In vitro thyroid hormone metabolism**:**
Effects of nuclear receptor activation on the metabolic profiles of thyroxine in rat and human hepatocytes

Vicki Michele Richardson

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum of Toxicology.

Chapel Hill
2013

Approved by:

Michael DeVito, PhD

Louise Ball, PhD

Kim Brouwer, Pharm.D., PhD

Edward LeCluyse, PhD

Mary Paine, PhD, RPh

ABSTRACT

VICKI RICHARDSON: *In Vitro* Thyroid Hormone Metabolism**:**
Effects of nuclear receptor activation on the metabolic profiles of thyroxine in rat and human hepatocytes
(Under the direction of Michael J. DeVito, PhD)

Thyroid hormones are critical in the normal growth and development of amphibians, birds, fish, and mammals. There are numerous xenobiotics that interfere with thyroid hormone homeostasis; therefore, exposure to these xenobiotics could be detrimental to the growth and development.. Xenobiotics categorized as thyroid hormone disruptors have been defined by their ability to reduce circulating concentrations of thyroid hormone in serum. It is has been proposed that thyroid hormone disruption occurs through the induction of thyroxine (T_4) glucuronidation and biliary elimination which ultimately results in reduced serum T_4 concentrations. This mode of action has been described using animal models, but the relevance to humans has not been determined. This research tests the hypothesis that there are species differences in the hepatic metabolism of thyroid hormones and these differences occur via nuclear receptor activation. Here we demonstrate the utility of sandwich-cultured rat and human hepatocytes in measuring T_4 metabolism following the activation of Aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) or pregnane x receptor (PXR). The relationship between T_4 metabolism and nuclear receptor activation was studied through the establishment of an *in vitro* assay for the qualitative and quantitative

measurement of T_4 metabolites. Here we report that hepatic glucuronidation may be a more important pathway for T_4 metabolism in rats whereas T_4 deiodination may be a favored pathway in humans. Following nuclear receptor activation, glucuronidation is a primary route of T_4 metabolism in rat and humans hepatocytes. Agonists of CAR/PXR are more consistent in the induction of T_4 glucuronidation in rat and human hepatocytes. We also show similarities in the *in vivo* and *in vitro* effect on T_4 metabolism in response to the environmental contaminant, 2,2′,4,4′-tetrabromodiphenyl ether (BDE 47). These results indicate possible species differences in hepatic T_4 metabolism and these differences may be based on nuclear receptor activation.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Dr. Michael DeVito, whose guidance on this project has been invaluable. To my committee members, Drs. Louise Ball, Kim Brouwer, Edward LeCluyse, and Mary Paine, thank you for your time and thoughtful criticism. I would especially like to thank Dr. Stephen Ferguson for his willingness to provide his support and expertise on numerous occasions. I must also express my appreciation to Dr. Linda Birnbaum for her encouragement and counsel over the years.

To my wonderful group of friends, thank you for your forgiveness and words of comfort. To David Ross and Dr. David Szabo, thank you for the laughter and friendship. To my dearest friend Monica Richard, thank you for being the voice of reason and my greatest cheerleader.

Finally, I would like to thank my parents for their love and inspiration. Knowing they were always in my corner gave me the courage to make my dreams come true.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

3MC	3-methylcholanthrene
ABC	Adenosine triphosphate binding cassette
ANOVA	Analysis of variance
ACN	Acetonitrile
AhR	Aryl hydrocarbon receptor
ALB	Albumin
BLQ	Below limits of quantitation
β-NF	Beta naphthoflavone
BDE-47	2,2′,4,4′-tetrabromodiphenyl ether
BSA	Bovine serum albumin
Cl_{int}	Intrinsic clearance
CAR	Constitutive androstane receptor
CITCO	6-(4-chlorophenyl)imidazo [2,1-*b*] thiazole-5-carbaldehyde *O*-(3,4 dichlorobenzyl) oxime
CO_2	Carbon dioxide
CYP	Cytochrome P450
D1	Type 1 deiodinase
D2	Type 2 deiodinase
D3	Type 3 deiodinase
DE-71	Pentabromodiphenyl ether
DMSO	Dimethyl sulfoxide
DMP 904	4-(3-pentylamino)-2,7-dimethyl-8-(2-methyl-4-methoxyphenyl)-pyrazolo-[1,5-a]-pyrimidine

EDTA	Ethylenediaminetetraacetic acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HCl	Hydrogen chloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPT	Hypothalamus-pituatary-thyroid
ITS+	Insulin, transferrin, and selenium complex], gentamicin, L-glutamine, and HEPES
MCT	Monocarboxylate transporters
MDR	Multidrug resistance proteins
$MgC1_2$	Magnesium chloride
mRNA	Messenger ribonucleic acid
MRP2	Mutidrug resistance-associated protein 2
MRP3	Mutidrug resistance-associated protein 3
N_2	Nitrogen
Na	Sodium
NaOH	Sodium hydroxide
NHANES	National health and nutrition examination survey
NTCP	Na+/taurocholate-cotransporting polypeptide
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
P450	Cytochrome P450
PAPS	3'-Phoshoadenosine-5'-phosphsulfate
PB	Phenobarbital
PBS	Phosphate buffered saline

PBDE	Polybrominated diphenyl ether
PCB	Polychlorinated biphenyl
PCB 153	2,2′,4,4′,5,5′-hexachlorobiphenyl
PCDD	Polychlorinated dibenzo-*p*-dioxins
PCDF	Polychlorinated dibenzofurans
PCN	Pregnenolone-16α-carbonitrile
PTU	Propylthiouracil
PXR	Pregnane x receptor
Rif	Rifampicin
RT-PCR	Real time polymerase chain reaction
SCH	Sandwich-cultured hepatocytes
SCRH	Sandwich-cultured rat hepatocytes
SCHH	Sandwich-cultured human hepatocytes
SULT	Sulfotransferase
$T_{1/2}$	Half-Life
T_3	3,3’,5-triiodothyronine
T_4	Thyroxine
rT_3	3,3’,5’-triiodothyronine
T_2	3,3’-diiodothyronine
T_4S	T_4-sulfate
T_4G	T_4-glucuronide
TAT	Tyrosine aminotransferase
TBG	Thyroid binding globulin

TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCPOBOP	1,4-bis[2-(3,5-dichloropyridyloxy)]benzene
Tg	Thyroglobulin
TH	Thyroid hormone
TPO	Thyroperoxidase
TRH	Thyrotropin-releasing hormone
TSH	Thyroid stimulating hormone
TTR	Transthyretin
UPLC	Ultra Performance Liquid Chromatography
UDP	Uridine diphosphate
UDPGA	Uridine diphosphate -glucuronic acid
UDPGT	Uridine-diphosphate glucuronosyl transferase
UGT	Uridine-diphosphate glucuronosyl transferase
XME	Xenobiotic metabolizing enzyme

CHAPTER 1

INTRODUCTION

A. Overview

Thyroid hormones (THs) are essential to development, growth and metabolism in humans with its most prominent effects occurring during fetal development and early childhood. The lack of TH in childhood delays growth and in adults the primary effect is an alteration in metabolism. A broad spectrum of xenobiotics decrease serum THs levels in rodents and these decreases are often associated with the induction of xenobiotics metabolizing enzymes which result in increases in thyroxine (T_4) metabolism and biliary elimination (Barter and Klaassen, 1992; Liu *et al.*, 1995; Kolaja and Klaassen, 1998; Hood *et al.*, 1999; Hood and Klaassen, 2000a; Hood and Klaassen, 2000b; Hood *et al.*, 2003). Xenobiotics that activate the nuclear receptors, such as aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), and pregnane X receptor (PXR), appear to consistently decrease circulating TH concentrations (Barter and Klaassen 1992; Kretschmer and Baldwin 2005; Qatanani *et al.* 2005). One commonality of these receptors is their regulation of xenobiotic metabolizing enzymes (XMEs), in particular uridine diphosphate glucuronosyltransferase (UGT) and sulfotransferase (SULT) and transporters. For example, through the activation of constitutive androstane receptor (CAR), phenobarbital (PB), induces hepatic UGTs in rat (Vansell and Klaassen, 2001; Vansell and Klaassen, 2002a),

which is associated with an increase in the biliary elimination of T_4-glucuronides (Oppenheimer *et al.*, 1968; McClain *et al.*, 1989; Wong *et al.*, 2005). PB increases [^{125}I] liver accumulation in rats (Kato *et al.*, 2010) and increases biliary elimination of [^{125}I]-T_4 and [^{125}I]-T_4G suggesting the involvement of hepatic transporters in cellular uptake and biliary excretion (Mitchell *et al.*, 2005; Visser *et al.*, 2011). PB also decreases serum T_4 concentrations in humans (Ohnhaus *et al.*, 1981; Eiris-Punal *et al.*, 1999). Mechanistic studies in humans are limited; consequently, the hypothyroid effect of PB in humans is thought to occur through the same mechanism as rats. Induction of hepatic UGTs by xenobiotics appears to be a common mechanism in thyroid hormone disruption in rodents; however, it is uncertain if this occurs in humans. The goals of this research are to examine the species differences in T_4 metabolism using human and rat hepatocytes and further explore how these differences are affected by exposure to AhR, CAR, and/or PXR agonists.

B. Nuclear receptors

B.1. Aryl Hydrocarbon Receptor

Aryl hydrocarbon receptor (AhR), also known as the dioxin receptor, is a ligand dependent basic helix-loop-helix/per-ARNT-Sim (bHLH/PAS) transcription factor and is activated by exogenous and endogenous compounds. Once activated, AhR induces or represses a large number of genes involved in biological or toxicological effects in several species and tissues (Hankinson, 2005; Beischlag *et al.*, 2008; Furness and Whelan, 2009). Ligands for AhR include a wide variety of hydrophobic environmental contaminants, including polycyclic aromatic hydrocarbons (PAH) and halogenated aromatic hydrocarbons (HAH). The most toxic and environmentally and biologically persistent compounds are

HAHs, which include polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDD), and polychlorinated dibenzofurans (PCDF) (Safe, 1990). There are species differences in the ligand binding domain of AhR, which may be responsible for species differences in ligand binding and response (Bisson *et al.*, 2009; Pandini *et al.*, 2009). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) a prototypical ligand for AhR, produces toxic effects some of which include tumor promotion, dermal toxicity, disruption of endocrine homeostasis, wasting, and lethality. In humans, a point mutation in the ligand binding domain was found to lower the ability of AhR to bind TCDD by 10-fold compared to mouse AhR (Ema *et al.*, 1994; Ramadoss and Perdew, 2004). TCDD and 3-methylcholanthrene (3MC) induced AhR-regulated genes, CYP1A1 and CYP1A2, to a greater degree in wild type mice compared to AhR humanized mice, demonstrating mouse AhR has a higher binding affinity for TCDD and 3MC than human AhR (Moriguchi *et al.*, 2003).

B.2. Constitutive Androstane Receptor

Constitutive androstane receptor (CAR) is a member of the NR1I3 subfamily of ligand activated transcription factors. CAR is a promiscuous receptor that binds to a wide range of structurally unrelated compounds and regulates numerous genes involved in the metabolism and transport of exogenous and endogenous compounds. Abundantly expressed in the liver, CAR is important in regulating the metabolism and elimination of xenobiotics. Unlike many nuclear receptor orthologs, which share more than 90% homology, human and rodent CAR orthologs share only about 70% amino acid identity in the ligand-binding domain (Moore *et al.*, 2002). This evolutionary divergence in the ligand-binding domain between humans and rodents is apparent with differences in response to xenobiotics. For

example, 1,4-bis-[2-(3,5,-dichloropyridyloxy)] benzene (TCPOBOP) is a potent CAR activator in rodents, but lacks activity in humans (Moore *et al.*, 2000). In contrast, 6-(4-chlorophenyl :imidazo [2,1-*b*] thiazole-5-carbaldehyde *O*-(3,4 dichlorobenzyl) oxime (CITCO) is a potent activator of CAR in human, yet it lacks activity in rats and mice (Scheer *et al.*, 2008). With the divergence of ligand-binding domains between the species, there are compounds such as phenobarbital (PB), which activate both human and rodent nuclear receptors orthologs (Moore, *et al.*, 2002); however this indirect activation is independent of the ligand binding domain (Sidhu and Omiecinski, 1995; Honkakoski and Negishi, 1998).

B.3. Pregnane X Receptor

Pregnane X receptor (PXR) is a member of the NR1I2 family of nuclear receptors and acts as a xenosensor and transcriptional activator (Kliewer and Willson, 2002). PXR is activated by a variety of naturally occurring steroids of which pregnanes are the most potent (Kliewer *et al.*, 1998; Lehmann *et al.*, 1998). Closely related, PXR and CAR share a variety of ligands and target genes (Maglich *et al.*, 2002); however, PXR is more promiscuous than CAR, because it binds to a wide range of compounds that are of different molecular weights and are structurally dissimilar (Jones *et al.*, 2000). PXR exhibits a marked divergence across species within the ligand binding domain, where human and mouse PXR is only 76% homologous; however, the DNA binding domain is 96% homologous. The divergence in the ligand binding domain results in different ligand binding specificities. Pregnenolone 16α-carbonitrile (PCN) is a ligand for rodent PXR, but not for human PXR. Human PXR is activated by rifampicin (Rif); however the rodent PXR is not activated by Rif. The species differences in PXR are consistent with the species differences in CYP3A induction. This

suggests that it is the species ortholog of the receptor and not the CYP3A gene promoter which determines the response.

C. Thyroid Hormones

C.1. Synthesis and Feedback

The main function of the thyroid gland is to produce hormones, T_4 and the active hormone, triiodothyronine (T_3) (Figure 1). T_4 and T_3 are synthesized in the thyroid gland by thyroperoxidase (TPO) where TPO converts iodide to iodine and then attaches it to tyrosine residues. Thyroglobulin (Tg), a large glycoprotein found within the thyroid follicular cells (thyrocytes), serves as a substrate for the synthesis and storage of THs and iodine. When thyroid hormone is needed, Tg is internalized at the apical pole of thyrocytes, where it is digested by proteases, resulting in free T_4 and T_3. After Tg digestion, T_4 and T_3 are released into the circulation.

TH is regulated by a negative feedback loop from the pituitary gland. When the pituitary gland detects too much Thyroid stimulating hormone (TSH) is the predominant regulator of thyroid hormone synthesis and release. Secreted from the pituitary, TSH interacts with its receptor (TSHR) in the thyrocytes to stimulate the accumulation of iodine and expression of the sodium/iodine symporter. T_4 and T_3 regulate the synthesis and release of TSH at the pituitary level, as well as indirectly by affecting TSH synthesis via their effects on the synthesis of TRH. TRH is the major positive regulator of TSH by activating the phospatidylinositol-protein kinase C pathway. Once activated, TRH acts on the anterior pituitary to stimulate the release of TSH. Collectively, these interrelated steps produce and

release thyroid hormones into the blood stream as well as regulate the amount of iodine available to the cells,

C.2. Production and Clearance in Humans and Rats

Although rats are often used to examine extrathyroidal TH metabolism, there are important differences in the production and kinetics of THs in rats and humans (Table 1). In rats, normal plasma T_4 concentrations are approximately 44nmol/L with a half-life ($t_{1/2}$) of 0.5-1 day. T_4 is cleared at a rate of about 50% per day in rats resulting in a daily production of approximately 1nmol/100g body weight(Bianco *et al.*, 2002). The mean normal concentration of total T_4 in human plasma is approximately 100nmol/L (Larsen PR, 1998) amid a daily T_4 production of 110 nmol/70kg body weight. With a half-life ($t_{1/2}$) of 5-9 days, about 10% or nearly 110nmol of T_4 is cleared from the circulation per day in humans. In rats and humans, plasma T_3 is derived from thyroid gland secretion and extrathyroidal deiodination; however, only 20% of plasma T_3 comes from thyroid secretions in humans, whereas 40% is secreted from the thyroid in rats. Mean plasma T_3 concentrations are approximately 750pmol/L in rats. With a turnover rate of over 200% per day ($t_{1/2}$ = 0.2 day), the daily production of T_3 in rats is 415pmol/100g. Humans have a mean normal plasma T_3 concentration of 1.8nmol/L with a daily production rate of 50nmol/70kg body weight and a $t_{1/2}$ of 1.5 days; consequently, the fractional turnover rate of T_3 in plasma is about 65% per day. In humans, of the daily production of T_4 (110nmol/day), 30-40% is converted to T_3 (40nmol) by peripheral deiodination, while the remaining amount (10nmol) is excreted directly from the thyroid(Larsen PR, 1998). In rats, approximately 415 pmol of T_3 is produced daily. About 20-25% of secreted T_4 (1nmol/day) in rats is deiodinated to produce

225 pmol of T_3 per day, while the remaining amount (190 pmol) is excreted directly from the thyroid (Oppenheimer *et al.*, 1972; Bianco, *et al.*, 2002). The molar ratio of T_4/T_3 in rat thyroid is 8:1 where as in humans the ratio is higher (15:1)(Abrams and Larsen, 1973; Izumi and Larsen, 1977). In comparing T_4/T_3 thyroidal secretion, the ratio is 5:1 in rats, while in humans the ratio is 11:1. This indicates there are small contributions of thyroidal deiodinases to T_4 to T_3 conversion in both species.

C.3. Serum Binding Proteins

Specific proteins carry thyroid hormones in the blood and their high affinity binding to T_4 and T_3 are essential to the availability of the hormones to target tissues. In humans, three major proteins bind (THs) in serum: thyroxine-binding globulin (TBG), transthyretin (TTR) and albumin (ALB) (Schussler, 2000). TBG is a low capacity-high affinity binder of thyroid hormones, albumin is a high capacity-low affinity binder and TTR is an intermediate capacity and affinity binder (Power *et al.*, 2000). T_4 affinity is greatest for TBG ($Ka=1x10^{-10}$ M), intermediate for TTR ($Ka=7x10^{-7}$ M), and lowest for ALB ($Ka= 7x10^{-5}$ M). The affinity of T_3 for the binding proteins is lower than that of T_4, where the affinity for TBG is approximately $5x10^{-8}$ M, for TTR it is $1x10^{-7}$ M, and $1x10^{-5}$ M for ALB (Robbins, 1991). All vertebrates have serum-binding proteins for thyroid hormone, but there are species differences in their specific composition within blood (Table 1). In humans, TBG is the least abundant but almost 70% of T_4 is bound to it (Benvenga, 2005). In rats, TBG expression peaks postnatally and is not detectable by early maturity at 8 weeks, but reappears in senescence (Savu *et al.*, 1991). Thus, through most of their lives rats lack one of the major carrier proteins for T_4, with TTR serving as the primary plasma transporter. The binding

affinity of TTR for THs is less than the binding affinity of THs for TBG (Benvenga *et al.*, 2002). In rodents, T_4 is bound to TTR where as T_3 is mainly bound to the least abundant TBG or albumin (Savu *et al.*, 1987). T_4-TTR binding is thought to be more susceptible to chemical interference than T_4-TBG binding (Munro *et al.*, 1989). This suggests that as a major circulating T_4 binding protein, TTR may be important in the disruption of TH homeostasis in rodents.

C.4. Metabolism

C.4.1 Deiodination

Tissue deiodinases are critical in the extrathyroidal conversion of T_4 into its biologically active form, T_3. There are two isoenzymes that convert T_4 to T_3: type I 5'-deiodinase (D1) and type 2 deiodinase (D2). D1 is located primarily in the liver, kidney and thyroid, and D2is located primarily in the brain, thyroid, anterior pituitary, brown adipose, placenta and skeletal muscle. D1 is responsible for most of the conversion of T_4 to T_3 in the blood, while D2 provides conversion of T_4 to T_3 for intracellular use. Type 3 deiodinase (D3) is located in the brain, placenta, fetal tissues and uterus during pregnancy and is responsible for inactivating thyroid hormones by converting T_4 to rT_3 and T_3 to T_2 (Figure 2). In humans, about 80% of circulating T_3 derives from peripheral 5´-monodeiodination of T_4, while the thyroid secretes 20% of the circulating T_3 (Larsen PR, 1998). Unlike humans, the intrathyroidal conversion of T_4 to T_3 provides 40% of the daily T_3 production in rats and the other 60% is derived peripherally (Chanoine *et al.*, 1993). This suggests that peripheral deiodination may play a larger role in TH homeostasis in humans as compared to rodents.

C.4.2. Glucuronidation

Glucuronidation involves the transfer of a sugar moiety on to uridine diphosphate (UDP)-glucuronic acid (UDPGA) to a substrate. The enzymes responsible for glucuronidation of THs are UDP-glucuronoysyltransferases (UGT), which are located mainly in the endoplasmic reticulum of liver cells. The TH glucuronide conjugate is excreted in bile, which may represent a reversible pathway as intestinal bacteria can hydrolyze the conjugates creating an enterohepatic cycle enabling reabsorption of free THs. Induction of UGTs by xenobiotics may play an important role in chemically induced decreases in circulating THs (Hood and Klaassen, 2000a; Klaassen and Hood, 2001; Zhou *et al.*, 2001). UGTs are also regulated by AhR, CAR and PXR (Maglich, *et al.*, 2002; Bock and Kohle, 2004; Wagner *et al.*, 2005). The degree to which chemicals reduce serum T_4 is not always correlated with the increase in T_4-UGT activity (Hood, *et al.*, 2003; Richardson *et al.*, 2008). There are also differences between rats and mice in which Kenechlor-500, a mixture of polychlorinated biphenyls (PCBs) with PB-like effects on XMEs, reduces circulating levels of T_4 in both rats and mice, but induces UGT activity in rats but not mice (Kato *et al.*, 2003). In addition, Kenechlor-500 causes decreases in circulating T_4 concentrations in the UGT1A deficient Gunn rats demonstrating that the decreases in circulating T_4 is not necessarily solely dependent upon the induction of TH glucuronidation dependent (Kato *et al.*, 2004). As a result, it can be argued that UGT induction alone is not a uniform marker of the ability of chemicals to cause a reduction in serum TH, which could explain the inconsistencies observed in T_4-UGT activity and T_4 serum concentrations (Table 2). Nonetheless, the ability of chemicals to reduce circulating levels of TH can be associated with UGT induction and an increase in fecal elimination of T_4 (de Sandro *et al.*, 1992; Vansell and Klaassen, 2001).

C.4.3. Sulfation

Sulfotransferases are a cytosolic group of phase II metabolizing enzymes important for the inactivation and elimination of endogenous and exogenous compounds. Sulfation is a conjugation reaction in which a sulfate group from a sulfate donor, 3-phoshoadenosine-5-phosphsulfate (PAPS), is transferred to a substrate. Sulfoconjugation of THs is an alternative metabolic pathway that enhances enzymatic deiodination and facilitates their biliary and urinary excretion. The TH-sulfate conjugate is rapidly deiodinated by type I deiodinase through successive deiodinations of the tyrosyl (inner) and phenolic (outer) rings, releasing iodine into the circulation for reutilization by the thyroid. Sulfated conjugates are rapidly cleared in rats when deiodinase activity is inhibited. TH sulfation is also thought to serve as a reservoir from which unconjugated hormone can be liberated through sulfatases in tissues or in intestinal bacteria (Hazenberg *et al.*, 1988; Kung *et al.*, 1988).

Because of their significance in xenobiotic metabolism and hormone metabolism, it has become important to understand the regulation of SULTs by nuclear receptors. SULTs are regulated by AhR, CAR and PXR (Sonoda *et al.*, 2002; Saini *et al.*, 2004); however, unlike UGTs, prototypical nuclear receptor activators may not markedly induce SULTs in rodents (Alnouti and Klaassen, 2008). Human and rat SULTs catalyze THs though studies show significant species differences in SULT activity. For example, even though there is an 80% amino acid sequence homology between human and rat SULT1A1 (Yamazoe *et al.*, 1994; Weinshilboum *et al.*, 1997), human SULT1A1 catalyzes THs while the rat isoform does not. Human SULT1A1 was also identified as a low Km sulfotransferase with similar Kms and TH substrate specificities as human hepatic and renal sulfotransferases (Visser *et al.*, 1998) (Kester *et al.*, 1999). This correlation between human SULT1A1 and sulfotranserase

activities toward THs suggests that human SULT1A1 is a prominent sulfotransferase in liver and kidney.

C.4.4. Transporters

Several AhR, CAR and PXR regulated transporters are known to actively transport glucuronides and/ or THs. These comprise major efflux transporters in the ATP binding cassette (ABC) gene family, including multidrug resistance-associated proteins (MRPs), multidrug resistance proteins (MDRs). There is also increasing evidence that uptake transporters such as, organic anion transport protein (OATP), and monocarboxylate transporter (MCT) are important in the intracellular access to THs for metabolism (Friesema *et al.*, 1999; Jansen *et al.*, 2005). There is a correlation between induction of hepatic UGTs (Ugt1a1), multidrug resistance protein-associated protein (Mrp2), and organic anion transporting proteins (Oatp1 and Oatp2) mRNA levels, with decreases in serum TH concentrations in treated with rats treated with 4-(3-pentylamino)-2, 7-dimethyl-8-(2-methyl-4-methoxyphenyl)-pyrazolo-[1,5-a]-pyrimidine (DMP 904)(Wong, *et al.*, 2005; Lecureux *et al.*, 2009). There are indications that multidrug resistance proteins (MDRs) are important in TH efflux (Mitchell, *et al.*, 2005). These studies indicate that active transport along with glucuronidation and altered serum binding are possibly involved in TH decreases.

Cellular entry is required for TH metabolism; therefore, transport can determine the availability of THs in tissues. Na+/taurocholate-cotransporting polypeptide (NTCP) ,expressed only in liver, and the Na+-independent) organic anion transporting polypeptides (OATPs), expressed in liver kidney and brain, are major influx transporters of thyronines and their metabolites in humans and rats (Hagenbuch, 1997; Kullak-Ublick, 1999). Studies

using oocytes injected with human and rat NTCP or OATP mRNA, observed a significant uptake of thyronines (Friesema, *et al.*, 1999). Conversely, rat hepatocytes incubated with a transport-blocking antibody resulted in a decrease clearance of the iodothyronines from the media and iodide into the media, confirming that active transport is essential to the uptake and metabolism of THs (Hennemann *et al.*, 1986). De Jong et al., (de Jong *et al.*, 1993) demonstrated that Ouabain, a Na+ gradient inhibitor, reduces the amount of T_4 taken up into human hepatocytes and reduces the amount of iodide cleared into the media. Together, these studies indicate that TH transport is rate limiting for subsequent metabolism and suggest that transporters may serve a regulatory role in bioavailability and metabolism.

D. Thyroid Hormone Disruption

D.1. Xenobiotics and Hepatic Thyroid Hormone Metabolism

Although xenobiotics can disrupt TH homeostasis by directly disrupting the functions of the thyroid, increases in extrathyroidal metabolism are also involved in facilitating changes in TH homeostasis. As a major site of xenobiotic metabolism, the liver is important in metabolism of THs in humans and rodents.

Xenobiotics decrease serum TH concentrations through hepatic mechanisms (McClain, *et al.*, 1989; Liu, *et al.*, 1995) and activators of AhR, CAR and PXR consistently affect TH homeostasis (Kretschmer and Baldwin, 2005; Qatanani *et al.*, 2005). PB and PCN, through activation of CAR and PXR respectively, induce rat hepatic microsomal enzymes, such as UGTs (Barter and Klaassen, 1992; Vansell and Klaassen, 2001; Vansell and Klaassen, 2002a). *In vivo* studies in rats also show that PB and PCN induce the biliary

elimination of T_4-glucuronides (Oppenheimer, *et al.*, 1968; McClain, *et al.*, 1989); confirming that enzyme inducers mediate the increase in biliary elimination of THs via the induction of hepatic metabolizing enzymes.

D.2. Animal to Human Extrapolation

There is data supporting the induction of hepatic metabolism by which xenobiotics decrease circulating TH concentrations in rats; however, the mechanisms in humans are unclear (Table 3). Specifically, PB induces CYP2B enzymes in rat and human hepatocytes, implicating CAR as a modulator of effects on circulating TH concentrations (Barter and Klaassen, 1994; Madan *et al.*, 2003). While PB decreases serum TH concentrations in humans and rats (McClain et al 1989, Benedetti et al., 2005) only the increased biliary elimination of T_4-glucuronide has been observed in rat models.

Although several studies show that UGT1A1 and UGT1A3 are important in hepatic metabolism of T_4, Tong et al., (2007) demonstrated that UGT activities toward THs were higher in mouse and rat liver microsomes as compared to human, suggesting that UGTs may play a more significant role in the metabolism of THs in rodents than in humans. Conversely, sulfotransferase may be more important in the metabolism of THs in humans. SULT1E1 conjugates THs in humans, yet not in rats (Kester et al., 1999; Kester et al., 2003). This divergence in substrate specificity between the species, suggests that sulfation may be more important in TH metabolism in humans than in rats. In general, human hepatic SULTs have lower Kms than UGTs toward THs, suggesting that sulfation may play a more important role than glucuronidation in metabolism of THs in humans. While hepatic UGTs and SULTs may be important in decreasing circulating TH concentrations, it is uncertain if

increased hepatic conjugation plays as large of a role as suspected. For instance, studies using UGT1A-deficient Gunn rats exposed to PB or PCBs demonstrate that decreases in serum total T_4 are not necessarily glucuronidation-dependent (Collins and Capen, 1980; Kato, *et al.*, 2004). Many studies have also reported the inconsistencies in hepatic T_4-UGT activity and decreases in circulations in which the degree of T_4 decreases do not always correlate with increases in T_4-UGT activity (see Table 3). Because of these inconsistencies, it is uncertain how relevant extrathyroidal TH disruption in animals is to humans.

Reports indicate that hepatic transport may also be responsible for decreases in serum TH concentrations. For example, correlations between the mRNA induction of hepatic UGTs and hepatic uptake (Oatp1 and Oatp2) transporters mRNA levels, with decreases in circulating TH concentrations were observed (Wong, *et al.*, 2005; Lecureux, *et al.*, 2009). Wong et al. (Wong, *et al.*, 2005) reported a greater biliary elimination of parent T_4 versus glucuronide conjugated T_4 following exposure to 4-(3-pentylamino)-2, 7-dimethyl-8-(2-methyl-4-methoxyphenyl)-pyrazolo-[1,5-a]-pyrimidine (DMP 904) along with an increase in hepatic Mrp2 mRNA levels in rats. DMP 904 also induced hepatic CYP2B1 and CYP3A1 mRNA levels in rats, suggesting that it acts as an activator of CAR or PXR. Together these studies show that hepatic uptake and efflux transporters play a role in increasing the metabolic availability of T_4 as well as facilitating the clearance of unconjugated hormone. TH homeostasis depends greatly on transport by serum binding proteins. In rats, TH is largely bound to transthyretin (TTR) while in humans; TH is mostly bound to thyroid binding globulin (TBG). The differences in protein binding may cause THs in rats to be more susceptible to hepatic metabolism. Binding to a higher affinity binding protein such as TBG may protect THs from metabolic degradation; therefore, species differences in serum binding

may be responsible for the sensitivity of rats to TH toxicants. Although hydroxylated PCBs and PBDEs have been shown to bind to human and rodent TTR *ex vivo*, there are fewer compounds that compete for T_4-TBG binding (Cheek *et al.*, 1999; Hallgren and Darnerud, 2002). This suggests that TH displacement from its binding protein may not necessarily be of concern for humans.

E. Rationale for the Proposed Project

TH concentrations are regulated by not only the hypothalamus-pituitary–thyroid axis, but also hepatic metabolism, and elimination; therefore, the liver is essential to the extrathyroidal regulation of THs. Decreases in circulating TH concentrations by the induction of microsomal enzyme inducers have been linked to the increase in TH metabolism and biliary elimination (Barter and Klaassen, 1992; Liu, *et al.*, 1995; Kolaja and Klaassen, 1998; Hood, *et al.*, 1999; Hood and Klaassen, 2000a; Hood and Klaassen, 2000b; Hood, *et al.*, 2003). The role of microsomal enzyme inducers on hepatic UGTs and their effects on circulating TH concentration have been thoroughly examined. THs are not only glucuronidated but are also sulfated in the liver, for possible elimination into the bile. Although xenobiotics can cause decreases in circulating THs, xenobiotics do not necessarily induce hepatic UGTs to the same degree; therefore, it is important to determine why this occurs. Attempts have been made to examine the hepatic metabolism of THs in rats and humans following xenobiotic exposures, but an investigation of species differences and what role the liver plays still need to be explored.

Several studies show that primary hepatocytes are useful for examining the metabolic profiles of drugs (Bort *et al.*, 1996a; Bort *et al.*, 1996b; Hewitt *et al.*, 2001; Ponsoda *et al.*,

2001). For example, Ponsoda *et al*. (Ponsoda, *et al.*, 2001), correlated the metabolites of aceclofenac, an anti-inflammatory analgesic drug, found in human urine, with the metabolites formed in human hepatocytes supporting the use of hepatocytes to predict what happens *in vivo.* Furthermore, hepatocytes express nuclear receptor and therefore respond to xenobiotics through the induction of metabolizing enzymes. Hepatocytes also express membrane bound transporters, which can influence the intracellular concentration of compounds therefore; hepatocytes can be used to define the mechanisms by which AhR, CAR, and PXR agonists can alter TH metabolism and clearance.

Decisions concerning risk assessment have been based on animal to human extrapolations; therefore *in vivo-in vitro* comparisons are very important in the decision process. *In vivo* responses to toxicity usually involve multiple mechanisms and multicellular interactions. However, by focusing on a single cell type, data from *in vitro* studies becomes invaluable in evaluating the assumptions of specific mechanisms of action between species in toxicity studies. The evaluation of potential species differences in hepatic T_4 metabolism and the effect AhR, CAR, and PXR activation have on T_4 metabolism were examined the following aims

Aim 1: Compare T_4 metabolic profiles and clearance in rat and human hepatocytes. T_4 is metabolized by deiodination, glucuronidation and sulfation in the liver of rats and humans. It has been hypothesized that glucuronidation is a key step in the metabolism of THs in rats, whereas in humans, there is some evidence that sulfation may play a more pertinent role. These potential species differences in T_4 metabolism in may quantitatively influence hepatic clearance of THs. In this study, the examination of metabolites formed following hepatic

thyroid hormone clearance will provide a better understanding of the differences between rat and human TH metabolism

a. Establish a radiometric UPLC method for determining T_4 metabolites (T_4G, T_4S, T_3, rT_3) in the media of hepatocytes.

b. Compare clearance and metabolic profile by incubating T_4 with rat and human hepatocytes**.**

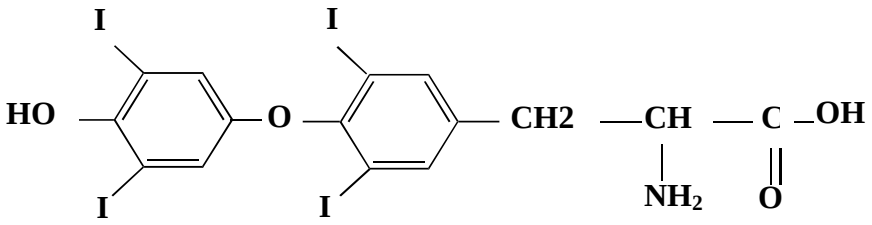
Aim 2: Determine hepatic clearance and the metabolic profile of THs in rat and human hepatocytes following exposure to Ahr, CAR, and PXR agonists. Metabolism plays a major role in the homeostasis of THs and because many xenobiotics can increase the metabolism of THs, it is important to understand the impact xenobiotics have on TH homeostasis. A major pathway of TH metabolism in the liver is the conjugation of the hormones to glucuroinides or sulfates. Uridine 5-diphosphate-glucuronosyltransferase (UGT) and sulfotransferase (SULT) mediate the conjugation of THs. The activation of these AhR, CAR or PXR by xenobiotics can induce metabolizing enzymes (UGT and SULT). The induction of UGTs and SULTs is thought to increase the metabolism and subsequent elimination of THs. To examine differences in human and rat hepatic metabolism of TH, this study will analyze metabolic profiles and gene involved in the metabolism of xenobiotics and THs.

a. Determine the effects of AhR, CAR, and PXR agonists (prototypical and environmental) on clearance and metabolic profiles of T_4 in rat and human hepatocytes.

b. Examine mRNA expression of genes related to xenobiotic and thyroid hormone metabolism. Correlate changes in mRNA expression with changes in the metabolic profile of T_4 following exposure to AhR, CAR and PXR agonists.

Aim 3: Compare the effects BDE-47 on T_4 metabolism in rats and metabolic profiles and clearance in rat and human hepatocytes.BDE-47, a CAR/PXR agonist, decreases T_4 serum concentrations in rodents. This decrease is thought to occur via: (1) the increase in hepatic T_4 metabolism and (2) the competitive binding of BDE-47 with TH binding proteins. Hepatocytes are a dependable model for studying the induction of xenobiotics metabolizing enzymes. In these experiments an *in vivo –in vitro* comparsion is made betweenTo compare the effects of BDE-47 Hepatocytes are used in the in vitro-in vivo extrapolation of metabolic activity toward a number of drugs. (LeCluyse, 2001; Hewitt *et al.*, 2007). Using primary rat and human sandwich-cultured hepatocytes this study makes comparisons of T_4 metabolism in response to nuclear receptor activation by BDE-47. The present study also compares the effects of BDE-47 on the genes involved in TH homeostasis in rat liver and hepatocytes.

Thyroxine
(T_4)



Triiodothyronine
(T_3)

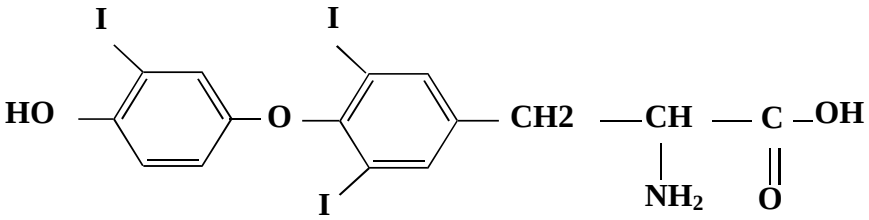
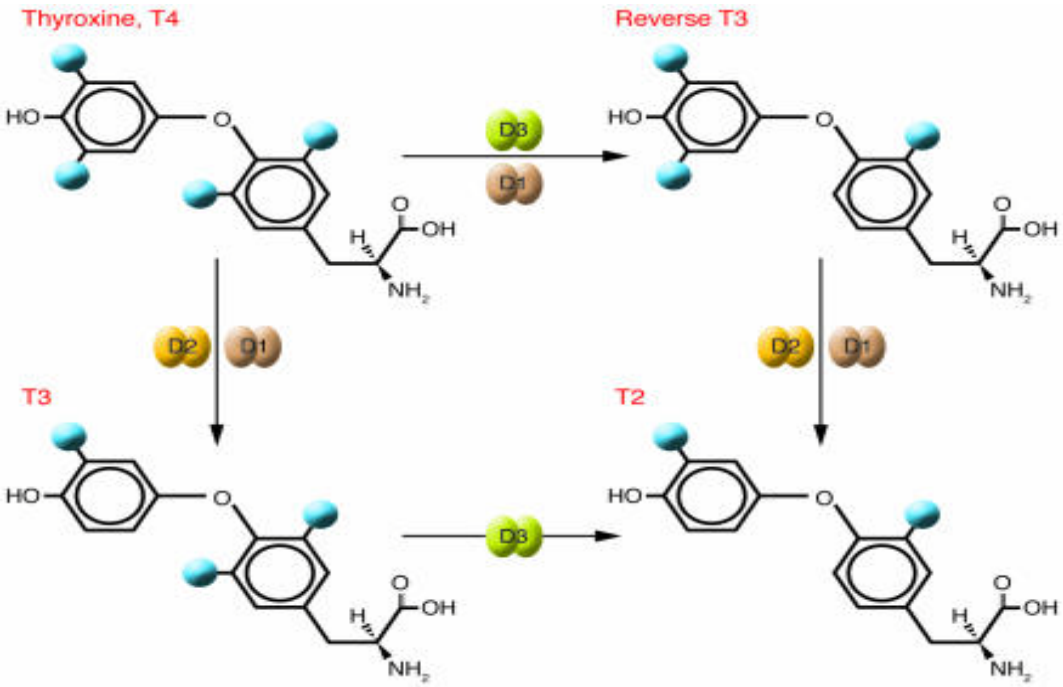


Figure 1.1 Stucture of thyroxine (T_4) and triiodothyronine (T_3)



From (Bianco and Kim, 2006)

Figure 1.2 Deiodinase conversion of thyroid hormones

Table 1.1

Physiological differences in thyroid hormone parameters

	Human	Rat	Reference
TBG (Serum concentration)	Present (0.02mg/ml)	Not Present	(Wade *et al.*, 1988)
TTR (Serum concentration)	Present (0.2mg/ml)	Present (0.5mg/ml)	(Benvenga and Robbins, 1998)
T_4 $t_{1/2}$ (days)	5-9	0.5- 1	(Capan, 2001; Bianco, *et al.*, 2002)
T_3 $t_{1/2}$ (days)	1	0.25	(Bianco, *et al.*, 2002)
Mean Serum T_4 (nM)	100.0[1,a]	43.8[2]	[1](Stockigt, 2003) [2](Woody *et al.*, 1998)
Mean Serum T_3 (nM)	1.9[1]	0.9[2]	[1](Stockigt, 2003) [2](Woody, *et al.*, 1998)

[a] Mean of reference range of 60-140nM in adult humans

Table 1.2

Inconsistencies in serum T_4 and T_4-UGT activity

Chemical	Nuclear Receptor	T_4-UGT Activity	Serum T_4	Reference
β-NF	AhR	↑↑↑	↓↓↓	(Hood and Klaassen, 2000)
3-MC	AhR	↑↑	↓↓	(Hood and Klaassen, 2000)
PCB	AhR/PXR	↑↑	↓↓↓	(Hood and Klaassen, 2000)
PCN	PXR	↑↑	↓↓	(Hood and Klaassen, 2000)
PB	CAR	↑↑	↓↓↓	(Hood and Klaassen, 2000)
DE 71	AhR/CAR/PXR	↑↑	↓↓	(Zhou *et al.*, 2002)
BDE 47	CAR	↔	↓	(Richardson *et al.*, 2008)
PB/PCB (Gunn Rat)	AhR/CAR/PXR	↔	↓↓↓	(Kato *et al.*, 2007)

↑ = increase
↓ = decrease
↔ = no change

Table 1.3

Key events in thyroid hormone disruption and relevance to humans

Key Event	Evidence in Rats	Evidence in Humans	Reference
Nuclear Receptor activation (CAR)	Yes *In vivo* and *in vitro*	Yes *In vitro*	(Barter and Klaassen, 1994; Hood and Klaassen, 2000a)
Hepatic UGT Induction	Yes *In vivo* and *in vitro*	Yes *In vitro*	(Barter and Klaassen, 1994; Hood and Klaassen, 2000a)
Increased TH or Conjugated TH Biliary Elimination	Yes *In vivo* and *in vitro*	No Data	(Kato *et al.*, 2005; Wong, *et al.*, 2005)
Hepatic Transporter Induction	Yes *In vivo*	No Data	(Ribeiro *et al.*, 1996; Mitchell, *et al.*, 2005; Wong, *et al.*, 2005)
TTR Binding	Yes *Ex vivo*; hydroxylated compounds bind to rTTR[1]	Yes *Ex vivo*; hydroxylated compounds bind to hTTR	(Cheek, *et al.*, 1999);(Hallgren and Darnerud, 2002; Meerts *et al.*, 2002)
TBG Binding	No Data (TBG not present)	Yes *Ex vivo*	(Cheek, *et al.*, 1999)
Serum TH Decrease	Yes *In vivo*	Yes *In vivo*	(Cavlieri *et al.*, 1973; Brucker-Davis, 1998)
Increased hepatic TH uptake/accumulation	Yes *In vivo*	No Data	(Kato, *et al.*, 2007)

REFERENCES

Abrams, G. M. and Larsen, P. R. (1973). Triiodothyronine and thyroxine in the serum and thyroid glands of iodine-deficient rats. *J Clin Invest* **52**(10), 2522-31.

Aleksunes, L. M. and Klaassen, C. D. (2012). Coordinated regulation of hepatic phase I and II drug-metabolizing genes and transporters using AhR-, CAR-, PXR-, PPARalpha-, and Nrf2-null mice. *Drug Metab Dispos* **40**(7), 1366-79.

Alnouti, Y. and Klaassen, C. D. (2008). Regulation of sulfotransferase enzymes by prototypical microsomal enzyme inducers in mice. *J Pharmacol Exp Ther* **324**(2), 612-21.

Barter, R. A. and Klaassen, C. D. (1992). UDP-glucuronosyltransferase inducers reduce thyroid hormone levels in rats by an extrathyroidal mechanism. *Toxicol Appl Pharmacol* **113**(1), 36-42.

Barter, R. A. and Klaassen, C. D. (1994). Reduction of thyroid hormone levels and alteration of thyroid function by four representative UDP-glucuronosyltransferase inducers in rats. *Toxicol Appl Pharmacol* **128**(1), 9-17.

Beischlag, T. V., Luis Morales, J., Hollingshead, B. D. and Perdew, G. H. (2008). The aryl hydrocarbon receptor complex and the control of gene expression. *Crit Rev Eukaryot Gene Expr* **18**(3), 207-50.

Benvenga, S. (2005). Peripheral Hormone Metabolism. In *The Thyroid: A Fundamental and Clinical Text* (L. E. Braverman, Utiger, R. D. , Ed.)^ Eds.)9 ed., pp. 97-108. Lippencott Williams and Wilkins, Philadelphia.

Benvenga, S., Lapa, D. and Trimarchi, F. (2002). Thyroxine binding to members and non-members of the serine protease inhibitor family. *J Endocrinol Invest* **25**(1), 32-8.

Benvenga, S. and Robbins, J. (1998). Thyroid hormone efflux from monolayer cultures of human fibroblasts and hepatocytes. Effect of lipoproteins and other thyroxine transport proteins. *Endocrinology* **139**(10), 4311-8.

Bianco, A. C., Salvatore, D., Gereben, B., Berry, M. J. and Larsen, P. R. (2002). Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. *Endocr Rev* **23**(1), 38-89.

Bisson, W. H., Koch, D. C., O'Donnell, E. F., Khalil, S. M., Kerkvliet, N. I., Tanguay, R. L., Abagyan, R. and Kolluri, S. K. (2009). Modeling of the aryl hydrocarbon receptor (AhR) ligand binding domain and its utility in virtual ligand screening to predict new AhR ligands. *J Med Chem* **52**(18), 5635-41.

Blondeau, J. P., Osty, J. and Francon, J. (1988). Characterization of the thyroid hormone transport system of isolated hepatocytes. *J Biol Chem* **263**(6), 2685-92.

Bock, K. W. and Kohle, C. (2004). Coordinate regulation of drug metabolism by xenobiotic nuclear receptors: UGTs acting together with CYPs and glucuronide transporters. *Drug Metab Rev* **36**(3-4), 595-615.

Bort, R., Ponsoda, X., Carrasco, E., Gomez-Lechon, M. J. and Castell, J. V. (1996a). Metabolism of aceclofenac in humans. *Drug Metab Dispos* **24**(8), 834-41.

Bort, R., Ponsoda, X., Carrasco, E., Gomez-Lechon, M. J. and Castell, J. V. (1996b). Comparative metabolism of the nonsteroidal antiinflammatory drug, aceclofenac, in the rat, monkey, and human. *Drug Metab Dispos* **24**(9), 969-75.

Capan, C. C. (2001). Toxic Responses of the Endocrine System. In *Casarett and Doull's Toxicology: The Basic Science of Poisons* (S. R. N. a. L. A. S. Andrea Seils, Ed.)^ Eds.), Vol. 6 6 ed., pp. 711-760. McGraw-Hill.

Chanoine, J. P., Braverman, L. E., Farwell, A. P., Safran, M., Alex, S., Dubord, S. and Leonard, J. L. (1993). The thyroid gland is a major source of circulating T3 in the rat. *J Clin Invest* **91**(6), 2709-13.

