The Effects of Six Organophosphate Flame Retardants on Endocrine Receptor Targets in Mammalian Cancer Cell Lines.

by

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Abstract

The effects of six selected organophosphate flame retardants (OPFRs) tris(2-butoxyethyl) phosphate (**TBOEP**), tris(2-chloroethyl) phosphate (**TCEP**), tris(1-chloro-2-propyl) phosphate (**TCIPP**), tris(methylphenyl) phosphate (**TMPP**), tris(1,3-dichloro-2-propyl) phosphate (**TDCIPP**) and triethyl phosphate (**TEP**) on the activities of the androgen-, estrogen- and aryl hydrocarbon receptors were assessed in human prostate cancer cells (LNCaP) and endometrial cancer cells (ECC-1 cells). Binding affinity for the estrogen receptor (ER) of the selected OPFRs appeared limited in ECC-1 cells, as no profound changes in ER inducible target gene expression were observed. Furthermore, the six selected OPFRs exerted few effects on the aryl hydrocarbon receptor-inducible CYP1A1 expression, although at high concentrations TMPP mildly induced gene expression. Messenger RNA and protein accumulation of androgen receptor (AR) target genes were examined for TDCIPP. Additionally, secretory PSA detection, chromatin immunoprecipitation and a ligand binding assay were performed using TDCIPP and the synthetic androgen methyltrienolone. AR inducible target gene and protein expression were significantly altered by TDCIPP exposure, as well as excreted prostate specific antigen. For the first time it was demonstrated that TDCIPP does not have binding affinity for the AR-ligand binding domain and appears to exert its anti-androgenic effects in LNCaP cells in a non-competitive fashion. Furthermore, TDCIPP exposure could adversely influence clinical outcomes for prostate cancer screenings, resulting in false negatives. Prolonged TDCIPP exposure could also carry the risk of exacerbating the progression of prostate cancer into a metastatic androgen-independent sub-type by simulating androgen deprivation.

Keywords: organophosphate flame retardants; endocrine disruptor; androgen receptor; estrogen receptor; aryl hydrocarbon receptor; LNCaP

For my parents Marylou and Martin

I owe you everything.

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List of Acronyms

1° Ab	Primary Antibody
11-KT	11 ketotestosterone
36B4	Acidic ribosomal protein P0
A	Ampère
ADT	Androgen Deprivation Therapy
AF	Activation Function
AhR	Aryl hydrocarbon Receptor
AhRC	Aryl hydrocarbon Receptor Complex
Akt	Protein kinase B
AR	Androgen Receptor
ARE	Androgen Response Element
ARNT	Aryl hydrocarbon Receptor Nuclear Translocator
bHLH	basic Helix-loop-Helix
bp	Base Pairs
BSA	Bovine Serum Albumin
c.s. FBS	Charcoal stripped Fetal Bovine Serum
cDNA	Complementary deoxyribonucleic acid
CEH	Chicken Embryonic Hepatocytes
CEN	Chicken Embryonic Neuronal Cells
CHO K1	Chinese hamster ovarian cells
CRPC	Castration Resistant Prostate Cancer
CXR	Chicken Xenobiotic Receptor
CYP11A2	Cytochrome P450 family 11, subfamily A, polypeptide 2
CYP11B2	Cytochrome P450 family 11, subfamily B, polypeptide 2
CYP19A1	Cytochrome P450 family 19, subfamily A, polypeptide 1
CYP1A1	Cytochrome P450 family 1, subfamily A, polypeptide 1
CYP1B1	Cytochrome P450 family 1, subfamily B, polypeptide 1
CYP2H1	Cytochrome P450 family 2, subfamily H, polypeptide 1
CYP3A37	Cytochrome P450 family 3, subfamily A, polypeptide 37
DBD	DNA binding domain
DHT	Dihydrostestosterone

DHT	5α-dihydrotestosterone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
E2	17-β estradiol
ECC-1	Endometrial Carcinoma Cells 1
EDC	Endocrine Disrupting Chemicals
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ER	Estrogen Receptor
ERE	Estrogen Response Element
ERK	Extracellular-Signal-Regulated Kinases
FBS	Fetal Bovine Serum
FP	Fluorescence Polarization
FSH	Follicle Stimulating Hormone
g	Gravitational force
GR	Glucocorticoid Receptor
h	Hour
H295R	Human Adrenal Corticocarcinoma
HA	Hemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His-GST	Histidine -Glutathione-S-transferase
HSD3β2	3β-hydroxysteroid dehydrogenases
Hsp	Heat Shock Protein
IGF-1	Insulin-like Growth Factor 1
IgG	Immunoglobulin G
kDa	kilo Dalton
L-FABP	Liver Fatty Acid-Binding Protein
LBD	Ligand binding domain
LBD	Ligand Binding Domain
LC50	Lethal concentration
LD50	Lethal dose
LH	Luteinizing Hormone

LiCl	Lithium Chloride
LNCaP	Lymph Node Cancer of the Prostate
MCF-7	Michigan Cancer Foundation-7, human breast cancer cell line
min	Minutes
mM	Milli Mole
mM	Milli Mole
mRNA	messenger Ribonucleic Acid
MVLN	Stably transfected MCF-7 cells with a luciferase reporter gene
NaCl	Sodium Chloride
$NaHCO_3$	Sodium Bicarbonate
NaOH	Sodium Hydroxide
nM	nano Mole
NOEL	No observed effects level
OPFR	Organophosphate Flame Retardant
P.I.	Protease Inhibitor
P/S	Penicillin/Streptomycin
PAS	PER/ ARNT/ the signal minded protein domain
PBDE	Polybrominated diphenyl ethers
PBS	Phosphate Buffered Saline
PC-12	Rat Adrenal Medulla Cell Line
PCR	Polymerase Chain Reaction
PFR	Phosphorous Flame Retardant
pg	piko grams
pH	Negative log of the activity of the hydrogen ion in aqueous solutions
PI3K	phosphoinositide 3 kinase
PPAR	Peroxisome Proliferator-Activated Receptors
pS2	Trefoil Factor 2
PSA	Prostate Specific Antigen
PSMA	Prostate Specific Membrane Antigen
PVDF	Polyvinylidene fluoride
PXR	Pregnane X receptor
R1881	Metribolone
rpm	Revolutions Per Minute

RPMI	Roswell Park Memorial Institute Medium
RTK	Receptor Tyrosine Kinases
s	Seconds
S9	Liver Tissue Homogenate to Assess Metabolism of Drugs and Xenobiotics
SDS	Sodium dodecyl sulfate
SEM	Standard error mean
SHGB	Sex hormone-binding globulin
src	A proto-oncogene tyrosine-protein kinase
SULT1E1	Sulfotransferase family 1E, estrogen-preferring, member 1
SULT2A1	Sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone -preferring, member 1
T	Testosterone
T_4	Serum Free Thyroxine
TBOEP	Tris(2-butoxyethyl) phosphate
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TCEP	Tris(chloroethyl) phosphate
TCIPP	Tris(2-chloroisopropyl) phosphate
TDCIPP	Tris(1,3-dichloroisopropyl) phosphate
TDI	Total Daily Intake
TE8	Tris-EDTA, pH 8
TEP	Tris(ethyl) phosphate
THR	Thyroid Hormone Receptor
TMPP	Tris (methlyphenyl) phosphate
TR	Thyroid hormone receptor
Tris-HCl	Tris(hydroxymethyl)aminomethane-hydrochloric acid
UGT1A9	Uridine 5'-diphospho-glucuronosyltransferase Glucuronosyltransferase 1 family, polypeptide A9
V	Volts
V79	Hamster Fibroblast Cell Line
VCaP	Vertebral Cancer of the Prostate
VGT	Vitellogenin
XRE	Xenobiotic Response Element

Chapter 1.

Introduction

1.1. Endocrine Disrupting Chemicals

Endocrine disrupting chemicals (EDCs) are exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body which are responsible for the maintenance or homeostasis, reproduction, development and or behavior (Kavlock et al., 1996). The United State Environmental Protection Agency adopted this definition with the addition that endocrine disruption is not considered an adverse effect *per se*, but rather to be a mode or mechanism of action potentially leading to other outcomes, for example carcinogenic, reproductive, or developmental effects (Crisp, Clegg, Cooper, & Anderson, 1997). Many humans are exposed to EDCs through occupational, accidental, environmental exposure and pharmaceutical usage (Hotchkiss et al., 2008). However, due to the ubiquitous usage of chemicals such as pesticides, plasticizers, flame retardants, lubricants and solvents in industry and the environment, common routes of exposure may occur through food, drinking water, air, soil, house dust, house hold materials and consumer goods (Meeker, 2012). Historically many chemical compounds were used for their beneficial properties to improve various aspects of human life, however often the knowledge on these chemicals in regards to their potential adverse effects on humans, biota and the environment were very limited or non-existent. Many compounds have since been identified as compounds with endocrine activity in humans and animals exerting adverse effects (Diamanti-Kandarakis et al., 2009). The endocrine system is involved in many regulating female and male reproductive and neuroendocrine systems as well as the thyroid hormone, metabolic and cardiovascular physiologies. Thus, EDCs can act on various targets.

Direct physiological effects due to EDC exposure in humans are difficult to deduce, as several factors convolute the potential causation (Gans & Crews, 1992):

1. Age at exposure. Individuals exposed during a critical life stage (sexual differentiation, puberty, etc) tend to be more susceptible to adverse outcomes than adult organisms.

2. Latency of effects. Sub-toxic exposure to developing organisms may not have acute effects, but may predispose the adult organism to diseases or disorders.

3. Sensitivity to EDCs. Species and strain dependent sensitivity to EDCs.

4. Degradation and Metabolism; Mixtures and Synergism of EDCs. The metabolites produced by the host of an EDC can be more reactive or toxic than the parent compound. Often organisms are exposed to a mixture of compounds that can interact with each other synergizing the adverse outcomes.

5. Non-traditional dose-response dynamics.

Thus, for most EDCs data on direct causation for physiological effects is limited and mostly correlative. However, some of the adverse effects of these compounds are more established. In humans, EDCs are associated with sperm abnormalities, shortened anogenital distance in boys, altered sex ratio, advanced onset of puberty, endometriosis, cryptorchidism, obesity, attention deficit disorder, allergies (Hotchkiss et al., 2008) and testis-, prostate-, breast-, and uterine cancer (Soto & Sonnenschein, 2010).

EDCs can exert their effect through various mechanisms such as non-nuclear steroid receptors (e.g. membrane bound ER), non-steroid receptors (e.g. neurotransmitter receptors), orphan receptors (e.g. aryl hydrocarbon receptor (AhR)) transcriptional co-activators or enzymatic pathways involved in steroid biosynthesis and/or metabolism (Diamanti-Kandarakis et al., 2009). More recently it was discovered EDCs can affect genes directly as well as their epigenetic disposition. In other words,

EDCs interfere with genes that are directly involved in DNA methylation and histone acetylation, thus altering the epigenome of the organism. These alterations may cause multi-generational and transgenerational physiological effects (Schug, Janesick, Blumberg, & Heindel, 2011).

Traditionally research has focused on the interaction between EDCs and the nuclear hormone family of transcription factors. EDCs can interact solely through the androgen receptor (AR), estrogen receptor (ER), progesterone receptor (PR) and the thyroid hormone receptor (TR) by acting either as direct antagonists or agonists. Adverse effects become apparent downstream and may affect homeostasis of the organism and/or contribute to disease. More detailed information on some of the nuclear hormone receptors will be provided in Section 1.4.1 and 1.4.2. Exogenous chemical compounds with endocrine activity are phthalates, polychlorinated biphenyls, polybrominated diphenyl esters (PBDEs), and dioxins activating multiple different hormone receptors (Table 1.1).

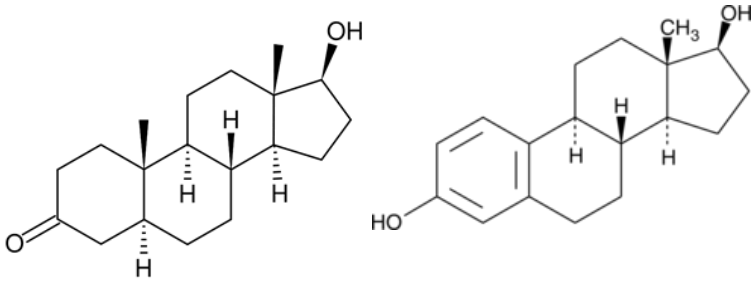
Table 1.1. Select human nuclear receptors and related functions.

Receptor	Abbreviation	Physiological Function	Endogenous ligand	Examples of endocrine Disrupting chemicals
Androgen	AR	Male sexual development	Testosterone	Pesticides
				Phtalates
				Plasticisers
				Polyhalogenated compounds
Estrogen	ERα, β	Female sexual development	Estradiol	Alkylphenols
	GPR30 (non-nuclear)			BPA
				Dioxins
				Furans
				Halogenated hydrocarbons
				Heavy metals
Aryl hydrocarbon	AhR	Circadian rhythm	Unknown	Dioxins
		Metabolism		Flavonoids
		Neurogenesis		Herbicides
		Organ development		Indoles

	Stress response	PCBs Pesticides

adapted from (Schug et al., 2011)

Structural similarity between EDCs and the endogenous ligands or between different EDCs itself are not always apparent. Yet many EDCs have lipophilic properties, are small in mass (< 1000 Daltons), have a phenolic moiety, which is thought to mimic endogenous hormone, and/or have halogenated substitutions such as chlorine or bromine (Diamanti-Kandarakis et al., 2009; Schug et al., 2011). Endogenous ligands, for the AR and ER receptor, for example, are dihydrotestosterone (DHT) and 17-β estradiol (E2), respectively (Figure 1.1).



Dihydrotestosterone 17-β estradiol

Figure 1.1. Chemical structures of endogenous hormones, DHT and E2.

1.2. Flame retardants

Flame retardants (FR) are chemical substances used in various products such as plastics, textiles and furnishing foam to reduce their fire hazards by interfering with the combustion of the polymeric materials (F. Rahman, Langford, Scrimshaw, & Lester, 2001). As early as 450 BC alum was used by the Egyptians to reduce the flammability of wood and roughly 200 BC a mixture of alum and vinegar was used to reduce the combustibility of wood (Alaee, 2003).

Today FRs can be grouped into four classes: inorganic, halogenated organic, organophosphorus and nitrogen based. The combustion of solid material or a gas occurs in four stages: preheating, volatilization/decomposition, combustion and propagation (Troitzch, 1990). FR can interfere with any of these processes. There are five

mechanisms by which flame retardancy is achieved: physical dilution (e.g. talc), chemical interaction (e.g. brominated FRs), inert gas dilution (e.g. metal hydroxides), thermal quenching (e.g. metal hydroxides and carbonates) or protective layering (e.g. phosphorous compounds) (USEPA, 2005). Organic halogenated and organophosphate based FRs mostly interfere with the radical formation in the propagation phase (chemical interaction), thus preventing a flame from spreading.

Halogenated FRs are extremely efficient in capturing free radicals and thus preventing the spread of the flame. It is essential that the FRs decompose at a slightly lower temperature than the host polymer to assure the decomposition/volatilization of the FR at the time before ignition of the host polymer. Hence thermal stability of the FR in respect to the polymer is of importance and aromatic FRs often are more stable than halogenated aliphatic compounds and therefore are the preferred compound (Alaee, 2003; F. Rahman et al., 2001). FRs can be incorporated into polymers additively or covalently. Additive FRs are more prone to leech or volatilize from the polymer into the environment over time and potentially cause adverse effects (de Wit, 2002; Marklund, Andersson, & Haglund, 2003; F. Rahman et al., 2001).

Polybrominated diphenyl ethers (PBDEs) are a group of chemicals that have been extensively used as FRs in various materials due to their advantageous properties in delaying the spread of fire. However, they have been now identified as endocrine disruptors and a ubiquitous contaminant in the environment (Darnerud, 2008; de Wit, 2002; Winter, Williams, & Elliott, 2013). PBDEs have historically been the most extensively used BFRs on the market since 1970 (Darnerud, 2008; de Wit, 2002; F. Rahman et al., 2001). Mainly PBDEs were used in electronic equipment (e.g. TV sets, computers, circuit card boards), building materials, high impact polystyrene and flexible polyurethane foam (Alaee, 2003; F. Rahman et al., 2001). Due to this wide and extensive use, multiple PBDE congeners are measurable in the environment (Darnerud, Eriksen, Jóhannesson, Larsen, & Viluksela, 2001), biota (Frouin, Lebeuf, Hammill, Sjare, & Fournier, 2011; Hale et al., 2001) and human tissues (e.g. breast milk, blood plasma, (Darnerud, 2008)) and will be for a considerable amount of time, as they are persistent and bioaccumulative (de Wit, 2002). In the early 1990's concern arose over the health safety of these compounds and nearly a decade later, sufficient data on endocrine

disruption (Meeker, Johnson, Camann, & Hauser, 2009a; Schreiber et al., 2009) had accumulated leading to a voluntary phase out of certain congeners by the largest manufacturer in 2004 (USEPA, 2005) and strict bans on the worldwide use shortly after (Directive ECC, cited in (Covaci et al., 2011). Consequently, replacement compounds were needed that would provide the equivalent fire hazard protection.

1.2.1. Organophosphate Flame Retardants - Emerging Contaminants

Phosphorous flame retardants are a group of chemicals that have been in use for over 150 years (Andrae, 2007). Flame reducing activities of organophosphate flame retardants (OPFRs) had been known and their first usage was recorded in the early 1970's for the treatment of children's sleepwear (Gold, Blum, & Ames, 1978). Since then OPFRs have been produced in high volumes and have been added to plastics, polymers, foams, textiles, electronics, construction material, floor polishes, waxes and furniture (Marklund, Andersson, & Haglund, 2005; Meeker & Stapleton, 2009; Reemtsma, Quintana, Rodil, Garcı a-López, & Rodrı guez, 2008; van der Veen & de Boer, 2012). The production of OPFRs has been increasing with the phase out of several BFRs (Reemtsma et al., 2008). OPFRs have been considered 'emerging pollutants' as they have not been included in previous national or international regulation and/or monitoring programs. However due to anthropogenic activities the compounds are being released into the environment continuously and extensively. Furthermore, data on their environmental fate and (eco)toxicological properties have not been evaluated sufficiently (Reemtsma et al., 2008).

When this project was initiated, studies on OPFRs were scarce. However over the last four years a considerable body of data has accumulated. The following Chapters and Sections will provide information available in the literature on six specific OPFRs (tris(2-butoxyethyl) phosphate (**TBOEP**), tris(2-chloroethyl) phosphate (**TCEP**), tris(1-chloro-2-propyl) phosphate (**TCIPP**), tris(methylphenyl) phosphate (**TMPP**), tris(1,3-dichloro-2-propyl) phosphate (**TDCIPP**) and triethyl phosphate (**TEP**); (Figure 1.2) and their effect in the environment, biota and humans. The six OPFRs of interest were

chosen due to their prioritization in the Chemical Management Plan by Environment Canada.

