The Effects of Legacy and Emerging Persistent Organic Pollutants on Endocrine Signalling Pathways in Mammalian Cancer Cell Lines

by

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B.Sc. (Biological Sciences), Simon Fraser University, 2010

Project Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Environmental Toxicology

in the
Department of Biological Sciences
Faculty of Science

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SIMON FRASER UNIVERSITY

Summer 2014

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Abstract

Despite its ban, DDT and DDE are still persistent in the environment. TBECH is a brominated flame retardant that has been detected in the biota. Here, we determined whether *p,p'*-DDT, *p,p'*-DDE and TBECH can negatively impact androgen receptor (AR)-regulated expression of prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA) and AR in human LNCaP cells. Real-time PCR, Western-blotting and COBAS PSA detection system were used to measure mRNA and protein levels. Chromatin-immunoprecipitation assay was used to determine AR-PSA promoter interaction. My results showed that *p,p'*-DDT and *p,p'*-DDE repressed R1881-inducible PSA mRNA and protein levels by blocking recruitment of AR to the PSA promoter. *p,p'*-DDT and *p,p'*-DDE can also significantly repress AR protein levels and relieve R1881-inducible PSMA repression. TBECH was found to repress R1881-inducible PSA protein levels. I conclude that men who have been exposed to either DDT or DDE may produce a false-negative PSA test when screening for prostate cancer.

Keywords: *p,p'*-DDT; *p,p'*-DDE; TBECH; prostate-specific antigen; LNCaP; androgen receptor

To my parents, Judy and Ken Wong and to my grandparents, for always believing in me

Acknowledgements

First and foremost, I would like to sincerely thank my senior supervisor, Tim Beischlag for his guidance and his incredible support throughout my graduate studies. I am also grateful to John Elliott for his support and for serving on my committee. In addition, I would like to thank Chris Kennedy for serving on my committee and for the many research opportunities he has given me during my undergraduate studies. I also like to thank Vicki Marlatt for serving as my external examiner and to Gerhard Gries for chairing my defense.

I would like to thank past and present members of the Beischlag lab, particularly Mark Labrecque and Mandeep Thakar for all their help during my time in the lab. Furthermore, I thank Mark for helping out with the Western blots and conducting the ChIP assays. I also thank members of the Lee and Prefontaine Lab, Beryl Luk, Shabnam Massah and Shazina Khan for their kindness and willingness to always lend a helping hand. I extend my thanks to all past and present MET colleagues for their friendship, particularly Alvin Louie, for all his help and encouragement. I thank Michael Cox and his laboratory at the Vancouver Prostate Cancer Centre for conducting the PSA COBAS assays. I thank Paul DeGrace for his assistance throughout the co-op application process.

Lastly, I would like to express my gratitude to my parents, Judy and Ken, and to my sister, Eva, for their love and support. I thank my grandparents for always believing in me. And to Wayne, thank you for your invaluable support and patience.

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List of Acronyms

µg	Microgram
µM	Micromolar
22Rv1	Human prostatic carcinoma xenograft, CWR22R, cell line
36B4	Acidic ribosomal phosphoprotein P0
3ß-Diol	5α androstane-3ß,17ß-diol
3α-Diol	5α androstane-3α,17ß-diol
AF	Activation function
AhR	Aryl-hydrocarbon receptor
AHRC	Aryl hydrocarbon receptor complex
ANOVA	Analysis of variance
AR	Androgen receptor
ARE	Androgen response element
AR_L	Full-length androgen receptor
ARNT2	Aryl-hydrocarbon receptor nuclear translocator 2
AR_s	Short androgen receptor isoform
bHLH-PAS	basic helix-loop-helix Per-ARNT-SIM
bis	Bisacrylamide
bp	Base pair
BSA	Bovine serum albumin
bw	Body weight
C.I.	Confidence interval
cAMP	Cyclic adenosine monophosphate
CATD	Cathepsin D
CBP	CREB-binding protein (coactivator)
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
c-Neu or erbB-2	Proto-oncogene that codes for an epidermal growth factor receptor family tyrosine kinase
COOH	Carboxyl group
COX-2	Cyclooxygenase-2

CRE	cAMP response element
CREB	cAMP response element binding protein (transcription factor)
CRPC	Castration-resistant prostate cancer
C-terminal	Carboxyl-terminal
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1
CYP450	Cytochrome P450
DBD	DNA-binding domain
DDA	2,2-bis(p-chlorophenyl)acetic acid
DDD	1,1-dichloro-2,2-bis(p-chlorophenyl)ethane
DDE	1,1-dichloro2,2-bis(p-chlorophenyl) ethylene
DDMU	1,1-di(p-chlorophenyl)-2chloroethylene
DDT	1,1,1- trichloro-2,2-bis(*p*-chlorophenyl)ethane
DHT	Dihydrotestosterone
Dioxin	Polychlorinated dibenzo-*p*-dioxin
DMEM	Dulbecco's Modified Eagle's Medium
E2	17ß-estradiol
E3	Ubiquitin-protein ligase
EC_{50}	Effective concentration
ECC-1	Human endometrial carcinoma cell line
EDC	Endocrine disrupting chemical
EDTA	Ethylenediaminetetraacetic acid
ER	Estrogen receptor
Erk	Extracellular signal-regulated kinase
EROD	7-ethoxyresorufin O-deethylase
ERß	Estrogen receptor ß
ERα	Estrogen receptor α
FBS	Fetal bovine serum
FSH	Follicle-stimulating hormone
g	Gram
g	Gravitational force
GPER-1	G-protein coupled estrogen receptor-1
G-protein	Guanosine nucleotide-binding protein
h	Hour

hAR	Human androgen receptor
HCl	Hydrochloric acid
Hepa-1c1c7	Mouse/murine hepatoma cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HepG2	Human hepatocellular carcinoma cell line
hER	Human estrogen receptor
HIF-1α	Hypoxia-inducible factor-1α
HSP	Heat shock protein
IC_{50}	Inhibitory concentration
IGEPAL	Octylphenoxypolyethoxyethanol
IGF-1	Insulin growth factor-1
IgG	Immunoglobulin G
kDa	Kilodalton
kg	Kilogram
L	Litre
l.w.	Lipid weight
LBD	Ligand binding domain
LH	Lutenizing hormone
LHRH	Lutenizing hormone-releasing hormone
LNCaP	Human prostate adenocarcinoma cell line
log K_{ow}	Octanol-water partition coefficient
logKoa	Octanol-air partition coefficient
logK_{oc}	Organic carbon partition coefficient
MAPK	Mitogen-activated protein kinase
MCF-7	Human breast adenocarcinoma cell line
mg	Milligram
MG132	26S proteasome inhibitor
MilliQ water	Ultrapure water type 1
mL	Millilitre
mm	Millimetre
mM	Millimolar
mmol	Millimole
mol	Mole