Cheek, A. O., Kow, K., Chen, J. and McLachlan, J. A. (1999). Potential mechanisms of thyroid disruption in humans: interaction of organochlorine compounds with thyroid receptor, transthyretin, and thyroid-binding globulin. *Environ Health Perspect* **107**(4), 273-8.

Collins, W. T., Jr. and Capen, C. C. (1980). Biliary excretion of 125I-thyroxine and fine structural alterations in the thyroid glands of Gunn rats fed polychlorinated biphenyls (PCB). *Lab Invest* **43**(2), 158-64.

Craft, E. S., DeVito, M. J. and Crofton, K. M. (2002). Comparative responsiveness of hypothyroxinemia and hepatic enzyme induction in Long-Evans rats versus C57BL/6J mice exposed to TCDD-like and phenobarbital-like polychlorinated biphenyl congeners. *Toxicol Sci* **68**(2), 372-80.

Davies, P. H., Sheppard, M. C. and Franklyn, J. A. (1996). Regulation of type I 5'-deiodinase by thyroid hormone and dexamethasone in rat liver and kidney cells. *Thyroid* **6**(3), 221-8.

De Jong, M., Docter, R., Van Der Hoek, H. J., Vos, R. A., Krenning, E. P. and Hennemann, G. (1992). Transport of 3,5,3'-triiodothyronine into the perfused rat liver and subsequent metabolism are inhibited by fasting. *Endocrinology* **131**(1), 463-70.

de Jong, M., Visser, T. J., Bernard, B. F., Docter, R., Vos, R. A., Hennemann, G. and Krenning, E. P. (1993). Transport and metabolism of iodothyronines in cultured human hepatocytes. *J Clin Endocrinol Metab* **77**(1), 139-43.

de Sandro, V., Catinot, R., Kriszt, W., Cordier, A. and Richert, L. (1992). Male rat hepatic UDP-glucuronosyltransferase activity toward thyroxine. Activation and induction properties--relation with thyroxine plasma disappearance rate. *Biochem Pharmacol* **43**(7), 1563-9.

Eiris-Punal, J., Del Rio-Garma, M., Del Rio-Garma, M. C., Lojo-Rocamonde, S., Novo-Rodriguez, I. and Castro-Gago, M. (1999). Long-term treatment of children with epilepsy with valproate or carbamazepine may cause subclinical hypothyroidism. *Epilepsia* **40**(12), 1761-6.

Ema, M., Matsushita, N., Sogawa, K., Ariyama, T., Inazawa, J., Nemoto, T., Ota, M., Oshimura, M. and Fujii-Kuriyama, Y. (1994). Human arylhydrocarbon receptor: functional expression and chromosomal assignment to 7p21. *J Biochem* **116**(4), 845-51.

Friesema, E. C., Docter, R., Moerings, E. P., Stieger, B., Hagenbuch, B., Meier, P. J., Krenning, E. P., Hennemann, G. and Visser, T. J. (1999). Identification of thyroid hormone transporters. *Biochem Biophys Res Commun* **254**(2), 497-501.

Furness, S. G. and Whelan, F. (2009). The pleiotropy of dioxin toxicity--xenobiotic misappropriation of the aryl hydrocarbon receptor's alternative physiological roles. *Pharmacol Ther* **124**(3), 336-53.

Hagenbuch, B. (1997). Molecular properties of hepatic uptake systems for bile acids and organic anions. *J Membr Biol* **160**(1), 1-8.

Hallgren, S. and Darnerud, P. O. (2002). Polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and chlorinated paraffins (CPs) in rats-testing interactions and mechanisms for thyroid hormone effects. *Toxicology* **177**(2-3), 227-43.

Hallgren, S., Sinjari, T., Hakansson, H. and Darnerud, P. O. (2001). Effects of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) on thyroid hormone and vitamin A levels in rats and mice. *Archives of toxicology* **75**(4), 200-8.

Hankinson, O. (2005). Role of coactivators in transcriptional activation by the aryl hydrocarbon receptor. *Arch Biochem Biophys* **433**(2), 379-86.

Hazenberg, M. P., de Herder, W. W. and Visser, T. J. (1988). Hydrolysis of iodothyronine conjugates by intestinal bacteria. *FEMS Microbiol Rev* **4**(1), 9-16.

Hennemann, G., Krenning, E. P., Polhuys, M., Mol, J. A., Bernard, B. F., Visser, T. J. and Docter, R. (1986). Carrier-mediated transport of thyroid hormone into rat hepatocytes is rate-limiting in total cellular uptake and metabolism. *Endocrinology* **119**(4), 1870-2.

Hewitt, N. J., Buhring, K. U., Dasenbrock, J., Haunschild, J., Ladstetter, B. and Utesch, D. (2001). Studies comparing in vivo:in vitro metabolism of three pharmaceutical compounds in rat, dog, monkey, and human using cryopreserved hepatocytes, microsomes, and collagen gel immobilized hepatocyte cultures. *Drug Metab Dispos* **29**(7), 1042-50.

Hewitt, N. J., Lechon, M. J., Houston, J. B., Hallifax, D., Brown, H. S., Maurel, P., Kenna, J. G., Gustavsson, L., Lohmann, C., Skonberg, C., Guillouzo, A., Tuschl, G., Li, A. P., LeCluyse, E., Groothuis, G. M. and Hengstler, J. G. (2007). Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metab Rev* **39**(1), 159-234.

Hites, R. A. (2004). Polybrominated diphenyl ethers in the environment and in people: a meta-analysis of concentrations. *Environ Sci Technol* **38**(4), 945-56.

Honkakoski, P. and Negishi, M. (1998). Protein serine/threonine phosphatase inhibitors suppress phenobarbital-induced Cyp2b10 gene transcription in mouse primary hepatocytes. *Biochem J* **330 (Pt 2)**, 889-95.

Hood, A., Allen, M. L., Liu, Y., Liu, J. and Klaassen, C. D. (2003). Induction of T(4) UDP-GT activity, serum thyroid stimulating hormone, and thyroid follicular cell proliferation in mice treated with microsomal enzyme inducers. *Toxicol Appl Pharmacol* **188**(1), 6-13.

Hood, A., Hashmi, R. and Klaassen, C. D. (1999). Effects of microsomal enzyme inducers on thyroid-follicular cell proliferation, hyperplasia, and hypertrophy. *Toxicol Appl Pharmacol* **160**(2), 163-70.

Hood, A. and Klaassen, C. D. (2000a). Differential effects of microsomal enzyme inducers on in vitro thyroxine (T(4)) and triiodothyronine (T(3)) glucuronidation. *Toxicol Sci* **55**(1), 78-84.

Hood, A. and Klaassen, C. D. (2000b). Effects of microsomal enzyme inducers on outer-ring deiodinase activity toward thyroid hormones in various rat tissues. *Toxicol Appl Pharmacol* **163**(3), 240-8.

Izumi, M. and Larsen, P. R. (1977). Triiodothyronine, thyroxine, and iodine in purified thyroglobulin from patients with Graves' disease. *J Clin Invest* **59**(6), 1105-12.

Jansen, J., Friesema, E. C., Milici, C. and Visser, T. J. (2005). Thyroid hormone transporters in health and disease. *Thyroid* **15**(8), 757-68.

Jemnitz, K. and Vereczkey, L. (1996). Ion-pair high-performance liquid chromatographic separation of two thyroxine glucuronides formed by rat liver microsomes. *J Chromatogr B Biomed Appl* **681**(2), 385-9.

Jones, S. A., Moore, L. B., Shenk, J. L., Wisely, G. B., Hamilton, G. A., McKee, D. D., Tomkinson, N. C., LeCluyse, E. L., Lambert, M. H., Willson, T. M., Kliewer, S. A. and Moore, J. T. (2000). The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Mol Endocrinol* **14**(1), 27-39.

Kato, Y., Haraguchi, K., Yamazaki, T., Ito, Y., Miyajima, S., Nemoto, K., Koga, N., Kimura, R. and Degawa, M. (2003). Effects of polychlorinated biphenyls, kanechlor-500, on serum thyroid hormone levels in rats and mice. *Toxicol Sci* **72**(2), 235-41.

Kato, Y., Ikushiro, S., Haraguchi, K., Yamazaki, T., Ito, Y., Suzuki, H., Kimura, R., Yamada, S., Inoue, T. and Degawa, M. (2004). A possible mechanism for decrease in serum thyroxine level by polychlorinated biphenyls in Wistar and Gunn rats. *Toxicol Sci* **81**(2), 309-15.

Kato, Y., Onishi, M., Haraguchi, K., Ikushiro, S., Ohta, C., Koga, N., Endo, T., Yamada, S. and Degawa, M. (2011). A possible mechanism for 2,2',4,4',5,5'-hexachlorobiphenyl-mediated decrease in serum thyroxine level in mice. *Toxicol Appl Pharmacol* **254**(1), 48-55.

Kato, Y., Suzuki, H., Haraguchi, K., Ikushiro, S., Ito, Y., Uchida, S., Yamada, S. and Degawa, M. (2010). A possible mechanism for the decrease in serum thyroxine level by phenobarbital in rodents. *Toxicol Appl Pharmacol* **249**(3), 238-46.

Kester, M. H., Kaptein, E., Roest, T. J., van Dijk, C. H., Tibboel, D., Meinl, W., Glatt, H., Coughtrie, M. W. and Visser, T. J. (1999). Characterization of human iodothyronine sulfotransferases. *J Clin Endocrinol Metab* **84**(4), 1357-64.

Klaassen, C. D. and Hood, A. M. (2001). Effects of microsomal enzyme inducers on thyroid follicular cell proliferation and thyroid hormone metabolism. *Toxicol Pathol* **29**(1), 34-40.

Kliewer, S. A. and Willson, T. M. (2002). Regulation of xenobiotic and bile acid metabolism by the nuclear pregnane X receptor. *J Lipid Res* **43**(3), 359-64.

Kolaja, K. L. and Klaassen, C. D. (1998). Dose-response examination of UDP-glucuronosyltransferase inducers and their ability to increase both TGF-beta expression and thyroid follicular cell apoptosis. *Toxicol Sci* **46**(1), 31-7.

Krenning, E., Docter, R., Bernard, B., Visser, T. and Hennemann, G. (1981). Characteristics of active transport of thyroid hormone into rat hepatocytes. *Biochim Biophys Acta* **676**(3), 314-20.

Kretschmer, X. C. and Baldwin, W. S. (2005). CAR and PXR: xenosensors of endocrine disrupters? *Chem Biol Interact* **155**(3), 111-28.

Kullak-Ublick, G. A. (1999). Regulation of organic anion and drug transporters of the sinusoidal membrane. *J Hepatol* **31**(3), 563-73.

Kung, M. P., Spaulding, S. W. and Roth, J. A. (1988). Desulfation of 3,5,3'-triiodothyronine sulfate by microsomes from human and rat tissues. *Endocrinology* **122**(4), 1195-200.

Larsen PR, D. T., Hay ID (1998). The thyroid gland. In *Williams textbook of endocrinology* (F. D. Wilson JD, Kronenberg HM, Larsen PR, Ed.)^ Eds.)9 ed., pp. 389-515. W.B. Saunders Co., Philadelphia.

LeCluyse, E. L. (2001). Human hepatocyte culture systems for the in vitro evaluation of cytochrome P450 expression and regulation. *Eur J Pharm Sci* **13**(4), 343-68.

Liu, J., Liu, Y., Barter, R. A. and Klaassen, C. D. (1995). Alteration of thyroid homeostasis by UDP-glucuronosyltransferase inducers in rats: a dose-response study. *J Pharmacol Exp Ther* **273**(2), 977-85.

Lorber, M. (2008). Exposure of Americans to polybrominated diphenyl ethers. *Journal of exposure science & environmental epidemiology* **18**(1), 2-19.

Madan, A., Graham, R. A., Carroll, K. M., Mudra, D. R., Burton, L. A., Krueger, L. A., Downey, A. D., Czerwinski, M., Forster, J., Ribadeneira, M. D., Gan, L. S., LeCluyse, E. L., Zech, K., Robertson, P., Jr., Koch, P., Antonian, L., Wagner, G., Yu, L. and Parkinson, A. (2003). Effects of prototypical microsomal enzyme inducers on cytochrome P450 expression in cultured human hepatocytes. *Drug Metab Dispos* **31**(4), 421-31.

Maglich, J. M., Stoltz, C. M., Goodwin, B., Hawkins-Brown, D., Moore, J. T. and Kliewer, S. A. (2002). Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. *Mol Pharmacol* **62**(3), 638-46.

McClain, R. M., Levin, A. A., Posch, R. and Downing, J. C. (1989). The effect of phenobarbital on the metabolism and excretion of thyroxine in rats. *Toxicol Appl Pharmacol* **99**(2), 216-28.

Mitchell, A. M., Tom, M. and Mortimer, R. H. (2005). Thyroid hormone export from cells: contribution of P-glycoprotein. *J Endocrinol* **185**(1), 93-8.

Moore, L. B., Maglich, J. M., McKee, D. D., Wisely, B., Willson, T. M., Kliewer, S. A., Lambert, M. H. and Moore, J. T. (2002). Pregnane X receptor (PXR), constitutive androstane receptor (CAR), and benzoate X receptor (BXR) define three pharmacologically distinct classes of nuclear receptors. *Mol Endocrinol* **16**(5), 977-86.

Moore, L. B., Parks, D. J., Jones, S. A., Bledsoe, R. K., Consler, T. G., Stimmel, J. B., Goodwin, B., Liddle, C., Blanchard, S. G., Willson, T. M., Collins, J. L. and Kliewer, S. A. (2000). Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J Biol Chem* **275**(20), 15122-7.

Moriguchi, T., Motohashi, H., Hosoya, T., Nakajima, O., Takahashi, S., Ohsako, S., Aoki, Y., Nishimura, N., Tohyama, C., Fujii-Kuriyama, Y. and Yamamoto, M. (2003). Distinct response to dioxin in an arylhydrocarbon receptor (AHR)-humanized mouse. *Proc Natl Acad Sci U S A* **100**(10), 5652-7.

Munro, S. L., Lim, C. F., Hall, J. G., Barlow, J. W., Craik, D. J., Topliss, D. J. and Stockigt, J. R. (1989). Drug competition for thyroxine binding to transthyretin (prealbumin): comparison with effects on thyroxine-binding globulin. *J Clin Endocrinol Metab* **68**(6), 1141-7.

Ohnhaus, E. E., Burgi, H., Burger, A. and Studer, H. (1981). The effect of antipyrine, phenobarbitol and rifampicin on thyroid hormone metabolism in man. *Eur J Clin Invest* **11**(5), 381-7.

Ohnhaus, E. E. and Studer, H. (1983). A link between liver microsomal enzyme activity and thyroid hormone metabolism in man. *Br J Clin Pharmacol* **15**(1), 71-6.

Oppenheimer, J. H., Bernstein, G. and Surks, M. I. (1968). Increased thyroxine turnover and thyroidal function after stimulation of hepatocellular binding of thyroxine by phenobarbital. *J Clin Invest* **47**(6), 1399-406.

Oppenheimer, J. H., Schwartz, H. L. and Surks, M. I. (1972). Propylthiouracil inhibits the conversion of L-thyroxine to L-triiodothyronine. An explanation of the antithyroxine effect of propylthiouracil and evidence supporting the concept that triiodothyronine is the active thyroid hormone. *J Clin Invest* **51**(9), 2493-7.

Pacyniak, E. K., Cheng, X., Cunningham, M. L., Crofton, K., Klaassen, C. D. and Guo, G. L. (2007). The flame retardants, polybrominated diphenyl ethers, are pregnane X receptor activators. *Toxicol Sci* **97**(1), 94-102.

Pandini, A., Soshilov, A. A., Song, Y., Zhao, J., Bonati, L. and Denison, M. S. (2009). Detection of the TCDD binding-fingerprint within the Ah receptor ligand binding domain by structurally driven mutagenesis and functional analysis. *Biochemistry* **48**(25), 5972-83.

Ponsoda, X., Pareja, E., Gomez-Lechon, M. J., Fabra, R., Carrasco, E., Trullenque, R. and Castell, J. V. (2001). Drug biotransformation by human hepatocytes. In vitro/in vivo metabolism by cells from the same donor. *J Hepatol* **34**(1), 19-25.

Power, D. M., Elias, N. P., Richardson, S. J., Mendes, J., Soares, C. M. and Santos, C. R. (2000). Evolution of the thyroid hormone-binding protein, transthyretin. *Gen Comp Endocrinol* **119**(3), 241-55.

Qatanani, M., Zhang, J. and Moore, D. D. (2005). Role of the constitutive androstane receptor in xenobiotic-induced thyroid hormone metabolism. *Endocrinology* **146**(3), 995-1002.

Ramadoss, P. and Perdew, G. H. (2004). Use of 2-azido-3-[125I]iodo-7,8-dibromodibenzo-p-dioxin as a probe to determine the relative ligand affinity of human versus mouse aryl hydrocarbon receptor in cultured cells. *Mol Pharmacol* **66**(1), 129-36.

Ribeiro, R. C., Cavalieri, R. R., Lomri, N., Rahmaoui, C. M., Baxter, J. D. and Scharschmidt, B. F. (1996). Thyroid hormone export regulates cellular hormone content and response. *J Biol Chem* **271**(29), 17147-51.

Richardson, V. M., Staskal, D. F., Ross, D. G., Diliberto, J. J., DeVito, M. J. and Birnbaum, L. S. (2008). Possible mechanisms of thyroid hormone disruption in mice by BDE 47, a major polybrominated diphenyl ether congener. *Toxicol Appl Pharmacol* **226**(3), 244-50.

Robbins, J. (1991). Thyroid hormone transport proteins and the physiology of hormone binding. In *Werner & Ingbars, The Thyroid.* 6 ed., pp. 111-125. J.B. Lippincott, Philadelphia, U.S.A.

Rutgers, M., Pigmans, I. G., Bonthuis, F., Docter, R. and Visser, T. J. (1989). Effects of propylthiouracil on the biliary clearance of thyroxine (T4) in rats: decreased excretion of 3,5,3'-triiodothyronine glucuronide and increased excretion of 3,3',5'-triiodothyronine glucuronide and T4 sulfate. *Endocrinology* **125**(4), 2175-86.

Safe, S. (1990). Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). *Crit Rev Toxicol* **21**(1), 51-88.

Saini, S. P., Sonoda, J., Xu, L., Toma, D., Uppal, H., Mu, Y., Ren, S., Moore, D. D., Evans, R. M. and Xie, W. (2004). A novel constitutive androstane receptor-mediated and CYP3A-independent pathway of bile acid detoxification. *Mol Pharmacol* **65**(2), 292-300.

Savu, L., Vranckx, R., Maya, M. and Nunez, E. A. (1987). A thyroxine binding globulin (TBG)-like protein in the sera of developing and adult rats. *Biochem Biophys Res Commun* **148**(3), 1165-73.

Savu, L., Vranckx, R., Rouaze-Romet, M., Maya, M., Nunez, E. A., Treton, J. and Flink, I. L. (1991). A senescence up-regulated protein: the rat thyroxine-binding globulin (TBG). *Biochim Biophys Acta* **1097**(1), 19-22.

Scheer, N., Ross, J., Rode, A., Zevnik, B., Niehaves, S., Faust, N. and Wolf, C. R. (2008). A novel panel of mouse models to evaluate the role of human pregnane X receptor and constitutive androstane receptor in drug response. *J Clin Invest* **118**(9), 3228-39.

Schussler, G. C. (2000). The thyroxine-binding proteins. *Thyroid* **10**(2), 141-9.

Sidhu, J. S. and Omiecinski, C. J. (1995). cAMP-associated inhibition of phenobarbital-inducible cytochrome P450 gene expression in primary rat hepatocyte cultures. *J Biol Chem* **270**(21), 12762-73.

Sonoda, J., Xie, W., Rosenfeld, J. M., Barwick, J. L., Guzelian, P. S. and Evans, R. M. (2002). Regulation of a xenobiotic sulfonation cascade by nuclear pregnane X receptor (PXR). *Proc Natl Acad Sci U S A* **99**(21), 13801-6.

Stockigt, J. (2003). Assessment of thyroid function: towards an integrated laboratory - clinical approach. *Clin Biochem Rev* **24**(4), 109-22.

Szabo, D. T., Richardson, V. M., Ross, D. G., Diliberto, J. J., Kodavanti, P. R. and Birnbaum, L. S. (2009). Effects of perinatal PBDE exposure on hepatic phase I, phase II, phase III, and deiodinase 1 gene expression involved in thyroid hormone metabolism in male rat pups. *Toxicol Sci* **107**(1), 27-39.

Tchaparian, E. H., Houghton, J. S., Uyeda, C., Grillo, M. P. and Jin, L. (2011). Effect of culture time on the basal expression levels of drug transporters in sandwich-cultured primary rat hepatocytes. *Drug Metab Dispos* **39**(12), 2387-94.

Tong, Z., Li, H., Goljer, I., McConnell, O. and Chandrasekaran, A. (2007). In vitro glucuronidation of thyroxine and triiodothyronine by liver microsomes and recombinant human UDP-glucuronosyltransferases. *Drug Metab Dispos* **35**(12), 2203-10.

Vansell, N. R. and Klaassen, C. D. (2001). Increased biliary excretion of thyroxine by microsomal enzyme inducers. *Toxicol Appl Pharmacol* **176**(3), 187-94.

Vansell, N. R. and Klaassen, C. D. (2002). Effect of microsomal enzyme inducers on the biliary excretion of triiodothyronine (T(3)) and its metabolites. *Toxicol Sci* **65**(2), 184-91.

Viluksela, M., Raasmaja, A., Lebofsky, M., Stahl, B. U. and Rozman, K. K. (2004). Tissue-specific effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the activity of 5'-deiodinases I and II in rats. *Toxicol Lett* **147**(2), 133-42.

Visser, T. J. (1996). Pathways of thyroid hormone metabolism. *Acta Med Austriaca* **23**(1-2), 10-6.

Visser, T. J., Kaptein, E., Gijzel, A. L., de Herder, W. W., Ebner, T. and Burchell, B. (1993). Glucuronidation of thyroid hormone by human bilirubin and phenol UDP-glucuronyltransferase isoenzymes. *FEBS Lett* **324**(3), 358-60.

Visser, T. J., Kaptein, E., Glatt, H., Bartsch, I., Hagen, M. and Coughtrie, M. W. (1998). Characterization of thyroid hormone sulfotransferases. *Chem Biol Interact* **109**(1-3), 279-91.

Wade, S., Bleiberg-Daniel, F., Le Moullac, B., Iyakaremye, D., Biou, D., Gauthier, F. and Lemonnier, D. (1988). Value of serum transthyretin measurements in the assessment of marginal protein-energy malnutrition in rats. *J Nutr* **118**(8), 1002-10.

Wagner, M., Halilbasic, E., Marschall, H. U., Zollner, G., Fickert, P., Langner, C., Zatloukal, K., Denk, H. and Trauner, M. (2005). CAR and PXR agonists stimulate hepatic bile acid and bilirubin detoxification and elimination pathways in mice. *Hepatology* **42**(2), 420-30.

Weinshilboum, R. M., Otterness, D. M., Aksoy, I. A., Wood, T. C., Her, C. and Raftogianis, R. B. (1997). Sulfation and sulfotransferases 1: Sulfotransferase molecular biology: cDNAs and genes. *Faseb J* **11**(1), 3-14.

Wong, H., Lehman-McKeeman, L. D., Grubb, M. F., Grossman, S. J., Bhaskaran, V. M., Solon, E. G., Shen, H. S., Gerson, R. J., Car, B. D., Zhao, B. and Gemzik, B. (2005). Increased hepatobiliary clearance of unconjugated thyroxine determines DMP 904-induced alterations in thyroid hormone homeostasis in rats. *Toxicol Sci* **84**(2), 232-42.

Woody, C. J., Weber, S. L., Laubach, H. E., Ingram-Willey, V., Amini-Alashti, P. and Sturbaum, B. A. (1998). The effects of chronic exercise on metabolic and reproductive functions in male rats. *Life Sci* **62**(4), 327-32.

Yamazoe, Y., Nagata, K., Ozawa, S. and Kato, R. (1994). Structural similarity and diversity of sulfotransferases. *Chem Biol Interact* **92**(1-3), 107-17.

Yanagiba, Y., Ito, Y., Kamijima, M., Gonzalez, F. J. and Nakajima, T. (2009). Octachlorostyrene induces cytochrome P450, UDP-glucuronosyltransferase, and sulfotransferase via the aryl hydrocarbon receptor and constitutive androstane receptor. *Toxicol Sci* **111**(1), 19-26.

Zhou, T., Ross, D. G., DeVito, M. J. and Crofton, K. M. (2001). Effects of short-term in vivo exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. *Toxicol Sci* **61**(1), 76-82.

CHAPTER 2

IN VITRO METABOLISM OF THYROXINE BY RAT AND HUMAN HEPATOCYTES[1]

A. INTRODUCTION

The liver has a major influence on plasma concentrations of thyroid hormones (THs) and their metabolites (Ohnhaus and Studer, 1983; Malik and Hodgson, 2002). Deiodinase I (DI), located primarily in the liver, is responsible for the extrathyroidal conversion of the prohormone, thyroxine (T_4) to its biologically active form 3,3',5-triiodothyronine (T_3). DI is also critical in the inactivation of T_3 to 3,3',5'-triiodothyronine (rT_3) and 3,3'-diiodothyronine (T_2). In the liver, THs also are metabolized to either glucuronide or sulfate conjugates and the resulting conjugates are excreted through the bile duct into the intestine. A portion of the conjugated hormone is hydrolyzed in the intestine and consequently the free hormones are reabsorbed into the systemic circulation while the conjugated portion is eliminated in the feces.

There are species differences in the rates of these reactions of TH conjugation. Tong *et al*., (2007) demonstrated that uridine diphosphate glucuronosyltransferase (UGT) activity toward T_3 was higher in mouse and rat liver microsomes than human liver microsomes and

---

[1]Richardson,V.M., Ferguson, S.S., Sey, Y.M., DeVito, M.J. (submitted) *Xenobiotica*

activity toward T_4 is higher in mouse liver microsomes compared to rat and human liver microsomes. This suggests that UGTs may play a more significant role in the metabolism of THs in rodents than in humans. In contrast, the literature suggests that sulfotransferase (SULT) may be more important in the metabolism of THs in humans, as SULT1E1 conjugates THs in humans, but not in rats (Kester, *et al.*, 1999; Kester *et al.*, 2003). These studies suggest that regulation of serum TH concentrations involve both hypothalamus-pituitary–thyroid (HPT) axis and hepatic metabolism and elimination.

Hepatic uptake and efflux transporters contribute to the transport of endogenous and exogenous compounds from the systemic circulation to bile (Arias *et al.*, 1993). Although it was once thought that THs traversed the plasma membrane by diffusion (Robbins and Rall, 1960) it is now known that THs and their conjugates are also actively transported. Specifically, multidrug resistance-associated proteins (MRPs) and multidrug resistance proteins (MDRs) play a major role in efflux transport of THs (Friesema, *et al.*, 1999; Mitchell, *et al.*, 2005). There is also evidence that uptake transporters such as organic anion transporting polypeptides (OATPs), and monocarboxylate transporters (MCTs) are important in the intracellular accumulation of THs (Friesema, *et al.*, 1999; Jansen, *et al.*, 2005). Because cellular entry is required for TH metabolism, it is likely that active transport is rate limiting with respect to bioavailability and metabolism of THs (de Jong, *et al.*, 1993; Friesema, *et al.*, 1999).

Xenobiotics can alter TH homeostasis at the point of its biosynthesis, release, transport, metabolism and excretion. A number of environmental chemicals decrease circulating TH concentrations through the induction of xenobiotic metabolizing enzymes resulting in increases in TH metabolism and biliary elimination (Barter and Klaassen, 1992;

Liu, *et al.*, 1995; Kolaja and Klaassen, 1998; Hood, *et al.*, 1999; Hood and Klaassen, 2000a; Hood and Klaassen, 2000b; Hood, *et al.*, 2003). For example, phenobarbital (PB), through the activation of the constitutive androstane receptor (CAR), induces rat hepatic xenobiotic metabolizing enzymes (XMEs), such as UGTs and increases the biliary elimination of T_4 or T_4-glucuronides (Oppenheimer, *et al.*, 1968; McClain, *et al.*, 1989; Barter and Klaassen, 1992; Vansell and Klaassen, 2001; Vansell and Klaassen, 2002a). While PB decreases serum TH concentrations in humans and rats (McClain, *et al.*, 1989; Benedetti *et al.*, 2005), mechanistic studies are only available for rats. A PB-like inducer, 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB 153), also decreases serum T_4 concentrations and induces hepatic microsomal T_4-UGT activity in rats (Craft *et al.*, 2002; He *et al.*, 2011). PCB 153 is a major component of Aroclor 1254 and is one of the PCBs with the highest human exposures (NHANES, 2012). Exposure to PCB 153 is associated with TH decreases in humans (Hagmar *et al.*, 2001), but *in vivo* induction of hepatic metabolizing enzymes by PCB 153 in humans has not been demonstrated. *In vitro*, PCB 153 induces Ugt2b1 mRNA in rat hepatocytes; however, effects on TH metabolism were not determined (Ganem *et al.*, 1999). Overall, xenobiotics that induce hepatic UGTs result in a concomitant decrease in circulating TH concentrations in rodents; however, the mechanism by which TH disruption occurs in humans is unclear.

To our knowledge, this is the first report to characterize the utility of sandwich-cultured hepatocytes (SCH) in studying TH metabolism. This study aims to compare pathways involved in the hepatic metabolism of T_4 in rats and humans, by examining the metabolic profiles of T_4 following incubation with fresh SCH from rats and humans. Parameters for analysis include comparative examinations of the metabolic profiles in rat and

human hepatocytes over incubation time, T_4 concentrations, culture days and following PCB 153 treatment. T_4 accumulation with hepatocytes was also explored to provide a better understanding of species differences in intercellular availability and hepatic metabolism of T_4.

B. MATERIALS AND METHODS

Chemicals

L-thyroxine (T_4) and phenobarbital were purchased from Sigma-Aldrich Co. (St. Louis, MO). PCB 153 was purchased from Radian Corporation (Austin, TX). [^{125}I]-T_4, -T_3, and -rT_3, (116 Ci/mmol) were purchased from Perkin Elmer Life Sciences Inc. (Waltham, MA) and were purified to >98% immediately before use with Sephadex LH-20 (Sigma-Aldrich) as described by Rutgers *et al.* (1989). All other reagents were of the highest grade commercially available.

T_4 glucuronidation assay

T_4 glucuronidation activity was evaluated in liver and intestinal microsomes from rat and humans (Zhou*, et al.*, 2001) based on a previously published method (Visser *et al.*, 1993b). Microsomes were obtained from CellzDirect or Xenotech (Table 2.2). A 100 µl aliquot of microsomes (2 mg protein per ml 1M Tris/HCl buffer pH 7.4) was incubated at 37°C with 4µM of cold T_4, [^{125}I]-T_4 (100,000 cpm), 0.1mM 6-n-propyl-2-thiouracil (PTU), alamethicin (50 µg/mg microsomal protein) and 5mM uridine diphosphoglucuronic acid (UDPGA) for 1 hour. Sample blanks (without UDPGA) were analyzed concurrently. The

reaction was stopped with methanol followed by centrifugation and mixing the supernatant with 0.1M HCl. T_4-glucuronide (T_4G) was separated by chromatography using lipophilic Sephadex LH-20 columns and eluent was measured for radioactivity as previously described (Zhou, *et al.*, 2001).

T_4 sulfation assay

Rat and human liver cytosol were assayed for SULT activities as previously described (Szabo *et al.*, 2009). Liver cytosols were obtained from CellzDirect (Table 2.2). 20 µg protein/ml of liver cytosol in the presence or absence (blank) of 50µM 3'-phosphoadenosine 5'-phosphosulfate (PAPS; Sigma) in 0.2 ml of 0.1M phosphate (pH 7.2) and 2mM EDTA (Sigma) (Kaptein *et al.*, 1997) was incubated with 4µM of T_4, and 100,000 cpm of [^{125}I]-T_4 for 30 min at 37°C. The reactions were applied to 1 ml of lipophilic sephadex (Sigma) LH-20 minicolumns (Superlco) equilibrated with 0.1M HCl. Iodine and T_4-sulfate (T_4S) were eluted with 2 X 1 ml of 0.1M HCl, and 6 X 1 ml of ethanol/water (20/80, vol/vol), respectively. Fractions were collected and 1 ml of the T_4S fraction was quantitated by gamma spectroscopy (Perkin-Elmer Life Sciences Inc., Waltham, MA).

Hepatocyte culture

Fresh primary sandwich-cultured rat hepatocytes (SCRH), isolated from male Sprague-Dawley rats, and sandwich-cultured human hepatocytes (SCHH) in 24-well plates were obtained on culture day 2 from Life Technologies (Durham, NC) and maintained in Williams' E medium with 0.1% dimethyl sulfoxide (DMSO) including Hepatocyte Maintenance Supplement (Life Technologies) which contains 0.1µM dexamethasone and

proprietary concentrations of penicillin-streptomycin, ITS+ (insulin, transferrin, selenium complex, BSA and linoleic acid), GlutaMAX™ and HEPES. Cultures were maintained for up to 6 days with medium replaced every 24 hours. Human donor demographics can be found in Table 2.1.

Thyroxine and thyroxine metabolite separation, identification and quantitation from medium and hepatocytes.

All medium samples were dried under N_2 and then reconstituted with 40µl mobile phase consisting of 18% acetonitrile in 0.02M ammonium acetate (pH=4). After addition of mobile phase, samples were centrifuged at 10,000 x g for 5 minutes at room temperature and the supernatants were placed in vials for UPLC analysis. UPLC equipped with a C18 – 2.1 x 50mm x 1.7µm (Waters Corp.; Milford, MA) resolution column and fraction collector were used for separation and identification of T_4 and T_4 metabolites. Gradient elution was performed using a modified version of a previously established method by Rutgers *et al*. (1987) with a 16 minute gradient of 18-40% acetonitrile in 0.02M ammonium acetate (pH=4). Solvent flow rate was 0.4ml/min and 0.25 minute fractions were collected in test tubes. Peaks were then identified by analyzing collected fractions for radioactivity by gamma spectroscopy. Retention times for T_4, T_3, rT_3, T_4-glucuronide (T_4G), and T4-sulfate (T_4S) were determined using synthetic and biosynthetic compounds. Peaks for T_3 and rT_3 were often inseparable; therefore these peaks were added together and are presented in this study as T_3+rT_3. Figure 2.1 shows the results of a typical UPLC separation from the collected fractions for T_4, T_4G, T_4S, T_3, and rT_3 from medium of SCRH. Protein content of the

hepatocytes was determined using a protein assay kit with bovine serum albumin as a standard (Bio-Rad Laboratories, Hercules, CA).

Commercially available [^{125}I]-T_4, -T_3, and -rT_3 were used as references to determine retention times using UPLC with fraction collections. T_4-glucuronide and -sulfate conjugates were not commercially available, therefore T_4G and T_4S references were prepared using recombinant human UGT1A8 from BD Biosciences (San Jose, CA) and recombinant human SULT1E (US Biologicals, Swampscott, MA), respectively.

T_4G was synthesized by incubating 4μM T_4 and 100,000 cpm of [^{125}I]-T_4 for 60 min at 37ºC with human recombinant UGT1A8 (BD Biosciences, San Jose, CA) (final concentration, 2 mg UGT1A8 protein/ml) in the presence or absence (blank) of 5mM uridine 5′-diphosphoglucuronic acid (UDPGA) in 0.2 ml (total reaction volume) 75mM Tris-HCl (pH 7.8), 7.5mM $MgC1_2$ with alamethicin (50 μg/mg UGT1A8 protein). The reactions were stopped by the addition of 0.2 ml ice-cold methanol, and after centrifugation (10,000 x g for 5 minutes at 4°C), supernatant was removed. The supernatant was then dried under N_2 at 40°C and prepared for UPLC analysis as mentioned above. T_4G peaks were confirmed by β-glucuronidase digestion (van der Heide *et al.*, 2002).

Recombinant human SULT1E (US Biologicals, Swampscott, MA) was used to synthesize T_4S references. T_4S was synthesized by incubating 4μM T_4 and 100,000 cpm [^{125}I]-T_4 incubated overnight at 37ºC with SULT1E (final concentration, 0.4 mg SULT1E protein /ml) in the presence or absence (blank) of 400μM adenosine 3′-phosphate 5′-phosphosulfate (PAPS; Sigma-Aldrich) in 0.2 ml (total reaction volume) 0.1M potassium phosphate (pH 7.2), 2mM EDTA. The reactions were stopped by the addition of 0.8 ml 0.1M HCl. Samples were then centrifuged at 10,000 x g for 5 minutes at 4°C. Supernatant

was collected, dried under N_2 at 40°C, and prepared for UPLC analysis as mentioned above. T_4S peaks were confirmed by acid solvolysis (van der Heide, *et al.*, 2002).

Comparisons of T_4 metabolism across days in culture

Fresh SCRH and SCHH were treated on culture day 3, 4, 5, or 6 with 0.05µM (rat) or 0.1µM (human) [^{125}I]-T_4 (500,000 cpm/well) for 24 hours, after which media were collected and analyzed for T_4 metabolites using the methods described above. Cells were washed twice with 0.5 ml/well of ice-cold PBS buffer. Hepatocytes were collected by adding 0.5ml/well of 0.1M NaOH and analyzed for protein content.

T_4 accumulation

SCRH or SCHH were used to measure [^{125}I]-T_4 association over time. Hepatocytes were incubated with supplemented Williams' E medium, as mentioned above, containing 0.0005µM (5000 cpm) per well of [^{125}I]-T_4 for up to 5 minutes on culture day 6. After incubation, media were collected and cells were washed twice with 0.5 ml/well of ice-cold PBS buffer. After washing, hepatocytes were collected by adding 0.5ml/well of 0.1M NaOH. ^{125}I-derived radioactivity in collected media and hepatocytes were analyzed by gamma spectroscopy.

Effects of incubation time on T_4 metabolite levels and T_4 depletion from medium

Fresh SCRH and SCHH were treated on culture day 6 with 0.05µM (rat) or 0.1µM (human) [^{125}I]-T_4 (500,000 cpm/well) for up to 24 hours. After 4, 8, 12, or 24 hours, media and hepatocytes were collected. Mass balance was determined by preparing and analyzing

media and hepatocytes for T_4 and T_4 metabolites using the methods described above. Hepatocytes were collected and analyzed for protein content.

Effects of T_4 concentration on metabolite levels

Fresh SCRH and SCHH were treated on culture day 6 with 0.05, 0.1, 5, 50, or 100 µM of [^{125}I]-T_4 (500,000 cpm/well) in supplemented Williams' E medium (0.5ml) for 24 hours. Time course studies were performed using 0.05µM (rat) or 0.1µM (human) [^{125}I]-T_4. After 24 hours, medium was collected, prepared, and analyzed for T_4 metabolites using the methods described above. Hepatocytes were collected and analyzed for protein content.

Effects of PCB 153 on T_4 metabolite levels

Stock solutions of PCB 153 were diluted in DMSO and added to Williams' E medium to a final DMSO concentration of 0.1%. On culture day 3, fresh SCRH and SCHH were exposed for 72 hours to the DMSO vehicle (0.1%) or PCB 153 (30µM). After 72 hours, medium was removed and replaced with Williams' E medium containing physiological concentrations [0.05µM (rat) or 0.1µM (human)] of [^{125}I]-T_4 (500,000 cpm/well) for 24 hours. Medium was collected, prepared and analyzed for T_4 metabolites by the method described above. Hepatocytes were collected and analyzed for protein content.

Data analysis

Linear and nonlinear regression analyses were used to assess the relationship between [^{125}I]-T_4 accumulation in hepatocytes and appearance of T_4 metabolites in medium and disappearance of T_4 from the medium. A one-phase association exponential equation

$Y=Y_0 + (Plateau-Y_0)*(1-exp (-K*x))$,

was fit to the accumulation data where *plateau* is the Y value at infinite time and *half time* is the time it takes to reach half the plateau and is computed as ln(2)/K. A one-phase decay exponential equation,

$Y= (Y_0-Plateau)*exp (-K*X) +Plateau$,

was fit to the [^{125}I]-T_4 disappearance data where the depletion rate constant (K) was determined. The half-life ($t_{1/2}$) of all reactions was then determined as ln(2)/K. Using the rate of [^{125}I]-T_4 depletion, intrinsic clearance (CL_{int}) estimates were determined as described by Obach (1997) using the equation,

CL_{int}, *in vitro*=KV/N,

expressed as µl/min/10^6 cells, where K is ln(2)/ $t_{1/2}$, V is the incubation volume and N is the number of hepatocytes used. Human hepatocyte CL_{int} (µl/min/10^6 cells) was scaled to *in vivo* CL_{int} (ml/min/kg body weight) using the physiological parameters, human liver weight (22 g/kg body weight (and hepatocellularity (120 X 10^6 cells/g of liver) (Bayliss *et al.*, 1999; Soars *et al.*, 2002). Rat hepatocyte CL_{int} (µl/min/10^6 cells) was scaled to *in vivo* CL_{int} (ml/min/kg body weight) using the physiological parameters, rat liver weight (40 g/kg body weight (and hepatocellularity (120 X 10^6 cells/g of liver) (Bayliss, *et al.*, 1999).

Intergroup comparisons of control versus PCB 153 for SCRH were determined using a t-test. GraphPad Prism 5.0 was used to analyze all data, (GraphPad Software San Diego, CA). The level of probability of statistical significance was $p < 0.05$.

C. RESULTS

Glucuronidation of T_4 in rat and human liver and intestinal microsomes

To begin to understand variability in human liver metabolism of T_4, human liver microsomes were obtained both pooled and individual, from two different suppliers (Table 2.2). T_4G activity in these microsomes range approximately 5-fold, with the highest activity found in the pooled microsomes obtained from Xenotech and the lowest from an individual donor from Invitrogen (Table 2.3). Compared to the pooled rat microsomes, T_4G activity was 2 to 17.5 times lower in the human microsomes. The T_4G activity in the pooled human intestinal microsomes was 3 to 15 times higher than the T_4G activity in the human liver microsomes. Compared to the pooled rat liver microsomal sample, the human liver microsomal samples were between 3 and 15 times lower for T_4G activity (Table 2.3). T_4 sulfation was also determined in rat and human liver cytosols. T_4 sulfation activity was approximately 10 times greater compared to T_4G activity based on mg protein assayed and ranged from 4.0 to 4.8 pmol/mg protein/minute. The pooled rat cytosol had a similar T_4 sulfation activity compared to the human cytosol (Table 2.3).

T_4 and T_4 metabolite separation, identification and quantitation

Retention times of commercially available [^{125}I]-T_4, -T_3, and -rT_3 were determined to be 12.75, 10.25, and 10.50 min, respectively. Conjugated metabolites of T_4 were not commercially available; therefore T_4G and T_4S were biosynthesized using recombinant human UGT1A8 and SULT1E, respectively. Retention times for T_4G and T_4S were determined to be 5.50 and 8.25 min, respectively. Figure 2.1 illustrates the separation of T_4

and its metabolites in medium of SCRH incubated with 5µM [^{125}I]-T_4 for 24 hours at 37ºC. The peaks correspond to the retention times of reference standards for T_4, T_3, rT_3, T_4G and T_4S.

Comparisons of T_4 metabolism across days in culture in SCRH and SCHH

Physiological serum concentrations of T_4 in rat (0.05µM) and human (0.10µM) were used to measure changes in metabolite levels in media of SCRH and SCHH over days in culture. T_4G and T_4S were not detected in cell lysates, but were present in media. Figure 2.2A shows T_4G accumulation in the medium increased in SCRH from culture day 3 to 4 and plateaued on days 4 through 6. T_4G in the medium of SCHH decreased until undetectable at day 5 (Hu8096) and day 6 (Hu8092). T_4S reached maximum at day 4 and day 5 in media from SCHH and SCRH, respectively (Figure 2.2B). By day 5 and 6, similar amounts of T_4S were found in the media of SCRH and SCHH. T_3+rT_3 levels plateaued between days 4 and 6 in the medium of SCRH (Figure 2.2C). In medium of Hu8092, T_3+rT_3 reached a maximum at day 5 and plateaus at day 6. T_3+rT_3 in the medium of Hu8096 plateaued between culture days 4 and 6. Overall, these data show that metabolic capacity differed quantitatively over culture days between species and between human donors, creating a challenge when examining species differences for TH metabolite appearance in this culture system. Given that intact canaliculi with increasing efflux transporter activity has been reported over culture days in SCH (Hoffmaster *et al.*, 2004) and that biliary transport is a component of TH association and TH metabolite elimination (Friesema, *et al.*, 1999; Mitchell, *et al.*, 2005), we decided to add [^{125}I]-T_4 to hepatocytes on culture day 6 for all subsequent experiments.

T_4 accumulation in SCRH and SCHH

Cellular accumulation of T_4 was evaluated by measuring radioactive [^{125}I]-T_4 from 1 to 30 minutes on culture day 6 (Figure 2.3). Preliminary studies showed [^{125}I]-T_4 did not significantly bind to BD Matrigel® (BD Biosciences) or plastic ware (data not shown). [^{125}I]-T_4 cellular associated radioactivity increased over time, but appeared to plateau between 10 and 20 minutes. An exponential one-phase association model fit to each set of data indicates [^{125}I]-T_4 accumulation into SCRH was significantly different from accumulation into SCHH ($F=5.46$ (3,24); $p=0.0053$). [^{125}I]-T_4 accumulation in SCHH was greater and more rapid than in SCRH. The accumulation into the SCHH plateaued at approximately 11.7 ± 3.9% of the [^{125}I]-T_4 dose. In SCRH, the cellular accumulation plateaued at 5.5 ± 0.9%. Differences were also observed in [^{125}I]-T_4 accumulation half times, where the halftime of the SCRH was 6.1 min and the SCHH was 16.0 min. To understand the accumulation at the early time points, a linear model was used to fit the data from 1 to 5 minutes. There was no significant difference in the accumulation of T_4 in rat and human hepatocytes based on a linear model of the early time points ($F=0.35$ (1,21); $p=0.56$).

Effects of incubation time on T_4 metabolite concentrations in SCRH and SCHH

On day 6 of culture, metabolite levels were measured in the media of SCRH and SCHH at 4, 8, 12 and 24 hours following exposure to physiological rat and human serum concentrations of T_4. In medium of SCRH, T_4G increased over time (Figure 2.4A). In rat 2, T_4G peaked at 12 hours while in rat 1, T_4G levels increased in a linear manner ($p < 0.001$; $r^2=0.98$) with a slope of 0.5 ± 0.05 pmol/hr/mg cellular protein. T_4G appearance in the medium of SCHH was only detected at 12 and 24 hours for Hu1362 and at 24 hours in the

medium of Hu1364. The amounts of T_4G were much higher (13 times) in medium of SCRH as compared to SCHH. In SCRH medium, T_4S was only detected at 24 hours (Figure 2.4B). In medium of Hu1362, T_4S was first detected by 8 hours but there was no consistent time related trend. In Hu1364, T_4S increased linearly ($p < 0.05$; $r^2 = 0.90$) with time starting at 4 hours at a rate of 0.6 ± 0.2 pmol/hr/mg cellular protein.

In the medium of SCRH, there were no consistent time related trends for T_3+rT_3 concentrations for either rat culture ($p > 0.05$; $r^2 = 0.18$). In medium of Hu1362, T_3+rT_3 concentrations decreased significantly in a linear manner ($p < 0.03$; $r^2 = 0.94$) between 4 to 24 hours. T_3+rT_3 concentrations decreased over time, but not in a linear manner ($p > 0.08$) in Hu1364 (Figure 2.4C).