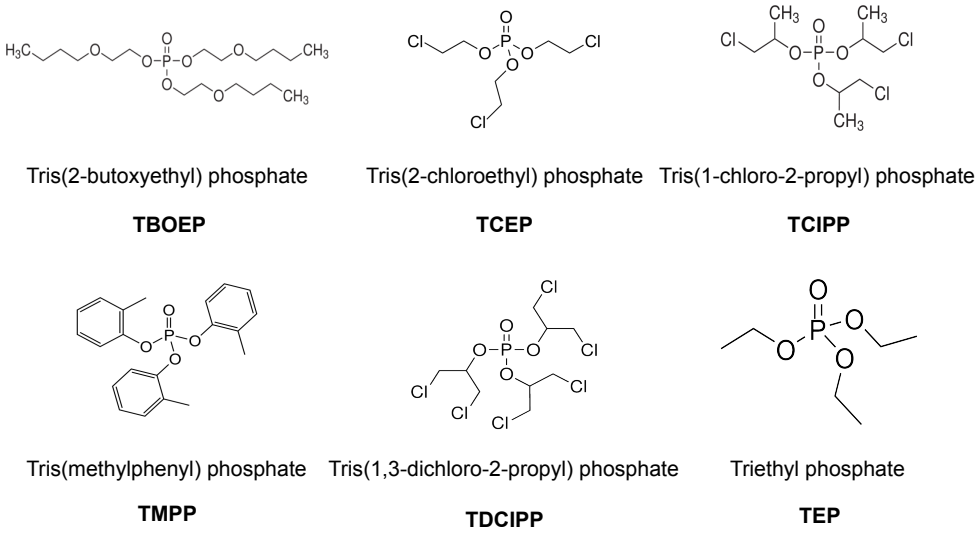


Figure 1.2. Six OPFRs of interest for this project.
Abbreviations for OPFRs are after the abbreviation standard by Bergman et al. (2012).

In the following Section, a literature review on the previously mentioned OPFRs are presented. Furthermore it includes theoretical background on selected transcription factor receptors. Chapter 2 describes the Materials and Methods applied in this project. Chapter 3 presents the results from the conducted experiments. Chapter 4 concludes the thesis with a discussion and analysis of the results including limitations and suggests direction for future work.

1.3. Summarizing Toxicological Information of OPFRs of Interest

In summary, OPFRs are increasingly used in a variety of products such as construction materials, furniture, plastics electronics, textiles and other materials and fluids (Meeker & Stapleton, 2009; Reemtsma et al., 2008; Stapleton et al., 2009). As OPFRs are not covalently bound to the treated materials, they are prone to leaching, volatilization and/or abrasion into the outdoor (air, soil and water) and indoor environment (house dust and air) (Marklund et al., 2003). Consequently, over recent

years OPFRs have been detected in the environment, biota and humans, such as in surface and sewage water (Regnery & Püttmann, 2010), house dust (Meeker & Stapleton, 2009; Stapleton et al., 2009), fish, mussels, breast milk (Sundkvist, Olofsson, & Haglund, 2010) and their metabolites in urine (E. M. Cooper, Covaci, Nuijs, Webster, & Stapleton, 2011; Meeker, Cooper, Stapleton, & Hauser, 2013). So far the bioaccumulation potential of OPFRs in the environment have not been studied extensively (Chen, Letcher, & Chu, 2012). However, several *ex/in vivo* studies including avian hepatocytes, neuronal cells, herring gull eggs and zebra fish larvae/embryos/adults revealed alterations in gene expression levels associated with steroidogenisis, lipid regulation, the thyroid hormone pathway and growth as well as adverse effects on fecundity, survival and hatching success when exposed to various OPFRs (Crump, Chiu, & Kennedy, 2012; C. Liu et al., 2013a; X. Liu, Ji, & Choi, 2012; X. Liu, Ji, Jo, Moon, & Choi, 2013b).

Other studies focusing on the effects of OPFRs on human blood samples and cell lines described alterations in (sex) hormone levels and synthesis, (nuclear) receptor related mRNA responses and sperm quality parameters (Kojima et al., 2013; X. Liu et al., 2012; Meeker & Stapleton, 2009; Stapleton et al., 2009).

Human exposure data is limited for a majority of the selected OPFRs. However, the estimated exposure to flame retardants by inadvertent dust ingestion is 1600 ng/day for children. The daily exposure for adults to flame retardants is considerably lower at 325 ng/day (Stapleton et al., 2009).

For the selected OPFRs urinary excretion appears to be the main pathway of excretion. Under metabolic conditions, the OPFRs are often reduced by one ester group to a dieester and consequently excreted via the urinary pathway (Sjögren, Iregren, & Järnberg, 2010).

Physico-chemical data on all OPFRs is presented in Table 1.2. For further detailed background information on the selected OPFRs see Sections 1.3.1 and 1.3.2.

Table 1.2. Physico-chemical data for selected OPFRs

OPFR	Halogenated?	Molecular Weight	Boiling point (°C)	Melting point (°C)	Solubility in water (mg/L)	Vapor pressure (mm Hg)	log K_{OW}	Bioconcentration factor
TBOEP	No	398.47	414.0	-70.0	62.80	2.6×10^{-8}	3.65	1080.00
TMPP	No	368.36	439.0	77.0	0.36	1.8×10^{-7}	5.11	8560.00
TEP	No	182.16	216.0	-56.0	5.0×10^{5}	0.29	0.80	3.88
TCEP	Yes	285.49	351.0	-55.0	7000.00	1.1×10^{-4}	1.44	1.37
TCIPP	Yes	327.56	359.0	72.0	1600.00	1.9×10^{-9}	2.59	42.40
TDCIPP	Yes	430.91	457.0	88.0	1.50	7.4×10^{-8}	3.80	13.50

1.3.1. Non-halogenated OPFRs

TBOEP, TMPPP and TEP are the non-halogenated OPFRs of interest and will be discussed individually in further detail in the following sections.

TBOEP

Physical and Chemical Properties. TBOEP is a light coloured, high boiling temperature, viscous liquid with a butyl-like odour. TBOEP dissolves well in non-polar solvents. The technical mixture may contain up to 3 % tri-bromo phenol, 2-butoxyethanol and phosphoric acid as impurities (Van Esch, 2000).

Occurrence in the Environment. TBOEP has been detected in fish samples taken downstream from a sewage treatment plant in Sweden with concentrations ranging from 36-140 ng/g lipid weight. Furthermore TBOEP has been measured in perch in a stream and snow samples taken from the roadside close to an airport in Sweden (Sundkvist et al., 2010). A study from China revealed high concentrations of TBOEP in lake sediment near densely populated, industrial and agricultural areas (Cao et al., 2012).

Further studies investigated the occurrence of TBOEP in muscle tissue from domestic chickens (*Gallus gallus domesticus*) and ducks (*Anas platyrhynchos domesticus*), in which high levels of TBOEP and other OPFRs were found (Yongqing Ma et al., 2013). A study conducted by McGoldrick and colleagues (2014) detected levels of TBOEP in Canadian fish in several lakes, albeit at low concentrations of < 10 ng/g wet weight. However, the suggestion from this study was to conduct further research on environmental degradation products and metabolites in aquatic biota. Letcher et al. (2011, 2012; cited in Egloff et al. 2014) monitoring several fish species and high trophic levels in avian species in the Great Lakes reported an increasing trend of TBOEP concentrations over the last two decades.

Human Exposure Route. TBOEP has been detected in indoor office space air in the United States with mean concentrations of 0.015 $\mu g/m^3$ (Van Esch, 2000). TBOEP is also found in floor polish, which may contain up to 1 % of the OPFR (Nakashima et al. 1993 cited in Sjögren, Iregren, & Järnberg, 2010). One example of particle exposure was demonstrated in Austria, where the floor dust of a new office building contained 4.3-7.8 g TBOEP/kg dust. After removal of the coating the TBOEP concentration in the dust gradually declined, until 3 months later the concentrations remained at 90 mg/kg (Hutter et al., 2006). Adipose tissues collected from male (30.9 %) and female (42.6 %) corpses contained traces of TBOEP (LeBel et al., 1986 cited in Sjögren, Iregren, & Järnberg, 2010). In a follow up study mean concentrations of 396 ng TBOEP/g tissue was detected in adipose tissues collected in municipalities in Ontario and Toronto (LeBel et al., 1989 cited in Sjögren, Iregren, & Järnberg, 2010). Furthermore, breast milk samples taken from primiparous women from 1997-2006 in Sweden revealed concentrations of TBOEP ranging from “not detected” to 63 ng/g lipid (Sundkvist et al., 2010). TBOEP concentrations were higher in breast milk samples collected earlier in the study than recently taken samples. Although the authors of this study could not exclude potential contamination of the older samples with OPFRs through laboratory equipment, their findings were in accordance with previous findings that TBOEP and other chlorinated OPFRs dominate in indoor dust, while TCIPP, TCEP and others are most abundant in indoor air (Sundkvist et al., 2010).

Toxicity Data. Toxicity data on TBOEP is limited, however some data exists from avian & mammalian species. An LD50 (lethal dose of 50 %) was established for adult hens at 5000 mg/kg body weight (Carrington et al., 1990). Porter et al. (2014) established an LC50 for chicken embryonic hepatocytes at 62 μM. High oral doses of TBOEP (15 000 mg/kg) to rats had no effect on reproductive system or embryo toxicity in rats (IPCS, 2000 cited in (Egloff et al., 2014).

Cellular and Molecular Effects. Data on endocrine disruption potential on TBOEP is scarce, however at 1 mg/mL TBOEP increased sex hormone production of E2 and testosterone (T), as well as increased the ratio of E2/T significantly in H295R cells. TBOEP also influenced the transcription of steroidogenic genes CYP11A1 and CYP11B2 in a concentration dependent manner. SULT1E1 and SULT2A1 genes were down regulated by TBOEP at 10 mg/L. CYP11B2 mainly regulates the synthesis of aldosterone, a steroid hormone involved in blood pressure regulation. Whereas, CYP11A1 is a mitochondrial enzyme involved in converting cholesterol to pregnenolone. SULT1E1 is major enzyme responsible for the inactivation of E2. SULT enzymes may inactivate E2 or precursors by sulfonation, therefore down regulation of such may suggest that TBOEP causes an increase in E2 concentration (X. Liu et al., 2012). The effects TBOEP displayed here were at extremely high concentrations and were not environmentally relevant (X. Liu et al., 2012).

TBOEP showed strong agonist activity for the human (pregnane X receptor) PXR in transiently transfected Chinese hamster ovary cells (CHO) K1 (Kojima et al., 2013). It did not reveal agonist or antagonist activity for the AR or ER. *In ovo* injections of TBOEP in chicken eggs did not affect embryonic viability, even at the highest dose of 45, 400 ng/g egg weight. However TBOEP did cause a relatively small, but significant decrease in chicken embryonic body mass of 9 % (Egloff et al., 2014). A slight mRNA up regulation of CYP3A37 was observed with a maximum 5-fold increase. Induction of CYP3A37 suggests the activation of the chicken xenobiotic receptor, which is the analog to the mammalian PXR and constitutive androstane receptor (CAR) (Egloff et al., 2014). No effects were observed for genes associated with the thyroid hormone pathway and a subtle effect was observed regarding genes associated with the xenobiotic metabolism

(Egloff et al., 2014). Finally TBOEP reduced cell viability below 80 % at 100 mg/mL and greater in human adrenocortical carcinoma (H295R) cells (X. Liu et al., 2012).

TMPP

Physical and Chemical Properties. TMPP (CAS 1330-78-5) is a non-flammable, almost colourless, viscous liquid with a subtle aromatic odour (Sjögren et al., 2010), which is a mixture of mainly three isomers: triortho cresylphosphate (TOCP) (CAS 78-30-8), trimeta cresylphosphate (TMCP) (CAS 563-04-2), and tripara cresylphosphate (p-TCP) (CAS 78–32-0). For this study the commercially available isomer mixture was used.

Human Exposure Route. Occupational exposure to TMPP has been recorded in the breathing zone of bench workers using a synthetic oil containing 1-5 % TMPP. The concentrations of TMPP were between 24-280 μg/m^3 (Solbu et al., 2007).

Toxicity Data. TOPC contained in the isomeric mixture of TMPP could be metabolized to neurotoxic derivatives (National Toxicology Program, 1994). TOPC is readily taken up dermally by cats (Schroeder et al., 1991 cited in Sjögren, Iregren, & Järnberg, 2010) and European ferrets (*Mustela putorius furo*) and resulted in neurological adverse effects, such as ataxia to partial paresis (Stumpf et al., 1989 cited in Sjögren, Iregren, & Järnberg, 2010). In cats the dermally given dose was recovered in the urine (28 %) and the feces (20 %) (Schroeder et al., 1991 cited in Sjögren, Iregren, & Järnberg, 2010). Treon et al. (1955) assessed the effects of commercially available TMPP mixtures in rabbits and found the minimum lethal dose varied between 0.4-3.2 mL/kg bw depending on the formulation (Sjögren et al., 2010). However, it should be noted that commercial mixtures at the time contained significant higher levels of TOCP and other toxic isomers compared to formulations available today (Sjögren et al., 2010).

Cellular and Molecular Effects. TMPP reduced cell viability below 80 % at 10 mg/mL and greater in H295R cells (X. Liu et al., 2012). TMPP up regulated HSD3β2, a steroid-metabolizing enzyme essential for adrenal production of mineralocorticoids and glucocorticoids, in H295R cells. This up regulation of HSD3β2 mRNA suggests the potential to interfere with cortisol production (X. Liu et al., 2012). TMPP increased sex

hormone production of E2 and testosterone (T), as well as shifted the ratio of E2/T significantly in H295R cells, although not in a dose-dependent manner (X. Liu et al., 2012). Furthermore CYP19A1 transcription was up regulated, the product of which is involved in converting T into E2. Congruously, these effects were observed in male adult zebra fish. Therefore, TMPP exerts endocrine disrupting activity in male zebra fish and suggests potential for endocrine disruption in humans due to effects observed in human H295R cell line (X. Liu et al., 2012). The endocrine disruption is established by a hormonal imbalance. In contrast, in MVLN cells (modified endometrial carcinoma cells stably expressing a luciferase reporter gene) TMPP revealed ER antagonist activity at concentrations 0.01 mg/L and higher (X. Liu et al., 2012).

TEP

Chemical and Physical Properties and Occurrence in the Environment. TEP is a colourless liquid. Reports on TEP in the environment were limited, but have been detected in indoor air and dust, in sediment and water (Bollmann, Möller, Xie, Ebinghaus, & Einax, 2012; Brommer, Harrad, Van den Eede, & Covaci, 2012; Marklund et al., 2003; Saito, Onuki, & Seto, 2007). TEP appears easily degradable *in vivo*, and potential in the environment and is consequently not readily found in the environment; see **Toxicity Data**.

Human Exposure Route. Human exposure to TEP is most likely through indoor air, as TEP migrates out of equipment and foams, such as mattresses. Commonly it is combined with polymers additively. Saito and colleagues (2007) assessed the leeching potential of TEP and other OPFRs out of electrical equipment, ceiling and wall coverings and mattresses and found the main route of exposure for humans is most likely through indoor air as TEP migrates easily out of the products.

Toxicity Data. TEP was given to rats and mice orally or intraperitoneally and a metabolite of TEP (90 % within 16 hours) was found in the urine (A. R. Jones, 1970). In chicken eggs that were injected with TEP and homogenized after pipping, TEP was not detectable suggesting complete metabolism/ degradation by the time of pipping (Egloff et al., 2014).

Cellular and Molecular Effects. TEP did not display agonist or antagonist activity for the human ERα, ERβ, glucocorticoid receptor (GR), thyroid hormone receptor α_1 ($TR\alpha_1$), $TR\beta_1$, retinoic acid receptor, retinoid X receptor α, PXR, peroxisome proliferator-activated receptor α (PPARα) and PPARβ in transiently transfected CHO K1 or COS (fibroblast like cells derived from monkey kidney cells) cells (Kojima et al., 2013).

TEP affected genes associated with phase I/II metabolism, lipid metabolism and oxidative stress in CEH at 1 μM and 300 μM (E. Porter, Crump, Egloff, Chiu, & Kennedy, 2014). Chicken egg injections with TEP at 241 000 ng/g resulted in a decrease of embryonic viability to 68 % (Egloff et al., 2014). This also resulted in reduced mean tarsus length and increased mean liver mass, thus leading to an increased liver somatic index (LSI). Sixty percent of livers in the high treatment groups also revealed a noticeable discolouring of the liver from the regular yellow to olive green (Egloff et al., 2014). Chicken embryos treated with more than 43,000 ng/g (equivalent to 2.36 mM/g) displayed a reduction in gall bladder size in a concentration dependent fashion and an increased level of circulating bile acid. These findings suggest impairment with the enterohepatic circulation system of bile acid to the gall bladder causing the discolouration. Furthermore, CYP3A37 and UGT1A9 mRNA were up regulated at high concentrations of TEP (Egloff et al., 2014). UGT1A9 decreases lipophilicity of xenobiotics and endogenous molecules by conjugating them with hydrophilic groups, thus expediting urinary and biliary excretion (Gamage et al., 2006). Induction of CYP3A37 suggests the activation of the chicken xenobiotic receptor, which is the analog to the mammalian PXR and CAR (Egloff et al., 2014). In the same study TEP reduced free circulating T_4 at all concentrations (8 -241, 500 ng/g) and increased the expression of liver fatty acid binding protein (LFABP) in chicken embryos. The LFABP is a protein in the liver that is associated with fatty acid transport, uptake and metabolism and has been linked to the thyroid hormone pathway (Q Wang, 2006).

1.3.2. Halogenated OPFRs

The halogenated OPFRs of interest are TCEP, TCIPP and TDCIPP and will be discussed individually in further detail in the following sections. These three halogenated

compounds are often the most prevalent OPFRs found in the environment (Salamova, Ma, Venier, & Hites, 2013), and are therefore of significant interest.

TCEP

Chemical and Physical Properties. TCEP is a colourless to pale yellow liquid with slight odour.

Occurrence in the Environment. Surprisingly, TCEP has been detected in fish from remote lakes in Canada with low human population densities, such as Great Bear Lake, Kusawa Lake and Lake Athabasca (McGoldrick et al., 2014). In the same study TCEP was not detected in fish samples taken from the Great Lakes. Fish sampled from the northern lakes were older than the fish collected from the Great Lakes, this could account for the absence of TCEP in the young fish. TCEP however has received a score, assessing its potential as a persistent organic pollutant, similar to that of known Arctic contaminants, suggesting TCEP is likely to undergo global transport via the atmosphere (McGoldrick et al., 2014; Reemtsma et al., 2008) and thus accumulate in the Northern Hemisphere.

Human Exposure Routes. TCEP was a commonly used OPFR, until in 1993 indoor air pollution emitted from wall coverings and potential carcinogenic effects were reported (Matthews, Eustis, & Haseman, 1993). In Japan TCEP was consequently replaced by TCIPP (Saito et al., 2007). In indoor environments, TCEP has been detected in acoustic ceilings (68 g/kg), polyurethane soft foam (20 g/kg) and wood preservation coatings (10 g/kg) (Ingerowski, Friedle, & Thumulla, 2001) as well as in indoor air in German homes and schools (Sjögren et al., 2010). TCEP levels of 2.1- 8.2 ng/g lipid has been detected in breast milk samples taken between 1997-2006 in Sweden from primiparous women (Sundkvist et al., 2010). Human exposure may vary geographically as exposure to TCEP from indoor air and dust as been lower in New Zealand for example compared to Belgium, Japan, Spain and Sweden. These differences may be due to the usage of different OPFRs for furniture in New Zealand (Ali et al., 2012).

Toxicity Data and Toxicokinetics. Single oral doses of 50 µM radiolabelled TCEP/kg body weight were administered to Wistar rats and absorption was calculated. As soon as three hours post treatment TCEP was measured throughout the body (urine, fecal matter, blood and exhaled air). 168 h after treatment 1.7 % had expired through exhalation and 96 % of the administered dose had been excreted via the urine (Minegishi et al., 1988). The metabolites in the urine had been identified as bis(2-chloroethyl) carboxymethyl phosphate, bis(2-chloroethyl) hydrogen phosphate, and bis(2-chloroethyl)-2-hydroxyethyl phosphate glucuronide (Minegishi et al., 1988). A similar study revealed 40 % excretion in rats and 70 % excretion in mice of a 175 mg/kg body weight dose 8 h post-treatment (Burka et al, 1991 cited in Sjögren, Iregren, & Järnberg, 2010) .