mRNA	Messenger ribonucleic acid
N/C	NH_2- and COOH-terminal
NaCl	Sodium chloride
$NaHCO_2$	Sodium bicarbonate
ng	Nanogram
nm	Nanometre
nM	Nanomolar
NTD	NH_2-terminal domain
o,o'-	*ortho, ortho;-*
o,p'-	*ortho, para'-*
OR	Odds ratio
p,p'-	*para, para'-*
P.I.	Protease inhibitor
P/S	Potassium penicillin/streptomycin sulphate
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
PKA	Protein kinase A
PKC	Protein kinase C
pM	Picomolar
pmol	Picomole
POP	Persistent organic pollutants
ppm	Parts per million
PR	Progesterone receptor
PSA	Prostate-specific antigen
PSMA	Prostate-specific membrane antigen
R1881	Synthetic androgen methyltrienolone
Ras	Guanosine triphosphate (GTP)-binding protein
RIPA	Radioimmunoprecipitation assay
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
SD	Standard deviation
SDS	Sodium dodecyl sulfate

Src	Tyrosine protein kinase
ß-TBECH	*rac*-(1*R*,2*R*)-1,2-dibromo-(4*S*)-4-((1*S*)-1,2-dibromoethyl)cyclohexane
T47D	Human breast ductal carcinoma cell line
TBECH	1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane or tetrabromoethylcyclohexane
TCDD	2,3,7,8-tetrachlorodibenzodioxin
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
TSE	Tris/sucrose/EDTA
v/v	volume/volume
VEGFA	Vascular endothelial growth factor
α-TBECH	*rac*-(1*R*,2*R*)-1,2-dibromo-(4*S*)-4-((1*R*)-1,2-dibromoethyl)cyclohexane
γ-TBECH	*rac*-(1*R*,2*R*)-1,2-dibromo-(4*R*)-4-((1*R*)-1,2-dibromoethyl)cyclohexane
δ-TBECH	*rac*-(1*R*,2*R*)-1,2-dibromo-(4*R*)-4-((1*S*)-1,2-dibromoethyl)cyclohexane

Chapter 1. Introduction

1.1. Persistent Organic Pollutants

Due to continued industrialization since World War II, numerous chemicals have been produced and released into the environment that are harmful to the environment and to human health. Persistent organic pollutants (POPs) are toxic chemicals that can adversely affect the environment and human health globally. In order for a chemical to be classified as a POP, it has to have the following four characteristics: persistence, bioaccumulation, toxicity and the capability for long range transport (Stockholm Convention, 2008). Many of the POPs are pesticides, industrial chemicals, flame retardants or by-products. The Stockholm Convention, which is an United Nations treaty, came into force in 2004 to help eliminate the production, use and/or release of POPs into the environment (Stockholm Convention, 2008). The first group of POPs to be recognized under the Stockholm Convention are known as the "dirty dozen" or "legacy" POPs which include 1,1,1- trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (dioxins) and polychlorinated dibenzofurans. Nine new POPs also known as the "emerging" POPs include the brominated flame retardants, tetrabromodiphenyl ether, pentabromodiphenyl ether, and hexabromobiphenyl, which were listed under the Stockholm Convention in 2009.

1.2. Endocrine Disrupting Chemicals

Increasing evidence since the 1980s suggested that adverse effects including a rise in hormone-dependent cancers in humans, reproductive and developmental abnormalities and declines in wildlife population, may be associated with environmental chemicals that disrupt endocrine systems (Colborn *et al,* 1993; Diamantai-Kandarakis *et*

al, 2009). Many chemicals, including some of the POPs, released into the environment since World War II are now recognized as suspected or known endocrine disrupting chemicals (EDCs). EDCs can be defined as any exogenous chemical that interferes with the body's endocrine system and disrupt normal hormone function, resulting in adverse health effects in the organism. Given that the endocrine system plays a major role in homeostasis, reproduction, developmental processes, neuroendocrine system, cardiovascular system and metabolism, EDCs can act on a variety of targets.

EDCs have different mechanisms of action including interfering with the synthesis, secretion, transport, metabolism, binding action or elimination of endogenous hormones in the body (US EPA, 2011). Some of the major molecular targets include nuclear receptors (androgen receptors (AR), estrogen receptors (ER), progesterone receptors (PR), thyroid receptors), non-genomic steroid nuclear receptors (membrane ERs), nonsteroid receptors (neurotransmitter receptors), orphan receptors (aryl hydrocarbon receptors (AhR)) and steroidogenic enzymes involved in the steroid biosynthetic pathway (Diamanti-Kandarakis *et al,* 2009). There is also increasing evidence that suggests that the actions of EDCs may also affect the exposed individual's offspring and subsequent generations due to epigenetic modifications such as alterations in DNA methylation and histone acetylation, which are involved in regulating gene expression (Anway and Skinner, 2006, Bruner-Tran *et al,* 2012).

EDCs can be either natural or derived from anthropogenic sources. Numerous synthetic EDCs are pesticides, industrial chemicals or their by-products, flame retardants and plasticizers (Diamanti-Kandarakis *et al,* 2009). Moreover, EDCs are also found in food and in our everyday consumer products such as plastic bottles, toys, household cleaning agents, cosmetics and personal care products. Natural chemicals called phytoestrogens that are found in plants such as soybean, also have the potential to be EDCs as they have been shown to bind weakly to ER (Kuiper *et al,* 1998; Cederroth *et al,* 2012).

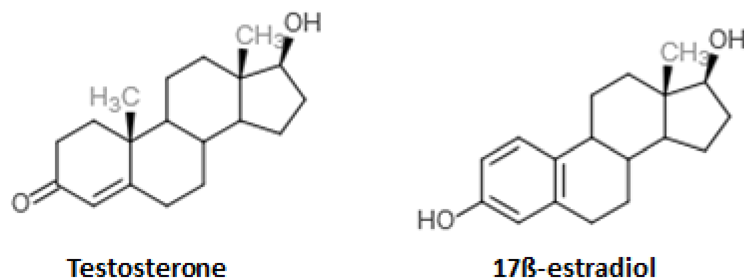


Figure 1-1. Chemical structures of the endogenous hormones, testosterone and 17ß-estradiol.

Many EDCs can exert their effect on the endocrine system due to their structural similarity to the body's endogenous hormones: testosterone in males and 17ß-estradiol (E2) in females (**Figure 1-1**). Based on some of the well-characterized EDCs such as flame retardants, PCBs and dioxin, it was discovered that these EDCs usually contain chlorine and bromine groups (Diamanti-Kandarakis *et al,* 2009). These halogen groups enable these chemicals to persist in the environment. EDCs also usually contain a phenolic group that is believed to mimic endogenous hormones, allowing them to bind to steroid nuclear receptors (Diamanti-Kandarakis *et al,* 2009). Another shared similarity between some EDCs and steroidal hormones is their lipophilic nature which allows them to bioaccumulate in adipose tissues of organisms (Schug *et al,* 2011). Furthermore, many EDCs have been found to exert their effects at low doses, similar to endogenous hormones (Vandenberg *et al,* 2012). There is also increasing evidence that suggests that EDCs may exert U-shaped and inverted U-shaped non-monotonic dose-response curves, which is also similar to the actions of hormones (Vandenberg *et al,* 2012).