The percent of [^{125}I] radioactivity for T_4 decreased over time as metabolites increased in the media of SCRH and SCHH. T_4 was consistently detectable in SCHH (Table 2.4) and SCRH (Table 2.5) at all time points tested. By 24 hours, a larger (2 times) percentage of T_4 accumulated in SCHH compared to SCRH. The percentage of T_4 increased over time in SCHH, but not in a linear manner ($p > 0.14$). T_4 in the medium of SCHH decreased; however, not in a linear manner ($p > 0.09$). T_4G and T_4S were not detectable in SCRH or SCHH. Initially at 4 hours, T_3+rT_3 were detectable in SCRH but not in SCHH. At 24 hours, T_3+rT_3 were detectable in SCHH and not detectable in SCRH.

Intrinsic clearance of T_4 from the media of SCRH and SCHH.

The clearance of T_4 from the media of SCRH and SCHH was evaluated between 4 and 24 hours. T_4 depletion profiles are shown in Figure 2.5, where the percent of T_4 remaining in media over time is shown for SCRH (A) and SCHH (B). A one-phase decay

model was used to estimate CL_{int} in both SCRH and SCHH. In SCRHs, T_4 concentrations significantly decreased from 4-24 hours. CL_{int} in the hepatocytes of rat 1 and rat 2 were 1.08 and 0.75 µl/min/10^6 cells, respectively. In humans, T_4 concentrations also significantly decreased from 4-24 hours. CL_{int} for Hu1362 and 1364 were 0.56 and 0.62 µl/min/10^6 cells, respectively.

Effects of T_4 concentration on metabolite levels

T_4 metabolite levels in media of SCRH (Figure 2.6A) or SCHH (Figure 2.6B) were examined following a 24 h incubation with increasing concentrations of [^{125}I]-T_4 on culture day 6. Metabolites levels increased with increasing [^{125}I]-T_4 concentrations in the media of SCRH and SCHH. At all concentrations, the rank order of metabolites in the medium of SCRH was: $T_4G > T_3+rT_3 > T_4S$. T_4G was undetectable at lower concentrations of [^{125}I]-T_4 in medium of SCHH. The rank order of metabolites in the medium of SCHH was $T_3+rT_3 > T_4G \approx T_4S$.

Effects of PCB 153 on T_4 metabolite levels and clearance

To study the effects of hepatic enzyme inducers on T_4 metabolism in hepatocytes, PCB 153 was used as a prototype environmental chemical. Following exposure to PCB 153 (72 hours), SCRH and SCHH were treated with physiological rat and human serum concentrations of T_4 for 24 hours. Treatment of SCRH with 30µM PCB 153 increased T_4G 2.9-fold (Figure 2.7A). T_4G in media of Hu8092 and Hu8096 was only detectable following PCB 153 treatment. Following PCB 153 treatment, T_4G levels increased in the media of Hu1362 and Hu1364 9.3- and 3.2-fold, respectively. T_4S levels did not change significantly

in the media of SCRH, Hu8092, Hu8096, Hu1362, and Hu1364 (Figure 2.7B). T_3+rT_3 levels following PCB 153 treatment were unchanged in the media of SCRH, Hu8092, Hu8096, Hu1362, and Hu1364 (Figure 2.7C). Increases in T_4G levels in media of rat and human hepatocytes were a more consistent response to PCB 153 treatment as compared to T_4S and T_3+rT_3 appearance.

Effect of PCB 153 on the intrinsic clearance of T_4 from the media of SCRH and SCHH.

Following 72 hours treatment with 30uM of PCB 153, T_4 clearance was evaluated between 4 and 24 hours for SCRH and SCHH (Table 2.6). A one-phase decay model was used to estimate the clearance rate in both SCRH and SCHH. CL_{int} in the hepatocytes of rat1 and rat 2 following PCB 153 treatment were 1.34 and 1.53 µl/min/10^6 cells, respectively. This represents between a 1.24- and 2.06-fold change in the clearance of T_4 in the rat hepatocytes compared to the control. CL_{int} for Hu1362 and Hu1364 were 0.47 and 0.61 µl/min/10^6 cells. This represents a 0.86-fold change for Hu1362 and no change in the clearance of T_4 in Hu1364 compared to the control.

D. DISCUSSION

Much of the studies examining thyroxine metabolism use either microsomal or cytosolic fractions of various tissues. Using isolated liver fractions such as microsomes and cytosols has limitations in understanding the overall metabolism of endogenous substances and xenobiotics. SCHs provide more *in vivo*-like metabolism, particularly when there are multiple enzymes involved with different intracellular locations. Differences in

glucuronidation and metabolism of THs between rats and humans were studied using liver and intestinal microsomal and liver cytosolic preparations and sandwich cultured hepatocytes. T_4G activity varied by a factor of 5 in human microsomes and varied by a less than a factor of two in the SCHH. The variation in human liver microsomal T_4G activity is similar to that observed by Yamanaka *et al* (2007) and Kato *et al* (2008). The rat liver microsomal T_4G activity was higher than the human microsomal activity by 2- to 17.5-fold. In the SCH, the basal T_4G activity was 2- to more than 13-fold greater in SCRH compared to SCHH, depending on the day in culture. The concordance between the microsomal and SCH results on species differences provides confidence that the culture conditions are representative of *in vivo* metabolism.

Pooled rat intestinal microsomes had greater T_4G activity than the pooled rat liver microsomes. Similar differences in T_4G activity were observed with the pooled human intestinal microsomes compared to the human liver microsomes. Yamanaka et al (2007) also demonstrated T_4 glucuronidation in human liver and intestinal microsomes and suggested that in humans, intestinal clearance of T_4 is half that of liver and that intestinal glucuronidation may play an important role in enterohepatic circulation. This is the first report to demonstrate that, similar to humans, rat intestinal microsomes have higher T_4G glucurondiation activity compared to liver microsomes and also suggests an important role of intestinal glucuronidation in the enterohepatic circulation of thyroxine in rats.

In contrast to glucuronidation, sulfation of T_4 in rat and human liver cytosols were similar and the variation in T_4 sulfation was less than 1.5-fold in the various human samples. In the SCRH, <1% of the T_4 is sulfated. In comparison, almost 25% of the T_4 is glucuronidated after 24 hours in culture. In SCHH, <1% of the T_4 is sulfated or

glucuronidated. While previous studies have examined T_3 sulfation in rat hepatocytes, this is the first study to examine T_4 sulfation in both SCRH and SCHH. The relative importance of T_4 metabolic pathways did not vary with substrate concentration (0.1 to 100 μM T_4) in either SCRH or SCHH. The similar proportions of metabolites produced as concentration increases is consistent with the similar K_m values ranging between 20-100 μM that various UGT and SULT isoforms exhibit towards T_4 (Li and Anderson, 1999; Yamanaka, *et al.*, 2007).

Results of the [^{125}I]-T_4 incubation time course studies show that T_4G does not appear in the medium of SCHH and T_4S does not appear in the medium of SCRH at early time points, but were measurable at the 24 hour time point. This is consistent with the intrinsic clearance of T_4 from media in the present study. T_3+rT_3 were detectable at each time point in the media of SCRH and SCHH. While T_3+rT_3 contaminants were found in stock solutions, they were determined to be less than 0.1% of the [^{125}I]-T_4. Data were then corrected for this contaminant, so it is unlikely that the appearance of T_3+rT_3 were due to impurities. Despite the appearance of T_3+rT_3 at all time points examined, we did not detect further conversion metabolites like T_2, T_3-glucuronide or T_3-sulfate in either medium or cells, but this may be due to detection limits for these metabolites.

In the present study, physiologically relevant concentrations of T_4 for rat (0.05μM) and human (0.1μM) were used to examine T_4 metabolite levels in media and cells of SCH. T_4G appearance in medium from SCRH is detectable on day 3 in culture and plateaus on days 4 through 6. In contrast, T_4G appearance diminished until undetectable in the medium of SCHH by as soon as the fourth day in culture. Amounts of T_4G in medium of SCRH were about 13-fold greater than in medium of SCHH. T_3+rT_3 levels increase from day 3 to day 4

in culture and plateaus between days 4 and 6 for SCRH and Hu8092. In medium of Hu8096, T_3+rT_3 levels remained constant over days in culture. In contrast, we found that T_4S amounts in the media of SCRH and SCHH were similar by culture days 5 and 6. Except for T_4G in the media of Hu8092 and Hu8096, metabolite levels were maintained out to culture day 6. In a study using monolayer cultures of rat hepatocytes, T_4G activity decreased over culture days (Viollon-Abadie *et al.*, 2000). This does not agree with the present study, however, the difference in culture systems between the studies suggests that the effects observed are related to culture conditions.

Transmembrane movement of thyroid hormones was thought to be a passive process; however, there is evidence that translocation occurs through active transport mechanisms (Blondeau *et al.*, 1988; De Jong *et al.*, 1992; de Jong, *et al.*, 1993). Specifically, members of the MCT and OATP family have been shown to facilitate TH transport (Pizzagalli *et al.*, 2002; Friesema *et al.*, 2003; Friesema *et al.*, 2008; van der Deure *et al.*, 2008). Active transport is likely the rate-limiting step for TH metabolism; therefore, SCRH and SCHH were used to examine T_4 accumulation over time. The rate of T_4 accumulation has been shown to be slower in human hepatocytes than rat hepatocytes (Krenning *et al.*, 1981; de Jong, *et al.*, 1993). In the present study, the rate of T_4 accumulation is similar between SCRH and SCHH up to 5 minutes. At the later time points in the accumulation studies, [^{125}I]-T_4 accumulation plateaued at lower concentrations in SCRH than SCHH. The lower accumulation of T_4 in SCRH compared to the SCHH is consistent with the greater T_4G activity of the SCRH compared to the SCHH. Transthyretin (TTR) and thyroid binding globulin (TBG) are major TH serum binding proteins and are thought to modulate the delivery of TH from blood to cells (Hennemann *et al.*, 2001; Choksi *et al.*, 2003). In

humans, the majority of T_4 is bound to TBG (Benvenga, 2005). In rats, TBG expression is not detectable in adults; therefore, the major carrier protein for T_4 is TTR (Savu, *et al.*, 1991). This difference in serum binding proteins between rat and human may be of important to T_4 accumulation due to the differences in T_4 affinity, where T_4 has approximately 140-times greater affinity for TBG ($Ka=1x10^{-10}$ M) than for TTR ($Ka=7x10^{-7}$ M) (Robbins, 1991). The T_4 concentrations used in the present study do not take into account the binding of T_4 to TTR or TBG; therefore, the species difference in TH binding proteins *in vivo* may indicate species differences in hepatic T_4 accumulation.

PCBs are environmental contaminates that were once used in capacitors and other industrial processes. In rats, PCBs decrease thyroid hormones and this effect is thought to be due to increased hepatic accumulation and glucuronidation (Bastomsky, 1974; Hood and Klaassen, 2000a; Klaassen and Hood, 2001; Craft, *et al.*, 2002; Crofton *et al.*, 2005; Martin *et al.*, 2012). PCB 153 is one of the predominate congeners found in the environment as well as in humans. It has been described as a phenobarbital-like PCB and decreases thyroid hormones in rats (Craft, *et al.*, 2002; Crofton, *et al.*, 2005). The current study shows that PCB 153 induced T_4 glucuronidation in both SCRH and SCHH. In comparison, T_4S and T_3+rT_3 production were unaffected by PCB 153 treatment in both SCRH and SCHH. T_4G in control media from Hu8092 and Hu8096 was not detectable; however, following PCB-153 treatment, T_4G accumulated in the media. In SCRH, T_4G accumulation in the medium from control and PCB153 treatment was greater than in SCHH. *In vivo* studies using the PCB mixture, Aroclor 1254, showed decreases in hepatic deiodinase I activity in rats (Hood and Klaassen, 2000b). In the present study, no change in T_3+rT_3 production following PCB 153 treatment of SCRH and SCHH was observed. The difference between the *in vivo* studies and

the *in vitro* studies may be that *in vivo*, reductions in hepatic deiodinase activity may not be due to direct effects of the chemical on hepatocytes, but a physiological response to decreased serum T_4 concentrations (Davies *et al.*, 1996). Therefore, if deiodinase activity reductions are a physiological response *in vivo*, it is likely that T_3+rT_3 production would not change in hepatocyte cultures at the concentrations of T_4 used in this study.

The present study assessed the utility of SCH for evaluating the species differences in TH metabolism changes following exposure to environmental chemicals. This is the first report, to our knowledge, characterizing inducibility, metabolism, and clearance of T_4 in an *in vitro* model system in both SCRH and SCHH. T_4 metabolism was measurable in untreated SCRH and SCHH. Exposure to PCB 153 increased metabolism of T_4, consistent with the effects observed *in vivo* in rats. This model is also consistent with other *in vivo* and *in vitro* data indicating that glucuronidation may be a predominant pathway for hepatic TH metabolism in rats. Previous studies have suggested that sulfation is the preferred pathway for TH metabolism in humans (Kester, *et al.*, 1999; Kester, *et al.*, 2003); however we find that deiodination of T_4 is the favored pathway in this model system. This *in vitro* system may be useful in evaluating a chemical's ability to increase hepatic T_4 metabolism *in vivo*. However, while the present model can be used to qualitatively assess whether a chemical can alter TH metabolism, quantitative extrapolation of this data to *in vivo* exposures requires further development.

REFERENCES

Arias, I. M., Che, M., Gatmaitan, Z., Leveille, C., Nishida, T. and St Pierre, M. (1993). The biology of the bile canaliculus, 1993. *Hepatology* **17**, 318-29.

Barter, R. A. and Klaassen, C. D. (1992). UDP-glucuronosyltransferase inducers reduce thyroid hormone levels in rats by an extrathyroidal mechanism. *Toxicol Appl Pharmacol* **113**, 36-42.

Bastomsky, C. H. (1974). Effects of a polychlorinated biphenyl mixture (aroclor 1254) and DDT on biliary thyroxine excretion in rats. *Endocrinology* **95**, 1150-5.

Bayliss, M. K., Bell, J. A., Jenner, W. N., Park, G. R. and Wilson, K. (1999). Utility of hepatocytes to model species differences in the metabolism of loxtidine and to predict pharmacokinetic parameters in rat, dog and man. *Xenobiotica; the fate of foreign compounds in biological systems* **29**, 253-68.

Benedetti, M. S., Whomsley, R., Baltes, E. and Tonner, F. (2005). Alteration of thyroid hormone homeostasis by antiepileptic drugs in humans: involvement of glucuronosyltransferase induction. *Eur J Clin Pharmacol* **61**, 863-72.

Benvenga, S. (2005). Peripheral Hormone Metabolism. The Thyroid: A Fundamental and Clinical Text.(L. E. Braverman, Utiger, R. D. Eds.), pp. 97-108.Lippencott Williams and Wilkins,Philadelphia.

Blondeau, J. P., Osty, J. and Francon, J. (1988). Characterization of the thyroid hormone transport system of isolated hepatocytes. *J Biol Chem* **263**, 2685-92.

Choksi, N. Y., Jahnke, G. D., St Hilaire, C. and Shelby, M. (2003). Role of thyroid hormones in human and laboratory animal reproductive health. *Birth defects research. Part B, Developmental and reproductive toxicology* **68**, 479-91.

Craft, E. S., DeVito, M. J. and Crofton, K. M. (2002). Comparative responsiveness of hypothyroxinemia and hepatic enzyme induction in Long-Evans rats versus C57BL/6J mice exposed to TCDD-like and phenobarbital-like polychlorinated biphenyl congeners. *Toxicol Sci* **68**, 372-80.

Crofton, K. M., Craft, E. S., Hedge, J. M., Gennings, C., Simmons, J. E., Carchman, R. A., Carter, W. H., Jr. and DeVito, M. J. (2005). Thyroid-hormone-disrupting chemicals: evidence for dose-dependent additivity or synergism. *Environ Health Perspect* **113**, 1549-54.

Davies, P. H., Sheppard, M. C. and Franklyn, J. A. (1996). Regulation of type I 5'-deiodinase by thyroid hormone and dexamethasone in rat liver and kidney cells. *Thyroid* **6**, 221-8.

De Jong, M., Docter, R., Van Der Hoek, H. J., Vos, R. A., Krenning, E. P. and Hennemann, G. (1992). Transport of 3,5,3'-triiodothyronine into the perfused rat liver and subsequent metabolism are inhibited by fasting. *Endocrinology* **131**, 463-70.

de Jong, M., Visser, T. J., Bernard, B. F., Docter, R., Vos, R. A., Hennemann, G. and Krenning, E. P. (1993). Transport and metabolism of iodothyronines in cultured human hepatocytes. *J Clin Endocrinol Metab* **77**, 139-43.

Friesema, E. C., Docter, R., Moerings, E. P., Stieger, B., Hagenbuch, B., Meier, P. J., Krenning, E. P., Hennemann, G. and Visser, T. J. (1999). Identification of thyroid hormone transporters. *Biochem Biophys Res Commun* **254**, 497-501.

Friesema, E. C., Ganguly, S., Abdalla, A., Manning Fox, J. E., Halestrap, A. P. and Visser, T. J. (2003). Identification of monocarboxylate transporter 8 as a specific thyroid hormone transporter. *J Biol Chem* **278**, 40128-35.

Friesema, E. C., Jansen, J., Jachtenberg, J. W., Visser, W. E., Kester, M. H. and Visser, T. J. (2008). Effective cellular uptake and efflux of thyroid hormone by human monocarboxylate transporter 10. *Mol Endocrinol* **22**, 1357-69.

Ganem, L. G., Trottier, E., Anderson, A. and Jefcoate, C. R. (1999). Phenobarbital induction of CYP2B1/2 in primary hepatocytes: endocrine regulation and evidence for a single pathway for multiple inducers. *Toxicol Appl Pharmacol* **155**, 32-42.

Hagmar, L., Rylander, L., Dyremark, E., Klasson-Wehler, E. and Erfurth, E. M. (2001). Plasma concentrations of persistent organochlorines in relation to thyrotropin and thyroid hormone levels in women. *Int Arch Occup Environ Health* **74**, 184-8.

He, P., Wang, A., Niu, Q., Guo, L., Xia, T. and Chen, X. (2011). Toxic effect of PBDE-47 on thyroid development, learning, and memory, and the interaction between PBDE-47 and PCB153 that enhances toxicity in rats. *Toxicology and industrial health* **27**, 279-88.

Hennemann, G., Docter, R., Friesema, E. C., de Jong, M., Krenning, E. P. and Visser, T. J. (2001). Plasma membrane transport of thyroid hormones and its role in thyroid hormone metabolism and bioavailability. *Endocr Rev* **22**, 451-76.

Hoffmaster, K. A., Turncliff, R. Z., LeCluyse, E. L., Kim, R. B., Meier, P. J. and Brouwer, K. L. (2004). P-glycoprotein expression, localization, and function in sandwich-cultured primary rat and human hepatocytes: relevance to the hepatobiliary disposition of a model opioid peptide. *Pharm Res* **21**, 1294-302.

Hood, A., Allen, M. L., Liu, Y., Liu, J. and Klaassen, C. D. (2003). Induction of T(4) UDP-GT activity, serum thyroid stimulating hormone, and thyroid follicular cell proliferation in mice treated with microsomal enzyme inducers. *Toxicol Appl Pharmacol* **188**, 6-13.

Hood, A., Hashmi, R. and Klaassen, C. D. (1999). Effects of microsomal enzyme inducers on thyroid-follicular cell proliferation, hyperplasia, and hypertrophy. *Toxicol Appl Pharmacol* **160**, 163-70.

Hood, A. and Klaassen, C. D. (2000a). Differential effects of microsomal enzyme inducers on in vitro thyroxine (T(4)) and triiodothyronine (T(3)) glucuronidation. *Toxicol Sci* **55**, 78-84.

Hood, A. and Klaassen, C. D. (2000b). Effects of microsomal enzyme inducers on outer-ring deiodinase activity toward thyroid hormones in various rat tissues. *Toxicol Appl Pharmacol* **163**, 240-8.

Jansen, J., Friesema, E. C., Milici, C. and Visser, T. J. (2005). Thyroid hormone transporters in health and disease. *Thyroid* **15**, 757-68.

Kaptein, E., van Haasteren, G. A., Linkels, E., de Greef, W. J. and Visser, T. J. (1997). Characterization of iodothyronine sulfotransferase activity in rat liver. *Endocrinology* **138**, 5136-43.

Kato, Y., Ikushiro, S., Emi, Y., Tamaki, S., Suzuki, H., Sakaki, T., Yamada, S. and Degawa, M. (2008). Hepatic UDP-glucuronosyltransferases responsible for glucuronidation of thyroxine in humans. *Drug Metab Dispos* **36**, 51-5.

Kester, M. H., Kaptein, E., Roest, T. J., van Dijk, C. H., Tibboel, D., Meinl, W., Glatt, H., Coughtrie, M. W. and Visser, T. J. (1999). Characterization of human iodothyronine sulfotransferases. *J Clin Endocrinol Metab* **84**, 1357-64.

Kester, M. H., Kaptein, E., Roest, T. J., van Dijk, C. H., Tibboel, D., Meinl, W., Glatt, H., Coughtrie, M. W. and Visser, T. J. (2003). Characterization of rat iodothyronine sulfotransferases. *Am J Physiol Endocrinol Metab* **285**, E592-8.

Klaassen, C. D. and Hood, A. M. (2001). Effects of microsomal enzyme inducers on thyroid follicular cell proliferation and thyroid hormone metabolism. *Toxicol Pathol* **29**, 34-40.

Kolaja, K. L. and Klaassen, C. D. (1998). Dose-response examination of UDP-glucuronosyltransferase inducers and their ability to increase both TGF-beta expression and thyroid follicular cell apoptosis. *Toxicol Sci* **46**, 31-7.

Krenning, E., Docter, R., Bernard, B., Visser, T. and Hennemann, G. (1981). Characteristics of active transport of thyroid hormone into rat hepatocytes. *Biochim Biophys Acta* **676**, 314-20.

Li, X. and Anderson, R. J. (1999). Sulfation of iodothyronines by recombinant human liver steroid sulfotransferases. *Biochem Biophys Res Commun* **263**, 632-9.

Liu, J., Liu, Y., Barter, R. A. and Klaassen, C. D. (1995). Alteration of thyroid homeostasis by UDP-glucuronosyltransferase inducers in rats: a dose-response study. *J Pharmacol Exp Ther* **273**, 977-85.

Malik, R. and Hodgson, H. (2002). The relationship between the thyroid gland and the liver. *QJM : monthly journal of the Association of Physicians* **95**, 559-69.

Martin, L. A., Wilson, D. T., Reuhl, K. R., Gallo, M. A. and Klaassen, C. D. (2012). Polychlorinated biphenyl congeners that increase the glucuronidation and biliary excretion of thyroxine are distinct from the congeners that enhance the serum disappearance of thyroxine. *Drug Metab Dispos* **40**, 588-95.

McClain, R. M., Levin, A. A., Posch, R. and Downing, J. C. (1989). The effect of phenobarbital on the metabolism and excretion of thyroxine in rats. *Toxicol Appl Pharmacol* **99**, 216-28.

Mitchell, A. M., Tom, M. and Mortimer, R. H. (2005). Thyroid hormone export from cells: contribution of P-glycoprotein. *J Endocrinol* **185**, 93-8.

Obach, R. S. (1997). Nonspecific binding to microsomes: impact on scale-up of in vitro intrinsic clearance to hepatic clearance as assessed through examination of warfarin, imipramine, and propranolol. *Drug Metab Dispos* **25**, 1359-69.

Ohnhaus, E. E. and Studer, H. (1983). A link between liver microsomal enzyme activity and thyroid hormone metabolism in man. *Br J Clin Pharmacol* **15**, 71-6.

Oppenheimer, J. H., Bernstein, G. and Surks, M. I. (1968). Increased thyroxine turnover and thyroidal function after stimulation of hepatocellular binding of thyroxine by phenobarbital. *J Clin Invest* **47**, 1399-406.

Pizzagalli, F., Hagenbuch, B., Stieger, B., Klenk, U., Folkers, G. and Meier, P. J. (2002). Identification of a novel human organic anion transporting polypeptide as a high affinity thyroxine transporter. *Mol Endocrinol* **16**, 2283-96.

Robbins, J. (1991). Thyroid hormone transport proteins and the physiology of hormone binding. Werner & Ingbars, The Thyroid.(R. D. Utiger and L. E. Braverman Eds.), pp. 111-125.J.B. Lippincott,Philadelphia, U.S.A.

Robbins, J. and Rall, J. E. (1960). Proteins associated with the thyroid hormones. *Physiol Rev* **40**, 415-89.

Rutgers, M., Bonthuis, F., de Herder, W. W. and Visser, T. J. (1987). Accumulation of plasma triiodothyronine sulfate in rats treated with propylthiouracil. *J Clin Invest* **80**, 758-62.

Rutgers, M., Pigmans, I. G., Bonthuis, F., Docter, R. and Visser, T. J. (1989). Effects of propylthiouracil on the biliary clearance of thyroxine (T4) in rats: decreased excretion of 3,5,3'-triiodothyronine glucuronide and increased excretion of 3,3',5'-triiodothyronine glucuronide and T4 sulfate. *Endocrinology* **125**, 2175-86.

Savu, L., Vranckx, R., Rouaze-Romet, M., Maya, M., Nunez, E. A., Treton, J. and Flink, I. L. (1991). A senescence up-regulated protein: the rat thyroxine-binding globulin (TBG). *Biochim Biophys Acta* **1097**, 19-22.

Soars, M. G., Burchell, B. and Riley, R. J. (2002). In vitro analysis of human drug glucuronidation and prediction of in vivo metabolic clearance. *J Pharmacol Exp Ther* **301**, 382-90.

Szabo, D. T., Richardson, V. M., Ross, D. G., Diliberto, J. J., Kodavanti, P. R. and Birnbaum, L. S. (2009). Effects of perinatal PBDE exposure on hepatic phase I, phase II, phase III, and deiodinase 1 gene expression involved in thyroid hormone metabolism in male rat pups. *Toxicol Sci* **107**, 27-39.

Tong, Z., Li, H., Goljer, I., McConnell, O. and Chandrasekaran, A. (2007). In vitro glucuronidation of thyroxine and triiodothyronine by liver microsomes and recombinant human UDP-glucuronosyltransferases. *Drug Metab Dispos* **35**, 2203-10.

van der Deure, W. M., Hansen, P. S., Peeters, R. P., Kyvik, K. O., Friesema, E. C., Hegedus, L. and Visser, T. J. (2008). Thyroid hormone transport and metabolism by organic anion transporter 1C1 and consequences of genetic variation. *Endocrinology* **149**, 5307-14.

van der Heide, S. M., Visser, T. J., Everts, M. E. and Klaren, P. H. (2002). Metabolism of thyroid hormones in cultured cardiac fibroblasts of neonatal rats. *J Endocrinol* **174**, 111-9.

Vansell, N. R. and Klaassen, C. D. (2001). Increased biliary excretion of thyroxine by microsomal enzyme inducers. *Toxicol Appl Pharmacol* **176**, 187-94.

Vansell, N. R. and Klaassen, C. D. (2002). Effect of microsomal enzyme inducers on the biliary excretion of triiodothyronine (T(3)) and its metabolites. *Toxicol Sci* **65**, 184-91.

Viollon-Abadie, C., Bigot-Lasserre, D., Nicod, L., Carmichael, N. and Richert, L. (2000). Effects of model inducers on thyroxine UDP-glucuronosyl-transferase activity in vitro in rat and mouse hepatocyte cultures. *Toxicol In Vitro* **14**, 505-12.

Visser, T. J., Kaptein, E., van Toor, H., van Raaij, J. A., van den Berg, K. J., Joe, C. T., van Engelen, J. G. and Brouwer, A. (1993). Glucuronidation of thyroid hormone in rat liver: effects of in vivo treatment with microsomal enzyme inducers and in vitro assay conditions. *Endocrinology* **133**, 2177-86.

Yamanaka, H., Nakajima, M., Katoh, M. and Yokoi, T. (2007). Glucuronidation of thyroxine in human liver, jejunum, and kidney microsomes. *Drug Metab Dispos* **35**, 1642-8.

Zhou, T., Ross, D. G., DeVito, M. J. and Crofton, K. M. (2001). Effects of short-term in vivo exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. *Toxicol Sci* **61**, 76-82.

Table 2.1

Donor information for human hepatocytes

Donor	Sex	Age	Smoke/Alcohol	Medications	Experimental Use
Hu1191	Male	19	No/No	UKN	T_4 Accumulation/ T_4 Clearance
Hu1193	Male	68	Yes/No	Aspirin Zolpidem Simvastatin Ranitidine	T_4 Accumulation/ T_4 Clearance
Hu1197	Female	29	No/Yes	Amphetamine Bupropion Cetirizine Clonazepam Lamotrigine	T_4 Accumulation/ T_4 Clearance
Hu1362	Female	57	No/No	Atenolol and Chlorthalidone Lorazepam Oxycodone	Time course/ PCB 153
Hu1364	Male	51	No/No	Cyanocobalamin Diltiazem Ferrous Sulfate Folic Acid Metoprolol Omeprazole Potassium Chloride Prednisone	Time course/ PCB 153
Hu8092 (non-transplantable)	Male	59	No/No	UKN	T_4 dose response/ culture days/ PCB 153
Hu8096 (non-transplantable)	Male	52	No/Yes	UKN	T_4 dose response/ culture days/ PCB 153

UKN=unknown

Table 2.2

Liver microsomes and cytosol

Species	Tissue	Fraction	Lot	Sex	Supplier	Number of subjects
Human	Liver	Cytosolic/ Microsomal	SD114	Male	CellzDirect	1
Human	Liver	Cytosolic/ Microsomal	SD119	Male	CellzDirect	1
Human	Liver	Cytosolic/ Microsomal	SD120	Female	CellzDirect	1
Human	Liver	Cytosolic/ Microsomal	SD122	Male	CellzDirect	1
Human	Liver	Cytosolic/ Microsomal	SD123	Female	CellzDirect	1
Human	Liver	Cytosolic/ Microsomal	SD129	Male	CellzDirect	1
Human	Liver	Microsomal	PL050	Mixed	CellzDirect	50
Human	Liver	Cytosolic	PL024	Mixed	CellzDirect	28
Rat	Liver	Cytosolic	RT042	Male	CellzDirect	50
Rat	Liver	Microsomal	RT046	Male	CellzDirect	50
Human	Liver	Microsomal	0710091	Mixed	XenoTech	50
Human	Intestine	Microsomal	0610108	Mixed	XenoTech	10
Rat	Liver	Microsomal	0710387	Male	XenoTech	400
Rat	Intestine	Microsomal	0410063	Male	XenoTech	148

Table 2.3

Comparison of hepatic and intestinal T_4G formation rates in microsomes and hepatic T_4S formation rates in cytosols from rats and humans[a].

Species	Tissues	Donor	T_4G pmol/mg/min	T_4S pmol/mg/min
Rat[b]	Liver	Pooled	2.0	4.4
Rat[c]	Liver	Pooled	3.5	ND
Rat[c]	Intestine	Pooled	7.2	ND[d]
Human[c]	Liver	Pooled	1.0	ND
Human[b]	Liver	Pooled	0.3	4.0
Human[c]	Intestine	Pooled	3.4	ND[d]
Human[b]	Liver	SD114	0.2	4.4
Human[b]	Liver	SD119	0.4	4.2
Human[b]	Liver	SD120	0.3	4.1
Human[b]	Liver	SD122	0.3	4.4
Human[b]	Liver	SD123	0.3	4.3
Human[b]	Liver	SD129	0.5	4.8

[a]Data are expressed as pmol/mg protein/min (mean). Data represent the average of duplicate experiments with variability of <30%. ND=not determined; T_4 conjugates were generated following an incubation with 4uM [^{125}I]-T_4

[b]Samples obtained from Invitrogen

[c]Samples obtained from Xenotech

[d]Cytosol not available

Table 2.4

Mass balance of T_4 and T_4 metabolites after exposure of human hepatocytes on culture day 6 to 0.1µM [^{125}I]-T_4

Time (hours)	T_4[a]		T_4G[a]		T_4S[a]		T_3+rT_3[a]	
	Cells	Media	Cells	Media	Cells	Media	Cells	Media
4	12.9	85.5	BLQ	BLQ	BLQ	BLQ	BLQ	1.5
8	14.2	84.2	BLQ	BLQ	BLQ	0.4	BLQ	1.4
12	17.4	80.7	BLQ	BLQ	BLQ	0.4	BLQ	1.7
24	17.8	79.5	BLQ	BLQ	BLQ	0.6	0.3	1.3

[a]Data are expressed as percent of total [^{125}I] activity (mean); Data represent the average of duplicate experiments; Limits of detection=0.4 pmol/mg cellular protein; BLQ=below limits of quantitation; n=2 (Hu1362 and Hu1364)

Table 2.5

Mass balance of T_4 and T_4 metabolites after exposure of rat hepatocytes on culture day 6 to 0.05µM [^{125}I]-T_4

Time (hours)	T_4[a]		T_4G[a]		T_4S[a]		T_3+rT_3[a]	
	Cells	Media	Cells	Media	Cells	Media	Cells	Media
4	10.5	83.8	BLQ	3.4	BLQ	BLQ	0.2	2.1
8	7.6	77.6	BLQ	12.7	BLQ	BLQ	BLQ	2.1
12	10.8	70.6	BLQ	16.8	BLQ	BLQ	BLQ	1.9
24	8.4	65.3	BLQ	23.7	BLQ	0.4	BLQ	2.3

Percentages of total metabolites, T_4G, T_4S and T_3+rT_3, in media and hepatocytes following exposure to [^{125}I]-T_4 for 4, 8, 12, or 24 hours.

[a]Data are expressed as percent of total [^{125}I] activity (mean); Data represent the average of duplicate from experiments; Limits of detection=0.5 pmol/mg cellular protein; BLQ=below limits of quantitation.; n=2.

Table 2.6

Intrinsic clearance of [^{125}I]-T_4 following treatment with PCB 153

		Control	PCB 153	Fold change
Rat 1	Rate constant K (1/min)	0.00075	0.00094	1.25
	Cl_{int} (µl/min/10^6 cells)	1.08	1.34	1.24
	Scaled Cl_{int} (ml/min/kg body weight)	5.16	6.41	1.24
Rat 2	Rate constant K (1/min)	0.00052	0.00107	2.06
	Cl_{int} (µl/min/10^6 cells)	0.75	1.53	2.04
	Scaled Cl_{int} (ml/min/kg body weight)	3.58	7.33	2.05
Hu 1362	Rate constant K (1/min)	0.00039	0.00033	0.84
	Cl_{int} (µl/min/10^6 cells)	0.56	0.47	0.86
	Scaled Cl_{int} (ml/min/kg body weight)	1.60	1.37	0.85
Hu 1364	Rate constant K (1/min)	0.00043	0.00043	0.98
	Cl_{int} (µl/min/10^6 cells)	0.61	0.61	1.00
	Scaled Cl_{int} (ml/min/kg body weight)	1.78	1.77	0.99

Data represent the average of duplicates from a single experiment.
SCH were incubated with 0.05µM [^{125}I]-T_4 (rat) or 0.1µM [^{125}I]-T_4 (human) for up to 24 hours.

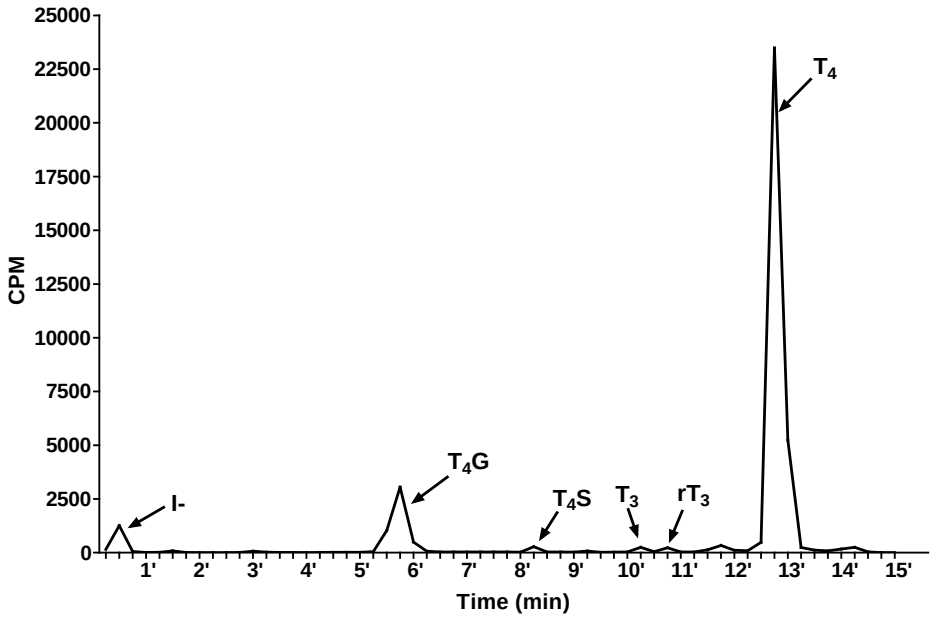
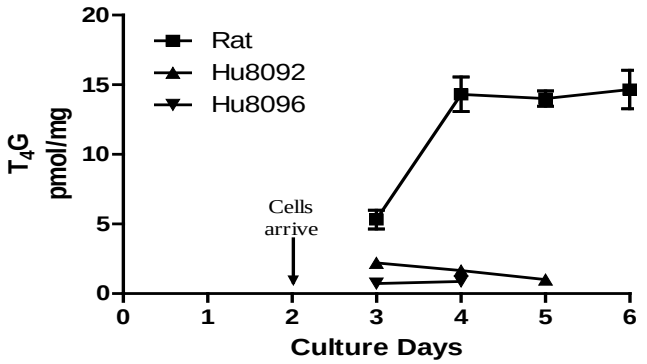
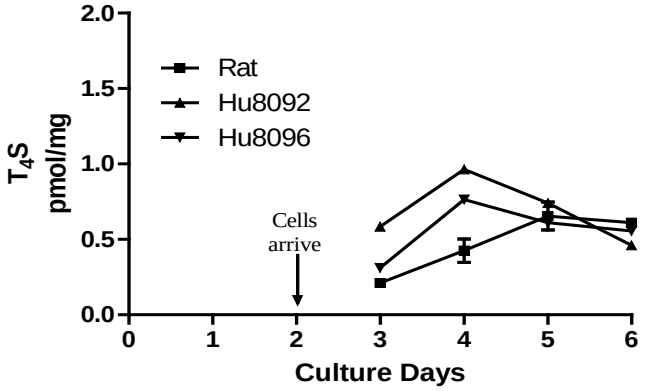


Figure 2.1 Representative chromatogram of T_4 and its metabolites in medium.
The graph shows metabolites [iodide (-I), T_4-glucuronide (T_4G), T_4-sulfate (T_4S), 3,3',5-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (rT_3)] in medium of SCRH following a 24 hour incubation with 5.0µM [^{125}I]-T_4 on culture day 6. Peaks were separated using UPLC. Fractions of eluent were collected and analyzed by gamma spectroscopy.

A.



B.



C.

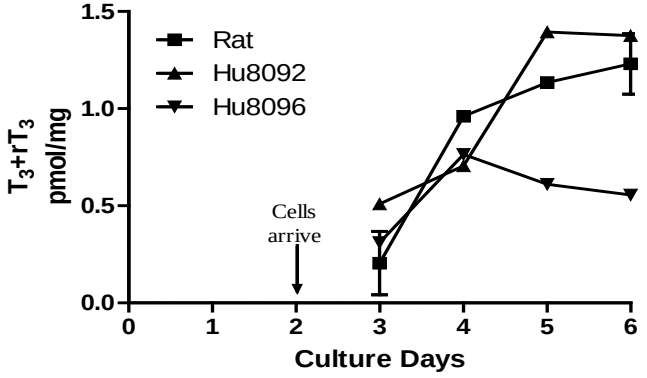


Figure 2.2 T_4 metabolite levels in media during time in culture.SCH arrive on culture day 2 and are untreated. SCH were incubated with 0.05µM [^{125}I]-T_4 (rat) or 0.1µM [^{125}I]-T_4 (human) for 24 hours on culture days 3, 4, 5, or 6. Metabolites were separated using the established UPLC method. Metabolites analyzed are (A) T_4G, (B) T_4S, and (C) T_3+rT_3. Data are expressed as pmol/mg cellular protein [mean (human hepatocytes)] or [mean±SD (rat hepatocytes)]. Human hepatocyte data represent the average of duplicates in a single experiment. Human hepatocytes are from two donors (Hu8092 and Hu8096). Rat hepatocytes are from 3 donors (n=3)

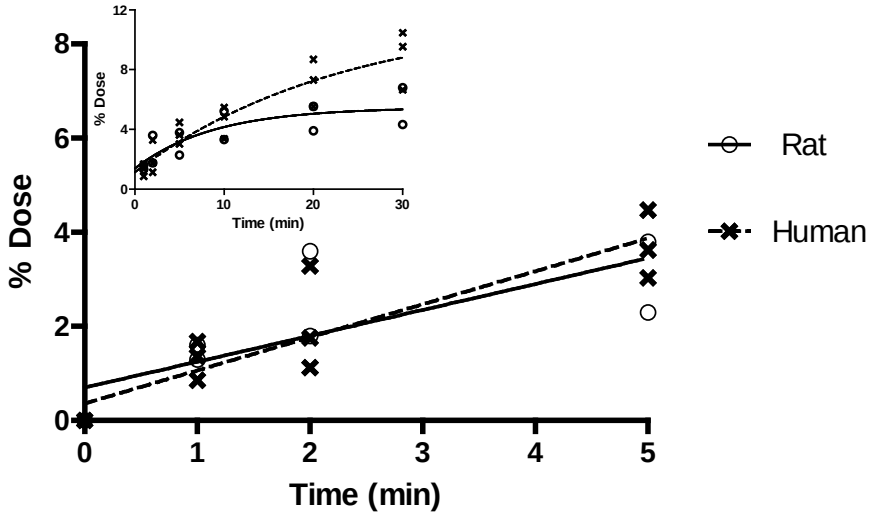


Figure 2.3. Accumulation of [^{125}I]-derived radioactivity in rat and human hepatocytes. Sandwich-cultured hepatocytes were incubated with 0.0005µM (5000 cpm) per well [^{125}I]-T_4 on culture day 6. The accumulation of [^{125}I]-T_4 was determined over time (1-5 min). Data represent the average of duplicates in a single experiment. Individual hepatocyte donor values are presented. Data are expressed as percentage of dose. The lines represent the linear regression of the data (1-5 min). The curved lines are the nonlinear regression of the data (1-30 min). Rat hepatocytes are from 2 donors (n=2) and human hepatocytes are from 3 donors (n=3).

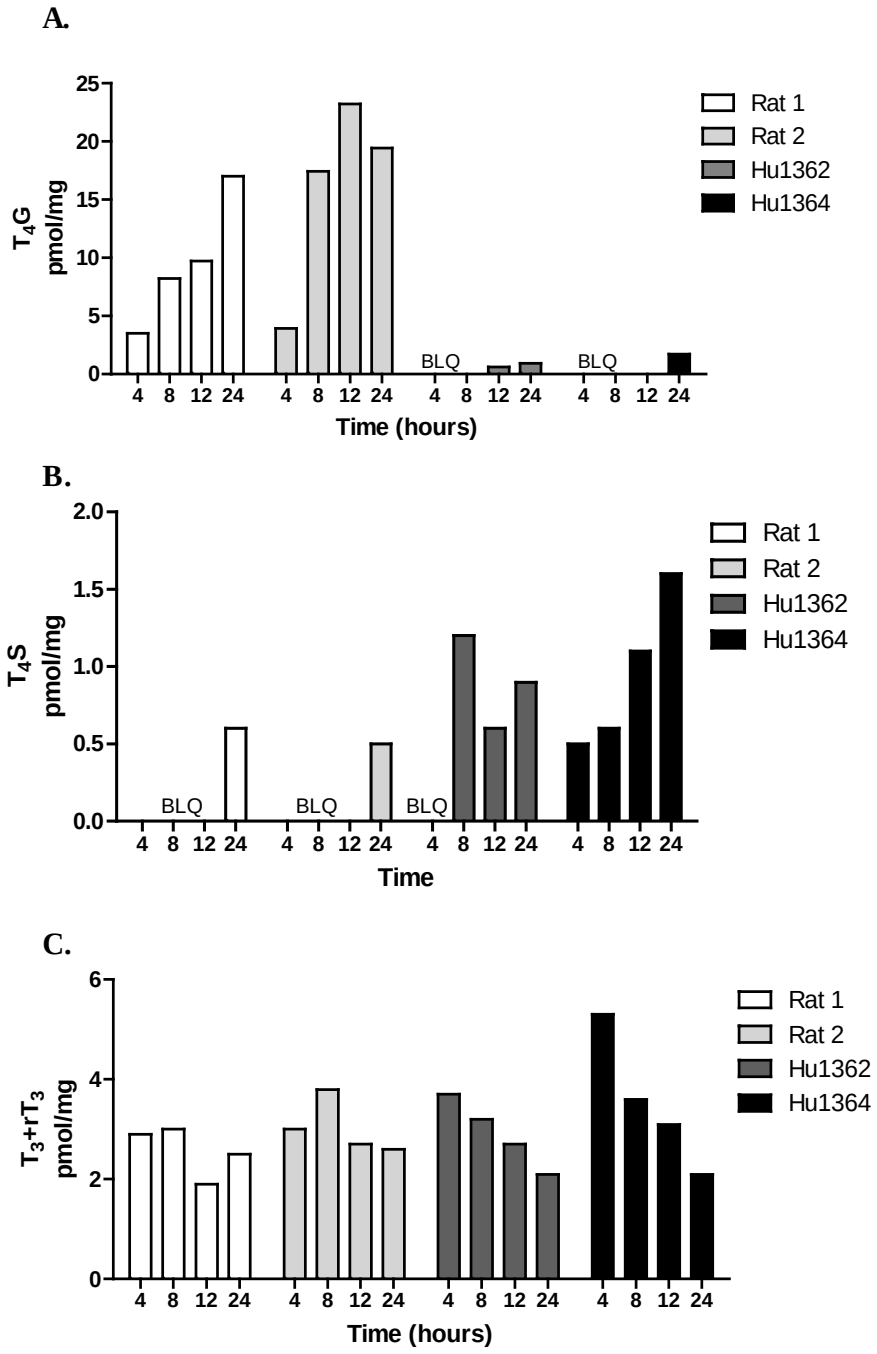
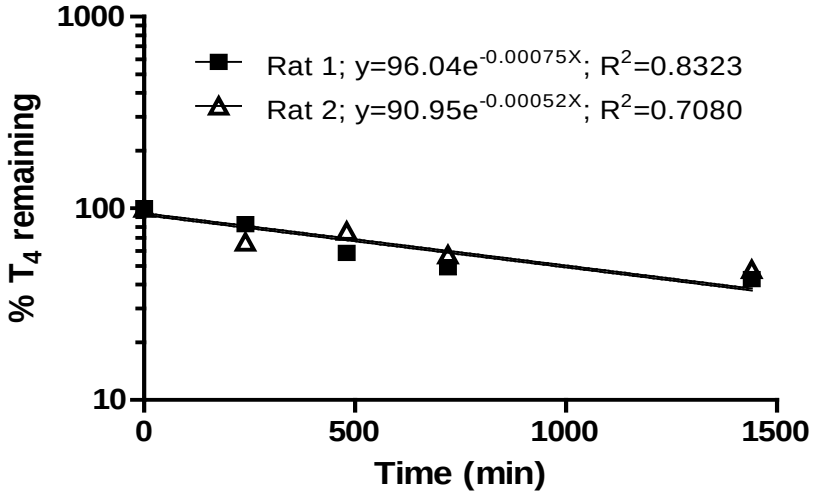


Figure 2.4. T_4 metabolite levels in media during incubation time. SCH were incubated with 0.05µM [^{125}I]-T_4 (rat) or 0.1µM [^{125}I]-T_4 (human) for on culture day 6. Metabolite appearance were determined over time (4-24).Metabolites were separated using the established UPLC method. Metabolites analyzed are (A) T_4G, (B) T_4S, and (C) T_3+rT_3. Data are expressed as pmol/mg cellular protein (mean). Data represent the average of duplicates in a single experiment. Limits of detection=0.5 pmol/mg cellular protein. BLQ=below limits of quantitation.