Cellular and Molecular Effects. TCEP was weakly cytotoxic above 10 µM in the presence of S9 fraction in V79 cells (hamster fibroblasts) and did not induce DNA strand breaks (Föllmann & Wober, 2006). Furthermore TCEP was not mutagenic assessed with the Ames test and did not have estrogenic or anti-estrogenic effects in human endometrial carcinoma cells modified with the recombinant yeast reporter (Föllmann & Wober, 2006). Chronic exposure to TCEP causes carcinogenicity and brain degenerative lesions in F344 rats (Matthews et al., 1993). Acute effects of TCEP exposure included an increase in ambulatory activity in male ICR mice caused by a neurochemical mechanism (Umezu, Yonemoto, Soma, & Suzuki, 1998). Thyroid hormone activity in undifferentiated PC-12 cells was not affected by TCEP exposure for 24 h at 50 µM and therefore shifted the differentiation towards the cholinergic phenotype (Dishaw, Macaulay, Roberts, & Stapleton, 2014).

A different study also investigating the effects of TCEP on PC-12 cells reported effects on cell viability at 80 µM and 150 µM (Ta et al., 2014). Apoptosis of PC-12 cells increased with increasing concentration of TCEP (40 µM – 200 µM). This study furthermore reported effects on cell morphology and neurotoxicity by assessing mRNA and protein levels. The effected genes and proteins were proposed as biomarkers for neurotoxicity, however a mechanism of action for TCEP could not be identified (Ta et al., 2014). TCEP reduced cell viability below 80 % at 100 mg/mL and greater in H295R cells (X. Liu et al., 2012). Exposure to TCEP for H295R cells TCEP (10 mg/mL) up regulated

HSD3β2, a steroid-metabolizing enzyme essential for adrenal production of mineralocorticoids and glucocorticoids, in H295R cells. This up regulation of HSD3β2 mRNA suggests the potential to interfere with cortisol production (X. Liu et al., 2012). Additionally CYP enzymes (CYP11A1, CYP11B2, CYP19A1) and SULT enzymes (SULT1E1 and SULT2A1) were up regulated or down regulated at the highest concentration of 10 mg/mL in H295R cells, respectively. E2, T and the E2/T ratio was significantly up regulated in H295R cells, indicating with the findings mentioned above, that TCEP causes an increase in the production and conversion of E2, as well as an inhibition of inactivating E2 (X. Liu et al., 2012). This research group did not continue to confirm these findings in vivo, as done for other OPFRs in zebrafish. TCEP did not display agonist or antagonist activity for the human ERα, ERβ, GR, $TR\alpha_1$, $TR\beta_1$, retinoic acid receptor, retinoid X receptor α, PXR, PPARα and PPARβ in transiently transfected CHO K1 or COS cells (Kojima et al., 2013).

TCIPP

Physical and Chemical Properties. TCIPP is a clear, colorless liquid, containing halogen substitutes. The vapor pressure is low (Table 1.2) and therefore transmission of TCPP from open sources into indoor air can not be excluded (van der Veen & de Boer, 2012). The trade product consists of an isomeric mixture.

Occurrence in the Environment. TCIPP has been extensively used as a substitute for the OPFR TCEP, as it is structurally very similar to TCEP. In 2006 between 4500 and 20 000 metric tons of TCIPP were produced in the United States (Stapleton et al., 2011). In 2000 European production encompassed nearly 36 000 metric tones/year of TCIPP (European Union, 2008 cited in (Farhat et al., 2013)). TCIPP is by volume the most important FR in Europe. TCIPP and other OPFRs have been detected in gull eggs from Lake Huron with concentrations ranging from not detectable to 4.1 ng/g wet weight. Although TCIPP has a fairly low K_{OW}, it appears that there is the potential for bioaccumulation (Chen et al., 2012). Sundkvist and colleagues (2010) identified TCIPP in perch collected from a Swedish stream and lake with concentrations ranging from 170 to 770 ng/g lipid. Human breast milk samples taken for the same study found TCIPP with a concentration median of 45 ng/g lipid, the most prevalent OPFR in the breast milk samples. The previously mentioned study by McGoldrick et al. (2014) found TCIPP

present in the fish samples taken from the Northern Lakes in Canada. (McGoldrick et al., 2014)

Human Exposure Routes. Up to 40 % of the added TCIPP may be released throughout the product's lifetime and as TCIPP is not readily degraded in water or soil, it may have the potential to persist and accumulate in the environment (European Union, 2008 cited in (Farhat et al., 2013). Outdoor air measurements in Tokyo were measured at 1260 ng.m^3 and were found to be the most commonly detected OPFR in indoor air samples. The maximal daily intake from indoor air was 11 % of total daily intake (TDI) (Saito et al., 2007).

Toxicity Data & Toxicokinetics. TCIPP was detected in the liver, cerebral hemisphere and yolk sac post treatment of injected chicken eggs. Nineteen days after injection less than 1 % of TCIPP was detectable in the eggs (Farhat et al., 2013).

The acute oral LD50 ranged from 500-4200 mg/kg body weight, the inhalative ranged from 4.6 mg/L 17.8 mg/L and the dermal toxicity ranged from 1230-5000 mg/kg body weight in rats (Leisewitz et al., 2000 cited in (van der Veen & de Boer, 2012). TCIPP is not acutely toxic, the chronic toxicity was determined as an no observed effect level (NOEL) at 36 mg/kg bodyweight and accumulates in the liver and kidneys, where it is metabolized into hydroxides of phosphorous acid (Leisewitz et al., 2000 cited in (van der Veen & de Boer, 2012). The 96 h LC50 for fat head minnow is determined at 51 mg/L and a no observed effects concentration at 9.8 mg/L (Fisk et al., 2003 cited in (van der Veen & de Boer, 2012).

Cellular and Molecular Effects. At high concentrations (10 and 100 mg/L) TCIPP up regulated the transcription of HSD3β2, CYP11A2 and most sensitively CYP11B2 and down regulated SULT1E1 and SULT2A1 in H295R cells (X. Liu et al., 2012). Again these findings indicate an increase in endogenous E2 as production and conversion are increased by the up regulation of the CYP enzymes and inactivation is decreased by the down regulation of the SULT enzymes. However, these alterations in enzyme transcription only translated to an increase in E2 and T in H295R cells at the highest concentration of 100 mg/L. The increase in E2 outweighed the increase in T,

resulting in a total increase of the E2/T ratio (X. Liu et al., 2012). TCIPP did not reveal cytotoxic activity even at 100 mg/L in H295R cells exposed for 24 h (X. Liu et al., 2012).

TCIPP was cytotoxic above 1 mM in V79 cells (hamster fibroblasts) in the presence of S9 fraction and did not induce DNA strand breaks (Föllmann & Wober, 2006). Furthermore, TCIPP was not mutagenic assessed via the Ames test and did not have estrogenic or anti-estrogenic effects in human endometrial carcinoma cells modified with the recombinant yeast reporter (Föllmann & Wober, 2006). TCIPP was shown to reduce the tarsus length of chicken embryos significantly when exposed *in ovo* to TCIPP, increase the liver somatic index (LSI), increase gene expression of CYP3A37 regulated by the CXR and an increase of LFABP regulated by the thyroid hormone receptor (THR). These effects were observed at concentrations usually not environmentally relevant. Pipping success of the chicken embryos were not affected, but was significantly delayed at high doses of TCIPP (Farhat et al., 2013). Crump et al. (2012) investigating the effects of TCIPP on gene expression in chicken embryonic hepatocytes (CEH) and chicken embryonic neuronal (CEN) cells, revealed alterations in gene expression profiles associated with the CXR activation, TH pathway, lipid regulation and growth. TCIPP did not have any cytotoxic effects on CEN cells and CEH (Crump et al., 2012). Thyroid hormone activity in PC-12 cells was not affected by TCEP exposure for 24 h and therefore shifted the differentiation towards the cholinergic phenotype (Dishaw et al., 2014).

TDCIPP

Chemical and Physical Properties. TDCIPP is a clear, viscous liquid with chloride substitutions and soluble in most organic solvents (Sjögren et al., 2010; van der Veen & de Boer, 2012).

Occurrence in the Environment. TDCIPP is added to resins, latexes and foams used in the automotive industry and furniture, such as sofas and chairs (Green, Schlabach, Bakke, Brevik, & Dye, 2008; Stapleton et al., 2009), and also baby products (Stapleton et al., 2011). TDCIPP has a higher production cost compared to TCIPP and is used in similar products as TCIPP, but generally appears in products that require a more effective FR. Stapelton and colleagues (2009) discovered TDCIPP in 26 foam samples

taken from furniture in the United States, whereas the average concentrations ranged between 1-5 % (w/w). Furthermore among other FRs TDCIPP and TCIPP were detected in baby foam products with concentrations of 1890 ng/g and 570 ng/g, respectively. These levels of OPFRs in the products were comparable to, or larger than PBDE concentrations found in house dust (Stapleton et al., 2011).

Human Exposure Routes. TDCIPP was detected in seminal fluid samples by mass spectral screening; concentrations in the seminal fluid were not determined (Hudec, Thean, Kuehl, & Dougherty, 1981). Estimated occupational inhalation exposure to TDCIPP ranges from 900–1350 ng/day for adults. This is in addition to exposure to TDCIPP via inadvertent dust ingestion accumulating to 38 and 195 ng/day for adults and children, respectively (Stapleton et al., 2009). Dust ingestion or inhalation can be due to occupational or domestic exposure, however regional differences in exposure are to be expected as large differences in concentrations were found between Belgium, Sweden, Japan and the United States (Ali et al., 2012). Men with infertility issues or in a relationship with conceiving issues were recruited for a study assessing a potential correlation between TDCIPP dust concentrations in their domestic environment and sperm parameters and hormone levels (Meeker & Stapleton, 2009). TDCIPP was detected in 96 % of all samples taken with up to 56 µg TDCIPP/g dust. Sperm samples were assessed for sperm concentration, motion parameters and morphology. Serum samples were analyzed for testosterone, sex hormone binding globulin (SHBG), inhibin B, luteinizing hormone (LH), follicle stimulating hormone (FSH), estradiol, prolactin, serum free thyroxine (T_4), total triiodithyronine and thyrotropin concentrations. No significant correlation was found between dust TDCIPP concentrations and semen parameters. However, an inverse association between TDCIPP concentrations in house dust and serum free T_4 levels and a positive association between TDCIPP and prolactin were found. TDCIPP was associated with a 3 % decline in T_4 and a 17 % increase in prolactin. Thyroid hormones play an important role in multiple physiological processes in the human body, such as reproduction, neurodevelopment, metabolism and cardio-vascular health. The regulation of serum thyroid hormones and their action is a complex interchange between multiple processes such as iodine uptake, hormone synthesis, storage, transport, release and degradation (Miller, Crofton, Rice, & Zoeller, 2009). These linked processes take place in multiple organs in the body and a slight disruption

or alteration in one location can have severe effects downstream (Miller et al., 2009). Prolactin on the other hand is a protein hormone involved in reproduction, metabolism, maintenance of homeostasis in immune responses, osmotic balance and angiogenesis (Freeman, Kanyicska, Lerant, & Nagy, 2000). Furthermore, it is recognized as an indicator for neuroendocrine/dopaminergic function, as dopamine with further factors regulates prolactin release (Meeker et al., 2009b). Increased levels of circulating prolactin may reflect a deficiency in dopamine release, transport and uptake (Ben-Jonathan & Hnasko, 2001).

Toxicity Data and Toxicokinetics. Radiolabelled TDCIPP was administered to rats orally at various doses. The majority (90 % of the given dose) was absorbed from the gastrointestinal tract within 24 h. 2 µmol/ kg bw in 60 µL methanol were applied to a 4 cm^2 area of the shaved back of rats and resulted in a blood concentration of 0.75 nmol/g after 4 h (Nomeir, Kato, & Matthews, 1981). After intravenous administration of 0.8 µmol of radiolabelled TDCIPP to male Sprague Dawley rats, the urine was collected over 24 h. The urine contained the major metabolite bis(1,3-dichloro-2-propyl) phosphate (BDCIPP) (Lynn, Wong, Garvie-Gould, & Kennish, 1981). In a study by Cooper et al. (2011), BDCIPP was detected in human urine samples from non-occupationally exposed individuals ranging from 47-1662 pg/mL. No extrapolation of initial TDCIPP exposure was made. A study screening for correlations between BDCIPP in human urine and TDCIPP in dust concentrations, were only able to find a weak correlation between the two parameters (Carignan et al., 2013). However BDCIPP was detected in all urine samples taken with concentrations ranging from 62.1 – 1760 pg/mL (Carignan et al., 2013). In vitro metabolism of TDCIPP by a NADPH-dependent microsomal mixed oxidase system and gluthathione S-transferase from rat liver resulted in the metabolites BDCIPP, 1,3-dichloro-2-propanol, 3-chloro-1,2-propanediol and a glutathione conjugate (Nomeir et al., 1981). Nomeir et al. (1981) reported that TDCIPP is readily absorbed dermally and via the gastrointestinal tract in rats. The half-life of TDCIPP in rats ranges from 1.5 – 5.4 h depending on the tissue. The half-life in humans is unknown, but can be expected to be similarly short (Carignan et al., 2013; Nomeir et al., 1981). TDCIPP was detected in the liver, cerebral hemisphere and yolk sac post treatment of TDCIPP injected chicken eggs. Nineteen days after injection less than 1 % of TDCIPP was detectable in the eggs (Farhat et al., 2013). (E. M. Cooper et al., 2011)

Cellular and Molecular Effects. TDCIPP first reported as weak mutagen by (Gold et al., 1978), however use continued, although it was banned from being used in children's sleepwear (Stapleton et al., 2009). In contrast, (Freudenthal & Henrich, 2000) concluded through extensive *in vitro* and *in vivo* testing that TDCIPP does not have genotoxic activity and is not a genotoxic carcinogen. The metabolites 1,3-dichloro-2-propanone and 1,3-dichloro-2- propanol were found to be direct-acting mutagens and weakly mutagenic in the Ames assay, respectively (Gold et al., 1978). In male Sprague-Dawley rats dosed with high doses of TDCIPP administered for 24 months appeared to exacerbate naturally occurring benign interstitial tumors (Freudenthal & Henrich, 2000). However, these tumors did not evolve into malignancies. A higher incidence of adrenal cortical carcinoma was observed in female rats exposed to high doses of TDCIPP. Overall the body weight of treated rats was significantly lower compared to the control rats. In an unpublished study by Bio/dynamics a statistically significant increase in mortality, in liver, kidney and thyroid weights were observed in Sprague-Dawley rats fed TDCIPP in their diet for 12 and 24 months (National Research Council (US) Subcommittee on Flame-Retardant Chemicals, 2000). Additionally abnormal histopathological findings were observed (National Research Council (US) Subcommittee on Flame-Retardant Chemicals, 2000).

Undifferentiated PC-12 cells (rat adrenal medulla cells) exposed to TDCIPP for 24 h inhibited DNA synthesis, decreased cell number, but increase cell growth and altered neuro-differentiation into dopaminergic and cholinergic neurophenotypes (Dishaw et al., 2011). Although in the same study oxidative stress was increased at 50 μM TDCIPP, cell viability appeared unaffected. Interestingly TDCIPP promoted both the emergence of dopaminergic and cholinergic phenotypes of the PC-12 cells (Dishaw et al., 2011). TDCIPP exposure in PC-12 cells caused dose-dependent changes in six genes and correlating protein expression, which are directly related to neuronal development, neuronal signal transduction and the neuronal cytoskeleton (Ta et al., 2014). However the authors of this study were not able to determine a mechanism of action or determine whether the observed effects were solely due to neurotoxic and cytotoxic effects.

TDCIPP decreased cell viability of undifferentiated PC-12 cells to 53.92 ± 1.3 % after exposure to 50 µM TDCIPP and increased the rate of apoptosis from 3.5 % at 5µM and to 13.8 % at 50 µM, thus exerting cytotoxic activity (Ta et al., 2014).

In CHO K1 cells transiently transfected with human AR and GR plasmids TDCIPP had strong and weak inhibitory effects at 1.9 ± 1.5 µM and 16.0 ± 4 µM in the presence of 0.001 µM dihydrotestosterone (DHT) and 0.03 µM hydrocortisone, respectively (Kojima et al., 2013). Out of eleven OPFRs this study assessed, TDCIPP induced the most potent anti-androgenic activity. TDCIPP at 1.4 ± 0.5 µM increased PXR dependent transcription significantly in the presence of rifampicin at 10 µM (Kojima et al., 2013). Significance is defined as the concentration of the OPFR showing 20 % activation of transcription induced by endogenous hormones (Kojima et al., 2013). No agonist or antagonist activity for TDCIPP was observed for the ERα, ERβ, and PPARα in the same study. In contrast, Liu, C. et al. (2013) observed induction of the genes associated with the PPARα network, such as interleukin 6 and 8. However, this study was conducted in zebra fish.

A study conducted by C. Liu et al. (2013) developed an *in vivo* (zebra fish) screening approach assessing 48 genes in 6 receptor-centered gene networks (aryl hydrocarbon receptor, PPARα, ER, TRα, GR and mineralocorticoid receptor; MR). Exposure to TDCIPP at 2 mg/mL altered transcriptional profiles of all six receptor-centered gene networks. AhR target genes were generally up regulated in a dose-dependent manner, such as CYP1A1 and CYP1B1 (C. Liu et al., 2013a). PPARα centered genes such as interleukin 6 and 8 were up regulated significantly. PPARα is involved in lipid homeostasis, inflammation and reproduction. Furthermore six ERα and ERβ inducible target genes, part of the ER centered gene network, were up regulated indicating potential estrogenicity of TDCIPP. Interestingly, only the low dose exposure initiated the change in the transcription profile; at the two highest concentrations (0.2 mg/L and 2 mg/L) no increase in ER inducible genes was observed (C. Liu et al., 2013a). Conversely, the authors concluded that the effects on all six receptor-centered gene networks were only observed at concentrations not pertinent to reported concentrations in the environment, i.e. (van der Veen & de Boer, 2012).

TDCIPP at 0.01 mg/L elicited an increase of E2 and T concentrations in H295R cells. Production of E2 exceeded the T concentration resulting in an overall increase of the E2/T ratio (X. Liu et al., 2012). Furthermore the same study identified significant transcription up-regulation for CYP11A1 and CYP11B2 in a concentration-dependent manner and for CYP19A1 at 1 mg/L. SULT1E1 and SULT2A1 were down regulated when exposed to 1 mg/L and 0.1 mg/L, respectively. Transcription of HSD3α2 was up regulated significantly in H295R cells at 1 mg/L. TDCIPP displayed ER antagonist activity in MVLN cells with in the presence of 0.1 μM E2. Cell viability of H295R cells decreased below 80 % when exposed to 10 mg/L TDCIPP or higher.

Plasma E2 and T increased in male fish after exposure to 1 mg/L TDCIPP, with the E2/T ratio also increasing. In male fish 11-KT (11 ketotestosterone) decreased, therefore E2/11-KT ratio increased significantly. A decrease in 11-KT levels may lead to an arrest in meiosis and germ cell maturation in male fish, as it plays an important role in spermatogonial proliferation (X. Liu et al., 2012). VTG1 (vitellogenin gene 1) gene transcription increased when exposed to 1 mg/L TDCIPP among male fish (X. Liu et al., 2012). In a follow up study these findings were confirmed at the plasma level in adult male zebra fish exposed for 21 days to TDCIPP (X. Liu et al., 2013b). VTG is an important biomarker for endocrine disruption in aquatic vertebrates. It is a yolk precursor protein produced in the liver after stimulation of hepatic estrogen receptors (Van der Ven et al., 2003). Following, it is released into the blood and incorporated in developing oocytes. Therefore, VTG is mostly present in sexually active female fish, as the male fish do not produce physiologically relevant concentrations of E2 for the induction of VTG protein (Van der Ven et al., 2003).