1.3. DDT and DDE

1.3.1. History of use/current use

DDT was first synthesized in 1874 but its insecticidal properties weren't discovered until 1939 by Paul Muëller (WHO, 1979). It was initially used as an insecticide for typhus and malaria control, and other insect-borne human diseases. However, its successful insecticidal properties subsequently led to the widespread use in

agriculture, gardens, homes and commercial buildings. In the late 1950s, increasing evidence suggested that DDT was causing eggshell thinning in birds, which led to the decline in their population. In the US, approximately 1.5 million tonnes of DDT were generated between 1945 and 1983 (CCME, 1999). The US continued to import DDT to Canada until 1985 (CCME, 1999). Due to the environmental effects and human health concerns, the use of DDT was first banned by Sweden in 1970, followed by restrictions in the US and Canada. In North America, DDT was banned in the US and Canada in the early 1990s and in Mexico in 2000. Under the Stockholm Convention, the use and production of DDT has been banned in most countries except for in third world countries where malaria is still prevalent.

Based on the 2012 DDT questionnaire distributed by the Stockholm Convention, the following countries have reported use of DDT between 2009-2011: India, Eritrea, Zambia, Swaziland, South Africa, Mozambique, Mauritius and Gambia. The current global production of DDT mainly comes from India, which produced 10246 tonnes of the active ingredient (*p,p'*-DDT) between 2009-2011. The following countries that are registered for acceptable use/production of DDT, did not submit their DDT questionnaire on time to be included in the 2012 DDT Expert group report: Botswana, China, Marshall Islands, Namibia, Senegal and Venezuela.

1.3.2. Chemical Structure

The technical-grade DDT formulation is a mixture of 80-85% of *p,p'*-DDT, 15-20% *o,p'*-DDT and trace amounts of o,o'-DDT (**Figure 1-2**) (ASTDR, 2002). The formulation may also have trace amounts of DDE (1,1-dichloro2,2-bis(p-chlorophenyl) ethylene) and/or DDD (1,1-dichloro-2,2-bis(p-chlorophenyl)ethane) as contaminants (ASTDR, 2002). The metabolites of DDT include *p,p'*-DDE, *o,p'*-DDE, *p,p'*-DDD and *o,p'*-DDD, but the primary metabolite is *p,p'*-DDE (**Figure 1-2**) (ASTDR, 2002).

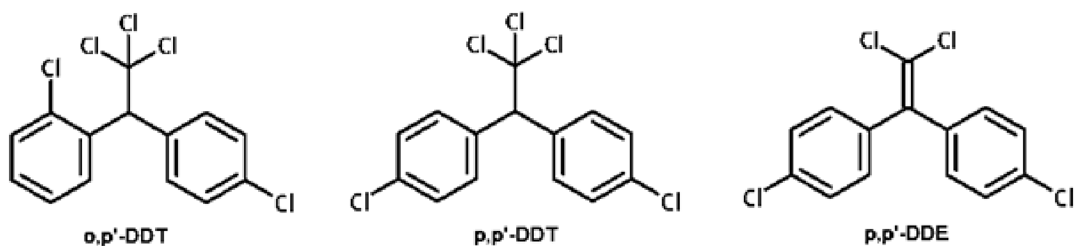


Figure 1-2. Chemical Structures of the DDT isomers and the primary metabolite, *p,p'*-DDE.

1.3.3. Environmental Fate and Transport

Despite its ban, DDT and its metabolite DDE continue to persist in the biota and environment today. Its chemical stability, lipophilic nature and resistance to metabolism, all contribute to its ability to bioaccumulate and biomagnify in living organisms, including humans (Evans *et al,* 1991; Leblanc, 1995, Jaga and Dharmani, 2003; Arrebola *et al,* 2012). Due to their high octanol-water partition coefficient (log K_{ow}) of 6.91 and 6.51 for *p,p'*-DDT and *p,p'*-DDE, respectively, they tend to accumulate in the adipose tissue of living organisms (Howard and Meylan, 1997). Their high organic carbon partition coefficients (log K_{oc}) of 5.18 and 4.70 for *p,p'*-DDT and *p,p'*-DDE, respectively, suggests that both chemicals can adsorb strongly to soil (Sabljic, 1984). The half-life of DDT and DDE in soil ranges from 2-15 years (Lichenstein and Shulz, 1959; Stewart and Chisholm, 1971; Racke *et al,* 1997). This variability in their half-life in soil can be due to several factors: temperature (Racke *et al,* 1997), sunlight (Racke *et al,* 1997), soil type (Dimond and Owen, 1996), bioavailability to microorganisms (Providenti *et al,* 1993), removal by nearby plants (Verma and Pillai, 1991) and flooding (Samuel and Pillai, 1989). The half-life of DDE in soil has been estimated to be 151-271 days in tropical regions and greater than 20 years in temperate regions. In soil, DDT can be degraded to DDE or DDD under certain conditions. DDT is converted to DDD via dechlorination under reducing environments, which is further degraded under aerobic conditions (Mwangi *et al,* 2010). In aerobic conditions, DDT is converted to DDE via biotic degradation, dehydrochlorination and photooxidation reactions (Mwangi *et al,* 2010). However, DDE has been found to be resistant to biodegradation under both aerobic and anaerobic environments (Strompl and Thiele, 1997), except in certain anaerobic marine

sediments, where DDE readily breaks down to 1,1-di(p-chlorophenyl)-2chloroethylene (DDMU) (Quensen *et al,* 2001). These chemicals attached to sediment or particulate matter can enter the aquatic environment via runoff from land or via atmospheric deposition.

In the aquatic environment, sediment can serve as a sink for DDT as it can adsorb strongly to particulate matter in water and sediment. The half-life of DDT is estimated to be between 14-21 years in sediment (Oliver *et al,* 1989). In water, direct photolysis is the major degradation pathway for DDT, with an estimated half-life of over 150 years (EPA, 1979). DDE can also undergo direct photolysis and photo-isomerization but its half-life in water is dependent on the amount of sunlight (EPA,1979). Biodegradation serves as a minor degradation pathway for DDT and DDE in water (Johnsen, 1976).