A.



B.

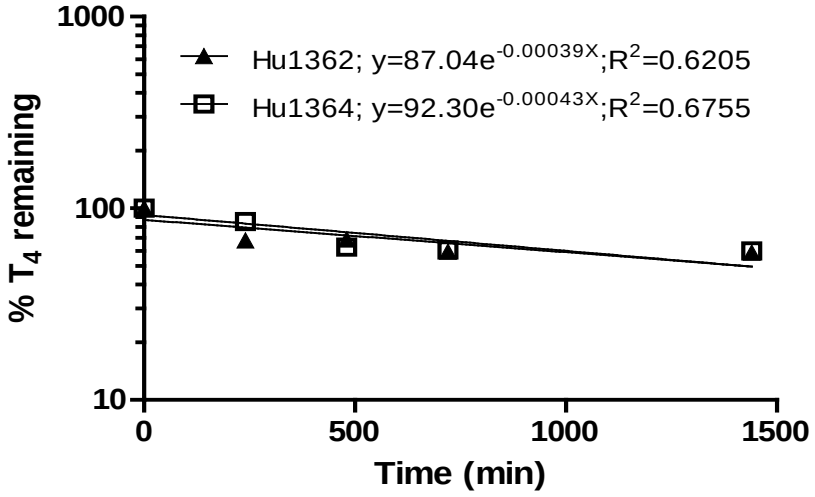
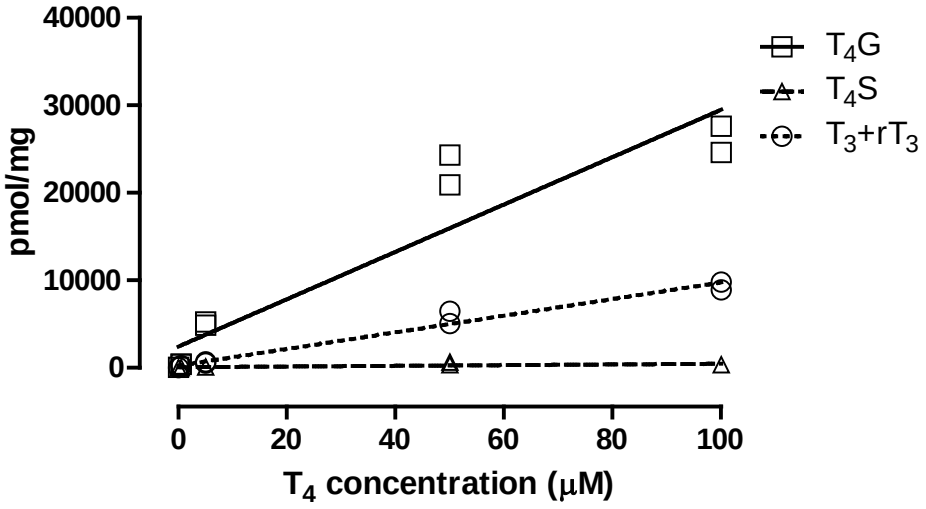


Figure 2.5. [^{125}I]-T_4 clearance in the media of rat (A) and human (B) hepatocytes. SCH were incubated with 0.05µM [^{125}I]-T_4 (rat) or 0.1µM [^{125}I]-T_4 (human) on culture day 6. The accumulation of [^{125}I]-T_4 was determined over time (4-24 hours). Data represent the average of duplicates in a single experiment. Data are expressed as percentage of T_4 remaining in media. The lines represent the linear regression of the data.

A.



B.

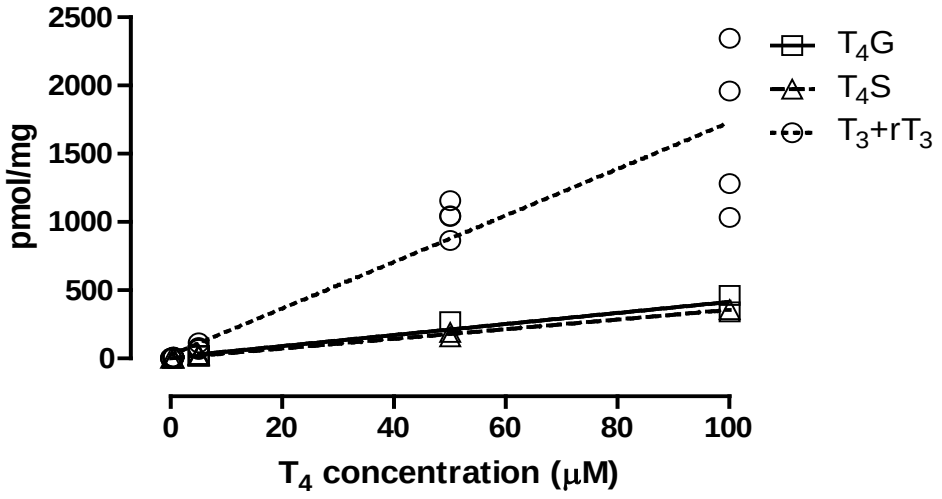
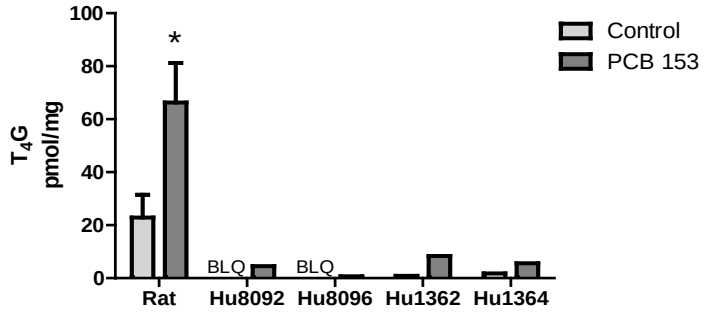
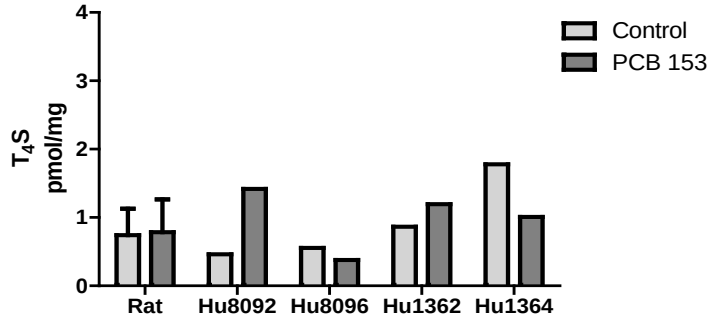


Figure 2.6. Metabolite levels in media of hepatocytes exposed to [^{125}I]-T_4 on culture day 6 for 24 hours. (A) Rat hepatocytes were incubated with 0.05-100μM [^{125}I]-T_4 and (B) human hepatocytes were incubated with 0.1-100μM [^{125}I]-T_4 for 24. Data are expressed as pmol/mg cellular protein (mean). Data represent the average of duplicate experiments. The lines represent the linear regression of the data. Limits of detection=0.5 pmol/mg cellular protein; BLQ=below limits of quantitation. n=2 for rat hepatocytes and human hepatocytes (Hu8092 and Hu8096).

A.



B.



C.

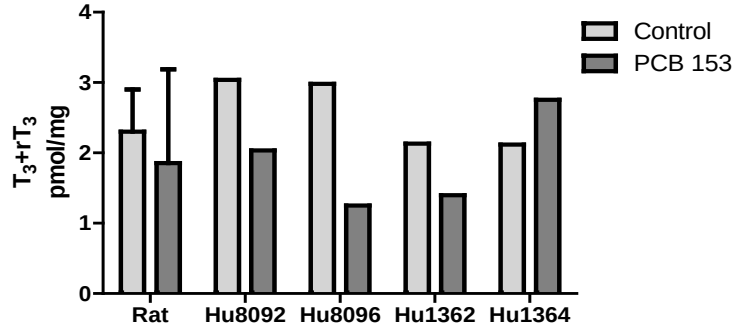


Figure 2.7. Comparison of metabolites in the media of SCRH and SCHH following treatment with PCB 153. Hepatocytes were incubated with 0.1% DMSO (control) or 30µM PCB 153 for 72 hours starting on culture day 3. Hepatocytes are then incubated for 24 hours on culture day 6 with 0.05µM (rat) or 0.1µM (human) [^{125}I]-T_4. Metabolites were separated using the established UPLC method. Metabolites analyzed are (A) T_4G, (B) T_4S, and (C) T_3+rT_3. Data represent the average of duplicate experiments. Data are expressed as pmol/mg cellular protein (mean±SD for rat hepatocytes and mean for human hepatocytes); Limits of detection= 0.5 pmol/mg cellular protein; BLQ=below limits of quantitation. n=3 for rat hepatocytes. Human hepatocytes are from 4 donors (Hu8092, Hu8096, Hu1362, and Hu1364). *Significantly different than control in rat hepatocytes; $p<0.05$.

CHAPTER 3

EFFECTS OF NUCLEAR RECEPTOR AGONISTS ON THYROXINE METABOLISM IN RAT AND HUMAN HEPATOCYTES

A. INTRODUCTION

Thyroid hormones (TH) are critical modulators of development in vertebrates. In humans, developmental hypothyroidism is associated with increased risk of neurological impairment and decreases in stature and hearing loss. The magnitude of these changes is dependent upon the magnitude of the developmental hypothyroidism. In amphibians and fish, metamorphosis occurs during peak plasma TH concentrations; therefore, decreases in TH delay metamorphosis. Because of its importance in development, chemicals that alter thyroid hormone concentrations are potential developmental toxicants.

Circulating TH concentrations are regulated, in part, by hepatic metabolism through pathways including: deiodination, glucuronidation and sulfation. In TH responsive tissues, thyroxine (T_4) is converted to triiodothyronine (T_3), the active hormone, by deiodinases. Deiodinases also convert T_4 and T_3 to 3, 3', 5'-triiodothyronine (rT_3) and 3, 3'-diiodothyronine (3, 3'-T_2), respectively. TH can be deactivated in the liver by, uridine diphosphate glucuronosyltransferase (UGT) and sulfotransferase (SULT) and these conjugates are then excreted through the bile into the intestines. There are significant species differences in thyroid hormone metabolism. For example, human SULT1A1 catalyzes TH

sulfation unlike the rat homolog (Visser, *et al.*, 1998; Kester, *et al.*, 1999). Although SULT1A1 is expressed in human and rat liver (Ozawa *et al.*, 1993; Runge-Morris *et al.*, 1998), the divergence of catalytic activity toward TH suggests SULT1A1 is important to TH sulfation in the human liver and not in rat liver. Primary rat hepatocytes produce approximately 13 times more T_4-glucuronide (T_4G) than T_4-sulfate (T_4S) when incubated with T_4 (Richardson *et al*., submitted). In contrast, primary human hepatocytes produced more T_4S and deiodination products, T_3 and rT_3 (Richardson *et al;* submitted). This suggests that T_4 glucuronidation is the predominate pathway of deactivation in rats, while humans utilize sulfation and to a greater extent deiodination to deactivate T_4.

There are multiple sites within the thyroid axis in which xenobiotics alter TH homeostasis. Although SULTs are involved in T_4 metabolism, most hypotheses on TH disruption focus on the increase in T_4 metabolism though the induction of hepatic UGTs. Many agents that decrease serum T_4 concentrations and induce hepatic UGTs activate nuclear receptors, such as constitutive androstane receptor (CAR), pregnane X receptor (PXR), and aryl hydrocarbon receptor (AhR) (Barter and Klaassen, 1992; Kretschmer and Baldwin, 2005; Qatanani, *et al.*, 2005). Phenobarbital (PB), a prototype activator of constitutive androstane receptor (CAR), decreases serum TH concentrations, increases hepatic T_4 glucuronidation (Hood and Klaassen, 2000a; Vansell and Klaassen, 2001; Vansell and Klaassen, 2002a; Hood, *et al.*, 2003; Kato, *et al.*, 2010) and increases the biliary elimination of T_4G in rodents (Oppenheimer, *et al.*, 1968; McClain, *et al.*, 1989; Wong, *et al.*, 2005). PB has been shown to decrease serum T_4 concentrations in humans (Tanaka *et al.*, 1987; Eiris-Punal, *et al.*, 1999); however, it is unclear by what mechanism the T_4 decrease occurs. Pregnenolone-16α-carbonitrile (PCN) and 3-methylcholanthrene (3MC),

prototype activators of PXR and AhR, respectively, also decrease serum T_4 concentrations and induce hepatic T_4-UGT activity in rats (Hood and Klaassen, 2000a; Hood, *et al.*, 2003; Richardson and Klaassen, 2010). While UGTs may play a role in decreasing circulating TH, it is not certain that the induction of hepatic T_4 glucuronidation alone is responsible for the effects on serum TH concentration following xenobiotic exposure. Research shows that serum T_4 decreases may not be dependent on increases in T_4 glucuronidation. For example, decreases in serum T_4 concentrations by phenobarbital (PB), pregnenolone-16α-carbonitrile (PCN), 3-methylcholanthrene (3MC) and polychlorinated biphenyls (PCBs) occur even in Ugt1a-deficient Gunn rats (Kato, *et al.*, 2004; Kato, *et al.*, 2005; Richardson and Klaassen, 2010). The experimental compound 4-(3-pentylamino)-2,7-dimethyl-8-(2-methyl-4-methoxyphenyl)-pyrazolo-[1,5-a]-pyrimidine (DMP 904) and the PCB mixture, Kanechlor-500 (KC500) decrease serum T_4 in rats, by increasing biliary elimination of unconjugated T_4, (Wong, *et al.*, 2005; Kato, *et al.*, 2007; Lecureux, *et al.*, 2009). Additionally, PB, DMP 904, and KC500 increase the accumulation of T_4 in the liver (Wong, *et al.*, 2005; Kato, *et al.*, 2007; Lecureux, *et al.*, 2009; Kato, *et al.*, 2010). This further suggests that serum T_4 decreases occur through a liver-selective accumulation and biliary elimination of T_4, rather than through the induction of hepatic UGT. This also indicates that the process for xenobiotic-mediated decreases in serum T_4 is controlled by uptake and efflux transporters in the liver.

In addition to UGTs, AhR, PXR and CAR regulate transporter proteins (Maglich, *et al.*, 2002; Bock and Kohle, 2004; Wagner, *et al.*, 2005; Jigorel *et al.*, 2006). Though much focus has been placed on the metabolism of TH by UGTs, it is not exactly clear what role hepatic transporters play in the decrease in serum TH. Multidrug resistance–associated

protein-2 (MRP2) and multidrug resistance protein-1 (MDR1) contribute to the excretion of a broad spectrum of substrates (Mitchell, *et al.*, 2005; Lecureux, *et al.*, 2009; Miyawaki *et al.*, 2012). Decreases in serum TH concentrations have been associated with increases in hepatic MRP2 mRNA and protein expression in rats treated with clobazam (CLB) (Miyawaki, *et al.*, 2012).

A potential difference between rodents and humans with respect to thyroid disruptors is the slight difference in the structure activity relationship for the activation of nuclear receptors across species. For example, in humans the PXR activator, rifampicin, does not activate PXR in rodents (Moore, *et al.*, 2000; Tirona *et al.*, 2004). Similarly, there are rodent and human specific activators of CAR. Activators of CAR and PXR affect TH homeostasis in rodents (Chen *et al.*, 2003; Kretschmer and Baldwin, 2005; Qatanani, *et al.*, 2005); thus, extrapolating xenobiotic-induced thyroid disruption by activators of hepatic nuclear receptors in rodents to humans is challenging due to the species differences in metabolism of the hormones and differences in the structure activity relationships for hepatic nuclear receptors across species.

To our knowledge, this is the first report to examine differences in rat and human hepatic T_4 metabolism as it relates to nuclear receptor activation. This study examines how prototype nuclear receptor agonists PB (human and rat CAR), PCN (rat PXR), Rif (human PXR), 3MC (human and rat AhR) change T_4 metabolism in rat and human sandwich-cultured hepatocytes (SCH). The PB-like inducer, PCB 153 was also used as a prototype environment contaminant to assess the effects on T_4 disposition in rat and human hepatocytes. In addition, mRNA expression for cytochrome P450s (CYPs) UGTs, SULTs, deiodinase 1 (D1), and

transporters, were also examined to determine other possible genes involved in TH disruption.

B. MATERIALS AND METHODS

Chemicals

L-thyroxine (T_4), phenobarbital (PB), 3-methylcholanthrene (3MC), pregnenolone-16α-carbonitrile (PCN), and rifampicin (Rif) were purchased from Sigma-Aldrich Co. (St. Louis, MO). 2, 2', 4, 4', 5, 5'-hexachlorobiphenyl (PCB 153) was purchased from Radian Corporation (Austin, TX). [^{125}I]-T_4, (116 Ci/mmol) was purchased from Perkin Elmer Life Sciences Inc. (Waltham, MA) and was purified to >98%) immediately before use with Sephadex LH-20 (Sigma-Aldrich) as described by Rutgers *et al.* (1989). All other reagents were of the highest grade commercially available.

Hepatocyte culture

Fresh primary male Sprague-Dawley sandwich-cultured rat hepatocytes (SCRH) and sandwich-cultured human hepatocytes (SCHH) in 24-well plates were received on culture day 2 from Life Technologies (Durham, NC). Hepatocytes were maintained for up to 6 days in culture with daily medium replacement. The maintenance medium consists of: Williams' E medium with 0.1% dimethyl sulfoxide (DMSO) including Hepatocyte Maintenance Supplement (Life Technologies), which contains 0.1 µM dexamethasone and proprietary concentrations of penicillin-streptomycin, ITS+ (insulin, transferrin, selenium complex, BSA

and linoleic acid), GlutaMAX™ and HEPES]. Human donor demographics can be found in Table 3.1.

T_4 and T_4 metabolite separation and quantitation

Samples were dried at 40°C under N_2 gas and then reconstituted with 40µl mobile phase consisting of 18% acetonitrile in 0.02M ammonium acetate (pH=4). After addition of mobile phase, samples were centrifuged at 10,000 x g for 5 minutes at room temperature and the supernatants were placed in vials for UPLC analysis. UPLC equipped with a C18 – 2.1 x 50mm x 1.7 µm (Waters Corp.; Milford, MA) resolution column and a fraction collector were used for identification of T_4 and T_4 metabolites. Gradient elution was performed using a method modified version of a previously established method by Rutgers *et al*. (1987) with a 16 minute gradient of 18-40% acetonitrile in 0.02M ammonium acetate (pH=4). Solvent flow rate was 0.4ml/min and 15 second fractions were collected in test tubes. Peaks were then identified by analyzing collected fractions for radioactivity by gamma spectroscopy. Retention times for T_4, T_3, rT_3, T_4-glucuronide (T_4G), and T_4-sulfate (T_4S) were determined using synthetic and biosynthetic compounds as described by Richardson *et al*. (submitted). Peaks for T_3 and rT_3 were often inseparable; as a result, these peaks were added together and are presented as T_3+rT_3. Hepatocyte protein content was determined using a protein assay kit with bovine serum albumin as a standard (Bio-Rad Laboratories, Hercules, CA).

T_4 accumulation in hepatocytes following treatment with nuclear receptor agonists

Stock solutions of each chemical were diluted in DMSO and added to maintenance medium to a final DMSO concentration of 0.1%. SCRH and SCHH were treated with species

appropriate prototypical nuclear receptor agonist starting on culture day 3. SCRH were treated for 72 hours with the DMSO vehicle (0.1%), PB (1000 µM), PCN (10 µM), 3MC (5 µM), or PCB 153 (30 µM) in maintenance medium for 72 hours. SCHH were treated for 72 hours with the DMSO vehicle (0.1%) or PB (1000 µM), Rif (10 µM), 3MC (5 µM), or PCB 153 (30 µM) in maintenance medium. Following the 72 hour incubation, medium was removed and replaced with maintenance medium containing 0.0005µM (5000 CPM) of [^{125}I]-T_4 for 1, 2, 5, 10, 20, or 30 minutes. After incubation, medium was collected and cells were washed twice with 0.5 ml/well of ice-cold PBS buffer. After washing, hepatocytes were collected by adding 0.5ml/well 0.1M NaOH. ^{125}I-derived radioactivity in collected medium and hepatocytes were analyzed by gamma spectroscopy.

Effects of hepatic enzyme inducers on T_4 metabolite levels

Stock solutions of PB, PCN, Rif, 3MC, and PCB 153 were diluted in dimethyl sulfoxide (DMSO) and added to Williams' E medium at a final DMSO concentration of 0.1%. On culture day 3, fresh SCRH were exposed for 72 hours to the DMSO vehicle (0.1%), PB (10, 100, 1000 µM), PCN (0.1, 1, 10 µM), 3MC (0.05, 0.5, 5 µM), or PCB 153 (0.3, 3, 30 µM). SCHH were treated for 72 hours to the DMSO vehicle (0.1%) or PB (10, 100, 1000 µM), Rif (0.1, 1, 10 µM), 3MC (0.05, 0.5, 5 µM), or PCB 153 (0.3, 3, 30 µM). After 72 hours, medium was removed and replaced with Williams' E medium containing 0.05 µM (rat) or 0.1 µM (human) [^{125}I]-T_4 (500,000 CPM) for 24 hours. Medium was collected and stored at 4°C until analysis. Hepatocytes were washed twice with 0.5 ml/well ice-cold PBS and collected by adding 0.5 ml/well of NaOH. Medium was prepared and analyzed for T_4 metabolites as the method describes above.

RNA isolation and real-time RT-PCR analysis.

Upon termination of the treatment period (72 hours), cells were washed once with ice-cold PBS (0.5ml/well), lysed by the addition of 0.7ml of RLT Buffer (Qiagen, Hilden, Germany) containing 1.0% β-mercaptoethanol (final concentration) and stored at -70° C until use. Lysates were thawed on ice and total RNA was isolated using the RNeasy Midi Kit with DNase I digestion performed during column purification (Qiagen, Hilden, Germany). The integrity of RNA samples were assessed using the 2100 Bioanalyzer was used (Agilent Technologies, Palo Alto, CA). RNA Integrity Numbers (RINs) greater than 8.0 are seen as acceptable for real-time RT-PCR analysis. Real-time RT-PCR was performed using the ABI Prism 7700 Sequence Detection System (ABI, Foster City, CA). Total RNA (100ng) was converted to cDNA using High Capacity cDNA Reverse Transcription Kits (ABI, Foster City, CA). PCR reactions were then performed on all cDNAs using TaqMan Universal PCR Master Mix and custom TaqMan Low Density Arrays (ABI, Foster City, CA) preloaded with target gene expression assays. Gene expression assay identifications are listed in Tables 3.2 and 3.3.

All RT-PCR data were quantified by the relative quantitation method $^{\Delta\Delta}$Ct (Applied Biosystems User Bulletin 2). These data were quantified relative to a control sample and an endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The control group is used as a reference point for all other dose groups; therefore, all samples are expressed as fold difference as compared with control.

Data analysis

The statistical intergroup comparisons were determined by using one-way analysis of variance (ANOVA) followed by Dunnet's Multiple Comparison and linear trend post tests. Nonlinear regression analysis was used to assess the relationship between [^{125}I]-T_4 accumulation and chemical treatments. A one-phase association exponential equation

$$Y=Y_0 + (Plateau-Y_0)*(1-exp(-K*x)),$$

was fit to the [^{125}I]-T_4 accumulation data where *plateau* is the Y value at infinite times and *half time* is the time (minutes) it takes to reach half the plateau and is computed as ln(2)/K. GraphPad Prism 5.0 was used to analyze all data (GraphPad Software San Diego, CA). The level of probability of statistical significance was $p < 0.05$.

C. RESULTS

T_4 metabolite levels following treatment with nuclear receptor activators

To study the effects of hepatic enzyme inducers on T_4 metabolism in hepatocytes, PB (CAR), and 3MC (AhR) were used as prototype nuclear receptor agonists for SCRH and SCHH. PCN and Rif were used as prototype PXR agonists for SCRH and SCHH, respectively. PCB 153 was used as a prototype environmental contaminant for SCRH and SCHH. T_4G in the medium of SCRH significantly increased with treatment of 1000 µM PB (1.7-fold), 1 and 10 µM PCN (1.7- and 2.0-fold), 0.05, 0.5, and 5 µM 3MC (1.6-, 2.2-, and 4.4-fold), and 0.3, 3, and 30 µM PCB 153 (1.6-, 2.3-, 4.8-fold) (Figure 3.1A). T_4G in medium of SCHH significantly increased following Rif (10 µM) and PCB 153 (30 µM) treatment 2.9- and 3.6-fold, respectively (Figure 3.2A). T_4G levels in the medium of SCHH

did not change with PB and 3MC treatment. T_4S (Figures 3.1B and 3.2B) and T_3+rT_3 (Figures 3.1C and 3.2C) levels in the medium of SCRH and SCHH were not significantly changed with chemical treatment compared to control.

Cytochrome P450 mRNA expression

To determine if chemical treatments effectively activated specific nuclear receptors, mRNA expression levels of target cytochrome P450s were measured by RT-PCR. CYP1A mRNA expression was used as a marker for Ah receptor activation in SCRH (Table 3.4) and SCHH (Table 3.5). 3MC significantly increased CYP1A1 mRNA in SCRH 270.2-fold (5 µM) and in SCHH 154.6-fold (0.5 µM) and 178.0 (5 µM). As markers for CAR activation, Cyp2b2 and CYP2B6 mRNA expression was used for SCRH and SCHH, respectively. PB (1000 µM) treatment significantly increased Cyp2b2 mRNA expression 197-fold in SCRH. CYP2B6 significantly increased following PCB 153 (3 and 30 µM) treatment in SCHH 21.7- and 31.4-fold. CYP3A mRNA expression was used as a marker for PXR activation. In SCRH, Cyp3a1 mRNA expression significantly increased 660.1-fold following treatment with PCN (10 µM). CYP3A4 mRNA expression significantly increased following PB (1000 µM) and Rif (1 and 10 µM) 20.0- 15.7- and 24.9-fold, respectively.

UGT, SULT and D1 mRNA expression

There was a significant 25.7-fold increase in Ugt1a6 mRNA expression with 3MC (5 µM) treatment of SCRH (Table 3.6). Ugt1a1, Ugt1a5, and Ugt2b mRNA expression in SCRH was not significantly altered with treatment. In SCHH, 1000 µM PB significantly increased UGT1A1, UGT1A4, UGT1A6, and UGT2B4 by approximately 6.4-, 9.6, 2.2-, and

2.0-fold (Table 3.7). SCHH treated with 1 and 10 µM Rif significantly increased UGT1A1 (4.7- and 6.6-fold) and UGT2B (2.1- and 2.4-fold) mRNA expression. UGT1A4 and UGT2B15 increased significantly in SCHH with Rif (10 µM) treatment by 7.2-fold and 3.1-fold, respectively. Expression levels for the UGT genes examined were not significantly changed in SCHH with 3MC or PCB 153 treatment. PB (1000 µM) significantly increased Sult1e1 mRNA expression in SCRH by 6.8-fold (Table 3.8). Sult1c1 mRNA expression in SCRH significantly decreased with 10 µM PCN by 80%. 3MC and PCB 153 treatment did not alter Sult mRNA expression in SCRH. In SCHH, SULT1A3, SULT1E1, and SULT2A1 were not significantly changed with chemical treatment (Table 3.9). Deiodinase 1 mRNA expression in SCRH was not significantly changed with chemical treatment (Table 3.10). In SCHH, deiodinase I mRNA expression increased 2.4-fold following treatment with 0.3 µM PCB 153, but was unchanged at the 2 higher doses of 3 and 30 µM PCB 153 (Table 3.11).

Cell-associated radioactivity of [^{125}I]-T_4 in fresh rat and human hepatocytes following xenobiotics treatment

Hepatocyte accumulation of T_4 following xenobiotic treatment (72 hours) was evaluated by measuring the amount of radioactivity in the hepatocytes after incubation with [^{125}I]-T_4 for 1 to 30 minutes (Figure 3.3). Preliminary studies showed [^{125}I]-T_4 did not significantly bind to BD Matrigel® (BD Biosciences) or plastic ware (data not shown). An exponential one-phase association model was used to fit accumulation data for control and treated hepatocytes. The data for SCRH fit one curve indicating no significant difference in [^{125}I]-T_4 accumulation between all treatment groups (Figure 3.3A). In SCRH, [^{125}I]-T_4 association plateau equaled 4.8% and half time was 4.0 minutes for all treatment groups.

[^{125}I]-T_4 accumulation was different between treatment groups in SCHH (Figure 3.3B). The model showed that [^{125}I]-T_4 accumulation into SCHH plateaued at 11.7, 11.5, 14.2, 11.7, and 10.9% for control, PB, Rif, 3MC, and PCB 153, respectively. Half times were determined to be 16.0, 13.9, 15.7, 14.2, and 20.6 minutes for control, PB, Rif, 3MC, and PCB 153, respectively. A linear model was used to fit the data from 1 to 10 minutes to analyze accumulation at early time points. The data for SCRH and SCHH fit one curve indicating no significant difference in [^{125}I]-T_4 accumulation between all treatment groups (SCRH: F=0.19 (4,76); p=0.9413 and SCHH: F=1.60 (4,110); p=0.1792).

Efflux and uptake transporter mRNA expression

Mdr1a mRNA expression increased significantly in SCRH with PB (1000 µM), 3MC (5 µM), and PCB 153 (30 µM) by 1.4-, 1.7-, and 1.6-fold (Table 3.12). PCN treatment did not significantly change Mdr1a mRNA expression in SCRH. In SCRH, Mdr1b, Mrp2, and Mrp3 were not significantly altered with chemical treatment. PB (1000 µM) treatment increased MDR1 and MRP2 mRNA expression in SCHH by 2.3- and 2.5-fold, respectively (Table 3.13). 1 µM and 10 µM Rif significantly increased MDR1 (2.0- and 2.0-fold, respectively) and MRP2 (2.0- and 2.1-fold, respectively) mRNA expression. MRP3 mRNA expression was not significantly altered with chemical treatment. Neither 3MC nor PCB 153 significantly changed MDR1, MRP2, and MRP3 mRNA expression in SCHH. Only 1000 µM PB significantly increased Oat2 mRNA expression in SCRH by 1.9-fold (Table 3.12). Ntcp1 mRNA expression in SCRH was unchanged with chemical treatment. In SCHH, NTCP1 and OAT2 were not significantly altered with chemical treatment (Table 3.13).

D. **DISCUSSION**

Through the activation of nuclear receptors such as AhR, CAR, and PXR, xenobiotics are thought to lower serum T_4 concentrations by increasing T_4 glucuronidation and biliary elimination (Barter and Klaassen, 1994; Klaassen and Hood, 2001; Kato *et al.*, 2011; Martin, *et al.*, 2012). However, xenobiotic-mediated increases in T_4 glucuronidation do not necessarily correlate with a change in serum T_4 concentrations in rodents (de Sandro, *et al.*, 1992; Hood and Klaassen, 2000a; Craft, *et al.*, 2002) and decreases in serum T_4 may occur independent of hepatic T_4 glucuronidation (Kato, *et al.*, 2004; Kato, *et al.*, 2005; Kato, *et al.*, 2007; Kato, *et al.*, 2010; Richardson and Klaassen, 2010). Previous studies also demonstrate that decreases in serum T_4 may occur as a result of increases in T_4 accumulation in the liver (Kato, *et al.*, 2011; Martin, *et al.*, 2012). The present study questions the importance of hepatic T_4 glucuronidation and nuclear receptor activation on T_4 clearance in rat and human hepatocytes. Prototype nuclear receptor agonists known to decrease serum T_4 concentrations and increase hepatic UGTs in rats were chosen for this study (Hood and Klaassen, 2000a; Craft, *et al.*, 2002). To examine the effects of nuclear receptor agonists on T_4 metabolism, sandwich-cultured Sprague-Dawley male rat (SCRH) and human (SCHH) hepatocytes were treated with PB and 3MC, the prototype agonists for CAR and AhR, respectively. Due to the species differences in PXR activation, SCRH were treated with PCN and SCHH were treated with Rif. PCB 153, often described as a PB-like inducer (Parkinson *et al.*, 1983; McFarland and Clarke, 1989), was used as a prototype persistent organic pollutant. PCB 153 mediates decreases in serum T_4 concentrations in rats and is found in high concentrations in human serum (NHANES, 2012) (Crofton, *et al.*, 2005; Liu *et al.*,

2012). The results of this study show that increases in T_4 glucuronidation are agonist- and species-specific. To our knowledge the present experiments were the first to examine species differences in xenobiotic-mediated increases in T_4 metabolism using rat and human hepatocytes.

To determine the functional integrity of the SCH used in this study, CYP1A, CYP2B, and CYP3A mRNA expression were measured, as classic targets for AhR, CAR, and PXR activation, respectively. As expected, the prototypical nuclear receptor agonists PB, PCN, and 3MC increased target P450 mRNA expression in SCRH. Consistent with previous studies, PB activates CAR to increase Cyp2b2 (CAR) and to a lesser degree Cyp3a1 (PXR) in SCRH (Frueh *et al.*, 1997; Meyer and Hoffmann, 1999; de Longueville *et al.*, 2003). In SCRH, PCN increased Cyp3a1 and to a much lesser degree Cyp2b2 (Smirlis *et al.*, 2001) and 3MC increased Cyp1a1 (AhR) (Hartley and Klaassen, 2000; Surry *et al.*, 2000). As previously reported, PB acted as a mixed activator of CAR and PXR through the induction of CYP2B6 and CYP3A4 in SCRH (Faucette *et al.*, 2007; Rotroff *et al.*, 2010). PXR activation by Rif resulted in the induction of both CYP3A4 and CYP2B6 in SCHH (Faucette *et al.*, 2006). An overlap in genes regulated by PXR and CAR has been previously demonstrated, where PXR regulates CYP2B genes and CAR regulates CYP3A genes and this crosstalk between receptors may explain the overlapping induction of CYP2B and CYP3A in this study (Maglich, *et al.*, 2002; Pascussi *et al.*, 2008). As expected, 3MC proved to be a strong activator of AhR through the increase of CYP1A1 in SCHH. The prototype environmental pollutant, PCB 153, is described as PB-like based on its ability to induce CYP2B (Parkinson, *et al.*, 1983; McFarland and Clarke, 1989). In the present study, PCB 153 was a potent inducer of Cyp2b2 in SCRH. In SCHH, PCB 153 was a more potent inducer of CYP2B6 and

CYP1A1, than CYP3A4, but the increase in CYP1A1 suggests the presence of a possible dioxin-like contaminant. However, the lack of a dose-dependent increase in Cyp1a1 in SCRH indicates the CYP1A1 increase in SCHH may be due to the overlapping regulation by CAR (Auyeung *et al.*, 2003; Nishimura *et al.*, 2005).

T_4G, T_4S, rT_3 and T_3 were all measured in the medium of SCRH and SCHH and only T_4G was altered by chemical treatment. T_4G increased in the medium of SCRH treated with PB, PCN, 3MC, and PCB 153 by as much as 1.7- , 2.0- , 4.4- , and 4.8-fold, respectively. These results are consistent with previous studies in which rat hepatocytes treated with PB (Viollon-Abadie, *et al.*, 2000) and 3MC (Jemnitz *et al.*, 2000) increased T_4-UGT activity. *In vivo* studies show that rats treated with PB, PCN, or 3MC resulted in increased hepatic T_4-UGT activity; however similar to the present study, PB-mediated increases in T_4-UGT activity were small compared to the other nuclear receptor agonists (Barter and Klaassen, 1992; Barter and Klaassen, 1994; Hood and Klaassen, 2000a). Kato *et al*. (2005) found that decreases in serum T_4 in Gunn rats treated with PB were independent of T_4 glucuronidation. In the present study, the small changes in T_4G levels in the medium of SCRH following PB treatment is consistent with studies using Gunn rats and suggests that hepatic T4 glucuronidation may play a smaller role in serum T_4 decreases in rats treated with PB compared to PCN and 3MC. In contrast to SCRH, SCHH treated with Rif and PCB 153 increased T_4G levels in the medium; however, PB and 3MC did not increase T_4G production. Studies by Ohnhaus *et al* (1981; 1983) showed that PB did not decrease serum T_4 in humans, but parameters for liver microsomal enzyme activity, antipyrene and 6-β-hydroxycortisol clearance were increased in humans. Compared to PB, Rif decreased serum T_4 and increased the metabolic clearance of antipyrene and 6-β-hydroxycortisol, but to a much greater extent

in humans. Although not exact evidence of T_4 metabolism, the data suggest that the decrease in serum T_4 followed by treatment with Rif may occur through mechanisms other than glucuronidation in humans.

In rat liver, Ugt1a1 and Ugt1a6 are thought to be the major isoforms responsible for T_4 glucuronidation (Vansell and Klaassen, 2002b) and UGT1A1, UGT1A3 and UGT1A9 are thought to be responsible for T_4 glucuronidation in human liver (Findlay *et al.*, 2000; Yoder Graber *et al.*, 2007). The differential induction of UGTs by AhR, CAR, and PXR is more apparent in rats, but not in humans. For example, AhR regulates Ugt1a6 in rats and to a lesser extent in humans (Auyeung, *et al.*, 2003; Bock, 2011). In rats, Ugt1a1 is regulated by CAR and PXR. In humans, UGT1A1 and UGT1A6 are regulated by AhR, CAR and PXR; however, UGT1A6 is not as inducible as UGT1A1 In the present study, not all of the agonists that increased T_4G levels in the medium of SCRH and SCHH increased UGT mRNA expression. The lack of UGT mRNA expression induction in rat hepatocytes treated with PB agrees with studies by Shelby and Klaassen (2006), which show hepatic Ugt1a1,Ugt1a5, and Ugt1a6 mRNA expression did not change in rats treated with PB. In contrast to the present study, hepatic Ugt1a1 and Ugt1a5 mRNA expression have been shown to increase in rats treated with PCN (Shelby and Klaassen, 2006). In the present study, the 25.7-fold increase in Ugt1a6 mRNA expression coincided with the 4.4-fold increase in T_4G levels in medium of SCRH treated with 3MC. Rat Ugt1a6 induction is dependent on AhR activation (Auyeung, *et al.*, 2003; Bock, 2011) and the lack of significant increases in Ugt1a6 mRNA expression confirms that PCB 153 is not activating AhR in this model system. In SCHH, UGT mRNA expression increased with PB treatment, although T_4G levels did not increase in the medium. Rif increased mRNA expression, which coincides with

increases in T_4G levels in the medium of SCHH. UGT mRNA expression levels were induced in SCHH treated with 3MC and PCB 153. Despite the increased UGT1A1 mRNA expression by 3MC, there was no increase in T_4G levels. PCB 153 increased T_4G levels by 3.6-fold, which coincided with increases in UGT1A1 ($p<0.01$) and UGT1A4 ($p<0.001$). These results show that UGT mRNA expression may not be a reliable indicator for the increase in T_4G levels in this system. However in SCRH, 3MC increased T_4G levels, which matched the increase in Ugt1a6 mRNA expression suggesting that potent activators of AhR may increase T_4G levels through the induction of UGTs to a greater extent than other nuclear receptor activators.

AhR, CAR and PXR also have been implicated in the regulation of SULTs (Saini, *et al.*, 2004; Yanagiba *et al.*, 2009; Aleksunes and Klaassen, 2012) and it has been proposed that SULTs may be more important in the metabolism of TH in humans than in rats (Kester, *et al.*, 1999; Kester, *et al.*, 2003). In the present study, there were no significant changes in T_4S levels following treatment of SCRH and SCHH with each nuclear receptor agonist. Because SULTs have a low affinity for T_4 and the evidence that T_4G is the major metabolite found in the bile in rats, it is not surprising that T_4 sulfation did not change in the medium of SCRH and SCHH (Rutgers, *et al.*, 1989; Visser, 1996). In addition, the lack of effect on T_4 sulfation may be partly due to the instability and poor capacity of the SULT cofactor, PAPS (Koster *et al.*, 1981; Kim *et al.*, 1995; Novakova *et al.*, 2004). Impaired hepatic DI activity is associated with hypothyroidism in rats treated with dioxin or dioxin-like chemicals (Hood and Klaassen, 2000b; Viluksela *et al.*, 2004; Szabo, *et al.*, 2009); however, in the present study, there was no change in T_3+rT_3 levels and no change in DI mRNA expression in both species of hepatocytes. This agrees with the premise that a reduction in deiodinase activity is

a physiological response and is not due to direct effects of microsomal enzyme inducers; therefore, changes in T_3+rT_3 production and DI mRNA expression may not be observed in an *in vitro* system (Davies, *et al.*, 1996).

Hepatic transporters are important in facilitating the absorption, distribution, and elimination of a variety of nutrients, drugs, and metabolites and are coordinately induced through the activation of nuclear receptors (Bock and Kohle, 2004; Kohle and Bock, 2009; Aleksunes and Klaassen, 2012). Furthermore, reports show that TH are actively transported into and out of cells (De Jong, *et al.*, 1992; de Jong, *et al.*, 1993; Friesema, *et al.*, 1999; Visser *et al.*, 2008; Lecureux, *et al.*, 2009). Kato *et al.* (2010; 2011) demonstrated that PB and PCB 153 increased [^{125}I]-T_4 liver accumulation in rodents. The increase [^{125}I]-T_4 accumulation in the liver resulted in increases in the biliary elimination of [^{125}I]-T_4 and [^{125}I]-T_4G, suggesting that hepatic transporters are involved in the xenobiotic-mediated cellular uptake and biliary excretion of ^{125}I]-T_4 and [^{125}I]-T_4G. Differences between rats and mice in the biliary elimination of [^{125}I]-T_4 and [^{125}I]-T_4G following treatment with PB and PCB 153 indicates a possible difference in the activity of transporters responsible for excretion into the bile duct (Kato, *et al.*, 2010; Kato, *et al.*, 2011). As a potential rate-limiting step for TH metabolism, we examined the species differences in accumulation of [^{125}I]-T_4 following treatment with different nuclear receptor agonists. In untreated hepatocytes, the rate of [^{125}I]-T_4 accumulation is slower in human hepatocytes than rat hepatocytes (Krenning, *et al.*, 1981; de Jong, *et al.*, 1993). Previous work in our laboratory (Richardson *et al;* submitted) showed that at early time points (1-5 minutes) [^{125}I]-T_4 accumulation in untreated SCRH and SCHH were not significantly different. In the present study, [^{125}I]-T_4 accumulation following treatment with each agonist was not significantly different in SCRH and SCHH; however,

mRNA expression for MDR1, an efflux transporter, increased significantly with PB, 3MC and PCB 153 in SCRH and with PB and Rif treatment in SCHH. MRP2 is also an efflux transporter and it increased in SCHH treated with PB and Rif. Oat2 mRNA expression, an uptake transporter, increased in SCRH treated with PB; however, no significant changes were found in SCHH. Collectively, the differences in transporter mRNA expression did not necessarily coincide with [^{125}I]-T_4 accumulation in SCRH and SCHH nor was it consistent with the differences in T_4G levels in the medium of SCRH and SCHH following treatment with nuclear receptor agonists. The main goal of the present study was to examine nuclear receptor-mediated increases in UGTs and T_4 metabolism; therefore our system was optimized for nuclear receptor activation (72 hours incubation with activator) and T_4 metabolism (24 hours incubation with T_4). Opposing expression profiles for uptake and efflux transporters were found over culture days in SCRH, suggesting optimal culture conditions for uptake transporters may not be optimal for efflux transporters (Tchaparian *et al.*, 2011). Because the system was optimized for induction and metabolism and not optimized for transporter activity, it is difficult to make assertions about T_4 transport in our system. In addition, mRNA expression is not a reliable indicator of transporter expression and therefore may explain the lack of consistency in the data (Johnson *et al.*, 2002b; Johnson and Klaassen, 2002; Kipp and Arias, 2002; Tchaparian, *et al.*, 2011). The *in vitro* system used in the present study may not be able to discriminate between T_4 accumulation and excretion; therefore, the use of transporter vesicles may be more suitable for the further exploration of all aspects of T_4 transport.

In the present study, PCB 153 increased T_4G levels in the medium of both SCRH and SCHH, whereas PB treatment caused a small increase in T_4G only in medium of SCRH.

Based on P450 induction, PB appears to be a CAR agonist at lower and possibly more physiologically relevant concentrations; PCB 153 appears to be a more potent CAR agonist in SCRH. In SCHH, PB is a mixed activator of CAR and PXR however; PCB 153 appears to be more of a potent CAR agonist. Overall, the data suggests that PCB 153 is not PB-like, due to the large increases in T_4G levels in medium of SCRH and SCHH; however, based on P450 induction PB and PCB 153 are similar. This suggests PCB 153 is a more potent inducer of T_4 glucuronidation than PB in SCRH and SCHH.

Species differences have been previously described in P450 induction and may explain species differences in T_4 metabolism found in the current study. For example, PB does not directly interact with CAR; however, human CYP2B6 is inducible by PB, although to a much lesser extent than rodent CYP2B. Since PB does not bind to CAR, this difference in induction may be due to differences in the proximal promoter for the CYP2B gene in rodents and humans (Faucette, *et al.*, 2006). In the current study, the species-specific differences in T_4 glucuronidation following treatment with nuclear receptor agonists may be due to alternative splicing, phosphorylation of the receptor, or through crosstalk from other signaling systems (Weigel, 1996; Crofts *et al.*, 1998; Fasco, 1998; Shao and Lazar, 1999). While it appears that UGT metabolism and nuclear receptor regulation may not explain decreases in serum T_4, further exploration of the role of transporters in this issue would be warranted.

The present study further supports previous findings, which propose that serum T_4 decreases may occur independent of glucuronidation. It has been suggested that increases in transporter-mediated accumulation of T_4 in the liver is a mechanism by which serum T_4 is metabolized or eliminated through the bile; however, we were unable to confirm that

differences in T_4 accumulation are linked to differences in T_4 metabolism in this system. Data from this study are in accordance with previous studies which show increases in T_4 glucuronidation are species- and agonist-specific and further confirms that PB mediated decreases in serum T_4 may be independent of hepatic T_4 glucuronidation in rats (Kato, *et al.*, 2005; Kato, *et al.*, 2010). This study shows that PCB 153 was PB-like in its ability to induce CYP2B in SCRH and SCHH; however, PCB 153 and PB are not similar in their ability to induce T_4 glucuronidation. Overall, we demonstrate that SCH are an appropriate tool for assessing TH disruption and human risk, although further exploration of the role of transporters in T_4 disposition is needed.