Plasma E2 and T increased in female fish after exposure to 1 mg/L TDCIPP, but E2/T ratio decreased in these fish opposed to an increase in male fish. Furthermore, in the female fish the 11-KT did not change significantly, while E2/11-KT ratio increased after exposure to 1 mg/L TDCIPP slightly, yet significantly. In female fish mRNA expression of CYP17 and CYP19A were significantly increased at 1 mg/L TDCIPP. VTG1 transcription decreased significantly when exposed to 0.2 mg/L TDCIPP. A decrease in VTG may lead to disruption in oocyte maturation. However, in a follow up study in adult female zebra fish exposed to TDCIPP for 21 days VTG plasma levels were

up regulated (X. Liu et al., 2013b). Furthermore, alterations in gene transcription levels of regulatory and steroidogenic genes of the HPG axis, an increase in E2 plasma concentrations in both sexes and impaired reproduction (decrease in egg production, number of eggs per spawning event, number of spawning events and decrease in fertilization and hatching success) were observed indicating a link between transcriptional/translational changes and physiological adverse effects for TDCIPP exposed fish (X. Liu et al., 2013b). A decrease in hatching success and survival in zebra fish exposed for 21 days was additionally confirmed including a mean LC50 of 7 mg/L by Liu C. et al. (2013).

Crump et al. (2012) investigating the effects of TDCIPP on gene expression in chicken embryonic hepatocytes (CEH) and chicken embryonic neuronal (CEN) cells, revealed alterations in gene expression profiles associated with the CXR activation, TH pathway, lipid regulation and growth. CEH cells exposed to 10 µM TDCIPP for 36 h revealed an increase of up to 13-fold CYP3A37 and CYP2H1 mRNA, two phase I metabolizing enzymes. A phase II metabolizing enzyme was significantly up regulated as well. Farhat et al. (2013) confirmed the increase of the same enzymes *in ovo* chicken embryos, although to a lesser extent. TDCIPP had cytotoxic effects on CEN cells and CEH (Crump et al., 2012). LFABP mRNA was significantly down regulated at all concentrations of TDCIPP (0.01 – 10 µM) in CEH cells. A follow-up study by Farhat et al. (2013) assessing these findings *in ovo* (chicken embryos) concluded that TDCIPP at high doses caused a delay in pipping time, significant reduction of head & bill length, embryo mass, a decrease in T_4 concentrations, a reduction and even an oblation, in gallbladder size of high dose treated embryos. Nevertheless no adverse morphological of developmental effects were observed at environmentally relevant concentrations of TDCIPP (12 ng/g egg) (Farhat et al., 2013).

1.4. Transcription Factor Receptors

1.4.1. Androgen Receptor

The AR is part of the nuclear receptor superfamily and two isoforms of the AR exist. Isoform A migrates with a mass of 110 kDa and comprises about 80 % of the

reactive receptor species. Isoform B has a mass of 87 kDa and constitutes about 20 % of the reactive receptor species (Hirawat, Budman, & Kreis, 2003). The AR is a modular protein with several different functional regions. The A/B domain or N-terminus domain, the DNA binding domain (DBD; C domain), the hinge region or D domain and the C-terminus also known as the E/F domain. The N-terminus domain contains several sequences important for AR conformation and activity as most phosphorylation sites are within this region (Hirawat et al., 2003). The DBD recognizes androgen response elements (ARE) in the regulatory regions of AR-inducible target genes. The hinge region is a short sequence of 50 amino acids that in part interacts with the cytoskeletal protein filamin A. Filamin A facilitates translocation into the nucleus and is accelerated upon androgen binding (Ozanne et al., 2000).

The ligand binding domain (LBD) is localized to the C-terminus region and androgens, predominantly testosterone (T) and dihydrotestosterone (DHT) have a binding affinity for the LBD. Both molecules interact with the LBD, however DHT has revealed a higher affinity and potency in mediating hormone responses compared to T. DHT is the product of enzymatic conversion of T by 5α- reductase (Hirawat et al., 2003; Keller, Ershler, & Chang, 1996). The un-activated AR is found in the cytoplasm accompanied by heat shock proteins (Hsp) 90 and other chaperones (Ozanne et al., 2000). Upon ligand binding the AR undergoes a conformational change, which allows it to disassociate from its stabilizing chaperones and to migrate into the nucleus. In the nucleus the AR is known to function as homodimers, but the AR also heterodimerizes with other hormone transcription factors such orphan testicular receptor 4 (Y.-F. Lee, Shyr, Thin, Lin, & Chang, 1999) and estrogen receptor α (Panet-Raymond, Gottlieb, Beitel, Pinsky, & Trifiro, 2000). The AR consequently interacts with a multitude of co-regulators, binds to the ARE located in the promoter or enhancer region and additional co-regulators are recruited to remodel the chromatin structure. Thereby transcription of AR inducible target genes, such as prostate specific antigen (PSA) and insulin-like growth factor 1 (IGF-1) are initiated (Hirawat et al., 2003). This pathway of direct ligand dependent genomic activation is depicted in Figure 1.3 (left side).

Activated AR does not only directly interact with DNA, but also with numerous signaling molecules on the cell membrane (Bennett et al., 2010). Some of these

molecules include kinases such as phosphoinositide 3 kinase (PI3K), src, extracellular signal regulated protein kinases (ERK) 1 and 2 and Akt. Growth factors such as IGF-1 and receptor-tyrosine kinases (RTK) can activate AR independently of a ligand via other signaling pathways in the cell (Figure 1.3 **right side**). These additional pathways can result in phosphorylation of the AR complex, preventing degradation and enhancing nuclear translocation and activity (Bennett et al., 2010).

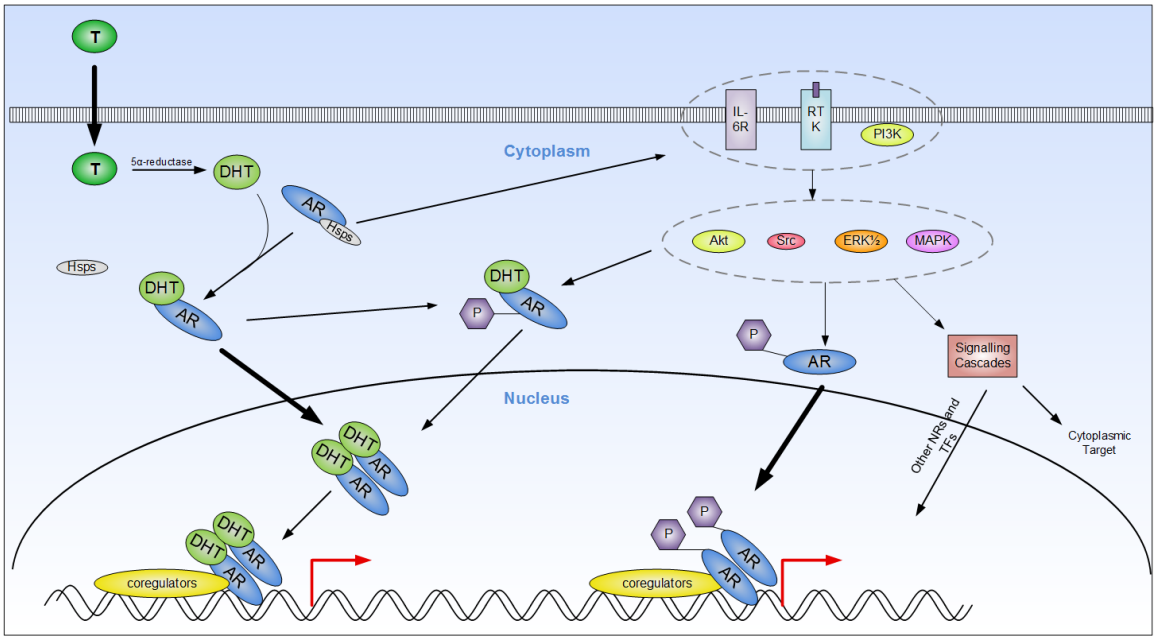


Figure 1.3. Schematic overview of androgen receptor activation.

The interaction of these membrane molecules and the (non-) ligand bound AR and can trigger a signaling cascade in the cell. This cascade may cause transcription factors, NRs and phosphorylated ligand or non-ligand bound AR to initiate the transcription of AR inducible target genes. Additionally activated AR can trigger a non-genomic signaling cascade in the cell resulting in the rapid release of calcium ions from the endoplasmic reticulum or the mitochondria (Bennett et al., 2010). These alternative signaling pathways enhance AR activity especially at low androgen levels.

The androgens are a class of hormones found in variety of tissues and organs in the human body and mediate a wide range of physiological responses. These tissues and organs include the skin, skeletal muscle, cardiac muscle, liver, kidney, central nervous system and the hematopoietic system (Keller et al., 1996). Androgens play an

essential role in sexual differentiation, sexual maturation and reproductive organ development (M. Rahman, Miyamoto, & Chang, 2004). The androgen receptor (AR) is a target for such androgens and mediates androgen related responses in the cell, such as cell proliferation, differentiation, apoptosis, prostate development and maintaining homeostasis in sexual organs (Bennett et al., 2010).

Male sexual differentiation and maintenance are mainly driven by androgens and are entirely androgen dependent, whereas female sexual differentiation is considered the default developmental pathway (Luccio-Camelo & Prins, 2011). Therefore, male sexual differentiation is especially sensitive to EDC interacting with the androgen signaling pathways and may be more severely impacted in sexual differentiation and maturation (Luccio-Camelo & Prins, 2011).

Some male reproductive abnormalities have been associated with the exposure to EDCs. These abnormalities can include altered semen quality (Stapleton et al., 2009), cryptorchidism, hypospadias, testicular and germ cell and prostate cancer (Alavanja, 2003; Maffini, Rubin, Sonnenschein, & Soto, 2006), as well as delayed puberty (Buck Louis et al., 2008) and reduced ano-genital distance in newborn male infants (Swan et al., 2005). A minority of these effects has been linked to pre-natal exposure to EDCs (Martin et al., 2008) and for the majority of these effects evidence remains associative (Luccio-Camelo & Prins, 2011).

1.4.2. Estrogen Receptor

Estrogens are essential for the development and maintenance of sexual and reproductive function, as well as for the cardiovascular, musculoskeletal, immune and central nervous system in men and women (Ascenzi, Bocedi, & Marino, 2006; Nilsson, Mäkelä, Treuter, & Tujague, 2001). A very potent estrogen is 17-β estradiol (E2). E2 metabolites, estrone and estriol, are significantly less potent in mammalian species. Estrogens have a high affinity for estrogen receptor (ER), which are transcription factors regulating target genes involved in development, metabolism and homeostasis in vertebrates (McKenna, Lanz, & O'Malley, 1999). In mammals two ER subtypes have been identified, namely ERα and ERβ; in fish a third sub type ERγ has been

characterized (Hawkins et al., 2000). This section focuses on ERα and from here on will be referred to as ER. The ER is a member of the nuclear receptor (NR) superfamily and is a modular protein sharing common regions, the A/B, C, D and E/F region (Ascenzi et al., 2006; Heldring et al., 2007).

The A/B region or the N-terminal transactivation domain is involved in protein-protein interaction (McInerney, Weis, Sun, Mosselman, & Katzenellenbogen, 1998), in transcriptional activation of target gene expression and contains the activation function (AF) 1. The DBD in the C region plays a pivotal role in receptor dimerization and binding of the receptor to its cognate DNA response sequence to initiate target gene expression (Nilsson et al., 2001), commonly referred to as estrogen response element (ERE). The hinge region (D region) is the most variable region between species and contains several sites for post-translational modifications (Sentis, Le Romancer, Bianchin, Rostan, & Corbo, 2005). The C-terminus (E/F region) contains the ligand-binding domain (LBD), the AF-2 domain, a region important in transactivation, and the nuclear localization signal sequence (Nilsson et al., 2001). Furthermore, the region is involved in binding protein chaperones such as heat-shock proteins (Hsp) 70 and 90. When the receptor is not activated, a protein complex containing Hsp 70 and 90 stabilizes the protein and sterically opens the un-occupied LBD facilitating steroid binding (Ascenzi et al., 2006). Four mechanistically different molecular pathways for the ER have been characterized. The tethered pathway includes protein-protein interaction of the ER with transcription factors, that bind to GC-rich promoters lacking an ERE (Saville et al., 2000), thus initiating transactivation mediated by the ER/transcription factor complex upon ligand binding. The second pathway includes non-genomic activation of the ER. Although these processes have been extensively studied, it remains unclear whether the ER or whether a distinct membrane receptor mediates the responses. (Simoncini et al., 2004). The ligand activates a membrane receptor or the ER, initiating a signal cascade caused by secondary messengers to ion channels for example. Rapid effects, such as vasodilation follow without involving gene regulation (Simoncini et al., 2004). Additionally to the ligand dependent activation, the ER can be activated ligand independently. Gene regulation occurs via excreted growth factors and kinases, causing the phosphorylation of the ER, dimerization and binding to DNA (Kato et al., 1995). Lastly, the classical pathway of ligand-dependent signaling begins by estrogen binding to the ER. The ER

undergoes rapid structural changes leading to homo-dimerization. The chaperone complex facilitates the translocation of the ER along microtubules to the nucleus (Pratt, Galigniana, Morishima, & Murphy, 2004). In the nucleus the homo-dimer can bind to the ERE on transcriptional regulatory regions of target genes, where co-activators or co-repressors are recruited to both AF in the LBD and N-terminus of the ER (Nilsson et al., 2001). Target gene transcription is either initiated or repressed depending on other active pathways in the cell at the time of hormone exposure (Nilsson et al., 2001). The previously mentioned mechanistic pathways will not be discussed in further detail for this project.

The LBD of the ER is not specific to estrogens, potentially due to its large ligand cavity (Brzozowski et al., 1997). A wide range of environmental contaminants, such as polyaromatic hydrocarbons, phthalates, (Brzozowski et al., 1997), bisphenol A, polychlorinated biphenyls (Lathers, 2002) have been known to activate the ER and cause a multitude of adverse effects in humans and wildlife (Sonnenschein & Soto, 1998).

It is not surprising that xenoestrogens can cause imbalances or deficiencies in the male and female body promoting the development and progression of various types of cancers, reproductive abnormalities, osteoporosis, neurodegenerative, cardiovascular diseases, endometriosis and obesity, as estrogens have a widespread role in the human physiology, (Deroo, 2006; Lathers, 2002).

1.4.3. Aryl Hydrocarbon Receptor

The aryl hydrocarbon receptor (AhR) is a member of the basic helix-loop-helix/PAS protein family of transcription factors (Hankinson, 1995; Kewley, Whitelaw, & Chapman-Smith, 2004). The PAS domain (for: PER/aryl hydrocarbon receptor nuclear translocator (ARNT)/ the single minded protein (SIM) domain) is essential for dimerization with other PAS proteins and DNA binding (Kewley et al., 2004). The bHLH/PAS proteins are ubiquitous transcription factors and one of the best characterized is the AhR in combination with ARNT (Kewley et al., 2004), also referred to as the AhR complex (AhRC) (Beischlag, Morales, Hollingshead, & Perdew, 2008).

Historically, the AhR has been considered an orphan receptor since endogenous ligands were unknown and limited to exogenous environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and dioxin like compounds (Hankinson, 1995; Kewley et al., 2004). However, endogenous ligands have been discovered, such as bilirubin (Sinal & Bend, 1997) and most recently the tryptophan catabolite kynurenine constitutively generated by human tumour cells (Opitz et al., 2011).

In the cytoplasm AhR exists in a stabilizing complex of proteins, Hsp 90, co-chaperone p23 and hepatitis B virus X associated protein. Upon ligand binding, AhR undergoes a conformational change and enters the nucleus. In the nucleus AhR dimerizes with ARNT and binds with xenobiotic response elements (XRE) to initiate transcription of target genes including cytochrome P450 (CYP), specifically CYP1A1 and CYP1B1 (Brokken & Giwercman, 2014; Kewley et al., 2004). However, the regulation of transcription of AhR inducible target genes is highly complex and does not rely solely on ligand binding, but on the recruitment of a battery of co-activators, co-repressors, chaperone proteins and other transcription factors (Beischlag et al., 2008).

Although the AhR is *per se* not a nuclear hormone receptor, its role in endocrine disrupting related adverse effects are of interest due to transcriptional cross-talk between nuclear hormone receptors, such as ER and AR. It has been demonstrated that TCDD exposure reduces expression of ER inducible target genes (Krishnan, Porter, Santostefano, Wang, & Safe, 1995; Zacharewski, Bondy, McDonell, & Wu, 1994). Ligands for the AhR can compete for shared co-factors (Nguyen, Hoivik, Lee, & Safe, 1999), decrease levels of ER (Safe et al., 1991), increase the rate of E2 metabolism (Hayes et al., 1996; Safe et al., 1991) and suppress the transcription of E2 inducible genes (Krishnan et al., 1995; Zacharewski et al., 1994).

AhR signaling may also interfere with the male reproductive system, reducing testosterone inducible transcription of genes such as PSA (Jana, Sarkar, Ishizuka, & Yonemoto, 1999). The same study demonstrated that TCDD exposure blocked androgen driven proliferation in LNCaP cells. This finding suggests that AR-AhR cross-talk may have a protective function for the prostate gland during adulthood (Brokken & Giwercman, 2014; Jana et al., 1999), however chronic effects for developing

reproductive systems may be adversely affected. Proposed cross-talk mechanisms for the AR and AhR are protein-protein interactions, competition for co-factors, competition for DNA binding, proteosomal degradation (Brokken & Giwercman, 2014) and alterations in the transduction cascade of downstream AR activity (Jana et al., 1999).

These cross-talk mechanisms are of specific interest as endocrine disruptors often interfere with the ER and AR causing ER/AR specific adverse effects. Consequently, these effects can be exacerbated and broadened if the disruptor is a ligand for the AhR.

1.5. Research Objective and Scope

As OPFRs are considered an emerging pollutants and data concerning their effects on the endocrine system were limited in the beginning of this project, it was deemed of interest to assess the effects of six selected OPFRs (**TBOEP, TCEP, TCIPP, TDCIPP, TEP and TMPP**; Figure 1.2) on the activities of the androgen-, estrogen- and aryl hydrocarbon receptors. Different human cancer cell lines, such as the endometrial cancer cell 1 (ECC-1) and lymph node cancer of the prostate (LNCaP), containing endogenous levels of the androgen, aryl hydrocarbon and estrogen receptor, respectively, were used as a model by measuring target gene mRNA and protein expression. Known target genes of the selected receptors were pre-selected prior to the beginning of the experiments. For putative endocrine disruptors further experiments were conducted to illuminate the mechanism of action. To our knowledge, no study has been conducted investigating direct target gene responses and physiological relevance in human cell lines containing endogenous nuclear receptors and investigating the mechanism of action.

This project focused on the interaction of six selected OPFRs and transcription factor receptor targets. To our knowledge, this is the first study to investigate direct target gene responses and physiological relevance in human cell lines containing endogenous levels of nuclear receptors and investigating the mechanism of action. It did not attempt to identify indirect endocrine disrupting targets and targets of

steroidogenesis. Additionally this project did not attempt to confirm any of the physiological findings *in vivo*.

Chapter 2.

Materials and Methods

2.1. Chemicals

TBOEP (CAS 78-51-3), TCEP (CAS 115-96-8), TMPP (CAS 1330-78-5), TEP (CAS 78-40-0) TDCIPP (CAS 13674-87-8) were purchased from Sigma Aldrich (ON, Canada). TCIPP (CAS 13674-84-5) was purchased from Pfaltz and Bauer (CT, Waterbury). The synthetic androgen, metribolone (R1881) was generously supplied by the Vancouver Prostate Center (Vancouver Coastal Health Research Institute). 17β-estradiol (E2) was purchased from Sigma (ON, Canada). Stock solutions of R1881, E2, TCDD and serial dilutions of all test chemicals were made in dimethyl sulfoxide (DMSO).

