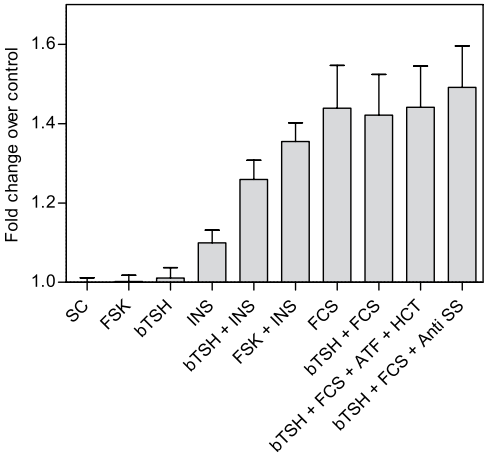

Fig. 2: FRTL-5 cell proliferation expressed as fold change over control upon exposure to 0.2% DMSO as solvent control (SC), 0.1 µM forskolin (FSK), 1 mIU/ml bovine thyrotopin (bTSH), 10 µg/ml insulin (INS), bTSH with INS, FSK and INS, 0.2% fetal calf serum (FCS), bTSH with 0.2% FCS, bTSH with 0.2% FCS in addition to 5 µg/ml apo-transferrin (ATF) and 10 nM hydrocortisone (HCT), bTSH with 0.2% FCS and 4 µg/ml anti-somatostatin antibody (Anti SS), and lastly bTSH, 0.2% FCS, FSK and Anti SS (n=1). Proliferation was measured as fluorescence resulting from the mitochondrial metabolism of resazurin to the fluorescent resorufin.

forskolin response closely mimicked that of TSH, and the combination of forskolin with insulin had an effect similar to that of TSH in combination with insulin, showing that the membrane receptor-mediated TSH-response was near optimal and reflects the differentiated characteristics of this cell line. As little as 0.2% FCS had a proliferative effect that was larger than bTSH. The addition of apo-transferrin and hydrocortisone, which are needed in the culture of FRTL-5 cells, had little effect in terms of TSH-responsiveness of the cells (Ambesi-Impiombato, 1986). The addition of a somatostatin antibody, to block any inhibitory action of residual somatostatin, also had little effect (Medina et al., 1999). Based on these results, further testing using the TSH-screen was performed in serum-free medium containing insulin (10 µg/ml). Figure 3 shows the TSH concentration-dependent proliferation of FRTL-5 cells in the newly developed TSH-screen. This response was achieved in cell culture medium completely devoid of serum but supplemented with 10 µg/ml insulin and 0.2% BSA. BSA is commonly added to serum-free media as it is thought to protect cells from agitation and aeration damage by biological and fluid-mechanical mechanisms (Papoutsakis, 1991; Michaels et al., 1995). The EC50 for TSH-dependent induction of cell proliferation was found to be 4.6 nM (95% confidence interval 1.7-12.6 nM).

3.4 Activity of selected compounds in the TSH-screen

In a next step, the series of model compounds was tested in the TSH-screen for either agonistic or antagonistic activity, the latter by testing cell proliferation in the presence of 1 mU bTSH (14.3 nM). The TSH-screen only gave a significant response with the endogenous agonist TSH while T4 showed antagonism with an IC50 of 0.11 nM (95% confidence interval 0-0.4 nM, Fig. 4).

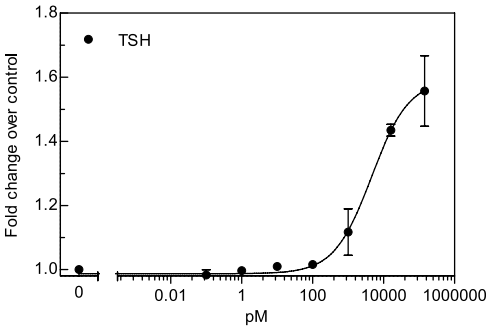

Fig. 3: TSH-induced FRTL-5 cell proliferation in the TSH screen. Proliferation is expressed as fold change over control, error bars represent the standard error mean (SEM) of two different experiments.

3.5 Correlation of in vitro thyroid cell proliferation with in vivo thyroid weight

Table 4 presents an overview of the effects of the selected model compounds on thyroid weight in in vivo studies, derived from literature, as well as the results obtained in the present study on the effects of the compounds in the TSH-screen. As can be observed from Table 4, the effects of the series of model compounds on in vitro FRTL-5 thyroid cell proliferation correlates in only a very limited number of cases with the effects of the compounds on thyroid weight in vivo. The endogenous agonist TSH was one of the two compounds that had a similar proliferative effect both in vitro and in vivo. The other compound, T4, acted in a potentially physiological manner since it reduced thyroid weight in vivo in female rats and showed an antagonistic activity in the TSH-screen, both with and without TSH (Fig. 5).

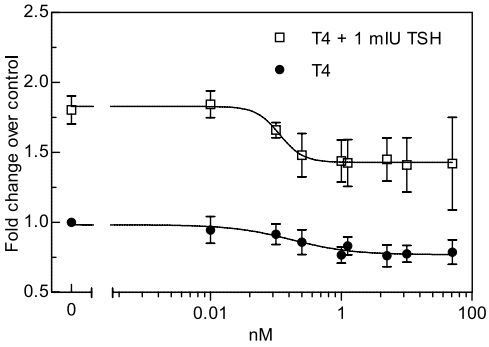

Fig. 4: Effect of T4 (n=3) on basal proliferation (closed circles) and TSH-induced proliferation (open circles) of FRTL-5 cells.

3.6 Correlation of T-screen with cardiac hypertrophy

Given that the effect of some of the thyroid-active model compounds on GH3 cell proliferation (T-screen) and THR activity (GH3-TRE-Luc reporter gene assay) could not be correlated to effects on pituitary weight in vivo, investigations into potential correlations with effects on other organs was carried out based on data retrieved from the literature. It was found that the proliferative response to thyroid-active compounds in the T-screen may correlate with the in vivo effects of these compounds on cardiac hyperthrophy. Figure 5 presents the plasma concentration-dependent effect of T4 and tetrac (Fig. 5A) and of T3 and triac (Fig. 5B) on relative heart weight as reported in literature (Liang et al., 1997;

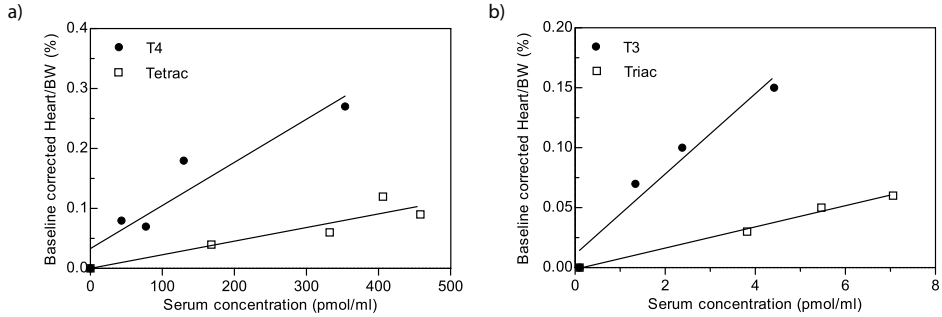

Fig. 5: Increase in relative heart weight (expressed as % of total body weight) with increasing serum levels of a) T4 and tetrac (data from Lameloise et al., 2001) and b) T3 and triac (data from Liang et al., 1997).

Lameloise et al., 2001). Comparison of these results with the results for T3, triac, T4 and tetrac mediated effects in the T-screen (Fig. 1A,C) indicates that the THR-mediated cell hyperplasia in the T-screen correlates well with the receptor-mediated cardiac hypertrophy (Fig. 6). This is corroborated by the results presented in Figure 6 in which the in vivo BMDL10 for 10% increase in relative heart weight in the in vivo studies was plotted against the EC50 for cell proliferation in the T-screen obtained in the present study, showing a correlation with a squared coefficient of correlation (r2) of 0.89. Thus, whereas activity of these thyroid-active compounds on pituitary cell proliferation in vitro in the T-screen did not correlate with their effects on pituitary weight in vivo, their activity in the T-screen did correlate with their effects on relative heart weight in vivo.

4 Discussion

The objective of the present study was to investigate a potential correlation between the effects of a series of model thyroidactive compounds on GH3 pituitary cell proliferation in the T-screen assay and their effects on pituitary weight in in vivo studies. Given that thyroid weight is one of the endpoints used in in vivo studies for thyroid hormone activity, an additional objective was to develop an in vitro thyroid cell proliferation assay and explore correlations between the in vitro data obtained and in vivo data for compounds known to affect thyroid weight (OECD, 2007). This was done in order to gain further insight into the relative value of these proliferative in vitro tests for an integrated testing strategy (ITS) for thyroid activity.

The newly developed TSH-screen was based on the proliferation of cells from the FRTL-5 cell line. The preservation of TSH-induced cell proliferation in the FRTL-5 cell line is a property exhibited by only three cell lines that originate from normal rat thyroid follicular epithelium. The other two cell lines, PC Cl3 and Wistar rat (WRT), have been

reported to have a suitable TSH-mediated proliferative response but are not as widely used and are not commercially available (Fusco et al., 1987; Brandi et al., 1987; Kimura et al., 2001). The reference compound used in this screen, TSH, has been shown to result in cell hyperplasia and hypertrophy, both in FRTL-5 cells in vitro and in the thyroid gland in vivo (Ossendorp et al., 1989; Ambesi-Impiombato et al., 1980; Brewer et al., 2007). Figure 2 shows that insulin is needed for the mitogenic action of TSH and was included in both culture and exposure media used in the TSH-screen. While TSH is the main hormone that regulates the growth of the thyroid gland, the requirement for insulin has been well documented in both rodents and humans (Felice et al., 2004).

In a previous study, it was demonstrated that an in vitro-in vivo correlation (IVIVC) for endocrine-active compounds exists between estrogen-mediated cell proliferation in the so-called E-screen and in vivo effects on uterine weight (Wang et al., 2012). The relative potency of a series of estrogens for estrogen-induced MCF-7 cell proliferation in the E-screen correlated with the relative potency of the same compounds in the in vivo uterotrophic assay (Wang et al., 2012). In a detailed review paper on thyroid hormone disruption assays, the OECD mirrors a widely-held notion among toxicologists that the T-screen is an in vitro bioassay that can detect compounds that interfere with THR signaling much the way the MCF-7 cells are used in the E-screen to detect compounds that interfere with estrogen receptor (ER) signaling (OECD, 2006a). However, results obtained in this study show that this comparison between the T-screen and the E-screen is inaccurate when it comes to in vitro-in vivo correlation (IVIVC), since the activity of the selected model compounds in the T-screen did not correlate well with their in vivo effect on pituitary weight. Likewise, the in vivo effects of thyroid-active compounds on thyroid weight did not exhibit a consistent correlation with thyroid cell proliferation in a newly developed TSH-screen. The discrepancy between the two endocrine systems in terms of the applicability of the in vitro-in vivo extrapolation may be due to the fact that the uterotrophic assay for estrogen activity is based on ER-mediated increases in uterine weight, while increases in pituitary or thyroid weight may reflect much more complex mechanisms. These mechanisms include, among others, acute xenobioticinduced dyshormogenesis, displacement of thyroid hormones from transport proteins, changes in metabolism and clearance, alterations in feedback mechanisms, onset of autoimmune thyroiditis, effects on cofactors and inhibitors, as well as crosstalk with other pathways (Roy and Mugesh, 2006b; Cao et al., 2010; Gayrard et al., 2011; Zabka et al., 2011; Kosuda et al., 1997; Grover et al., 2007; Liu and Brent, 2010). Thus, with the exception of T4 and TSH, the effects of the selected thyroid-active compounds on pituitary and thyroid organ weights in vivo are not likely to be due to a direct effect on cell proliferation. The T-screen and TSH-screen are therefore less predictive for the effects of thyroid-active

compounds on pituitary and thyroid weight in vivo than the E-screen is for the effects of estrogenic compounds on uterine weight in the uterotrophic assay. Altogether, the results obtained indicate that while the Tscreen and the TSH-screen accurately reflect TH and TSH receptor-mediated cell proliferation, respectively, an integrated testing strategy for replacement of these in vivo endpoints by a battery of in vitro tests will need additional assays to cover mechanisms of action that are neither THR nor TSHR mediated. The known modes of action underlying the effects of these compounds are presented in the following text, thereby highlighting some of the critical issues that have to be accounted for with additional in vitro assays. Recently, a mechanism-based testing strategy has been proposed using a battery of in vitro assays for the identification of thyroid hormone disrupting chemicals (Murk et al., in press). The TSH-screen presented in this study is already included in the proposed battery as well, in addition to tests for NIS and cAMP production that play an important role in TH biosynthesis in thyrocytes.

TRH is a hypothalamic hormone whose main role is to stimulate the release of TSH and prolactin from the pituitary gland. TRH reaches the pituitary through the hypothalamic-hypophyseal portal system, triggering a signaling cascade by binding to the TRH receptor that eventually leads to TSH and prolactin secretion (De Léan et al., 1977). TRH is rapidly metabolized, resulting in a half-life of 4 minutes, rendering it undetectable in the systemic circulation (Redding and Schally, 1972; Bassiri and Utiger, 1973). It was previously shown in in vivo experiments performed on rats that TRH alone does not have a proliferative effect on pituitary cells, which is not in accordance with the results of the present study in which TRH did have an agonistic activity on GH3 cell proliferation (Quintanar-Stephano and Valverde, 1997). TRH did not have any proliferative effect on FRTL-5 thyroid follicular cells in the TSH-screen even though in vivo thyroid weight is increased. The in vivo effect can be simply an indirect effect, whereby TRH leads to an increase in TSH secretion which, in turn, is mitogenic to thyroid cells, a sequence of events that is not easily mimicked in vitro.

TSH, the pituitary hormone whose main role is to stimulate thyroid growth and thyroid hormone production, had no effect on GH3 cell proliferation in the T-screen, which is in agreement with previous studies (Felice et al., 2004; Theodoropoulou et al., 2000). It did, nonetheless, exhibit a physiologically relevant and concentration-dependent mitogenic effect on FRTL-5 cells in the TSH-screen.

T3 and T4, the thyroid hormones, resulted in a concentrationdependent increase in GH3 pituitary cell proliferation, which is the basis of the T-screen (Gutleb et al., 2005). However, only T4 leads to a significant increase in pituitary weight in vivo, and this increase was found to be heavily dependent on the duration of exposure (Tab. 3). The endogenous THR agonist precursor T4 causes a significant increase in pituitary weight

but only after long-term exposure of more than one year, while in the OECD TG 407, which is based on subacute exposure (28 days), no significant effect is found (Sellers and Schänbaum, 1965; OECD, 2006b). This stands in contrast to the 4-fold increase in GH3 pituitary cell proliferation observed upon exposure to T4 for only 4 days (Fig. 1A). The effect of T3 on FRTL-5 rat thyroid cell proliferation was indistinguishable from the controls, and this does compare well with the lack of change in thyroid weight upon T3 administration. T4 had an antagonistic effect in the TSH-screen, which correlated significantly but only with female rats used in one of the in vivo studies conducted by the OECD to validate the updated TG 407 (OECD, 2006b). It is possible that T4, an iodinated hormone precursor that is deiodinated intrathyroidally, acts in a similar way to iodide, which is known to lead to cell cycle arrest in FRTL-5 cells (Laurberg, 1988; Smerdely et al., 1993). Moreover, amiodarone, a T4 analogue, has an inhibitory effect on TSH-induced cAMP production at concentrations that are lower than iodide, suggesting a direct mechanism of action (Pitsiavas et al., 1999). This in vitro effect could indicate a physiologically relevant short-feedback regulatory loop.

Antithyroid drugs PTU and MMI, the degradation product of ethylene bis-dithiocarbamate fungicide ETU, as well as the herbicide 3-AT, decrease serum levels of thyroid hormones by inhibiting thyroperoxidase (TPO), a critical enzyme in thyroid hormone synthesis (Roy and Mugesh, 2006a; Marinovich et al., 1997; Hurley, 1998). The increase in thyroid weight resulting from the in vivo administration of these compounds and the increase in pituitary weight resulting from the administration of PTU and 3-AT are not related to a direct effect on cell proliferation as was reflected in this study by the T-screen and the TSH-screen. It can therefore be concluded that their effect may rather be due to a drop in thyroid hormone production, which in turn diminishes the inhibitory effect of T4 on pituitary weight and triggers an increase in TSH secretion by the pituitary and a subsequent mitogenic effect on the thyroid.

$NaClO_4$, a non-reactive electrolyte under physiological conditions, exerts its effect on the thyroid by inhibiting iodide transport through the sodium iodide symporter (NIS), resulting in a drop in the iodide-dependent thyroid hormone production (Yoshida et al., 1998). In vivo, this drop in thyroid hormone levels results in decreased negative feedback on the thyroid and a concomitant increase in TSH secretion, which in turn results in an increase in thyroid weight. Pituitary weight is not affected, nor is pituitary or thyroid cell proliferation as assessed by the T-screen and the TSH-screen, respectively.

BPF, widely used in the production of polycarbonate and epoxy resins, causes liver toxicity and was found to be estrogenic in the uterotrophic assay. Following the OECD TG 407 which includes endpoints for thyroid hormone disruption, Higashihara et al. found effects on thyroid hormone level in addition to changes in thyroid and pituitary

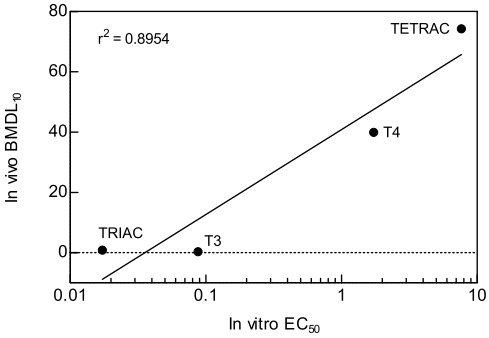

Fig. 6: Correlation between EC50 for in vitro thyroid hormone-dependent GH3 pituitary cell proliferation (T-Screen) and BMDL10 for in vivo change in relative heart weight as induced by the compounds T3, T4, triac and tetrac.

weight (Higashihara et al., 2007). While the exact mechanism of action is not yet elucidated, BPF did not affect cell proliferation in both the T-screen and the TSH-screen, suggesting that its main mechanism of action is not THR or TSHR-mediated. Initial data, as suggested by the authors of the in vivo study, pointed at a possible effect on thyroid hormone metabolism in the liver which can lead to compensatory TSH production which could ultimately lead to goiter and thyroid hyperplasia (Curran and DeGroot, 1991).

Altogether, it is concluded that unlike the direct in vitro-in vivo correlation that can be made with the E-screen, which tests for the estrogenic activity of compounds, proliferation of pituitary and thyroid cells is representative of only a small part of an array of mechanisms of actions involved in thyroid hormone disruption.

Interestingly, however, additional results of the present study indicated a possible correlation between the effects of thyroidactive compounds on cell proliferation in the T-screen and relative heart weight in vivo (Fig. 6). This correlation between the T-screen and cardiac hypertrophy may align well with studies on both GH3 cells and the heart. The adult rodent heart expresses 70% THRα1 and 30% THRβ1. It was found that THRβ knockout (KO) mice did not exhibit a T3-mediated increase in left ventricular heart mass whereas THRα KO mice did (Swanson et al., 2003; Weiss et al., 2002). This indicates that THRβ expression is a requirement in the well-established thyroid hormoneinduced cardiac hypertrophy.

There are more factors affecting cardiac hypertrophy such as the renin-angiotensin system. However, this system is itself regulated by thyroid hormones (Diniz et al., 2009). Makino et al. (2009) reported that cardiac capillary networks are reduced in THRβ KO mice but not in THRα KO mice. Taken together, these studies indicate that THRβ-regulated cardiac hypertrophy acts together with THRβ-mediated angiogenesis to result in an overall effect on heart weight that is dominated by thyroid hormone agonism of THRβ. In GH3 cells, these effects are likely to be mediated by THRβ2 – the predominant isoform in pituitary cells (Lazar, 1990; Ball et al., 1997; Hahn et al., 1999). Regardless of the mechanism of action, the thyroid hormone-dependent cell proliferation that is the

hallmark of the T-screen correlates more closely with in vivo heart weight than with in vivo pituitary weight. This correlation between in vivo BMDL10 and in vitro EC50, could be expanded to compare absolute effect levels by taking toxicokinetics into account whereby the in vivo blood levels are calculated from the in vivo exposure levels, as has been done recently for estrogenic effects (Punt et al., 2013).

5 Conclusion

In vitro pituitary and thyroid cell proliferation assays are not viable substitutes for assessing pituitary and thyroid organ weight change, respectively. This calls into question the use of thyroid hormone-dependent cell proliferation assays and related TR-mediated reporter gene assays as alternative in vitro tests for the in vivo effect of chemicals on the HPT axis. When these THR-activation tests turn out negative, this study indicates that there is a considerable chance of a false negative result with the compound still affecting thyroid or pituitary weight in vivo. Nonetheless, the present study has found that the T-screen has potential applications in assessing the effects of compounds on thyroid hormone-mediated cardiac hypertrophy. In the context of the 3Rs (refinement, reduction, or replacement of animal studies), it is concluded that it is not currently feasible to have simple standalone in vitro replacements for in vivo tests for the disruption of the thyroid system. The results of the present study indicate that a complex interplay between factors within the HPT axis may underlie the effects of thyroid-active compounds on thyroid and pituitary organ weight endpoints in vivo. Therefore, it is concluded that the development of future alternative tests, aiming at refinement, reduction, or replacement of animal studies should include a broad battery of in vitro tests that cover the various modes of action of thyroid-active compounds as an initial screen and/or use more complex model systems that more closely reflect an intact HPT axis such as the nematode Caenorhabditis elegans or the vertebrate Danio rerio. Tests with both organisms can be performed in such a way that they are still considered alternatives to animal testing in higher vertebrates such as mouse and rat (Van der Ven, 2009).

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Chapter 3

Simple and rapid in vitro assay for detecting human thyroid peroxidase disruption

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Summary

A simple and rapid luminometric assay for the detection of chemical inhibitors of human thyroid peroxidase (hTPO) activity was developed and validated with 10 model compounds. hTPO was derived from the human thyroid follicular cell line Nthy-ori 3-1 and its activity was quantified by measuring the oxidation of luminol in the presence of hydrogen peroxide (H_2O_2), which results in the emission of light at 428 nm. In this assay, hTPO activity was shown to be inhibited by 5 known TPO inhibitors and not inhibited by 5 non-inhibitors. Similar results were obtained with porcine TPO (pTPO). The inhibition of hTPO by the model compounds was also tested with guaiacol and Ampliflu Red as alternative indicator substrates. While all substrates allowed the detection of pTPO activity and its inhibition, only the Ampliflu Red and luminol-based methods were sensitive enough to allow the quantification of hTPO activity from Nthy-ori 3-1 cell lysates. Moreover, luminol gave results with a narrower 95% confidence interval and therefore more reliable data. Whole extracts of fast-growing Nthy-ori 3-1 cells circumvent the need for animal-derived thyroid organs, thereby reducing costs, eliminating potential contamination and providing the possibility to study human instead of porcine TPO. Overall, the application of luminol and Nthy-ori 3-1 cell lysate for the detection of the disruption of hTPO activity was found to represent a valuable in vitro alternative and a possible candidate for inclusion within a high throughput integrated testing strategy for the detection of compounds that potentially interfere with normal thyroid function in vivo.

1 Introduction

Thyroid hormones are essential for normal development and the regulation of basal metabolism. A deficiency in these hormones due to a lack of iodine during the first two trimesters of pregnancy is prevalent in landlocked areas and leads to cretinism (Cao et al., 1994). The tell-tale signs of cretinism include short stature and mental retardation. Later on in life, thyroid hormone deficiency or excess lead to goitre, which is a swelling of the neck due to the enlargement of the thyroid gland. In the case of deficiency, the symptoms include weight gain, constipation and lethargy while in the case of excess, the symptoms include adrenergic stimulation as well as hypermetabolism (Dogra et al., 2006). The turn of the 21st century witnessed a worldwide drop in iodine deficiency due to decades of table salt fortification with iodine and a rise in concern over the ability of manmade chemicals to disrupt the thyroid hormone system (Delange et al., 2001; Jomaa, 2014). Addressing endocrine disruption, including the disruption of the thyroid hormone system, has been mandatory in the US since 1996 and the EU since 2006 (USEPA, 1996a; b; European Commission, 2006). Testing for thyroid hormone disrupters has been challenging due to the long duration, costs and unethical nature of the animal experiments involved. This has prompted the Organisation for Economic Co-operation and Development (OECD) to search for high-throughput in vitro alternatives (Jacobs et al., 2013; OECD, 2014).

The in vivo tests to be replaced include the OECD test guideline number 407 (TG 407) for a repeated dose 28-day oral toxicity study in rodents that defines serum thyroid hormone levels, thyroid histopathology, as well as pituitary and thyroid organ weights as endpoints (OECD, 2007 p. 407). While it is currently not possible to replicate thyroid histopathology in vitro, in a previous study, we have searched for potential correlation between the effects of chemicals on pituitary and thyroid organ weights in vivo and the effects of the same set of chemicals on cellular proliferation in vitro using pituitary and thyroid model cell lines. To this end, 11 thyroid-active compounds were tested and it was concluded that in vitro cellular proliferation alone correlates poorly with pituitary and thyroid organ weight change in vivo (Jomaa et al., 2013). This is likely due to the complex multi-organ paradigm that the thyroid system represents (Jomaa, 2014). A remaining in vivo endpoint that may prove suitable for the development of in vitro alternatives is the serum level of thyroid hormones, which is under the control of a feedback mechanism that accounts for hormonogenesis, metabolism and excretion.

Maternal thyroid deficiency is associated with subtle deficits in the neuropsychological development of the fetus (Haddow et al., 1999). Thyroid dyshormonogenesis can be due to either genetic mutations or chemicals present in our food and environment. There is a high prevalence of thyroid peroxidase (TPO) mutations in patients with low levels of

thyroid hormones at birth, a condition known as congenital hypothyroidism (Avbelj et al., 2007). Left untreated, this disease negatively affects growth and leads to mental retardation (Rose and Brown, 2006). The environment, through chemical inhibitors, can also affect TPO function. These chemical TPO inhibitors include the anti-thyroid drugs propylthiouracil (PTU) and methimazole (MMI), the industrial chemicals benzophenone 2 (BP2) and bisphenol A (BPA), and also foods rich in glucosinolates and flavonoids (Alexander and Zenker, 1986; Divi and Doerge, 1996; Schmutzler et al., 2007). Eating large quantities of cabbage, a source of glucosinolates, can lead to hypothyroidism and in extreme cases even coma (Chu and Seltzer, 2010; Dolan et al., 2010). Moreover, infants fed soy formula, which are rich in flavonoids, can develop soybean goiter (Shepard et al., 1960).