Table 3.1

Donor information for human hepatocytes

Donor	Gender	Age	Smoke/Alcohol	Medications	Experimental Use
Hu1191	Male	19	No/No	UKN	T_4 accumulation
Hu1193	Male	68	Yes/No	Aspirin Zolpidem Simvastatin Ranitidine	T_4 accumulation
Hu1197	Female	29	No/Yes	Amphetamine Bupropion Cetirizine Clonazepam Lamotrigine	T_4 accumulation
Hu1236	Female	68	No/No	Multivitamin Vitamin D	Dose response
Hu1248	Male	63	Yes/Yes	Sertraline Vicodin Docusate Sodium	Dose response
Hu1362	Female	57	No/No	Atenolol and Chlorthalidone Lorazepam Oxycodone	Dose response
Hu1364	Male	51	No/No	Cyanocobalamin Diltiazem Ferrous Sulfate Folic Acid Metoprolol Omeprazole Potassium Chloride Prednisone	Dose response

UKN=unknown

Table 3.2

TaqMan rat gene expression assays

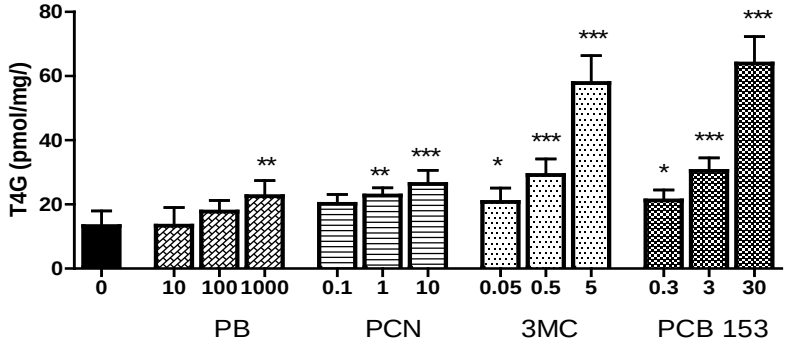
Group	Gene	TaqMan Gene Expression Assay ID
Endogenous control	Gapdh	Rn99999916_s1
Nuclear receptor activation	Cyp1a1	Rn00487218_m1
	Cyp2b2	Rn02786833_m1
	Cyp3a1	Rn01640761_gH
Phase II metabolism	Ugt1a1	Rn00754947_m1
	Ugt1a5	Rn01427785_m1
	Ugt1a6	Rn00756113_mH
	Ugt2b	Rn02349650_m1
	Sult1a1	Rn00582915_m1
	Sult1b1	Rn00673872_m1
	Sult1c1	Rn00581955_m1
	Sult1e1	Rn00820646_g1
Transporter	Mdr1a	Rn00591394_m1
	Mdr1b	Rn00561753_m1
	Mrp2	Rn00563231_m1
	Mrp3	Rn00589786_m1
	Ntcp1	Rn00566894_m1
	Oat2	Rn00585513_m1
Thyroid hormone responsive	D1	Rn00572183_m1

Table 3.3

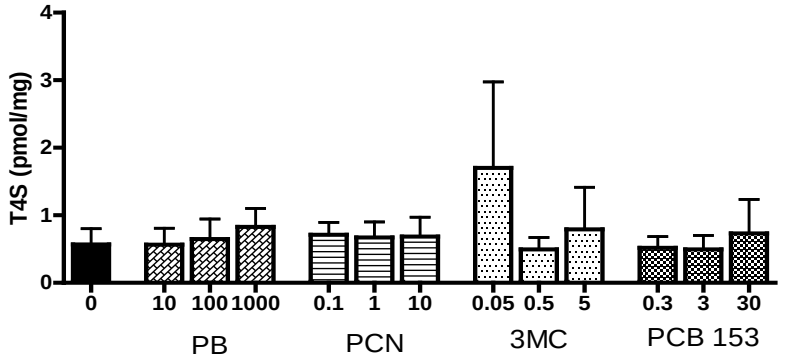
TaqMan human gene expression assays

Group	Gene	TaqMan Gene Expression Assay ID
Endogenous control	GAPDH	Hs99999905_m1
Nuclear receptor activation	CYP1A1	Hs00153120_m1
	CYP2B6	Hs03044634_m1
	CYP3A4	Hs00430021_m1
Phase II metabolism	UGT1A1	Hs02511055_s1
	UGT1A4	Hs01592480_m1
	UGT1A6	Hs01592477_m1
	UGT1A9	Hs02516855_sH
	SULT1A3	Hs00413970_m1
	SULT1E1	Hs00193690_m1
	SULT2A1	Hs00234219_m1
Transporter	MDR1B	Hs00184500_m1
	MRP2	Hs00166123_m1
	MRP3	Hs00358656_m1
	NTCP1	Hs00161820_m1
	OAT2	Hs00185140_m1
Thyroid hormone responsive	D1	Hs01554724_m1

A.



B.



C.

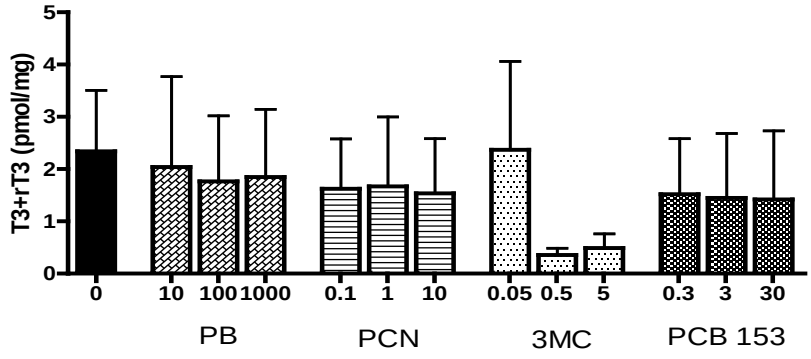
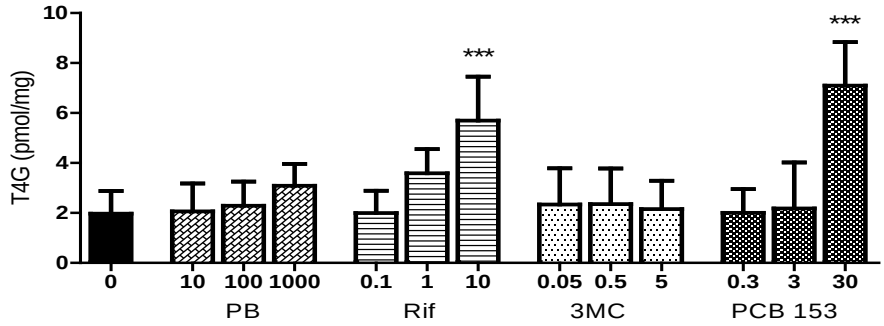
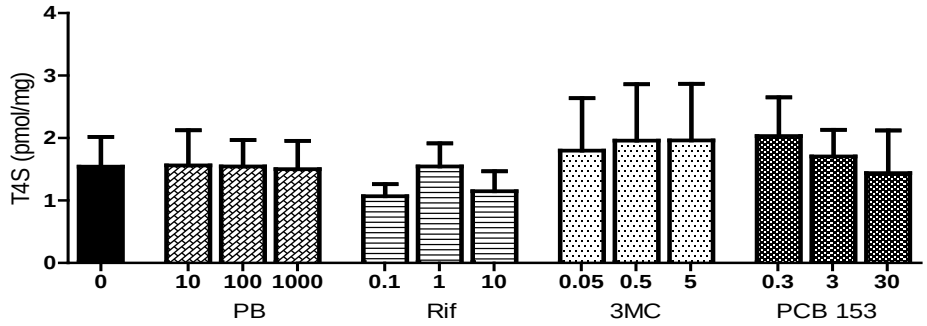


Figure 3.1. Comparison of appearance of metabolites in medium of SCRH following treatment with nuclear receptor agonists. Hepatocytes were incubated with 0.1% DMSO (control), PB, PCN, 3MC, or PCB 153 for 72 hours. Hepatocytes are then incubated for 24 hour with 0.05 µM [^{125}I]-T_4. Metabolites were separated using the established UPLC method. Metabolites analyzed are T_4G (A), T_4S (B), and T_3+rT_3 (C). Experiments were performed in duplicate Data are expressed as mean±standard deviation. n=4/group. *Significantly different than control; $p<0.05$. **Significantly different than control; $p<0.01$. ***Significantly different than control; $p<0.001$.

A.



B.



C.

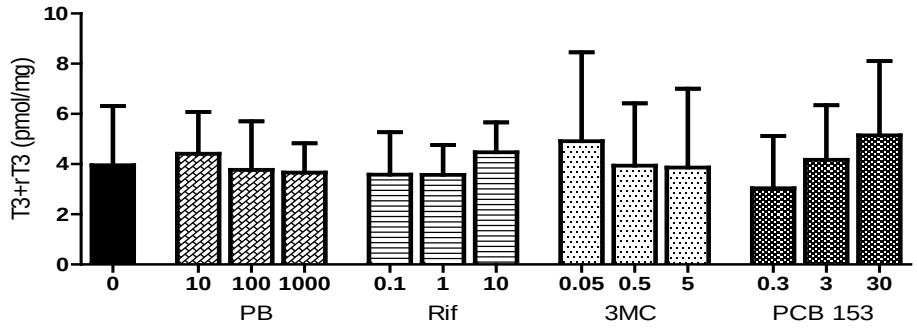


Figure 3.2. Comparison of appearance of metabolites in medium of SCHH following treatment with nuclear receptor agonists. Hepatocytes were incubated with 0.1% DMSO (control), PB, Rif, 3MC, or PCB 153 for 72 hours. Hepatocytes are then incubated for 24 hour with 0.1 µM [^{125}I]-T_4. Metabolites were separated using the established UPLC method. Metabolites analyzed are T_4G (A), T_4S (B), and T_3+rT_3 (C). Experiments were performed in duplicate Data are expressed as mean±standard deviation. n=4/group. ***Significantly different than control; p<0.001.

Table 3.4

Fold change in rat hepatic P450 mRNA expression

Chemical	Dose (μM)	Cyp1a1[a]	Cyp2b2[a]	Cyp3a1[a]
PB	0	1.0±0.0	1.0±0.0	1.0±0.0
	10	1.8±0.8	10.1±14.9	11.2±18.2
	100	1.8±1.4	28.3±19.9	18.2±28.7
	1000	4.7±2.4	197.0±78.6[b]	60.4±51.8
PCN	0	1.0±0.0	1.0±0.0	1.0±0.0
	0.1	2.8±3.8	0.8±0.6	17.9±27.6
	1	1.7±1.8	4.2±2.9	213.9±347.2
	10	2.8±2.3	7.4±2.9	660.7±489.1[b]
3MC	0	1.0±0.0	1.0±0.0	1.0±0.0
	0.05	33.8±52.8	0.9±0.9	7.8±12.2
	0.5	30.9±24.6	0.8±0.9	5.3±7.2
	5	270.2±204.9[b]	1.0±0.4	4.4±4.2
PCB 153	0	1.0±0.0	1.0±0.0	1.0±0.0
	0.3	2.1±1.2	20.9±12.2	7.2±10.8
	3	4.2±2.8	27.4±19.4	21.1±34.0
	30	3.6±0.5	19.0±18.7	78.6±94.0

[a]Data are expressed as fold change mean±standard deviation (n=3/group) relative to control.
[b]Significantly different from control group (p<0.001).

Table 3.5

Fold change in human hepatic P450 mRNA expression

Chemical	Dose (μM)	CYP1A1[a]	CYP2B6[a]	CYP3A4[a]
PB	0	1.0±0.0	1.0±0.0	1.0±0.0
	10	0.5±0.3	1.3±0.2	1.0±0.4
	100	0.8±0.5	5.7±2.1	3.7±2.4
	1000	1.3±0.5	17.1±7.4	20.0±10.8[d]
Rif	0	1.0±0.0	1.00±0.01	1.00±0.00
	0.1	0.6±0.4	2.34±1.61	5.10±4.20
	1	1.0±0.5	6.80±5.56	15.65±9.33[c]
	10	0.7±0.4	15.29±9.46	24.87±12.09[d]
3MC	0	1.0±0.0	1.0±0.0	1.0±0.0
	0.05	9.8±6.4	1.3±0.3	1.0±0.3
	0.5	154.6±132.8[c]	1.2±0.4	0.7±0.5
	5	178.0±162.4[c]	1.0±0.1	0.9±0.3
PCB 153	0	1.0±0.0	1.0±0.0	1.0±0.0
	0.3	0.8±0.3	4.6±2.5	1.4±0.8
	3	5.2±5.6	21.7±11.0[b]	2.9±0.7
	30	19.6±22.5	31.4±24.1[d]	5.3±2.4

[a]Data are expressed as fold change mean±standard deviation (n=4/group) relative to control.
[b]Significantly different from control group (p<0.05).
[c]Significantly different from control group (p<0.01).
[d]Significantly different from control group (p<0.001).

Table 3.6

Fold change in rat hepatic UGT mRNA expression

Chemical	Dose (μM)	Ugt1a1[a]	Ugt1a5[a]	Ugt1a6[a]	Ugt2b[a]
PB	0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
	10	1.2±0.8	2.0±1.0	1.4±0.3	2.8±2.9
	100	1.9±1.7	2.0±0.7	2.0±1.2	3.1±2.7
	1000	2.4±0.6	1.4±0.1	1.9±0.4	2.3±1.1
PCN	0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
	0.1	1.4±0.8	1.4±0.8	1.6±0.2	2.0±1.5
	1	2.5±1.9	2.6±2.6	1.8±0.8	3.1±2.9
	10	2.3±0.8	2.4±1.0	0.9±0.1	1.3±0.4
3MC	0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
	0.05	1.5±1.0	1.3±0.6	2.3±0.9	3.1±2.9
	0.5	1.0±0.2	1.2±0.3	8.2±1.7	2.7±1.8
	5	0.5±0.2	0.5±0.3	25.7±21.2[b]	1.1±0.3
PCB 153	0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
	0.3	1.3±0.5	1.5±1.1	1.7±0.4	2.7±2.5
	3	1.8±1.1	1.6±1.2	2.1±0.1	2.7±2.7
	30	1.5±0.3	2.1±0.4	1.9±0.9	0.9±0.3

[a]Data are expressed as fold change mean±standard deviation (n=3/group) relative to control.

[b]Significantly different from control group (p<0.001).

Table 3.7

Fold change in human hepatic UGT mRNA expression

Chemical	Dose (μM)	UGT1A1[a]	UGT1A4[a]	UGT1A6[a]	UGT1A9[a]	UGT2B15	UGT2B4
PB	0	1.0±0.0	1.0±0.1	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
	10	0.9±0.1	1.2±0.3	1.1±0.1	0.9±0.1	0.9±0.1	1.3±0.0
	100	2.2±0.7	1.7±0.3	1.6±0.7	1.0±0.3	1.6±0.5	1.5±0.4
	1000	6.4±2.6[d]	9.6±6.2[d]	2.2±0.3[d]	1.0±0.2	1.9±0.8	2.0±0.5[b]
Rif	0	1.0±0.0	1.0±0.1	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
	0.1	1.5±0.3	1.5±0.4	1.2±0.1	1.1±0.3	1.5±0.3	1.5±0.3
	1	4.7±2.2[b]	3.3±0.5	1.2±0.0	0.8±0.3	2.7±0.6	2.1±0.5[c]
	10	6.6±3.6[d]	7.2±0.7[d]	1.6±0.2	0.9±0.3	3.1±1.3[d]	2.4±0.9[d]
3MC	0	1.0±0.0	1.0±0.1	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
	0.05	1.5±0.2	1.7±0.7	1.2±0.3	1.0±0.2	1.2±0.6	1.3±0.3
	0.5	3.3±0.6	2.8±1.6	1.3±0.4	0.7±0.0	1.1±0.7	1.2±0.4
	5	2.9±1.1	1.8±0.6	1.1±0.3	1.0±0.3	0.9±0.3	1.1±0.3
PCB 153	0	1.0±0.0	1.0±0.1	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
	0.3	1.3±0.2	1.3±0.5	1.1±0.3	1.1±0.3	1.3±0.6	1.3±0.3
	3	2.8±1.3	1.4±0.6	0.9±0.1	1.3±0.1	1.2±0.2	1.4±0.4
	30	3.5±1.5	2.1±0.1	1.3±0.2	1.0±0.1	1.2±0.3	1.4±0.2

[a]Data are expressed as fold change mean±standard deviation (n=4/group) relative to control.
[b]Significantly different from control group ($p<0.05$).
[c]Significantly different from control group ($p<0.01$).
[d]Significantly different from control group ($p<0.001$).

Table 3.8

Fold change in rat hepatic SULT mRNA expression

Chemical	Dose (μM)	Sult1a1[a]	Sult1b1[a]	Sult1c1[a]	Sult1e1[a]
PB	0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
	10	1.7±1.7	1.5±1.2	0.9±0.3	0.8±0.6
	100	1.8±1.2	1.7±0.6	1.7±0.2	2.5±1.3
	1000	2.1±0.6	1.4±1.3	0.9±0.1	6.8±4.2[c]
PCN	0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
	0.1	1.7±0.8	1.3±0.5	1.1±0.2	1.3±0.3
	1	2.2±1.7	2.3±1.0	0.7±0.1	1.7±1.1
	10	0.9±0.3	1.5±0.5	0.2±0.1[b]	1.1±0.5
3MC	0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
	0.05	1.7±1.5	1.4±1.0	0.6±0.2	0.9±0.4
	0.5	1.9±0.5	2.0±0.7	1.3±0.8	1.5±0.3
	5	1.1±0.6	1.0±0.0	1.2±0.5	1.2±0.4
PCB 153	0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
	0.3	1.7±0.5	1.7±0.9	1.2±0.3	2.4±1.0
	3	1.9±0.9	1.7±0.9	0.9±0.2	1.7±0.6
	30	0.7±0.2	0.9±0.2	0.8±0.8	0.8±0.4

[a]Data are expressed as fold change mean±standard deviation (n=3/group) relative to control.
[b]Significantly different from control group ($p<0.05$).
[c]Significantly different from control group ($p<0.001$).

Table 3.9

Fold change in human hepatic SULT mRNA expression

Chemical	Dose (μM)	SULT1A3[a]	SULT1E1[a]	SULT2A1[a]
PB	0	1.0±0.0	1.0±0.0	1.0±0.0
	10	0.9±0.4	1.0±0.3	0.9±0.0
	100	1.1±0.3	0.5±0.1	1.0±0.7
	1000	1.3±0.4	0.3±0.2	1.0±0.4
Rif	0	1.0±0.0	1.0±0.0	1.0±0.0
	0.1	0.9±0.5	1.1±0.7	1.1±0.7
	1	1.1±0.5	0.7±0.5	0.9±0.3
	10	1.0±0.4	0.7±0.7	0.9 ±0.7
3MC	0	1.0±0.0	1.0±0.0	1.0±0.0
	0.05	0.8±0.3	1.1±0.3	1.0±0.2
	0.5	0.9±0.4	0.7±0.2	1.0±0.2
	5	0.7±0.3	0.9±0.1	1.0±0.2
PCB 153	0	1.0±0.0	1.0±0.0	1.0±0.0
	0.3	0.8±0.2	1.0±0.3	0.9±0.3
	3	0.9±0.3	1.0±0.3	1.2±0.4
	30	1.4±0.9	0.6±0.3	0.8±0.4

[a]Data are expressed as fold change mean±standard deviation (n=4/group) relative to control.

Table 3.10

Fold change in rat hepatic deiodinase I mRNA expression

Chemical	Dose (μM)	D1[a]
PB	0	1.0±0.0
	10	0.8±0.2
	100	1.1±0.0
	1000	1.4±0.2
PCN	0	1.0±0.0
	0.1	0.9±0.3
	1	1.1±0.3
	10	0.8±0.2
3MC	0	1.0±0.0
	0.05	0.8±0.3
	0.5	1.2±0.6
	5	1.3±1.0
PCB 153	0	1.0±0.0
	0.3	0.9±0.3
	3	1.3±0.1
	30	0.8±0.1

[a]Data are expressed as fold change mean±standard deviation (n=4/group) relative to control.

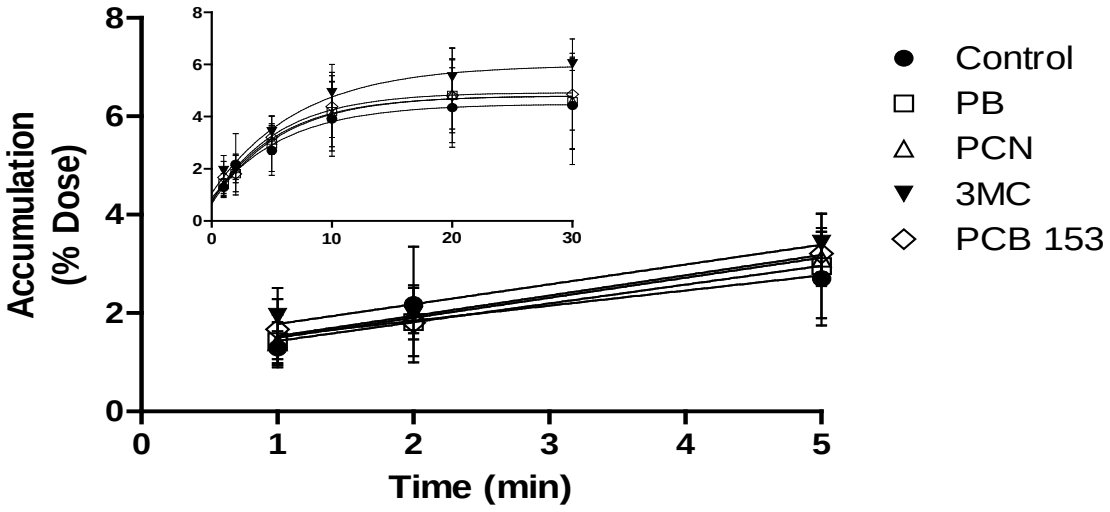
Table 3.11

Fold change in human hepatic deiodinase I mRNA expression

Chemical	Dose (μM)	D1[a]
PB	0	1.0±0.0
	10	1.0±0.2
	100	1.6±1.0
	1000	2.0±0.5
Rif	0	1.0±0.0
	0.1	1.7±0.7
	1	1.9±0.4
	10	1.6±0.1
3MC	0	1.0±0.0
	0.05	1.1±0.4
	0.5	0.9±0.0
	5	1.2±0.7
PCB 153	0	1.0±0.0
	0.3	2.4±1.3[b]
	3	1.9±1.1
	30	1.5±0.3

[a]Data are expressed as fold change mean±standard deviation (n=4/group).

A.



B.

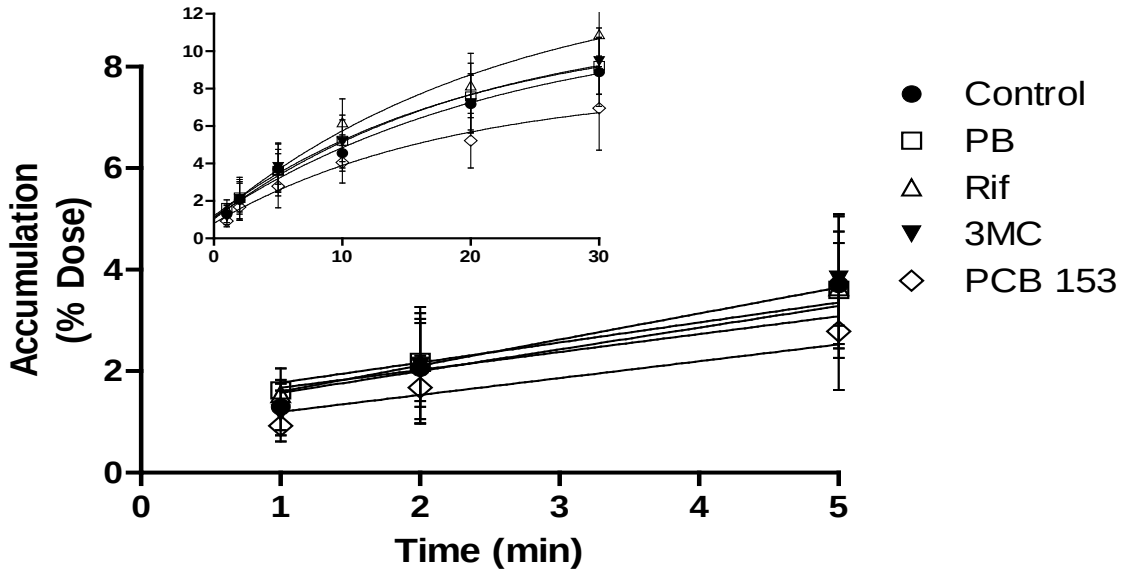


Figure 3.3. Accumulation of [^{125}I]-derived radioactivity in rat (A) and human (B) hepatocytes following treatment with nuclear receptor agonists..Sandwich-cultured hepatocytes were plated at 3.5x10^5 cells per well and incubated with 0.0005µM (5000 CPM) [^{125}I]-T_4. The accumulation of [^{125}I]-T_4 was determined over time (1-30 min) following treatment. Data are expressed as percentage of dose mean ± standard deviation. The lines represent the linear regression of the data. The curved lines represent the nonlinear regression of the data. n=3/group.

Table 3.12

Fold change in rat hepatic efflux and uptake transporter mRNA expression

Chemical	Dose (μM)	Mdr1a[a]	Mdr1b[a]	Mrp2[a]	Mrp3[a]	Ntcp1[a]	Oat2[a]
PB	0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
	10	0.9±0.1	0.9±0.4	2.0±1.8	1.1±0.4	1.2±1.1	1.2±0.5
	100	0.9±0.2	0.8±0.5	2.2±1.7	1.1±0.3	1.5±0.8	1.4±0.2
	1000	1.4±0.5[d]	1.3±0.7	2.4±0.8	1.5±0.5	1.6±0.6	1.9±0.7[b]
PCN	0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
	0.1	0.7±0.3	0.7±0.3	2.2±1.4	1.1±0.3	1.3±0.7	1.1±0.3
	1	1.2±0.2	1.1±0.4	3.9±4.3	1.4±0.1	1.9±1.6	1.5±0.5
	10	1.2±0.1	1.3±0.2	4.0±1.8	1.2±0.1	0.8±0.2	0.8±0.2
3MC	0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
	0.05	0.9±0.1	0.9±0.1	2.1±1.8	0.9±0.1	1.3±1.4	1.0±0.4
	0.5	0.9±0.4	0.8±0.4	1.7±0.1	1.6±0.9	1.4±0.4	1.4±0.4
	5	1.7±0.2[c]	1.0±0.4	1.8±0.3	2.0±0.7	1.2±0.9	1.4±0.2
PCB 153	0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
	0.3	0.9±0.4	0.7±0.5	1.9±0.9	1.1±0.5	1.5±0.9	1.3±0.3
	3	1.1±0.2	0.8±0.4	2.5±1.6	1.3±0.5	1.2±0.7	1.3±0.1
	30	1.6±0.1[b]	1.1±0.1	2.8±1.4	1.3±0.1	0.7±0.1	1.0±0.3

[a]Data are expressed as fold change mean±standard deviation (n=3/group) relative to control.

[b]Significantly different from control group (p<0.05).

[c]Significantly different from control group (p<0.01).

[d]Significantly different from control group (p<0.001).

Table 3.13

Fold change in human hepatic efflux and uptake transporter mRNA expression

Chemical	Dose (μM)	MDR1[a]	MRP2[a]	MRP3[a]	NTCP1[a]	OAT2[a]
PB	0	1.0±0.1	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
	10	0.9±0.2	1.0±0.1	0.7±0.2	0.9±0.3	0.9±0.4
	100	1.2±0.3	1.3±0.4	0.7±0.2	0.8±0.5	1.0±0.30
	1000	2.3±0.6[c]	2.5±0.0[c]	0.9±0.3	0.6±0.1	0.7±0.4
Rif	0	1.0±0.1	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
	0.1	1.1±0.2	1.2±0.3	0.9±0.1	0.8±0.1	1.4±0.4
	1	2.0±0.7[b]	1.8±0.3[c]	1.0±0.3	1.3±0.9	1.1±0.1
	10	2.0±0.7[b]	2.1±0.3[c]	0.8±0.2	1.1±0.5	1.3±0.2
3MC	0	1.0±0.1	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
	0.05	0.9±0.3	1.0±0.2	0.7±0.1	1.0±0.5	1.0±0.5
	0.5	0.8±0.2	0.9±0.2	0.7±0.2	0.6±0.1	0.7±0.4
	5	0.8±0.1	0.9±0.2	0.7±0.3	0.8±0.2	0.7±0.3
PCB 153	0	1.0±0.1	1.0±0.0	1.0±0.0	1.0±0.03	1.0±0.0
	0.3	1.1±0.1	1.1±0.0	0.9±0.2	0.9±0.3	1.0±0.2
	3	1.1±0.3	1.1±0.2	0.9±0.3	1.1±0.0	1.2±0.3
	30	1.4±0.6	1.4±0.3	0.9±0.3	0.7±0.2	0.8±0.2

[a]Data are expressed as fold change mean±standard deviation (n=4/group) relative to control.
[b]Significantly different from control group (p<0.01).
[c]Significantly different from control group (p<0.001).

REFERENCES

Aleksunes, L. M. and Klaassen, C. D. (2012). Coordinated regulation of hepatic phase I and II drug-metabolizing genes and transporters using AhR-, CAR-, PXR-, PPARalpha-, and Nrf2-null mice. *Drug Metab Dispos* **40**(7), 1366-79.

Auyeung, D. J., Kessler, F. K. and Ritter, J. K. (2003). Mechanism of rat UDP-glucuronosyltransferase 1A6 induction by oltipraz: evidence for a contribution of the Aryl hydrocarbon receptor pathway. *Mol Pharmacol* **63**(1), 119-27.

Barter, R. A. and Klaassen, C. D. (1992). UDP-glucuronosyltransferase inducers reduce thyroid hormone levels in rats by an extrathyroidal mechanism. *Toxicol Appl Pharmacol* **113**(1), 36-42.

Barter, R. A. and Klaassen, C. D. (1994). Reduction of thyroid hormone levels and alteration of thyroid function by four representative UDP-glucuronosyltransferase inducers in rats. *Toxicol Appl Pharmacol* **128**(1), 9-17.

Bock, K. W. (2011). From differential induction of UDP-glucuronosyltransferases in rat liver to characterization of responsible ligand-activated transcription factors, and their multilevel crosstalk in humans. *Biochem Pharmacol* **82**(1), 9-16.

Bock, K. W. and Kohle, C. (2004). Coordinate regulation of drug metabolism by xenobiotic nuclear receptors: UGTs acting together with CYPs and glucuronide transporters. *Drug Metab Rev* **36**(3-4), 595-615.

Chang, T. K. and Waxman, D. J. (2006). Synthetic drugs and natural products as modulators of constitutive androstane receptor (CAR) and pregnane X receptor (PXR). *Drug Metab Rev* **38**(1-2), 51-73.

Chen, C., Staudinger, J. L. and Klaassen, C. D. (2003). Nuclear receptor, pregname X receptor, is required for induction of UDP-glucuronosyltranferases in mouse liver by pregnenolone-16 alpha-carbonitrile. *Drug Metab Dispos* **31**(7), 908-15.

Craft, E. S., DeVito, M. J. and Crofton, K. M. (2002). Comparative responsiveness of hypothyroxinemia and hepatic enzyme induction in Long-Evans rats versus C57BL/6J mice exposed to TCDD-like and phenobarbital-like polychlorinated biphenyl congeners. *Toxicol Sci* **68**(2), 372-80.

Crofton, K. M., Craft, E. S., Hedge, J. M., Gennings, C., Simmons, J. E., Carchman, R. A., Carter, W. H., Jr. and DeVito, M. J. (2005). Thyroid-hormone-disrupting chemicals: evidence for dose-dependent additivity or synergism. *Environ Health Perspect* **113**(11), 1549-54.

Crofts, L. A., Hancock, M. S., Morrison, N. A. and Eisman, J. A. (1998). Multiple promoters direct the tissue-specific expression of novel N-terminal variant human vitamin D receptor gene transcripts. *Proc Natl Acad Sci U S A* **95**(18), 10529-34.

Davies, P. H., Sheppard, M. C. and Franklyn, J. A. (1996). Regulation of type I 5'-deiodinase by thyroid hormone and dexamethasone in rat liver and kidney cells. *Thyroid* **6**(3), 221-8.

De Jong, M., Docter, R., Van Der Hoek, H. J., Vos, R. A., Krenning, E. P. and Hennemann, G. (1992). Transport of 3,5,3'-triiodothyronine into the perfused rat liver and subsequent metabolism are inhibited by fasting. *Endocrinology* **131**(1), 463-70.

de Jong, M., Visser, T. J., Bernard, B. F., Docter, R., Vos, R. A., Hennemann, G. and Krenning, E. P. (1993). Transport and metabolism of iodothyronines in cultured human hepatocytes. *J Clin Endocrinol Metab* **77**(1), 139-43.

de Longueville, F., Atienzar, F. A., Marcq, L., Dufrane, S., Evrard, S., Wouters, L., Leroux, F., Bertholet, V., Gerin, B., Whomsley, R., Arnould, T., Remacle, J. and Canning, M. (2003). Use of a low-density microarray for studying gene expression patterns induced by hepatotoxicants on primary cultures of rat hepatocytes. *Toxicol Sci* **75**(2), 378-92.

de Sandro, V., Catinot, R., Kriszt, W., Cordier, A. and Richert, L. (1992). Male rat hepatic UDP-glucuronosyltransferase activity toward thyroxine. Activation and induction properties--relation with thyroxine plasma disappearance rate. *Biochem Pharmacol* **43**(7), 1563-9.

Eiris-Punal, J., Del Rio-Garma, M., Del Rio-Garma, M. C., Lojo-Rocamonde, S., Novo-Rodriguez, I. and Castro-Gago, M. (1999). Long-term treatment of children with epilepsy with valproate or carbamazepine may cause subclinical hypothyroidism. *Epilepsia* **40**(12), 1761-6.

Fasco, M. J. (1998). Estrogen receptor mRNA splice variants produced from the distal and proximal promoter transcripts. *Mol Cell Endocrinol* **138**(1-2), 51-9.

Faucette, S. R., Sueyoshi, T., Smith, C. M., Negishi, M., Lecluyse, E. L. and Wang, H. (2006). Differential regulation of hepatic CYP2B6 and CYP3A4 genes by constitutive androstane receptor but not pregnane X receptor. *J Pharmacol Exp Ther* **317**(3), 1200-9.

Faucette, S. R., Zhang, T. C., Moore, R., Sueyoshi, T., Omiecinski, C. J., LeCluyse, E. L., Negishi, M. and Wang, H. (2007). Relative activation of human pregnane X receptor versus constitutive androstane receptor defines distinct classes of CYP2B6 and CYP3A4 inducers. *J Pharmacol Exp Ther* **320**(1), 72-80.

Findlay, K. A., Kaptein, E., Visser, T. J. and Burchell, B. (2000). Characterization of the uridine diphosphate-glucuronosyltransferase-catalyzing thyroid hormone glucuronidation in man. *J Clin Endocrinol Metab* **85**(8), 2879-83.

Friesema, E. C., Docter, R., Moerings, E. P., Stieger, B., Hagenbuch, B., Meier, P. J., Krenning, E. P., Hennemann, G. and Visser, T. J. (1999). Identification of thyroid hormone transporters. *Biochem Biophys Res Commun* **254**(2), 497-501.

Frueh, F. W., Zanger, U. M. and Meyer, U. A. (1997). Extent and character of phenobarbital-mediated changes in gene expression in the liver. *Mol Pharmacol* **51**(3), 363-9.

Hartley, D. P. and Klaassen, C. D. (2000). Detection of chemical-induced differential expression of rat hepatic cytochrome P450 mRNA transcripts using branched DNA signal amplification technology. *Drug Metab Dispos* **28**(5), 608-16.

Hood, A., Allen, M. L., Liu, Y., Liu, J. and Klaassen, C. D. (2003). Induction of T(4) UDP-GT activity, serum thyroid stimulating hormone, and thyroid follicular cell proliferation in mice treated with microsomal enzyme inducers. *Toxicol Appl Pharmacol* **188**(1), 6-13.

Hood, A. and Klaassen, C. D. (2000a). Differential effects of microsomal enzyme inducers on in vitro thyroxine (T(4)) and triiodothyronine (T(3)) glucuronidation. *Toxicol Sci* **55**(1), 78-84.

Hood, A. and Klaassen, C. D. (2000b). Effects of microsomal enzyme inducers on outer-ring deiodinase activity toward thyroid hormones in various rat tissues. *Toxicol Appl Pharmacol* **163**(3), 240-8.

Jemnitz, K., Veres, Z., Monostory, K. and Vereczkey, L. (2000). Glucuronidation of thyroxine in primary monolayer cultures of rat hepatocytes: in vitro induction of UDP-glucuronosyltranferases by methylcholanthrene, clofibrate, and dexamethasone alone and in combination. *Drug Metab Dispos* **28**(1), 34-7.

Jigorel, E., Le Vee, M., Boursier-Neyret, C., Parmentier, Y. and Fardel, O. (2006). Differential regulation of sinusoidal and canalicular hepatic drug transporter expression by xenobiotics activating drug-sensing receptors in primary human hepatocytes. *Drug Metab Dispos* **34**(10), 1756-63.

Johnson, D. R., Habeebu, S. S. and Klaassen, C. D. (2002). Increase in bile flow and biliary excretion of glutathione-derived sulfhydryls in rats by drug-metabolizing enzyme inducers is mediated by multidrug resistance protein 2. *Toxicol Sci* **66**(1), 16-26.

Johnson, D. R. and Klaassen, C. D. (2002). Regulation of rat multidrug resistance protein 2 by classes of prototypical microsomal enzyme inducers that activate distinct transcription pathways. *Toxicol Sci* **67**(2), 182-9.

Kato, Y., Ikushiro, S., Haraguchi, K., Yamazaki, T., Ito, Y., Suzuki, H., Kimura, R., Yamada, S., Inoue, T. and Degawa, M. (2004). A possible mechanism for decrease in serum thyroxine level by polychlorinated biphenyls in Wistar and Gunn rats. *Toxicol Sci* **81**(2), 309-15.

Kato, Y., Ikushiro, S., Takiguchi, R., Haraguchi, K., Koga, N., Uchida, S., Sakaki, T., Yamada, S., Kanno, J. and Degawa, M. (2007). A novel mechanism for polychlorinated biphenyl-induced decrease in serum thyroxine level in rats. *Drug Metab Dispos* **35**(10), 1949-55.

Kato, Y., Onishi, M., Haraguchi, K., Ikushiro, S., Ohta, C., Koga, N., Endo, T., Yamada, S. and Degawa, M. (2011). A possible mechanism for 2,2',4,4',5,5'-hexachlorobiphenyl-mediated decrease in serum thyroxine level in mice. *Toxicol Appl Pharmacol* **254**(1), 48-55.

Kato, Y., Suzuki, H., Haraguchi, K., Ikushiro, S., Ito, Y., Uchida, S., Yamada, S. and Degawa, M. (2010). A possible mechanism for the decrease in serum thyroxine level by phenobarbital in rodents. *Toxicol Appl Pharmacol* **249**(3), 238-46.

Kato, Y., Suzuki, H., Ikushiro, S., Yamada, S. and Degawa, M. (2005). Decrease in serum thyroxine level by phenobarbital in rats is not necessarily dependent on increase in hepatic UDP-glucuronosyltransferase. *Drug Metab Dispos* **33**(11), 1608-12.

Kester, M. H., Kaptein, E., Roest, T. J., van Dijk, C. H., Tibboel, D., Meinl, W., Glatt, H., Coughtrie, M. W. and Visser, T. J. (1999). Characterization of human iodothyronine sulfotransferases. *J Clin Endocrinol Metab* **84**(4), 1357-64.

Kester, M. H., Kaptein, E., Roest, T. J., van Dijk, C. H., Tibboel, D., Meinl, W., Glatt, H., Coughtrie, M. W. and Visser, T. J. (2003). Characterization of rat iodothyronine sulfotransferases. *Am J Physiol Endocrinol Metab* **285**(3), E592-8.

Kim, H. J., Cho, J. H. and Klaassen, C. D. (1995). Depletion of hepatic 3'-phosphoadenosine 5'-phosphosulfate (PAPS) and sulfate in rats by xenobiotics that are sulfated. *J Pharmacol Exp Ther* **275**(2), 654-8.

Kipp, H. and Arias, I. M. (2002). Trafficking of canalicular ABC transporters in hepatocytes. *Annu Rev Physiol* **64**, 595-608.

Klaassen, C. D. and Hood, A. M. (2001). Effects of microsomal enzyme inducers on thyroid follicular cell proliferation and thyroid hormone metabolism. *Toxicol Pathol* **29**(1), 34-40.

Kohle, C. and Bock, K. W. (2009). Coordinate regulation of human drug-metabolizing enzymes, and conjugate transporters by the Ah receptor, pregnane X receptor and constitutive androstane receptor. *Biochem Pharmacol* **77**(4), 689-99.

Koster, H., Halsema, I., Scholtens, E., Knippers, M. and Mulder, G. J. (1981). Dose-dependent shifts in the sulfation and glucuronidation of phenolic compounds in the rat in vivo and in isolated hepatocytes. The role of saturation of phenolsulfotransferase. *Biochem Pharmacol* **30**(18), 2569-75.

Krenning, E., Docter, R., Bernard, B., Visser, T. and Hennemann, G. (1981). Characteristics of active transport of thyroid hormone into rat hepatocytes. *Biochim Biophys Acta* **676**(3), 314-20.

Kretschmer, X. C. and Baldwin, W. S. (2005). CAR and PXR: xenosensors of endocrine disrupters? *Chem Biol Interact* **155**(3), 111-28.

Lecureux, L., Dieter, M. Z., Nelson, D. M., Watson, L., Wong, H., Gemzik, B., Klaassen, C. D. and Lehman-McKeeman, L. D. (2009). Hepatobiliary disposition of thyroid hormone in Mrp2-deficient TR- rats: reduced biliary excretion of thyroxine glucuronide does not prevent xenobiotic-induced hypothyroidism. *Toxicol Sci* **108**(2), 482-91.

Liu, C., Wang, C., Yan, M., Quan, C., Zhou, J. and Yang, K. (2012). PCB153 disrupts thyroid hormone homeostasis by affecting its biosynthesis, biotransformation, feedback regulation, and metabolism. *Horm Metab Res* **44**(9), 662-9.

Maglich, J. M., Stoltz, C. M., Goodwin, B., Hawkins-Brown, D., Moore, J. T. and Kliewer, S. A. (2002). Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. *Mol Pharmacol* **62**(3), 638-46.

Martin, L. A., Wilson, D. T., Reuhl, K. R., Gallo, M. A. and Klaassen, C. D. (2012). Polychlorinated biphenyl congeners that increase the glucuronidation and biliary excretion of thyroxine are distinct from the congeners that enhance the serum disappearance of thyroxine. *Drug Metab Dispos* **40**(3), 588-95.

McClain, R. M., Levin, A. A., Posch, R. and Downing, J. C. (1989). The effect of phenobarbital on the metabolism and excretion of thyroxine in rats. *Toxicol Appl Pharmacol* **99**(2), 216-28.

McFarland, V. A. and Clarke, J. U. (1989). Environmental occurrence, abundance, and potential toxicity of polychlorinated biphenyl congeners: considerations for a congener-specific analysis. *Environ Health Perspect* **81**, 225-39.

Meyer, U. A. and Hoffmann, K. (1999). Phenobarbital-mediated changes in gene expression in the liver. *Drug Metab Rev* **31**(2), 365-73.

Mitchell, A. M., Tom, M. and Mortimer, R. H. (2005). Thyroid hormone export from cells: contribution of P-glycoprotein. *J Endocrinol* **185**(1), 93-8.

Miyawaki, I., Tamura, A., Matsumoto, I., Inada, H., Kunimatsu, T., Kimura, J. and Funabashi, H. (2012). The effects of clobazam treatment in rats on the expression of genes and proteins encoding glucronosyltransferase 1A/2B (UGT1A/2B) and multidrug resistance-associated protein-2 (MRP2), and development of thyroid follicular cell hypertrophy. *Toxicol Appl Pharmacol* **265**(3), 351-9.

Moore, L. B., Parks, D. J., Jones, S. A., Bledsoe, R. K., Consler, T. G., Stimmel, J. B., Goodwin, B., Liddle, C., Blanchard, S. G., Willson, T. M., Collins, J. L. and Kliewer, S. A. (2000). Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J Biol Chem* **275**(20), 15122-7.

Nishimura, N., Yonemoto, J., Miyabara, Y., Fujii-Kuriyama, Y. and Tohyama, C. (2005). Altered thyroxin and retinoid metabolic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin in aryl hydrocarbon receptor-null mice. *Archives of toxicology* **79**(5), 260-7.

Novakova, S., Van Dyck, S., Glatz, Z., Van Schepdael, A. and Hoogmartens, J. (2004). Study of enzyme kinetics of phenol sulfotransferase by electrophoretically mediated microanalysis. *J Chromatogr A* **1032**(1-2), 319-26.

Ohnhaus, E. E., Burgi, H., Burger, A. and Studer, H. (1981). The effect of antipyrine, phenobarbitol and rifampicin on thyroid hormone metabolism in man. *Eur J Clin Invest* **11**(5), 381-7.

Ohnhaus, E. E. and Studer, H. (1983). A link between liver microsomal enzyme activity and thyroid hormone metabolism in man. *Br J Clin Pharmacol* **15**(1), 71-6.

Oppenheimer, J. H., Bernstein, G. and Surks, M. I. (1968). Increased thyroxine turnover and thyroidal function after stimulation of hepatocellular binding of thyroxine by phenobarbital. *J Clin Invest* **47**(6), 1399-406.

Ozawa, S., Nagata, K., Gong, D. W., Yamazoe, Y. and Kato, R. (1993). Expression and functional characterization of a rat sulfotransferase (ST1A1) cDNA for sulfations of phenolic substrates in COS-1 cells. *Jpn J Pharmacol* **61**(2), 153-6.

Parkinson, A., Safe, S. H., Robertson, L. W., Thomas, P. E., Ryan, D. E., Reik, L. M. and Levin, W. (1983). Immunochemical quantitation of cytochrome P-450 isozymes and epoxide hydrolase in liver microsomes from polychlorinated or polybrominated biphenyl-treated rats. A study of structure-activity relationships. *J Biol Chem* **258**(9), 5967-76.

Pascussi, J. M., Gerbal-Chaloin, S., Duret, C., Daujat-Chavanieu, M., Vilarem, M. J. and Maurel, P. (2008). The tangle of nuclear receptors that controls xenobiotic metabolism and transport: crosstalk and consequences. *Annu Rev Pharmacol Toxicol* **48**, 1-32.

Qatanani, M., Zhang, J. and Moore, D. D. (2005). Role of the constitutive androstane receptor in xenobiotic-induced thyroid hormone metabolism. *Endocrinology* **146**(3), 995-1002.

Richardson, T. A. and Klaassen, C. D. (2010). Disruption of thyroid hormone homeostasis in Ugt1a-deficient Gunn rats by microsomal enzyme inducers is not due to enhanced thyroxine glucuronidation. *Toxicol Appl Pharmacol* **248**(1), 38-44.

Richardson, V. M., Ferguson, S.S., Sey, Y.M., and DeVito, M.J. (submitted). In vitro metabolism of thyroxine by rat and human hepatocytes.

Rotroff, D. M., Beam, A. L., Dix, D. J., Farmer, A., Freeman, K. M., Houck, K. A., Judson, R. S., LeCluyse, E. L., Martin, M. T., Reif, D. M. and Ferguson, S. S. (2010). Xenobiotic-metabolizing enzyme and transporter gene expression in primary cultures of human hepatocytes modulated by ToxCast chemicals. *Journal of toxicology and environmental health. Part B, Critical reviews* **13**(2-4), 329-46.

Runge-Morris, M., Rose, K., Falany, C. N. and Kocarek, T. A. (1998). Differential regulation of individual sulfotransferase isoforms by phenobarbital in male rat liver. *Drug Metab Dispos* **26**(8), 795-801.

Rutgers, M., Bonthuis, F., de Herder, W. W. and Visser, T. J. (1987). Accumulation of plasma triiodothyronine sulfate in rats treated with propylthiouracil. *J Clin Invest* **80**(3), 758-62.

Rutgers, M., Pigmans, I. G., Bonthuis, F., Docter, R. and Visser, T. J. (1989). Effects of propylthiouracil on the biliary clearance of thyroxine (T4) in rats: decreased excretion of 3,5,3'-triiodothyronine glucuronide and increased excretion of 3,3',5'-triiodothyronine glucuronide and T4 sulfate. *Endocrinology* **125**(4), 2175-86.

Saini, S. P., Sonoda, J., Xu, L., Toma, D., Uppal, H., Mu, Y., Ren, S., Moore, D. D., Evans, R. M. and Xie, W. (2004). A novel constitutive androstane receptor-mediated and CYP3A-independent pathway of bile acid detoxification. *Mol Pharmacol* **65**(2), 292-300.