2.2. Cell Culture

LNCaP cells were cultured in RPMI 1640 media with L-Glutamine (CORNING cellgro) with 10 % (v/v) FBS (Gibco) and supplemented with 100 units/mL potassium penicillin – 100 units /mL streptomycin (1 %, P/S; BioWhittaker, Lonza) at 37 °C, 20 % O_2 and 5 % CO_2.

ECC-1 cells were maintained in Dulbecco's Modified Eagle's Medium with 4.5 g/L glucose, 4.5 g/L L-glutamine and sodium pyruvate (DMEM; CORNING cellgro) with 10 % (v/v) FBS (Gibco) and supplemented with 1 % P/S (BioWhittaker, Lonza) at 37 °C, 20 % O_2 and 5 % CO_2.

VCaP cells were cultured in DMEM with 1.5 g/L sodium bicarbonate (ATCC) in 12 % (v/v) FBS (Gibco) and supplemented with 100 units/mL potassium penicillin – 100 units /mL streptomycin (1 %, P/S; BioWhittaker, Lonza) at 37 °C, 20 % O_2 and 5 % CO_2.

2.3. Reverse Transcription and Real-Time PCR

2.3.1. Chemical Treatment

LNCaP and VCaP cells in Multiwell™ 6-well cell culture plates were starved in RPMI media (Corning cellgro) and phenol-free DMEM with 3 % charcoal stripped (c.s.) FBS (Gibco®) and 1 % P/S, respectively. Cells were starved for 24 h prior to treatment. ECC-1 cells were serum starved in phenol-free DMEM media with 4.5 g/L glucose and sodium pyruvate (Corning cellgro) with 1 x L-Glutamine and 1 % P/S.

For initial screening LNCaP cells were treated with vehicle (DMSO; 0.1 v/v %), R1881 at 1 nM and a flame retardant alone at final concentrations of 10 nM, 100 nM, 1 µM, 10 µM and 20 µM and in combination with R1881 at 1 nM for 24 h. For the concentration-response curves LNCaP and VCaP cells were treated with TDCIPP with increasing concentrations from 1 nM to 50 µM for 24 h.

For initial screening ECC-1 cells were treated with vehicle (DMSO; 0.1 v/v %), E2 at 10 nM, a flame retardant alone at final concentrations of 10 nM, 100 nM, 1 µM, 10 µM and 20 µM and in combination with E2 at 10 nM for 24 h. MCF-7 cells were treated with vehicle (DMSO), TCDD at 2.5 nM, a flame retardant alone at final concentrations of 10 nM, 100 nM, 1 µM, 10 µM and 20 µM and in combination with TCDD at 2.5 nM for 24 h. For ECC-1 cells just prior to chemical treatment 30 % bovine serum albumin (BSA) in phosphate buffered saline (PBS) was added to each well in a 5:1 ratio.

2.3.2. RNA Extraction

After 24 h of chemical treatment the culture media was removed from the cells and discarded of appropriately. Following two washes of PBS the cells were transferred to 1.5 mL centrifuge tubes with 750 µL of TRI Reagent® (Sigma). 200 µM of chloroform (Caledon) was added to each tube, after which the tubes were vigorously shaken 15-20 seconds and rested for 5 min at RT. The tubes were centrifuged at 11,000 x g for 10 min at 4 °C followed by removing up to 280 µL from the aqueous layer into new microcentrifuge tubes on ice. In the next step isopropanol was added in a 1:1 ratio and the tubes were inverted several times. After 5 min on the bench at RT the samples were

centrifuged at 12,000 x g for 10 min at 4 °C. Consequently the supernatant was removed and the RNA pellet was re-suspended in 1 mL 75 % ethanol. The samples were either stored at – 80 °C or were processed immediately. The samples were then centrifuged at 14,000 x g for 15 min at 4 °C. The ethanol was removed and the pellet was allowed to air-dry for several minutes until re-suspension in up to 20 µL RNase and DNase free water (Sigma).

2.3.3. Production of cDNA

RNA concentration and purity was determined using a NanoDrop Lite (Thermo Scientific) in order to reverse transcribe and amplify 2 µg of RNA per treatment group. Each samples were subjected to a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) containing: 1 x RT Buffer, 1 x dNTP, 1 x random primers and 50 units MultiScribe™ Reverse Transcriptase totaling for a 20 µL reaction. Under the following conditions the reverse transcription reaction was carried out with a Veriti, 96 well Thermo Cycler (Applied Biosystems): 25 °C for 5 min, 37 °C for 120 min, 85 °C for 5 min and 4 °C until storage at - 20 °C.

2.3.4. Quantitative Real-Time PCR

Each cDNA was diluted 1:15 and 4 µL of sample was added to master mix containing: 1 x Power SYBR Green (Applied Biosystems), 3.0 pM primer and water. The primer sequences for CYP1A1, CYP1B1, IGF-1, pS2, PSA, PSMA and 36B4 (Integrated DNA Technologies) are listed in Table 2.1.

Table 2.1. List of oligonucleotide primer pairs for real-time PCR

Gene	Gene Name	Direction	Sequence
36B4	Acidic ribosomal phosphoprotein P0	Forward	5'-CCACGGTGCTGAACATGCT-3'
		Reverse	5'TCGAACACCTGCTGGATGAC-3'
CYP1A1	Cytochrome P450, family1 subfamily A, polypeptide 1	Forward	5'-CACTCTTCCTTCGTCCCCCT-3'
		Reverse	5'-TGGTTGATCTGCCACTGGTT-3'
CYP1B1	Cytochrome P450, family1 subfamily B, polypeptide 1	Forward	5'-CATGCGCTTCTCCAGCTTTGT-3'
		Reverse	5'-GGCCACTTCACTGGGTCATGA-3'
IGF-1	Insulin growth factor 1	Forward	5'-GACAGGCATCGTGGATGAG-3'
		Reverse	5'-GACAGAGCGAGCTGACTTG-3'
pS2	Trefoil factor 1	Forward	5'-GAGGCCCAGACAGAGACGTG-3'
		Reverse	5'-CCCTGCAGAAGTGTCTAAAATTCA-3'
PSA	Prostate specific antigen	Forward	5'-GACCACCTGCTACGCCTCA-3'
		Reverse	5'-GGAGGTCCACACTGAAGTTTC-3'
PSMA	Prostate specific membrane antigen	Forward	5'-AACTGGACCCCAGGTCTGGA-3'
		Reverse	5'GAGGATTTTATAAACCACCCGAA-3'

Standards were made using the respective positive control with the following dilutions: 1: 10, 1:100, 1:1000, 1:10,000 and 1:100,000. Each sample was place in a MicroAmp® Fast Optical 96-well reaction plate (Applied Biosystems) and subjected to the following conditions for quantitative real-time PCR: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute and ending with 1 cycle of 95°C for 15 seconds, 60°C for 1 minute and 95°C for 15 seconds in a StepOne Plus™ Real-Time PCR system (Applied Biosystems). Reactions were performed in triplicate for each sample and data were averaged and normalized to the mean of the expression of the endogenous control gene, 36B4. A non-template control was included in each assay.

2.4. Protein Extraction and Immunoblotting

2.4.1. Chemical Treatment

LNCaP cells were serum-starved in RPMI (CORNING cellgro) media with 3 % c.s. FBS and 1 % P/S. All cells were starved for 24 h before treatment and were cultured in 150 mm dishes.

For subcellular protein fractionation LNCaP cells were starved in phenol free DMEM media with 4.5 g/L glucose and sodium pyruvate (Corning cellgro) with 3 % c.s. FBS, 1 x L-Glutamine and 1 % P/S. These cells were either treated for 1 h or 24 h. LNCaP cells were treated with DMSO, R1881 and increasing concentrations of TDCIPP from 1 nM to 50 µM in combination with 1 nM R1881 for 24 h.

2.4.2. Whole Cell and Sub-Cellular Protein Extractions

After washing the cells twice with 1 x ice-cold PBS whole cell lysates were collected with 500 µL of lysis buffer (1 M Tris pH 8, 400 mM NaCl, 0.5 M EDTA, 0.1% (v/v) glycerol, 0.1% IGEPAL® CA-630, autoclaved milliQ water; per 1 mL lysis buffer 20 µL of 100 x complete protease inhibitor cocktail (P.I.; Bioshop) and 0.34 µL of β-mercaptoethanol was added) in 1.7 mL tubes on ice. Every 5 min the samples were vortexed while incubating on ice for 20 min, followed by centrifugation at 14,000 rpm for 15 min at 4 °C. The supernatant was then collected in aliquots and stored at – 80 °C.

For cytosolic and nuclear protein extractions the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) was used. The manufacturer's protocol was generally followed with a few additional modifications. The cells were washed twice with 1 x ice-cold PBS. The cells were then harvested with 1 mL ice cold 1 x PBS and transferred into a 1.7 mL tube and centrifuged at 500 x *g* for 3 min. The supernatant was carefully removed and depending on the cell pellet size an appropriate amount of ice-cold CER I solution and P.I. (Bioshop) was added. A reduced volume of CER I solution was added to the pellet than recommend by the manufacturer, in order to increase protein concentration, albeit still maintaining recommended ratios of reagents. Samples were vigorously vortexed for 15 s to fully suspend the cell pellet and

consequently incubated on ice for 10 min. An appropriate volume of ice-cold CER II solution was added and samples were vortexed for 5 s on the highest setting, incubated for 1 min, vortexed for 5 s and finally centrifuged at 16,000 x *g* for 5 min. Immediately after centrifugation the supernatant/cytoplasmic extract was transferred in aliquots into pre-chilled 1.7 ml tubes and kept on ice and stored at – 80 °C. The remaining nuclei pellet was re-suspended in in ice-cold NER and P.I. Samples were vortexed for 15 s every 10 min for 1 h and additionally the cell slurry was pipetted up and down to optimize membrane rupture. Tubes were then centrifuged at 16,000 x *g* for 10 min at 4 °C and the supernatant/nuclear extract was transferred in aliquots into pre-chilled 1.7 mL tubes and stored at – 80 °C until quantification.

2.4.3. Protein Quantification

The RC DC protein assay (BioRad) was used according to the manufacturer's protocol to determine protein concentrations in the samples. Standards were made from a BSA stock (2mg/mL, BioRad) with concentrations at 0.2 mg/mL, 0.5 mg/mL, 0.75 mg/mL, 1.0 mg/mL, 1.5 mg/mL and 2.0 mg/mL. Samples were diluted 1:3 with lysis buffer and 125 µL of Reagent I was added to each sample, standard and the blank containing just lysis buffer. All samples were vortexed and incubated for 1 min followed by the addition of 125 µL of Reagent II. Once again the sample was vortexed and centrifuged at 15,000 x *g* for 5 min at room temperature. The supernatant was poured off and the tubes were inverted for several minutes and pulsed down for a few seconds. The remaining supernatant was carefully pipetted off and 127 µL of Reagent A' was added to each sample. Reagent A' contained per sample 5 µL of DC Reagent S and 250 µL of DC Reagent A. After vortexing, incubation at room temperature for 5 min and further vortexing, 1 mL of DC Reagent B was added to each sample and immediately vortexed. Samples and standards were then incubated for 15 min at room temperature after which a SmartSpec™ Plus Spectrophotometer (Bio-Rad) was used to measure absorbance at 750 nm.

2.4.4. Immunoblotting

Equal amount of protein were resolved on an 8 % or 10 % SDS-polyacrylamide gel using 40 % acrylamide/bis solution (29:1; BioRad) in 1.0 M Tris, 5.0 mM SDS, 0.1% ammonium persulfate and 0.01% tetramethylethylenediamine solution. Samples were denatured in 5x SDS sample buffer consisting of 33.33 mM Tris-HCl, pH 6.8, 5.3 % glycerol, 1.0 % SDS, 26.6 mM β-mercaptoethanol and 0.006 % bromophenol blue at 100 °C for 5 min. PageRuler Plus Prestained Protein Ladder (Thermo Scientific) and samples were loaded and gel electrophoresis was conducted in 1 x Running Buffer (1 M Tricine, 1 M Tris-Cl, 50 mM SDS) between 39 - 41 V for approximately 20 h. Consequently the protein was transferred to a polyvinylidene fluoride (PVDF) at 0.6 A for 2 h. After the completed transfer the membrane was re-activated with 100 % methanol for 30 s and was blocked with 1x TBST with 5 % non-fat powdered milk (2.5 mM Tris, 140 mM NaCl, 2.5 mM KCl, 0.5% Tween20) for 1 h at room temperature. The membrane was then probed with several primary antibodies (1° Ab; Table 2.) in 1x TBST with 5 % milk separately overnight at 4 °C.

Table 2.2. List of 1° Ab used for Immunoblotting

1° Ab	Dilution	Species	Supplier
anti-AR N20	1/5000	rabbit polyclonal IgG	Santa Cruz Inc.
anti-AR PG21	1/1500	rabbit monoclonal IgG	Millipore
anti-CYP1A1	1/1000	rabbit polyclonal IgG	Santa Cruz Inc.
anti-Histone H1	1/500	rabbit polyclonal IgG	Santa Cruz Inc.
anti-PSA C-19	1/500	goat polyclonal IgG	Santa Cruz Inc.
anti-PSMA	1/2500	mouse monoclonal IgG	BC Cancer Agency
anti-α-tubulin	1/1500	mouse monoclonal IgG	Santa Cruz Inc.

Blots were then washed 3 times with 1 x TBST with 5 % milk for 10 min at room temperature followed by incubation with a horseradish-peroxidase conjugated secondary antibody (anti-goat, anti-mouse or anti rabbit IgG) for 1 h at room temperature. Afterwards the blots were washed 3 times with 1 x TBST for 5 min at room temperature. Protein was visualized with the ECL Prime Detection Kit (GE Healthcare) in a Dyversity 2D-image analysis system (Syngene).

2.5. Measurement of Extracellular PSA Accumulation

Culture media of LNCaP cells was collected after 24 h treatment with increasing concentrations of TDCIPP individually (1 nM to 50 µM) or in combination with R1881at 1 nM. Alternatively, LNCaP cells were pre-treated with increasing concentrations of TDCIPP (1 nM to 50 µM) for 30 min followed by R1881 at 1 nM for 24 h. PSA concentrations in the conditioned media were quantified using a COBAS® e 411 analyzer at the Vancouver Prostate Center, BC, Canada.

2.6. Androgen Receptor Competitor Assay

PolarScreen™ Androgen Receptor Competitor Assay, Green was purchased from Invitrogen. This competition assay determines the relative affinity of a test compound to the AR ligand binding domain (AR-LBD). It consists of a tagged androgen receptor ligand binding domain (AR-LBD (His-GST)) and a Fluoromone™ AL Green when combined result in high fluorescence polarization values. TDCIPP was serial diluted in AR Green Assay Buffer including 2 mM dithiothreitol (DTT) to 2x concentration. The test compound was consequently added to a multiwell plate (CORNING 3676). A competitor will displace the Fluoromone™ AL Green resulting in a low polarization value, whereas a non-competitor will not displace the Fluoromone™ AL Green remaining in a high polarization value. To triplicate wells of TDCIPP (0.5 nM – 100 µM) AR-LBD (His-GST)/Fluoromone AL Green Complex mixture was added for a final concentration of 25 nM/1 nM. The plate was incubated for 4 h at RT in the dark before reading fluorescence polarization (FP) values using a TECAN Infinite® F500. The instrument was equipped with 485 nm excitation and 535 nm FP emission interference filters. R1881 at a final concentration of 25 nM served as a positive control, FP One-Step Reference Kit (Invitrogen) was used as a control for the plate reader. A non-template control was included in each assay.

2.7. Chromatin Immunoprecipitation Assay

2.7.1. Chemical Treatment

LNCaP cells were cultures in 150 mm plates and serum starved for 24 h prior to chemical treatment. LNCaP cells were starved in phenol free DMEM media with 4.5 g/L glucose and sodium pyruvate (Corning cellgro) with 3 % c.s. FBS, 1x L-Glutamine and 1 % P/S. LNCaP cells were treated with DMSO, R1881 and TDCIPP (1 µM and 10 µM) alone and in combination R1881 (1 nM) for 45 min.

2.7.2. Chromatin Immunoprecipitation Assay

Chromatin Immunoprecipitation (ChIP) assay was performed as described in (Shang, Myers, & Brown, 2002) with minor modifications. After chemical treatment the cells were washed with ice-cold PBS and cross-linked with the addition of 1 mL of 11 % formaldehyde in 1 M HEPES, pH 7.8 per 10 mL of PBS for 10 min at room temperature. The fixing solution was aspirated off and the plates were washed twice with ice-cold PBS. Cells were harvested with 1 mL of Tris/DTT solution (100 mM Tris, pH 8.3, 10 mM DTT and 150 mM NaOH) and transferred into 1.7 mL tubes. Tubes were incubated at 30 °C in a water bath for 15 min and followed by centrifugation at 2,000 x *g* for 5 min. To the tubes 1 mL ice-cold PBS was added to re-suspend the pellet. Samples were centrifuged again at 2000 x *g* for 5 min and re-suspended in 600 µL of ice-cold lysis buffer (1 % SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1). Samples were sonicated three times for 8-9 s to yield DNA fragments of 500-600 bp size while kept cold on ice. Thereafter the samples were loaded on a 1 % agarose gel with 1x SYBR® Safe (Life Technologies) at 90 V for approximately 30 min to assess the DNA fragment sizes. Meanwhile the samples were kept on ice to precipitate the SDS out. If the DNA fragments were the right size, the samples were centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was transferred in aliquots to 1.7 mL tubes to be mixed in 600 µL dilution buffer with P.I. (1 % TRITON X, 2 mM EDTA, 150 mM NaCl, 20 mM TRIS-HCl, pH 8.1) followed by immunoclearing with 10 µg salmon sperm DNA and protein A-Sepharose (100 µL of 25 % slurry in 10 mM Tris-HCl, pH 8.1, and 1 mM EDTA; TE8 buffer) for 90 min at 4 °C. After transferring the supernatant into 1.7 mL tubes immunoprecipitation was performed

over night at 4 °C with 2 µg AR PG21 antibody (Santa Cruz Inc.) or hemagglutinin antibody as negative control. After immunoprecipitation 50 µL of pre-cleared A/G-Sepharose beads was added and samples were further incubated for 2.5 h. Samples were pulsed down and the supernatant was aspirated off. Samples were then washed sequentially for 7.5 min each with 1 mL RIPA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mL EGTA, 140 mM NaCl, 1 % Triton X 100, 0.1 % Na-deoxycholate, 0.1 % SDS and 1 x P.I.), TSE II buffer (0.1 % SDS, 1 % Triton X 100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl, pH 8.1 and 1 x P.I.) and Buffer III (250 mM LiCl, 1 % IGEPAL CA 630, 1 % Na-deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). The beads were then washed three times with TE8 buffer and extracted with 300 µL elution buffer (1 % SDS, 100 mM $NaHCO_3$) overnight in a 65 °C water bath. Samples were extracted with phenol: chloroform:isoamyl alcohol (25:24:1; Sigma) and DNA was then precipitated with 2 µL pellet paint (Novagen), 30 µL 3 M Na-acetate, pH 5.2 and 600 µL of 100% ethanol. Consequently the pellet was washed repeatedly with 70 % and 100 % ethanol finally re-suspended in 50 µL water. PCR amplification was carried out with 4 µL DNA extract and the TAQ DNA polymerase kit (Invitrogen). Primers for the ARE I and II promoter region and ARE III enhancer region were used as shown in Table 2.3.

Table 2.3. Primer sequences for PSA promoter and enhancer regions

Primer pair	Direction	Sequence*
ARE I	Forward	5'-TCTGCCTTTGTCCCCTAGAT-3'
	Reverse	5'-AACCTTCATTCCCCAGGACT-3'
ARE II	Forward	5'-AGGGATCAGGGAGTCTCACA-3'
	Reverse	5'-GCT AGCACTTGCTGTTCTGC-3'
ARE III	Forward	5'-CCTCCCAGGTTCAAGTGATT-3'
	Reverse	5'-GCCTGTAATCCCAGCACTTT-3'

* Shang et al., 2002

Chapter 3.