TPO is a heme-dependent enzyme that, in the presence of H_2O_2, catalyses the iodination and coupling of tyrosyl residues on thyroglobulin (TG), which are important steps in the biosynthesis of the thyroid hormones triiodothyronine (T3) and tetraiodothyronine (T4).

Even though the reduction in thyroid hormone levels due to the inhibition by chemicals of human TPO (hTPO) activity is a cause of concern, there is still no effective assay suited for hTPO-based high throughput screening (HTS). In a new scoping document on in vitro and ex vivo assays for the identification of modulators of thyroid hormone signaling, the OECD concluded that it was necessary to develop a screening assay for the inhibition of TPO that does not use animal tissue and is suited for HTS (Jacobs et al., 2013; OECD, 2014). Moreover, the OECD mentioned a study by Takamaya et al. that found rat TPO to be in some cases more than 455 times more sensitive to xenobiotic inhibitors than monkey TPO, emphasizing the potential for substantial interspecies differences (Takayama et al., 1986). The aim of the present study is to address these issues by developing an assay that a) uses hTPO instead of TPO from animal tissue, and b) is suited for HTS.

2 Materials and methods

Chemicals

Methimazole (MMI), propylthiouracil (PTU), quercetin (QE), naringenin (NAR), resorcinol (RES), triiodothyronine (T3), tetraiodothyronine (T4), ouabain (OUB), benzyl butyl phthalate (BBP), and amiodarone hydrochloride (AMI) were of high purity and were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). Unless otherwise specified, all reagents were also obtained from Sigma-Aldrich Chemie.

Cell culture

Nthy-ori 3-1, a normal thyroid follicular epithelial cell line, was obtained from the European Collection of Cell Cultures (EACC, Wiltshire, UK) and grown as a monolayer in RPMI-1640 medium (Gibco, Paisley, Scotland) supplemented with 10% Fetal Calf Serum (FCS). The cells were cultured at 37 °C in a humid atmosphere containing 5% (v/v) CO_2 and passaged enzymatically twice a week.

FRTL-5, a rat thyroid cell line (obtained from CLS, Germany), was cultured as a monolayer in tissue-culture flasks (obtained from Corning, Badhoevedorp, The Netherlands) at 37 °C in a humid atmosphere containing 5% (v/v) CO_2 and passaged once a week with an interim refresh of the medium, which consisted of Coon's Modified F-12 medium (Biochrom, Berlin, Germany), supplemented with 5% FCS, and a mixture of six hormones/growth factors (this medium was referred to as 6H5, reflecting the presence of 6 hormones/growth factors and 5% FCS). The six hormones/growth factors included bovine thyroid stimulating hormone (1 mIU/ml), insulin (10 µg/ml), hydrocortisone (10 nM), apo-transferrin (5 µg/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 ng/ml). All these hormones/growth facors were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). This 6H5 cell culture medium was supplemented with 1 mM non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Difco, Amsterdam, The Netherlands). Enzymatic passaging was performed using a cell detachment and disaggregation solution containing 20 Units/ml collagenase, 0.075% trypsin, and 2% chicken serum (CTC) in PBS. Collagenase and chicken serum were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands) while trypsin was obtained from Difco (Amsterdam, The Netherlands). The FRTL-5 cell line was used as a reference cell line that is positive for TPO (Suzuki et al., 1998).

The human hepatocellular carcinoma (HepG2) cell line was used as a reference cell line that is negative for TPO activity and cultured in DMEM/F12 medium containing 10% FCS, passaged twice a week and kept at 37 °C in a humid atmosphere containing 5% (v/v) CO_2.

TPO preparation

TPO from Nthy-ori 3-1 cells was prepared as a whole cell extract. Briefly, cells were washed with phosphate buffered saline (PBS), scraped, resuspended in a small volume of PBS and spun down for 5 minutes at 150 g. The supernatant was discarded and the cell pellet lysed by resuspension in 0.1 % sodium deoxycholate (DC, in PBS) and incubation on ice for 20 minutes. The lysed cells were centrifuged for 5 minutes at 11,350 g to separate the soluble protein fraction from unlysed cells and debris. Protein

concentration of the supernatant was then measured using the Pierce BCA protein assay (Pierce, Etten-Leur, The Netherlands) following the manufacturer's protocol. Cell lysates from one 75 cm^2 cell culture flask that is ≥ 80% confluent were resuspended in 2 ml of 0.1% DC and had a protein content of 0.1 – 0.2 mg/ml. With 100 µl used per 96-well plate quadruplicate, one 75 cm^2 cell culture flask was therefore sufficient for testing ~ 20 different concentrations of a test compound or controls. For long-term storage, cell lysates were kept at -80 °C. Relative TPO gene expression in the Nthy-ori 3-1 cell line has been previously quantified by others (Tuncel et al., 2007). In our experiments, TPO activity in cell extracts, which is proportional to relative fluorescence (for Ampliflu Red) or relative luminescence (for luminol) obtained from cell lysates containing 0.1 – 0.2 mg/ml total protein, was consistently above the limit of quantitation (LOQ) assessed as the mean of the blank + 10 times the standard deviation.

TPO from porcine thyroid glands was prepared essentially as described previously (Hosoya et al., 1985), with some modifications. Briefly, porcine thyroid tissue was obtained at the slaughterhouse (Visser, Lunteren, The Netherlands) and homogenized in sterile buffer (0.25 M sucrose; 20 mM Tris/HCl, pH 7.4; 100 mM KCl; 40 mM NaCl; 10 mM $MgCl_2$) with 10 strokes at 24,000 rpm using an Ultra-Turrax. The sample was homogenized further by 10 strokes in a Potter-Elvehjem tissue grinder (VWR, Amsterdam, The Netherlands). The ground tissue was centrifuged for 10 minutes at 375 g. The supernatant was then diluted to 50 mg/ml of tissue in sterile buffer.

Guaiacol and Ampliflu Red assays for TPO activity

Reactions were conducted in 96-well plates with a volume of 200 µl per well containing a thyroid tissue protein content of 0.6 - 1 mg/ml or a cell lysate protein content of 0.1 – 0.2 mg/ml, 0.1 M potassium phosphate (pH 7.4), and the desired concentration of the test compound added from a 100 times concentrated stock solution in DMSO (1% DMSO final concentration). 1% DMSO does not interfere with either the guaiacol or the Ampliflu Red assays for peroxidase activity (Vogt et al., 2002; Schmutzler et al., 2007; Dabir et al., 2013). The mixture was incubated for 30 minutes with gentle shaking at 37 °C after which the reaction was initiated by addition of 20 µl of either 1 M guaiacol or 1 mM Ampliflu Red and 5 µl of 80 mM H_2O_2. Guaiacol absorbance was measured at 470 nm ($\varepsilon = 2.66 \times 10^4\ M^{-1}\ cm^{-1}$ (Chance and Maehly, 1955)) and Ampliflu Red fluorescence was measured at 544/590 nm excitation/emission wavelengths, with readings every 30 seconds for 10 minutes on a SpectraMax M2 microplate reader (Molecular Devices, Menlo Park, CA, USA). The reaction rate was calculated as the slope of a linear fit of the 21 absorbance values using the SoftMax Pro software provided with the microplate reader.

Luminol assay for TPO activity

Reactions were conducted in 96-well plates with a volume of 200 µl per well containing a thyroid tissue protein content of 0.6 - 1 mg/ml or a cell lysate protein content of 0.1 – 0.2 mg/ml, 1M glycine-NaOH (pH 9.0), 1 mM EDTA, and the desired concentration of the test compound added from a 100 times concentrated stock solution in DMSO (1% DMSO final concentration). 1% DMSO does not affect luminol luminescence (Shertzer et al., 2004). The mixture was incubated for 30 minutes with gentle shaking at 37 °C after which the reaction was initiated by the addition of 20 µl of luminol mix containing 1M glycine-NaOH (pH 9.0), 1 mM EDTA and 400 µM luminol. Following a 4 second delay, 5 µl of 80 mM H_2O_2 was automatically added. Luminescence was measured as relative luminescence units (RLUs) integrated over 10 seconds using a Luminoskan Ascent luminometer from Labsystems (Helsinki, Finland).

Data analysis

In the guaiacol, Ampliflu Red and luminol assays for TPO activity, data points are representative of three independent experiments (N = 3) and four replicate wells per data point in each experiment. Raw data from quadruplicate wells were averaged, converted to percent of solvent control and represented graphically as the means of independent experiments with bars representing the standard error of that mean (SEM). Nonlinear curve-fitting described in the results was done using the Hill equation with the help of GraphPad Prism software version 5.04 (GraphPad Software, San Diego, CA, USA). In vitro, relative IC50 values were calculated from the fitted models as the concentration of the tested compound that gave 50% of the maximal response. A response was considered positive when the squared Pearson's coefficient of correlation (r^2), as an indication of goodness-of-fit of a logistic dose-response model, had a value of 0.8 or higher. The dynamic range, Z′ factor (Zhang et al., 1999) and average assay coefficient of variation (CV) were calculated in order to describe the performance of the luminol assay for TPO activity. The Z′ factor is defined in terms of the standard deviations (SD) and the means of the positive and negative (DMSO) controls with the following formula:

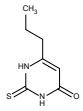
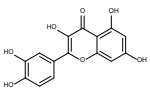
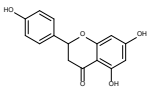
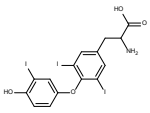
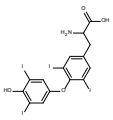
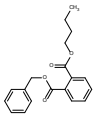
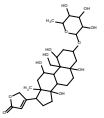
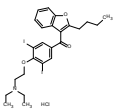
$$Z' = 1 - \left(\frac{3SD\ of\ positive\ control\ signal + 3SD\ of\ DMSO\ control}{|mean\ of\ positive\ control\ signal - mean\ of\ DMSO\ control|} \right)$$

3 Results

3.1 Compound selection

Five model compounds known to inhibit TPO in vitro were selected and these are MMI, PTU, QE, NAR and RES. Five thyroid-active compounds that interfere with the thyroid hormone system through a non-TPO mode of action were also selected and these are T3,

Tab. 1: Test compounds used in this study with a brief description of their use or nature and their chemical structure. A dash represents a lack of literature on the compound's effect on TPO.

Compound	Abbr.	CAS No.	Description	Chemical structure	In vitro TPO inhibition literature (Guaiacol assay)	In vivo thyroid disruption literature
Methimazole	MMI	60-56-0	Antithyroid drug		(Taurog et al., 1996)	(Hood et al., 1999)
Propylthiouracil	PTU	51-52-5	Antithyroid drug		(Hosoya, 1963)	(OECD, 2006)
Quercetin	QE	117-39-5	Plant copigment		(Divi and Doerge, 1996)	(Giuliani et al., 2014)
Naringenin	NAR	480-41-1	Plant copigment		(Divi and Doerge, 1996)	(Panda and Kar, 2014)
Resorcinol	RES	108-46-3	Antiseptic used in cosmetics		(Divi and Doerge, 1994)	(Berthezéne et al., 1979)
Triiodothyronine	T3	6893-02-3	Endogenous hormone		-	(Soukup et al., 2001)
Thyroxine	T4	51-48-9	Endogenous hormone		-	(OECD, 2006)
Benzyl butyl phthalate	BBP	85-86-7	Plasticizer		-	(Nagao et al., 2000)
Ouabain	OUB	630-60-4	Cardiotonic and antiarrhythmic drug		-	(Wardlaw, 1986)
Amiodarone HCl	AMI	19774-82-4	Antiarrhythmic drug		-	(Safran et al., 1986)

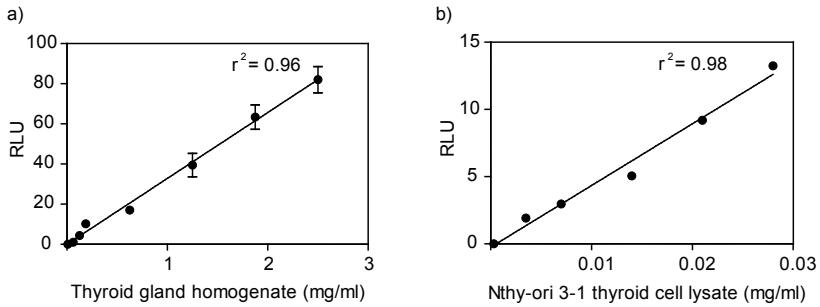


Fig. 1: TPO-mediated light emission upon oxidation of luminol. a) Porcine thyroid gland homogenate and b) human Nthy-ori 3-1 thyroid cell lysate dependent enzymatic activity in terms of RLU emitted upon oxidation of luminol in the presence of H_2O_2.

T4, BBP, OUB and AMI. Table 1 details the CAS numbers, common use or origin, chemical structure and a representative literature reference pertaining to existing guaiacol-based in vitro tests of effects of these compounds on TPO activity as well as the in vivo effects of these compounds on the thyroid hormone system. All test compounds and reagents were of high purity and were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands).

3.2 Luminol assay for TPO activity

Figure 1 shows that the protein content of the porcine thyroid homogenate and Nthy-ori 3-1 lysates correlate linearly with the luminescence that is being produced in the luminol assay. For thyroid homogenate the squared coefficient of correlation (r^2) was 0.96 and for the Nthy-ori 3-1 lysate the r^2 was 0.98. Based on results obtained with MMI (N = 3) as a positive control, the luminol assay for TPO activity had a dynamic range of 25.4 ± 4.3 (mean ±SEM), a Z' factor of 0.74 ± 0.021 (mean ±SEM) and an average CV (%) of 4.94. In the luminol assay, the reaction was started with H_2O_2 that was added by the automatic dispenser in the luminometer followed by a 2 second delay before measurement and integration of the signal over 10 seconds. The luminol signal decays more quickly than ampliflu red, however, the integration procedure enables reproducible results (%CV = 4.94 for MMI) irrespective of the decay of the luminescence signal. The fact that the luminol signal can be measured within a shorter period of time allowed for a more rapid assay. In case of the Ampliflu Red assay, the reaction proceeded linearily over the measured reaction period of 10 min.

Tab. 2: IC50 values of the compounds as TPO inhibitors in the guaiacol assay, Ampliflu Red assay and luminol assay. CI stands for confidence interval (n=3). r^2 is indicated for the goodness-of-fit of a logistic dose-response model.

Compound	IC50 µM (95% CI or reference) guaiacol assay		IC50 µM (95% CI) Ampliflu Red assay		IC50 µM (95% CI) luminol assay			
	pTPO	r^2	hTPO	r^2	pTPO	r^2	hTPO	r^2
MMI	4.0 (Taurog, 1970)	-	2.7 (0.4 - 19.9)	0.95	5.7 (3.0 - 10.7)	0.95	4.0 (1.6 - 10.1)	0.91
RES	6.5 (Taurog, 1970)	-	18.7 (6.8 – 51.9)	0.84	5.8 (4.0 - 8.3)	0.99	5.0 (3.6 to 6.9)	0.98
QE	8.4 (1.2 – 60.1)	0.78	7.7 (3.9 - 15.4)	0.96	10.0 (6.7 - 14.9)	0.99	4.1 (3.2 to 5.3)	0.99
PTU	10.7 (5.1 – 22.2)	0.92	35.2 (10.4 - 119.1)	0.86	41.6 (24.9 - 69.5)	0.94	16.4 (10.0 - 27.0)	0.91
NAR	10 - 150 (Divi and Doerge, 1996)	-	59.0 (30.4 - 114.6)	0.94	67.5 (29.24 - 155.6)	0.95	55.5 (43.2 to 71.3)	0.98

3.3 TPO inhibition

In figure 2 dose response curves for the 5 tested TPO inhibitors are presented based on three separate experiments with four replicate wells per concentration. It can be seen that the luminol assay detects the disruption of TPO activity by the 5 selected TPO inhibitors in a dose-dependent manner. Moreover, while the Ampliflu Red (Paul et al., 2014), guaiacol and luminol (Table 1) methods are all able to detect pTPO activity that is present in porcine thyroid tissue samples, the guaiacol assay was negative with hTPO from the Nthy-ori 3-1 human thyroid cell line (data not shown), and only the Ampliflu Red and luminol methods were able to detect hTPO activity in lysates from Nthy-ori 3-1 human thyroid cells cultured in vitro (Table 1). The selected non-inhibitors of TPO, namely, T3, T4, BBP, OUB, and AMI were also all correctly identified as non-inhibitors by both the Ampliflu Red method and the luminol method (figure 3).

A comparison of the concentrations resulting in 50% inhibition (IC50s) presented in table 2 reveals that the luminol method detects the effects of the tested compounds with a narrower 95% confidence interval (CI). The overall potency ranking for pTPO inhibition was MMI > RES > QE > PTU > NAR. The potency ranking for hTPO inhibition was MMI > QE > RES > PTU > NAR.

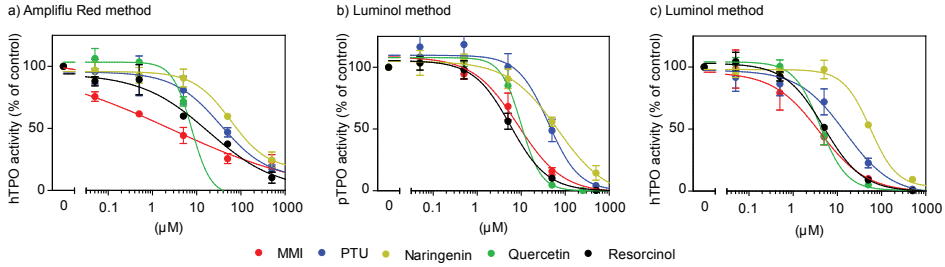


Fig. 2: Effect of MMI, PTU, quercetin, naringenin and resorcinol on a) hTPO activity in the Ampliflu Red assay, and on b) pTPO, and c) hTPO activity in the luminol assay.

3.4 Confounders of peroxidase activity measurements in samples from in vivo or in vitro origin

In order to find out whether contamination of thyroid tissue with blood or muscle tissue could be a confounder when analysing thyroid peroxidase activity in samples from animal origin, hemoglobin-rich muscle tissue homogenates were tested for peroxidase activity and compared to results obtained for thyroid homogenates. Figure 4a shows that hemoglobin-rich muscle tissue has peroxidase activity as well.

Confounding of measurement of TPO activity when using samples from in vitro cell lines could come from non-TPO based activity in cells from non-thyroid origin. The cell lines used in this experiment were grown under controlled conditions to ensure uniformity and kept at below passage 30 in order to retain as much of the characteristics of their organ of origin as possible. Figure 4b shows that in the luminol assay using cell lysates from follicular rat thyroid cells grown using 5% serum (FRTL-5), from human Nthy-ori 3-1 cells or from human hepatocellular carcinoma (HepG2) cells, only cell lysates from cells of thyroidal origin have peroxidase activity.

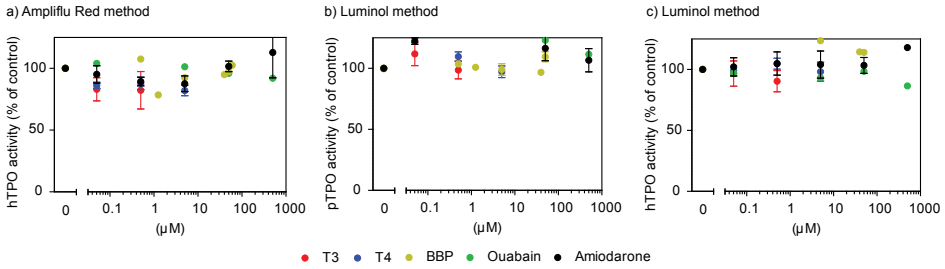


Fig. 3: Effect of T3, T4, ouabain, BBP and amiodarone on a) hTPO activity in the Ampliflu Red assay, and on b) pTPO, and c) hTPO activity in the luminol assay.

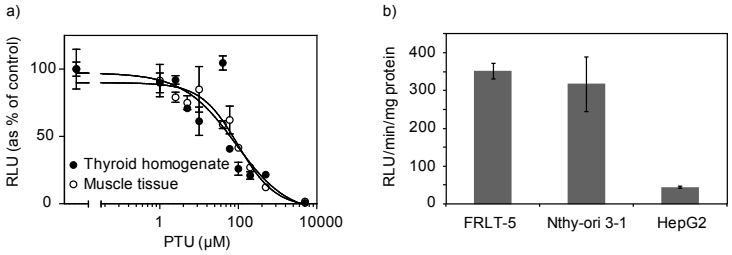


Fig. 4: In vivo and in vitro TPO activity. a) Peroxidase activity can be detected from both thyroid homogenates and muscle tissue. b) In vitro peroxidase activity is present in thyroid cell lines only.

4 Discussion

The aim of the present study was to address the pitfalls of animal tissue-based assays using guaiacol as a substrate by developing an assay that a) uses human TPO (hTPO) instead of TPO from animal tissue, and b) uses a substrate that is suited for HTS.

The results obtained reveal that lysates of the Nthy-ori 3-1 human thyroid cell line retain peroxidase activity that can be detected in the Ampliflu Red assay and luminol assay but not in the guaiacol assay. The greater sensitivity of luminol in comparison with guaiacol supports previous findings where luminol was found to be 3-fold more sensitive than guaiacol in detecting TPO activity (Kaczur et al., 1997). While already used for the detection of TPO inhibition by anti-TPO antibodies and in the detection of the inhibition of HRP by chemical inhibitors (Kaczur et al., 1997; Navas Díaz et al., 1998), the present study reports the application of luminol as a highly sensitive and reliable substrate that can be applied to detect the effect of chemical inhibitors on TPO activity. Figure 2 shows that all compounds that tested positive for inhibition in the guaiacol assay also tested positive for inhibition in the luminol assay. These results are in accordance with the known in vitro effect of these compounds on TPO activity (table 1). Moreover, while the Ampliflu Red assay was also able to detect compounds known to inhibit TPO activity, the 95% CI of the IC50s of the compounds tested was wider and therefore the Ampliflu Red-based assay is regarded to be less reliable than the luminol-based assay (table 2).

Even though the guaiacol oxidation assay is widely used to detect peroxidase activity, the apparent lack of sensitivity is one of several drawbacks since guaiacol is also readily oxidized upon exposure to air and light, which means that necessary precautions have to be put in place to avoid premature color change. Moreover, the measurement time needed is 2 - 10 minutes, compared to 10 seconds for the luminol assay. Compound purity could be yet another drawback as it has been previously reported that guaiacol from Aldrich Chemical Co. contained contaminants that lead to low values for peroxidase activity (Taurog et al., 1992).

The successful use of TPO derived from the human follicular thyroid cell line Nthy-ori 3-1 avoids the use of animal tissue and eliminates interspecies differences when evaluating risks for the human population. Such interspecies differences exist and have been previously reported (Takayama et al., 1986; Paul et al., 2013) and are also illustrated in the present study by the difference in potency ranking observed between pTPO and hTPO in the luminol assay. Moreover, animal sources of TPO are often contaminated with blood hemoglobin that can also act as a peroxidase, a fact that can be attributed to the catalytic heme moiety present in both hemoglobin and TPO (Harauchi and Yoshizaki, 1982). In this study, it was demonstrated that hTPO from Nthy-ori 3-1 cells can be easily used in combination with a luminol assay method to detect thyroid-active compounds whose mechanism of action involves TPO inhibition. While other studies have reported increased in vitro TPO protein levels and activity in cells transfected with a TPO expression construct (Marinovich et al., 1995), the present study shows that the luminol assay is sensitive enough to detect the activity of endogenously expressed TPO from cells of the commercially-available human Nthy-ori 3-1 cell line. This overcomes the inability to measure TPO activity in samples derived from this cell line when using guaiacol as a substrate. It can therefore be concluded that there is no apparent need for using porcine TPO instead of human TPO. The relative high speed, stability and sensitivity of the luminol-based assay relative to the guaiacol-based assay fulfilled our objective to have an assay suited for HTS.

The results obtained with hTPO also mirror the effect of the TPO inhibitors tested in humans (Divi and Doerge, 1994; Nakamura et al., 2007). hTPO inhibition profiles derived from both the luminol and Ampliflu Red assay showed that MMI followed by quercetin and resorcinol were the most potent of the inhibitors tested. PTU was less potent than MMI, a finding that is supported in clinical studies (Nakamura et al., 2007), whereas naringenin was the least potent TPO inhibitor tested, showing no reported effects in humans. The association between the topical application of resorcinol in ointments and thyroid dysfunction in humans has a long history but even though rodent studies support causality, the higher sensitivity of rodents to thyroid disruption means that causality in humans is still considered inconclusive (Lynch et al., 2002). The resorcinol derivative quercetin, which is present in plant components of the human diet, is thought to be offsetting the benefit of iodized salt in parts of India that are affected by endemic goitre and while further human studies are necessary to confirm causality, recent rodent studies support the notion that quercetin inhibits thyroid function (Divi and Doerge, 1994; Giuliani et al., 2014). Overall, these antioxidants inhibit TPO in vitro and are associated with thyroid dysfunction in vivo and this is accurately reflected in the luminol assay. In conclusion, the combination of luminol as a chemiluminescent

indicator of TPO activity and the Nthy-ori 3-1 cell line as a source of hTPO results in an alternative assay for the detection of TPO inhibitors that holds promise for inclusion within a high throughput integrated testing strategy.

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Chapter 4

Identification of thyroid hormone receptor coregulator interactions and profiling of their modulation by T3, T4, amiodarone and compound 1-850

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Summary

Nuclear receptor coregulators play a central role in mediating the effect of endocrine-active compounds. Here we report the application of a microarray assay for real-time coregulator-nuclear receptor Interaction (MARCoNI) for the characterisation of coregulator recruitment by thyroid hormone receptor alpha (TRα) and beta (TRβ) induced upon binding of the thyroid hormones T3 and T4 acting as receptor agonists, and of the thyroid hormone antagonists amiodarone and compound 1-850. The modulation, by the test compounds, of TRα and TRβ binding to 154 nuclear receptor interaction motifs of 66 coregulators was measured relative to the solvent control, and analysed by hierarchical clustering. The agonists T3 and T4 clustered separately from the antagonists amiodarone and compound 1-850 for both receptor isoforms tested. The results of the current study also reveal new TR-interacting coregulators including candidate coactivators BL1S1 and WIPI1 as well as candidate corepressors RBL2, DHX30 and LCOR. The results were further supported by full dose-response curves for the compounds tested and underlined the potential of the MARCoNI assay as an alternative screening test for thyroid-active compounds.