DDT and DDE can enter the atmosphere following volatilization from soil and surface water. In the atmosphere, approximately 50% of DDT is bound to particulate matter with the remaining 50% in the vapor phase (Bidleman, 1988). The DDT that remains in the vapor phase can undergo rapid photooxidation, with an estimated half-life of 1.5 days (Meylan and Howard, 1993). DDE has a higher vapor pressure, with an estimated half-life of 17 days (Meylan and Howard, 1993). However, DDT and DDE may not be subjected to photooxidation if they are bound to particulate matter in the atmosphere (Meyland and Howard, 1993). These chemicals can then be deposited back on land or surface water. DDT and DDE have been found in various environmental media in the Arctic regions, which suggests that DDT is capable of long range transport (Anthony *et al,* 1999; Harner, 1997).

1.3.4. Toxicokinetics

DDT and DDE can enter the body via oral, dermal and inhalation exposures. The major route of exposure for humans is from consuming food contaminated with DDT and DDE. Inhalation is assumed to be minor route of exposure except for people who are involved in the handling and application of DDT. However, in malaria prevalent countries, the main routes of exposure for humans are inhalation and dermal contact,

though dermal route of exposure is considered to be negligible because they are poorly absorbed through the mammalian skin (Wolfe *et al,* 1971).

In humans, following oral exposure, DDT is absorbed by the lymphatic system in the intestine, followed by distribution via the lymph and blood to the rest of the body where it ultimately accumulates in the adipose tissue (Morgan and Roan, 1971). In humans, DDT can be metabolized to either DDE via dehydrochlorination or to DDD via reductive dechlorination. DDD can undergo another dechlorination step to generate DDMU, which can be further broken down into DDA (2,2-bis(p-chlorophenyl)acetic acid) via dechlorination and oxidation reactions (Morgan and Roan, 1971). Very little DDE is converted to DDA (Roan *et al,* 1971). DDA is readily excreted in the urine due to its solubility in water (Roan *et al,* 1971). The metabolism of DDT to DDE is much slower compared to the metabolism of DDT to DDD (Morgan and Roan, 1971). Further metabolism of DDE is even slower as it ends up stored in the adipose tissue (Morgan and Roan, 1971). It is estimated to take 10-20 years for DDT to be eliminated from the body in the absence of subsequent exposure (Smith AG, 1991). In humans, DDE has a longer half-life than DDT, which is estimated to be 10 years in the plasma (Hunter *et al,* 1997). DDT and DDE can also be eliminated from the body via the placenta (Al-Saleh *et al,* 2011) and in breast milk (Vuori *et al,* 1977; Bouwman *et al,* 2012). Some DDT can be excreted in the feces via biliary excretion (Jensen *et al,* 1957).

1.3.5. Endocrine Disruption Evidence

Cellular and Molecular

Estrogenic Effects: ER-dependent mechanisms

Early studies have established that *o,p'*-DDT, *p,p'*-DDT, *p,p'*-DDE and *o,p'*-DDE are ER agonists (Soto *et al,* 1995, Chen *et al,* 1997, Danzo, 1997 and Zava *et al* 1997). Among the DDT isomers, *o,p'*-DDT is the most estrogenic (Soto *et al,* 1995; Danzo *et al,* 1997). Despite that, the relative binding affinity of *o,p'*-DDT to the human ER (hER) has been shown to be 8000-30,000 times weaker compared to E2, in the human breast cancer cell line, MCF-7 (Soto *et al,* 1995; Zava *et al,* 1997). Further studies showed that *o,p'*-DDT (but not *p,p'*-DDT) can downregulate ER protein expression at 1 μM and 10 μM

in MCF-7 cells (Chen *et al,* 1997; Zava *et al,* 1997; Diel *et al,* 2002). Chen *et al* (1997) also showed that in the presence of E2 and either *o,p'*-DDT, *o,p'*-DDE or *p,p'*-DDT at 100 nM and 1 μM, significant additive transcriptional activation response was observed in yeast cells expressing LexA-hER. Moreover, *o,p'*-DDT at 1 μM induced cellular proliferation in two human breast cancer cell lines, T47D and MCF-7 (Zava *et al,* 1997; Diel *et al,* 2002) and also reduced the rate of cellular apoptosis in MCF-7 (Diel *et al,* 2002). The increased cellular proliferation effects was shown to be ER-mediated as hydroxytamoxifen (an ER antagonist), when added together with 1 nM E2, nearly completely abolished the proliferation effects of *o,p'*-DDT (Zava *et al,* 1997).

More studies are currently focused on unravelling the molecular mechanisms of the estrogenic effects of DDT and other xenoestrogens. A recent study demonstrated the ability of *o,p'*-DDT to downregulate the aryl-hydrocarbon receptor nuclear translocator 2 (ARNT2) mRNA expression via the ERα in MCF-7 cells (Qin *et al,* 2011). The ARNT2 is a basic helix-loop-helix-Per-ARNT-SIM (bHLH-PAS) transcription factor that is involved in the activation of the aryl hydrocarbon receptor and hypoxia-inducible factor-1α (HIF-1α). In addition, it is involved in numerous physiological pathways including metabolism of xenobiotics, tumor angiogenesis and response to hypoxia (Maltepe *et al,* 2000). They demonstrated that ARNT2 mRNA levels were significantly reduced in a dose-dependent exposure to *o,p'*-DDT in MCF-7 and BG1Luc4ER cells (BG-1 ovarian cancer cell line stably transfected with a luciferase reporter gene that responds to estrogen or estrogenic chemicals), with maximal reduction at 10μM. Moreover, ARNT2 protein expression was significantly reduced by 10μM *o,p'*-DDT. Finally, they determined that the reduction of ARNT2 mRNA expression was mediated by the ERα. They showed this by co-administering MPP (an ERα antagonist) with 10μM *o,p'*-DDT in MCF-7 cells, which resulted in the complete abolishment of the suppressive effects of *o,p'*-DDT. These results suggests that the suppressive effects of *o,p'*-DDT on ARNT2 expression could affect AhR-mediated detoxification of xenobiotics (Qin *et al,* 2011).

In addition to the classic genomic ER signalling pathway, in which E2 binds to nuclear ER to regulate gene expression, studies have also focused on the non-genomic ER signalling pathways. Non-genomic ER signalling involves the activation of

membrane-bound or cytosolic ER by E2 resulting in brief but rapid second messenger signalling events including the activation of membrane adenylate cyclase and cyclic AMP (cAMP) levels, mitogen-activated protein kinase (MAPK) pathway and the Src/Ras/Erk pathway (Migliaccio *et al,* 1996; Aronica *et al,* 1999; Improta-Brears *et al,* 1999). A study conducted by Silvia *et al* (2010) demonstrated that the estrogenic effects of *o,p'*-DDT involves both the genomic and non-genomic ER pathways (Src/Ras/Erk pathway) in MCF-7 cells. The Src/Ras/Erk pathway is of particular interest due to their role in cellular division, differentiation and apoptosis. Furthermore, the activation of Src has been suggested to play a role in breast cancer carcinogenesis (Finn R, 2008).