Shao, D. and Lazar, M. A. (1999). Modulating nuclear receptor function: may the phos be with you. *J Clin Invest* **103**(12), 1617-8.

Shelby, M. K. and Klaassen, C. D. (2006). Induction of rat UDP-glucuronosyltransferases in liver and duodenum by microsomal enzyme inducers that activate various transcriptional pathways. *Drug Metab Dispos* **34**(10), 1772-8.

Smirlis, D., Muangmoonchai, R., Edwards, M., Phillips, I. R. and Shephard, E. A. (2001). Orphan receptor promiscuity in the induction of cytochromes p450 by xenobiotics. *J Biol Chem* **276**(16), 12822-6.

Surry, D. D., Meneses-Lorente, G., Heavens, R., Jack, A. and Evans, D. C. (2000). Rapid determination of rat hepatocyte mRNA induction potential using oligonucleotide probes for CYP1A1, 1A2, 3A and 4A1. *Xenobiotica; the fate of foreign compounds in biological systems* **30**(5), 441-56.

Szabo, D. T., Richardson, V. M., Ross, D. G., Diliberto, J. J., Kodavanti, P. R. and Birnbaum, L. S. (2009). Effects of perinatal PBDE exposure on hepatic phase I, phase II, phase III, and deiodinase 1 gene expression involved in thyroid hormone metabolism in male rat pups. *Toxicol Sci* **107**(1), 27-39.

Tanaka, K., Kodama, S., Yokoyama, S., Komatsu, M., Konishi, H., Momota, K. and Matsuo, T. (1987). Thyroid function in children with long-term anticonvulsant treatment. *Pediatric neuroscience* **13**(2), 90-4.

Tchaparian, E. H., Houghton, J. S., Uyeda, C., Grillo, M. P. and Jin, L. (2011). Effect of culture time on the basal expression levels of drug transporters in sandwich-cultured primary rat hepatocytes. *Drug Metab Dispos* **39**(12), 2387-94.

Tirona, R. G., Leake, B. F., Podust, L. M. and Kim, R. B. (2004). Identification of amino acids in rat pregnane X receptor that determine species-specific activation. *Mol Pharmacol* **65**(1), 36-44.

Vansell, N. R. and Klaassen, C. D. (2002a). Increase in rat liver UDP-glucuronosyltransferase mRNA by microsomal enzyme inducers that enhance thyroid hormone glucuronidation. *Drug Metab Dispos* **30**(3), 240-6.

Vansell, N. R. and Klaassen, C. D. (2001). Increased biliary excretion of thyroxine by microsomal enzyme inducers. *Toxicol Appl Pharmacol* **176**(3), 187-94.

Vansell, N. R. and Klaassen, C. D. (2002b). Effect of microsomal enzyme inducers on the biliary excretion of triiodothyronine (T(3)) and its metabolites. *Toxicol Sci* **65**(2), 184-91.

Viluksela, M., Raasmaja, A., Lebofsky, M., Stahl, B. U. and Rozman, K. K. (2004). Tissue-specific effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the activity of 5'-deiodinases I and II in rats. *Toxicol Lett* **147**(2), 133-42.

Viollon-Abadie, C., Bigot-Lasserre, D., Nicod, L., Carmichael, N. and Richert, L. (2000). Effects of model inducers on thyroxine UDP-glucuronosyl-transferase activity in vitro in rat and mouse hepatocyte cultures. *Toxicol In Vitro* **14**(6), 505-12.

Visser, T. J. (1996). Pathways of thyroid hormone metabolism. *Acta Med Austriaca* **23**(1-2), 10-6.

Visser, T. J., Kaptein, E., Glatt, H., Bartsch, I., Hagen, M. and Coughtrie, M. W. (1998). Characterization of thyroid hormone sulfotransferases. *Chem Biol Interact* **109**(1-3), 279-91.

Visser, W. E., Friesema, E. C., Jansen, J. and Visser, T. J. (2008). Thyroid hormone transport in and out of cells. *Trends Endocrinol Metab* **19**(2), 50-6.

Wagner, M., Halilbasic, E., Marschall, H. U., Zollner, G., Fickert, P., Langner, C., Zatloukal, K., Denk, H. and Trauner, M. (2005). CAR and PXR agonists stimulate hepatic bile acid and bilirubin detoxification and elimination pathways in mice. *Hepatology* **42**(2), 420-30.

Weigel, N. L. (1996). Steroid hormone receptors and their regulation by phosphorylation. *Biochem J* **319 (Pt 3)**, 657-67.

Wong, H., Lehman-McKeeman, L. D., Grubb, M. F., Grossman, S. J., Bhaskaran, V. M., Solon, E. G., Shen, H. S., Gerson, R. J., Car, B. D., Zhao, B. and Gemzik, B. (2005). Increased hepatobiliary clearance of unconjugated thyroxine determines DMP 904-induced alterations in thyroid hormone homeostasis in rats. *Toxicol Sci* **84**(2), 232-42.

Yanagiba, Y., Ito, Y., Kamijima, M., Gonzalez, F. J. and Nakajima, T. (2009). Octachlorostyrene induces cytochrome P450, UDP-glucuronosyltransferase, and sulfotransferase via the aryl hydrocarbon receptor and constitutive androstane receptor. *Toxicol Sci* **111**(1), 19-26.

Yoder Graber, A. L., Ramirez, J., Innocenti, F. and Ratain, M. J. (2007). UGT1A1*28 genotype affects the in-vitro glucuronidation of thyroxine in human livers. *Pharmacogenet Genomics* **17**(8), 619-27.

CHAPTER 4

EFFECTS OF BDE 47 ON THYROXINE METABOLISM

A. INTRODUCTION

Polybrominated diphenyl ethers (PBDEs) are a class of flame retardants used in various consumer products including polyurethane foam, electronics, and textile coating. Due to their persistence and lipophilicity, PBDEs bioaccumulate and have been detected in biota, environmental samples and human biological samples (Focant *et al.*, 2004a; Schecter *et al.*, 2010; Krol *et al.*, 2012). Pentabromodiphenyl ether (DE-71), is a commercial mixture of tribromodiphenyl ether to hexabromodiphenyl ether congeners, of which approximately 30% consists of 2,2′,4,4′-tetrabromodiphenyl ether (BDE 47). BDE 47 is the predominant congener found in most environmental and human samples and usually accounts for half of the total PBDEs measured (Hites, 2004; Lorber, 2008). Although found in human tissues, the health risks from exposure to PDBEs are uncertain.

Similar to polychlorinated biphenyls (PCBs) and dioxins, PBDEs disrupt thyroid hormone (TH) homeostasis (Zhou, *et al.*, 2001; Hallgren and Darnerud, 2002; Richardson, *et al.*, 2008; Szabo, *et al.*, 2009). Although there are many sites within the thyroid axis in which xenobiotics such as PBDEs can interrupt TH homeostasis, the literature focuses on two possible mechanisms: (1) the increase in hepatic TH metabolism and (2) the competitive

binding of PBDEs with TH binding proteins. In rodents, decreases in thyroxine (T_4) are often associated with xenobiotic-mediated increases in T_4 glucuronidation and the subsequent elimination of T_4-glucuronide (T_4G) into bile (Vansell and Klaassen, 2002b; Hood, *et al.*, 2003; Martin, *et al.*, 2012). Decreases in serum T_4 in rodents treated with PBDEs often have been linked to inductions of hepatic uridinediphosphate glucuronosyltransferases (UGTs). In rat pups, gestational and lactational exposures to PBDEs result in decreased serum T_4 concentrations and inductions in hepatic UGT activity (Zhou, *et al.*, 2002; Szabo, *et al.*, 2009). Exposure to BDE 47 resulted in a 50% decrease in serum T_4 concentrations and increases in hepatic Ugt1a1 and Ugt1a7 mRNA expression in female mice; however liver T_4-UGT activity did not increase (Richardson, *et al.*, 2008). Although it appears that serum T_4 decreases are associated with increases in hepatic UGTs, there is evidence that T_4 decreases may be independent of UGT induction. Ugt1a-deficient Gunn rats fed, 3-methylcholanthrene (3MC), pregnenolone-16α-carbonitrile (PCN), or Arochlor 1254 resulted in decreases in serum T_4 concentrations 19, 38, and 91%, respectively; however, hepatic T_4-UGT activity was unchanged and the magnitude of the decrease in serum T_4 was similar to that observed in the Wistar rat (Richardson and Klaassen, 2010). Kenechlor-500 administered to Wistar and Gunn rats decreased serum T_4 and increased the accumulation of [^{125}I]-T_4 in several tissues including the liver, but only increased T_4 glucuronidation in the Wistar rat (Kato, *et al.*, 2007). In Wistar rats treated with phenobarbital (PB), decreases in serum T_4 is associated with increased hepatic T_4-UGT activity and increased biliary elimination of [^{125}I]-T_4 and [^{125}I]-T_4G, but in the Gunn rat, PB decreases serum T_4 concentrations without a concomitant increase in T_4 glucuronidation or biliary elimination (Kato, *et al.*, 2010).

In addition to UGTs, Sulfotransferases (SULTs) are a pathway for TH metabolism. SULTs are inducible in rat liver following xenobiotics treatment and may be linked to enhanced TH metabolism in the liver and biliary elimination (Szabo, *et al.*, 2009; Paul *et al.*, 2010). Sulfation of TH facilitates their further degradation by deiodinase I (DI) (Visser *et al.*, 1990; Visser, 1994). Deiodinases also are involved in TH metabolism in their conversion of T_4 and T_3 to 3, 3', 5'-triiodothyronine (rT_3) and 3, 3'-diiodothyronine (3, 3'-T_2), respectively. Together these studies show that decreases in serum T_4 are not completely associated with hepatic T_4 glucuronidation, but also may involve sulfation, deiodination, serum-to-liver accumulation and biliary elimination of T_4.

Transthyretin (TTR), is a major plasma TH binding protein found in all vertebrates. Hydroxylated PBDEs have been shown to competitively interact with TTR and it is hypothesized that this interaction displaces T_4 from binding to TTR (Meerts *et al.*, 2000; Hallgren and Darnerud, 2002; Hamers *et al.*, 2008). Hydroxylated metabolites of BDE 47, -51, -51, -75, and -77 displaced more than 60% of [^{125}I]-T_4 from TTR *in vitro* (Meerts, *et al.*, 2000). Hamers *et al.* (2008) reported that six different hydroxylated metabolites of BDE 47 had TTR-binding potencies 160–1600 times higher than the parent compound (BDE 47). This suggests that decreases in serum T_4 may result from the displacement of T_4 from TTR by BDE 47 metabolites.

Aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR) regulate genes such as cytochrome P450s (CYPs), UGTs, SULTs and transporters which are all involved in the metabolism and elimination of xenobiotics (Bock and Kohle, 2004; Wagner, *et al.*, 2005; Kohle and Bock, 2009; Tolson and Wang, 2010). Correlations between inductions in hepatic UGTs and multidrug resistance protein-

associated protein (MRP) with decreases in serum T_4 have been observed. In mice, BDE 47 induces CAR regulated genes such as Cyp2b10 mRNA expression (Richardson, *et al.*, 2008). BDE 47 also increased CAR regulated genes possibly involved in the metabolism and transport of THs. For example, hepatic Ugt1a1 and Ugt1a7 mRNA expression increases correlated with serum T_4 decreases in mice treated with BDE 47. There is evidence that transporters regulated by nuclear receptors are responsible for transporting T_4 or conjugated compounds such as T_4G. Multidrug resistance proteins (MDRs) are important in TH efflux (Mitchell, *et al.*, 2005). Several studies report a correlation between the induction of hepatic UGT (Ugt1a1 and Ugt1a6), multidrug resistance protein-associated protein (Mrp2), and organic anion transporting proteins (Oatp1 and Oatp2) mRNA expression levels with decreases in serum TH concentrations in rodents following chemical exposure (Ribeiro, *et al.*, 1996; Mitchell, *et al.*, 2005; Wong, *et al.*, 2005; Miyawaki, *et al.*, 2012). Friesema *et al* (2005) used Xenopus laevis oocytes to identify Na(+) taurocholate cotransporting polypeptide (NTCP) and organic anion transporting polypeptide (OATP) as TH uptake transporters. In mice, BDE 47 increases Mdr1a and Mrp3 mRNA expression (Richardson, *et al.*, 2008). These results suggest that the upregulation of hepatic UGTs in concert with the upregulation in hepatic transporters may enhance the elimination of TH by BDE 47. Collectively, these studies show that hepatic T_4 metabolism, displaced T_4-TTR binding, and the active transport of THs may play a part in disrupting TH homeostasis.

The use of in vitro approaches to predict aspects of human drug metabolism has been of great interest. Hepatocytes are used in the *in vitro-in vivo* extrapolation of metabolic activity toward a number of drugs. As biologically relevant tools in the study of metabolism, hepatocytes are a dependable model for predicting the induction of xenobiotics metabolizing

enzymes (LeCluyse, 2001; Hewitt, *et al.*, 2007). Using primary rat and human sandwich-cultured hepatocytes this study makes comparisons of T_4 metabolism in response to nuclear receptor activation by BDE 47. The present study also compares the effects of BDE 47 on the genes involved in TH homeostasis in rat liver and hepatocytes.

B. MATERIALS AND METHODS

Chemicals

2, 2', 4, 4'-Tetrabromodiphenyl ether (BDE 47) was provided by Battelle Memorial Institute (Columbus, OH). Purity was >98% as determined by reverse-phase-performance liquid chromatography (HPLC). L-thyroxine (T_4) was purchased from Sigma-Aldrich Co. (St. Louis, MO). [^{125}I]-T_4, (116 Ci/mmol) was purchased from Perkin Elmer Life Sciences Inc. (Waltham, MA) and was purified to >98%) immediately before use with Sephadex LH-20 (Sigma-Aldrich) as described by Rutgers *et al.* (1989). All other chemicals used were of the highest grade commercially available.

Animals and Treatment

Adult (7 -9 week old) male Sprague-Dawley rats were purchased from Harlan Laboratories (Dublin, VA).Animals were maintained on a 12h light/dark cycle at ambient temperature (22ºC) and relative humidity (55 ± 5%), and were provided with NTP 2000 (Zeigler Bros. Inc., Gardner, PA) and tap water *ad libitum*.. All procedures comply with the Animal Welfare Act Regulations, 9 CFR 1-4 and animals will be handled and treated according to the *Guide for the Care and Use of Laboratory Animals* (ILAR, 1996). Animals

were treated with a single daily dose for 4 consecutive days. Rats were randomly selected for each treatment group (n=6/group). Rats were administered BDE 47 in corn oil (0, 3, 30, or 100 mg/kg/day for 4 days) by oral gavage (5 ml/kg). Dosing solutions were prepared by dissolving BDE 47 in acetone followed by the addition of corn oil. The acetone was removed from the dosing solutions by evaporation with a speed vacuum device

Serum and Tissue Collection

24 Hours after the final dose, mice were euthanized by CO_2 asphyxiation followed by exsanguinations via cardiac puncture; and blood and livers were collected. Blood was allowed to clot for 1 hour on ice in serum separator tubes (Becton Dickinson, Franklin Lakes, NJ). Blood was centrifuged at 1300 x g for 30 min to obtain serum. Serum was frozen and held at -80°C until analyzed. Livers were removed and weighed. Approximately 200 mg of liver was placed in a separate tube and frozen at -80°C for subsequent RNA purification. The remaining liver was frozen at -80°C for further enzyme activity analysis.

Serum T_4 concentration

Serum T_4, was measured in duplicate using Coat-a-Count radioimmunoassay kits (Diagnostic Products Corporation, Los Angeles, CA) according to the method of Craft *et al*.,(2002)

T_4-UGT Activity Assay

Liver microsomal fractions were prepared (Zhou, *et al.*, 2001) and protein concentrations were measured using a protein assay kit with bovine serum albumin as a

standard (Bio-Rad Laboratories, Hercules, CA). Hepatic T_4-UGT activity was determined by using a modified assay (Zhou, *et al.*, 2001; Zhou, *et al.*, 2002) based on a previously published method (Visser *et al.*, 1993a). All data are expressed as fold change compared to control, where controls for T_4-UGT are expressed as pMol T_4-UGT/min/mg protein.

T_4-SULT activity assay

Liver cytosol was collected at the same time the microsomes were collected. SULT activities were assayed by incubation of 4µM of T_4, and 100,000 cpm of ^{125}I-labeled T_4 (Perkin-Elmer) for 30 min at 37°C with 20 ug protein/ml of liver cytosol in the presence or absence (blank) of 50µM 3'-phosphoadenosine 5'-phosphosulfate (PAPS; Sigma) in 0.2 ml of 0.1M phosphate (pH 7.2) and 2mM EDTA (Sigma) (Kaptein, *et al.*, 1997). The mixtures were applied to 1 ml of lipophilic sephadex (Sigma) LH-20 minicolumns (Superlco), and equilibrated in 0.1M HCl. Iodine, and sulfated T_4 were successively eluted with 2 X 1 ml of 0.1M HCl, and 6 X 1 ml of ethanol/water (20/80, vol/vol), respectively. Fractions were collected and 1 ml of the T_4 sulfate (T_4S) fraction was quantitated for radioactivity.

Hepatocyte culture and BDE 47 treatment

Fresh primary male Sprague-Dawley sandwich-cultured rat hepatocytes (SCRH) and sandwich-cultured human hepatocytes (SCHH) in 24-well plates were received on culture day 2 from Life Technologies (Durham, NC). Hepatocytes were maintained for up to 6 days in culture with medium replaced daily. The maintenance medium consists of: Williams' E medium with 0.1% dimethyl sulfoxide (DMSO) including Hepatocyte Maintenance Supplement (Life Technologies) which contains 0.1µM dexamethasone and proprietary

concentrations of penicillin-streptomycin, ITS+ (insulin, transferrin, selenium complex, BSA and linoleic acid), GlutaMAX™ and HEPES].

Stock solutions of BDE 47 were diluted to 0.3, 3, and 30µM in DMSO and added to maintenance medium at a final DMSO concentration of 0.1%. On culture day 3, fresh SCRH and SCHH were exposed for 72 hours to the DMSO vehicle (0.1%) or BDE 47 (0.3, 3 30µM). After 72 hours, medium was removed and replaced with maintenance medium containing physiological concentrations [0.05µM (rat) or 0.1µM (human)] of [^{125}I]-T_4 (500,000 cpm/well) for 24 hours. Medium was collected, prepared and analyzed for T_4 metabolites by the method described below. Hepatocytes were collected and analyzed for protein content or frozen at -70°C for RNA isolation. Human donor demographics can be found in Table 4.1.

T_4 and T_4 metabolite separation and quantitation

All media samples were dried at 40°C under N_2 gas and then reconstituted with 40µl mobile phase consisting of 18% acetonitrile in 0.02M ammonium acetate (pH=4). After addition of mobile phase, samples were centrifuged at 10,000 x g for 5 minutes at room temperature and the supernatants were placed in vials for UPLC analysis. UPLC equipped with a C18 – 2.1 x 50mm x 1.7µm (Waters Corp.; Milford, MA) resolution column and fraction collector were used for identification of T_4 and T_4 metabolites. Gradient elution was performed using a method modified version of a previously established method by Rutgers *et al*. (1987) with a 16 minute gradient of 18-40% acetonitrile in 0.02M ammonium acetate (pH=4). Solvent flow rate was 0.4ml/min and 15 second fractions were collected in test tubes. Peaks were then identified by analyzing collected fractions for radioactivity by gamma

spectroscopy. Retention times for T_4, T_3, rT_3, T_4-glucuronide (T_4G), and T_4 sulfate (T_4S) were determined using synthetic and biosynthetic compounds as described by Richardson *et al.*(submitted). Peaks for T_3 and rT_3 were often inseparable; as a result, these peaks were added together and are presented as T_3+rT_3. Hepatocyte protein content was determined using a protein assay kit with bovine serum albumin as a standard (Bio-Rad Laboratories, Hercules, CA).

RNA Isolation and relative real-time RT-PCR

Total RNA was isolated using the RNeasy Mini Plus Kit with gDNA eliminator columns. (Qiagen, Hilden, Germany). To assess the integrity of the RNA samples, the 2100 Bioanalyzer was used (Agilent Technologies, Palo Alto, CA). RNA with RNA Integrity Numbers (RINs) greater than 8.1 were used for RT-PCR. Real-time RT-PCR was performed using the ABI Prism 7700 Sequence Detection System (ABI, Foster City, CA). 100 ng of total RNA was used for each reaction. cDNA was synthesized using the High Capacity RNA-to-cDNA Kit (ABI, Foster City, CA). PCR was then performed on all cDNAs using TaqMan Universal PCR Master Mix and Custom TaqMan Array Micro Fluidic Cards (ABI, Foster City, CA). TaqMan gene expression assays are listed in Tables 4.2 and 4.3.

Physiologically Based Pharmacokinetic Modeling

In order to compare media concentrations to peak blood concentrations, a physiologically based pharmacokinetic model was used to estimate peak blood concentrations in the *in vivo* studies. Using the PBPK model of Emond *et al*. (2010), peak blood and liver concentrations were estimated for each dose of BDE 47 administered to the

rats. The Emond *et al*. (2010) model was developed based on data from male and female Sprague-Dawley rats following single and multiple dose exposures.

Data Analysis

All data are presented as the mean ± standard deviation. The statistical intergroup comparisons were determined by using one-way analysis of variance (ANOVA) followed by Dunnet's Multiple Comparison post-hoc test (GraphPad Prism 5.0, GraphPad Software San Diego, CA). The levels of probability of statistical significance are $p < 0.05$. A one-phase decay exponential equation,

$$Y= (Y_0\text{-Plateau})*\exp(-K*X) +\text{Plateau},$$

was fit to the [^{125}I]-T_4 disappearance data where the depletion rate constant (K) was determined. The half-life ($t_{1/2}$) of all reactions was then determined as ln(2)/K. Using the rate of [^{125}I]-T_4 depletion, intrinsic clearance (CL_{int}) estimates were determined as described by Obach (1997) using the equation,

$$CL_{int}, \textit{ in vitro}=KV/N,$$

expressed as µl/min/10^6 cells, where K is ln(2)/ $t_{1/2}$, V is the incubation volume and N is the number of hepatocytes used. Human hepatocyte CL_{int} (µl/min/10^6 cells) was scaled to *in vivo* CL_{int} (ml/min/kg body weight) using the physiological parameters, human liver weight 22 g/kg body weight and hepatocellularity 120 X 10^6 cells/g of liver (Bayliss, *et al.*, 1999; Soars, *et al.*, 2002). Rat hepatocyte CL_{int} (µl/min/10^6 cells) was scaled to *in vivo* CL_{int} (ml/min/kg body weight) using the physiological parameters, rat liver weight 40 g/kg body weight and hepatocellularity 120 X 10^6 cells/g of liver (Bayliss, *et al.*, 1999)

C. RESULTS

Rat body weight, liver weight and serum total T_4

No obvious signs of toxicity and no treatment effect on body weight were noted between treatment groups of BDE 47. Liver weights increased 11%, 19%, and 21% at 10, 30, and 100 mg/kg/day BDE 47, respectively (Table 4.5). Similarly, liver-to-body weight ratios increased 9%, 17%, and 22% at 10, 30, and 100 mg/kg/day BDE 47, respectively. Decreases in serum total T_4 of 76% and 96% were observed at 30 and 100 mg/kg/day of BDE 47 (Figure 4.1). There were no significant effects on serum total T_4 at the lowest dose tested (10 mg/kg/day BDE 47)

Intrinsic clearance of T_4 from the media of SCRH and SCHH

The clearance of T_4 from the media of SCRH and SCHH treated with 30µM BDE 47 was evaluated between 4 and 24 hours (Table 4.4). A one-phase decay model was used to estimate intrinsic clearance (CL_{int}) in both SCRH and SCHH. BDE 47 did not significantly change Cl_{int} of T_4 in the media of SCRH and SCHH. CL_{int} in the untreated hepatocytes of rat1 and rat 2 was 1.08 and 0.75 µl/min/10^6 cells, respectively. In BDE 47 treated SCRH, CL_{int} was 0.93 and 0.86 µl/min/10^6 cells for rat 1 and rat 2, respectively. In untreated SCHH, CL_{int} for Hu1362 and 1364 was 0.56 and 0.62 µl/min/10^6 cells, respectively. Following BDE 47 treatment, CL_{int} was 0.56 and 0.52 µl/min/10^6 cells for Hu1362 and Hu1364, respectively.

CYPs: P450 mRNA expression

Rats treated with BDE 47 (100 mg/kg/day) resulted in increased hepatic Cyp1a1 mRNA expression by 44.9-fold (Table 4.6). Cyp1a1 did not change with 10 or 30 mg/kg/day BDE 47. At 30 and 100 mg/kg/day BDE 47, Cyp2b2 increased 16.4- and 21.0-fold and increased Cyp3a1 6.2-and 8.9-fold, respectively. In rat liver, Cyp2b2 and Cyp3a1 did not change at the lowest dose (10 mg/kg/day).

BDE 47 did not change Cyp1a1 mRNA expression in SCRH (Table 4.7). In SCRH, Cyp2b2 increased 45.1- and 40.6-fold following treatment with 3 and 30µM BDE 47. 0.3µM BDE 47 did not change Cyp2b2. Cyp3a1 mRNA expression increased in SCRH 7.2-fold only at 30 µM BDE 47 and not at the lower concentrations. BDE 47 did not change CYP1A1 in SCHH (Table 4.8). BDE 47 (30µM) increased CYP2B6 and CYP3A4 in SCHH by 15.7- and 10.9-fold, respectively. CYP2B6 and CYP3A4 mRNA expression did not change with 0.3 or 3µM BDE 47.

UGTs: T_4G levels, enzyme activity and mRNA expression

BDE 47 increased hepatic microsomal T_4-UGT activity by 60%, 50%, and 50% at 10, 30, and 100 mg/kg/day (Table 4.9). In rat liver, Ugt1a1 mRNA expression increased approximately 1.7- and 1.7-fold at 30 and 100 mg/kg/day, respectively. Hepatic Ugt1a5and Ugt1a6 mRNA expression increased 1.5- and 2.0-fold, respectively in rats treated with 100 mg/kg/day BDE 47.

In the medium of SCRH, T_4G levels were unchanged (Figure 4.2A) and UGT mRNA expression in rat hepatocytes was unchanged following BDE 47 treatment (Table 4.10). In the medium of SCHH, T_4G increased 2.0-fold at 30µM BDE 47 (Figure 4.3A). UGT1A1

increased 3.6-fold in human hepatocytes exposed to 30µM BDE 47 (Table 4.11). UGT1A4, UGT1A6, and UGT1A9 mRNA expression did not change with BDE 47 treatment.

SULTs: T_4S levels, enzyme activity and mRNA expression

BDE 47 did not change T_4-SULT activity or Sult mRNA expression in rat liver (Table 4.12). T_4S levels in the media of SCRH and SCHH did not change following treatment with BDE 47 treatment (Figure 4.2B and Figure 4.3B). SULT mRNA expression also did not change in SCRH and SCHH with BDE 47 (Table 4.13 and 4.14).

Transporters: mRNA expression

In rat liver, Mdr1a and Mdr1b mRNA expression increased following treatment with 100 mg/kg/day BDE 47 1.6- and 2.5-fold, respectively (Table 4.15). BDE 47 increased Mrp3 mRNA expression 10.4- and 15.0-fold at 30 and 100mg/kg/day BDE 47 in rat liver. BDE 47 did not change Mrp2, Ntcp1, and organic anion transporter (Oat2) in rat liver.Mrp2 increased 1.6-fold mRNA expression in SCRH treated with 30µM BDE 47 (Table 4.16). BDE 47 did not change mRNA expression for Mdr1a, Mdr1b, Mrp3, Ntcp1, and Oat2 in SCRH. In SCHH, MRP2 mRNA expression increased 1.6-fold with 30µM BDE 47 (Table 4.17). MRP3 mRNA expression in human hepatocytes decreased 31% with 3 and 30µM BDE 47. OAT2 mRNA expression in human hepatocytes decreased 54% and 63% with 3 and 30µM BDE 47, respectively. MDR1 and NTCP1 mRNA expression in SCHH did not increase with BDE 47.

Thyroid responsive genes.

In rat liver and SCRH, Dio1 and Ttr mRNA expression did not change with BDE 47 treatment (Table 4.18 and Table 4.19). In SCHH DIO1 did not change with BDE 47 treatment, but decreased 56% with 30uM BDE 47 (Table 4.20).

Physiologically Based Pharmacokinetic Modeling.

A physiologically based pharmacokinetic model was used to estimate peak blood concentrations in the *in vivo* studies. Using the PBPK model of Emond *et al.* (2010), peak blood and liver concentrations were estimated for each dose of BDE 47 administered to the rats. The model predicts that the range of peak blood concentrations of BDE 47 in rats treated with 10-100 mg/kg of BDE 47 is similar to the range of media concentrations used in the in vitro studies (Figure 4.4).

D. DISCUSSION

It has been hypothesized that in rodents, PBDEs disrupt TH homeostasis by the induction of hepatic T_4-glucuronidation and the competition between PBDE metabolites and T_4 for binding to the serum transport protein, TTR. The effects of PBDEs on TH homeostasis in humans are limited and it is unclear if the mechanisms underlying the decrease in serum T_4 in rodents is the same for humans. This study further investigates the alteration of thyroid hormone homeostasis of BDE 47. In particular, this study aims to identify nuclear receptor regulated genes altered by BDE 47, which may help understand the mechanisms by which BDE 47 decreases serum T_4 in rats. Using rat and human hepatocytes, we also make species

comparisons on the effects of BDE 47 on hepatic T_4 metabolism and the genes involved. In this study, BDE 47 decreased serum T_4 in rats and increased T_4G in the medium of SCHH. In addition, BDE 47 altered the mRNA expression of recognized hepatic UGTs and transporters of TH or glucuronides.

After a 4-day treatment, BDE 47 decreased circulating total serum T_4 concentration in rats by as much as 96% at the highest dose of 100 mg/kg/day. This observation supports our recent findings in which mice treated with 100mg/kg/day BDE 47 for 4 days decreased serum T_4, although to a smaller degree in mice with a 43% decrease (Richardson, *et al.*, 2008). Decreases in serum T_4 in rats treated with BDE 47 have previously been associated with moderate increases in hepatic T_4-UGT activity (Hallgren *et al.*, 2001; Hallgren and Darnerud, 2002). In the present study, BDE 47 increased T_4-UGT activity by approximately 50% from controls. It seems unlikely that a small increase in T_4-UGT activity could, by itself, result in over a 90% decrease in hormone concentrations. In SCRH, BDE 47 did not increase T_4G in the medium nor Ugt mRNA. In contrast, T_4G levels increased in the medium of SCHH as was UGT1A1 mRNA expression.

The present *in vivo* and *in vitro* data in rats is inconsistent with the hypothesis that the large decrease in serum T_4 in rats are due solely to increases in Ugt mRNA expression and T_4-UGT activity in liver. The lack of change in T_4G levels in the medium or Ugt mRNA expression in SCRH following BDE 47 treatment supports the findings in the *in vivo* study and suggests that T_4 glucuronidation has only a small effect, if any on the observed decreases in serum T_4 in rats. In SCHH, the increase in T_4G levels in the medium may be due to the nearly 4-fold increase in UGT1A1. This also suggests that BDE 47 may have a greater effect on human UGTs compared to rat UGTs.

It is hypothesized that SULTs may be more important in the metabolism of THs in humans than rats, because SULT1E1 conjugates THs in humans, but not in rats (Kester, *et al.*, 1999; Kester, *et al.*, 2003). Rutgers *et al.* (1989) reports that in untreated rats, 7 times more T_4G was excreted into the bile than T_4S. In this study, T_4G levels were about 25 times greater in the medium of SCHH compared to T_4S. In contrast, T_4G and T_4S levels in the medium of untreated SCHH were similar. BDE 47 had no effect on T_4-SULT activity and SULT mRNA expression in rat liver. There also was no effect on T_4S medium levels and SULT mRNA expression in SCRH and SCHH. SULT mRNA expression agreed with the T_4-SULT activity and T_4S levels and confirms that BDE 47 has no effect on T_4 sulfation.

DI is responsible for most T_3 production peripherally. However, when serum T_4 decreases, DI also decreases in an attempt to preserve serum T_3 concentrations suggesting that the decrease in DI is mediated by a feedback mechanism through the thyroid axis (Zavacki *et al.*, 2005). Hypothyroidism in rats treated with dioxin or dioxin-like chemicals is often associated with inhibited hepatic DI activity (Hood and Klaassen, 2000b; Viluksela, *et al.*, 2004; Szabo, *et al.*, 2009). In this study, DI mRNA expression and T_3+rT_3 levels in media did not change in SCRH and SCHH treated with BDE 47. This agrees with the idea that decreases in DI are a physiological response to decreases in serum T_4 and therefore may not be detected in an *in vitro* system. DI mRNA expression also did not change in rat liver which did not agree with the hypothesized association between decreases in DI and serum T_4.

CYP1A, CYP2B and CYP3A are classic targets for AhR, CAR, and PXR activation. In rats and mice, BDE 47 does not increase CYP1A mRNA, suggesting it is not an AhR agonist in rodents (Pacyniak *et al.*, 2007; Richardson, *et al.*, 2008). HepG2 cells treated with BDE 47 also showed no AhR activation (Peters *et al.*, 2006). Liver from F344 rats BDE 47

had a significant effect on the level of Cyp1a1 mRNA expression (2.4-fold) only at the highest dose, (100 μmol/kg-day for 3 days) indicating an activation of the Ah receptor (Sanders *et al.*, 2005). In the present study, BDE 47 induced Cyp1a1 mRNA expression in rat liver, but not in SCRH and SCHH. The effect of BDE 47 on CYP1A1 in SCRH and SCHH agrees with previous studies showing BDE 47 is not an AhR agonist. In contrast, increases in Cyp1a1 in rat liver suggest that the AhR is activated. Ugt1a6 expression is regulated, in part by the AhR (Auyeung, *et al.*, 2003; Nishimura, *et al.*, 2005). In this study, Cyp1a1 mRNA expression increase 44.8-fold while Ugt1a6 mRNA expression increased only 2.0-fold in the liver of rats treated with 100 mg/kg/day. The increase in Cyp1a1 and Ugt1A6 mRNA indicates the activation of AhR may come from an unidentified contaminant in the BDE 47 (Wahl *et al.*, 2008).

BDE 47 increased CYP2B2 and CYP3A1 in rat liver and SCRH and SCHH which agrees with previous studies in mice and rats (Sanders, *et al.*, 2005; Pacyniak, *et al.*, 2007; Richardson, *et al.*, 2008). As common targets of CAR and PXR, the increases in CYP2B2 and CYP3A1 indicate BDE 47 is a mixed inducer in rats, SCRH and SCHH. In particular, the increases in P450 mRNA expression support the idea that the hepatocytes used in this study are functional and viable.

In general, it is hypothesized that hydroxylated PBDEs bind to TTR may decrease serum T_4 concentrations by increase the hepatic uptake and biliary elimination of T_4 in rodents (Hallgren and Darnerud, 2002). The present study showed no change in TTR mRNA expression in rat liver and SCHR. This does not agree with previous studies in which Ttr mRNA expression in mouse liver decreases with BDE 47 (Richardson, *et al.*, 2008). TTR mRNA expression decreased in SCHH treated with BDE 47 (30μM), which agrees with

previous studies in mice (Richardson, *et al.*, 2008). Thyroxine-binding globulin (TBG) is the major T_4 binding protein in humans; therefore it is unclear how significant a decrease in hepatic TTR mRNA would be to TH homeostasis in humans.

Like other xenobiotic metabolizing enzymes, transporters are also regulated by nuclear receptor activation. In this study, we examined the effects of BDE 47 on hepatic uptake and efflux transporters and the role they may play in TH disruption. MDR1, MRP2, and MRP3 are efflux transporters and are regulated by AhR, CAR, and PXR (Cherrington *et al.*, 2002; Maglich, *et al.*, 2002; Kohle and Bock, 2009). MDR1 encodes for the efflux transporter P-glycoprotein (P-gp) involved in the transport of hormones, steroids and a wide range of xenobiotics. Cells transfected with Mdr1 cDNA increased the efflux of T_3 compared to cells without Mdr1 (Mitchell, *et al.*, 2005). In mice, glucuronidated compounds are substrates for Mdr1. Like MDR1, MRP2, is a canalicular efflux transporter and is responsible for the transport of conjugated organic anions into bile (Leslie *et al.*, 2005; Nies and Keppler, 2007). Increases in MRP2 in rodents have been linked to UGT induction andT_4 elimination following treatment with PB or PCN (Johnson *et al.*, 2002a; Miyawaki, *et al.*, 2012). Szabo *et al.* (2009) demonstrated that rat pups exposed to DE-71 decreased serum T_4 and increased hepatic Mrp2 and UGTs. MRP3, a sinusoidal efflux transporter, exports glucuronides from the liver to the blood. NTCP, a sinusoidal uptake transporter, is known as a transporter of T_4 and T_4S (Friesema, *et al.*, 1999; Visser, *et al.*, 2011). OAT2 is a basolateral uptake transporter and mediates the transport of exogenous and endogenous compounds from blood to liver.

Mdr1a and Mdr1b encode P-gp in rodents. In mice treated with BDE 47, hepatic Mdr1a decreased and Mdr1b mRNA expression did not change (Richardson, *et al.*, 2008). In

the present study, hepatic Mdr1a and Mdr1b increased in rats treated with 100mg/kg/day BDE 47. In SCRH, Mdr1a and Mdr1b mRNA expression did not change. MDR1 encodes P-gp in humans and was unchanged in SCHH treated with BDE 47. This suggests MDR1 in rats may mediate the biliary elimination of T_4 or T_4G.

BDE 47 did not change Mrp2 mRNA expression in rat liver. Mrp2 increased in SCRH. Although linked with T_4 biliary elimination; our results suggest that Mrp2 mRNA expression may not be responsible for the observed decrease in serum T_4 in rats. In contrast, MRP2 increased in hepatocytes from rats and humans following BDE 47 treatment. In response to BDE 47 treatment, MRP2 in rat liver may play a different role in the transport of T_4, T_4G and xenobiotics when compared to MRP2 in the hepatocytes.

NTCP1 did not change with BDE 47 treatment of rats, SCRH and SCHH. OAT2 did not change in rat liver and SCRH. OAT2 mRNA expression in SCHH decreased at 3 and 30µM BDE 47. These results suggest BDE 47 did not induce a NTCP1- or OAT2-mediated increase in hepatic T_4 uptake. In general, the effects of BDE 47 on transporter mRNA expression are inconsistent between rat and human hepatocytes and between rat liver and rat hepatocytes. There are inconsistencies between transporter mRNA expression and T_4G levels in media. Tchaparian *et al*.(2011) found a lack of concordance in the expression profiles for uptake and efflux transporters in SCRH over days in culture, indicating there may be different culture conditions for uptake transporters and efflux transporters .The aim of the present study was to examine increases in UGTs and T_4 metabolism in response to BDE 47; as a result, our goal was to optimize our system for nuclear receptor activation by BDE 47 and T_4 metabolism. To optimize our system we determined that it was best to use an induction phase (BDE 47 incubation) of 72 hours followed by a metabolism phase (T_4

incubation) of 24 hours. Transporter activity or expression was not optimized in this study and may explain the contrasting results observed in this study. Overall, transporter function were not confirmed; therefore, conclusions about the effect of BDE 47 on T_4 uptake or biliary excretion.

A PBPK model was used to estimate the peak blood concentrations of BDE 47 from animals treated with 10, 30, or 100 mg/kg of BDE 47 for 4 consecutive days. Model predictions of blood concentrations were within the range of the *in vitro* medium concentrations evaluated in the *in vitro* component of this study. The modest effects on the induction of T_4 glucuronidation occurred both *in vitro* and *in vivo* and at similar exposure concentrations. In contrast, *in vivo* BDE 47 induced over a 90% decrease in serum T_4 concentrations. These data suggest that the *in vitro* model recapitulates the *in vivo* response at similar concentrations and that it is unlikely that T_4 glucuronidation plays a large part of this effect. In the latest NHANES survey, the mean human serum concentrations of BDE 47 are 2.1 nM with the 95^{th} percentile at 17.8 nM. These concentrations are approximately 1000 fold lower than the exposures examined in these studies and suggest that effects on hepatic enzyme induction or changes in serum TH concentrations are unlikely to occur at background human exposure.

The present study investigated the effects of BDE 47 on T_4 serum concentrations and hepatic genes involved in T_4 metabolism and transport in rats. Species differences in T_4 metabolism also were examined using SCRH and SCHH treated with BDE 47. The TH disrupting effects of BDE 47 in rats is likely due to multiple mechanisms in the liver; including T_4 glucuronidation and/or biliary efflux of T_4 and is mediated through CAR/PXR pathways. In SCRH, BDE 47 appears to activate the CAR/PXR pathway and the mechanism

may be different than that of rats, because T_4 glucuronidation appears not to be involved. In SCHH, T_4 glucuronidation, biliary efflux and decreases in T_4-TTR binding may be involved and is mediated through a CAR/PXR pathway. The effects observed in rats may be a result of a furan contaminant, which makes it difficult to attribute all of the observed effects to BDE 47 alone. There were some inconsistencies in some of the transporter mRNA expression levels in the hepatocytes. This may be due to the culture days chosen for sample collection which may affect transporter mRNA expression or activity; therefore, transport efflux and uptake should not be ruled out as a mechanism of action. Overall, this study shows the utility of SCH in the continuing investigation of TH disruption and human relevance.

Table 4.1

Donor information for human hepatocytes

Donor	Gender	Age	Smoke/Alcohol	Medications	Experimental Use
Hu1236	Female	68	No/No	Multivitamin Vitamin D	BDE 47 Treatment
Hu1248	Male	63	Yes/Yes	Sertraline Vicodin Docusate	BDE 47 Treatment
Hu1362	Female	57	No/No	Atenolol and Chlorthalidone Lorazepam Oxycodone	BDE 47 Treatment/ T_4 Clearance
Hu1364	Male	51	No/No	Cyanocobalamin Diltiazem Ferrous Sulfate Folic Acid Metoprolol Omeprazole Potassium Chloride Prednisone	BDE 47 Treatment/ T_4 Clearance

Table 4.2

Taqman rat gene expression assays

Group	Gene	Taqman Gene Expression Assay ID
Endogenous control	Gapdh	Rn99999916_s1
Nuclear receptor activation	Cyp1a1	Rn00487218_m1
	Cyp2b2	Rn02786833_m1
	Cyp3a1	Rn01640761_gH
Phase II metabolism	Ugt1a1	Rn00754947_m1
	Ugt1a5	Rn01427785_m1
	Ugt1a6	Rn00756113_mH
	Ugt2b	Rn02349650_m1
	Sult1a1	Rn00582915_m1
	Sult1b1	Rn00673872_m1
	Sult1c1	Rn00581955_m1
	Sult1e1	Rn00820646_g1
Transporter	Mdr1a	Rn00591394_m1
	Mdr1b	Rn00561753_m1
	Mrp2	Rn00563231_m1
	Mrp3	Rn00589786_m1
	Ntcp1	Rn00566894_m1
	Oat2	Rn00585513_m1
Thyroid hormone responsive	Dio1	Rn00572183_m1
	Ttr	Rn00562124_m1

Table 4.3

Taqman human gene expression assays

Group	Gene	Taqman Gene Expression Assay ID
Endogenous control	GAPDH	Hs99999905_m1
Nuclear receptor activation	CYP1A1	Hs00153120_m1
	CYP2B6	Hs03044634_m1
	CYP3A4	Hs00430021_m1
Phase II metabolism	UGT1A1	Hs02511055_s1
	UGT1A4	Hs01592480_m1
	UGT1A6	Hs01592477_m1
	UGT1A7	Hs02517015_s1
	UGT1A9	Hs02516855_sH
	UGT1A10	Hs02516990_s1
		Hs00413970_m1
	SULT1A3	Hs00193690_m1
	SULT1E1	Hs00234219_m1
	SULT2A1	
Transporter	MDR1B	Hs00184500_m1
	MRP2	Hs00166123_m1
	MRP3	Hs00358656_m1
	NTCP1	Hs00161820_m1
	OAT2	Hs00185140_m1
Thyroid hormone responsive	D1	Hs01554724_m1
	TTR	Hs00174914_m1

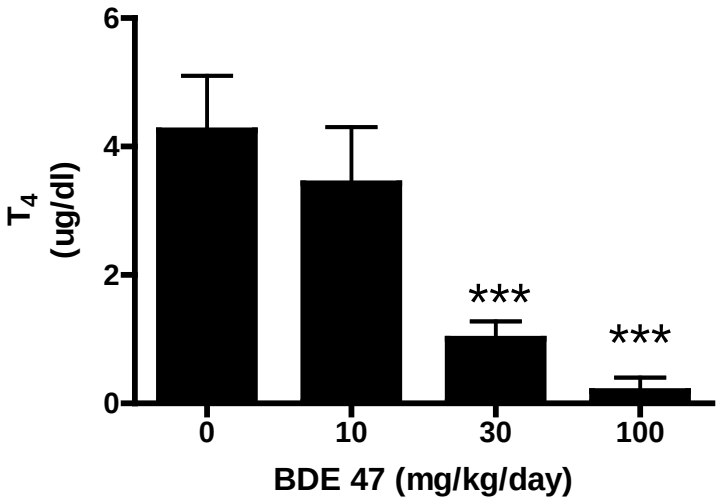
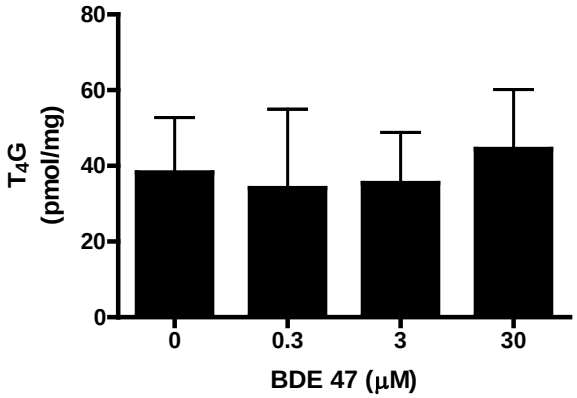
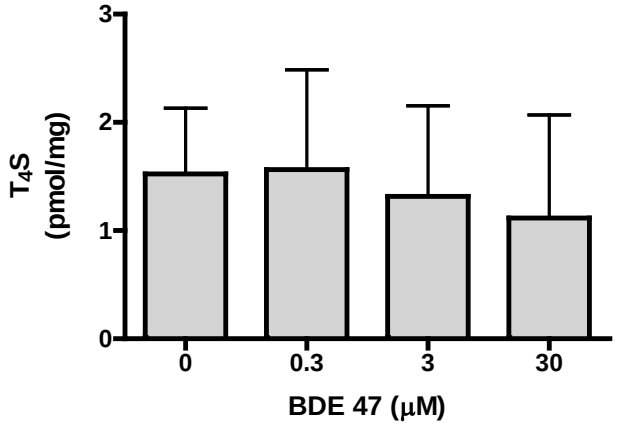


Figure 4.1. Total serum T_4 concentrations in rats treated with BDE 47.
Data are expressed as mean ± standard deviation (n=6/group).
***Significantly different from control group ($p < 0.001$).

A.


80
60
40
20
0
T4G
(pmol/mg)
0
0.3
3
30
BDE 47 (μM)

B.


3
2
1
0
T4S
(pmol/mg)
0
0.3
3
30
BDE 47 (μM)

C.