1 Introduction

Thyroid hormones are essential for normal physical growth and mental development (Rastogi and LaFranchi, 2010). They exert their action by binding to the thyroid hormone receptor alpha and beta subtypes (TRα and TRβ) that are situated inside the nucleus of most of the body's cells (Abu et al., 1997; Shahrara et al., 1999). As nuclear receptors, TRs are transcription factors that can be activated by ligands – in this case thyroid hormones. However, unlike type I nuclear receptors such as the estrogen receptor (ER) and androgen receptor (AR), type II nuclear receptors, which include TRs and the retinoic acid receptor (RAR), form heterodimers with the retinoid x receptor (RXR). In the unliganded state, type II receptor-RXR heterodimers bind to DNA within a corepressor complex of proteins that condenses chromatin and actively suppresses gene transcription. Whereas type I nuclear receptors bind their ligand in the cytoplasm and are translocated to the nucleus to activate gene transcription, type II nuclear receptors bind their ligand within the nucleus which causes a structural change that dissociates corepressors and recruits coactivator proteins that decondense chromatin and mediate gene transcription (Gronemeyer et al., 2004; Germain et al., 2006). Coactivators and corepressor proteins, are therefore integral and essential coregulators of TR-mediated gene transcription.

The 1990s witnessed the public scrutiny of marketed products after the discovery of endocrine-disrupting chemicals in household items (Feldman and Krishnan, 1995). This has led to the establishment of regulations that require the testing of chemicals for endocrine activity. With increased testing came increased concern for the high cost, long duration, and large number of animals needed for safety testing and this increased the need for alternative in vitro testing methods (van der Jagt et al., 2004). In the case of estrogenic activity, alternative in vitro testing strategies have proven promising. In vitro proliferation of uterine cells after exposure to estrogenic compounds was found to correctly predict changes in uterine weight after exposure to estrogenic compounds in vivo (Wang et al., 2012). On the other hand, we have shown in a previous study that the cellular proliferation of pituitary and thyroid cells after exposure to thyroid-active compounds in vitro correlates poorly to the weight change of their respective organ in an in vivo test (Jomaa et al., 2013). This lack of correlation is likely due to the complex multi-organ interaction that controls the thyroid hormone system. Therefore, an elaborate in vitro test battery representing the various in vivo endpoints would be required to screen for thyroid-active compounds (Jomaa, 2014). One type of assay that would contribute to such an integrated testing strategy is an in vitro assay for detecting the effects of compounds on TR-mediated gene transcription. To this end, a TR-based reporter gene assay has been developed by stably transfecting the rat pituitary tumour cell line GH3, that consitutively expresses both TRα and TRβ, with a thyroid hormone response element-driven luciferase

gene (Freitas et al., 2011). Among the disadvantages of such reporter gene assays are the use of luciferase as the reporter gene as it may generate false positives (Sotoca et al., 2010), the lack of distinction between receptor isoforms and the interference that is exhibited in some cases due to other receptors (Bhattacharjee and Khurana, 2014). Recently a novel approach to detect nuclear receptor binding was introduced, being the so-called microarray assay for real-time coregulator-nuclear receptor interaction (MARCoNI). The MARCoNI holds promise as a candidate for inclusion within a high-throughput testing strategy (HTS) for endocrine-active compounds. However, studies on nuclear receptor ligand interactions using MARCoNI have so far focused mainly on the estrogen receptor (ER), the glucocorticoid receptor (GR) and the Pregnane X receptor (Wang et al., 2013; Desmet et al., 2014; Evers et al., 2014; Murayama et al., 2014).

As the name suggests, the principle of MARCoNI is based on measuring the interaction of a nuclear receptor with its coregulators and studying how a ligand, either an agonist or antagonist, affects this interaction. To do this, an array of coregulator peptides, each representing a specific coregulator-nuclear receptor interaction motif is first printed with a piezoelectric print head on a chip composed of 60 µm-wide aluminum oxide capillaries (van Beuningen et al., 2001). Then, capillary flow-through exposure to a nuclear receptor with or without ligands is performed and the interaction between the LBD of the nuclear receptor of interest and each of the coregulator peptides is quantitated based on fluorescence-based immunochemistry.

Previous studies with the ER receptors revealed that MARCoNI is able to measure the potency of different agonists and to distinguish known transcriptional agonists from antagonists based on coregulator modulation profiles even without the addition of an endogenous ligand (Wang et al., 2013).

The aim of the present study is to investigate whether MARCoNI is able to correctly identify TR-agonists and TR-antagonists and to investigate possible differences in ligand-induced coregulator activation by TRα and TRβ. The results obtained will provide an insight into the usefulness of this assay within an integrated testing strategy for thyroid-active compounds.

2 Materials and methods

Compounds

Dimethyl sulfoxide (DMSO), T3, T4 and amiodarone were obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). TR antagonist compound 1-850 (CAS 251310-57-3) was obtained from Merck Millipore (Amsterdam, The Netherlands).

MARCoNI

Human GST-tagged TRα LBD (Invitrogen, Breda, The Netherlands) and human GST-tagged TRβ LBD (AB Vector (San Diego, CA, USA) were diluted in reaction buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 0.2% BSA, 0.05% Tween-20) containing 50 mM D-dithiothreitol (DTT) to a final assay concentration of 5 nM. 25 µl of this thyroid receptor LBD solution with 25 nM Alexa 488-conjugated GST antibody (Invitrogen, Breda, The Netherlands), either with or without ligand (agonist or antagonist) in 2% DMSO, were added to a MARCoNI PamChip® containing 154 NR coregulator peptides (Pamgene International BV 's-Hertogenbosch, The Netherlands). All steps were performed on ice. Measurements were performed automatically using a PamStation®-12 (PamGene International B.V.) at a constant temperature of 20°C according to the provided protocol. Briefly, 80 cycles of capillary flow-through and charge-coupled device (CCD) camera-based fluorescence measurement were performed at a rate of 2 cycles / min. To minimize non-specific binding of the antibody, each chip was first incubated for 20 cycles at a rate of 2 cycles / min in blocking buffer which consisted of TBS with 1% BSA and 0.01% Tween-20. Final exposure concentrations were equal to 0.1 µM, 1 µM, 10 µM, 50 µM and 100 µM for amiodarone and compound 1-850; 0.001 µM, 0.01 µM, 0.1 µM, and 10 µM for T3; 0.001 µM, 0.01 µM, 0.1 µM, 1 µM and 10 µM for T4.

Data analysis

Uncompressed tagged image file format (TIFF) images obtained from the PamStation®-12 were analyzed using BioNavigator software (PamGene International B.V.). Automated array grid detection and median signal-minus-background calculation was performed with a cut-off value of 5 arbitrary units (AU) for each fluorescent peptide spot and the resulting value was used for deriving the modulation index (MI). The MI was calculated as the log10 change in binding (relative fluorescence) of the receptor LBD to the coregulator in the presence of a ligand over the binding of the receptor LBD to the coregulator in solvent only. For each compound-receptor pair, a Student's t-test was used to determine whether the fluorescence measured at the 2 highest concentrations (ligand-saturated concentrations) is significantly different from the fluorescence measured from 4 separate solvent controls.

3 Results

The modulation of TRα-LBD and TRβ-LBD binding to the 154 motifs of 66 coregulators with respect to the solvent control (DMSO) was tested for T3, T4, amiodarone and compound 1-850. An overview based on literature of all 66 coregulators present on the peptide array and their function or known interaction with hormone receptors is presented in Tab. 1.

Tab. 1: Coregulators on the peptide array and their function.

Protein	Uniprot Accession	Full name (alias)	Function/structure	Reference
ANDR	P10275	Androgen receptor	Transcription factor	(Lee and Chang, 2003)
BL1S1	P78537	Biogenesis of lysosome-related organelles complex-1 subunit 1 (MTA1-interacting coactivator)	ER coactivator	(Mishra et al., 2003)
BRD8	Q9H0E9	Bromodomain-containing protein 8 (thyroid hormone receptor coactivating protein 120kDa)	TR coactivator	(Monden et al., 1997)
CBP	Q92793	CREB-binding protein	TR, RXR, RAR, ER coactivator	(Kamei et al., 1996)
CCND1	P24385	G1/S-specific cyclin-D1	TRβ corepressor, AR corepressor, ERα coactivator	(Petre-Draviam et al., 2004)
CENPR	Q13352	Centromere protein R (Nuclear receptor-interacting factor 3)	TR and RXR coactivator	(Li et al., 1999)
CHD9	Q3L8U1	Chromodomain-helicase-DNA-binding protein 9 (Peroxisomal proliferator-activated receptor A-interacting complex 320 kDa protein)	PPARα, CAR, ERα, and RXR coactivator	(Surapureddi et al., 2006)
CNOT1	A5YKK6	CCR4-NOT transcription complex subunit 1 (Negative regulator of transcription subunit 1 homolog)	TRβ interacting protein; ERα, and RXR ligand-dependent corepressor	(Winkler et al., 2006 p. 4)
DDX5	P17844	Probable ATP-dependent RNA helicase DDX5	AR coactivator	(Clark et al., 2008)
DHX	Q7L2E3	Putative ATP-dependent RNA helicase DHX30	NR2E3 interacting protein	(Qin et al., 2013)
EP300	Q09472	Histone acetyltransferase p300	TRα, ERα, AR, PPARα, PPARγ coactivator	(Dowell et al., 1997; Kodera et al., 2000; Fan et al., 2002; De Luca et al., 2003; Dai et al., 2008)
GELS	P06396	Gelsolin precursor	AR coactivator	(Nishimura et al., 2003)

GNAQ	P50148	Guanine nucleotide-binding protein G(q) subunit alpha	Mediates stimulation of phospholipase C beta	(Dong et al., 1995)
HAIR	O43593	Protein hairless	TR coregulator	(Thompson and Bottcher, 1997)
IKBB	Q15653	NF-kappa-B inhibitor beta (Thyroid receptor-interacting protein 9, TRIP-9)	TR-interacting protein	(Lee et al., 1995)
ILK	Q13418	Integrin-linked protein kinase	ERα coregulator	(Acconcia et al., 2006)
JHD2C	Q15652	Probable JmjC domain-containing histone demethylation protein 2C (Thyroid receptor-interacting protein 8, TRIP-8)	TR-interacting protein	(Lee et al., 1995)
KIF11	P52732	Kinesin-like protein KIF11 (Thyroid receptor-interacting protein 5, TRIP-5)	TR-interacting protein	(Lee et al., 1995)
L3R2A	Q9Y6C7	Loss of heterozygosity 3 chromosomal region 2 gene A protein	ERα corepressor	(Meng et al., 2004)
LCOR	Q96JN0	Ligand-dependent corepressor	ERα, GR, PR, VDR ligand-dependent corepressor (recruits factors that repress transcription); ERβ, RAR and RXRα interacting protein	(Fernandes et al., 2003)
MAPE	P78395	Melanoma antigen preferentially expressed in tumors	RAR ligand-dependent corepressor	(Epping et al., 2005)
MED1	Q15648	Mediator of RNA polymerase II transcription subunit 1 (Thyroid receptor-interacting protein 2, TRIP-2, Thyroid hormone receptor-associated protein complex 220 kDa component, TRAP220)	TRα, ER coactivator; VDR-, RARα-, RXRα-, PPARα-, PPARγ- interacting protein	(Yuan et al., 1998; Kang et al., 2002)
MEN1	O00255	Menin	TGF-β signalling regulator	(Kaji et al., 2001)
MGMT	P16455	Methylated-DNA{protein-cysteine methyltransferase	ERα ligand-dependent corepressor	(Teo et al., 2001)
MLL2	O14686	Myeloid/lymphoid or mixed-lineage leukemia protein 2	ERα coactivator	(Mo et al., 2006)
MTA1S	Q13330-2	Metastasis-associated protein MTA1	ER ligand-dependent corepressor	(Mazumdar et al., 2001)

Protein	Uniprot Accession	Full name (alias)	Function/structure	Reference
NCOA1	Q15788	Nuclear receptor coactivator 1 (Steroid receptor coactivator 1, SRC-1)	AR, ERα, PPARα, RXR and TRα coactivator	(Kalkhoven et al., 1998; Treuter et al., 1998; Kucera et al., 2002; Masiello et al., 2004)
NCOA2	Q15596	Nuclear receptor coactivator 2	AR, ER and PR coactivator. GR-, RXR-, RAR,-TR-interacting protein.	(Voegel et al., 1996)
NCOA3	Q9Y6Q9	Nuclear receptor coactivator 3 (Thyroid hormone receptor activator molecule 1, TRAM-1)	TR coactivator	(Takeshita et al., 1997)
NCOA3 MOUSE	O09000	Nuclear receptor coactivator 3 (Thyroid hormone receptor activator molecule 1, TRAM-1)	TR coactivator	(Takeshita et al., 1997)
NCOA4	Q13772	Nuclear receptor coactivator 4	AR and PPARγ coactivator	(Yeh and Chang, 1996; Heinlein et al., 1999)
NCOA6	Q14686	Nuclear receptor coactivator 6 (Thyroid hormone receptor-binding protein)	TR, ERα, AR, RAR, coactivator	(Lee et al., 1999 p. -2; Ko et al., 2002; Goo et al., 2004)
NCOR1	O75376	Nuclear receptor corepressor 1 (Thyroid Hormone- And Retinoic Acid Receptor-Associated Corepressor 1)	AR, GR, PPARα, RAR corepressor	(Dowell et al., 1999; Cheng et al., 2002 p. -; Stevens et al., 2003; Shimizu et al., 2015 p. 1)
NCOR2	Q9Y618	Nuclear receptor corepressor 2 (Silencing mediator of retinoic acid and thyroid hormone receptor, SMRT)	TR, AR, RARα and PPARγ corepressor	(Hong et al., 1997; Shi et al., 2002; Liao et al., 2003; Shimizu et al., 2015)
NELFB	Q8WX92	Negative elongation factor B	Transcriptional repressor	(Yamaguchi et al., 2002)
NR0B1	P51843	Nuclear receptor subfamily 0 group B member 1 (DSS-AHC critical region on the X chromosome protein 1, DAX1)	LRH-1 corepressor	(Sablin et al., 2008)
NR0B2	Q15466	Nuclear receptor subfamily 0 group B member 2	TR-, RXR-, RAR-interacting protein	(Seol et al., 1996)
NRBF2	Q96F24	Nuclear receptor-binding factor 2 (Comodulator of PPAR and RXR, COPR)	RXRα ligand-dependent corepressor; PPARα-interacting protein.	(Flores et al., 2004)

NRIP1	P48552	Nuclear receptor-interacting protein 1 (receptor interacting protein 140, RIP140)	ERα coactivator. TR, ERβ RAR, AR and GR ligand-dependent corepressor.	(Lee and Wei, 1999; Subramaniam et al., 1999; Wei and Hu, 2004; Carascossa et al., 2006; Docquier et al., 2013; Rosell et al., 2014)
NSD1	Q96L73	Histone-lysine N-methyltransferase	TR-, ER-, RAR- and RXR-interacting protein	(Huang et al., 1998 p. 1)
PAK6	Q9NQU5	Serine/threonine-protein kinase PAK 6	AR corepressor	(Yang et al., 2001 p. 6)
PCAF	Q92831	Histone acetyltransferase KAT2B (P300/CBP-associated factor, PCAF)	Transcriptional coactivator with histone acetyl transferase activity	(Ogryzko et al., 1996)
PELP1	Q8IZL8	Proline-, glutamic acid- and leucine-rich protein 1	ERα coactivator, GR corepressor	(Vadlamudi et al., 2001; Choi et al., 2004)
PIAS2	O75928	Protein inhibitor of activated STAT (Androgen receptor-interacting protein 3, ARIP3)	AR coactivator	(Lin et al., 2004)
PNRC1	Q12796	Proline-rich nuclear receptor coactivator 1	TR, PR and ERRα1 coactivator; AR-, GR-, RAR-, and RXR-interacting protein	(Zhou et al., 2000)
PNRC2	Q9NPJ4	Proline-rich nuclear receptor coactivator 2	ERα and ERRα1 coactivator. TR-, ER-, GR-, PR-, RAR- and RXR-interacting protein	(Zhou and Chen, 2001)
PPRC1	Q5VV67	Peroxisome proliferator-activated receptor gamma coactivator-related protein 1	Coactivator of Nuclear Respiratory Factor 1-Dependent Transcription	(Andersson and Scarpulla, 2001)
PR285	Q9BYK8	Peroxisomal proliferator-activated receptor A-interacting complex 285kDa protein	TRα, TRβ, PPARγ, PPARα, and RXRα coactivator	(Tomaru et al., 2006)
PRDM2	Q13029	PR domain zinc finger protein 2	ER-interacting protein	(Abbondanza et al., 2000)
PRGC1	Q9UBK2	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	TRβ, ER, GR, RXRα and PPARγ coactivator	(Puigserver et al., 1999; Knutti et al., 2000; Delerive et al., 2002; Wu et al., 2002)
PRGC2	Q86YN6	Peroxisome proliferator-activated receptor gamma coactivator 1-beta	TRβ, ERα, PPARα, and PPARγ coactivator	(Meirhaeghe et al., 2003)
PRGR	P06401	Progesterone receptor	Transcription factor	(Jacobsen and Horwitz, 2012)
PROX1	Q92786	Prospero homeobox protein 1	LRH-1 and ROR corepressor	(Qin et al., 2004; Takeda and Jetten, 2013 p. 1)

Protein	Uniprot Accession	Full name (alias)	Function/structure	Reference
RAD9A	Q99638	Cell cycle checkpoint control protein (RAD9A)	HDAC1-interacting protein	(Cai et al., 2000)
RBL2	Q08999	Retinoblastoma-like protein 2	ER-β-interacting protein	(Macaluso et al., 2006)
TF65	Q04206	Transcription factor p65 (TF65, Nuclear factor NF-kappa-B p65 subunit)	Transcription factor	(Ruben et al., 1992)
TGFI1	O43294	Transforming growth factor beta-1-induced transcript 1 protein	AR coactivator	(Fujimoto et al., 1999)
TIF1A	O15164	Transcription intermediary factor 1-alpha (Tripartite motif-containing protein 24, TRIM24)	AR coactivator; VDR-, TRα-, RXRα-, and RARα-interacting protein	(Thénot et al., 1997 p. 1; Kikuchi et al., 2009)
TIP60	Q92993	Histone acetyltransferase KAT5	TR, AR and ERα coactivator; ERβ coregulator	(Miyajima et al., 2008; Govindan et al., 2009; Jeong et al., 2011; Lee et al., 2013)
TREF1	Q96PN7	Transcriptional-regulating factor 1	CBP/p300-interacting protein	(Gizard et al., 2001)
TRIP4	Q15650	Activating signal cointegrator 1 (Thyroid receptor-interacting protein 4, TRIP-4)	TRβ, ERα coactivator	(Kim et al., 1999)
TRRAP	Q9Y4A5	Transformation/transcription domain-associated protein (TRRAP)	LXR and FXR coactivator	(Unno et al., 2005)
TRXR1	Q16881	Thioredoxin reductase 1	ER coregulator	(Damdimopoulos et al., 2004)
UBE3A	Q05086	Ubiquitin-protein ligase E3A	TR, ER, AR, RARα, PR and GR coactivator	(Nawaz et al., 1999)
WIPI1	Q5MNZ9	WD repeat domain phosphoinositide-interacting protein 1	AR-, ER-, RAR-, RXR-interacting protein	(Proikas-Cezanne et al., 2004)
ZNHI3	Q15649	Zinc finger HIT domain-containing protein 3 (Thyroid receptor-interacting protein 3, TRIP-3)	TR-interacting protein	(Lee et al., 1995)
ZNT9	Q6PML9	Zinc transporter 9	GRIP1-dependent nuclear receptor coactivator	(Chen et al., 2005)

Dose response curves for the modulation of the coregulator interactions induced by the different model compounds for both TRα and TRβ are presented as supplementary Fig. 1 - 8. This evaluation involved fitting of a logistic dose-response model and a goodness of fit of 0.9 or higher was used as a cut-off for coregulators to be considered to be modulated in a dose-dependent manner by the test compounds.

The antagonist compound 1-850 dose-dependently modulates 11 out of 154 TRα-LBD-coregulator interactions and 10 out of 154 TRβ-LBD-coregulator interactions, 4 of which are modulated for both TRs, namely the interactions with ANDR, EP300, NCOR1 and NELFB (Supplementary Fig. 1 and 5). Amiodarone on the other hand dose-dependently modulates 21 out of 154 TRα-LBD-coregulator interactions and 22 of the 154 TRβ-LBD-coregulator interactions with 5 coregulator interactions being modulated for both TRs, namely ANDR, MAPE and NRIP (motif # 173_195 and 173_195_C177S) (Supplementary Fig. 2 and 6). T3 dose-dependently modulates 49 of the 154 TRα-LBD-coregulator interactions and 28 out of 154 TRβ-LBD-coregulator interactions, with 11 coregulator interactions being modulated for both TRs (Supplementary Fig. 3 and 7), while T4 dose-dependently modulates 43 of the 154 TRα-LBD-coregulator interactions and 81 of the 154 TRβ-LBD-coregulator interactions, 32 of which are modulated by both TRs (Supplementary Fig. 4 and 8).

The dose response results revealed that for certain TR-coregulator interations, dose response curves did not fit the goodness of fit criteria that were set since T3 and T4 produced a saturated signal within the greater part of the concentration range tested. Therefore, modulation index (MI) values were calculated as complementary analysis to the dose response analysis. MI values were calculated as the ratio of the binding (fluorescence) of the receptor LBD to the coregulator in the presence of the test compound to the binding observed for the solvent control. DMSO solvent controls and the highest two (ligand-saturated) concentrations of the test compounds were used to calculate the MI patterns displaying the effect on all coregulators tested in alphanumerical order as shown in Fig. 1-3. From the MI results presented it can be derived that the MI patterns for T3 and T4 partly overlap (Fig. 1). For TRα, T4 recruits most coregulators to a lower extent than T3 but both thyroid hormones inhibit the binding of this receptor isoform to corepressors NCOR1 and NCOR2 to the same extent (Fig. 1a).The opposite is true for TRβ since the only marked difference between T3- and T4-mediated effects on TRβ-LBD-coregulator interactions is in their inhibition of NCOR1 and NCOR2 binding to the receptor, with reduced binding in the case of T3 but a strikingly lower level of modulation by T4 (Fig. 1b). Fig. 2 presents a comparison of the binding of T3 (Fig. 2a) and T4 (Fig. 2b) for both receptors. This comparison reveals that the TR-LBD-coregulator interactions affected follow patterns that are comparable for both receptor isoforms tested. T4 is

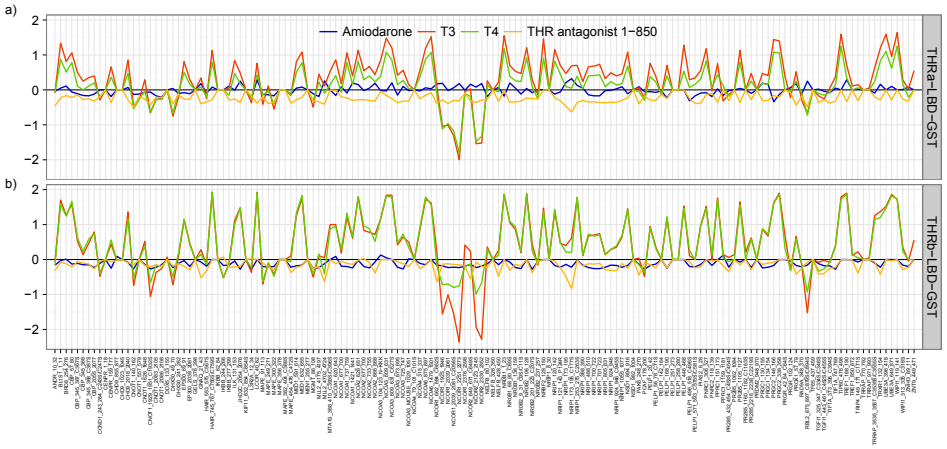

Fig. 1: T3, T4, amiodarone and compound 1-850 modulation of coregulator binding to TRα (upper panel) and TRβ (lower panel) relative to the solvent control (DMSO), expressed as MI values. The 154 coregulator motifs present on the pamchip are represented on the horizontal axis in alphanumeric order from left to right. Detailed background information on the coregulators and their function can be found in Tab. 1.

more active in recruiting coregulators to TRβ than to TRα and inhibits the binding of corepressors NCOR1 and NCOR2 to TRβ less than it does with TRα. The differences between TRα and TRβ with respect to T3-mediated coregulator recruitment are less pronounced (Fig. 2a).

The antagonists amiodarone and compound 1-850 modulated LBD-coregulator interactions in both a compound-specific and receptor-specific manner (Fig. 1a and Fig.1b) with a level of modulation that was much lower than for the agonists T3 and T4. Compound 1-850 only inhibited the interaction with the tested coregulators for both receptor isoforms, whereas amiodarone showed both stimulatory and inhibitory modulation, including stimulation for TRα and inhibition for TRβ of NCOR1 and NCOR2 corepressor binding, pointing to a different mode of action of the two antagonists tested.

In order to find out whether agonists and antagonists could be clustered separately, a heat map visualising hierarchical clustering of the coregulator binding profile versus the test compound was created for both TRα-LBD (Fig. 3a) and TRβ-LBD (Fig. 3b). Hierarchical clustering of the observed binding profiles showed not only that receptor agonists and antagonists clustered separately but also that both TR isoforms have different patterns of modulation.