Results

3.1. Effects on Messenger RNA expression of ER inducible target gene pS2 in ECC-1 cells

NOTE: These experiments were conducted once and measured in triplicate. For this reason no statistical analysis was performed.

The effects of all six OPFR's on the estrogen receptor were evaluated. ECC-1 cells were either dosed individually with an OPFR or in combination with E2. No profound changes in pS2 mRNA expression in ECC-1 cells were observed for any of the OPFRs of interest (Figure 3.1). Mild repression of pS2 transcription was observed after 24 h treatment with TCEP, however the effect did not occur in a concentration-dependent manner. No agonist activity was observed for TCEP at concentrations up to 20 μM. A mild repression pS2 mRNA was observed after exposure TMPP and E2 (0.01 μM) at high concentrations. This did not occur in a concentration dependent manner. At 20 μM TEP pS2 transcription appeared increased, suggesting weak ER agonist activity. No further experiments were conducted assessing the direct effects of OPFRs on ER target gene expression or its proteins, as none of the effects appeared profound.

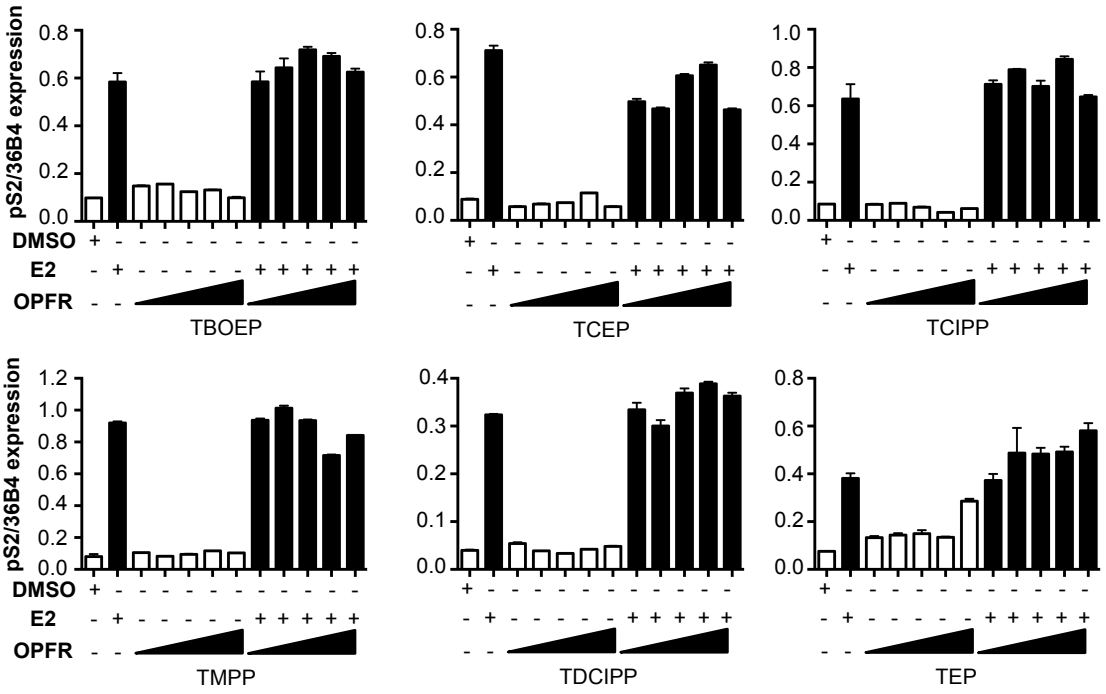


Figure 3.1. Effects of OPFR on pS2 mRNA expression levels.
ECC-1 cells treated with 0.01 µM, 0.1 µM, 1 µM, 10 µM and 20 µM of an OPFR individually or in combination with the ER ligand E2 (10nM). The mRNA levels for pS2 and 36B4 were determined by real-time PCR and normalized to constitutively expressed 36B4 gene. Values are represented as mean value ± standard deviation (SD).

3.2. Effects on Messenger RNA and Protein Expression of AhR Inducible Target Genes

NOTE: These experiments were conducted once and measured in triplicate. For this reason no statistical analysis was performed.

The effects of all six OPFRs on the AhR were assessed by treating ECC-1 cells with the flame retardants individually or in combination with TCDD, a potent agonist of the AhR and its target gene CYP1A1. Figure 3.2 displays the normalized CYP1A1 mRNA expression after 24 h treatment. TEP, TCEP, TCIPP and TDCIPP had no effect on CYP1A1 mRNA expression in ECC-1 cells. However TMPP and TBOEP increased CYP1A1 expression at concentrations of 10 and 20 µM slightly compared to the basal

levels of the solvent control, DMSO, suggesting TBOEP and TMPP may function as weak agonists for the AhR.

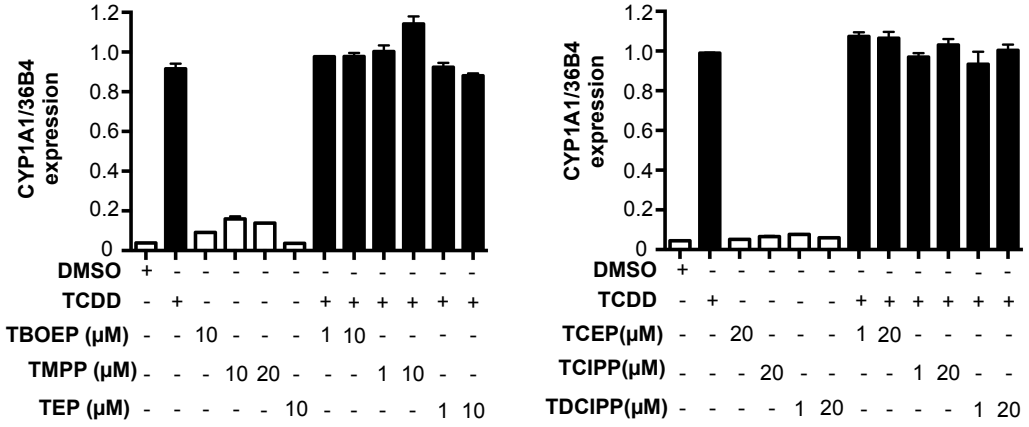


Figure 3.2. CYP1A1 mRNA expression after OPFR exposure in ECC-1 cells.
ECC-1 cells were treated with 0.01 µM, 0.1 µM, 1 µM, 10 µM and 20 µM of an OPFR individually or in combination with TCDD (2.5 nM). The mRNA expression of AhR target gene CYP1A1 were determined by real-time PCR and normalized to constitutively expressed 36B4. Values are represented as mean value ± SD.

Although TEP revealed no changes and TBOEP and TMPP only revealed marginal changes in mRNA expression experiments were conducted exposing ECC-1 cells to selected OPFRs at concentrations ranging over several orders of magnitude. Exposure of ECC-1 cells to higher concentrations (0.1 – 100 µM) of TBOEP and TEP had no effect on CYP1A1 expression, leading to the assumption that TBOEP and TEP do not function as agonists for the AhR in ECC-1 cells (Figure 3.3).

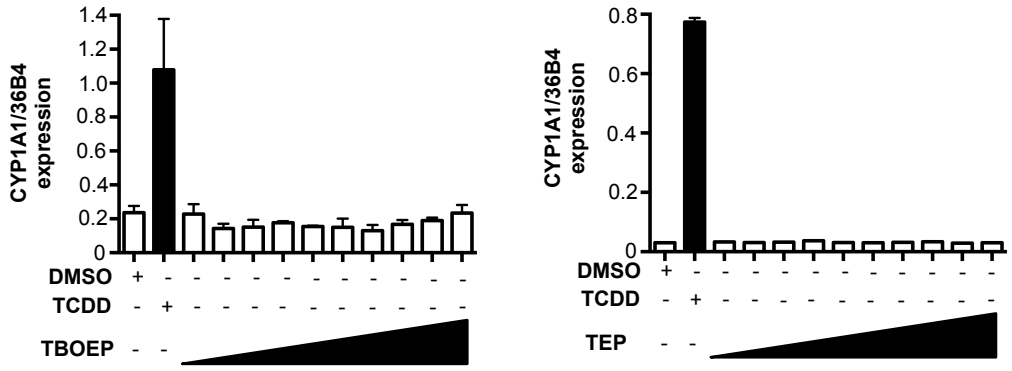


Figure 3.3. CYP1A1 mRNA expression after TBOEP & TEP exposure in ECC-1 cells.

ECC-1 cells were treated with TBOEP or TEP individually with concentrations ranging from 0.01μM to 100 μM. The mRNA expression of AhR target gene CYP1A1 & CYP1B1 was determined by real-time PCR and normalized to constitutively expressed 36B4. Values are represented as mean value ± SD.

TMPP exposure at higher concentrations (5 μM – 100 μM) appeared to increase CYP1A1 mRNA expression profoundly **(**Figure 3.4), indicating a weak agonist effect of TMPP on the AhR. Therefore it was of interest to assess effects on a different AhR target gene such as CYP1B1. There was no effect of TMPP on CYP1B1 gene expression at all concentrations tested (Figure 3.4).

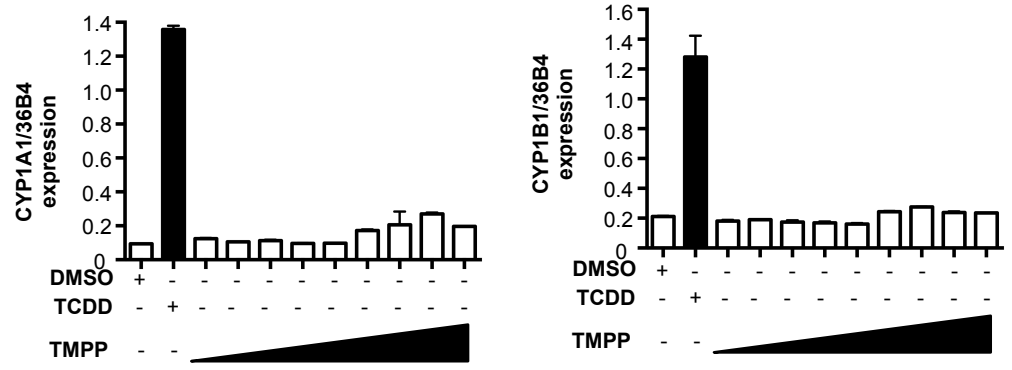


Figure 3.4. CYP1A1 mRNA expression after TMPP exposure in ECC-1 cells.

ECC-1 cells were treated with TMPP individually with concentrations ranging from 0.01μM to 100 μM. The mRNA expression of AhR target gene CYP1A1 & CYP1B1 was determined by real-time PCR and normalized to constitutively expressed 36B4. Values are represented as mean value ± SD.

3.3. Effects on Messenger RNA and Protein Expression of AR Inducible Target Genes

NOTE: These experiments were conducted once and measured in triplicate. For this reason no statistical analysis was performed.

Additionally it was of interest to elicit the interaction of the OPFR's with the AR. LNCaP cells were used to model the interaction of individual OPFRs or in combination with the AR ligand R1881 (Figure 3.5). None of the six OPFRs appeared to have any agonist effects on AR inducible target gene PSA. Exposure to R1881 + TMPP and R1881 + TCIPP moderately repressed PSA mRNA expression suggesting mild antagonist activity. R1881 + TDCIPP repressed mRNA expression of PSA at concentrations 10 μM and 20 μM, suggesting strong antagonist effects on the androgen receptor and its inducible gene, PSA.

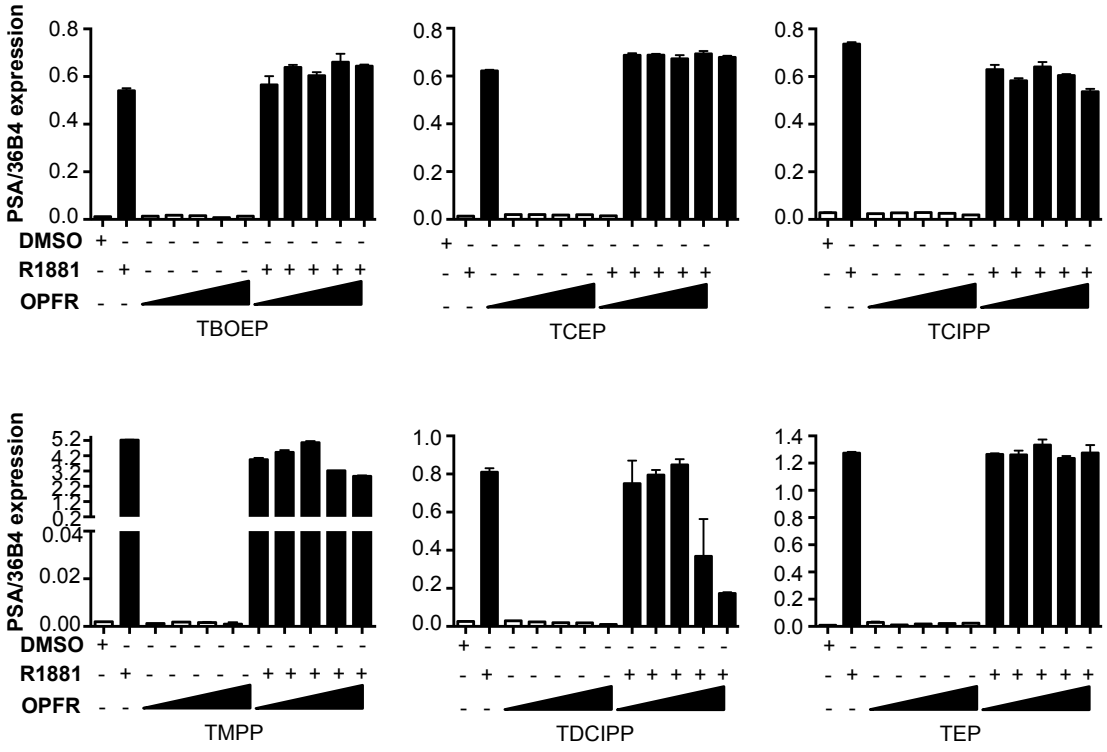


Figure 3.5. Effects of OPFR on PSA mRNA expression levels.
LNCaP cells treated with 0.01 μM, 0.1 μM, 1 μM, 10 μM and 20 μM of an OPFR individually or in combination with the AR ligand R1881 (0.001 μM). The mRNA levels for PSA and 36B4 were determined by real-time PCR and normalized to constitutively expressed 36B4 gene. Values are represented as mean value ± SD.

While several selected OPFRs revealed mild antagonist effects on the androgen receptor, further investigations were focused on TDCIPP and its effect on the AR, as the effects appeared most profound.

3.3.1. Effects of TDCIPP on AR Inducible Target Gene mRNA & Protein Expression

In order to investigate the effects of TDCIPP on AR regulated target gene expression, mRNA accumulation of PSA, IGF-1 and PSMA was determined Figure 3.6**.** In a concentration-dependent manner TDCIPP repressed PSA and IGF-1 mRNA when activated with R1881. Significant repression of PSA and IGF-1 was observed at concentrations of TDCIPP from 1 µM – 50 µM ($p < 0.01$). PSMA expression is repressed in LNCaP cells with ligand activated AR compared to inactivated AR (DMSO control). Thus, PSMA mRNA expression was initially repressed as the cells were treated combinatorial with R1881 and TDCIPP. At higher concentrations (5 µM – 50 µM; $p < 0.01$) repression of PSMA mRNA accumulation by R1881 was significantly decreased by TDCIPP.

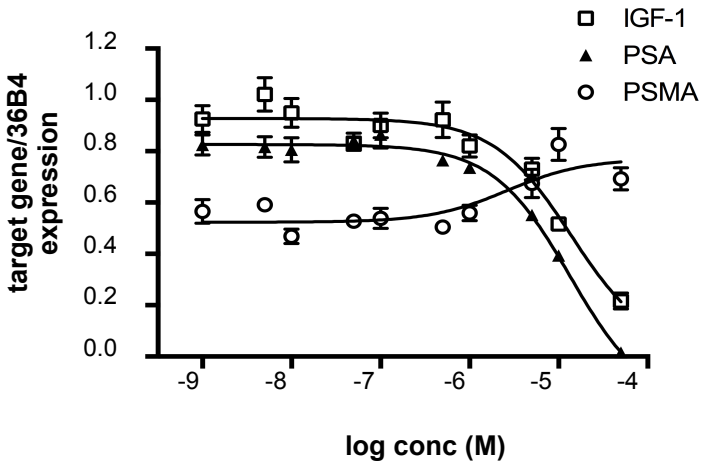


Figure 3.6. Effects of TDCIPP on PSA, PSMA and IGF-1 mRNA levels.
Cells were co-treated with 0.001 µM R1881 and varying concentrations of TDICPP (0.001 µM, 0.005 µM, 0.01 µM, 0.05 µM, 0.1 µM, 0.5 µM, 1 µM, 5 µM, 10 µM and 50 µM). The mRNA levels for PSA, PSMA and IGF-1 and 36B4 were determined by real-time PCR and normalized to constitutively expressed 36B4 gene (n=3). Treatments are expressed as mean values ± standard error mean (SEM). Treatment groups from 1 – 50 µM for PSA and IGF-1 mRNA expression were statisticaly significant ($p < 0.001$). PSMA mRNA was statistically signicfiant for concentrations of TDCIPP from 5 – 50 µM ($p < 0.05$).

Subsequently, it was of interest to determine if TDCIPP inducible alterations in AR-target gene mRNA accumulation resulted in a concomitant change of intracellular protein levels. Whole cell protein extracts were prepared from LNCaP cells treated with TDCIPP in combination with R1881 (Figure 3.7 **A**) and protein levels of PSA and PSMA were determined. These results were in concert with the effects seen on the mRNA level. PSA was repressed at lower concentrations of TDCIPP (0.05 μM – 10 μM) and below basal levels at 50 μM. The repression of PSMA protein was reversed by TDCIPP at the high concentrations of 10 μM and 50 μM. These results provide additional evidence that TDCIPP is an antagonist for the AR and exerts anti-androgenic effects.