Estrogenic Effects: ER-independent mechanisms

DDT at 10 nM was first shown to stimulate cellular proliferation in the human breast epithelial cell line, MCF-10A (that do not express ER) via activation of growth factor receptors and cytokine receptor-mediated signalling pathways (Shen and Novak, 1997). These results provided evidence that DDT can stimulate cellular proliferation via multiple signalling pathways independent of the ER, which can potentially lead to tumor formation. A subsequent study demonstrated that *o,p'*-DDT at 0.1-1 nM can activate and increase the activity of the c-Neu proto-oncogene in an ER-independent mechanism, in MCF-7 cells (Enan and Matsumura, 1998). c-Neu also known as erbB-2, codes for an epidermal growth factor receptor family tyrosine kinase. This oncogene is involved in various mitotic signalling pathways, including the MAPK pathway and is overexpressed in different types of cancers, including breast and prostate (Tessier and Matsumura, 2001).

Moreover, the effects of DDT on the steroidogenesis pathway have been investigated. One of the targets, aromatase, is of particular interest. Aromatase is responsible for catalyzing the irreversible conversion of testosterone to estradiol. Moreover, the potential for DDT and DDE to play a role in the development and/or progression of estrogen-dependent diseases such as breast cancer is of considerable interest. It is suggested that increased aromatase levels can result in increased levels of estrogen in the target tissue, which could lead to activation of ER-dependent or ER-independent pathways (Holloway *et al,* 2005). The persistent metabolite, *p,p'*-DDE has been shown to induce aromatase activity in human endometrial stromal cells (Holloway

et al, 2005). Another study conducted by Han *et al* (2010) discovered that *o,p'*-DDT can increase aromatase levels in two human breast cancer cells (MCF-7 and MDA-MB-231), similar to two ER-independent mechanisms by which estrogens increase aromatase levels in breast cancer tumors. COX-2 (cyclooxygenase-2) is normally over-expressed in breast cancer tumors. They found that *o,p'*-DDT at 1 μM can bind to the promoter, cAMP response element (CRE) which induces the transcription of the COX-2 and aromatase genes. COX-2 in turn, leads to the production of prostaglandin E2, which results in the activation of aromatase transcription and activity. Aromatase expression can also be regulated via cAMP-mediated pathways in breast cancer tissue (Zhao et al, 1996). Han et al (2010) also showed that *o,p'*-DDT can increase intracellular cAMP levels which can increase the binding of CREB (cAMP response element binding protein) to CRE, in order for transcription to occur.

Lastly, DDT and its metabolites have been demonstrated to induce transcription through the activation of several components of the transcriptional machinery including the nuclear transcription factor, activator protein-1 (Frigo *et al,* 2002) and the transcriptional coactivators p300 (Bratton *et al,* 2009) and the glucocorticoid receptor-interacting protein 1 (Frigo *et al,* 2006). A recent study conducted by Bratton *et al* (2012) showed that *o,p'*-DDT at 10 μM activated several genes involved in breast cancer signalling in MCF-7 cells. One of these genes was the vascular endothelial growth factor (VEGFA), which is involved in cellular angiogenesis and cellular differentiation (Zhang *et al,* 1995). This study showed that *o,p'*-DDT stimulated VEGFA gene transcription independent of ERα, but could not rule out that the effects were also independent of E2 . This novel mechanism involves activation of p38 kinase through phosphorylation in the cytoplasm, which in turns, phosphorylates the CREB-binding protein (CBP). This results in CBP translocating to the nucleus, where it binds to HIF-1α. The CBP-HIF-1α complex then binds to the HIF-1 response element within the VEGFA promoter, which activates the transcription of the VEGFA gene (Bratton *et al,* 2012).

Anti-estrogenic Effects

In human placental explants, DDT and DDE isomers (*p,p'*-DDT, *o,p'*-DDT, *p,p'*-DDE, *o,p'*-DDE) at concentrations ranging from 1-100 ng/mL and 1 μg/mL, all reduced

estradiol secretion. This effect was due to the inhibitory action of DDT and DDE on aromatase activity, which was measured using the fluorometric substrate dibenzylfuorescein (Wojtowicz *et al,* 2007). Western blot analysis demonstrated that 100 ng/mL of *p,p'*-DDT and *o,p'*-DDE reduced aromatase protein levels significantly more than 100 ng/mL of *p,p'*-DDT and *p,p'*-DDE. It has been suggested that *p,p'*-DDT and *p,p'*-DDE may be slowing down the degradation of aromatase by forming a complex with the enzyme (Harada and Hatano, 1998; Wojtowicz et al, 2007). Furthermore, o,'p-DDT, *p,p'*-DDE and *o,p'*-DDE at concentrations of 100 ng/mL and 1 µg/mL, significantly increased progesterone secretion by 1.9-2.5-fold (Wojtowicz *et al,* 2007). As progesterones are important in the maintenance of pregnancy, any alteration in its secretion could potentially result in an abortion or preterm birth (Spencer and Bazer, 2004).

Androgenic Effects

In terms of androgenic effects, Tessier and Matsumura (2001) demonstrated that *o,p'*-DDT at 10-1000 nM can induce cellular proliferation via activation of erbB-2 kinase, which results in increased phosphorylation of MAPK in LNCaP cells. However, in another human prostate cancer cell line, PC-3, that is androgen-independent, *o,p'*-DDT was unable to induce cellular proliferation. These investigators demonstrated that this proliferative effect was not due to the direct binding of *o,p'*-DDT to AR, as *p,p'*-DDE failed to block this effect (Tessier and Matsumura, 2001). Again, these results suggest that DDT can play a role in the development and/or progression of hormonal carcinogenesis.

Anti-androgenic Effects

The persistent DDT metabolite, *p,p'*-DDE was first shown to be a potent AR antagonist using a competitive ligand binding assay in monkey kidney CV-1 cells transiently co-transfected with the human AR expression vector and a mouse mammary tumor virus promoter-luciferase reporter vector (Kelce *et al,* 1995). Competitive binding assays with the rat AR using ^{3}[H]R1881 showed that the IC_{50} for *p,p'*-DDE was 5 µM whereas it was 75 µM, 95 µM and 90µM for *p,p'*-DDT, *o,p'*-DDT and *p,p'*-DDD, respectively (Kelce *et al,* 1995). These results showed that the parent compounds, *o,p'*-