A closer look at the coregulators that showed the most significantly increased recruitment by both T3 and T4 in both TR isoforms underscores the physiological relevance of the results obtained. CREB-binding protein (CBP), mediator of RNA polymerase II

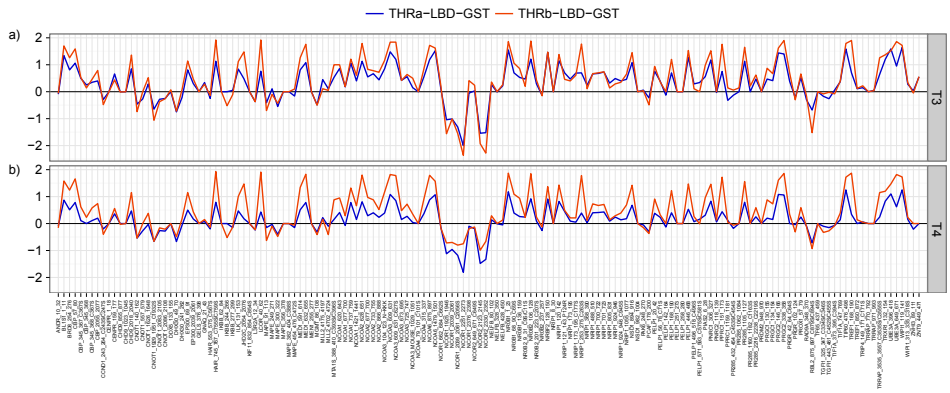

Fig. 2: Comparison of TRα and TRβ coregulator MI profiles for a) T3 and b) T4.

transcription subunit 1 (MED1) and nuclear receptor coactivators (NCoAs) 1, 2 and 3, which are the principal thyroid hormone coactivators (Ito and Roeder, 2001) were among the coactivators whose binding to both receptor isoforms is significantly modulated by T3 and T4 (Fig. 3). The other coregulators whose binding to both receptor isoforms was significantly modulated by T3 and T4 were histone acetyltransferase HTATIP (TIP60), protein hairless (HAIR), ligand-dependent corepressor (LCOR), peroxisome proliferator-activated receptor gamma coactivator 1-beta (PRGC-2), nuclear receptor-interacting protein 1 (NRIP1), proline-rich nuclear receptor coactivator 1 (PNRC1), nuclear receptor subfamily 0 group B member 1 (NR0B1), bromodomain-containing protein 8 (BRD8), mediator of RNA polymerase II transcription subunit 1 (MED1), proline-, glutamic acid- and leucine-rich protein 1 (PELP1) and protein inhibitor of activated STAT (PIAS2). Binding of NRIP1 and LCOR was found to be modulated by T3 and T4 in a dose-dependent manner (Fig. 4). Some coregulators appeared to be recruited by one of the two TRs upon binding to either T3 or T4, for example biogenesis of lysosome-related organelles complex-1 subunit 1 (BL1S1) binding to TRα is significantly modulated by T4 and WD repeat domain phosphoinositide-interacting protein 1 (WIPI1) binding to TRβ and TRα is significantly modulated by T3 and T4, respectively.

Among the coregulators that showed decreased recruitment by both T3 and T4 in both TR isoforms were nuclear receptor corepressor 1 (NCOR1) and its paralog nuclear receptor corepressor 2 (NCOR2). These are known to be the canonical nuclear receptor corepressors associated with the transcriptional silencing of thyroid hormone receptors in the absence of a ligand. NCOR2 is also known by the alias silencing mediator for retinoid and thyroid hormone receptors (SMRT) (Moore and Guy, 2005). NCOR1 and NCOR2 act as scaffold proteins that recruit histone deacetylase complexes (HDACs) (Ito

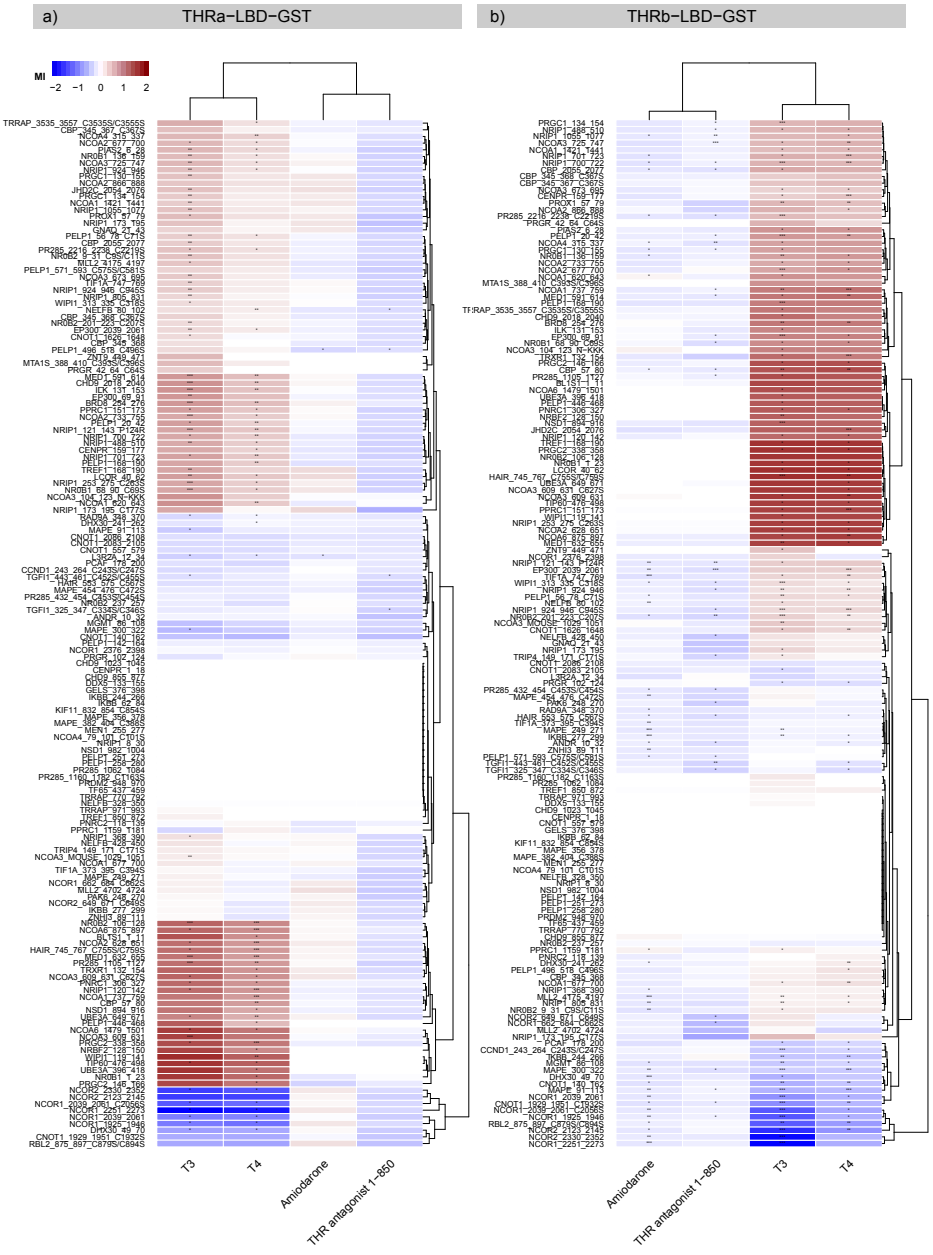

Fig. 3: A heat map for TRα-LBD a) and TRβ-LBD b) MI values and hierarchic clustering of the data showing that agonists and antagonists cluster separately. The significance level of the observed MI values based on Student's t-test are indicated (*P≤0.05; **P≤0.01; ***P≤0.001).

and Roeder, 2001). It can be seen from Fig. 3 that the endogenous ligands T3 and T4 significantly reduce the binding of TRα and TRβ to the majority of NCOR1 and NCOR2 binding motifs tested an observation that is in line with activation of TR mediated gene expression by T3 and T4. As shown in Fig. 5 this inhibition occurred in a dose-dependent fashion. Other coregulators for which the binding of certain motifs to TR was significantly inhibited by T3 and T4 included DHX30, retinoblastoma-like protein 2 (RBL2) and CCR4-NOT transcription complex subunit 1 (CNOT1) (Fig. 3). The inhibition of binding of DHX30 and RBL2 (the latter inhibited only in the case of TRβ) was found to occur in a dose-dependent manner (Fig. 6).

The CNOT1 motifs tested displayed divergent results, CNOT1 motifs # 1929_1951, 140_162 and 2083_2105 showing a significant decrease in TRβ binding upon exposure to T3 with the prior 2 motifs exhibiting a similar interaction upon exposure to T4 (Fig. 3). On the other hand, motif # 1626_1648 showed a significant increase in TRα binding with the addition of T3 and a significant increase in TRβ binding with the addition of T3 and T4.

4 Discussion

Due to the complex nature of the thyroid hormone system, an in vitro alternative for current animal tests for the detection of thyroid-active compounds must include a mechanism-based battery of tests (Murk et al., 2013; Jomaa, 2014). This battery can include both kinetics and dynamics-based tests, the latter of which could be focused on the transcriptional effect of thyroid hormones at the cellular level. Uncovering the role of specific coregulator-nuclear receptor interactions in mediating transcription has enabled the use of MARCoNI peptide microarray-based assays as a substitute for reporter gene assays. The advantages conferred by MARCoNI assays include the use of a cell free system which eliminates interference caused by other receptors and reporter-related artifacts (Sotoca et al., 2010; Bhattacharjee and Khurana, 2014).

Peptide microarrays are an up and coming technology that has been successfully used in detecting protein kinase activity in drug screening, epitope mapping of food allergens, defining patient antibody signatures and most recently in MARCoNI assays detecting the modulation of nuclear receptor-coregulator binding by endocrine-active compounds (Shigaki et al., 2007; Quintana et al., 2008; Lin et al., 2009; Aarts et al., 2013; Wang et al., 2013; Evers et al., 2014). Here we report the first application of a high-throughput peptide microarray to test the interaction of TRα and TRβ with 154 coregulator motifs as well as the modulation of this interaction by the thyroid hormones T3 and T4 in addition to the two known TR antagonists amiodarone and 1-850 (Drvota et al., 1995; Schapira et al., 2003).

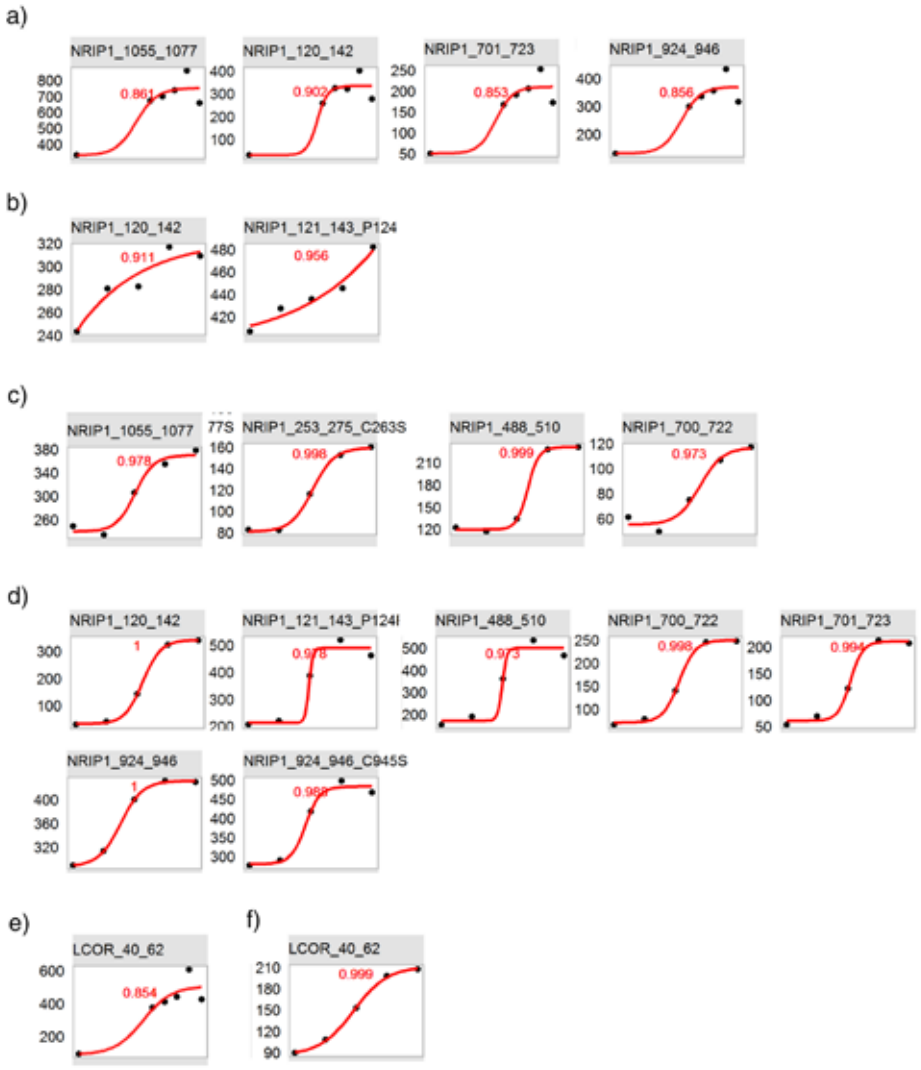

Fig. 4: Concentration response curves for the T3-mediated inhibition of the binding of NRIP1 to a) TRα and b) TRβ and of the T4-mediated inhibition of NRIP1 for c) TRα and d) TRβ; T3-mediated e) and T4-mediated f) inhibition of binding of LCOR binding to TRα. The decimal number in red in each plot indicates the Pearson correlation coefficient for goodness of fit of a logistic dose-response model.

In addition to the improved speed and scalability, microarrays generate relatively large data sets that can be used to perform meta-analyses and thus reveal toxicity profiles that would otherwise not be readily available. This became evident when test compound modulation indices (MIs) were compared among the different ligands and hierarchic

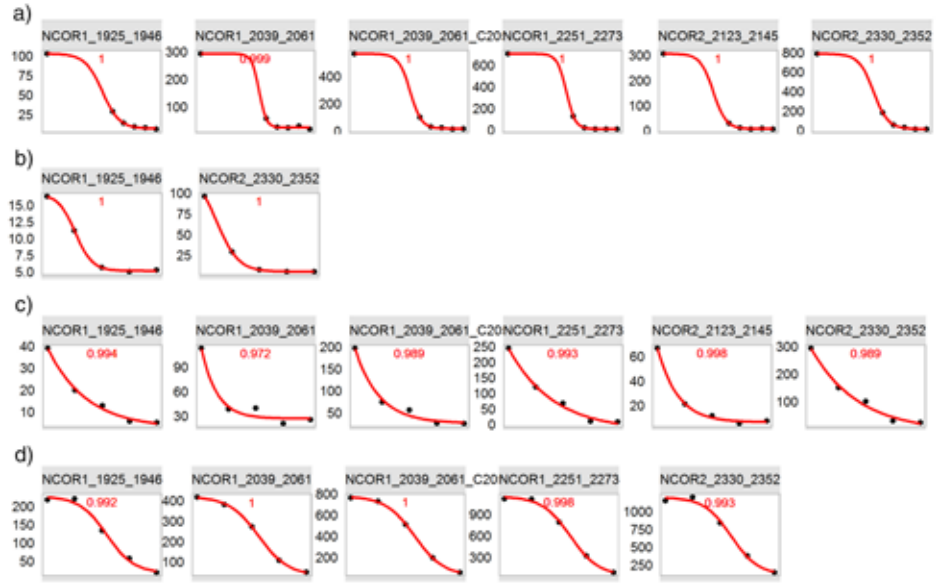

Fig. 5: Concentration-response curves for the T3-mediated inhibition of the binding of N-CoR1 and N-CoR2 to a) TRα and b) TRβ and the T4 mediated inhibition of the binding of N-CoR1 and N-CoR2 to c) TRα and d) TRβ. The decimal number in red in each plot indicates the Pearson correlation coefficient for goodness of fit of a logistic dose-response model.

clustering of the data revealed that agonists T3 and T4 clustered separately from the antagonists amiodarone and compound 1-850. Results presented in this paper indicate that thyroid receptor antagonists amiodarone and compound 1-850 display a different pattern of modulation of TR-coactivator interactions. In addition both antagonists tested showed a trend to inhibit or significantly inhibited the binding of TRβ to the tested motifs of corepressors NCOR1 and NCOR2 (Fig. 3). However, in view of their TR antagonist properties, this supposedly transactivation-stimulating effect is apparently outweighed by the overall inhibitory effects on coactivator binding.

With two thyroid hormone receptor isoforms and 66 individual coregulators represented by a total of 154 motifs, the resolution provided by the MARCoNI assay used in this paper has provided a detailed interaction signature that not only helped discriminate between agonists and antagonists but also revealed differences between individual agonists and antagonists. Importantly, this can help explain the differing physiological effects of some thyroid-active compounds in vivo (Stoykov et al., 2007).

The results of the current study have revealed coregulators whose interaction with both TRs is significantly modulated by both thyroid hormones T3 and T4. These included coregulators known to interact with TRs including TIP60, HAIR, PRGC-2, NRIP, PNRC1,

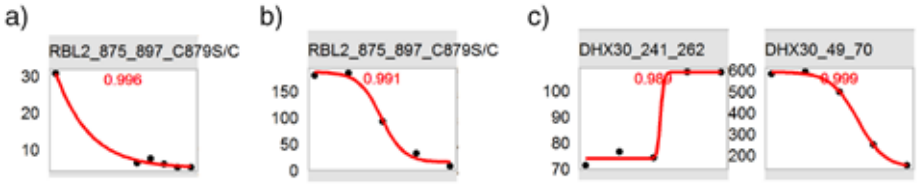

Fig. 6: Concentration response curves for the T3 and T4-mediated inhibition of the binding of RBL2 to a) TRα and b) TRβ and of the T4-mediated inhibition of DHX30 for c) TRβ. The decimal number in red in each plot indicates the Pearson correlation coefficient for goodness of fit of a logistic dose-response model.

BRD8 and MED1 (see Tab. 1 for references) as well as coregulators that have not yet been show to interact with TRs including NROB1, PELP1 and PIAS2 and LCOR.

While NROB2 has been shown to interact with TR, little is known of the interaction of NROB1 with TRs (Seol et al., 1996). However, in research conducted by Ito et al. (1997), NROB1 was found not to repress but to activate the T3-mediated transcriptional activation by TRα and TRβ, but this coactivator effect was just presented and not discussed (Ito et al., 1997). PELP1 is involved in DNA and histone modification and interacts with several steroid receptors including the estrogen receptor (ER), androgen receptor (AR), glucocorticoid receptor (GR), progesterone receptor (PR) as well as the vitamin D3 and mineralocorticoid (MR) receptors (Gonugunta et al., 2014). PIAS2 is an inhibitor of signal transducer and activator of transcription (STAT) and a coactivator of AR (Lin et al., 2004). LCOR is discussed later in the text along with other corepressors based on its established role as a corepressor of other nuclear receptors (Tab. 1).

MI values obtained in this study have also indicated that biogenesis of lysosome-related organelles complex-1 subunit 1 (BL1S1) binding to TRα is significantly modulated by T4 and WD repeat domain phosphoinositide-interacting protein 1 (WIPI1) motif # 313_335 binding to TRβ and TRα is significantly modulated by T3. BL1S1 and WIPI1 are known coactivators of other nuclear receptors (Mishra et al., 2003; Proikas-Cezanne et al., 2004) but have not yet been reported to interact with TR.

T3 and T4 also modulated the binding of TR corepressors. T3 and T4 inhibited the binding of TRα and TRβ, respectively, to RBL2 in a dose dependent manner with a goodness of fit of 1.0 (Fig. 6 a and b). In the case of TRβ, this inhibition was found to also be of statistical significance (Fig. 3). RBL2, also known as p130, is a transcriptional repressor and a key regulator of cell cycle progression that has not yet been associated to TR-mediated transcriptional silencing. The decreased interaction between TRs and RBL2 upon exposure to thyroid hormones is likely of physiological relevance as decreased expression of RBL2 has been associated with anaplastic thyroid cancer (Ito

et al., 2003). DHX30, also known as Ret-COR, interacts with the orphan nuclear receptor NR2E3 and is speculated to be a corepressor of that receptor (Takezawa et al., 2007; Qin et al., 2013). Here we have found DHX30 to interact with TRα and TRβ and this interaction shows significant inhibition by the endogenous ligands T3 and T4 in a way that is similar to the cognate corepressors (Fig. 3 and 5c).

Ligand-dependent corepressors are a separate class of corepressors that inhibit the transcriptional activation in the presence of a ligand. These includes nuclear receptor-interacting protein 1 (NRIP1) and ligand-dependent corepressor (LCOR) (Leonardsson et al., 2004). While NRIP has previously been associated as a TH-dependent corepressor of TR (Wei and Hu, 2004), here we confirm this interaction (Fig. 3 and Fig. 4a for T3 TRα, 4b for T3 TRβ, 4c for T4 TRα and 4d for T4 TRβ) and report for the first time that T3 and T4 are capable of significantly stimulating LCOR-TR binding in the MARCoNI assay (Fig. 3) and in a dose dependent manner for TRα (Fig. 6e for T3 and 6f for T4), indicating that LCOR is likely to act as a TH-dependent corepressor.

Overall, the results have shown that the MARCoNI assay can be used to discriminate between thyroid hormone receptor agonists and antagonists and initial results indicate that it holds promise for inclusion as a screen within an integrated testing strategy for the detection of thyroid-active compounds. Moreover, this study has shed light on new TR-interacting coregulators, namely, candidate coactivators BL1S1 and WIPI1, candidate corepressors RBL2 and DHX30 as well as candidate ligand-mediated corepressor LCOR.

Acknowledgements

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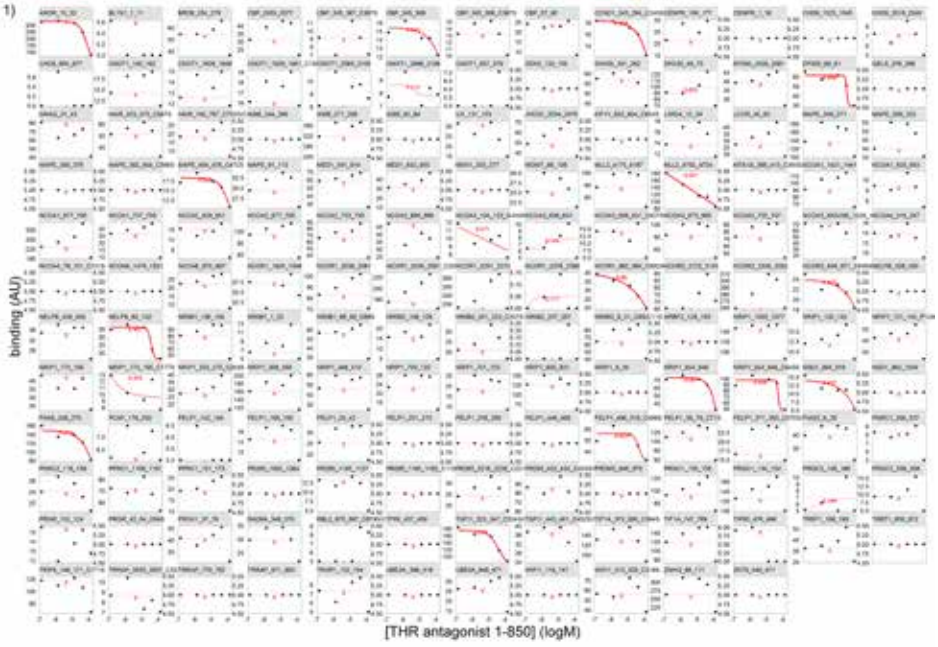
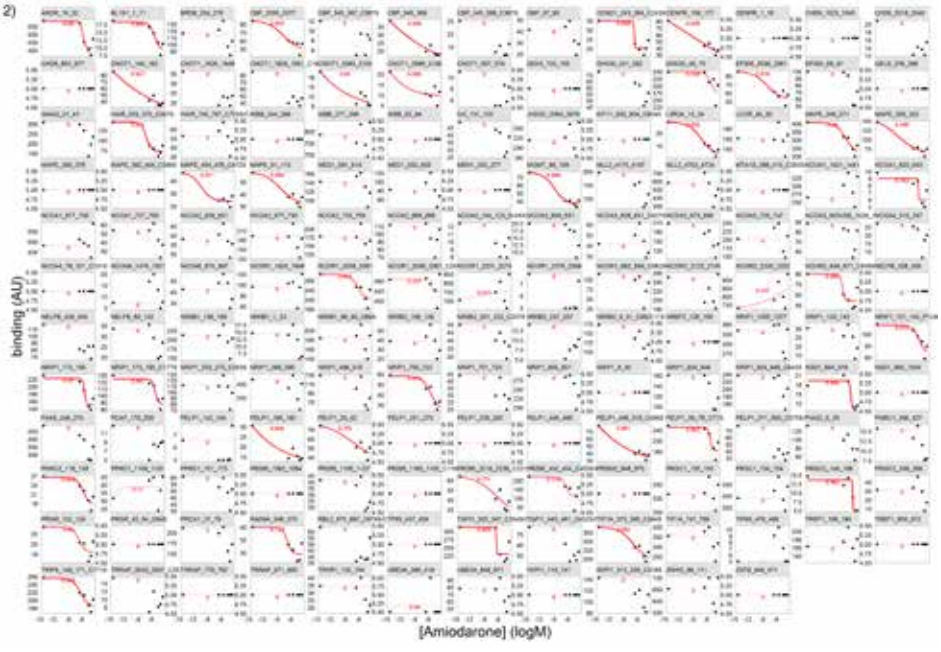
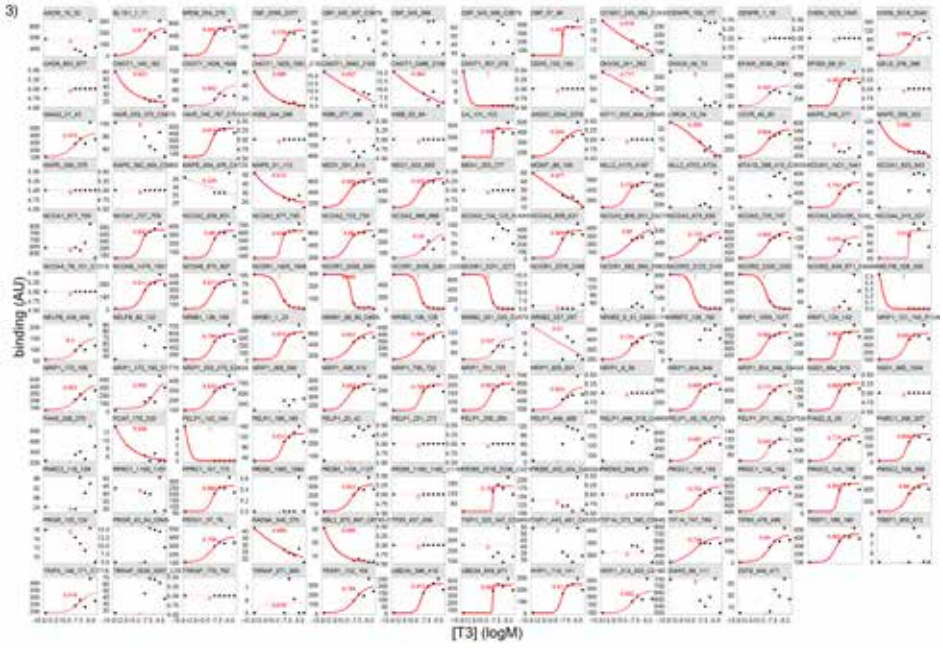
Supplementary materials



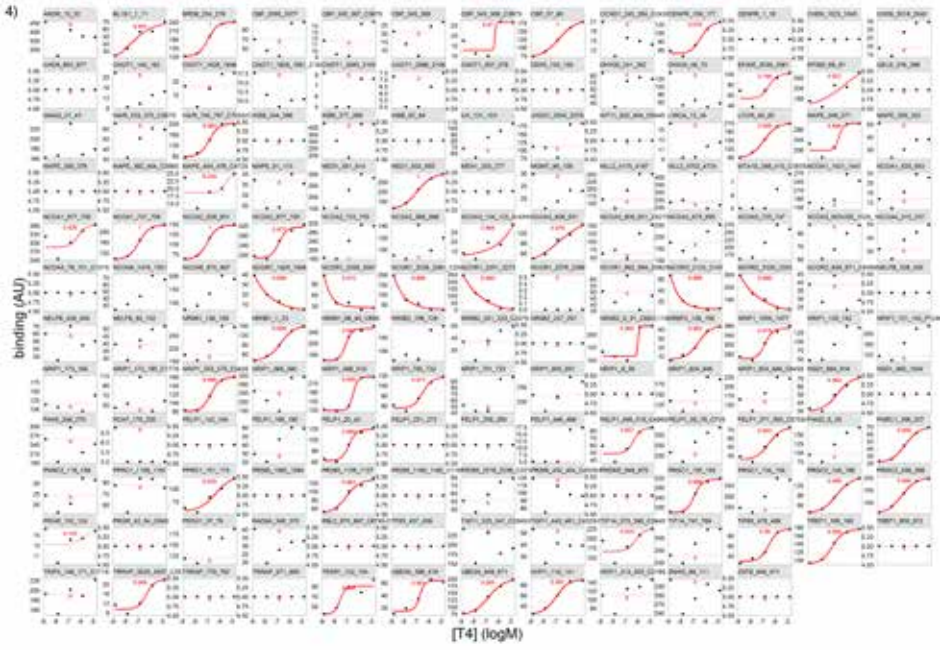
Supplementary Fig. 1: Concentration response curves for compound 1-850-mediated modulation of coregulator-TRα binding. The decimal number in red in each plot indicates the Pearson correlation coefficient for goodness of fit of a logistic dose-response model.