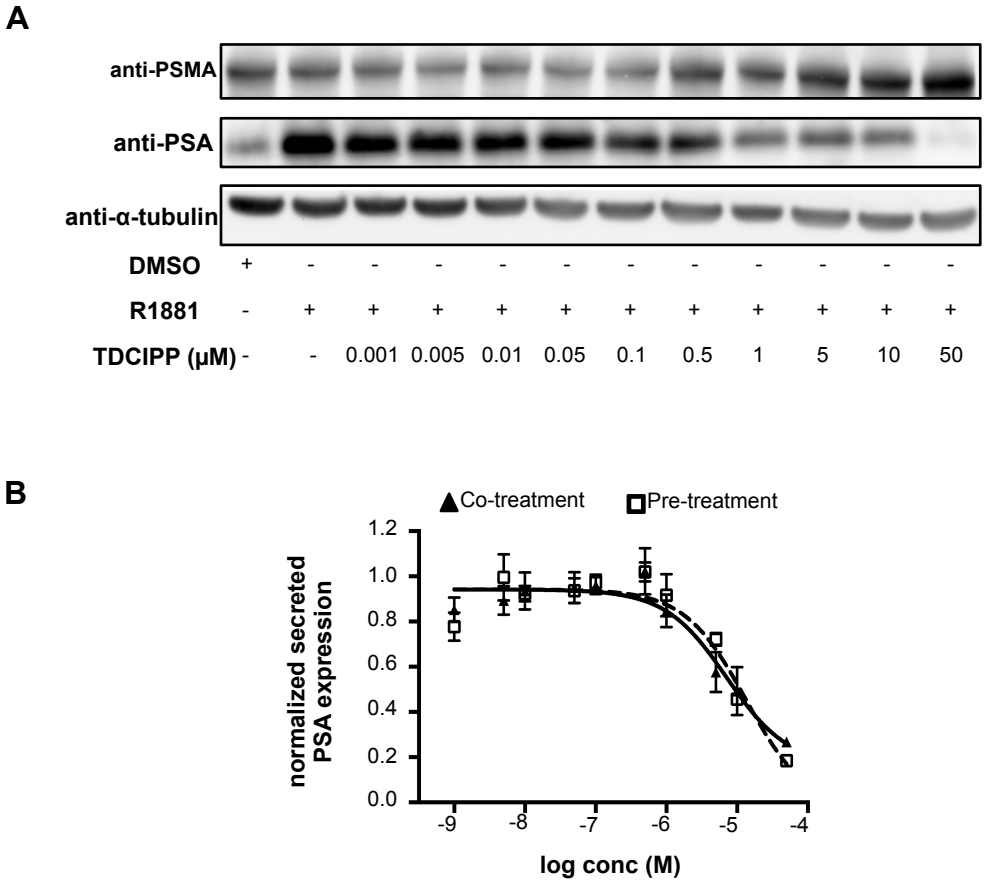


Figure 3.7. (A)Effects of TDCIPP on PSMA and PSA protein expression. Immunoblots of PSMA, PSA and α-tubulin protein levels after chemical treatments for 24 h. Cells were treated with vehicle, R1881 at 0.001 μ M, TDCIPP at 0.001 μM, 0.005 μM, 0.01 μM, 0.05 μM, 0.1 μM, 0.5 μM, 1 μM, 5 μM, 10 μM and 50 μM in combination with 0.001 μM R1881. Immunoblotting was performed using antibodies directed against PSMA, PSA and α-tubulin. **(B) Comparison of excretory PSA protein: Co-treatment versus pre-treatment.** LNCaP cells were cultured and treated as described in Figure 3.6. Culture media was collected 24 h after treatment. LNCaP cells were pre-treated with TDCIPP at the same concentrations as the co-treated cells for 30 min followed by R1881. The cells were then incubated for 24 h and culture media was collected. Excretory PSA levels were quantified using an ultrasensitive COBAS® e 411 CORE II immune-detection system. The overall course and slope of the repression appears almost identical for both treatments. Treatments are expressed as mean values ± standard error mean (SEM); one way ANOVA; n=4.

PSA is an excretory protein and thus, it was of interest to assess the effect of TDCIPP on extracellular PSA protein expression in conditioned culture media. Conditioned culture media contained significantly less PSA when cells were co-treated with 5 μM, 10 μM and 50 μM TDCIPP (Figure 3.7 **B**; n=4, $p < 0.05$). LNCaP cells were also pre-treated with the concentrations mentioned above for 30 min before R1881 (0.01

μM) was added for 24 h (Figure 3.7 **B**). Extracellular PSA protein was statistically significantly repressed at 10 μM and 50 μM TDCIPP, no differences in the trend of the concentration response curve were observed concluding R1881 can still activate the AR, initial presence is not required for the inhibitory effects of TDCIPP on PSA protein expression.

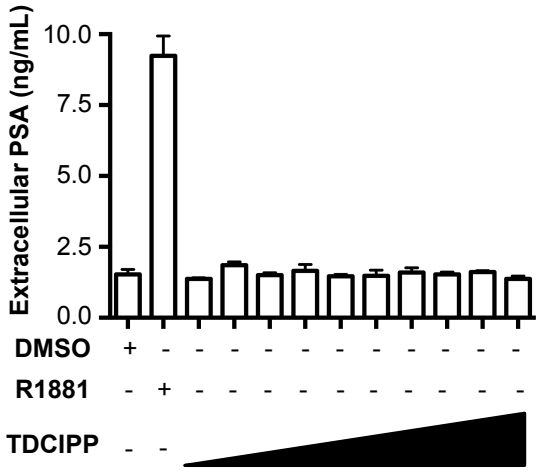


Figure 3.8. Effect of TDCIPP on excretory PSA protein.
Cells were treated with various concentrations of TDICPP (0.001 μM, 0.005 μM, 0.01 μM, 0.05 μM, 0.1 μM, 0.5 μM, 1 μM, 5 μM, 10 μM and 50 μM) and DMSO and R1881 (0.001 μM). Excretory PSA concentrations were not affected by increasing concentrations of TDCIPP. Treatments are expressed as mean values ± standard error mean (SEM); differences in treatment groups were not found to be statistically significant, n=4.

While some receptor antagonists have shown to act as partial agonists for the same receptor at high concentrations, no such effect was observed in LNCaP cells, as excretory PSA protein was not affected by increasing concentration of TDCIPP (Figure 3.8).

It has been reported that AR mRNA accumulation is repressed by exposure to androgens and AR protein levels are stabilized in LNCaP cells (Blok et al., 1992; Wolf, Herzinger, Hermeking, Blaschke, & Hörz, 1993). When LNCaP cells were treated with TDCIPP and R1881 in combination, we observed significant lower AR protein levels (Figure 3.9) with increasing TDCIPP concentrations. The amount of AR protein levels slightly decreased at concentrations ranging from 1 μM to 50 μM, indicating that TDCIPP may have an effect on AR protein abundance in LNCaP cells.

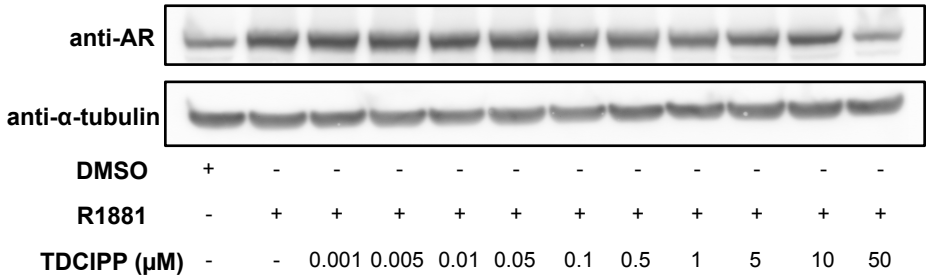


Figure 3.9. Effects of TDCIPP on AR protein expression in LNCaP cells.
Immunoblot of AR and α-tubulin protein levels after chemical treatments for 24 h. Cells were treated with vehicle, R1881 at 0.001 μM, TDCIPP at 0.001 μ M, TDCIPP at 0.001 μM, 0.005 μM, 0.01 μM, 0.05 μM, 0.1 μM, 0.5 μM, 1 μM, 5 μM, 10 μM and 50 μM in combination with 0.001 μM R1881. Immunoblotting was performed using antibodies directed against AR and α-tubulin. An observable reduction in AR protein was revealed with co-treatment of R1881 and TDCIPP at 10 μM and 50 μM.

The effects of TDCIPP were assessed in a second prostate cancer cell line (VCaP). Basal levels of PSA mRNA expression were reduced with increasing concentrations of TDCIPP alone, these effects were not significant ($p > 0.05$; Figure 3.10 **A**). PSA expression induced by R1881 was repressed by TDCIPP at 1 μM and 50 μM. However only the latter was statistically significant ($p < 0.001$). When the effects of TDCIPP on R1881 –inducible AR target gene mRNA accumulation in LNCaP and VCaP cells were compared, it appeared that the mRNA accumulation in LNCaP cells was more sensitive to the effects of TDCIPP. PSA protein expression in VCaP cells was not as drastically repressed at 50 μM as the mRNA transcription indicated. In fact, PSA protein repression only seemed to decrease marginally at 50 μM TDCIPP (Figure 3.10 **B**).

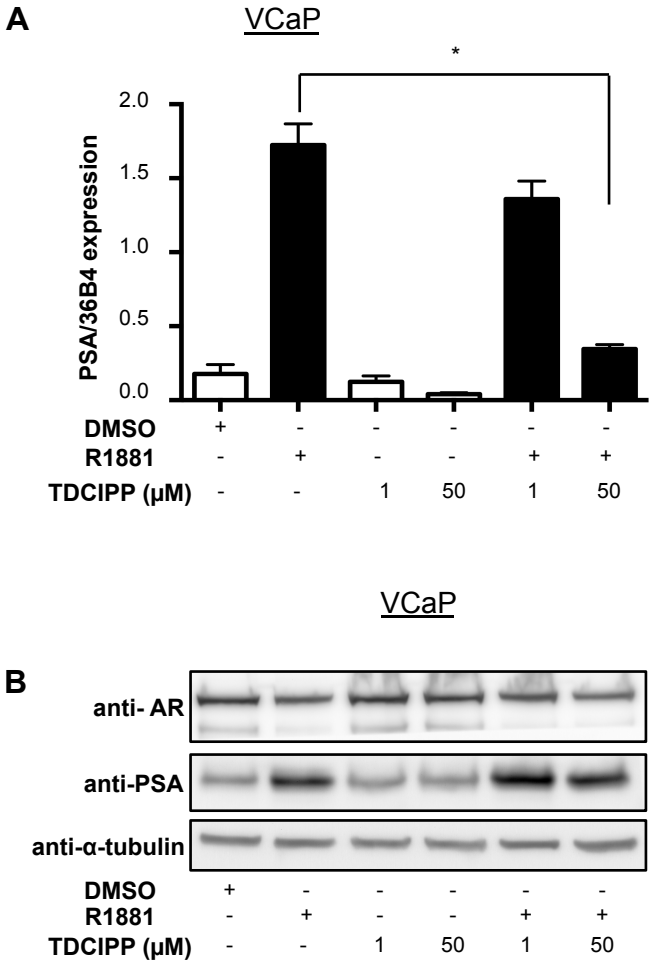


Figure 3.10. (A) Effects of TDCIPP on PSA mRNA expression in VCaP cells. VCaP cells were treated with TDCIPP at 1 μM and 50 μM alone and in combination with R1881 (0.001 μM) for 24 h. Basal levels of PSA mRNA expression were reduced with increasing concentrations of TDCIPP alone, although none of the effects were significant. PSA expression induced by R1881 was reduced by TDCIPP at 1 μM and significantly at 50 μM. Treatments are expressed as mean values ± standard error mean (SEM); * $p < 0.05$, one way ANOVA; n=3. **(B) Effects of TDCIPP on PSA and AR protein expression in VCaP cells.** VCaP cells were treated with TDCIPP at 1 μM and 50 μM alone and in combination with R1881 (0.001 μM). Whole cell lysate was collected and probed for anti-AR, anti-PSA and anti-α-tubulin. It appeared that the overall AR protein level remained unaffected.

Furthermore, the effect of TDCIPP on the abundance of AR itself was investigated in LNCaP and VCaP cells (Figure 3.9 and Figure 3.10 **B**, respectively). In VCaPs in contrast to LNCaP cells, AR protein is decreased with exposure to androgens (Cai et al., 2011). This phenomenon was confirmed as with exposure to R1881 (lane 2), AR abundance is slightly repressed compared to the DMSO control (lane 1). However, when VCaP cells were treated with TDCIPP at 1 μM and 50 μM alone or in combination

with R1881 it appeared that the overall AR protein level remained unaffected (Figure 3.10 **B**).

3.3.2. Recruitment of AR to PSA promoter

As the molecular mechanism of the anti-androgenic actions of TDCIPP are not currently known, a ChIP assay was conducted to determine whether TDCIPP represses the AR complex recruitment to the AREs of AR target genes. There are three well characterized proximal ARE in the PSA promoter, ARE I, and II (Shang et al., 2002). Oligonucleotides to amplify the DNA were directed against the above mentioned response elements. The concentrations (1 & 10 μM) were chosen for TDCIPP treatments, as this was the concentration that was shown to significantly reduce AR protein. An enrichment of ARE I and II promoter chromatin was observed for LNCaP cells treated with R1881 alone for 1 h (Figure 3.11). Cells dosed with R1881 and TDCIPP in combination at 1 μM and 10 μM showed a decrease in ARE I chromatin enrichment compared to R1881 treatment. TDCIPP appeared to reduce chromatin enrichment to basal levels for this response element. TDCIPP alone did not affect chromatin enrichment at either concentration. For the ARE II PSA promoter a concentration dependent repression of the chromatin signal was observed for combinatorial treatment of R1881 and TDCIPP at 1 and 10 μM, suggesting that R1881-inducible recruitment of AR to this region is impeded in the presence of TDCIPP. Chromatin enrichment observed in precipitates from cells treated with TDCIPP at 1 μM alone increased surprisingly. The mechanisms behind this observation remain unclear.

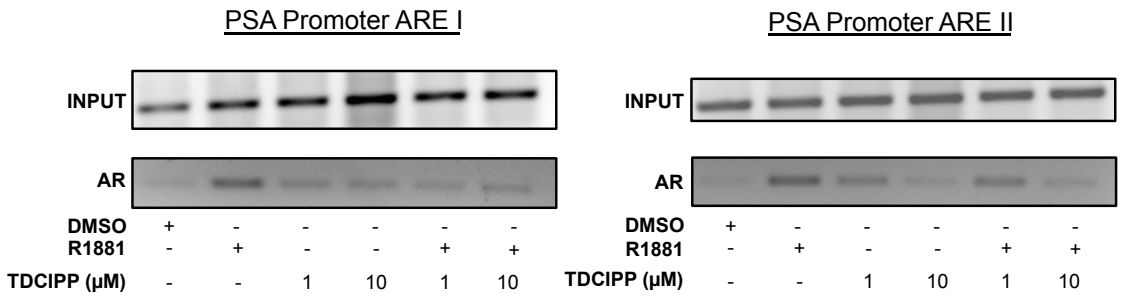


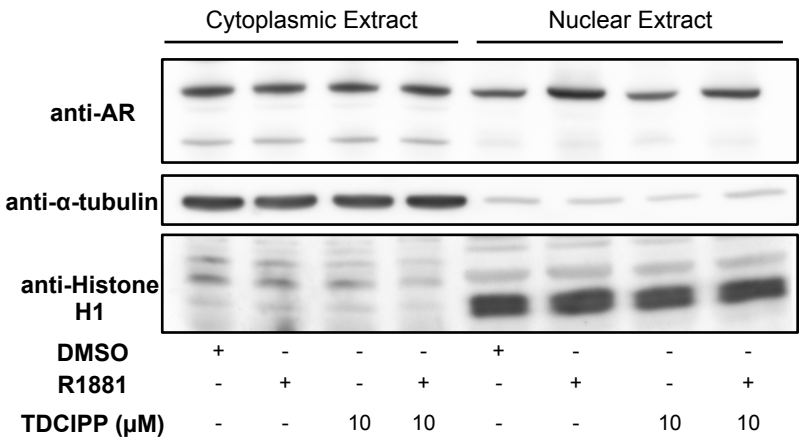
Figure 3.11. Effects on the R1881-inducible recruitment of AR at the PSA gene in LNCaP cells.

Chromatin immunoprecipitation assays of PSA promoter regions in LNCaP cells using antibodies targeting AR. Cells were treated with vehicle, R1881 at 1 nM, TDCIPP alone at 1 & 10 μM and in combination with R1881 for 45 min.

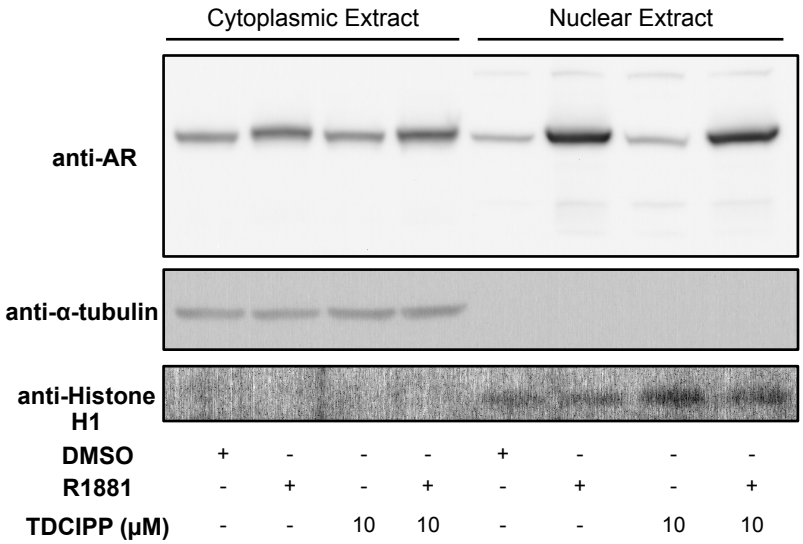
Subcellular fractionation was performed to determine if TDCIPP interfered with the translocation of the AR-complex into the nucleus in LNCaP cells. Following a 1 h treatment, TDCIPP alone at 10 μM had no effect on AR translocation into the nucleus (Figure 3.12 **A**). Co-treatment of R1881 and TDCIPP at 10 μM partially blocked the localization of AR in the nucleus compared to the positive control 0.001 μM R1881. Densitometric analysis of three separate observations confirmed a significant reduction of nuclear AR accumulation, when treated with R1881 and TDCIPP at 10 μM (Figure 3.12 **B**). The blocking of the AR protein complex could partially account for the altered PSA and PSMA protein expression (Figure 3.7 **A**). Cytosolic AR abundance is comparatively even over all treatment groups (Figure 3.12 **A**). Generally AR induction by R1881 occurs within an hour of exposure and the AR complex is shuttled continuously from the cytoplasm to the nucleus, where gene transcription is initiated. This dynamic process would explain the even distribution of AR between DMSO and R1881 as well as the TDCIPP treatment groups (Figure 3.12 **A**).

Interestingly, LNCaP cells treated for 24 h revealed a different pattern of AR protein distribution in the cytosol (Figure 3.12 **C**) compared to the 1 h treatment (Figure 3.12 **B**). The cytosolic distribution of AR protein accumulation increased for R1881 treated and TDCIPP + R1881 treated samples. There was no apparent difference between DMSO and TDCIPP treated cells in AR abundance.

A


Cytoplasmic Extract
Nuclear Extract
anti-AR
anti-α-tubulin
anti-Histone H1
DMSO + - - - + - - -
R1881 - + - + - + - +
TDCIPP (μM) - - 10 10 - - 10 10

B


Cytoplasmic Extract
Nuclear Extract
anti-AR
anti-α-tubulin
anti-Histone H1
DMSO + - - - + - - -
R1881 - + - + - + - +
TDCIPP (μM) - - 10 10 - - 10 10

C

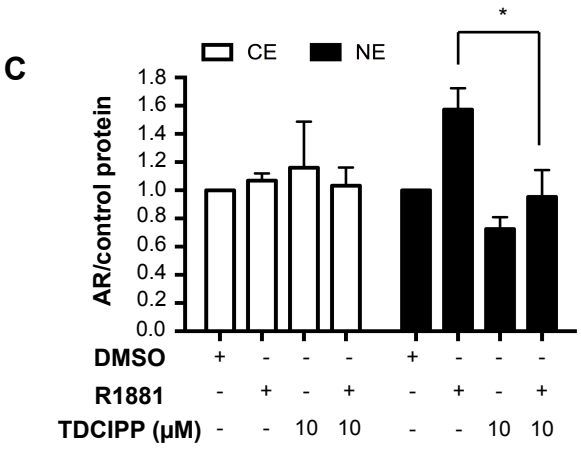

CE
NE
*
AR/control protein
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
DMSO + - - - + - - -
R1881 - + - + - + - +
TDCIPP (μM) - - 10 10 - - 10 10

Figure 3.12. (A) Subcellular fractionation of AR after 1 h treatment.
Immunoblotting of fractionated cytoplasmic and nuclear LNCaP cell lysates following treatment with vehicle, R1881 at 0.001 μM, TDCIPP (10 μM) alone or in combination with R1881 for 1 h was performed using antibodies directed against AR, α-tubulin (cytoplasmic marker) and histone H1 (nuclear marker). (B) Subcellular fractionation of AR after 24 h treatment. LNCaP cells were treated identically as the experiment described in (A), except cells were treated for 24. (C) Densitometry analysis of one h treated cells. Densitometry analysis was performed on AR immune blots (normalized against α-tubulin). Data represents three different observations and values are express as mean values ± SEM. (*) $p<0.05$ compared to R1881 alone, one way ANOVA.