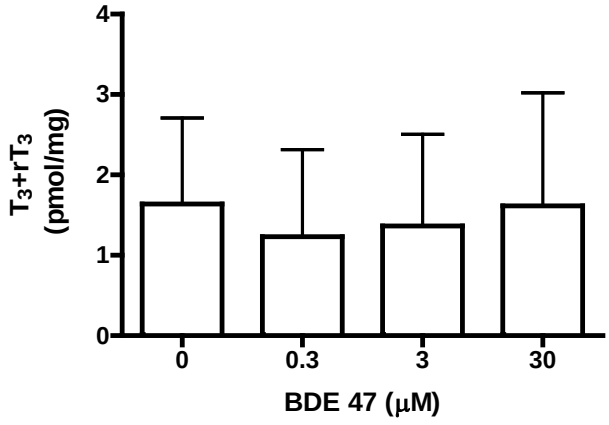
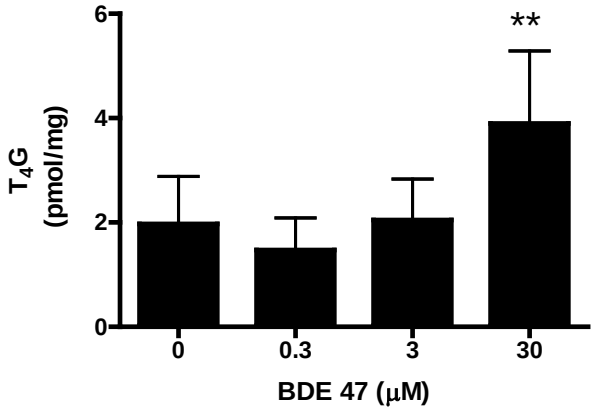
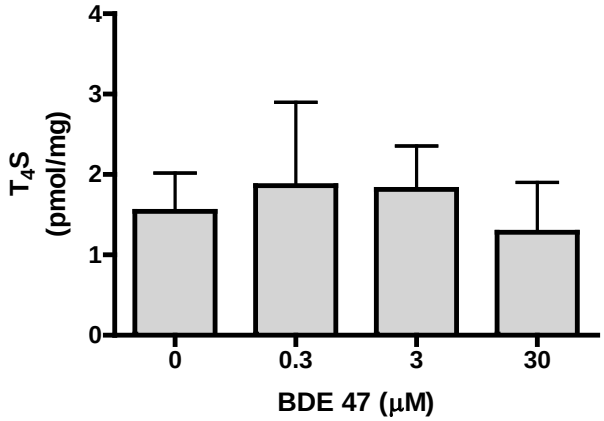

4
3
2
1
0
T3+rT3
(pmol/mg)
0
0.3
3
30
BDE 47 (μM)

Figure 4.2. Comparison of metabolites in the medium of SCRH treated with BDE 47. Hepatocytes were incubated with 0.1% DMSO (control), 0.3, 3, or 30µM BDE 47 for 72 hours starting on culture day 3.Hepatocytes are then incubated for 24 hours on culture day 6 with 0.05µM [^{125}I]-T_4. Metabolites were separated using the established UPLC method. Metabolites analyzed are (A) T_4G, (B) T_4S, and (C) T_3+rT_3. Data represent the average of duplicate experiments. Data are expressed as pmol/mg cellular protein (mean±SD). Limits of detection= 0.5 pmol/mg cellular protein. n=4 for rat hepatocytes.

A.


6
4
2
0
**
T4G
(pmol/mg)
0
0.3
3
30
BDE 47 (μM)

B.


4
3
2
1
0
T4S
(pmol/mg)
0
0.3
3
30
BDE 47 (μM)

C.

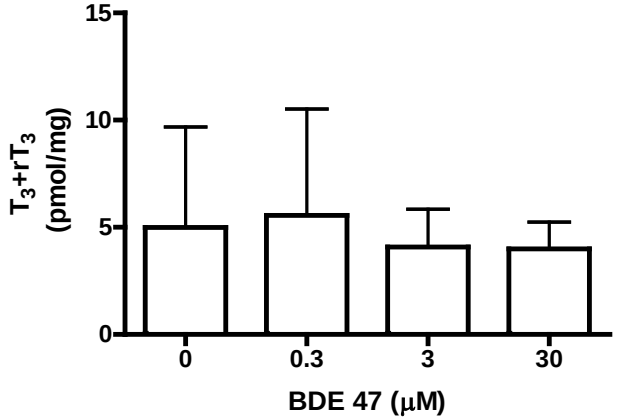

15
10
5
0
T3+rT3
(pmol/mg)
0
0.3
3
30
BDE 47 (μM)

Figure 4.3. Comparison of metabolites in the medium of SCHH treated with BDE 47. Hepatocytes were incubated with 0.1% DMSO (control), 0.3, 3, or 30µM BDE 47 for 72 hours starting on culture day 3.Hepatocytes are then incubated for 24 hours on culture day 6 with 0.1µM [^{125}I]-T_4. Metabolites were separated using the established UPLC method. Metabolites analyzed are (A) T_4G, (B) T_4S, and (C) T_3+rT_3. Data represent the average of duplicate experiments. Data are expressed as pmol/mg cellular protein (mean±SD) Limits of detection= 0.5 pmol/mg cellular protein. n=4 human donors. Human hepatocytes are from 4 donors (Hu1236, Hu1248, Hu1362, and Hu1364). **Significantly different than control; $p<0.01$.

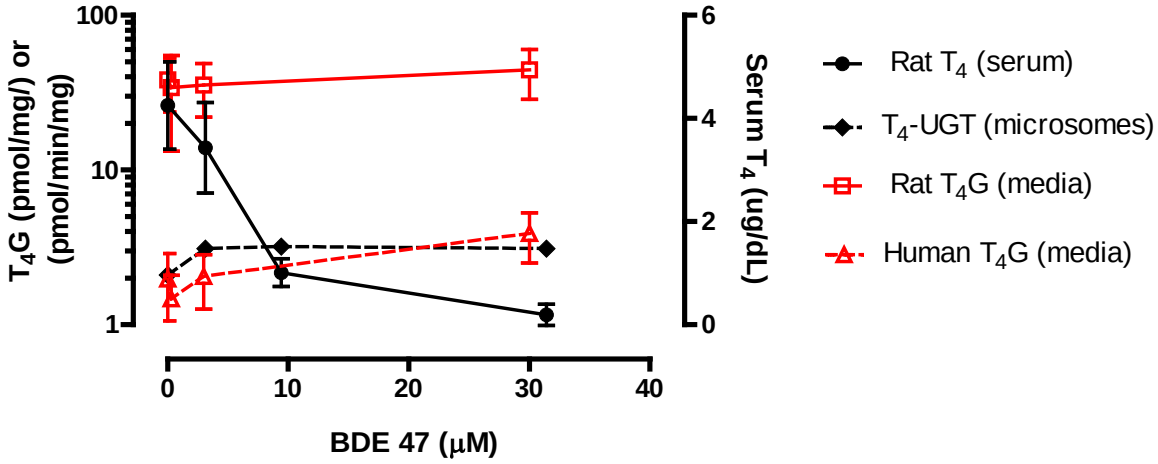


Figure 4.4. Comparison of *in vivo* and *in vitro* responses based on either medium concentrations (*in vitro*) or estimated blood concentrations (*in vivo*). T_4G activity is presented as either T_4G accumulation in medium (T_4G pmol/mg protein) or T_4G activity in hepatic microsomal fractions (T_4G pmol/min/mg protein)

Table 4.4

Intrinsic clearance of [^{125}I]-T_4 following treatment with BDE 47

		Control	BDE 47	Fold Change
Rat 1	Rate constant K (1/min)	0.00075	0.00065	0.86
	Cl_{int} (µl/min/10^6 cells)	1.08	0.93	0.86
	Scaled Cl_{int} (ml/min/kg body weight)	5.16	4.47	0.87
Rat 2	Rate constant K (1/min)	0.00052	0.00060	1.15
	Cl_{int} (µl/min/10^6 cells)	0.75	0.86	1.15
	Scaled Cl_{int} (ml/min/kg body weight)	3.58	4.11	1.15
Hu 1362	Rate constant K (1/min)	0.00039	0.00039	1.00
	Cl_{int} (µl/min/10^6 cells)	0.56	0.56	1.00
	Scaled Cl_{int} (ml/min/kg body weight)	1.60	1.61	1.00
Hu 1364	Rate constant K (1/min)	0.00043	0.00037	0.86
	Cl_{int} (µl/min/10^6 cells)	0.62	0.52	0.86
	Scaled Cl_{int} (ml/min/kg body weight)	1.78	1.50	0.84

Table 4.5

Effects of BDE 47 on rat body weight, organ weights, and liver-to-body weight ratio

Treatment (mg/kg/day)	Body (g) [a]	Liver (g) [a]	Liver-to-body wt. ratio [a]
0	234.7±8.0	10.0±0.5	0.04±0.0
10	238.5±9.5	11.2±0.8[b]	0.05±0.0[c]
30	237.8±7.3	12.3±0.6[d]	0.05±0.0[d]
100	232.1±10.7	12.7±0.9[d]	0.06±0.0[d]

[a]Data are expressed as mean ± standard deviation (n=6/group)
[b]Significantly different from control group ($p< 0.05$)
[c]Significantly different from control group ($p< 0.01$)
[d]Significantly different from control group ($p< 0.001$)

Table 4.6

Effects of BDE 47 on P450s in rat liver

Treatment (mg/kg/day)	Cyp1a1[a]	Cyp2b2[a]	Cyp3a1[a]
0	1.1±0.6	1.2±0.8	1.0±0.3
10	13.5±12.5	4.7±1.5	2.2±0.7
30	24.1±27.0	16.4±6.7[d]	6.2±2.8[c]
100	44.8±45.4[b]	21.0±7.1[d]	8.9±3.1[d]

[a]Data are expressed as mean ± standard deviation (n=6/group)
[b]Significantly different from control group ($p< 0.05$)
[c]Significantly different from control group ($p< 0.01$)
[d]Significantly different from control group ($p< 0.01$)

Table 4.7

Effects of BDE 47 on P450s in rat hepatocytes

Treatment (μM)	Cyp1a1[a]	Cyp2b2[a]	Cyp3a1[a]
0	1.0±0.0	1.0±0.0	1.0±0.0
0.3	1.6±1.7	7.3±4.7	1.7±1.5
3	1.2±1.4	45.1±36.5[b]	1.3±0.1
30	0.6±0.4	40.6±25.7[b]	7.2±4.7[c]

[a]Data are expressed as mean ± standard deviation (n=5/group)
[b]Significantly different from control group ($p< 0.05$)
[c]Significantly different from control group ($p< 0.01$)

Table 4.8

Effects of BDE 47 on P450s in human hepatocytes

Treatment (μM)	CYP1A1[a]	CYP2B6[a]	CYP3A4[a]
0	1.0±0.0	1.0±0.0	1.0±0.0
0.3	1.0±0.6	4.1±4.7	1.4±1.1
3	1.0±1.2	5.0±3.3	2.1±1.2
30	2.4±1.7	15.7±12.1[b]	11.0±8.4[b]

[a]Data are expressed as mean ± standard deviation (n=4/group)
[b]Significantly different from control group ($p< 0.05$)

Table 4.9

Effects of BDE 47 on T_4-UGT activity and Ugt mRNA expression in rat liver

Treatment (mg/kg/day)	T_4-UGT[a]	Ugt1a1[a]	Ugt1a5[a]	Ugt1a6[a]
0	1.0±0.1	1.1±0.4	1.0±0.3	1.0±0.3
10	1.6±0.1[c]	1.3±0.2	1.0±0.3	1.3±0.2
30	1.5±0.2[c]	1.7±0.4[b]	1.2±0.0	1.6±0.3
100	1.5±0.1[c]	1.6±0.4[b]	1.5±0.3[b]	2.0±0.6[c]

[a]Data are expressed as fold change mean ± standard deviation (n=5-6/group)
[b]Significantly different from control group ($p< 0.05$)
[c]Significantly different from control group ($p< 0.001$)

Table 4.10

Effects of BDE 47 on Ugt mRNA expression in rat hepatocytes

Treatment (μM)	Ugt1a1[a]	Ugt1a5[a]	Ugt1a6[a]
0	1.0±0.0	1.0±0.0	1.0±0.0
0.3	1.3±0.5	1.3±0.5	1.2±0.5
3	0.9±0.1	1.3±0.5	1.1±0.1
30	1.1±0.4	1.8±0.8	1.7±0.8

[a]Data are expressed as fold change mean ± standard deviation (n=5/group)

Table 4.11

Effects of BDE 47 on UGT mRNA expression in human hepatocytes

Treatment (μM)	UGT1A1[a]	UGT1A4[a]	UGT1A6[a]	UGT1A9[a]
0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.00
0.3	1.2±0.4	1.4±0.3	1.1±0.3	1.0±0.2
3	1.7±0.7	1.6±1.3	1.2±0.5	1.0±0.2
30	3.6±1.6[b]	2.3±0.6	1.5±0.6	1.0±0.2

[a]Data are expressed as fold change mean ± standard deviation (n=4/group)
[b]Significantly different from control group ($p< 0.01$)

Table 4.12

Effects of BDE 47 on T_4-SULT activity and SULT mRNA expression in rat liver

Treatment (mg/kg/day)	T_4-SULT[a]	Sult1a1[a]	Sult1b1[a]	Sult1c1[a]	Sult1e1[a]
0	1.0±0.3	1.0±0.2	1.0±0.1	1.0±0.2	1.0±0.2
10	1.0±0.3	0.9±0.1	1.1±0.3	1.0±0.2	1.0±0.2
30	1.0±0.5	0.8±0.1	1.0±0.2	1.0±0.2	0.9±0.1
100	0.8±0.3	0.8±0.3	1.0±0.2	1.0±0.2	0.9±0.1

[a]Data are expressed as fold change mean ± standard deviation (n=5-6/group)
[a]Significantly different from control group ($p< 0.05$)
[b]Significantly different from control group ($p< 0.01$)

Table 4.13

Effects of BDE 47 on and SULT mRNA expression in rat hepatocytes

Treatment (μM)	Sult1a1[a]	Sult1b1[a]	Sult1c1[a]	Sult1e1[a]
0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
0.3	1.2±0.5	1.2±0.2	1.0±0.5	1.4±1.0
3	0.9±0.3	1.0±0.2	0.9±0.2	1.1±0.5
30	0.7±0.3	1.0±0.3	1.0±1.0	1.4±0.5

[a]Data are expressed as fold change mean ± standard deviation (n=5/group)

Table 4.14

Effects of BDE 47 on SULT mRNA expression in human hepatocytes

Treatment (μM)	SULT1A3[a]	SULT1E1[a]	SULT 2A1[a]
0	1.0±0.0	1.0±0.0	1.0±0.0
0.3	1.6±1.0	2.0±1.7	1.0±0.4
3	1.1±0.6	0.9±0.6	1.1±0.8
30	0.6±0.2	0.4±0.5	1.1±0.6

[a]Data are expressed as fold change mean ± standard deviation (n=4/group)

Table 4.15

Effects of BDE 47 on efflux and uptake transporter mRNA expression in rat liver

Treatment (mg/kg/day)	Mdr1a[a]	Mdr1b[a]	Mrp2[a]	Mrp3[a]	Ntcp1[a]	Oat2[a]
0	1.0±0.1	1.1±0.5	1.0±0.2	1.0±0.4	1.0±0.3	1.0±0.2
10	1.3±0.3	1.2±0.4	1.1±0.3	4.2±2.5	1.2±0.4	0.9±0.2
30	1.3±0.3	1.8±0.6	1.2±0.2	10.4±3.2[d]	1.1±0.3	0.9±0.1
100	1.6±0.4[b]	2.5±0.9[c]	1.4±0.5	15.0±2.8[d]	1.1±0.4	0.9±0.2

[a]Data are expressed as fold change mean ± standard deviation (n=6/group)
[b]Significantly different from control group ($p< 0.05$)
[c]Significantly different from control group ($p< 0.01$)
[d]Significantly different from control group ($p< 0.001$)

Table 4.16

Effects of BDE 47 on efflux and uptake transporter mRNA expression in rat hepatocytes

Treatment (μM)	Mdr1a[a]	Mdr1b[a]	Mrp2[a]	Mrp3[a]	Ntcp1[a]	Oat2[a]
0	1.0±0.00	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
0.3	1.4±0.6	1.4±0.5	1.3±0.5	1.3±0.4	1.2±0.5	1.3±0.5
3	1.0±0.3	0.9±0.3	1.1±0.3	0.9±0.1	0.7±0.2	1.0±0.2
30	1.5±0.6	1.3±0.7	1.6±0.5[b]	1.3±0.5	0.7±0.4	1.1±0.3

[a]Data are expressed as fold change mean ± standard deviation (n=5/group)
[b]Significantly different from control group ($p< 0.05$)

Table 4.17

Effects of BDE 47 on efflux and uptake transporter mRNA expression in human hepatocytes

Treatment (uM)	MDR1[a]	MRP2[a]	MRP3[a]	NTCP1[a]	OAT2[a]
0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
0.3	1.1±0.3	0.9±0.6	0.9±0.3	1.3±0.6	0.9±0.4
3	1.1±0.0	1.0±0.2	0.7±0.1[b]	1.0±0.5	0.5±0.1[b]
30	1.5±0.6	1.6±0.3[c]	0.7±0.1[b]	0.4±0.2	0.4±0.2[b]

[a]Data are expressed as fold change mean ± standard deviation (n=4/group)
[b]Significantly different from control group ($p< 0.05$)
[c]Significantly different from control group ($p< 0.01$)

Table 4.18

Effects of BDE 47 on thyroid hormone responsive genes in rat liver

Treatment (mg/kg/day)	Dio1[a]	Ttr[a]
0	1.0±0.2	1.0±0.3
10	1.1±0.2	1.0±0.4
30	1.1±0.1	1.0±0.2
100	1.1±0.4	0.9±0.4

[a]Data are expressed as mean ± standard deviation (n=6/group)

Table 4.19

Effects of BDE 47 on thyroid hormone responsive genes in rat hepatocytes

Treatment (μM)	Dio1[a]	Ttr[a]
0	1.0±0.0	1.0±0.0
0.3	1.2±0.6	1.2±0.6
3	0.9±0.4	1.0±0.5
30	0.7±0.2	1.1±0.9

[a]Data are expressed as mean ± standard deviation (n=5/group)

Table 4.20

Effects of BDE 47 on thyroid hormone responsive genes in human hepatocytes

Treatment (μM)	DIO1[a]	TTR[a]
0	1.0±0.0	1.0±0.0
0.3	1.5±0.5	1.1±0.4
3	1.3±0.3	0.8±0.0
30	1.7±0.6	0.4±0.1[b]

[a]Data are expressed as mean ± standard deviation (n=4/group)
[b]Significantly different from control group ($p< 0.01$)

REFERENCES

Auyeung, D. J., Kessler, F. K. and Ritter, J. K. (2003). Mechanism of rat UDP-glucuronosyltransferase 1A6 induction by oltipraz: evidence for a contribution of the Aryl hydrocarbon receptor pathway. *Mol Pharmacol* **63**, 119-27.

Bayliss, M. K., Bell, J. A., Jenner, W. N., Park, G. R. and Wilson, K. (1999). Utility of hepatocytes to model species differences in the metabolism of loxtidine and to predict pharmacokinetic parameters in rat, dog and man. *Xenobiotica; the fate of foreign compounds in biological systems* **29**, 253-68.

Bock, K. W. and Kohle, C. (2004). Coordinate regulation of drug metabolism by xenobiotic nuclear receptors: UGTs acting together with CYPs and glucuronide transporters. *Drug Metab Rev* **36**, 595-615.

Cherrington, N. J., Hartley, D. P., Li, N., Johnson, D. R. and Klaassen, C. D. (2002). Organ distribution of multidrug resistance proteins 1, 2, and 3 (Mrp1, 2, and 3) mRNA and hepatic induction of Mrp3 by constitutive androstane receptor activators in rats. *J Pharmacol Exp Ther* **300**, 97-104.

Craft, E. S., DeVito, M. J. and Crofton, K. M. (2002). Comparative responsiveness of hypothyroxinemia and hepatic enzyme induction in Long-Evans rats versus C57BL/6J mice exposed to TCDD-like and phenobarbital-like polychlorinated biphenyl congeners. *Toxicol Sci* **68**, 372-80.

Emond, C., Raymer, J. H., Studabaker, W. B., Garner, C. E. and Birnbaum, L. S. (2010). A physiologically based pharmacokinetic model for developmental exposure to BDE-47 in rats. *Toxicol Appl Pharmacol* **242**, 290-8.

Focant, J. F., Pirard, C. and De Pauw, E. (2004). Automated sample preparation-fractionation for the measurement of dioxins and related compounds in biological matrices: a review. *Talanta* **63**, 1101-13.

Friesema, E. C., Docter, R., Moerings, E. P., Stieger, B., Hagenbuch, B., Meier, P. J., Krenning, E. P., Hennemann, G. and Visser, T. J. (1999). Identification of thyroid hormone transporters. *Biochem Biophys Res Commun* **254**, 497-501.

Friesema, E. C., Jansen, J., Milici, C. and Visser, T. J. (2005). Thyroid hormone transporters. *Vitam Horm* **70**, 137-67.

Hallgren, S. and Darnerud, P. O. (2002). Polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and chlorinated paraffins (CPs) in rats-testing interactions and mechanisms for thyroid hormone effects. *Toxicology* **177**, 227-43.

Hallgren, S., Sinjari, T., Hakansson, H. and Darnerud, P. O. (2001). Effects of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) on thyroid hormone and vitamin A levels in rats and mice. *Archives of toxicology* **75**, 200-8.

Hamers, T., Kamstra, J. H., Sonneveld, E., Murk, A. J., Visser, T. J., Van Velzen, M. J., Brouwer, A. and Bergman, A. (2008). Biotransformation of brominated flame retardants into potentially endocrine-disrupting metabolites, with special attention to 2,2',4,4'-tetrabromodiphenyl ether (BDE-47). *Mol Nutr Food Res* **52**, 284-98.

Hewitt, N. J., Lechon, M. J., Houston, J. B., Hallifax, D., Brown, H. S., Maurel, P., Kenna, J. G., Gustavsson, L., Lohmann, C., Skonberg, C., Guillouzo, A., Tuschl, G., Li, A. P., LeCluyse, E., Groothuis, G. M. and Hengstler, J. G. (2007). Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metab Rev* **39**, 159-234.

Hites, R. A. (2004). Polybrominated diphenyl ethers in the environment and in people: a meta-analysis of concentrations. *Environ Sci Technol* **38**, 945-56.

Hood, A., Allen, M. L., Liu, Y., Liu, J. and Klaassen, C. D. (2003). Induction of T(4) UDP-GT activity, serum thyroid stimulating hormone, and thyroid follicular cell proliferation in mice treated with microsomal enzyme inducers. *Toxicol Appl Pharmacol* **188**, 6-13.

Hood, A. and Klaassen, C. D. (2000). Effects of microsomal enzyme inducers on outer-ring deiodinase activity toward thyroid hormones in various rat tissues. *Toxicol Appl Pharmacol* **163**, 240-8.

Johnson, D. R., Guo, G. L. and Klaassen, C. D. (2002). Expression of rat Multidrug Resistance Protein 2 (Mrp2) in male and female rats during normal and pregnenolone-16alpha-carbonitrile (PCN)-induced postnatal ontogeny. *Toxicology* **178**, 209-19.

Kaptein, E., van Haasteren, G. A., Linkels, E., de Greef, W. J. and Visser, T. J. (1997). Characterization of iodothyronine sulfotransferase activity in rat liver. *Endocrinology* **138**, 5136-43.

Kato, Y., Ikushiro, S., Takiguchi, R., Haraguchi, K., Koga, N., Uchida, S., Sakaki, T., Yamada, S., Kanno, J. and Degawa, M. (2007). A novel mechanism for polychlorinated biphenyl-induced decrease in serum thyroxine level in rats. *Drug Metab Dispos* **35**, 1949-55.

Kato, Y., Suzuki, H., Haraguchi, K., Ikushiro, S., Ito, Y., Uchida, S., Yamada, S. and Degawa, M. (2010). A possible mechanism for the decrease in serum thyroxine level by phenobarbital in rodents. *Toxicol Appl Pharmacol* **249**, 238-46.

Kester, M. H., Kaptein, E., Roest, T. J., van Dijk, C. H., Tibboel, D., Meinl, W., Glatt, H., Coughtrie, M. W. and Visser, T. J. (1999). Characterization of human iodothyronine sulfotransferases. *J Clin Endocrinol Metab* **84**, 1357-64.

Kester, M. H., Kaptein, E., Roest, T. J., van Dijk, C. H., Tibboel, D., Meinl, W., Glatt, H., Coughtrie, M. W. and Visser, T. J. (2003). Characterization of rat iodothyronine sulfotransferases. *Am J Physiol Endocrinol Metab* **285**, E592-8.

Kohle, C. and Bock, K. W. (2009). Coordinate regulation of human drug-metabolizing enzymes, and conjugate transporters by the Ah receptor, pregnane X receptor and constitutive androstane receptor. *Biochem Pharmacol* **77**, 689-99.

Krol, S., Zabiegala, B. and Namiesnik, J. (2012). PBDEs in environmental samples: sampling and analysis. *Talanta* **93**, 1-17.

LeCluyse, E. L. (2001). Human hepatocyte culture systems for the in vitro evaluation of cytochrome P450 expression and regulation. *Eur J Pharm Sci* **13**, 343-68.

Leslie, E. M., Deeley, R. G. and Cole, S. P. (2005). Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicol Appl Pharmacol* **204**, 216-37.

Lorber, M. (2008). Exposure of Americans to polybrominated diphenyl ethers. *Journal of exposure science & environmental epidemiology* **18**, 2-19.

Maglich, J. M., Stoltz, C. M., Goodwin, B., Hawkins-Brown, D., Moore, J. T. and Kliewer, S. A. (2002). Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. *Mol Pharmacol* **62**, 638-46.

Martin, L. A., Wilson, D. T., Reuhl, K. R., Gallo, M. A. and Klaassen, C. D. (2012). Polychlorinated biphenyl congeners that increase the glucuronidation and biliary excretion of thyroxine are distinct from the congeners that enhance the serum disappearance of thyroxine. *Drug Metab Dispos* **40**, 588-95.

Meerts, I. A., van Zanden, J. J., Luijks, E. A., van Leeuwen-Bol, I., Marsh, G., Jakobsson, E., Bergman, A. and Brouwer, A. (2000). Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin in vitro. *Toxicol Sci* **56**, 95-104.

Mitchell, A. M., Tom, M. and Mortimer, R. H. (2005). Thyroid hormone export from cells: contribution of P-glycoprotein. *J Endocrinol* **185**, 93-8.

Miyawaki, I., Tamura, A., Matsumoto, I., Inada, H., Kunimatsu, T., Kimura, J. and Funabashi, H. (2012). The effects of clobazam treatment in rats on the expression of genes and proteins encoding glucronosyltransferase 1A/2B (UGT1A/2B) and multidrug resistance-associated protein-2 (MRP2), and development of thyroid follicular cell hypertrophy. *Toxicol Appl Pharmacol* **265**, 351-9.

Nies, A. T. and Keppler, D. (2007). The apical conjugate efflux pump ABCC2 (MRP2). *Pflugers Arch* **453**, 643-59.

Nishimura, N., Yonemoto, J., Miyabara, Y., Fujii-Kuriyama, Y. and Tohyama, C. (2005). Altered thyroxin and retinoid metabolic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin in aryl hydrocarbon receptor-null mice. *Archives of toxicology* **79**, 260-7.

Obach, R. S. (1997). Nonspecific binding to microsomes: impact on scale-up of in vitro intrinsic clearance to hepatic clearance as assessed through examination of warfarin, imipramine, and propranolol. *Drug Metab Dispos* **25**, 1359-69.

Pacyniak, E. K., Cheng, X., Cunningham, M. L., Crofton, K., Klaassen, C. D. and Guo, G. L. (2007). The flame retardants, polybrominated diphenyl ethers, are pregnane X receptor activators. *Toxicol Sci* **97**, 94-102.

Paul, K. B., Hedge, J. M., DeVito, M. J. and Crofton, K. M. (2010). Short-term exposure to triclosan decreases thyroxine in vivo via upregulation of hepatic catabolism in Young Long-Evans rats. *Toxicol Sci* **113**, 367-79.

Peters, A. K., Nijmeijer, S., Gradin, K., Backlund, M., Bergman, A., Poellinger, L., Denison, M. S. and Van den Berg, M. (2006). Interactions of polybrominated diphenyl ethers with the aryl hydrocarbon receptor pathway. *Toxicol Sci* **92**, 133-42.

Ribeiro, R. C., Cavalieri, R. R., Lomri, N., Rahmaoui, C. M., Baxter, J. D. and Scharschmidt, B. F. (1996). Thyroid hormone export regulates cellular hormone content and response. *J Biol Chem* **271**, 17147-51.

Richardson, T. A. and Klaassen, C. D. (2010). Disruption of thyroid hormone homeostasis in Ugt1a-deficient Gunn rats by microsomal enzyme inducers is not due to enhanced thyroxine glucuronidation. *Toxicol Appl Pharmacol* **248**, 38-44.

Richardson, V. M., Staskal, D. F., Ross, D. G., Diliberto, J. J., DeVito, M. J. and Birnbaum, L. S. (2008). Possible mechanisms of thyroid hormone disruption in mice by BDE 47, a major polybrominated diphenyl ether congener. *Toxicol Appl Pharmacol* **226**, 244-50.

Rutgers, M., Bonthuis, F., de Herder, W. W. and Visser, T. J. (1987). Accumulation of plasma triiodothyronine sulfate in rats treated with propylthiouracil. *J Clin Invest* **80**, 758-62.

Rutgers, M., Pigmans, I. G., Bonthuis, F., Docter, R. and Visser, T. J. (1989). Effects of propylthiouracil on the biliary clearance of thyroxine (T4) in rats: decreased excretion of 3,5,3'-triiodothyronine glucuronide and increased excretion of 3,3',5'-triiodothyronine glucuronide and T4 sulfate. *Endocrinology* **125**, 2175-86.

Sanders, J. M., Burka, L. T., Smith, C. S., Black, W., James, R. and Cunningham, M. L. (2005). Differential expression of CYP1A, 2B, and 3A genes in the F344 rat following exposure to a polybrominated diphenyl ether mixture or individual components. *Toxicol Sci* **88**, 127-33.

Schecter, A., Colacino, J., Sjodin, A., Needham, L. and Birnbaum, L. (2010). Partitioning of polybrominated diphenyl ethers (PBDEs) in serum and milk from the same mothers. *Chemosphere* **78**, 1279-84.

Soars, M. G., Burchell, B. and Riley, R. J. (2002). In vitro analysis of human drug glucuronidation and prediction of in vivo metabolic clearance. *J Pharmacol Exp Ther* **301**, 382-90.

Szabo, D. T., Richardson, V. M., Ross, D. G., Diliberto, J. J., Kodavanti, P. R. and Birnbaum, L. S. (2009). Effects of perinatal PBDE exposure on hepatic phase I, phase II, phase III, and deiodinase 1 gene expression involved in thyroid hormone metabolism in male rat pups. *Toxicol Sci* **107**, 27-39.

Tchaparian, E. H., Houghton, J. S., Uyeda, C., Grillo, M. P. and Jin, L. (2011). Effect of culture time on the basal expression levels of drug transporters in sandwich-cultured primary rat hepatocytes. *Drug Metab Dispos* **39**, 2387-94.

Tolson, A. H. and Wang, H. (2010). Regulation of drug-metabolizing enzymes by xenobiotic receptors: PXR and CAR. *Adv Drug Deliv Rev* **62**, 1238-49.

Vansell, N. R. and Klaassen, C. D. (2002). Increase in rat liver UDP-glucuronosyltransferase mRNA by microsomal enzyme inducers that enhance thyroid hormone glucuronidation. *Drug Metab Dispos* **30**, 240-6.

Viluksela, M., Raasmaja, A., Lebofsky, M., Stahl, B. U. and Rozman, K. K. (2004). Tissue-specific effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the activity of 5'-deiodinases I and II in rats. *Toxicol Lett* **147**, 133-42.

Visser, T. J. (1994). Role of sulfation in thyroid hormone metabolism. *Chem Biol Interact* **92**, 293-303.

Visser, T. J., Kaptein, E., Gijzel, A. L., de Herder, W. W., Ebner, T. and Burchell, B. (1993). Glucuronidation of thyroid hormone by human bilirubin and phenol UDP-glucuronyltransferase isoenzymes. *FEBS Lett* **324**, 358-60.

Visser, T. J., van Buuren, J. C., Rutgers, M., Eelkman Rooda, S. J. and de Herder, W. W. (1990). The role of sulfation in thyroid hormone metabolism. *Trends Endocrinol Metab* **1**, 211-8.

Visser, W. E., Friesema, E. C. and Visser, T. J. (2011). Minireview: thyroid hormone transporters: the knowns and the unknowns. *Mol Endocrinol* **25**, 1-14.

Wagner, M., Halilbasic, E., Marschall, H. U., Zollner, G., Fickert, P., Langner, C., Zatloukal, K., Denk, H. and Trauner, M. (2005). CAR and PXR agonists stimulate hepatic bile acid and bilirubin detoxification and elimination pathways in mice. *Hepatology* **42**, 420-30.

Wahl, M., Lahni, B., Guenther, R., Kuch, B., Yang, L., Straehle, U., Strack, S. and Weiss, C. (2008). A technical mixture of 2,2',4,4'-tetrabromo diphenyl ether (BDE47) and brominated furans triggers aryl hydrocarbon receptor (AhR) mediated gene expression and toxicity. *Chemosphere* **73**, 209-15.

Wong, H., Lehman-McKeeman, L. D., Grubb, M. F., Grossman, S. J., Bhaskaran, V. M., Solon, E. G., Shen, H. S., Gerson, R. J., Car, B. D., Zhao, B. and Gemzik, B. (2005). Increased hepatobiliary clearance of unconjugated thyroxine determines DMP 904-induced alterations in thyroid hormone homeostasis in rats. *Toxicol Sci* **84**, 232-42.

Zavacki, A. M., Ying, H., Christoffolete, M. A., Aerts, G., So, E., Harney, J. W., Cheng, S. Y., Larsen, P. R. and Bianco, A. C. (2005). Type 1 iodothyronine deiodinase is a sensitive marker of peripheral thyroid status in the mouse. *Endocrinology* **146**, 1568-75.

Zhou, T., Ross, D. G., DeVito, M. J. and Crofton, K. M. (2001). Effects of short-term in vivo exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. *Toxicol Sci* **61**, 76-82.

Zhou, T., Taylor, M. M., DeVito, M. J. and Crofton, K. M. (2002). Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption. *Toxicol Sci* **66**, 105-16.

CHAPTER 5

CONCLUSIONS

This study builds upon previous investigations into the mechanism of T_4 disruption. It is hypothesized that through the activation of nuclear receptors such as, AhR, CAR, and PXR, hepatic UGTs are induced resulting in the enhancement in hepatic T_4 glucuronidation and T_4 clearance from the serum. There are findings that show that the magnitude of serum T_4 decreases does not correlate with the magnitude of inductions in hepatic T_4-UGT activity. Further reports indicate that the inconsistencies in serum T_4 concentrations may be due the increased hepatic accumulation and elimination of T_4 instead of increases in T_4 glucuronidation. Overall, this work shows that the induction of T_4 glucuronidation is species- and agonist-specific. In addition, because of the small changes in T_4 glucuronidation, the role of hepatic T_4 glucuronidation in the disruption of T_4 homeostasis is uncertain.

The liver is a major site of xenobiotic and TH metabolism; therefore, hepatocytes were used because of their biological relevance. Hepatocytes also offered a direct comparison of hepatic T_4 metabolism between rats and humans. Sandwich cultured hepatocytes (SCH) were specifically chosen because hepatocytes in a sandwich configuration have been shown to extend the metabolism and transport functions of the cells. To achieve the goals set forth in this study, an analytical method for the detection of THs and their metabolites was developed. Most importantly, the radiometric detection method developed

for these studies simultaneously detect and quantify radiolabelled THs and metabolites in media and in hepatocytes. To my knowledge, these are the first in vitro experiments to use physiologically relevant concentrations of T_4 for rat (0.05µM) and human (0.1µM) to examine T_4 metabolite levels in media. In an effort to create a straightforward method for measuring T_4 metabolism TH serum binding proteins were not added to the media. To make the experiments more relevant, future studies should use serum binding proteins (TTR and TBG) in the media, at species relevant concentrations would be helpful in examining the influence of T_4 binding on metabolism. TTR and TBG are produced in the liver and are presumed to be produced in hepatocytes; therefore, by adding TTR and TBG to the media in addition to what is produced by the hepatocytes may result in serum protein concentrations greater than those found in rat and human serum.

With the knowledge that TH translocation occurs through active transport mechanisms (Blondeau, *et al.*, 1988; De Jong, *et al.*, 1992; de Jong, *et al.*, 1993), it is reasonable to assume that active transport is likely the rate-limiting step for TH metabolism in hepatocytes. T_4 uptake has been shown to be slower in human hepatocytes than rat hepatocytes (Krenning, *et al.*, 1981; de Jong, *et al.*, 1993). In agreement with previous studies, I found that [^{125}I]-T_4 accumulation was a slower process in SCHH than in SCRH at longer time points; however at shorter time points accumulation did not change. Hepatic elimination may be rate-limiting due to the interplay of metabolic and transport processes; therefore, the greater T_4 metabolite production of SCRH in comparison to SCHH may be partially explained by the more rapid uptake of T_4 into SCRH.

Agonists of hepatic nuclear receptors CAR, PXR, and AhR, consistently cause decreases in circulating TH concentrations in rodents (Barter and Klaassen 1992; Kretschmer

and Baldwin 2005; Qatanani *et al.* 2005). Prototypical agonists were used to examine the effects of nuclear receptor agonists on T_4 metabolism. SCRH and SCHH hepatocytes were treated with PB and 3MC, the prototypical agonists for CAR and AhR, respectively. Due to the species differences in PXR activation, rat hepatocytes were treated with PCN and human hepatocytes were treated with Rif. The prototypical persistent organic pollutant, PCB 153 was used in these studies because as a PB-like PCB, it induces the CAR P450 target, CYP2B. SCRH and SCHH were treated with PCB 153 to compare with the effects of PB treatment on T_4 metabolism. T_4G in the media of SCRH increased with all of the nuclear receptor activators; however T_4G only increased in the media of SCHH following treatment with Rif and PCB 153. Only PCB 153 increased T_4G levels in the media of both SCRH and SCHH. This suggests that PCB 153 may be similar to PB in its ability to increase CYP2B; however PCB 153 is not PB-like when comparing T_4G levels in the media of SCRH and SCHH. The results in SCRH are similar to results *in vivo* which show increases in hepatic T_4-UGT activity or biliary T_4G elimination (McClain, *et al.*, 1989; Hood and Klaassen, 2000a; Craft, *et al.*, 2002; Kato, *et al.*, 2004; Kato, *et al.*, 2011). Data on xenobiotic-mediated increases in T_4 metabolism in humans is limited; however, the results in SCRH are similar to previous human *in vivo* studies, in which Rif decreased serum T_4 and increased liver metabolic activity to a greater extent than PB (Ohnhaus, *et al.*, 1981; Ohnhaus and Studer, 1983). There is some consensus with the results from previous *in vivo* studies; however, it was difficult to correlate the UGT mRNA expression with T_4G levels in media of SCRH and SCHH. The most important finding from these data is that while PCB 153 is PB-like in its ability to induce CYP2B, PCB 153 is different from PB as it is a more efficacious inducer of

T_4 glucuronidation than PB. Future studies should focus on the differences in UGT induction between PB and PCB 153.

AhR, CAR and PXR have been shown to regulate SULTs (Saini, *et al.*, 2004; Yanagiba, *et al.*, 2009; Aleksunes and Klaassen, 2012); however, T_4S levels in the media of SCRH and SCHH did not change with treatment of each agonist. MRNA expression of SULTs involved with T_4 sulfation also did not change. The lack of change in T_4S levels is not exactly surprising, because T_4G is the major metabolite found in the bile of rats. Also, SULTs have a low affinity for T_4 so it is reasonable that T_4S levels do not change whereas T_4G levels increase (Rutgers, *et al.*, 1989; Visser, 1996). In this study, there was no change in T_3+rT_3 levels and no change in DI mRNA expression in both species of hepatocytes. Hepatic deiodinase I activity often decreases with hypothyroidism in rats treated with AhR agonists (Hood and Klaassen, 2000b; Viluksela, *et al.*, 2004; Szabo, *et al.*, 2009). Reductions in deiodinase activity is thought to be a physiological response to decreases in serum T_4 and is not mediated by nuclear receptor agonist; therefore, changes in T_3+rT_3 production and DI mRNA expression may not be observed in an *in vitro* system(Davies, *et al.*, 1996).

Increases in [^{125}I]-T_4 liver accumulation and biliary elimination of [^{125}I]-T_4 and [^{125}I]-T_4G occurs in rodents treated with PB or PCB 153 (Kato, *et al.*, 2010; 2011). I found that [^{125}I]-T_4 accumulation is slower in SCHH than in SCRH. This shows that uptake and efflux transporter may play an important part in the metabolism of T_4. The effects of nuclear receptor agonists on uptake and efflux transporter mRNA expression were mixed. mRNA expression for efflux transporters MDR1, an efflux transporter, increased with PB, 3MC and PCB 153 in SCRH and with PB and Rif in SCHH. MRP2, also an efflux transporter

increased in SCHH treated with PB and Rif. The only change in uptake transporters was with the increase in Oat2 mRNA expression, in SCRH treated with PB. These results indicate that treatment with nuclear receptor agonists may alter efflux and uptake transporter activity; however, it is difficult to correlate T_4G changes with transporter mRNA expression in SCRH and SCHH. It could be explained that the lack of consistency between transporter mRNA expression and treatment with nuclear receptor agonist is due to the culture day in which the cells were collected for mRNA analysis. Hepatocytes were treated for 72 with an agonist; however, it is possible that treating the hepatocytes for 48 hours would result in more consistency in transport mRNA expression. Tchaparian, *et al.*(2011) demonstrated that in SCRH, expression profiles for uptake and efflux transporters do not predict transport activity. This suggests that optimal culture conditions for uptake transporters are not necessarily optimal for efflux transporters. The *in vitro* method described in this report was optimized for T_4 metabolism and not for transporter activity; therefore, it is difficult to make conclusions about T_4 transport in our system. Further work describing the uptake and efflux of T_4 by transporters is needed.

BDE 47 is a major congener in the PBDE class of brominated flame retardants. It is also the predominant congener found in most environmental and human samples (Hites, 2004; Lorber, 2008). BDE 47 has been shown to be an agonist for CAR and to a lesser extent PXR in rodents (Pacyniak, *et al.*, 2007; Richardson, *et al.*, 2008). Previous studies, show BDE 47 decreases serum T_4 in rodents and increases T_4-UGT activity or UGT mRNA expression (Hallgren, *et al.*, 2001; Hallgren and Darnerud, 2002; Richardson, *et al.*, 2008). To my knowledge this is the first time *in vivo-in vitro* comparisons have been made concerning BDE 47 and its effect on T_4 metabolism. Using SCRH and SCHH, we also

investigated the changes in T_4G levels in response to BDE 47. To examine effects on T_4 glucuronidation, T_4-UGT activity and UGT mRNA expression were analyzed in the liver of rats treated with BDE 47. T_4-UGT activity did not change; however, UGT mRNA expression increased. BDE 47 also did not change T_4G levels or UGT mRNA expression in SCRH. The increase in hepatic UGT mRNA expression did not coincide with the lack of T_4-UGT activity increase in rat liver or T_4G levels in the media of rat hepatocytes. This discrepancy could be due to an inability of the T_4-UGT enzyme assay to accurately measure T_4 glucuronidation. It is possible that the T_4-UGT assay was not optimized for different UGT isoforms; therefore, changes in T_4 glucuronidation may not be measured accurately (Visser, *et al.*, 1993a; Hood and Klaassen, 2000a). Also, certain UGT isoforms may be necessary to adequately measure increases in T_4-UGT activity or in T_4G levels in the media. The rat and SCRH data is inconsistent with the large decrease (96%) in serum. In SCRH, BDE 47 did not change T_4G levels or UGT mRNA expression which supports the findings in the *in vivo* rat study. This suggests that hepatic T_4 glucuronidation has only a minor effect on the decrease in serum T_4 in rats. In contrast, BDE 47 increased T_4G levels in the media of SCHH and increased UGT1A1 mRNA expression which shows a potential species difference in the BDE 47 effect on T_4 metabolism in rodents and humans.

The response of transport mRNA expression to BDE 47 was mixed. Overall, the results suggests MDR1 in rats may mediate the biliary elimination of T_4 or T_4G following BDE 47 treatment; however, this same result may not be observed in SCRH or SCHH. Mrp2 mRNA expression did not change with BDE 47; therefore, it may not be responsible for the decrease in serum T_4 observed in rats. In general, transporter mRNA expressions following treatment with BDE 47 are mixed between the species of hepatocytes and rat liver which

makes it difficult to make conclusions about BDE 47 and T_4 transport. To better answerer questions concerning transporters, further studies on the elimination or accumulation of T_4 in rats should be conducted.

In conclusion, there were consistent differences in the levels of T_4G in the media of untreated SCRH and SCHH. Species differences were also observed for [^{125}I]-T_4 uptake and T_4G levels in treated hepatocytes which may give insight into the effects of nuclear receptor agonists on TH disruption. It is understood that there are species differences in nuclear receptor activation and ligand binding and these species difference were apparent in this study. There were some inconsistencies in some of the UGT, SULT and transporter mRNA expression levels which are difficult to explain; but they should not be ruled out as a method to describe increases in T_4 metabolism or biliary elimination. Future studies may be able to resolve these inconsistencies by confirming the gene expression data with protein analysis. To my knowledge this is the first study to examine multiple T_4 metabolites and induction by AhR, CAR, and PXR agonist in rat and human hepatocytes. While there are limitations in the quantitation of T_4 uptake, further work is warranted. Overall, these studies show the utility of SCH in the study of hepatic T_4 metabolism in the continuing investigation of thyroid hormone disruption and human relevance.

REFERENCES

Aleksunes, L. M. and Klaassen, C. D. (2012). Coordinated regulation of hepatic phase I and II drug-metabolizing genes and transporters using AhR-, CAR-, PXR-, PPARalpha-, and Nrf2-null mice. *Drug Metab Dispos* **40**(7), 1366-79.

Blondeau, J. P., Osty, J. and Francon, J. (1988). Characterization of the thyroid hormone transport system of isolated hepatocytes. *J Biol Chem* **263**(6), 2685-92.

Craft, E. S., DeVito, M. J. and Crofton, K. M. (2002). Comparative responsiveness of hypothyroxinemia and hepatic enzyme induction in Long-Evans rats versus C57BL/6J mice exposed to TCDD-like and phenobarbital-like polychlorinated biphenyl congeners. *Toxicol Sci* **68**(2), 372-80.

Davies, P. H., Sheppard, M. C. and Franklyn, J. A. (1996). Regulation of type I 5'-deiodinase by thyroid hormone and dexamethasone in rat liver and kidney cells. *Thyroid* **6**(3), 221-8.

De Jong, M., Docter, R., Van Der Hoek, H. J., Vos, R. A., Krenning, E. P. and Hennemann, G. (1992). Transport of 3,5,3'-triiodothyronine into the perfused rat liver and subsequent metabolism are inhibited by fasting. *Endocrinology* **131**(1), 463-70.

de Jong, M., Visser, T. J., Bernard, B. F., Docter, R., Vos, R. A., Hennemann, G. and Krenning, E. P. (1993). Transport and metabolism of iodothyronines in cultured human hepatocytes. *J Clin Endocrinol Metab* **77**(1), 139-43.

Hallgren, S. and Darnerud, P. O. (2002). Polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and chlorinated paraffins (CPs) in rats-testing interactions and mechanisms for thyroid hormone effects. *Toxicology* **177**(2-3), 227-43.