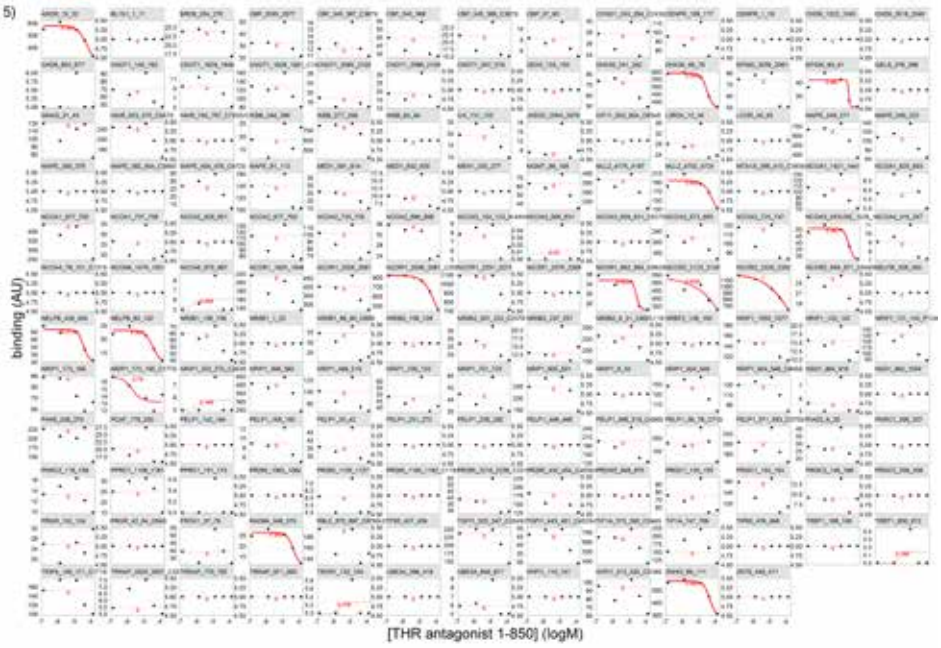
Supplementary Fig. 2: Concentration response curves for amiodarone-mediated modulation of coregulator-TRα binding. The decimal number in red in each plot indicates the Pearson correlation coefficient for goodness of fit of a logistic dose-response model.



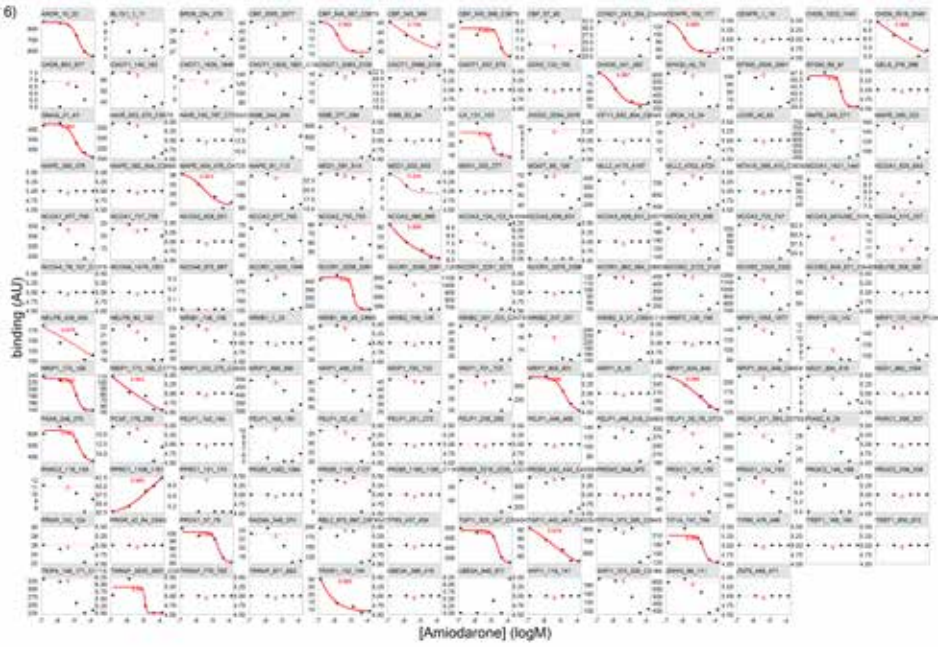
Supplementary Fig. 3: Concentration response curves for T3-mediated modulation of coregulator-TRα binding. The decimal number in red in each plot indicates the Pearson correlation coefficient for goodness of fit of a logistic dose-response model.



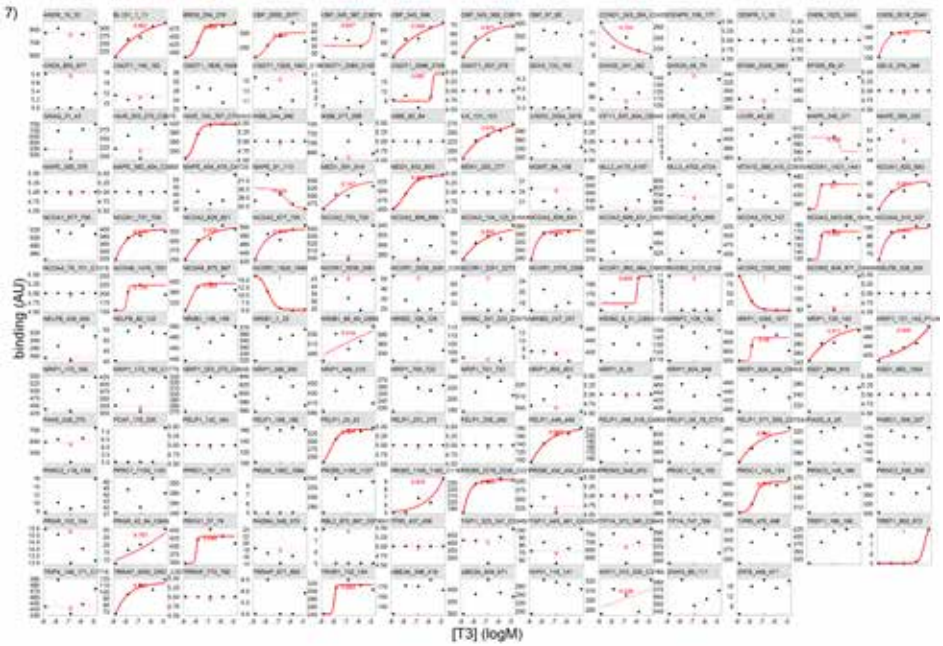
Supplementary Fig. 4: Concentration response curves for T4-mediated modulation of coregulator-TRα binding. The decimal number in red in each plot indicates the Pearson correlation coefficient for goodness of fit of a logistic dose-response model.



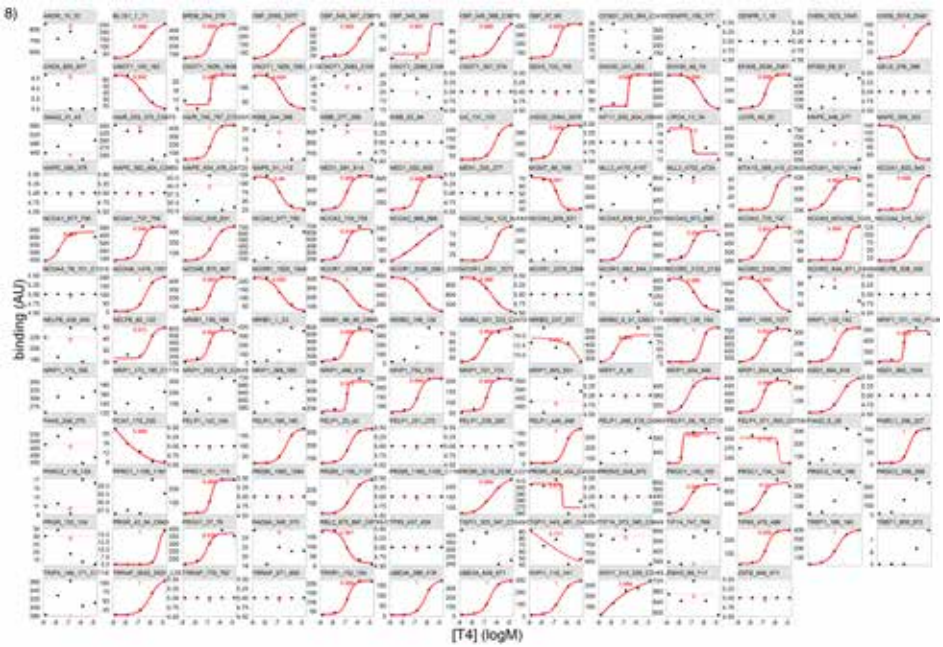
Supplementary Fig. 5: Concentration response curves for compound 1850-mediated modulation of coregulator-TRβ binding. The decimal number in red in each plot indicates the Pearson correlation coefficient for goodness of fit of a logistic dose-response model.



Supplementary Fig. 6: Concentration response curves for compound amiodarone-mediated modulation of coregulator-TRβ binding. The decimal number in red in each plot indicates the Pearson correlation coefficient for goodness of fit of a logistic dose-response model.



Supplementary Fig. 7: Concentration response curves for compound T3-mediated modulation of coregulator-TRβ binding. The decimal number in red in each plot indicates the Pearson correlation coefficient for goodness of fit of a logistic dose-response model.



Supplementary Fig. 8: Concentration response curves for compound T4-mediated modulation of coregulator-TRβ binding. The decimal number in red in each plot indicates the Pearson correlation coefficient for goodness of fit of a logistic dose-response model.

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Chapter 5

Developmental toxicity of thyroid-active compounds in a zebrafish embryotoxicity test

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Summary

Zebrafish embryos were exposed to concentration ranges of selected thyroid-active model compounds in order to assess the applicability of zebrafish-based developmental scoring systems within an alternative testing strategy to detect the developmental toxicity of thyroid-active compounds. Model compounds tested included triiodothyronine (T3), propylthiouracil (PTU), methimazole (MMI), sodium perchlorate ($NaClO_4$) and amiodarone hydrochloride (AMI), selected to represent different modes of action affecting thyroid activity. Tested time windows included 48-120 hours post fertilization (hpf), 0-72 hpf and 0-120 hpf. All tested compounds resulted in developmental changes, with T3 being the most potent. The developmental parameters affected included reflective iridophores, beat and glide swimming, inflated swim bladders, as well as resorbed yolk sacs. These effects are only evident by 120 hpf and therefore an existing General Morphology Score (GMS) system was extended to create a General Developmental Score (GDS) that extends beyond the 72 hpf scoring limit of GMS and includes additional parameters that are affected by exposure to model thyroid-active compounds. Moreover, the GDS is cumulative as it includes not only the scoring of developmental morphologies but also integrates developmental dysmorphologies. Exposures from 48-120 hpf did not provide additional information to exposures from 0-120 hpf. The results indicate that the zebrafish GDS can detect the developmental toxicity of thyroid toxicants and may be of use in an integrated testing strategy to reduce, refine and, in certain cases, replace animal testing.

1 Introduction

The EU Regulation on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) requires all substances marketed or manufactured in quantities above 10 tons per annum to be tested for developmental toxicity (OECD 421 or OECD 422). Substances that are marketed or manufactured in quantities above 100 tons per annum are required to be tested for pre-natal developmental toxicity (OECD 414) (REACH, 2006). Based on data on chemical production from 1991 to 1994, the EU estimates that 2.6 million animals will be needed for all REACH testing or a total of 9 million when offspring produced during reproductive and developmental toxicity testing are taken into account. While some studies have suggested much higher numbers, the EU is sticking to its initial predictions (ECHA, 2009; Hartung and Rovida, 2009; van der Jagt et al., 2004; Pedersen et al., 2003). The issues associated with these tests include ethical acceptability considerations, high costs and long duration. This has prompted research into the development of suitable high-throughput alternative tests that, once validated, could reduce animal testing within an integrated testing strategy (ITS).

In a previous study we investigated to what extent thyroid (TSH-screen) and pituitary (T-screen) cell proliferation assays could be used as in vitro screens for detecting the effects of thyroid hormone-disrupting compounds on thyroid and pituitary weights used as endpoints in in vivo assays (Jomaa et al., 2013). While the effect of estrogen-mimicking compounds on cell proliferation in the in vitro E-screen was found to correlate with their effects on in vivo uterine weight (r^2=0.85) (Wang et al., 2012), the effects of thyroid-active compounds on thyroid (TSHscreen) and pituitary (T-screen) cell proliferation was found to have poor correlation with their effects on the weight of these respective organs in vivo. The results of the study indicated that a complex interplay between factors within the hypothalamuspituitary-thyroid (HPT) axis may underlie the effects of thyroidactive compounds on thyroid and pituitary organ weight endpoints in vivo. Therefore, it was proposed that the development of future alternative tests, aiming at the refinement, reduction, or replacement of animal studies, should include as an initial screen a broad battery of in vitro tests that cover the various modes of action of thyroid-active compounds and should in addition be based on more complex model systems that better integrate the various processes involved in the HPT axis. The vertebrate *Danio rerio* (zebrafish) may represent such a complex model system that includes an intact HPT axis. Given that alterations in the thyroid system can have severe effects on development (Boyages and Halpern, 1993; Haddow et al., 1999; Utiger, 1999), the aim of the present study was to investigate whether a zebrafish-based developmental toxicity assay would be able to detect thyroid hormone-active compounds.

Hermsen et al. (2011) developed a zebrafish-based quantitative scoring system for developmental and teratogenic endpoints, with the former called the general morphology score (GMS). The developmental endpoints include abnormalities related to the completion of gastrulation, formation of somites, development of the eyes, spontaneous movement, blood circulation, pigmentation, edemata, malformations of the chorda structure, spinal cord (scoliosis, rachitis), head, sacculi/otoliths, tail, heart, yolk sac, growth retardation, and tail length (Nagel, 2002). The GMS is meant to be semi-quantitative and hence more efficient than more complex scoring systems while maintaining the same relevance in terms of output (Hermsen et al., 2011). The GMS scoring system uses the 0-72 hpf time window and was validated using eight glycol ethers and six 1,2,4-triazole antifungals (Hermsen et al., 2011). While alternative methods for developmental toxicity testing are unlikely to replace testing on rodents, they can potentially be used in pre-screening in order to reduce the need for consecutive in vivo testing (Piersma, 2006).

In order to investigate whether a zebrafish-based developmental toxicity assay would be able to detect thyroid hormoneactive compounds, the effects in the zebrafish-based bioassay of a series of model thyroid-active compounds representing different modes of thyroid action in vivo (Tab. 1) was investigated. The selected compounds were triiodothyronine (T3), sodium perchlorate ($NaClO_4$), propylthiouracil (PTU), methimazole (MMI) and amiodarone (AMI). All selected compounds are known to affect thyroid hormone levels in vivo albeit by different modes of action (Grover et al., 2007; Hood et al., 1999; de Sandro et al., 1991; Stoker et al., 2006; Yamasaki et al., 2002).

2 Materials and methods

Compounds

All compounds were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands) and were of high purity (≥95%). All test chemicals were added from 500-fold concentrated stock solutions in dimethylsulfoxide (DMSO, Acros Organics, Geel, Belgium) to Dutch Standard Water (DSW; demineralised water supplemented with $NaHCO_3$ (100 mg/l), $CaCl_2 \cdot H_2O$ (200 mg/l), $KHCO_3$ (20 mg/l), and $MgSO_4 \cdot 7H_2O$ (180 mg/l)). The exposure medium was then aerated for 24 h at 27 °C, the pH was adjusted to 7.4-8.4 and O_2 concentration was above 6.5 mg/l. The concentration range that was selected for the model compounds was based on reported effective concentrations for inducing reduction in T4 levels in zebrafish (Raldúa and Babin, 2009).

Tab. 1: List of compounds used in this study with their main mode of action on the thyroid hormone system based on rodent studies, and concentration ranges used in the experiments. logP was predicted based on structure using chemaxon software.

Compound	Abbreviation	CAS No.	Description	logP	Mode of Action	Concentration Range (µM)
Triiodothyronine	T3	6893-02-3	Endogenous ligand	2.80	Thyroid hormone receptor agonist	0.00005 – 0.5
Propylthiouracil	PTU	51-52-5	Antithyroid drug	1.2	Inhibits thyroid peroxidase and deiodinase type 1	25 - 400
Methimazole	MMI	60-56-0	Antithyroid drug	0.75	Inhibits thyroid peroxidase	125 - 2,000
Sodium perchlorate monohydrate	$NaClO_4 \cdot H_2O$	7791-07-3	Environmental contaminant	-0.10	Inhibits iodide uptake	25 - 400
Amiodarone hydrochloride	AMI	19774-82-4	Antiarrhythmic drug	7.64	Increases iodine load	0.001 - 10

Zebrafish embryos

Zebrafish, obtained commercially (Ruinemans Aquarium BV, Montfoort, The Netherlands), were maintained and bred at RIVM (Bilthoven, The Netherlands) for more than five years. Their daily diet consisted of two servings of dry flakes (Special Diet Services, Tecnilab-BMI BV, The Netherlands) and one serving of Artemia (Landman BV, The Netherlands). Constant filtering and permanent water flow-through kept contaminants to a minimum. Male and female adult zebrafish free from externally visible diseases were housed together in 7.5 l ZebTEC aquaria at 27 °C ±1 °C with a light/dark (LD) cycle of 14-hour light/10-hour dark. Three days prior to mating, males and females were placed in spawning chambers within separate tanks. Mating involved placing two males with two females in the spawning chambers with reduced water flow and keeping them in the dark overnight after which turning on the lights induced mating, spawning and fertilization within 30 minutes. The spawning chambers have a perforated bottom surface that allows the eggs to fall through, thus preventing the predation of eggs by adult zebrafish.

Exposure

Spawned eggs were rinsed in DSW and unfertilized eggs, less than 10% of total, were removed. Embryos were selected at the 4 to 32-cell stage and one embryo was added per well of a 24-well plate containing 2 ml of test medium per well. Alternatively, the transfer to the 24-well plates containing 2 ml of the test medium was performed at

48 hpf. The 24-well plates were kept in an incubator at 26.5 °C ±1 °C with a light/dark (LD) cycle of 14-hour light/10-hour dark. Exposure was performed at the start of the light cycle.

TSH-screen

FRTL-5 cells at 70% confluence and below passage 30 were incubated in serum-free, phenol red-free DMEM containing 0.2% BSA for a period of 96 h. The cells were then harvested and plated at a density of 5,000 cells/well on a 96-well plate in phenol red-free DMEM with insulin (10 µg/ml) and, following an attachment period of 2 to 3 h, exposed to a concentration range of the chemicals to be tested alone or in combination with 1 mIU/ml bTSH, in triplicate, for 72 h. Following this incubation, cell proliferation was measured 4 h after the addition of 10 µl/well of 0.1 mg/ml resazurin as described above.

General morphology score (GMS)

Scoring of the developmental morphology was performed using a Leica Labovert FS microscope as outlined by Hermsen et al. (2011). Briefly, an incremental binary scoring system was used to assign a 0 to a non-event and a 1 to each normal morphologic developmental event that is easy to visualize under a regular stereo microscope. Sets of developmental events included detachment of tail, somite formation, eye development, movement, heartbeat, blood circulation, pigmentation of the head and body, pigmentation of the tail, pectoral fin development, protruding mouth, and hatching. The subtotals of all sets of developmental events were added towards a general morphology score (GMS), which in normal non-exposed embryos amounts to 15 at the 72 hour time point. In a separate binary scoring system, teratogenicity was scored, whereby normal embryos were assigned a 1 and malformed embryos were given a 0 for every malformation studied. These malformations included pericardial edema, yolk sac edema, eye edema, malformation of the head, absence/malformation of sacculi/otoliths, malformation of tail, malformation of heart, modified chorda structure, scoliosis, rachischisis and yolk deformation. Testing was considered valid when no more than 10% of control embryos were coagulated, underdeveloped or malformed.

Immunohistochemistry

Whole-mount immunohistochemistry of T4 was performed as previously described by Raldúa and Babin (2009), with slight modifications. Briefly, zebrafish embryos at 5 dpf were sacrificed by incubation on ice for 5 min and then fixed overnight in 4% paraformaldehyde at 4 °C. They were then washed repeatedly with PBS, dehydrated

with a methanol row (30%, 60%, 100%) and stored overnight or longer at -20°C. The embryos were then rehydrated with a methanol row (60%, 30%) and washed several times with PBS containing 1% Triton X-100. Permeabilization was achieved by 15 minute incubation at room temperature with 0.1% collagenase in PBS. Immediately after the incubation, the embryos were washed several times with PBS containing 1% Triton X-100 and blocked in blocking buffer containing 4% goat serum, 1% BSA, 1% DMSO, 0.8% Triton X-100 and 0.1% Tween-20 in PBS for 2 h. This was followed by two washes in 1% BSA in PBS and an overnight incubation in a solution consisting of a polyclonal antibody against T4 (MP Biochemicals, Amsterdam, The Netherlands) diluted 1:4,000 in blocking buffer containing 0.02% sodium azide. The following day, the embryos were washed several times in 1% BSA in PBS and incubated on a gentle rotation device for 2 h in a solution containing a 1:300 dilution of the secondary antibody conjugated to the fluorescent label Alexa Fluor 488 (Life Technologies, Breda, The Netherlands). Right after the incubation, the embryos were either stored in glycerol or embedded in 1% gelatine for imaging on an a Zeiss Axiovert 100 M confocal microscope (Zeiss Netherlands bv, Weesp, The Netherlands).

Data analysis

Non-linear curve fitting was done using the Hill equation with the help of GraphPad Prism software version 5.04 (GraphPad Software, San Diego, CA, USA). For the in vivo data that were collected from literature, a dose of 10 ppm MMI, equivalent to 10 mg/kg, was converted to 0.5 mg/kg bw/day based on the assumption that rats consume 5% of their body weight of food each day.