DDT and *p,p'*-DDT are only weak AR antagonists. Furthermore, they demonstrated that *p,p'*-DDE did not exert its anti-androgenic effects via inhibition of 5α-reductase (which converts testosterone to dihydrotestosterone) as the concentrations required to inhibit this enzyme, were 200-5000 times higher than the concentrations to inhibit AR binding. In a subsequent study, Kelce *et al* (1997) showed that *p,p'*-DDE exposed to male rats at 200 mg/kg/day, can alter two AR target genes, prostate specific binding protein and testosterone-repressed prostatic message-2 in rat ventral prostates. Another study later demonstrated that *o,p'*-DDT, *p,p'*-DDT, *o,p'*-DDE, *o,p'*-DDD and *p,p'*-DDD are weakly AR antagonists at concentrations 10^{-6} M or higher, in a human hepatoma cell line (HepG2) transfected with the human AR and an androgen-responsive reporter (Maness *et al* 1998). Recently, a study demonstrated that exposure to *p,p'*-DDE at 1 pM and 1 nM for 3 days, can alter human sperm function by increasing cytosolic [Ca2+] via the opening of a sperm specific ion channel, the CatSper channel in the plasma membrane (Tavares *et al,* 2013). The CatSper channel has been shown to be activated by progesterone, which is secreted by the egg (Strünker et al, 2011). Moreover, this study showed that exposure to higher concentrations at 25 and 50 μM for 2 days, can stimulate the loss of the sperm's acrosome. The acrosome is the organelle that contains enzymes required for egg penetration. The 3 day exposure period mimics the typical time frame sperm is spent in a female reproductive tract. This study suggests that *p,p'*-DDE could potentially indirectly affect male fertility (Tavares *et al,* 2013).

Furthermore, androgen and estrogen signalling pathways have been shown to have counteractive effects on the proliferation of CAMA-1 breast cancer cell line (Aube *et al,* 2008). It has been reported that plasma levels of *p,p'*-DDE were linked to tumor development in breast cancer patients (Demers *et al,* 2000). Aube *et al* (2008) found that exposure to anti-androgens such as *p,p'*-DDE can disrupt the androgen signalling pathways that inhibits cellular proliferation initiated by the estrogen signalling pathways in breast cancer cells. Using the CAMA-1 cells (that express both ERα and AR), they showed that in the presence of physiological levels of E2 and DHT, *p,p'*-DDE at 10 μM, can promote the cells to enter the S phase of the cell cycle. This resulted in increased cellular proliferation, which in turn favors breast cancer development or progression (Aube *et al,* 2008).

Effects on AhR Function

O,p'-DDT has been shown to inhibit 2,3,7,8-tetrachlorodibenzodioxin (TCDD)-inducible CYP1A1 expression in murine Hepa-1c1c7 cells via a mechanism independent of ER (Jeong and Kim, 2002). These results may be cell-specific as the Hepa-1c1c7 cell line lack functional ER. It was shown that *o,p'*-DDT downregulated CYP1A1 mRNA expression and suppressed 7-ethoxyresorufin O-deethylase (EROD) activity in a dose-dependent manner (0.1-20 μM) when co-administered with 0.5 nM TCDD in Hepa-1c1c7 cells. EROD activity is a measure of CYP450 induction. These results led to the hypothesis that *o,p'*-DDT may be inhibiting AhR-mediated gene expression. Before the AhR can be activated, it needs to undergo a transformation that is ligand-dependent. Using an electrophoretic mobility shift assay, it was shown that *o,p'*-DDT reduced the TCDD-induced transformation of the AhR. Another study also demonstrated that *p,p'*-DDT, *o,p'*-DDT, *p,p'*-DDE and *o,p'*-DDE suppressed EROD activity and decreased AhR protein expression at concentrations ranging from 1 to 100 ng/mL in human placental cells (Wojtowicz *et al,* 2011). As CYP1A1 is involved in biotransformation and detoxification of xenobiotics, the reduction in CYP1A1 expression could increase the susceptibility of the individual, including a developing fetus to the toxicity of the non-metabolized xenobiotics. This may serve as a risk factor for pregnancy loss and other health concerns (Wojtowicz *et al,* 2011).

Physiological

Experimental Animal Studies

The estrogenic effects of DDT have been demonstrated in several *in vivo* studies. DDT has been demonstrated to significantly increase uterine weights of ovariectomized mice and rats exposed to doses ranging from continuous low doses to 100-500 mg of *o,p'*-DDT/kg body weight (bw) via various exposure routes (subcutaneous implantation, intraperitoneal injection, oral administration and gastric tube feeding) (Singhal *et al,* 1970; Bigsby *et al,* 1997; Diel *et al,* 2000; Ulrich *et al,* 2000). As evidenced by the molecular effects of DDT and DDE, endocrine disruption appears to be one of the mechanisms for causing or promoting hormone-responsive cancers.

Based on numerous animal studies that demonstrated that both DDT and DDE can increase the incidence of tumors in rats and mice, DDT and DDE have been classified as probable human carcinogens by the US E.P.A and the International Agency for Research on Cancer (US EPA, 2014; IARC, 1991). These studies showed that mice and rats exposed to 100-250 ppm and 500 ppm of DDT, respectively, via diet and subcutaneous injections, had an increased incidence of hepatocellular carcinoma (Terracini *et al,* 1973; Thorpe and Walker, 1973; Turusov *et al,* 1973; Kashyap *et al,* 1977; Rossi *et al,* 1977; Cabral *et al,* 1982a). Furthermore, mice exposed to 2.8-50 ppm of DDT via diet, also had an increased incidence of lung tumors (Tarjan and Kemeny, 1969; Shabad *et al,* 1973). DDT did not increase tumor incidence in other species such as the hamster as they are less efficient in metabolizing DDT to DDE (Cabral *et al,* 1982b; Gingell and Wallcave, 1974). However, DDE did increase incidence of hepatocellular tumors in hamsters and mice exposed to 500-1000 ppm and 250 ppm of DDE, respectively, via diet (Tomatis *et al,* 1974; Rossi *et al,* 1983). Furthermore, DDT and *p,p'*-DDE have been shown to accelerate mammary tumorigenesis in rats exposed to another carcinogen, 2-acetamidophenanthrene (Scribner *et al,* 1981) and in a transgenic mouse model expressing the oncogene, c-Neu (Johnson *et al,* 2012).

In addition to estrogenic effects, DDT and DDE also exhibit anti-androgenic properties. Kelce *et al* (1995) were the first to show that *in utero* exposure to *p,p'*-DDE at 100 mg/kg/day resulted in male offspring with reduced anogenital distance and retention of thoracic nipples. Furthermore, exposure to the same concentration of *p,p'*-DDE impeded the onset of puberty by 5 days in weanling male rats and significantly reduced the weights of the androgen-dependent seminal vesicles and prostates in adult castrated male rats (Kelce *et al,* 1995). However, *p,p'*-DDE did not reduce serum levels of testosterone in the adult rats (Kelce *et al,* 1995). Their subsequent study later showed that this seminal vesicle and prostate weight reduction in castrated male rats was due to the alteration of androgen-dependent gene expression (Kelce *et al,* 1997). Furthermore, adult male rats had higher incidences of chronic suppurative prostatistis following *in utero* exposure to 100 mg *p,p'*-DDE/kg/day (You *et al,* 1999). It was also discovered that a single dose of *p,p'*-DDE at 500 mg/kg via intraperitoneal injection, decreased erectile function in castrated adult male rats (Brien *et al,* 2000). Lastly, other studies have shown that *p,p'*-DDE can induce testicular toxicity in male rats by inducing

apoptosis of the Sertoli cells, which plays a crucial role in spermatogenesis (Shi *et al,* 2010; Song *et al,* 2011). In another species, *p,p'*-DDT was found to induce cryptochidism (undescended testes) in male rabbit dosed with either 25 or 250 mmol/kg/day for the first 4 weeks after birth (Veeramachaneni *et al,* 2007). The higher dose also resulted in morphologically defective sperm (Veeramachaneni *et al,* 2007).