Hallgren, S., Sinjari, T., Hakansson, H. and Darnerud, P. O. (2001). Effects of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) on thyroid hormone and vitamin A levels in rats and mice. *Archives of toxicology* **75**(4), 200-8.

Hites, R. A. (2004). Polybrominated diphenyl ethers in the environment and in people: a meta-analysis of concentrations. *Environ Sci Technol* **38**(4), 945-56.

Hood, A. and Klaassen, C. D. (2000a). Differential effects of microsomal enzyme inducers on in vitro thyroxine (T(4)) and triiodothyronine (T(3)) glucuronidation. *Toxicol Sci* **55**(1), 78-84.

Hood, A. and Klaassen, C. D. (2000b). Effects of microsomal enzyme inducers on outer-ring deiodinase activity toward thyroid hormones in various rat tissues. *Toxicol Appl Pharmacol* **163**(3), 240-8.

Jemnitz, K. and Vereczkey, L. (1996). Ion-pair high-performance liquid chromatographic separation of two thyroxine glucuronides formed by rat liver microsomes. *J Chromatogr B Biomed Appl* **681**(2), 385-9.

Kato, Y., Ikushiro, S., Haraguchi, K., Yamazaki, T., Ito, Y., Suzuki, H., Kimura, R., Yamada, S., Inoue, T. and Degawa, M. (2004). A possible mechanism for decrease in serum thyroxine level by polychlorinated biphenyls in Wistar and Gunn rats. *Toxicol Sci* **81**(2), 309-15.

Kato, Y., Onishi, M., Haraguchi, K., Ikushiro, S., Ohta, C., Koga, N., Endo, T., Yamada, S. and Degawa, M. (2011). A possible mechanism for 2,2',4,4',5,5'-hexachlorobiphenyl-mediated decrease in serum thyroxine level in mice. *Toxicol Appl Pharmacol* **254**(1), 48-55.

Kato, Y., Suzuki, H., Haraguchi, K., Ikushiro, S., Ito, Y., Uchida, S., Yamada, S. and Degawa, M. (2010). A possible mechanism for the decrease in serum thyroxine level by phenobarbital in rodents. *Toxicol Appl Pharmacol* **249**(3), 238-46.

Krenning, E., Docter, R., Bernard, B., Visser, T. and Hennemann, G. (1981). Characteristics of active transport of thyroid hormone into rat hepatocytes. *Biochim Biophys Acta* **676**(3), 314-20.

Lorber, M. (2008). Exposure of Americans to polybrominated diphenyl ethers. *Journal of exposure science & environmental epidemiology* **18**(1), 2-19.

McClain, R. M., Levin, A. A., Posch, R. and Downing, J. C. (1989). The effect of phenobarbital on the metabolism and excretion of thyroxine in rats. *Toxicol Appl Pharmacol* **99**(2), 216-28.

Ohnhaus, E. E., Burgi, H., Burger, A. and Studer, H. (1981). The effect of antipyrine, phenobarbitol and rifampicin on thyroid hormone metabolism in man. *Eur J Clin Invest* **11**(5), 381-7.

Ohnhaus, E. E. and Studer, H. (1983). A link between liver microsomal enzyme activity and thyroid hormone metabolism in man. *Br J Clin Pharmacol* **15**(1), 71-6.

Pacyniak, E. K., Cheng, X., Cunningham, M. L., Crofton, K., Klaassen, C. D. and Guo, G. L. (2007). The flame retardants, polybrominated diphenyl ethers, are pregnane X receptor activators. *Toxicol Sci* **97**(1), 94-102.

Richardson, V. M., Ferguson, S.S., Sey, Y.M., and DeVito, M.J. (submitted). In vitro metabolism of thyroxine by rat and human hepatocytes.

Richardson, V. M., Staskal, D. F., Ross, D. G., Diliberto, J. J., DeVito, M. J. and Birnbaum, L. S. (2008). Possible mechanisms of thyroid hormone disruption in mice by BDE 47, a major polybrominated diphenyl ether congener. *Toxicol Appl Pharmacol* **226**(3), 244-50.

Rutgers, M., Pigmans, I. G., Bonthuis, F., Docter, R. and Visser, T. J. (1989). Effects of propylthiouracil on the biliary clearance of thyroxine (T4) in rats: decreased excretion of 3,5,3'-triiodothyronine glucuronide and increased excretion of 3,3',5'-triiodothyronine glucuronide and T4 sulfate. *Endocrinology* **125**(4), 2175-86.

Saini, S. P., Sonoda, J., Xu, L., Toma, D., Uppal, H., Mu, Y., Ren, S., Moore, D. D., Evans, R. M. and Xie, W. (2004). A novel constitutive androstane receptor-mediated and CYP3A-independent pathway of bile acid detoxification. *Mol Pharmacol* **65**(2), 292-300.

Szabo, D. T., Richardson, V. M., Ross, D. G., Diliberto, J. J., Kodavanti, P. R. and Birnbaum, L. S. (2009). Effects of perinatal PBDE exposure on hepatic phase I, phase II, phase III, and deiodinase 1 gene expression involved in thyroid hormone metabolism in male rat pups. *Toxicol Sci* **107**(1), 27-39.

Tchaparian, E. H., Houghton, J. S., Uyeda, C., Grillo, M. P. and Jin, L. (2011). Effect of culture time on the basal expression levels of drug transporters in sandwich-cultured primary rat hepatocytes. *Drug Metab Dispos* **39**(12), 2387-94.

Tong, Z., Li, H., Goljer, I., McConnell, O. and Chandrasekaran, A. (2007). In vitro glucuronidation of thyroxine and triiodothyronine by liver microsomes and recombinant human UDP-glucuronosyltransferases. *Drug Metab Dispos* **35**(12), 2203-10.

Viluksela, M., Raasmaja, A., Lebofsky, M., Stahl, B. U. and Rozman, K. K. (2004). Tissue-specific effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the activity of 5'-deiodinases I and II in rats. *Toxicol Lett* **147**(2), 133-42.

Visser, T. J. (1996). Pathways of thyroid hormone metabolism. *Acta Med Austriaca* **23**(1-2), 10-6.

Visser, T. J., Kaptein, E., Gijzel, A. L., de Herder, W. W., Ebner, T. and Burchell, B. (1993). Glucuronidation of thyroid hormone by human bilirubin and phenol UDP-glucuronyltransferase isoenzymes. *FEBS Lett* **324**(3), 358-60.

Yanagiba, Y., Ito, Y., Kamijima, M., Gonzalez, F. J. and Nakajima, T. (2009). Octachlorostyrene induces cytochrome P450, UDP-glucuronosyltransferase, and sulfotransferase via the aryl hydrocarbon receptor and constitutive androstane receptor. *Toxicol Sci* **111**(1), 19-26.

APPENDIX

POSSIBLE MECHANISMS OF THYROID HORMONE DISRUPTION IN MICE BY BDE 47, A MAJOR POLYBROMINATED DIPHENYL ETHER CONGENER[2]

A. INTRODUCTION

Polybrominated diphenyl ethers (PBDEs) have been commercially used as fire retardants in consumer products such as polyurethane foam, electronics, and textile coating. PBDEs are lipophilic compounds that have been detected in human serum and breast milk as well as in various types of environmental media around the world (Petreas *et al.*, 2003; Schecter *et al.*, 2003; Focant *et al.*, 2004b). 2, 2', 4, 4'-Tetrabromodiphenyl ether (BDE 47) is the predominant congener found in most wildlife and human samples and generally accounts for half of the total PBDEs measured (Birnbaum and Staskal, 2004; Hites, 2004). Although health risks to humans following PBDE exposure are unknown, several studies in rodents report potential developmental, reproductive, neurological, and endocrine toxicity (Zhou, *et al.*, 2001; de Wit, 2002; Zhou, *et al.*, 2002; Birnbaum and Staskal, 2004).

Due to their structural similarity to polychlorinated biphenyls (PCBs) and thyroid hormones (THs), PBDEs may act as TH disruptors. Developmental exposures to commercial

---

[2]Richardson, V.M., Staskal, D.F., Ross, D.G., Dilliberto, J.J., DeVito, M.J., and Birnbaum, L.S. *Toxicol Appl Pharmacol* **226**(3), 244-50. 2008

PBDE mixtures decreased circulating TH concentrations in rat pups (Zhou, *et al.*, 2002; Ellis-Hutchings *et al.*, 2006). Additionally, short term exposures to PBDEs during puberty resulted in decreases in TH in rats (Zhou, *et al.*, 2001; Stoker *et al.*, 2004). While there is sufficient evidence that PBDEs decrease circulating TH concentrations, the mechanisms involved are unclear.

There are multiple sites within the thyroid axis in which xenobiotics can alter the hormonal balance, such as changes in binding proteins and hormone metabolism. In rodents, decreases in TH concentrations are often associated with induced hepatic thyroxine (T_4)-glucuronidation followed by increased biliary elimination of the conjugated hormone (Barter and Klaassen, 1992; Vansell and Klaassen, 2002a). Rats exposed to PBDEs have decreased concentrations of circulating T_4 which are linked to moderate induction in hepatic uridinediphosphate glucuronosyltransferases (UGTs) (Hallgren and Darnerud, 2002; Zhou, *et al.*, 2002). While UGTs play a role in decreasing circulating TH, it is not certain that the induction of hepatic T_4-glucuronidation alone is responsible for the effects on TH concentration following PBDE exposures. For instance, studies using UGT1A-deficient Gunn rats exposed to phenobarbital (PB) or PCBs demonstrate that decreases in serum total T_4 were not necessarily glucuronidation dependent (Collins and Capen, 1980; Kato, *et al.*, 2004). *In vivo* studies show that PCB metabolites can bind to transthyretin (Ttr), a major TH transport protein in plasma, (Kato, *et al.*, 2005) and also cause increases in biliary T_4 excretion which in combination may result in decreases in circulating total T_4. Hydroxylated PBDEs incubated with T_4 and human transthyretin show decreases in *ex-vivo* T_4-Ttr binding (Hallgren and Darnerud, 2002; Hamers *et al.*, 2006). This suggests that alterations in Ttr-mediated transport may also contribute to the observed decrease in T_4.

Xenobiotic metabolizing enzymes (XMEs) such as UGTs and cytochrome P450s (P450s), as well as transport proteins, are induced through the activation of a variety of nuclear receptors including the aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR), and constitutive androstane receptor (CAR) (Maglich, *et al.*, 2002; Auyeung, *et al.*, 2003; Bock and Kohle, 2004; Wagner, *et al.*, 2005). The role of UGTs in the PBDE–mediated decrease in TH has been studied, but the function transporters play in this decrease has yet to be determined. In addition to UGTs, PBDEs induce cytochrome P450s (Sanders, *et al.*, 2005; Staskal *et al.*, 2005), but it is uncertain if transport proteins are also altered, and, if so, what role they play in the decrease in circulating T_4. Studies have shown a correlation between induction of hepatic UGTs (Ugt1a1), multidrug resistance protein associated protein (Mrp2), and organic anion transporting proteins (Oatp1 and Oatp2) mRNA levels, with decreases in serum TH concentrations (Ribeiro, *et al.*, 1996; Mitchell, *et al.*, 2005; Wong, *et al.*, 2005). There is also evidence that multidrug resistance proteins (MDRs) are important in TH efflux (Mitchell, *et al.*, 2005). Collectively, these studies indicate that active transport along with glucuronidation and altered serum binding are possibly involved in TH decreases.

In an effort to understand the mechanisms involved with alterations in TH concentrations following exposure to PBDEs, this study examines the effects of BDE 47 on TH homeostasis in adult female mice. Multiple parameters including enzymatic activities and gene expression were assessed to identify possible mechanisms of TH disruption. In particular, genes related to TH transport and metabolism were analyzed for changes following BDE 47 exposure.

B. MATERIALS AND METHODS

Chemicals

2, 2', 4, 4'-Tetrabromodiphenyl ether (BDE 47) was generously provided by Great Lakes Chemical Corporation (Indianapolis, IN). Purity was >98% as determined by reverse-phase-performance liquid chromatography (HPLC). All other chemicals used were of the highest grade commercially available.

Animals and Treatment

Female C57BL/6 mice (9 weeks old, 19-22g) were obtained from Charles River Breeding Laboratories (Raleigh, NC). Animals were maintained on a 12- hour light/dark cycle at ambient temperature (22°C) and relative humidity (55±5%). They were provided with Purina 5001 Rodent Chow (Ralston Purina Co., St. Louis, MO) and tap water *ad libitum.* Mice were housed individually and allowed to acclimate for one week before study commencement. Animals were held in a facility approved by the American Association for Accreditation of Laboratory Animal Care (AAALAC). The Animal Care and Use Committee of NHEERL (U.S. EPA, RTP, NC) approved all animal protocols. Animals were treated with a single daily dose for 4 consecutive days. Mice were randomly selected for each treatment group (n=10/group). Mice were administered BDE 47 in corn oil (0, 3, 10, or 100 mg/kg/day for 4 days) by oral gavage (10 ml/kg). Dosing solutions were prepared by dissolving BDE 47 in hexane followed by the addition of corn oil. Hexane was removed from the dosing solutions by evaporation with a speed vacuum device (Speed Vac, Savant Instruments, Inc., Farmingdale, NY).

Serum and Tissue Collection

24 hours after the final dose, mice were euthanized by CO_2 asphyxiation followed by exsanguination via cardiac puncture; and blood, liver, and kidneys were collected. Blood was allowed to clot for 1 hour on ice in serum separator tubes (Becton Dickinson, Franklin Lakes, NJ). Blood was then centrifuged at 1200 x g for 30 min to obtain serum. Serum was frozen and held at -80°C until analyzed. Livers and kidneys were removed and weighed. Between 100 and 150 mg of liver or kidney were placed in RNA*later* solution (Ambion, Inc., Austin, TX) and frozen at -80°C for subsequent RNA purification. The remaining liver was frozen at -80°C for further enzyme activity analysis.

Thyroid Hormone Analysis

Serum total thyroxine (T_4) concentrations were measured using the Coat-a-Count radioimmunoassay (RIA) kit (Diagnostic Products Corporation, Los Angeles, CA) according to the manufacturer's protocol. Each sample was measured in duplicate. All data is expressed as ng/ml serum T_4.

EROD, PROD, and T_4-UGT assays

Liver microsomal fractions were prepared (Zhou, *et al.*, 2001; Zhou, *et al.*, 2002) and protein concentrations were measured using a protein assay kit with bovine serum albumin as a standard (Bio-Rad Laboratories, Hercules, CA). Enzymatic activities for ethoxyresorufin O-deethylase (EROD), a marker for CYP1A1, and pentoxyresorufin O-deethylase (PROD), a marker for CYP2B, were measured using a spectrofluorimetric assay (DeVito *et al.*, 1994). Hepatic T_4-UGT activity was determined by using a modified assay (Zhou, *et al.*, 2001;

Zhou, *et al.*, 2002) based on a previously published method (Visser, *et al.*, 1993b). All data is expressed as fold change as compared to control, where controls for EROD and PROD are expressed as nMol resorufin/min/mg protein and controls for T_4-UGT are expressed as pMol T_4-UGT/min/mg protein.

RNA isolation, relative real-time RT-PCR, and RT-PCR data analysis

Total RNA was isolated using the RNeasy Midi Kit with DNase I digestion performed during column purification (Qiagen, Hilden, Germany). To assess the integrity of the RNA samples, the 2100 Bioanalyzer was used (Agilent Technologies, Palo Alto, CA) using RNA with RNA Integrity Numbers (RINs) greater than 8.1. Samples with RINs less than 8.1 were not analyzed by RT-PCR, therefore, the sample size used for many of the genes examined varied (n=4-6/group). Real-time RT-PCR was performed using the ABI Prism 5700 Sequence Detection System (ABI, Foster City, CA). 100 ng of total RNA was used for each reaction. cDNA was synthesized using TaqMan Reverse Transcriptase Kits (ABI, Foster City, CA). PCR was then performed on all cDNAs using TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays (ABI, Foster City, CA). These assays are Cyp1a1 (ABI# Mm00487218_m1), Cy2b10 (Mm00456591_m1), Ugt1a1 (Mm0129059_m1), Ugt1a6 (Mm01290954_m1), Ugt1a7 (Mm01967851_s1), Ugt2b5 (Mm01623253_s1), Mdr1a (Mm00440761_m1), Mdr1b (Mm0040736_m1), Mrp3 (Mm00551550_m1), Mct8 (Mm00486202_m1), Ttr (Mm00443267_m1). The thermal cycle condition for the RT reaction was as follows: 10 min at 25°C, 30 min at 48°C, and then 5 min at 95°C. The PCR reaction was performed as follows: 2 min at 50°C (uracil-DNA glycosylase activation), 10 min at 95°C (activation of *Taq* DNA polymerase), and 40 cycles

of denaturation at 95°C for 15 s followed by annealing and extension at 60°C for 1 min. Data were analyzed using the Sequence Detection Systems software (ABI, Foster City, CA). All RT-PCR data were quantified by the $\Delta\Delta C_t$ method (Applied Biosystems User Bulletin 2) relative to a calibrator sample and an endogenous control (18S). In the $\Delta\Delta C_t$ method, the control group is used as a reference point for all other dose groups; therefore, all samples are expressed as fold difference as compared to control.

Data Analysis

All data are presented as the mean ± standard deviation. The statistical intergroup comparisons were determined by using one-way analysis of variance (ANOVA) followed by Dunnet's Multiple Comparison post test (GraphPad Prism 3.0, GraphPad Software San Diego, CA). The levels of probability of statistical significance are $p < 0.05$. Data were transformed (Y=1/Y), where intergroup variability was deemed significantly different by Bartlett's test for equal variances**.** Data transformed due to intergroup variability include EROD, PROD, Cyp2b10, Ugt1a7, Mrp3, and Mct8.

C. RESULTS

Body weight, liver weight, and serum total T_4

There were no obvious signs of toxicity and no significant treatment effect on body weight, or kidney weight between treatment groups. Liver weights and liver-to-body weight ratios significantly increased 14% and 10%, respectively, at 100 mg/kg/day of BDE 47 (Table 1). A maximal decrease in serum total T_4 of almost 43% was observed at 100

mg/kg/day of BDE 47 with respect to controls (Figure 1). There were no significant effects on liver weight or serum total T_4 at the lower doses tested (3 and 10 mg/kg/day of BDE 47) (Table 1).

Hepatic UGT induction

To determine which UGT isoforms were induced with exposure to BDE 47, liver mRNA expression was analyzed by real time RT-PCR. Treatment with BDE 47 increased hepatic mRNA expression of Ugt1a1, Ugt1a7 and Ugt2b5. Specifically, 100 mg/kg/day of BDE 47 significantly increased hepatic Ugt1a1 and Ugt2b5 mRNA expression 1.2- and 1.3-fold, respectively. Ugt1a7 mRNA expression also significantly increased 1.3- and 1.7-fold at 10 mg/kg/day and 100 mg/kg/day of BDE 47, respectively. Ugt1a1, Ugt1a7, and Ugt2b5 correlated with the observed decreases in T_4 (R^2=0.27, 0.46, and 0.22, respectively and p<0.05, 0.001, and 0.05, respectively). Liver microsomes were used to determine UDP-glucuronosyltransferase (UGT) activity with respect to T_4. Hepatic T_4-UGT enzyme activity was unchanged with BDE 47 treatment and therefore, did not correspond with the changes observed in UGT mRNA expression. The T_4-UGT enzyme assay does not detect specific UGT isoforms. This suggests that our assay may not be a sensitive measure of actual changes in individual UGT isoform activity (Table 2*).*

Hepatic Cytochrome P450 induction

Effects on major xenobiotic metabolizing enzymes (XMEs) for phase I metabolism, were examined. Cyp1a1 mRNA expression and EROD activity were used as markers for Ah receptor activation (Whitlock, 1990). BDE 47 significantly increased hepatic EROD, a

marker for Cyp1a1 activity, 1.4-fold at the highest dose (100 mg/kg/day); however, hepatic Cyp1a1 mRNA expression was not significantly changed with treatment. Hepatic PROD and Cyp2b10 mRNA expression were used as markers for CAR activation (Waxman, 1999; Maglich, *et al.*, 2002; Xiong *et al.*, 2002; Yamada *et al.*, 2006). Hepatic PROD, a marker for CYP2B activity, increased significantly at the 3, 10, and 100 mg/kg/day doses, 1.2- , 1.8-, and 4.8- fold, respectively. Hepatic Cyp2b10 mRNA expression was increased significantly at 10 and 100 mg/kg/day, 2.5- and 19.9-fold, respectively. Correlations with T_4 decreases were significant for PROD (R^2=0.57, p<0.0001) and Cyp2b10 (R^2=0.44, p<0.005). Hepatic Cyp3a11 mRNA expression, a marker for PXR activation (Waxman, 1999; Xie *et al.*, 2000), showed no significant changes with treatment.(Table 3).

Transporter mRNA expression

Several transporters known to transport glucuronides or thyroid hormones were analyzed. Specifically, major efflux transporters in the ATP binding cassette (ABC) gene family, including multidrug resistance-associated proteins (MRPs), and multidrug resistance proteins (MDRs), were analyzed for changes in mRNA expression in liver *(Table 3).* The expression of hepatic Mrp3 mRNA, a major sinusoidal efflux transporter of glucuronides, showed a significant 47% increase at 100 mg/kg/day of BDE 47. Overall, Mrp3 mRNA expression correlated significantly with decreases in **T_4** (R^2=0.46, p<0.001). Mdr1a and Mdr1b encode P-glycoproteins (P-gp) in mice and can transport several substrates including glucuronides and thyroid hormones (Ribeiro, *et al.*, 1996; Mitchell, *et al.*, 2005). Exposures to BDE 47 caused a significant dose-dependent decrease at all doses tested in hepatic Mdr1a mRNA expression; however, the decreases did not correlate with **T_4** decrease (R^2= 0.17,

p=0.08). Mdr1b mRNA expression was unchanged in liver (Table 4). MCT8, a membrane bound thyroid hormone uptake transporter (Friesema, *et al.*, 2003) was examined for BDE 47- related changes in mRNA expression. Hepatic Mct8 mRNA expression significantly decreased 0.8-fold at 100 mg/kg/day; however, this decrease did not correlate significantly with decreases in $\mathbf{T_4}$, (R^2= 0.02, *p*=0.56) (Table 5).

Mdr1a, Mdr1b, and Mrp3 mRNA expression was also measured in kidney. BDE 47 had no observed effect on mRNA expression of these transporters analyzed.

Transthyretin mRNA expression

Transthyretin (Ttr), a major serum transport protein in rodents was analyzed for effects on mRNA expression following exposures to BDE 47. Ttr mRNA expression was significantly decreased at the highest dose tested (100 mg/kg/day) and correlated well with the observed decrease in serum T_4 (R^2=0.61; *p*<0.0001) (Table 5). .

D. DISCUSSION

Previous hypotheses on TH disruption by BDE 47 have focused on two mechanisms: 1) induction of hepatic UGT-mediated TH metabolism resulting in decreased circulating T_4 and 2) competitive binding of BDE 47 and thyroid hormones to serum transport proteins. This study further investigates parameters involved in the alteration of thyroid hormone status. Specifically, this study aims to identify the genes activated by BDE 47, which may provide information as to the mechanisms by which PBDEs exert their effects. In this study, BDE 47 decreased T_4 and increased hepatic UGT mRNA expression in female mice. In

addition, BDE 47 altered the hepatic mRNA expression of putative transporters of TH or glucuronides.

In vivo studies show that decreases in TH concentrations in rats treated with BDE 47 (Hallgren, *et al.*, 2001; Hallgren and Darnerud, 2002) coincide with increases in hepatic T_4-UGT activity. In this study, BDE 47 exposures increased Ugt1a1, Ugt1a7, and Ugt2b5 mRNA expression in mouse liver, while Ugt1a6 remained unchanged. Changes in specific UGT isoforms, such as Ugt1a7 mRNA expression correlated well with decreases in T_4. (R^2= 0.46, p<0.001)

There was no increase in hepatic T_4-UGT activity, which does not agree with the observed increases in UGT mRNA expression. This may be because the T_4-glucuronidation enzyme assay does not evaluate activity for specific UGT isoforms. Alternatively, increases in UGT mRNA expression may not result in adequate increases of the respective enzyme proteins. Some UGT activity induction can also be dependent on AhR activation. For example, Ugt1a6 induction is dependent on AhR activation (Auyeung, *et al.*, 2003; Nishimura, *et al.*, 2005); therefore, the lack of significant increases in hepatic Ugt1a6 mRNA expression in our study further supports the idea that BDE 47 is not an agonist for AhR and may explain the lack of hepatic UGT activity.

Once activated, nuclear receptors coordinately regulate genes involved in the detoxification (phase I and II) and elimination (phase III/transport) of xenobiotics, steroid hormones, and bile acids (Bock and Kohle, 2004; Wagner, *et al.*, 2005). With the induction of genes involved in phase I and phase II metabolism, this study examined the effects of BDE 47 on the expression of uptake and efflux transporters in the liver. MCT8 is a major thyroid hormone uptake transporter. MCT8-null mice exhibit abnormal thyroid hormone

levels (decreased serum T_4 and elevated serum T_3) (Trajkovic *et al.*, 2007) suggesting that the decrease in hepatic Mct8 mRNA expression found in the present study following BDE 47 exposure may play an important role in the serum T_4 decrease.

P-gp, encoded by Mdr1a and Mdr1b in mice, is a major canalicular efflux transporter and plays a role in the transport of a diverse range of xenobiotics and steroids into the bile. There is also evidence that MDR transporters are important in TH efflux, in which cells transfected with Mdr1 cDNA increased the efflux of T_3 as compared to control cells (Mitchell, *et al.*, 2005). (Nishio *et al.*, 2005)also examined changes in P-gp in hyperthyroid rats. They demonstrated that rats in a hyperthyroid state have increased levels of Mdr1a and P-gp in the liver. They also found that hyperthyroid rats administered digoxin had lower concentrations of digoxin in serum, which may be attributable to the P-gp increases. Studies from our laboratory show a decrease in the urinary elimination of BDE 47 (single, oral dose of 100 mg/kg) in Mdr1a/b deficient mice as compared to wild type mice[1]. Together, these studies show that P-gp also plays an important role in defining the toxicokinetics of many xenobiotics. Furthermore, these studies show that P-gp may be regulated by TH levels. In the present study, hepatic Mdr1b mRNA expression was unchanged with exposure to BDE 47, while Mdr1a mRNA expression decreased significantly, even at the lowest dose tested (3 mg/kg/day). Based on the cited studies, there are two possible explanations for the decrease in hepatic Mdr1a following BDE 47 exposure. First, it is possible that once in a hypothyroid state, hepatic Mdr1a expression will decrease in response to the decrease in circulating serum T_4. On the other hand, decreases in hepatic P-gp may result in a longer half-life of BDE 47 in liver, resulting in increased opportunity for induction of UGTs, consequentially decreasing serum T_4.

There were no changes in hepatic mRNA expression of sinusoidal efflux transporters, Mrp1 and Mrp5. However, Mrp3 mRNA expression increased with BDE 47 exposures. Mrp3, a sinusoidal efflux transporter, exports glucuronides from the liver and into the blood. It has been suggested that the induction of Mrp3 results from the need for an alternative protective mechanism (Inokuchi *et al.*, 2001; Wagner, *et al.*, 2005). The increase in Mrp3 mRNA expression, suggests that high doses of BDE 47 may saturate hepatic efflux transporters. The saturation of hepatic efflux transporters would require the induction of a back-up transporter to move glucuronides from the liver into the bloodstream. Altogether, the altered expression in hepatic transporter mRNA expression suggests that BDE 47 may mediate changes in biliary elimination of glucuronides. The induction of Mrp3 also shows that increases in glucuronidation may be a major cause of the TH decreases even though T_4-UGT enzymatic activity was unchanged as measured by our assay.

CYP1A1, CYP2B10, and CYP3A11, classic targets for AhR, CAR, and PXR activation, respectively, (Maglich, *et al.*, 2002), were measured to establish which nuclear receptor pathway BDE 47 may activate. Cyp1a1 mRNA expression and associated enzyme activity (EROD) was used to determine the ability of BDE 47 to act through the AhR. The slight increase in EROD and lack of hepatic Cyp1a1 mRNA induction at the highest dose tested supports data that shows BDE 47 is not an AhR agonist (Peters *et al.*, 2004; Sanders, *et al.*, 2005). The modest increase in EROD activity may also be due to overlapping regulation by CAR (Auyeung, *et al.*, 2003; Nishimura, *et al.*, 2005). Other studies suggest that BDE 47 may be an AhR antagonist in rats, and this may explain the small decrease in mouse Cyp1a1 mRNA expression seen in this study (Peters, *et al.*, 2004; Hamers, *et al.*, 2006). Common targets for CAR activation, Cyp2b10 mRNA expression and associated

enzyme activity (PROD), increased significantly in liver. The sensitive dose-dependent increases in hepatic PROD (3 mg/kg/day) and Cyp2b10 mRNA expression (10 mg/kg/day) suggests that CAR may be one of the major nuclear receptors involved in the regulation of the observed responses. Hepatic Cyp3a11 mRNA expression, a common target for PXR activation was unchanged following BDE 47 exposure. In contrast to the reported increase in male mice exposed to 50 mg/kg/day BDE 47 by intraperitoneal injections for 4 days (Pacyniak, *et al.*, 2007)**,** we saw no increase in hepatic Cyp3a11 mRNA expression. Whether or not this result is due to differences in exposure routes or the sex of the mice used is unclear.

It is hypothesized that hydroxylated PCB metabolites (Brouwer *et al.*, 1988; Lans *et al.*, 1994) and hydroxylated PBDE metabolites (Hallgren and Darnerud, 2002) bind to transthyretin causing an increase in the hepatic uptake and biliary elimination of T_4. The present study showed decreases in hepatic Ttr mRNA expression that correlated well with the observed T_4 decreases (R^2=0.61; p<0.0001). This further supports the hypothesis that BDE 47 may alter T_4 transport by Ttr in rodents. Further investigation is needed to examine what mechanisms may cause the decrease in Ttr mRNA expression. Considering that thyroxine-binding globulin (TBG) is the major TH serum carrier protein in humans and although Ttr may be critical during human development, it is unclear if alteration in hepatic Ttr would play a significant role in TH homeostasis in adult humans.

In conclusion, this study was designed to examine TH decreases following a short-term exposure; therefore, the doses chosen are relatively high in comparison to daily human exposure and are known to be non-linear in toxicokinetic mouse studies (Staskal *et al.*, 2006). This report shows that active transport along with glucuronidation and alterations in TH

binding in serum may be involved in the TH decreases at 100 mg/kg/day BDE 47 in adult female mice. Decreases in total T_4 after BDE 47 exposure may be mediated through CAR/PXR pathways and this is evident by the induction of genes regulated by CAR/PXR, in particular, Cyp2b10, Ugt1a1, Ugt1a7, Ugt2b5 and Mrp3. Although mRNA expression was unchanged for several of the transport proteins important in glucuronide or TH transport, this does not rule out possible changes in regulation at the translational or posttranslational levels (Peeters *et al.*, 2002). Together our data demonstrates that the coordinated regulation of phase I, phase II, phase III/transporters, serum binding proteins and thyroid hormone clearance following exposures to environment contaminants, such as BDE 47 are complicated processes, which require further examination.

Table A.1.

Body weight, organ weights, and liver-to-body weight ratio

Treatment (mg/kg/day)	Body (g) [a]	Liver (g) [a]	Kidney (g) [a]	Liver-to-body wt. ratio [a]
0	20.60±0.63	0.96±0.07	0.23±0.02	0.046±0.003
3	21.33±0.72[a]	0.99±0.08	0.23±0.01	0.046±0.003
10	21.03±0.60	0.92±0.07	0.23±0.02	0.044±0.002
100	21.52±0.51[b]	1.09±0.06[b]	0.24±0.01	0.051±0.002[b]

[a]Data are expressed as mean ± standard deviation (n=10/group)
[a]Significantly different from control group ($p< 0.05$)
[b]Significantly different from control group ($p< 0.01$)

Table A.2.

Fold change in hepatic UDPGT activity and mRNA expression

Treatment (mg/kg/day)	UDPGT[a]	Ugt1a1[a]	Ugt1a6[a]	Ugt1a7[a]	Ugt2b5[a]
0	1.0±0.2	1.0±0.0	1.0±0.2	1.0±0.1	1.0±0.1
3	1.1±0.4	1.0±0.1	1.2±0.3	1.0±0.1	0.9±0.2
10	1.1±0.2	1.1±0.1	1.2±0.3	1.3±0.2[c]	1.2±0.2
100	1.1±0.3	1.2±0.1[b]	1.4±0.3	1.7±0.1[c]	1.3±0.2[b]

[a]Data are expressed as fold mean change ± standard deviation (n=4-5/group)
[b]Significantly different from control group ($p< 0.05$)
[c]Significantly different from control group ($p< 0.01$)

Table A.3.

Fold change in hepatic cytochrome P450 activity and mRNA expression

Treatment (mg/kg/day)	Cyp1a1[a]	EROD[a]	Cyp2b10[a]	PROD[a]	Cyp3a11[a]
0	1.0±0.3	1.0±0.1	1.0±0.1	1.0±0.2	1.0±0.1
3	0.8±0.3	0.9±0.1	1.2±0.4	1.2±0.2[b]	0.9±0.1
10	0.8±0.3	1.0±0.1	2.5±0.8[c]	1.8±0.2[c]	1.1±0.1
100	0.6±0.1	1.4±0.5[c]	19.9±2.9[c]	4.8±0.5[c]	1.2±0.2

[a]Data are expressed as fold change mean ± standard deviation (n=5-10/group)
[b]Significantly different from control group ($p < 0.05$)
[c]Significantly different from control group ($p < 0.01$)

Table A.4.

Fold change in hepatic efflux transporter mRNA expression

Treatment (mg/kg/day)	Mrp3[a]	Mdr1a[a]	Mdr1b[a]
0	1.00±0.07	1.00±0.23	1.00±0.33
3	0.80±0.05	0.66±0.10[c]	1.23±0.56
10	1.19±0.14	0.61±0.36[b]	1.37±0.43
100	1.47±0.20[b]	0.60±0.06[b]	1.27±0.59

[a]Data are expressed as fold change mean ± standard deviation (n=5-6/group)
[b]Significantly different from control group ($p< 0.01$)
[c]Significantly different from control group ($p< 0.05$)

Table A.5.

Fold change in hepatic thyroid hormone transporter mRNA expression

Treatment (mg/kg/day)	Ttr [a]	Mct8 [a]
0	1.00±0.06	1.00±0.20
3	0.91±0.04	1.02±0.16
10	0.93±0.11	1.05±0.16
100	0.73±0.04[b]	0.80±0.04[c]

[a]Data are expressed as fold change mean ± standard deviation (n=5-6/group)
[b]Significantly different from control group ($p< 0.01$)
[c]Significantly different from control group ($p< 0.05$)

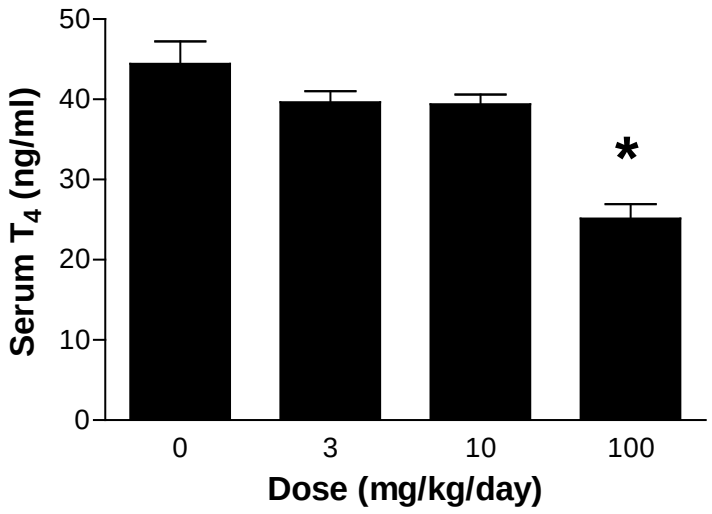


Figure A.1. Total serum T_4 concentrations in mice treated with BDE 47
*Significantly different from control group ($p < 0.0001$)
Data are expressed as mean ± standard deviation (n=9-10/group).

REFERENCES

Auyeung, D. J., Kessler, F. K., and Ritter, J. K., 2003. Mechanism of rat UDP-glucuronosyltransferase 1A6 induction by oltipraz: evidence for a contribution of the Aryl hydrocarbon receptor pathway. Mol Pharmacol. 63, 119-127.

Barter, R. A., and Klaassen, C. D., 1992. UDP-glucuronosyltransferase inducers reduce thyroid hormone levels in rats by an extrathyroidal mechanism. Toxicol Appl Pharmacol. 113, 36-42.

Birnbaum, L. S., and Staskal, D. F., 2004. Brominated flame retardants: cause for concern? Environ Health Perspect. 112, 9-17.

Bock, K. W., and Kohle, C.,2004. Coordinate regulation of drug metabolism by xenobiotic nuclear receptors: UGTs acting together with CYPs and glucuronide transporters. Drug Metab Rev. 36, 595-615.

Brouwer, A., Blaner, W. S., Kukler, A., and Van den Berg, K. J., 1988. Study on the mechanism of interference of 3,4,3',4'-tetrachlorobiphenyl with the plasma retinol-binding proteins in rodents. Chem Biol Interact. 68, 203-217.

Collins, W. T., Jr., and Capen, C. C., 1980. Biliary excretion of 125I-thyroxine and fine structural alterations in the thyroid glands of Gunn rats fed polychlorinated biphenyls (PCB). Lab Invest. 43, 158-164.

de Wit, C. A., 2002. An overview of brominated flame retardants in the environment. Chemosphere. 46, 583-624.

DeVito, M. J., Ma, X., Babish, J. G., Menache, M., and Birnbaum, L. S., 1994. Dose-response relationships in mice following subchronic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin: CYP1A1, CYP1A2, estrogen receptor, and protein tyrosine phosphorylation. Toxicol Appl Pharmacol. 124, 82-90.

Ellis-Hutchings, R. G., Cherr, G. N., Hanna, L. A., and Keen, C. L., 2006. Polybrominated diphenyl ether (PBDE)-induced alterations in vitamin A and thyroid hormone concentrations in the rat during lactation and early postnatal development. Toxicol Appl Pharmacol. 215, 135-145.

Focant, J. F., Sjodin, A., Turner, W. E., and Patterson, D. G., Jr., 2004. Measurement of selected polybrominated diphenyl ethers, polybrominated and polychlorinated biphenyls, and organochlorine pesticides in human serum and milk using comprehensive two-dimensional gas chromatography isotope dilution time-of-flight mass spectrometry. Analytical chemistry. 76, 6313-6320.

Friesema, E. C., Ganguly, S., Abdalla, A., Manning Fox, J. E., Halestrap, A. P., and Visser, T. J., 2003. Identification of monocarboxylate transporter 8 as a specific thyroid hormone transporter. J Biol Chem. 278, 40128-40135.

Hallgren, S., and Darnerud, P. O., 2002. Polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and chlorinated paraffins (CPs) in rats-testing interactions and mechanisms for thyroid hormone effects. Toxicology. 177, 227-243.

Hallgren, S., Sinjari, T., Hakansson, H., and Darnerud, P. O., 2001. Effects of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) on thyroid hormone and vitamin A levels in rats and mice. Archives of toxicology. 75, 200-208.

Hamers, T., Kamstra, J. H., Sonneveld, E., Murk, A. J., Kester, M. H., Andersson, P. L., Legler, J., and Brouwer, A., 2006. In vitro profiling of the endocrine-disrupting potency of brominated flame retardants. Toxicol Sci. 92, 157-173.

Hites, R. A., 2004. Polybrominated diphenyl ethers in the environment and in people: a meta-analysis of concentrations. Environmental science & technology. 38, 945-956.

Inokuchi, A., Hinoshita, E., Iwamoto, Y., Kohno, K., Kuwano, M., and Uchiumi, T., 2001. Enhanced expression of the human multidrug resistance protein 3 by bile salt in human enterocytes. A transcriptional control of a plausible bile acid transporter. J Biol Chem. 276, 46822-46829.

Kato, Y., Ikushiro, S., Haraguchi, K., Yamazaki, T., Ito, Y., Suzuki, H., Kimura, R., Yamada, S., Inoue, T., and Degawa, M., 2004. A possible mechanism for decrease in serum thyroxine level by polychlorinated biphenyls in Wistar and Gunn rats. Toxicol Sci. 81, 309-315.

Kato, Y., Suzuki, H., Ikushiro, S., Yamada, S., and Degawa, M., 2005. Decrease in serum thyroxine level by phenobarbital in rats is not necessarily dependent on increase in hepatic UDP-glucuronosyltransferase. Drug metabolism and disposition: the biological fate of chemicals. 33, 1608-1612.

Lans, M. C., Spiertz, C., Brouwer, A., and Koeman, J. H., 1994. Different competition of thyroxine binding to transthyretin and thyroxine-binding globulin by hydroxy-PCBs, PCDDs and PCDFs. Eur J Pharmacol. 270, 129-136.

Maglich, J. M., Stoltz, C. M., Goodwin, B., Hawkins-Brown, D., Moore, J. T., and Kliewer, S. A., 2002. Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. Mol Pharmacol. 62, 638-646.

Mitchell, A. M., Tom, M., and Mortimer, R. H., 2005. Thyroid hormone export from cells: contribution of P-glycoprotein. J Endocrinol. 185, 93-98.

Nishimura, N., Yonemoto, J., Miyabara, Y., Fujii-Kuriyama, Y., and Tohyama, C., 2005. Altered thyroxin and retinoid metabolic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin in aryl hydrocarbon receptor-null mice. Archives of toxicology. 79, 260-267.

Nishio, N., Katsura, T., Ashida, K., Okuda, M., and Inui, K., 2005. Modulation of P-glycoprotein expression in hyperthyroid rat tissues. Drug metabolism and disposition: the biological fate of chemicals. 33, 1584-1587.

Pacyniak, E. K., Cheng, X., Cunningham, M. L., Crofton, K., Klaassen, C. D., and Guo, G. L., 2007. The flame retardants, polybrominated diphenyl ethers, are pregnane x receptor activators. Toxicol Sci. 97, 94-102.

Peeters, R. P., Friesema, E. C., Docter, R., Hennemann, G., and Visser, T. J., 2002. Effects of thyroid state on the expression of hepatic thyroid hormone transporters in rats. Am J Physiol Endocrinol Metab. 283, E1232-1238.

Peters, A. K., van Londen, K., Bergman, A., Bohonowych, J., Denison, M. S., van den Berg, M., and Sanderson, J. T., 2004. Effects of polybrominated diphenyl ethers on basal and TCDD-induced ethoxyresorufin activity and cytochrome P450-1A1 expression in MCF-7, HepG2, and H4IIE cells. Toxicol Sci. 82, 488-496.

Petreas, M., She, J., Brown, F. R., Winkler, J., Windham, G., Rogers, E., Zhao, G., Bhatia, R., and Charles, M. J., 2003. High body burdens of 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) in California women. Environmental health perspectives. 111, 1175-1179.

Ribeiro, R. C., Cavalieri, R. R., Lomri, N., Rahmaoui, C. M., Baxter, J. D., and Scharschmidt, B. F., 1996. Thyroid hormone export regulates cellular hormone content and response. The Journal of biological chemistry. 271, 17147-17151.

Sanders, J. M., Burka, L. T., Smith, C. S., Black, W., James, R., and Cunningham, M. L., 2005. Differential expression of CYP1A, 2B, and 3A genes in the F344 rat following exposure to a polybrominated diphenyl ether mixture or individual components. Toxicol Sci. 88, 127-133.

Schecter, A., Pavuk, M., Papke, O., Ryan, J. J., Birnbaum, L., and Rosen, R., 2003. Polybrominated diphenyl ethers (PBDEs) in U.S. mothers' milk. Environmental health perspectives. 111, 1723-1729.

Staskal, D. F., Diliberto, J. J., DeVito, M. J., and Birnbaum, L. S., 2005. Toxicokinetics of BDE 47 in female mice: effect of dose, route of exposure, and time. Toxicol Sci. 83, 215-223.

Staskal, D. F., Hakk, H., Bauer, D., Diliberto, J. J., and Birnbaum, L. S., 2006. Toxicokinetics of polybrominated diphenyl ether congeners 47, 99, 100, and 153 in mice. Toxicol Sci. 94, 28-37.

Stoker, T. E., Laws, S. C., Crofton, K. M., Hedge, J. M., Ferrell, J. M., and Cooper, R. L., 2004. Assessment of DE-71, a commercial polybrominated diphenyl ether (PBDE) mixture, in the EDSP male and female pubertal protocols. Toxicol Sci. 78, 144-155.

Trajkovic, M., Visser, T. J., Mittag, J., Horn, S., Lukas, J., Darras, V. M., Raivich, G., Bauer, K., and Heuer, H., 2007. Abnormal thyroid hormone metabolism in mice lacking the monocarboxylate transporter 8. J Clin Invest. 117, 627-635.

Vansell, N. R., and Klaassen, C. D., 2002. Effect of microsomal enzyme inducers on the biliary excretion of triiodothyronine (T(3)) and its metabolites. Toxicol Sci. 65, 184-191.

Visser, T. J., Kaptein, E., van Toor, H., van Raaij, J. A., van den Berg, K. J., Joe, C. T., van Engelen, J. G., and Brouwer, A., 1993. Glucuronidation of thyroid hormone in rat liver: effects of in vivo treatment with microsomal enzyme inducers and in vitro assay conditions. Endocrinology. 133, 2177-2186.

Wagner, M., Halilbasic, E., Marschall, H. U., Zollner, G., Fickert, P., Langner, C., Zatloukal, K., Denk, H., and Trauner, M., 2005. CAR and PXR agonists stimulate hepatic bile acid and bilirubin detoxification and elimination pathways in mice. Hepatology. 42, 420-430.

Waxman, D. J., 1999. P450 gene induction by structurally diverse xenochemicals: central role of nuclear receptors CAR, PXR, and PPAR. Archives of biochemistry and biophysics. 369, 11-23.

Whitlock, J. P., Jr., 1990. Genetic and molecular aspects of 2,3,7,8-tetrachlorodibenzo-p-dioxin action. Annual review of pharmacology and toxicology. 30, 251-277.

Wong, H., Lehman-McKeeman, L. D., Grubb, M. F., Grossman, S. J., Bhaskaran, V. M., Solon, E. G., Shen, H. S., Gerson, R. J., Car, B. D., Zhao, B., and Gemzik, B., 2005. Increased hepatobiliary clearance of unconjugated thyroxine determines DMP 904-induced alterations in thyroid hormone homeostasis in rats. Toxicol Sci. 84, 232-242.

Xie, W., Barwick, J. L., Downes, M., Blumberg, B., Simon, C. M., Nelson, M. C., Neuschwander-Tetri, B. A., Brunt, E. M., Guzelian, P. S., and Evans, R. M., 2000. Humanized xenobiotic response in mice expressing nuclear receptor SXR. Nature. 406, 435-439.

Xiong, H., Yoshinari, K., Brouwer, K. L., and Negishi, M., 2002. Role of constitutive androstane receptor in the in vivo induction of Mrp3 and CYP2B1/2 by phenobarbital. Drug metabolism and disposition: the biological fate of chemicals. 30, 918-923.

Yamada, H., Ishii, Y., Yamamoto, M., and Oguri, K., 2006. Induction of the hepatic cytochrome P450 2B subfamily by xenobiotics: research history, evolutionary aspect, relation to tumorigenesis, and mechanism. Curr Drug Metab. 7, 397-409.

Zhou, T., Ross, D. G., DeVito, M. J., and Crofton, K. M., 2001. Effects of short-term in vivo exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. Toxicol Sci. 61, 76-82.

Zhou, T., Taylor, M. M., DeVito, M. J., and Crofton, K. M., 2002. Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption. Toxicol Sci. 66, 105-116.