3 Results

3.1 Zebrafish thyroid development within selected exposure period

A limit of exposure was set at 120 hpf (the end of the embryonic phase), which is the regulatory limit to in vitro experimentation as defined by Directive 86/609/EEC (European Council, 1986). To place the exposure regimen into perspective, an analysis of zebrafish, rat and human developmental staging was performed (Fig. 1a). This comparison was based on O'Rahilly and Müller's developmental stages of human embryos, Witschi's development of the rat, and Kimmel's stages of embryonic development of the zebrafish (Kimmel et al., 1995; O'Rahilly and Müller, 1987; Witschi, 1962). Carnegie stage 24 is the last stage of embryonic development and occurs at 33 days for humans, the equivalent of 16 days for rat and 72 h for zebrafish. Additional stages are added as indicated in the figure. The results obtained reveal many parallels between the different

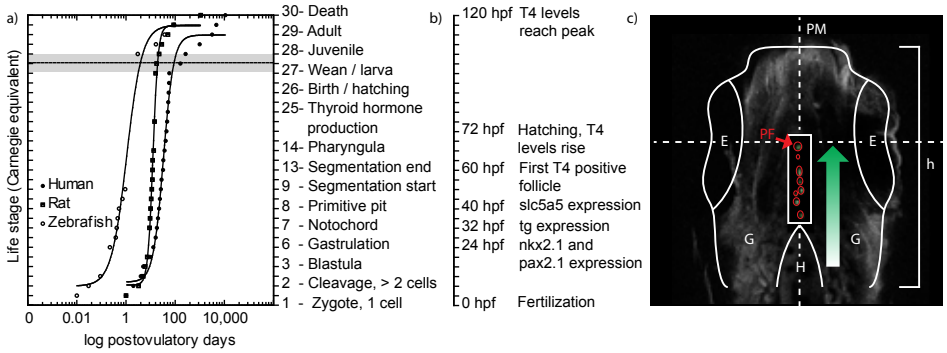

Fig.1: a) Human, rat and zebrafish developmental stages (Carnegie equivalent) plotted against time in postovulatory days. Carnegie stage 24 is the last stage of embryonic development and occurs at 33 days for humans, the equivalent of 16 days for rat and only 72 hours for zebrafish. b) Developmental landmarks of the thyroid hormone system in zebrafish from 0 to 120 hpf., including the expression of the differentiation markers nkx2.1 and pax2.1, expression of thyroglobulin (tg), the sodium iodide symporter (slc5a5), the first follicle that can be detected by T4 IHC, as well as the rise and peak of T4 levels. c) Schematic representation of thyroid follicle development. PF stands for primary follicle (additional follicles are circled in red); H for heart; PM for pharyngeal midline (represented by a dotted line); E for eyes; G for gills; h for head region. The green arrow indicates the direction of PF development from 0 to 120 hpf. In the background, T4 IHC of a 120 hpf zebrafish provides a visual reference.

species. Thyroid hormone production starts after 71 days in man, 20 days in rat and 2.5 days (~ 60 hpf) in zebrafish (Bohnsack et al., 2011; Contempré et al., 1993; Strum et al., 1971).

Figure 1b provides a schematic overview of landmarks associated with the development of the thyroid and the production of T4 in zebrafish from 0 until 120 hpf. Briefly, the zebrafish thyroid gland develops from endodermal cells through a process that is in large part evolutionarily conserved (Wendl et al., 2002). The two critical transcription factors nkx2.1 and pax2.1 (functionally similar to human pax 8) are already expressed at 24 hpf (Antonica et al., 2012; Wendl et al., 2002). A fate mapping study showed that the thyroid precursor cells are located near cardiac lateral plate mesoderm (Wendl et al., 2007). Thyroglobulin gene expression starts around 32 hpf and the expression of the sodium iodide symporter (slc5a5) at 40 hpf. A first follicle is positive for the thyroglobulin protein by 55 hpf and for T4 by 60 hpf and expands anteriorly away from the heart, leaving a trail of thyroid follicles along the pharyngeal midline (Alt et al., 2006; Wendl et al., 2007). By 120 hpf, 6-10 thyroid follicles span along this axis from the gill region until they reach an axis that intersects the middle of the eyes. This is a time of peak T4 production, with the rise starting at around the time of hatching (72 hpf) (Chang et al., 2012). Live imaging using a transgenic zebrafish line expressing fluorescent

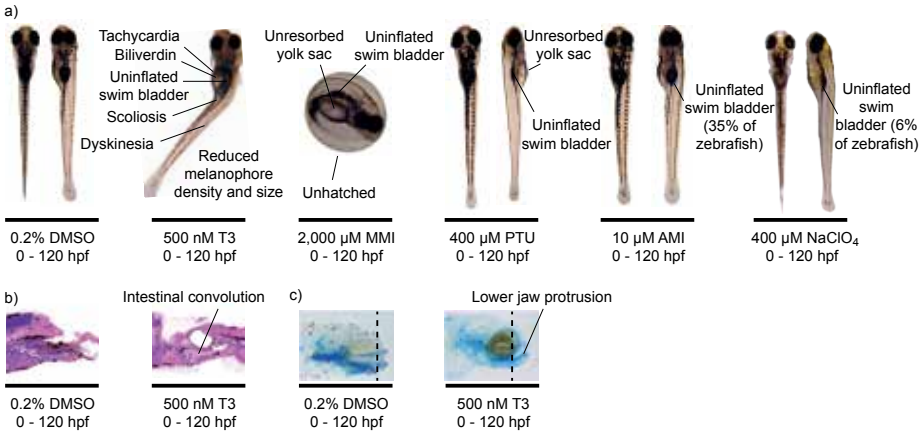

Fig. 2: a) Zebrafish morphology upon exposure to solvent control (0.2% DMSO), 500 nM T3, 2,000 µM MMI, 400 µM PTU, 10 µM AMI and 400 µM ClO_4. b) Sagittal section of zebrafish exposed to 500 nM T3 stained with hematoxylin and eosin (H&E) reveals a high degree of intestinal convolution that is absent in the solvent control. c) Alcian blue staining of the cartilage revealing the anterior-dorsal protrusion of the lower jaw following exposure to 500 nM T3 from 0 – 120 hpf.

thyroglobulin has recently provided further insight into the development of the thyroid, suggesting a role for the hypobranchial artery in guiding late thyroid expansion (Opitz et al., 2012). Figure 1c provides a schematic overview of thyroid tissue development with T4 immunohistochemistry (IHC) at 120 hpf providing a visual reference. The first follicle to be formed (primary follicle) is formed near the heart during early development and expands anteriorly along the pharyngeal midline until it reaches the mid-region of the eyes, leaving behind a trail of follicular cells that later form additional follicles.

Based on this analysis, two time windows of exposure, namely 48 hpf to 120 hpf and 0 to 120 hpf were chosen, with the prior avoiding the potential effect of compounds on morphogenesis of the first thyroid follicle and the second one including such effects. These thyroid-specific time windows were further compared with the exposure time window of 0-72 hpf, which was used by Hermsen et al. (2011) in order to assess the GMS of developmental toxicants. Exposures were conducted with 12 replicates per compound concentration for all time windows in one experiment. In addition, for the 0-120 hpf time window, an additional experiment with 8 replicates was performed resulting in a total of 20 replicates per compound concentration for the 0-120 hpf time window.

3.2 Morphological effects of thyroid-active model compounds

Figure 2 shows an overview of the altered morphologies of the zebrafish exposed from 0 to 120 hpf to the highest tested concentrations of the test substances. The highest

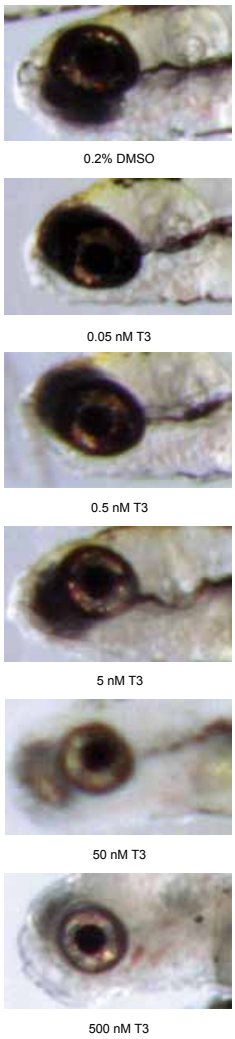

Fig. 3: Lateral view of 120 hpf zebrafish showing iridophore pigmentation (reflective pigments) increasing dose-dependently upon exposure to T3.

tested concentration of T3 (500 µM) affected zebrafish embryos dramatically with an observed reduction in melanophore density and size, abnormal iridophores, deflated swim bladder, accumulation of biliverdin around the liver, and an indication of an adrenergic response – mainly tachycardia, which is a well documented effect of T3 – as well as dyskinesia observed as a lack of swimming behavior. In terms of teratogenicity, an observation was made for kyphosis (hunchback), however since the teratogenicity parameters used only included scoliosis (oblique bending), it was scored as such. Most effects on morphology could already be seen at 50 nM T3. The effects were similar in the 48-120 hpf exposure window while for the 0-72 hpf time window only abnormal pigmentation, kyphosis and a mean percentage failed or delayed hatching of 41.7 ±1.2% (mean ±SEM) could be observed. Intestinal convolution could be clearly observed with histological slices (Fig. 2b) but could not be clearly viewed under a stereo microscope and hence was not included as a parameter in the scoring system. Lower jaw protrusion was also clearly visible, especially with Alcian blue staining (Fig. 2c).

At doses of 250 µM MMI and higher, failure to hatch was observed for the exposure periods 0-72 hpf and 0-120 hpf. For the exposure period from 48-120 hpf, only the highest tested concentration of 2 mM led to the failed hatching of 16.7 ±0.9% of the zebrafish embryos, compared to 100.0 ±0.0% failed hatching at the highest tested concentration for 0-72 hpf and 84.2 ±0.4% for 0-120 hpf. Unhatched embryos had deflated swim bladders, unresorbed yolk sacs and, as can be expected from unhatched embryos, a lack of swimming behavior. PTU also led to failed hatching at the highest tested concentration of 400 µM at a rate of 27.3 ±1.2% for exposures from 0-72 hpf, 11.1 ±0.5% from 0-120 hpf while no failed hatching was observed for the exposure period from 48-120 hpf.

Upon exposure to $NaClO_4$ from 0-120 hpf, deflated swim bladders were observed starting at 25 µM up to the highest tested concentration of 400 µM in an average of 12.0 ±0.4% of embryos, while exposure to AMI at the highest tested concentration of 10 µM led to deflated swim bladders in 35.3 ±0.7% of embryos exposed during the same time period. No significant effects could be

observed during exposures to either compound using the other time windows tested (0-72 hpf or 48-120 hpf) suggesting exposure duration to influence the sensitivity of the assay and 0-120 hpf to be the preferred time window for exposure.

3.3 Adjusted morphological scoring: general development score

The GMS scoring system was developed to look at the effect of chemicals on zebrafish development at 72 hpf (Hermsen et al., 2011). Based on the results obtained here with the selected thyroid modulating compounds, four main adjustments were made to the GMS scoring system. First, 120 hpf-specific developmental endpoints affected by thyroid-active compounds were added to the GMS. These endpoints included reflective iridophores (Fig. 3), beat and glide swimming, inflation of swim bladder and resorption of yolk sac (Fig. 2). Since these endpoints are not included in the GMS for 0-72 hpf, they were incorporated in a new scoring system denoted general development score (GDS). Secondly, a refinement was made in the newly defined GDS by combining both morphology and teratogenicity endpoints in a similar way to the total morphological score (TMS) applied in the method of Piersma et al. (2004) for the rat whole embryo culture (WEC) which combines teratogenicity and developmental toxicity (Fig. 4). Thirdly, a refined scoring approach was also implemented similar to that used by Teixidó et al. (2013) whereby developmental abnormalities for an endpoint are treated as a lack of development and therefore result in a score of 0 for that developmental endpoint compared to a score of 1 per event for normal development. Fourthly, the terms used for developmental abnormalities are compliant with major databases with the use of the Entity Quality Ontology (EQO) convention suggested by the Open Biomedical Ontologies (OBO) Consortium, which involves describing an entity (e.g. tail vasculature) and a quality (e.g., edema) (Balhoff et al., 2011; Dahdul et al., 2010). This leaves room for the standardized annotation of additional abnormalities based on anatomical location or feature (such as movement). Database compliance also enables computable phenotypes as well as the development of iterative models that improve the accuracy of future chemoinformatic predictions. Examples of commonly observed abnormalities are included in Figure 4 for reference purposes. Minor adjustments to the highlighted abnormalities include the addition of visible biliverdin or bilirubin as well as the changing of the term scoliosis to spinal curvature in order to encompass all forms of a bent spine, which include kyphosis and lordosis in addition to scoliosis (Fig. 4).

3.4 GMS compared to GDS

The GMS for the highest concentration of T3 (500 µM) decreased to 12.9 ±0.7 (n=12) for the exposure period from 0-72 hpf, 11.3 ±0.5 (n=12) from 48-120 hpf and 11.1 ±2.6 from 0-120 hpf (n=20), as compared to a value of 15.0 ±0.0 for the unexposed control.

General Development Score - Part 1 of 3

	Abnormality	*12 hpf*	*24 hpf*	*48 hpf*	*72 hpf*	*120 hpf*
Detachment of tail	All tail malformations including: □ tail, malformation □ tail vasculature, edema □ vertebral column, curvature (scoliosis, kyphosis, lordosis) □ neural tube, malformation (rachischisis, anencephaly) □ notochord, malformation 0	 0  18hpf 1	 2	 3	 3	 3
Somite formation	All somite malformations including: □ somite border, incomplete 0	No = 0	Yes = 1	Yes = 1	Yes = 1	Yes = 1
Eye development	All eye malformations including: □ eye, single (cyclopia) □ eye, absent □ eye, edema 0	 1	 2	 2 + 1 for pigment	 2 + 1 for pigment	 3 + 1 for reflective iridophores
Movement	All movement abnormalities including: □ organismal movement, abnormal (spasms) 0	No = 0	Yes = 1	Yes = 1	Yes = 1	Yes = 1 + 1 for beat and glide swimming

General Development Score - Part 2 of 3

	Abnormality	*12 hpf*	*24 hpf*	*48 hpf*	*72 hpf*	*120 hpf*
Heartbeat	All heart malformations including: □ heart, malformation □ pericardial sac, edema 0	No = 0	Yes = 1	Yes = 1	Yes = 1	Yes = 1
Blood circulation	All circulation abnormalities including: □ blood coagulation, present □ bilirubin oxidase activity, increased in magnitude 0	No = 0	No = 0	Yes = 1	Yes = 1	Yes = 1
Pigmentation head - body	All pigmentation abnormalities in head body region (except eyes) including: □ pigmentation, irregular spacial pattern □ pigmentation, decreased □ pigmentation, increased 0	0	0	1	1	1
Pigmentation tail	All pigmentation abnormalities in tail including: □ pigmentation, irregular spacial pattern □ pigmentation, decreased □ pigmentation, increased 0	0	0	1	1	1

General Development Score - Part 3 of 3

	Abnormality	*12 hpf*	*24 hpf*	*48 hpf*	*72 hpf*	*120 hpf*
Pectoral fin	0	0	0	0	 1	1
Head: Protruding mouth	All head malformations (except for eyes) including: □ head, malformation □ head, edema □ saccule / otolith, malformation □ brain, small volume					
	0	0	0	0	1	1
Hatching	0	No = 0	No = 0	No = 0	Yes = 1	Yes = 1
Inflated swim bladder	All swim bladder malformations					
	0	No = 0	No = 0	No = 0	No = 0	Yes = 1
Yolk sac resorbed	All yolk malformations including: □ yolk, malformation □ yolk extension, malformation					
	0	No = 0	No = 0	No = 0	No = 0	Yes = 1
GDS	***0***	***1***	***7***	***12***	***15***	***19***

Fig. 4: GDS system for the assessment of general developmental toxicity at 120 hpf. The system takes into account morphology, dysmorphology as well as non-morphological parameters such as swimming. Additions made to the GMS scoring system (Hermsen et al., 2011) in order to better accommodate effects of thyroid-active compounds are highlighted with a bold outline.

The GDS for the highest concentration of T3 was 12.2 ±3.7 (n=20) from 0-120 hpf as compared to18.8 ±0.7 for the control. For better comparison, normal growth was set as a common denominator with a baseline value of 1, and the fold change relative to the controls was also calculated. The fold change relative to the controls for the two highest concentrations of T3 (1-120 hpf, n=20) went from 0.9 and 0.7 in the GMS to 0.8 and 0.6 in the GDS, respectively, thereby increasing the statistical significance from $p \leq 0.01$ to $p \leq 0.0001$.

The GMS at the highest tested concentration of MMI (2,000 µM) was 13.1 ±0.3 as an average of all exposure periods, which represents a fold change of 0.9 relative to

the control (GMS = 15.0 ±0.3). The GDS at the highest tested concentration of MMI was 12.8 ±2.7 (0-120 hpf), which represents a fold change of 0.7 relative to the control (GDS = 18.4 ±1.6; $p \leq 0.0001$). Moreover, statistical significance could be reached at the lower concentration of 1,000 µM ($p \leq 0.05$) with the GDS but not the GMS.

Figure 5 gives an overview of the effects of T3, MMI, PTU, and ClO_4 on zebrafish development, in both GMS and GDS, represented as fold change relative to the controls. These tested compounds led to developmental abnormalities at the highest tested concentration and the GDS was better able to reflect these abnormalities than the GMS.

A comparison of the exposure time windows of 0-72 hpf and 0-120 hpf, reveals that the most significant results were achieved with exposures from 0-120 hpf (Fig. 5). The time window of 48-120 hpf did not add any additional information or significance compared to the other time windows. In the GDS, T3 and MMI exposure resulted in a no observed adverse effect level (NOAEL) of 5 nM and 500 µM, respectively. The GDS for PTU, amiodarone and ClO_4 did not change significantly and therefore a NOAEL could not be established for these compounds.

3.5 T4 immunohistochemistry

The model compounds tested were selected based on their known ability to alter thyroid homeostasis and only those that significantly affected the GDS score, namely T3 and MMI, were further tested for thyroid status by T4 immunohistochemistry (IHC) in order to confirm that the developmental toxicity detected by the GDS for these compounds was accompanied by an effect on the thyroid system (Fig. 6). Under control conditions, the thyroid follicles are well spaced out anteriorly by 120 hpf. In zebrafish exposed to T3 both number of follicles and their spread were affected compared to the control (Fig. 6a), indicating potential thyroid dysmorphogenesis, while MMI exposure led to a clear drop in signal intensity and in the number of follicles compared to the control (Fig. 6b,c), confirming a drop in follicular T4 levels.

4. Discussion

Critical to the proper development of the vertebrate embryo, thyroid homeostasis is prone to disruption by man-made chemicals with an ability to act through very diverse modes of action and within multiple organs (Hartoft-Nielsen et al., 2011; Jomaa, 2014; Tan et al., 2007). Given that alterations in the thyroid system can have severe effects on development (Boyages and Halpern, 1993; Haddow et al., 1999; Utiger, 1999), the aim of the present study was to investigate whether a zebrafish-based developmental toxicity assay would be able to detect thyroid hormone-active compounds.

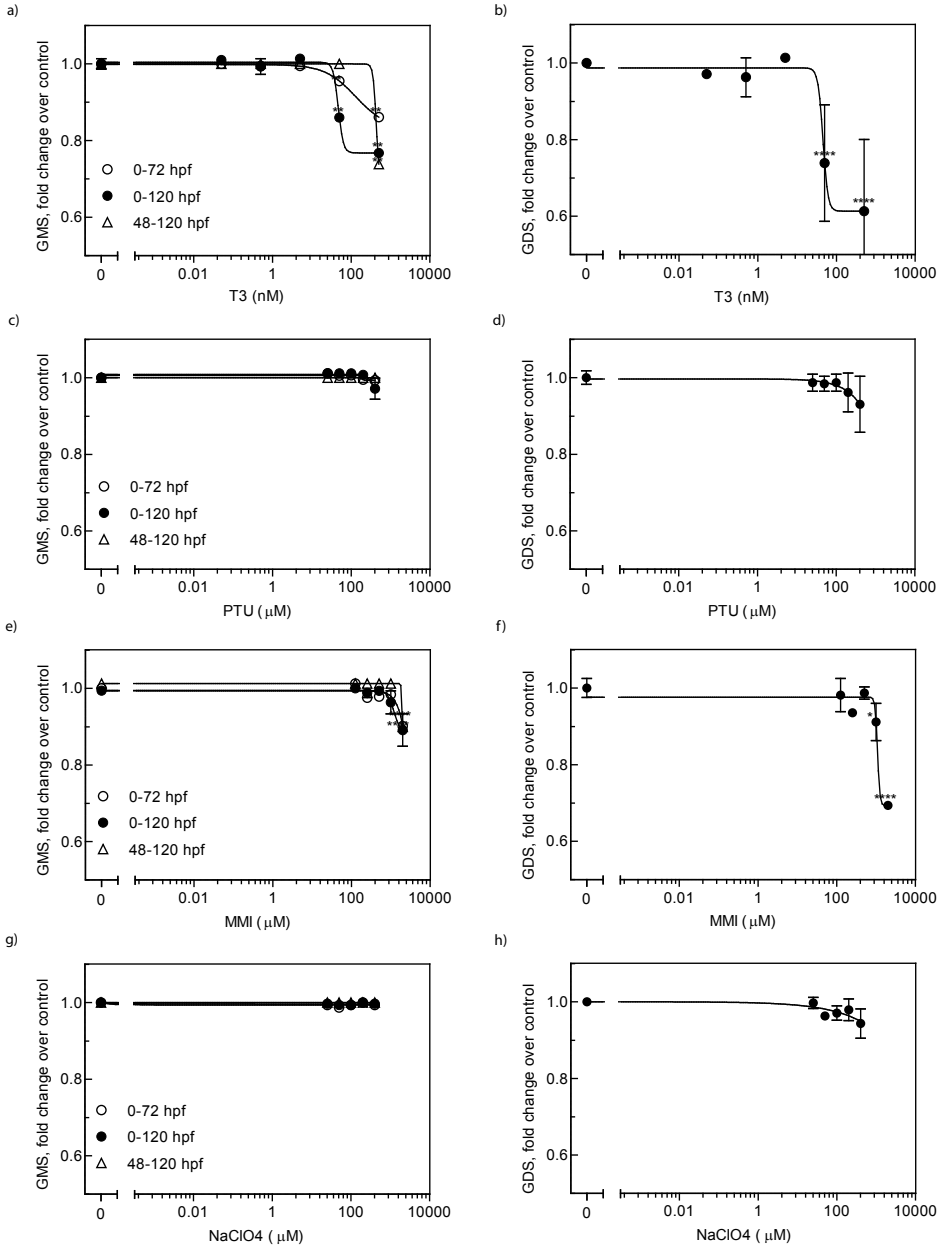

Fig. 5: Effect of T3 exposure on a) GMS b) GDS; PTU exposure on c) GMS d) GDS; MMI exposure on e) GMS f) GDS ClO_4 exposure on g) GMS h) GDS. Results are based on 12 replicates per concentration (n = 12) and since the total scores are different, these are expressed as fold decrease relative to the full score (normal development). A top down approach was used to minimize the number of embryos used and the 0 – 120 hpf exposure window, which was found to be the most appropriate time window for the thyroid-related parameters studied, was selected for an additional independent experiment at a separate laboratory with 8 replicates per concentration (n = 8; a total of n = 20 for 0-120 hpf experiments). The error bars are shown for only this time window as the standard error of the mean of these two experiments (N = 2). The calculation of statistical significance was done by pooling all replicates from each experiment for each concentration and comparing it to the 0.2% DMSO solvent controls in a two tailed student's t-test. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$.

The pathways involved in human thyroid development are conserved in the zebrafish and so are its tissue architecture, function and feedback regulation by the HPT axis, making it a promising model for the study of thyroid-active compounds (Bourque and Houvras, 2011; Jomaa et al., 2013; Raldúa and Babin, 2009).

T3, acting through its nuclear receptor, is a morphogenic agent in certain animal species, controlling the metamorphosis of tadpoles and frogs (Shi et al., 1996). In the present study, T3 exposed zebrafish clearly showed significant developmental toxicity evident as scoliosis, abnormal iridophores, reduced melanophore density and size as well as a lack of an inflated swim bladder (Fig. 4). Moreover, T3 exposed zebrafish showed signs of acute toxicity such as excess biliverdin that is visible under a stereo microscope, tachycardia and dyskinesia. The latter two are symptomatic of an adrenergic response and are likely a result of T3 increasing beta-adrenergic receptor density (Mooradian and Scarpace, 1993). A high rate of 42% failed hatching occurred only at the 0-72 hpf exposure window. Increased sensitivity for hatching failure is expected at 72 hpf since that is around the time hatching occurs. However, as can be seen in Figure 5, overall sensitivity to thyroid-active compounds is higher in the GDS at 0-120 hpf as it includes additional parameters that are specific to that time window.

PTU and MMI have a direct effect on thyroglobulin iodination by thyroid peroxidase, thereby inhibiting thyroid hormone synthesis (Taurog, 1976). These compounds are used medically to treat hyperthyroidism. A decrease in the circulating T3 levels can affect various developmental endpoints including intestines, brain and reproductive capacity. Raldúa and Babin (2009) reported a complete lack of T4 immunoreactivity beyond 0.75 mM and 0.18 mM in zebrafish for MMI and PTU, respectively. This reduction in thyroid hormone levels was accompanied by a reduction in the GDS (Fig. 5). Moreover, the hatching rate was strikingly low in the MMI-treated group and this was deemed significant, not only statistically, but also physiologically. This result is in line with the observation that chicken embryos have been reported to have delayed hatching upon exposure to MMI (Haba et al., 2011). Interestingly, another compound shown to affect zebrafish hatching rate is perfluorooctanoic acid (PFOA), an established thyroid-active compound (Lopez-Espinosa et al., 2012; Melzer et al., 2010; Zheng et al., 2011). Hence, the change in zebrafish hatching rate that was induced by thyroid active compounds was accurately reflected in the GDS developmental toxicity assay (Fig. 5). Moreover, MMI exposure resulted in a NOAEL of 500 µM in the GDS, which is several orders of magnitude higher than the T3 NOAEL of 5 nM, and this relative potency compares well with the NOAEL based on changes in thyroid stimulating hormone (TSH) levels derived from rodent studies (a NOAEL of 0.5 mg/kg bw/day for MMI and a NOAEL of less than 10 µg/kg bw/day for T3) (Grover et al., 2007; Hood et al., 1999).

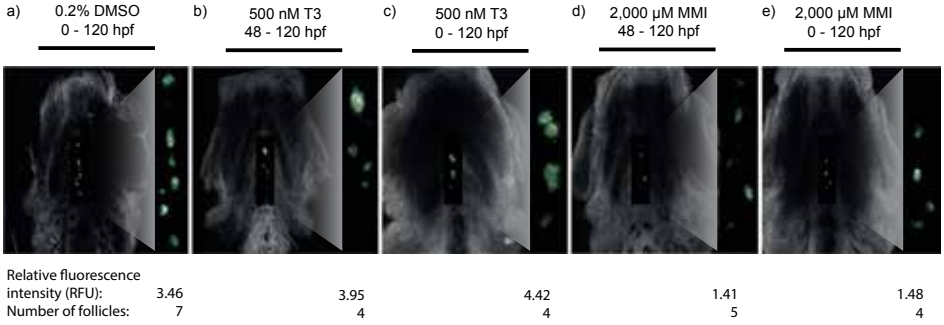

Fig. 6: T4 whole mount immunohistochemistry upon exposure of zebrafish to a) 0.2% DMSO as the solvent control from 0 – 120 hpf; 500 nM T3 from b) 48 – 120 hpf and c) 0 – 120 hpf; and 2,000 µM MMI from d) 48 – 120 hpf and e) 0 – 120 hpf. Thyroid follicles were tested for the signal intensity of T4 immunofluorescence and expressed as relative fluorescence units (RFU). The number of follicles was counted based on the images taken with the confocal laser scanning microscope (the region of interest is enlarged and presented at the right side of each panel). T3 exposure at the highest tested concentration and both time points resulted in a decrease in the number of follicles. MMI exposure at the highest concentration resulted in both lower T4 signal intensity and lower number of follicles.