3.3.3. Affinity of TDCIPP for AR Ligand Binding Domain

Thus far the assumption was that TDCIPP exerted its effects by directly inhibiting ligand binding to the AR-LBD. This assumption was tested using the PolarScreen™ Androgen Receptor Competitor Assay. The positive control R1881 successfully replaced the AL-Green Fluoromone and thus significantly repressed fluorescent polarization values (mFP) compared to the vehicle control. This observation confirmed the affinity of R1881 for the AR-LBD. Contrary to our expectations the polarization values were not affected by increasing concentrations of TDCIPP (Figure 3.13) leading to the hypothesis that TDCIPP is not a ligand for the AR-LBD, but possibly exerts its antagonist effects on the AR by non-competitive inhibition.

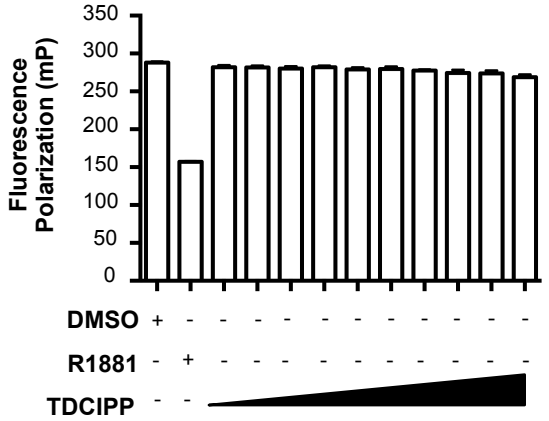


Figure 3.13. TDCIPP binding affinity for AR-LBD.
Fluorescent polarization values were measured after treatment of AR-LBD/AL-Green Fluoromone (0.025 μM/0.001 μM) with TDCIPP from 0.0005 μM – 10 μM. No effect of TDCIPP on the AR-LBD was observed, as the polarization values remained the same over all doses. Treatments are expressed as mean values ± standard error mean (SEM); n=2.

Chapter 4. Discussion

4.1. Effects of OPFRs on Aryl hydrocarbon and Estrogen Receptor Inducible Genes and their Proteins

The six OPFRs tested did not exert profound effects on estrogen receptor signaling. TCEP displayed mild antagonist effects on the ER in ECC-1 cells (Figure 3.1). These results are supported by findings in Follmann and colleagues (2006) and Kojima and colleagues (2013) that assessed the effect of TCEP in ECC-1 cells and in transiently transfected CHO K1 cells, respectively. TCEP does not appear to exert endocrine disrupting effects directly via hormone receptors (Kojima et al., 2013), however, does exert endocrine disrupting effects in steroidogenesis in H295R cells (X. Liu et al., 2012). In chicken embryos TEP reduced free circulating T_4 at concentrations as low as 9 ng/g (equivalent to 494 nM/g) and activated the transcription of CXR responsive genes, which is the avian analog to the mammalian PXR. Kojima et al. (2013) did not observe any affect of TEP on the PXR, AR or ER. In this study however, TEP increased pS2 mRNA transcription in ECC-1 cells at 20 µM (Figure 3.1). Different observations may be due to concentration differences or species and model (*in vitro* versus *in vivo*) specificity.

Many CYP enzymes are induced by a variety of substrates including pharmaceutical drugs, industrial chemicals but also endogenous compounds (Tompkins & Wallace, 2007) and are regulated by receptor transcription factors. None of the six selected OPFRs exhibited major effects on CYP1A1 gene expression. Therefore, it was concluded that the six OPFRs do not have a high binding affinity for the AhR in ECC-1 cells. However, subtle CYP1A1 induction via the AhR in ECC-1 cells was observed for TMPP at concentrations of 5 – 100 µM, no such induction was observed for CYP1B1 mRNA expression. TBOEP and TEP in a dose-response experiment had no effect on the AhR (Figure 3.3**)**. Observations regarding TMPP, TBOEP and TEP and their effects on AhR and its inducible target genes have not been reported. However many OPFRs including those selected for this study, have been shown to induce the CYP11A,

CYP11B, CYP19A (involved in steroidogenisis) CYP3A, CYP2H (oxidation of lipophilic compounds, such as steroids and xenobiotics) and CYP17 (glucuronidation pathway for urinary excretion) families in human cancer cell lines, zebrafish and chicken embryos (Crump et al., 2012; Egloff et al., 2014; Farhat et al., 2013; X. Liu et al., 2012; 2013b; E. Porter et al., 2014). Direct interaction with the respective key receptor transcription factors for the CYP genes were not assessed in the aforementioned studies, however it has been well established that these CYP enzymes can be induced by the PXR/CXR, CAR, PPAR, GR and liver X receptor (Tompkins & Wallace, 2007). Thus, OPFRs' affinity for these transcription factors are likely and could pose downstream endocrine disrupting risks for humans and wildlife.

In this study TDCIPP had no effect on AhR activity in ECC-1 cells (Figure 3.2). Similarly TDCIPP did not alter expression of CYP1A1 in zebrafish (C. Liu et al., 2013a), although, other AhR inducible target genes, such as CYP1B1, AhR itself and related co-regulators were up regulated in the same study. The regulation of transcription of AhR inducible target genes is highly complex and does not rely solely on ligand binding but on the recruitment of a battery of co-activators, co-repressors, chaperone proteins and other transcription factors (Beischlag et al., 2008). Thus, species-specific responses involving complex signaling interactions are plausible. Crosstalk between the AhR and other receptor transcription factors is plausible and transactivation and repression potential of OPFR's and the AhR ought to be further investigated.

4.2. Effects of TBOEP, TCEP, TCIPP, TEP and TMPP on Androgen Receptor Inducible Genes and their Proteins

The results demonstrate that the selected OPFRs did not display agonist activity for the AR in LNCaP cells. To my knowledge no other study has found AR agonist properties of these OPFRs. However PXR agonist properties were found for TBOEP, TCIPP and TDCIPP in transiently transfected CHO K1 cells (Kojima et al., 2013). TMPP and TCIPP exhibited mild AR antagonist properties by repressing PSA mRNA expression (Figure 3.5), but did not show a concentration-response relationship. To my knowledge, this is the first study to investigate directly the effects of TBEOP, TCEP, TCIPP, TEP and TMPP exposure on R1881-inducible target genes.

4.3. Effects of TDCIPP on AR inducible genes and their proteins and mechanism of action

As demonstrated in this study, TDCIPP significantly repressed the transcription of PSA and IGF-1 in LNCaP cells. A previous study by Kojima et al. (2013) revealed TDCIPP's antagonist effects against the human AR in transiently transfected CHO K1 cells with a luciferase reporter gene assay. The 20 % relative inhibitory concentration in this cell line was calculated at 1.9 ± 1.5 µM. In this study effective repression of PSA mRNA and protein in LNCaP cells was observed at 1 µM TDCIPP and higher (Figure 3.6 **&** Figure 3.7 **A**). It was demonstrated that internal and excretory PSA protein expression decreased with increasing TDCIPP concentrations suggesting anti-androgen activity of TDCIPP in LNCaP cells. A further indication of TDCIPP functioning via the AR, is the repression of R1881-inducible IGF-1 expression, when exposed to high concentrations of TDCIPP. Crump et al. (2012) recently reported a 10-fold reduction of IGF-1 transcription in CEH treated for 36 h with TDCIPP (10 µM). This study however did not assess whether this effect was mediated via the AR. The effect of TDCIPP on PSA in a second prostate cancer cell line (VCaP) showed similar results to those obtained in LNCaP cells. Repression of PSA mRNA, although significant, was only achieved at a much higher TDCIPP concentration (50 µM, Figure 3.10 **A**). PSA protein repression in VCaP cells was not apparent. Although both cell lines are androgen sensitive, AR abundance is repressed in VCaPs (Cai et al., 2011) and stabilized in LNCaP cells upon androgen exposure (Blok et al., 1992; Wolf et al., 1993). Different mechanisms of AR regulation may alter PSA gene and protein expression, thus causing cell dependent sensitivities to TDCIPP.

PSMA is a cell surface protein that is expressed in both healthy prostate tissue and in prostate cancers. It can be expressed up to a 1000 fold higher in prostate cancer tissue than in healthy tissue (Ghosh & Heston, 2004). For this reason PSMA has been used as a visualizing biomarker for monitoring prostate cancer progression or reoccurrence (Osborne et al., 2013). In cancer cell lines, such as LNCaP cells, it has been demonstrated that PSMA is repressed by androgens, even though PSMA is normally up regulated by androgens in prostate cancer. Similarly, in this study PSMA expression is repressed when treated with R1881 and up regulated in the untreated

group (Figure 3.7 **A**). Co-treatment of TDCIPP and R1881 blocked R1881- inducible repression of PSMA in a concentration-dependent fashion, thus strengthening the hypothesis that TDCIPP exerts its affects via the AR.

The conformational change of ligand-activated AR takes place on average within 3.5 min (J. O. Jones et al., 2009). This change initiates the translocation of the AR complex into the nucleus. The same research group demonstrated that the abundance of conformational changed AR peaked after 1 h treatment with DHT and remained stable up to 4 h later (J. O. Jones & Diamond, 2008). Yet, no later time points were taken. Upon androgen exposure translocation into the nucleus can occur within 15 min and accumulates almost primarily in the nucleus after 60 min depending on the cell species (Tyagi et al., 2000). Although, conformational change and nuclear translocation due to androgen exposure in the cell is almost immediate, the transcriptional machinery for gene expression requires a 12-24 h period for protein levels to reflect the exposure condition. Therefore, the immunoblot after 1 h exposure (Figure 3.12 **A**) depicts a snap shot of the dynamic and continuing process of protein translation. Hence, AR protein accumulation appears equal between DMSO and R1881 treatment in the cytoplasm. After 24 h – R1881 exposure, AR protein accumulation has increased in the cytoplasm, but is predominant in the nucleus (Figure 3.12 **B**).

TDCIPP reduced AR translocation within the 1 h treatment **(**Figure 3.12 **A, right lane in nuclear extract**) compared to R1881. After 24 h the ratio of R1881/R1881 + TDCIPP was unaffected (Figure 3.12 **B, right lane in nuclear extract)**. Interestingly, the translocation of the AR to the nucleus upon antagonist activation still occurs, yet a slower rate compared to agonist activation (Tyagi et al., 2000); potentially explaining reduced AR protein accumulation in the nucleus after R1881 + TDCIPP (10 μM) treatment for 1 h. Bicalutamide and hydroxyflutamide, AR antagonists, have been shown to significantly delay translocation, with predominant nuclear localization only being observed 120 min after ligand addition (Klokk et al., 2007; Tyagi et al., 2000). Furthermore, AR-ARE binding stability and time was significantly reduced, thus reducing gene transcription. Overall these results indicate that TDCIPP causes alterations in AR-inducible genes most likely during early exposure and the AR target genes do not recover after 24 h, even though AR balance in the cell is restored. Furthermore, reduced

chromatin enrichment at the PSA promoter (ARE I; Figure 3.11) after 1 h treatment indicates a blockage of the transcription complex to the promoter. Thereby, PSA gene expression was reduced. ChIP was not performed after a 24 h treatment with TDCIPP. This could elicit if the reduction in ARE enrichment persisted even though nucleic AR levels had equated after 24 h -TDCIPP treatment. The persistence of this reduction could explain the reduced concomitant gene transcription observed in this study. Overall, these results suggest that TDCIPP likely exerts its transcriptional anti-androgenic effects by reducing AR protein accumulation (Figure 3.9), impeding translocation of the AR into the nucleus (Figure 3.12) and blocking the binding of AR to the PSA promoter (Figure 3.11).

Lastly, an AR ligand binding assay was employed to assess TDCIPP's affinity for the AR-LBD. This assay utilized the AR-LBD from the rat. The LBD sequence of the rat and human share 100 % sequence identity (Chang, Kokontis, & Liao, 1988). Surprisingly, TDCIPP did not reveal any affinity for the LBD, as fluorescence polarization values were stable over all concentrations. This is indicative of TDCIPP not binding to the LBD when exerting its anti-androgenic effects in LNCaP cells. It has been well documented that the human cancer cell line, LNCaP, expresses a mutated form of the AR (Veldscholte et al., 1992). This missense mutation in the LBD interchanges the amino acid Threonine to Alanine at position 877. The AR is then sensitive to progesterone, estrogen, anti-estrogens, adrenal androgens and hydroxyflutamide as ligands for activation (Veldscholte et al., 1992). The rat AR- LBD used in the binding assay does not contain this mutation (Chang et al., 1988). Therefore it could be argued that TDCIPP's lack of affinity for the rat LBD is due to the missing point mutation. However, Montgomery et al. (1992) did not find a difference in androgen affinity between the wild type AR derived from ventral prostate cytosol from rats and the AR –T877A harbored by LNCaP cells. Additionally, transcriptional effects of TDCIPP were reproduced in VCaP cells, which express the wild type AR (Figure 3.10 A). Furthermore, ECC-1 cells for this project did not confirm estrogenic or anti-estrogenic activity for TDCIPP (Figure 3.1). Hence, it is conceivable that TDCIPP is not interacting with the AR in LNCaP cells due to the point mutation, and TDCIPP is not exerting its effects as an (anti-) estrogenic compound.

Based on the results presented, this study proposes that TDCIPP acts as a non-competitive inhibitor on the AR. Several pathways for non-competitive inhibition are possible. For example TDCIPP binds to the AR other than the LBD, causing a conformational change in

1. the AR-LBD, thereby preventing ligands, such as R1881 from binding, or,
2. the AR protein itself, impeding or preventing the interaction of chaperone proteins or co-factors with the AR, or,
3. the AR protein, impeding or preventing the AR complex to bind to DNA in the nucleus.

The combination of these effects can theoretically lead to the anti-androgenic effects of TDCIPP observed in this study. However, with the data set obtained from this study it is not possible to determine the exact mechanisms of action. Lastly, the possibility must be considered, that all of the above mentioned results are merely a downstream effect of TDCIPP and are not due to direct interaction with the AR.

DDT/DDE (Danzo, 1997), vinclozolin (Monosson, Kelce, Lambright, Ostby, & Gray, 1999), linuron (Lambright et al., 2000), lindan (Danzo, 1997) and bisphenol A (Wetherill et al., 2006) are all environmental contaminants that have been proven to exert their anti-androgenic effects through binding of the AR to alter folding of the LBD and blocking recruitment of co-activators or by directly competing with ligands for the LBD. Interestingly, bisphenol A known for its estrogenic effects, exerts strong anti-androgenic effects on the AR by non-competitive inhibition and inhibits ligand binding by either using a different binding site or altering the conformation of the AR complex (Wetherill et al., 2005). The authors further proposed that bisphenol A serves as a sensitizer for mutant ARs in prostate cancer and thus can initiate or exacerbate a relapse into castration resistant prostate cancer (CRPC). Androgen deprivation therapy (ADT) is the first line of treatment for patients diagnosed with prostate cancer. This line of treatment is most successful for 2-3 years, before the majority of patients develop advanced prostate cancer or CRPC (Tilki & Evans, 2014). Cancerous prostate cells can resume growth after ADT via multiple adaptive mechanisms. These mechanisms include among others AR amplification (Koivisto et al., 1997), AR mutations (B. J. Feldman & Feldman, 2001; Marcelli et al., 2000) and ligand independent AR activation (Culig et al.,

1994; B. J. Feldman & Feldman, 2001). Ultimately, ADT increases the pressure of the malignancy to develop towards a castration-resistant and lethal phenotype.

Treatment for CRPC remains palliative. Prolonged exposure to TDCIPP may potentially mimic androgen deprivation in existing prostate cancer and can accelerate the progression of the disease to CRPC. Prostate cancer is the most commonly diagnosed cancer in men in North America. The diagnostic biomarker for prostate cancer is assessed via the serum PSA levels usually determined with a COBAS PSA detection system. Wong et al. (2015) revealed that DDT/DDE at concentrations reported in human tissues can effectively decrease PSA concentrations and therefore could mask the presence of prostate cancer in patients tested for PSA levels via a COBAS detection system. Thus false negatives could be produced. TDCIPP has been detected in multiple human tissues at varying concentrations (Carignan et al., 2013; E. M. Cooper et al., 2011; Hudec et al., 1981) and in this study, it has displayed strong anti-androgenic properties, by repressing excreted PSA accumulation in conditioned culture media (Figure 3.7 B). It is comprehensible that TDCIPP can not only hinder early prostate cancer detection efforts, but also increase selective pressure on cancers to develop to a therapeutically refractile disease state.

4.4. Limitations and Future Perspective

In general limitations of this study are:

1. Using transformed cell lines such as ECC-1, LNCaP and VCaP cells. Transformed cells often have a higher resistance to the effects of chemicals, thus underestimation of effective concentrations for a chemical are possible (Dishaw et al., 2011).

2. Using a synthetic androgen, such as R1881 opposed to DHT or T. R1881 has a 1.5-2.0 fold higher binding affinity for the AR compared to DHT (T. R. Brown, Rothwell, & Migeon, 1981). Consequently the effective concentration of TDCIPP in LNCaP cells could be underestimated.

3. A limited sample size. Statistical analysis due to small sample size for some experiments was not possible,

4. Concentrations of the OPFRs in the culture media were not determined. This information could have given evidence on uptake into the cell and chemical stability of the OPFR. For this study it was assumed that the total concentration equaled the effective concentration in the cell.

5. Experimentation with exposure time to simulate chronic exposure.

Future work regarding TDCIPP ought to include:

1. Assessing the half-life of TDCIPP in cells, which will allow to estimate the duration of action.

2. Expanding the knowledge on non-competitive inhibition by:

    a. co-incubating TDCIPP and known competitive inhibitor, such as bicalutamide to investigate potential synergism of both antagonists.

    b. co-incubating the AR-ligand at varying concentrations with TDICPP to investigate if the known maximum response is repressed or shifted with increasing concentrations of the ligand.

    c. using green fluorescent protein (GFP) labeled AR to investigate real-time translocation. These results could give indication on the delay or prevention of translocation.

3. Target co-factors of AR-complex for ChIP analysis, could give indication if TDCIPP impedes co-factor binding.

4.5. Conclusion

In conclusion, binding affinity of the six selected OPFRs for the ER was limited in ECC-1 cells as no profound changes in ER inducible target gene expression were observed. Furthermore, the six selected OPFRs exerted few effects on the AhR-inducible CYP1A1 gene, although at high concentrations TMPP mildly induced gene expression. Although this study did not reveal significant endocrine disrupting potential concerning the human estrogen and aryl hydrocarbon receptor in ECC-1 cells, their widespread use and ubiquitous detection of OPFRs in the environment and biota remains a concern (Dishaw et al., 2014; McGoldrick et al., 2014). Data surrounding OPFR bioaccumulation potential is also scarce. Additional effects on other hormone

receptors, steroidogenisis, and neurotoxicity have been reported suggesting that additional research on these OPFRs is warranted, particularly, because some of the observed effects appear species-specific.

The data from this study on TDCIPP's anti-androgenic activity is compelling and is supported by similar findings in the literature. AR inducible target gene and protein expression were significantly altered by TDCIPP exposure. For the first time it was demonstrated that TDCIPP does not bind to the AR-LBD and appears to exert its ant-androgenic effects in LNCaP cells via non-competitive inhibition. Furthermore, TDCIPP exposure could potentially adversely influence clinical outcomes for prostate cancer screenings, resulting in false negatives. Prolonged TDCIPP exposure could also carry the risk of exacerbating the progression prostate cancer into a metastatic androgen-independent sub-type by simulating androgen deprivation.

This project focused on the direct effects of the six selected OPFRs on receptor targets and their target genes and proteins and did not focus on any indirect and downstream effects on the complex endocrine system. Therefore adverse effects along other endocrine signaling pathways cannot be excluded and should be investigated further.

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