Human Epidemiological Studies

There have been numerous epidemiologic studies linking organochlorine pesticide exposure, including DDT and DDE, to various human health effects. However, results have remained inconclusive. The link between DDT/DDE exposure and breast cancer has been extensively studied since the 1990s. To date, only several epidemiological studies have found a positive association between serum/adipose tissue levels of *p,p'*-DDT and *p,p'*-DDE, and increased breast cancer risk (Aronson *et al,* 2000; Cohn *et al,* 2007; Ociepa-Zawal *et al,* 2010). These studies found that exposure early in life and higher concentrations of *p,p'*-DDE may contribute to breast carcinogenesis in older women (Cohn *et al,* 2007; Ociepa-Zawal *et al,* 2010).

Pesticide exposure has also been linked to higher risk of prostate cancer in agricultural workers and farmers (Alavanja *et al,* 2003; Settimi *et al,* 2003; Meyer *et al,* 2007; Band *et al,* 2011; Alavanja *et al,* 2013; Koutros *et al,* 2013). Some studies have found a positive association between organochlorine pesticides use/exposure (including DDT) and increased risk of prostate cancer in agricultural workers based on case-control studies and cohort studies in BC, Canada (Band *et al,* 2011), in Italy (Settimi *et al,* 2003) and in Iowa and North Carolina in the US (Alavanja *et al,* 2003).

Exposure to DDT/DDE has also been linked to other types of cancers and diseases including liver cancer (McGlynn *et al,* 2006; Persson *et al,* 2012), non-Hodgkin lymphoma (Spinelli *et al,* 2007; Viel *et al,* 2011; Brauner *et al,* 2012), Alzheimer disease (Richardson *et al,* 2014), hypertension (La Merrill *et al,* 2013; Lind *et al,* 2014), diabetes (Lee *et al,* 2006; Everett *et al,* 2007; Arrebola *et al,* 2012) and impaired semen quality (De Jager *et al,* 2006; Aneck-Hahn *et al,* 2007; Messaros *et al,* 2009).

The growing fetus can be exposed to DDT/DDE via the placenta and infants can be exposed via breast milk. Numerous epidemiological studies have found a link between elevated maternal serum and breast milk of *p,p'*-DDT and *p,p'*-DDE and pregnancy loss (Korrick *et al,* 2001; Venners *et al,* 2005), fetal growth (Al-Saleh *et al,* 2011; Kezios *et al,* 2013), preterm birth (Longnecker *et al,* 2001; Wolff *et al,* 2007), urogenital birth defects in males (including cryptochidism and decreased testicular size) (Andersen *et al,* 2008; Brucker-Davis *et al,* 2008), impaired cognitive functioning (Ribas-Fito *et al,* 2006; Torres-Sanchez *et al,* 2012) and obesity (Mendez *et al,* 2011; Valvi *et al,* 2012).

1.4. TBECH

1.4.1. History of use/current use

The addition of some of the widely used brominated flame retardants on the Stockholm Convention, led to the search for alternative flame retardants. One of these alternatives was 1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane, which is also known as tetrabromoethylcyclohexane (TBECH). TBECH is an additive brominated flame retardant that is not bound to polymers and therefore, can leach out of consumer products. It is primarily used in expandable polystyrene beads, which are used in the construction of polystyrene bead boards for thermal insulation in housing (US EPA, 1985). TBECH is also used in extruded polystyrene foams, fabric and vinyl adhesives, electrical cable coatings, high-impact plastic parts of appliances and construction materials (US EPA, 1985). In the U.S., production volumes of TBECH in 1986, 1990, 1994, 1998 and 2002 ranged from 4.5 to 226 tonnes per year (US EPA, 2002).

1.4.2. Chemical Structure

Due to the four chiral carbons present in its structure, TBECH can exist as four diasteromers: α-, ß-, γ- and δ-TBECH (**Figure 1-3**). The technical TBECH mixture contains near equimolar amounts of *rac*-(1*R*,2*R*)-1,2-dibromo-(4*S*)-4-((1*R*)-1,2-dibromoethyl)cyclohexane (α-TBECH) and *rac*-(1*R*,2*R*)-1,2-dibromo-(4*S*)-4-((1*S*)-1,2-dibromoethyl)cyclohexane (ß-TBECH). During the manufacturing process at

temperatures of 125°C or higher , some of α- and ß-TBECH can isomerize into *rac*-(1*R*,2*R*)-1,2-dibromo-(4*R*)-4-((1*R*)-1,2-dibromoethyl)cyclohexane (γ-TBECH) and *rac*-(1*R*,2*R*)-1,2-dibromo-(4*R*)-4-((1*S*)-1,2-dibromoethyl)cyclohexane (δ-TBECH) (Arsenault *et al,* 2008). In a thermal equilibration mixture, the proportions of α-, ß-, γ- and δ-TBECH isomers exist as 33%, 33%, 17% and 17%, respectively (Arsenault *et al,* 2008).

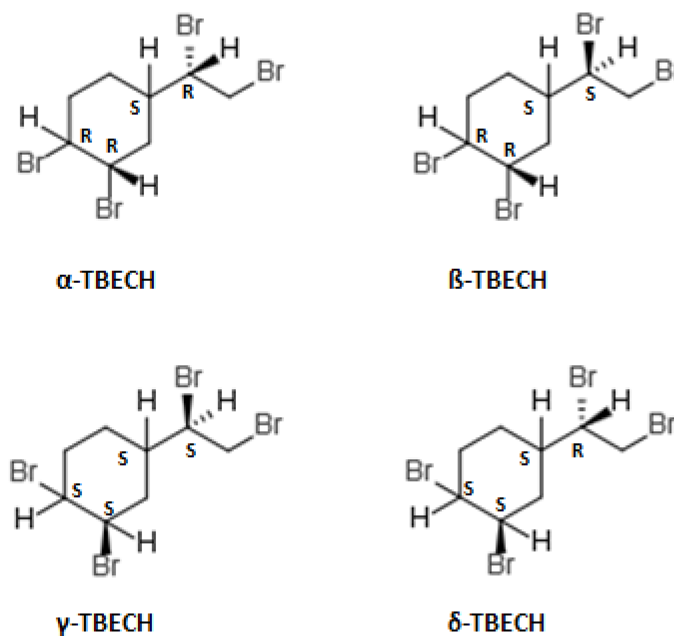


Figure 1-3. Chemical structures of the four TBECH diastereomers.