$NaClO_4$ inhibits sodium iodide symporter (NIS) transport of iodide into the thyroid follicular cells, thereby resulting in a decline in thyroid hormones and a rise in thyrotropin (Stoker et al., 2006). The morphological changes observed in the developing zebrafish embryo included uninflated swim bladder and incomplete resorption of the yolk sac. While these were consistent observations that could only be made by including 120 hpf-specific parameters, they did not have enough weight within either the GMS or GDS system to significantly change the overall score. This corroborates in vivo data on altered thyroid hormone levels in rodent studies showing that $NaClO_4$ is less potent than either PTU or MMI (Hood et al., 1999; Stoker et al., 2006; Yamasaki et al., 2002). Moreover, a human epidemiological study failed to find an association between perchlorate levels in drinking water and congenital defects of the thyroid (Crump et al., 2000). In another study with volunteers given perchlorate in drinking water the administered dose was negatively correlated with thyroidal iodide uptake but even a 70% decrease in iodide transport did not result in altered T4 levels (Greer et al., 2002; Strawson et al., 2004). The relatively low developmental toxicity of perchlorate is supported by animal studies showing that pregnant rats and rabbits exposed to a perchlorate dose as high as 30 mg/ kg bw/ day do not give birth to offspring with signs of developmental toxicity and changes in thyroid hormone levels were seen as adaptive and reversible (York et al., 2003, 2005).

Amiodarone is an anti-arrhythmic agent (heart), beta blocker and calcium blocker. Its chemical structure resembles that of T4 and the compound alters thyroid hormone

levels in vivo (de Sandro et al., 1991), likely due to its high iodide content (Pitsiavas, 1999). In zebrafish, amiodarone was reported to lead to a significant reduction in T4 immunoreactivity at 1 μM (Raldúa and Babin, 2009). However, while morphological changes were observed in the present study upon exposure to amiodarone, such as the lack of an inflated swim bladder in some animals, which is included in the GDS, statistical significance could not be reached in both the GMS and GDS (data not shown). Indeed, human studies on amiodarone treated mothers indicate that transient hypothyroidism in the newborn may occur but there is no adverse effect on development (Matsumura et al., 1992; Valensise et al., 1992).

The thyroid-active compound associated phenotypes observed in 120 hpf zebrafish include 1) hatching (discussed in the previous section), 2) yolk sac resorption, 3) iridiophore pigmentation, 4) melanophore pigmentation, 5) swim bladder inflation, 6) bent spines, 7) beat and glide swimming, 8) lower jaw cartilage protrusion and 9) maturation of the gastrointestinal system, with the last two being better suited for staining techniques or high resolution microscopy for accurate assessment. These developments occur concurrently with a surge in thyroid hormone levels, which reach a peak at 120 hpf (Chang et al., 2012). The overall results have indicated that the time window of 0-120 hpf is better able to reflect the effect of thyroid-active compounds on zebrafish development than the other time windows studied (Fig. 5) and support the suggestion by Liu and Chan that there is a distinct thyroid hormone-mediated embryonic to larval transition in zebrafish that is analogous to the thyroid hormone-mediated larval to juvenile transition originally proposed by Brown (Brown, 1997; Liu and Chan, 2002).

The period from the time the zebrafish embryo has hatched (72 hpf) until the time it is free swimming (120 hpf) is termed the yolk-sac larva (Balon, 1990). The resorption of the yolk sac and hence the maternal food supply is only evident at around 120 hpf, with total resorption evident by around 168 hpf (Jardine and Litvak, 2003; Kimmel et al., 1995). The role of thyroid hormones in this developmental process has been previously documented whereby MMI co-administered with AMI resulted in the retardation of yolk sac resorption and this was rescued with T4 (Liu and Chan, 2002).

Overall, the results obtained in this paper show the importance of the 0-120 hpf time window of zebrafish exposure in the assessment of the developmental toxicity of thyroid-active compounds. The newly developed GDS scoring system takes into account parameters that occur after 72 hpf. The defined GDS can successfully detect zebrafish developmental toxicity induced by thyroid-active compounds and can benefit from the model organism's potential for high-throughput, which, for screening purposes, is a key advantage over rodent assays. The parameters affected, which include reflective iridophores, beat and glide swimming, inflated swim bladders, and resorbed yolk sacs,

are shown to be sensitive towards toxicity induced by TH disrupting compounds. One could envisage that other compounds causing developmental toxicity by different modes of action may also affect these parameters. Since this opens the way to false positives if the assay were to be used in isolation to detect TH activity, we envision that the GDS could be part of an ITS that includes specific biomarkers of thyroid hormone disruptor activity. Although the GDS will detect developmental toxicity resulting from various modes of action, it is shown in the present study that the GDS can be used to flag compounds as potential thyroid disruptors. Due to the presence of an intact thyroid hormone feedback loop, the zebrafish represents an experimental in vitro platform that is vastly more comprehensive than individual cell lines. Moreover, the zebrafish embryo up to 120 hpf is more socially-acceptable as a test organism than higher vertebrates. When applied in combination with other in vitro tests within an ITS, the zebrafish GDS holds promise to reduce, refine and, in certain cases, replace animal testing.

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Chapter 6

General discussion and future perspectives

The aim of this thesis was to find in vitro and toxicogenomics-based alternatives to in vivo thyroid hormone disruption tests. Thyroid hormone receptor (TR) and thyroid stimulating hormone (TSH)-mediated cellular proliferation, thyroid peroxidase (TPO) inhibition, nuclear receptor-coregulator binding, and developmental toxicity were chosen as endpoints for this research based on their relevance for the thyroid hormone system. In vitro alternatives can help reduce the amount of animal testing required under the European Union regulation for the registration, evaluation, authorization and restriction of chemicals (REACH). Moreover, with the use of human cell lines and human-identical synthetic proteins, interspecies differences can be reduced and in some cases eliminated.

In Chapter 1, a mode-of-action-based alternative strategy focused on the in vitro testing for thyroid activity was introduced and set the framework upon which complementary alternative tests can be developed. This strategy followed a multi-compartmental approach, as explained below, which divides the thyroid hormone system into 6 main compartments, starting with the thyroid itself, and subsequently expanding to other organs and tissues that play a role in thyroid hormone signaling:

1) Thyroidal hormonogenesis: TSH receptor (TSHR) activation, iodination, coupling of iodinated tyrosyl residues, transport of iodide, proteolysis of iodinated thyroglobulin, iodide scavenging and thyroid hormone (TH) synthesis
2) Pituitary control: Thyrotropin (TSH)-releasing hormone receptor (TRHR) activation, TSH synthesis
3) Hypothalamic CNS control: Thyrotropin-releasing hormone (TRH) synthesis
4) Thyroid hormone activity at the target tissue: TR activation and coregulator interaction
5) Kinetics: TH blood transport, cellular uptake of TH and TH metabolism
6) Epigenetics, immune response and other mechanisms: ITG αvβ3 activation

The above-listed compartments will be discussed in this chapter and will cover the research that has been presented in this thesis as well as new avenues of research that are part of a proposed future perspective for testing thyroid-active compounds. Overall, the aforementioned compartments will form the foundation upon which an integrated testing strategy for the testing of thyroid-active compounds can be built.

1) Thyroidal hormonogenesis

The first compartment that is outlined in this approach focuses on the effects of compounds on the thyroid and includes TSHR activation, which was tackled with the TSH-screen in Chapter 2; iodination & coupling, which was tackled with the luminol-

based TPO assay in Chapter 3; and TH synthesis, which was tested in zebrafish in Chapter 5 using immunochemistry techniques. Iodide transport, proteolysis of iodinated thyroglobulin and iodide scavenging will be discussed at the end of this section as topics of future research.

While not many compounds have yet been found to act through **TSHR activation**, it is presently unclear to what extent this is due to lack of knowledge. Therefore, we developed a new assay enabling the measurement of chemical interference with normal TSH signaling, called the TSH-screen and described in Chapter 2. This assay might facilitate the identification of thyroid disruptors acting through this membrane receptor (Jomaa et al., 2013). The assay measures TSHR activation-dependent thyroid cell proliferation without added TSH (to detect agonist activity) as well as in the presence of 1 milli-international unit per milliliter (mIU/ml) TSH (to detect antagonist activity). The cell proliferation signal quantified in this simple and inexpensive assay reflects the integrated effects of the binding of TSH to TSHR, second messenger activity, and downstream signal transduction on gene expression, which is finally determining the level of hormone-mediated mitogenesis. One of the objectives of the study reported in Chapter 2 was to find out whether the TSH-screen can predict in vivo thyroid organ weight change, which is an endpoint used in rodent toxicity assays to detect thyroid-active compounds (OECD test guidelines (TG) 407, 416, 443, 452 and 453). To this end, 11 compounds found to be thyroid-active in rodents were tested in the TSH-screen. Results indicated that the in vitro - in vivo correlation was poor and highlighted the shortcoming of current in vitro tests such as the lack of an intact hormonal feedback system.

Not all potential effects on the thyroid are related to TSH signaling. In this regard, a transcriptomics test using microarray technology might be a promising addition to a battery of assays in order to uncover the effects of test chemicals that modulate physiologically relevant thyroid endpoints that are not, or not only regulated by TSH. Furthermore, nongenetic endpoints may also be affected by thyroid active compounds. An example of an important nongenetic parameter is the inhibition of thyroid function-related enzyme activities. One of the most important enzymes for the thyroid hormone system is thyroid peroxidase (TPO), which is a critical factor in the iodination and coupling of tyrosyl residues on thyroglobulin in order to produce the thyroid hormones T3 and T4. Important for toxicity testing is that, a large number of compounds that have been found to be thyroid-active in rodent toxicity assays appear to exert their effect by inhibiting the activity of this enzyme (Jomaa et al., 2013; OECD, 2014).

Iodination and coupling of iodinated tyrosyl residues are carried out by TPO and in Chapter 3, a TPO inhibition assay was developed as a refinement of existing assays (Jomaa et al., 2015). TPO assays are routinely performed using extracts from porcine

thyroid glands based on the principle that TPO will oxidize a substrate such as guaiacol leading to a color change that can be quantified (Hosoya, 1963). Guaiacol is a small volatile organic compound that gives a smoky aroma to tomatoes and roasted coffee (Mageroy et al., 2012). During testing, guaiacol is unstable and is readily oxidized upon exposure to air leading to a change in color (Sr, 2008), which can either jeopardize the reliability of experimental results or necessitate precautions being put in place to avoid artifacts due to guaiacol instability. The other downside of traditional TPO tests is the use of an enzyme derived from an animal's thyroid tissue. Beyond ethical concerns, this also raises the question of inter-species differences and potential contamination with hemoglobin, which itself can act as a peroxidase (Harauchi and Yoshizaki, 1982). Recently, there have been efforts to overcome the obstacles posed by guaiacol using an alternative substrate, namely Ampliflu Red, but this study was using TPO originating from rat thyroids (Paul et al., 2014), compromising the extrapolation to the human situation. The assay that we developed addresses the shortcomings of the porcine TPO / guaiacol assay by using luminol, which is a luminescent substrate that oxidizes TPO in the presence of hydrogen peroxide, in combination with human TPO derived from a commercially available thyroid cell line. Five TPO inhibitors were tested and compared with the guaiacol and the Ampliflu Red TPO assays. The results indicated that the luminol assay is successful in detecting TPO inhibitors and does so within a smaller 95% confidence interval than with guaiacol or Amplifu Red TPO assays, which is an indication of reliability. Moreover, the luminol assay proved to be simple and rapid. It did not require the overexpression of TPO and was suited for high-throughput applications, since the experiment was performed in 96-well plates with the reagents being dispensed automatically by a Luminoskan Ascent luminometer from Labsystems (Helsinki, Finland). The measurement time for guaiacol and Ampliflu Red was 10 minutes whereas the measurement time for the luminol assay was only 10 seconds. The luminol assay shows potential as a simple, rapid and reliable TPO assay with added value as compared to the existing TPO assays for an integrated testing strategy for thyroid-active compounds.

The sodium/iodide symporter (NIS) mediates the **transport of iodide** into thyroid follicles (Dohán et al., 2003) and this can be detected in radioactive iodide (I-131) transport studies (Tonacchera et al., 2004). In general, there is a general move away from radioisotopes in testing, when possible, in favor of fluorescence and hence the development of radioisotope-free alternative assays has high priority (Sundberg, 2000). This has recently been the direction taken by another group that performed NIS inhibition studies by measuring the inhibition of yellow fluorescent protein variant YFP–H148Q/I152L expressed in FRTL-5 rat thyroid cells by anions including thiocyanate and perchlorate (Di Bernardo et al., 2011). The sodium potassium pump (Na/K-ATPase)

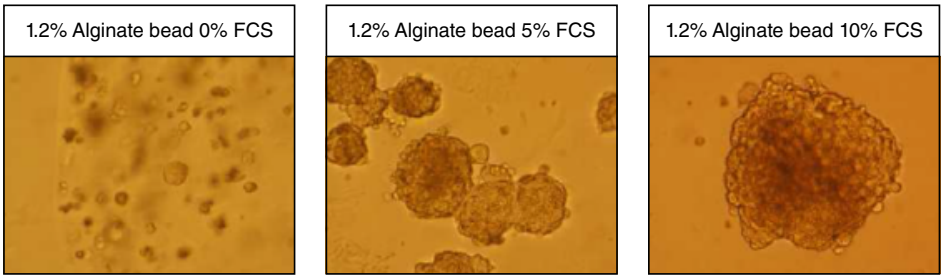

Fig. 1: Morphology of Nthy ori 3-1 cells in alginate bead cultured in depletion medium (phenol red free DMEM F12 supplemented with 10 µg/ml insulin, 10 ng/ml sodium selenite, 10 µg/ml apotransferrin, 0.2% BSA, 500 nM NaI and 10 nM hydrocortisone) with different concentrations of FCS for 10 days as seen under light microscopy, 10x magnification. In the presence of 5% and 10% FCS the formation of follicle-like spheroids is observed.

provide the sodium gradient that is needed for NIS to function as a symporter and compounds affecting the Na/K-ATPase can in turn inhibit NIS-mediated iodide transport (Josefsson et al., 2006). Promising high throughput assays for Na, K-ATPase function have been developed by another group using nonradioactive rubidium ion uptake (Gill et al., 2004).

Another parameter to be considered within the framework of possible effects on thyroidal hormonogenesis is **proteolysis by lysosomal proteases**, which is a critical factor in the release of T3 and T4 following the iodination and coupling of tyrosyl residues on thyroglobulin (Selmi and Rousset, 1988). The process is inhibited by excess iodine and iodine-rich compounds, including amiodarone (Radvila et al., 1976). Research into the inhibition of this process by thyroid-active compounds is scant and based on porcine thyroglobulin. While an in vitro protease inhibition assay is feasible, in vitro production of thyroid hormones is still a challenge that needs to be overcome. **Iodide scavenging** by iodotyrosine deiodinase (IYD) ensures that waste iodide that is produced as a byproduct of proteolysis is reused by the thyroid (Gnidehou et al., 2004 p. 1). While mutations in the IYD genes have been associated with hypothyroidism, little is known of its possible inhibition by thyroid-active compounds and this would be an interesting topic of future research (Moreno et al., 2008).

In Chapter 5, T4 immunochemistry (IHC) was used to obtain an indication of the inhibition of **TH synthesis** in zebrafish by model compounds, which was subsequently correlated to symptoms of developmental toxicity scored up to 120 hours post fertilization (hpf). The scoring system initially used was developed for the 0 – 72 hpf time window, but since results indicated that the morphological parameters affected upon exposure to thyroid active compounds develop after 72 hpf, an adjusted scoring system was developed and named the General Development Score (GDS). These developmental

parameters were shown to be regulated at least in part by thyroid hormones (Jomaa et al., 2014) indicating that they can be used in combination with other thyroid-regulated phenotypical changes to flag compounds as potentially thyroid-active and used within a wider testing strategy. A two step bioassay combining thyroid-related zebrafish development parameters, which encompass transcriptional and non-transcriptional effects, and T4 IHC, which can detect TPO inhibition, can be more informative than either test on its own. Most importantly, when development is affected in a GDS screen, T4 IHC can reveal whether this is due to an effect related to T4 levels.

To date there are no in vitro methods to produce thyroid hormones and this is due to the three dimensional morphology of thyroid follicles which concentrate thyroglobulin into a central lumen that acts as a bioreactor for the production of thyroid hormones which are later absorbed by pinocytosis into the cell for cleavage and eventual secretion into the blood stream. Within the framework of the present PhD thesis, efforts to mimic such morphology using alginate beads imbedded with human Nthy-ori 3-1 cells were undertaken and revealed serum-dependency for the formation of spheroids (Fig. 1, part of MSc thesis work by Qian Zhang). As serum can contain thyroid hormones and growth factors, future research should focus on the development of serum-free methods with a defined medium.

2) Pituitary control

The pituitary regulates the levels of **TSH synthesis** and secretion based on circulating levels of thyroid hormones. In vitro models for TSH secretion include the human T-cell leukemia cell line Molt 4, the mouse thyrotropic pituitary tumor cell line TtT-97 and the immortalized mouse pituitary TαT1 cell line (Martin et al., 1988; Alarid et al., 1996; James et al., 1997). Molt 4 cells were grown but did not produce levels of TSH that are above the limit of quantitation using standard ELISA

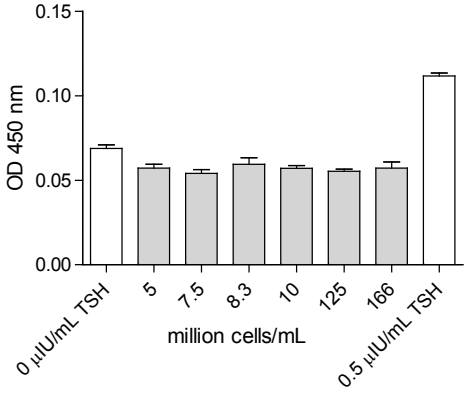

Fig. 2: TSH enzyme-linked immunosorbent assay (ELISA) performed to test TSH secretion by the Molt 4 cell line after 24 h exposure to 1 nM TRH (grey bars, n = 3). TSH concentrations were quantified by measuring optical density at 450 nm (OD450 nm), which is proportional to the amount of TSH in solution. 0 µIU/mL and 0.5 µIU/mL TSH (open bars, n = 2), tested from standard solutions, represent the negative and positive control, respectively. It can be derived from the bar graph that no detectable levels of TSH were measured in MOLT-4 cells. The analytical sensitivity of the human TSH ELISA kit (Calbiotech Inc., CA, USA), with which the experiments were performed, is 0.5 µIU/ml according to the manufacturer.

tests (Fig. 2, part of MSc thesis work by Katerina Kademoglou). On the other hand, TαT1 (kindly provided to us by the Mellon lab at the University of California, San Diego) cell culture required matrigel, led to low cell densities and was difficult to maintain – casting doubt on its use within a high throughput screen. Further research needs to be conducted in order to derive a pituitary cell line that is suitable for high throughput screening and is of human origin. Instead of measuring TSH activity levels, an alternative approach would be to quantitate levels of mRNA encoding the peptide hormone TSH. Microarrays have previously been used by other groups in in vivo experiments to show the up-regulation of TSHB mRNA in the pituitary by glutamine and glutamic acid (Aizawa et al., 2012). The use of microarrays in unraveling the interference of compounds with TSH expression has great potential, especially within a broader battery of tests aimed at detecting other endpoints that are relevant to thyroid disruption.

3) Hypothalamic CNS control

It is very difficult to mimic in vitro the potential effect of compounds on the control of TRH production in vivo at the level of the hypothalamus due to the complex signaling networks that are present in the central nervous system. The secretion of TRH is regulated by thyroid hormones by a negative feedback mechanism but is also regulated by neuropeptide Y, melanocortin-stimulating hormone, and agouti-related peptide (Nillni, 2010). Perhaps the biggest obstacle of a purely in vitro test system is the lack of a hormonal feedback system. To this end, we explored the application of zebrafish embryos for the testing of developmental anomalies caused by thyroid-active compounds since the disturbance of normal embryonic development is a major adverse effect that is caused by thyroid-active compounds (Atkins et al., 2000). Moreover, zebrafish have an intact thyroid hormone feedback mechanism and experiments using zebrafish embryos are considered in vitro up to 120 hpf.

4) Thyroid hormone activity at the target tissue

The inhibition of T3-mediated **TR activation** and subsequent pituitary cell proliferation was assessed in a test denoted T-screen. This simple and inexpensive assay encompasses the binding of a test compound with TR agonist or antagonist activity to TRs endogenously expressed in GH3 rat pituitary cells, second messenger activity, downstream signal transduction, and gene expression, in the end resulting in mitogenesis dependent on the level of TR activation. The aim of the study reported in Chapter 2 was to find out whether the T-screen in combination with the developed TSH-screen (discussed in section 1 above) can predict in vivo thyroid and pituitary organ weight changes – the latter being parameters used in rodent toxicity assays to

detect thyroid-active compounds. To this end, 11 compounds found to be thyroid-active in rodent tests were tested in both screens. Results indicated that the in vitro in vivo correlation was poor and highlighted the shortcoming of in vitro tests such as the lack of an intact hormonal feedback system. This is in contrast with the E-screen, which successfully correlated the compound-mediated inhibition of estrogen-induced uterine cell proliferation to in vivo uterine weight change (Wang et al., 2012). Another factor that has to be taken into account is the complexity of the thyroid hormone system which relies on multi-organ interactions to maintain hormonal homeostasis (Jomaa, 2014). Nonetheless, further analysis of the data revealed that the EC50s for T3, T4, triac and tetrac-mediated pituitary cell proliferation in the T-screen correlated with the BMDL10 for in vivo change in relative heart weight as induced by these compounds. Indeed, thyroid hormones have previously been reported to be linked with cardiac hypertrophy (Ojamaa, 2010). This raises the possibility of using the T-screen to predict the adverse effect of thyroid-active compounds on heart weight.

In Chapter 4, we focused our research on **coregulator interaction**. Coregulators are mediators of transcription, and the effect of ligands in modulating TR-coregulator interactions was evaluated as a way of detecting thyroid-active compounds. Protein-protein interactions were previously studied by other groups with the use of the yeast-two hybrid technique, which is a type of protein fragment complementation assay that is carried out in yeast (Brückner et al., 2009). While yeast two-hybrid screening helped identify SMRT and NCoR as proteins showing decreased interaction with TR upon T3 binding, new high-throughput methods are also available such as peptide arrays (Chen and Evans, 1995; Hörlein et al., 1995). These are mostly based on tagged TR ligand-binding domains (LBDs) that are added to a library/array of known co-regulator peptides that contain the LXXLL binding motif (L for leucine, X for any amino acid) (Moore et al., 2004; Johnson et al., 2011). Peptides are easier to handle yet maintain, by encompassing complete functional motifs, some of the essential features of protein function (Volkmer et al., 2012). In chapter 4, we have used the so-called microarray assay for real-time coregulator-nuclear receptor interaction (MARCoNI) (Houtman et al., 2012) using the LBDs of TR isoforms alpha and beta in conjunction with 4 thyroid-active compounds, namely, T3, T4, amiodarone and compound 1-850. The results underlined the power of such an experimental platform in delivering insights into the mechanism underlying thyroid receptor agonists and antagonists action by unraveling the coregulators involved. In addition, hierarchical clustering of the modulation index, which is defined as the log ratio of the binding of the receptor to the coregulator in the presence of a ligand over that of the solvent control (Koppen et al., 2009), resulted in agonists T3 and T4 clustering separately from antagonists amiodarone and 1-850. The

MARCoNI assay therefore has the potential to be used in the detection of thyroid-active compounds within a wider battery of tests that include other endpoints. The MARCoNI assay has several advantages over reporter gene assays since the use of luciferase as the reporter gene may generate false positives and the endogenous expression of other receptors or receptor isoforms can confound the results generated (Sotoca et al., 2010; Bhattacharjee and Khurana, 2014).

5) Kinetics

Other endpoints that have to be included within a comprehensive integrated testing strategy for thyroid-active compounds should include **TH blood transport** by binding to the transport proteins transthyretin (TTR) and thyroxine binding globulin (TBG), **cellular uptake of TH** by monocarboxylate transporter 8 (MCT8) and organic anion-transporting polypeptide (OATP) and **TH metabolism** by deiodinases (DIOs) and liver enzymes including UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs). The following text in this section will list some promising assays that are likely candidates for inclusion within an integrated testing strategy for thyroid-active compounds.

The 8-anilino-1-naphthalenesulfonic acid ammonium (ANSA) assay can be used to detect compounds that bind to the transport proteins TTR and TBG and hence have the potential to compete with thyroid hormones in blood transport. When ANSA binds to TTR or TBG, the fluorescence peak of ANSA shifts from 515 to 465 nm and the fluorescence intensity is substantially increased. By placing an emission filter near 465 nm and a defined ratio of ANSA–TTR, the decrease in fluorescence at that wavelength due to increasing concentrations of T4 is assessed (Cao et al., 2010). This gives a dose–response curve from which a half maximal inhibitory concentration (IC50) can be calculated.

IC50 values for the displacement of ANSA from TTR by test compounds are calculated in a similar manner. The obtained data can then be used to rank the test compounds in terms of T4 equivalency factors (TEQ) by dividing the T4 IC50 by the test compound IC50. In other words, the higher the TEQ the more potent the test compound is at displacing T4 from its binding proteins. Such a method has been successfully applied to test the potential of hydroxylated PCBs (polychlorinated biphenyls) and polybrominated diphenyl ethers (PBDEs) for competitive binding to TTR (Montaño et al., 2012).

MCT8- and OATP-mediated cellular uptake of thyroid hormones can be assessed by transport studies with the radiolabeled hormones [125I]T4 and [125I]T3 (Kinne et al., 2009; Westholm et al., 2009). However, since the target tissues are many and the cell types that they contain are diverse, mimicking the physiological setting in an exact manner is inefficient while screening for thyroid hormone disrupters. Hence, an

artificial system has been created with controlled settings. Cells are selected based on low endogenous expression of the transporters being studied and then they are stably transfected with an expression vector conferring a constitutive high level of transporter expression (Kinne et al., 2009; Westholm et al., 2009). In this way, significant amounts of thyroid hormones can be transported across the cell membrane and by comparing with the wild-type cell line it can be concluded that the effect is specific to the studied transporters. It can be deduced that transport studies measuring the uptake of [125I] T4 and [125I]T3 by cells stably transfected with thyroid hormone transporters have the potential to be used to quantify the interference with thyroid hormone transport by thyroid-active compounds. Radioisotope-free alternative assays still have to be developed for the inhibition of MCT8- and OATP-mediated thyroid hormone transport by thyroid-active compounds.

Assessing the deiodination of T4 to the more active T3 by deiodinase enzymes (DIOs) using in vitro testing is currently being tested by measuring [125I] levels (Kuiper et al., 2005). Such tests are not suitable for high throughput testing due to the radioactive waste produced. With improvements in techniques and hardware, it is becoming increasingly likely that future tests might be based on tandem mass spectrometry as is being done for TH measurement in the diagnosis of thyroid disease (van Deventer and Soldin, 2013).

The last part to be considered in the kinetics compartment is TH metabolism and clearance. In metabolism and clearance studies, liver microsomes are currently the gold standard for testing for the inhibition of liver enzymes. The enzyme incubation is usually followed by an analytical technique, such as high-performance liquid chromatography (HPLC), in order to quantitatively determine the metabolite profile of TH (Shah et al., 2008). The applicability of such assays for thyroid metabolism in a high throughput setting still has to be evaluated and forms yet another necessary avenue for future research.

6) Epigenetics, immune response and other mechanisms: ITG αvβ3 activation

Epi is Greek for above and epigenetics literally means above genetics, which is a term used to describe heritable changes in gene expression that are not due to an altered sequence of DNA. This change in gene expression can be due to a variety of factors including DNA methylation, histone modification and non-coding RNA-associated gene silencing. This field has unraveled in the past decade and the role of epigenetics in mediating the toxicity of environmental chemicals is the subject of a growing amount of research (Baccarelli and Bollati, 2009; Li et al., 2014). It has recently been shown that the phenolic herbicide ioxynil (IOX) and the brominated flame retardant tetrabromobisphenol A (TBBPA) affect

TH-induced histone modifications, leading the authors to conclude that these compounds exert an epigenetic effect on TH receptor-mediated gene expression (Otsuka et al., 2014). The inclusion of epigenetic testing within an integrated testing strategy for thyroid-active compounds requires high throughput techniques, which can include quantitative mass spectrometry (MS)-based proteomics and genomic sequencing (Stunnenberg and Vermeulen, 2011). Future research could be conducted on the effect of IOX and TBBPA on coregulator modulation in a MARCoNI assay since, in Chapter 4, we have found histone modifying coregulators among the coregulators that interact with thyroid hormones.

Thyroid-active compounds can also affect immune response as was found in a study showing a link between iodine and autoimmune thyroditis (Rose et al., 1999). Moreover, soy isoflavones have been found to be involved in the formation of TPO neoantigens during their covalent binding to and inactivation of the enzyme (Doerge and Sheehan, 2002). It is still unknown what other compounds that inhibit TPO by covalent binding can also lead to the formation of neoantigens and more importantly, it is necessary to study the clinical relevance of these findings. Hence more research is necessary on this topic before it can be deemed a priority for inclusion within a wider panel of tests for thyroid-active compounds.

The modulation by T4 of the cell surface receptor integrin alpha v beta 3 (ITG αvβ3) is the least understood and perhaps the most intriguing mechanism of action of thyroid hormones and it has been found to induce a downstream Mitogen-activated protein kinase (MAPK) signaling cascade (Bergh et al., 2005). While this might help explain some of the in vivo effects of T4 that cannot be accounted for by other mechanisms, it is not yet understood if the disruption of T4-mediated ITG αvβ3 signaling cascades is a main target of thyroid-active compounds. By establishing the profile of T4-medated ITG αvβ3 kinase activation using a kinase array, it is possible for future research to determine the modification of this profile by thyroid-active compounds. Kinase array technology is high throughput and can enable kinetic monitoring of kinase activity from cell lysates (Hilhorst et al., 2009).

Conclusions

In the pursuit of an in vitro ITS for the testing of thyroid-active compounds, the present thesis:

1. Assessed the in vivo relevance of a newly developed TSH-screen and other currently available thyroid and pituitary cellular proliferation assays.
2. Outlined the development of a new assay for the detection of chemical inhibitors of TPO.

3. Reported the application of MARCoNI for the unraveling of TR-coregulator modulation by ligands.
4. Set up a new zebrafish general development screen that includes endpoints affected by thyroid active compounds.
5. Proposed a multi-compartment model approach that may be of help in the further development of an in vitro integrated testing strategy for the detection of thyroid-active compounds - based on the in vitro assays studied as well as complementary future assays.

The findings described in this thesis have contributed to the current search for alternatives to animal testing and not only adhered to the 3R principles of ethical animal testing, but also improved the human relevance of current tests.

In view of the severity of the adverse effects that can be expected from chemical disruption of the thyroid system, there is an urgent need to follow an integrated testing strategy for the testing of thyroid-active compounds. The set of in vitro tests that we developed and those that still need further development in the future shows that there is still a lot of work to be done in order to develop comprehensively predictive in vitro models for thyroid disruption.

The inherent complexity of the thyroid hormone system makes it unlikely that one in vitro test alone will encompass the multi-organ interactions that result in thyroid hormone homeostasis in man. Moreover, the important role that thyroid hormones play during development makes finding a simple in vitro alternative even more challenging. A reliable in vitro screening strategy for thyroid-active compounds must therefore include a battery of in vitro tests within a comprehensive integrated testing strategy. Thyroid activity can be affected at various levels through a wide range of modes of action and this thesis illustrates that some of these modes of action can be successfully modeled in in vitro systems, while for others this is more difficult. In particular, the zebrafish, from 0–5 days post-fertilization, may prove to be an invaluable addition within an ITS as an in vitro model with intact feedback mechanisms that can be used to screen thyroid-acting compounds for developmental toxicity.

Further research into suitable in vitro models and the development of high-throughput screens might lead to a comprehensive testing program, decreased expenditure for government agencies, and a decreased need to test on rodents and other mammals. Suggestions include the use of DNA microarrays, further development of peptide microarrays and computer modeling of individual assay results in order to recreate in vivo feedback mechanisms in silico.

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Chapter 7

Summary

The aim of this thesis was to find in vitro and toxicogenomics-based alternatives to in vivo thyroid hormone disruption tests. In vitro alternatives can help reduce the amount of animal testing required under the European Union regulation for the registration, evaluation, authorization and restriction of chemicals (REACH). Moreover, with the use of human cell lines and human-identical synthetic proteins, interspecies differences can be reduced and in some cases eliminated.

Chapter 1 introduces thyroid disruption and sets the scene for the rest of the thesis. It starts with a brief overview of the historical events that have shaped our understanding of the thyroid hormone system and have culminated in public concern over endocrine-active chemicals. One such decisive event was the so-called 'cranberry scare', in which a herbicide, aminotriazole, was shown in a rodent study to increase the incidence of thyroid cancer. Today, the fear is focused on compounds that may alter thyroid homeostasis in a subtle way at much lower doses than those causing cancer but still have the potential to affect the hormone system's well-documented orchestration of sexual and mental development. Regulatory authorities have acknowledged the potential dangers to public health and have included endpoints related to endocrine disruption in chemical safety testing using experimental animal models. Rodent assays, the golden standard for the detection of thyroid-active compounds, are costly, have disputable ethicality and do not always represent effects seen in humans. It is for these reasons that in vitro alternatives would be desirable. Chapter 1 then presents the current state of the art in thyroid toxicity testing, followed by the objective of the studies described in this thesis, which is to identify and develop in vitro and toxicogenomics-based alternatives to in vivo thyroid hormone disruption tests.

Chapter 2 details the testing of eleven thyroid-active compounds that have been documented to result in pituitary and/or thyroid weight change in in vivo studies, in in vitro pituitary and thyroid cell-proliferation assays denoted T-screen and TSH-screen, respectively. Analysis of the results revealed that in vitro-in vivo correlation is poor, and this implies that these assays, on their own, cannot be representative of the thyroid system. It was concluded that further research is required in order to model the thyroid system, which represents a complex multi-organ paradigm. Two approaches were proposed and tested to a further extent in the present thesis, the first involving a wider battery of in vitro tests and the second involving in vitro zebrafish embryo-based tests. Nonetheless, a good correlation was observed between TR-mediated pituitary cell proliferation in the T-screen and increase in heart weight, suggesting that the T-screen might represent TH-mediated increases in heart weight.

In **Chapter 3**, an alternative luminescence-based in vitro test for thyroid peroxidase (TPO) inhibitors was developed which uses human TPO derived from a cultured cell line.

This test was evaluated using 10 model compounds which were correctly identified as either inhibitors or non-inhibitors of TPO and was found to provide results with a smaller 95% confidence interval than two other currently-available tests. Another important advantage of the newly developed in vitro test is that it enables the use of human TPO as an alternative to porcine TPO.

In **Chapter 4**, the microarray assay for real-time coregulator-nuclear receptor interaction (MARCoNI) was applied to test the effect of two known thyroid hormone receptor (TR) agonists and two known TR antagonists in order to assess the usefulness of this in vitro test as part of an integrated testing strategy for thyroid-active compounds. In the MARCoNI assay, a peptide microarray with 154 motifs representing 66 different nuclear receptor coregulators is exposed to TR isoform α or β with and without ligand. The MARCoNI assay indicated that ligand-induced modulation of TR-coregulator interactions is a potentially useful parameter to detect TR receptor agonists and antagonists and also to distinguish agonists from antagonists.

However, an integrated testing strategy for thyroid-active compounds must also include a more complete test system, preferably with an intact hormonal feedback loop. This is explored in **Chapter 5** with the use of zebrafish embryos aged from 0 – 120 hours post-fertilization (hpf). Zebrafish development was scored using the general morphology score (GMS) which was developed for the 0 – 72 hpf time window. The results indicated that the morphological parameters affected upon exposure to thyroid active compounds develop after 72 hpf, and thus an adjusted scoring system was developed and named the general development score (GDS). In addition to the thyroid-related morphological parameters, dysmorphologies were also integrated into the same scoring system. The results of the study suggested that this new zebrafish-based GDS morphological scoring system would indeed present a valuable contribution to an integrated testing strategy for thyroid-active compounds, specifically addressing their potential developmental toxicity.

Chapter 6 goes on to discuss the results presented in this thesis in context of the initial outline and objectives presented in Chapter 1. A number of in vitro tests were successfully developed covering TSH-dependent cellular proliferation, TPO inhibition and zebrafish developmental toxicity including thyroid-related endpoints, in addition to the application of the MARCoNI assay to measure interference with TR-coregulator interaction. However, attention was also given to the challenges ahead, mainly the lack of suitable tests for high throughput screens covering many relevant endpoints that are important for the thyroid hormone system. The development of a cell-based system that produces thyroid hormones and the replication of multi-organ hormonal feedback mechanisms are among the most important challenges that need to be overcome. Suggestions include the use of DNA microarrays, further development of peptide

microarrays and computer modeling of individual assay results in order to recreate in vivo feedback mechanisms in silico.

In conclusion, this thesis has shed light on the relevance of current in vitro assays for thyroid and pituitary cell proliferation, has led to the development of the TSH screen, a luminol-based TPO inhibition assay and the zebrafish-based GDS for the detection of developmental toxicants, including those that act through the thyroid hormone system. Moreover, the MARCoNI assay was used to reveal the modulating effects of thyroid-active compounds on TRα and TRβ interactions with a peptide array representing 66 different coregulators. These developments along with an in-depth analysis of the thyroid hormone system and the presentation of the state of the art in thyroid disruption testing have highlighted the progress made and at the same time have underlined the challenges that lay ahead.

Chapter 8

Samenvatting

Het doel van dit proefschrift was om in vitro en op toxicogenomics-gebaseerde alternatieven te ontwikkelen voor in vivo proeven met betrekking tot ontregelde schildklierhormoonwerking. In vitro alternatieven kunnen leiden tot een vermindering van het aantal dierexperimenten die noodzakelijk zijn in het kader van de regelgeving van de Europese Unie voor de registratie, evaluatie, autorisatie en beperking van chemische stoffen (REACH). Bovendien, door gebruik te maken van humane cellijnen en aan de menselijke variant-identieke relevante eiwitten kan interspecies variatie worden verminderd en in sommige gevallen zelfs volledig worden geëlimineerd.

In **Hoofdstuk 1** wordt het onderwerp verstoorde schildklierhormoonwerking geïntroduceerd. Dit hoofdstuk begint met een kort overzicht van de historische gebeurtenissen die ons begrip van het schildklierhormoonsysteem hebben gevormd en hebben geleid tot publieke bezorgdheid over endocrien-actieve stoffen. Een voorbeeld van een dergelijke beslissende historische gebeurtenis was de zogenaamde “cranberry scare”, waarbij het gebruik van een herbicide, aminotriazole, in een proefdierstudie met knaagdieren geassocieerd bleek te zijn met een verhoogde incidentie van schildklierkanker. Tegenwoordig gaat de aandacht vooral uit naar stoffen die de schildklierhomeostase op een subtiele manier veranderen, bij veel lagere doseringen dan die kanker veroorzaken, maar nog steeds het potentieel hebben om het hormonale systeem, dat verantwoordelijk is voor de orkestratie van seksuele en geestelijke ontwikkeling, negatief te beïnvloeden.

Regelgevende instanties hebben de potentiële gevaren voor de volksgezondheid erkend en hebben eindpunten toegevoegd met betrekking tot de verstoring van de hormoonhuishouding aan de proefdiertesten voor chemische veiligheid. Proeven met knaagdieren zijn de gouden standaard voor de detectie van schildklier-actieve verbindingen maar ze zijn erg duur, roepen ernstige ethische vragen op en geven niet altijd een goede voorspelling van de effecten in de mens. Om deze redenen zouden in vitro alternatieven dus wenselijk zijn. Hoofdstuk 1 geeft de huidige stand van zaken weer met betrekking tot schildklier toxiciteitstesten, gevolgd door de doelstelling van de in dit proefschrift beschreven studies; namelijk het identificeren en ontwikkelen van in vitro en op toxicogenomics-gebaseerde alternatieven voor in vivo testmethoden met betrekking tot ontregelde of verstoorde schildklierhormoonwerking.

Hoofdstuk 2 beschrijft het testen in in vitro hypofyse- en schildkliercelproliferatie assays, respectievelijk aangeduid als T-screen en TSH-screen, van 11 schildklier-actieve stoffen waarvan bekend is dat zij leiden tot gewichtsveranderingen van de hypofyse en/of de schildklier in vivo. Uit analyse van de resultaten bleek dat de in vitro-in vivo correlatie slecht was, hetgeen impliceert dat deze testen op zichzelf niet representatief zijn voor het schildkliersysteem. We concludeerden dat er verder onderzoek nodig is om

de schildklier, een paradigma van een complex multi-orgaansysteem, te “modelleren”. In dit proefschrift werden 2 benaderingen voorgesteld en verder getest. De eerste benadering bestond uit een bredere reeks van in vitro testen en het tweede behelsde in vitro proeven met zebravisembryo’s. Overigens beschrijft hoofdstuk 2 dat wel een goede correlatie werd gevonden tussen-TR gemedieerde hypofysecelproliferatie in de T-screen en een toename van het hartgewicht, wat suggereert dat de T-screen een TH-gemedieerde toename in gewicht van het hart zou kunnen detecteren.

In **Hoofdstuk 3** wordt een alternatieve op luminescentie gebaseerde in vitro test voor schildklierperoxidase(TPO)-remmers ontwikkeld, waarbij gebruik gemaakt werd van humaan TPO, verkregen uit gekweekte cellen. Deze test werd vervolgens geëvalueerd met een behulp van een panel van 10 modelverbindingen die correct werden geïdentificeerd als remmers of niet-remmers van TPO. Een belangrijke bevinding was dat de resultaten van deze test een kleiner 95% betrouwbaarheidsinterval opleverden dan twee andere momenteel beschikbare testen. Een ander voordeel van deze nieuwe in vitro assay was dat er gebruik gemaakt werd van humaan TPO in plaats van TPO verkregen uit varkens.

In **Hoofdstuk 4** werd gebruik gemaakt van de microarray assay voor real-time coregulator-nucleaire receptor interactie (MARCoNI) om het effect van twee bekende schildklierhormoonreceptor (TR)-agonisten en twee bekende TR-antagonisten te testen, en zo het nut van deze in vitro test te evalueren als onderdeel van een geïntegreerde teststrategie voor schildklier-actieve verbindingen. In de MARCoNI test wordt een peptide microarray met 154 motieven die 66 verschillende nucleaire receptor coregulatoren representeren, blootgesteld aan TR-isovorm α of β met en zonder ligand. De MARCoNI-test gaf aan dat ligand-geïnduceerde modulatie van TR-coregulatorinteracties mogelijk een nuttige parameter is om TR-agonisten en -antagonisten te detecteren en ook om agonisten te onderscheiden van antagonisten.

Een geïntegreerde teststrategie voor schildklieractieve verbindingen dient echter ook een compleet testsysteem te bevatten, bij voorkeur met een intacte hormonale feedback loop. Dit werd onderzocht in **Hoofdstuk 5** door gebruik te maken van zebravisembryo’s met een leeftijd van 0 tot 120 uur postfertilisatie (HPF). De ontwikkeling van de zebravis werd gescoord met behulp van de algemene morfologiescore (GMS), die werd ontwikkeld voor de 0 - 72 HPF periode. De resultaten gaven duidelijk aan dat de morfologische parameters pas na 72 HPF beïnvloed werden door blootstelling aan schildklieractieve stoffen. Naar aanleiding hiervan werd een aangepast scoresysteemontwikkeld, namelijk de algemene ontwikkelingsscore (GDS). Naast de aan de schildklier gerelateerde morfologische parameters werden ook dysmorfologieën geïntegreerd in hetzelfde scoresysteem. De resultaten van deze studie gaven aan dat dit nieuwe GDS scoresysteem inderdaad een waardevolle bijdrage kan leveren aan een

geïntegreerde teststrategie voor schildklieractieve verbindingen, in het bijzonder voor het identificeren van mogelijke toxische effecten op de ontwikkeling.

In **Hoofdstuk 6** worden de in de voorgaande hoofdstukken verkregen resultaten verder bediscussieerd in de context van het overzicht en de doelstellingen gepresenteerd in Hoofdstuk 1. Er werden met succes een aantal in vitro testen ontwikkeld voor door TSH-gereguleerde cellulaire proliferatie, TPO-inhibitie en ontwikkelingstoxiciteit bij de zebravis, via schildklier-gerelateerde eindpunten, en bovendien de toepassing van de MARCoNI-assay om interferentie met TR-coregulatorinteractie te meten. Er werd ook aandacht besteed aan de uitdagingen voor de toekomst, met name het opzetten van geschikte testen waarmee het mogelijk is om stoffen high-throughput te screenen op de vele eindpunten die belangrijk zijn voor het schildklierhormoonsysteem. Tevens behoren het ontwikkelen van een cel-gebaseerd systeem om schildklierhormonen te produceren en het in vitro simuleren van multi-orgaan feedbackmechanismen tot de belangrijkste uitdagingen. In het licht hiervan worden een aantal suggesties gedaan, zoals de verdere ontwikkeling van peptide microarrays en het genereren van computermodellen waarmee op basis van individuele assayresultaten, de in vivo feedbackmechanismen in silico nagebootst kunnen worden.

Uit de bevindingen, beschreven in de onderzoekshoofdstukken 2, 3, 4 en 5, kan geconcludeerd worden dat dit proefschrift licht geworpen heeft op de relevantie van de huidige in vitro testen voor schildklier en hypofyse celproliferatie. Voorts hebben de bevindingen uit dit proefschrift geleid tot de ontwikkeling van een TSH screeningmethode, een op luminol gebaseerde TPO inhibitie-assay en de op zebravis gebaseerde GDS voor de detectie van toxische stoffen die de ontwikkeling verstoren, met inbegrip van toxines die werken via het schildklierhormoonsysteem. Bovendien is de MARCoNI-assay geschikt gebleken om de modulerende effecten van schildklieractieve verbindingen op interacties van TRα- en TRβ met transcriptionele coregulatoren aan te tonen. Deze nieuwe ontwikkelingen, samen met de diepgaande analyse van het schildklierhormoon systeem en het overzicht van de huidige state-of-the-art in het testen van verstoringen van de schildklier, geven een beeld van de geboekte vooruitgang en onderstrepen tegelijkertijd de uitdagingen voor de toekomst.

Appendix

Acknowledgements, curriculum vitae, list of publications and overview of completed training activities

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Curriculum Vitae

Barae Georges Jomaa was born in Beirut, Lebanon on January 31, 1978. He attended pre-university education in both Lebanon and Montréal, Canada. In 2002, he received a Bachelor of Science degree as well as a diploma in Agricultural Engineering. During that time he conducted research on the effect of ethanol on the social behavior of honey bees with the help of Professor Charles Abramson of Ohio State University. Jomaa also gave a seminar entitled "Update on Bovine Spongiform Encephalopathy". From 2002 until 2006, he worked in journalism. In 2006, he enrolled at Wageningen University, Wageningen, The Netherlands, in the Master's of Science in Medical Biotechnology program. He wrote his Master's thesis entitled "Construction and Evaluation of FOXO3 Luciferase Reporter Vectors and the Identification of Stimuli that Activate FOXO3 in Neuroblastoma" at the Human Genetics Department of the Academic Medical Centre of Amsterdam University under the leadership of Professor Rogier Versteeg and the supervision of PhD candidate Evan Santo. He conducted his Master's internship working on "Identification and Evaluation of the ECT2 Binding Site on Anillin" at the Department of Biology of Concordia University, Montréal, Canada under the mentorship of Associate Professor Alisa Piekny. During that time he also followed a course with Oxford York Health Care Services (OYHC) to become an Accredited, Health Care Provider BLS/AED-Level C+ CPR. Back in The Netherlands, he completed his Master's degree in 2009 and then followed the course "Systems Biology" where he met his soon-to-be co-promoter Jac Aarts as a classmate. With the guidance of Jac Vervoort, who was instructing the course, he found an interesting PhD research topic entitled "Toxicogenomics-based In Vitro Alternatives to In Vivo Thyroid Hormone Disruption Tests". From 2010 until the completion of this thesis he worked on this project, which is funded by the Netherlands Toxicogenomics Centre, under the direction of Professor Ivonne Rietjens. Alongside his PhD research, he followed postgraduate education in toxicology, resulting in his certification as a European Registered Toxicologist (ERT) in 2015.

List of publications

Jomaa, B., Houtman, R., de Haan, L.H.J., Peijnenburg, A.A.C.M., Aarts, J.M.M.J.G., Rietjens, I.M.C.M. Identification of thyroid hormone receptor coregulator interactions and profiling of their modulation by T3, T4, amiodarone and compound 1-850. In preparation for submission.

Jomaa, B., de Haan, L.H.J., Peijnenburg, A.A.C.M., Bovee, T.F.H., Aarts, J.M.M.J.G., Rietjens, I.M.C.M. (2015). Simple and rapid in vitro assay for detecting human thyroid peroxidase disruption. ALTEX.

Jomaa, B., Hermsen, S.A.B., Kessels, M.Y., van den Berg, H.H.J., Peijnenburg, A.A.C.M., Aarts, J.M.M.J.G., Piersma, A.H., Rietjens, I.M.C.M. (2014). Developmental toxicity of thyroid-active compounds in a zebrafish embryotoxicity test. ALTEX 31, 303-17.

Jomaa, B. (2014). Thyroid toxicogenomics: A multi-organ paradigm. In Kleinjans, J. [ed.], Toxicogenomics-Based Cellular Models: Alternatives to Animal Testing for Safety Assessment, 159–180. Academic Press.

Jomaa, B., Aarts, J.M.M.J.G., de Haan, L.H.J., Peijnenburg, A.A.C.M., Bovee, T.F.H., Murk, A.J., Rietjens, I.M.C.M. (2013). In vitro pituitary and thyroid cell proliferation assays and their relevance as alternatives to animal testing. ALTEX 30, 293–307.

Overview of completed training activities

Discipline specific activities

- Course Pathobiology, Postgraduate Education in Toxicology (PET), 2011
- Course Cell Toxicology, PET, 2012
- Course Epidemiology, PET, 2012
- Course Medical and Forensic Toxicology, PET, 2012
- Course Organ Toxicology, PET, 2013
- Course Risk Assessment, PET, 2013
- Course Molecular Toxicology, PET, 2014
- Course Immunotoxicology, PET, 2014

Meetings

- Netherlands Toxicogenomics Centre (NTC) Annual Meeting, 2011, poster presentation
- NVT days, Nederlandse Vereniging Toxicology, 2011, poster presentation
- NVT days, Nederlandse Vereniging Toxicology, 2012, poster presentation
- Netherlands Toxicogenomics Centre (NTC) Annual Meeting, 2013, oral presentation
- NVT days, Nederlandse Vereniging Toxicology, 2013, poster presentation
- Netherlands Toxicogenomics Centre (NTC) Final Conference, 2013, poster presentation
- Congress of the European Societies of Toxicology (Eurotox), Switzerland, 2013, poster presentation
- NVT days, Nederlandse Vereniging Toxicology, 2014, oral presentation and poster presentation
- Congress of the European Societies of Toxicology (Eurotox), United Kingdom, 2014, poster presentation

General courses

- VLAG PhD week, VLAG, 2010
- Course Genomics in Molecular Med., NIHES, 2012
- Course Principles of Research in Medicine and Epidemiology, NIHES, 2012
- Course Principles of Genetic Epidemiology NIHES, 2012
- Course Lab. Animal Science, PET, 2011

Optional activities

- Preparation Research Proposal, 2010
- Attending scientific presentations, toxicology, WUR, 2010 - 2014
- International Toxicology PhD Excursion, Italy and Switzerland, 2011
- MSc course Environmental Toxicology, WUR, 2014
- MSc course General Toxicology, WUR, 2014
- Organizing Int. PhD Excursion, 2014

Approved by the graduate school VLAG

Colophon

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