1.4.3. Environmental Fate and Transport

The environmental concern for TBECH has been growing due to its ability to leach out of consumer products and release into the environment. Furthermore, in 2006, TBECH was identified as a high priority chemical with bioaccumulation, persistence and long range atmospheric transport potential (Muir and Howard, 2006). Currently, little is known about the environmental fate and transport of TBECH, but modelling data has provided some estimation for the physiochemical properties of TBECH. The $\log K_{ow}$ and log bioconcentration factor (BCF) of TBECH (based on the non-isomer structure) is estimated to be 5.25 and 4.4, which suggests TBECH is lipophilic and has the potential to bioaccumulate (Muir and Howard, 2006; Howard and Muir, 2010). It is not surprising that TBECH has low water solubility at 0.06915 mg/L (Ruan *et al,* 2009). The

degradation and atmosphere oxidation half-lives are estimated to be 37.5 and 2.2 days, which is indicative for long range atmospheric transport (Muir and Howard, 2006; Howard and Muir, 2010). Modelling data has estimated the octanol-air partition coefficient ($logK_{oa}$) to be 8.01 for TBECH (Howard and Muir, 2010). It has been predicted that chemicals with $logK_{ow}$ >5 and $logK_{oa}$ > 8, are more likely to adhere to particles in the atmosphere and in the water (Brown and Wania, 2008). Nyholm *et al* (2010) demonstrated that TBECH can degrade rapidly in both aerobic and anaerobic soil, with half-lives between 21-36 days at room temperature. However, Nyholm *et al* (2010) also showed that the biodegradation rate decreased by five-fold at a lower temperature of 8°C, which reflects countries with moderate climates.

1.4.4. Toxicokinetics

The α/ß-TBECH diastereomers were found to be equally metabolized to approximately 40% via hydroxylation in an enzyme-mediated biotransformation assay using rat liver microsomes over a 60 min incubation period (Chu *et al,* 2012). The metabolites of TBECH were identified to be α- and β-monohydroxy-TBECH (OH-TBECH) and dihydroxy-TBECH ($(OH)_2$-TBECH) (Chu *et al,* 2012). These results are also consistent with the findings from a ß-TBECH feeding study using captive American Kestrels (Marteinson *et al,* 2012). This feeding study found that concentrations of ß-TBECH were below the detection limit in the liver, fat, plasma and egg samples after 28 days of exposure (Marteinson *et al,* 2012). Furthermore, no detectable levels of ß-TBECH metabolites were found in the Kestrels. A recent TBECH *in ovo* study using zebra finch eggs showed that α-/ß-TBECH (2.3-94 ng/g) were rapidly metabolized between the 3-day and 14-day old embryos (Currier *et al,* 2013). They also found that ß-TBECH depleted more rapidly than α-TBECH in the embryos. Another recent *in ovo* study using chicken embryos showed evidence of isomerization of α/ß-TBECH at pipping (α-isomer increased from 57% to 72%, whereas ß-isomer decreased from 43% to 28%) (Crump et al, 2014). Crump et al (2014) also showed that α-/ß-TBECH was detected in the chicken embryos at the highest dose of 54,900 ng/g, with the highest accumulation in the yolk sac (5604 ng/g), followed by the liver (1069 ng/g).

Several fish bioaccumulation studies using TBECH have been conducted in the past few years. In a zebrafish uptake and depuration study, the uptake efficiency for TBECH was found to be over 60% with half-lives of less than 2 days for their low- (0.36 nmol/g) and high-dose (34 nmol/g) dietary exposure groups (Nyholm *et al,* 2009). However, another study using juvenile brown trout found that the depuration half-life of ß-TBECH was between 13.5-22.5 days in all three treatment groups (0.5, 5.4 or 54 μg into 20 mL of corn oil), but no significant differences were found (Gemmill *et al,* 2011). Furthermore, there were no detectable levels of debrominated TBECH metabolites in the liver or whole-fish extracts, and no evidence of isomerization to α-TBECH (Gemmill *et al,* 2011). Lastly, there is evidence that TBECH can be maternally transferred to offspring, which was detected in zebrafish eggs following parental exposure via diet for 42 days (Nyholm *et al,* 2008).

1.4.5. Endocrine Disruption Evidence

Cellular and Molecular

TBECH was first identified as a potent human AR (hAR) agonist through theoretical modelling and a series of *in vitro* receptor-binding and AR activation assays (Larsson *et al,* 2006). The same investigators later showed that γ/δ-TBECH were more potent agonists of the hAR in the HepG2 hepatocellular carcinoma and LNCaP prostate cancer cell lines, compared to α/ß-TBECH (Khalaf *et al,* 2009). The ligand binding affinity of γ/δ-TBECH was found to be very similar to DHT with EC_{50} of 14.9 nM and 10.5 nM, respectively. The EC_{50} for α/ß-TBECH is 174 nM. Furthermore, they showed that α/ß- and γ/δ-TBECH mixtures alone at 1 μM, were able to significantly activate basal PSA protein expression in LNCaP cells, which is an AR target gene.

TBECH has also been shown to be partial agonist of the zebrafish AR, with γ/δ-TBECH being the more potent partial agonist (Pradhan *et al,* 2013). Their quantitative real-time polymerase chain reaction (qRT-PCR) results showed that TBECH can cause transcriptional alterations in genes involved in chondrogenesis, drug metabolism, inflammation, apoptosis and stress in the zebrafish liver cell line, ZFL. *In vivo* exposure also showed that TBECH at both concentrations can cause downregulation in the transcription of genes involved in sex differentiation. More recently, a study showed that

TBECH can only weakly bind to the chicken AR, but can also activate the estrogen receptor and thyroid receptor in the chicken LMH cell line (Asnake *et al,* 2013). Collectively, these studies show that different species respond differently to TBECH.

Furthermore, ß-TBECH has been shown to disrupt the thyroid axis in juvenile brown trout via diet at concentrations ranging from 2.02 to 118.4 pmol/g lipid weight for 56 days (Park *et al,* 2011). Epithelial cell hypertrophy was observed in the thyroid of the brown trout at all three concentrations (2.02, 14.7 and 118.4 pmol/g). However, at the highest concentration, ß-TBECH resulted in a decrease in plasma total T4 (thyroxine) levels.

Physiological

As of August 2014, there have only been three avian studies evaluating the potential effects of TBECH *in vivo* (Marteinson *et al,* 2012; Currier *et al,* 2013; Crump et al, 2014). Adverse reproductive effects have been observed in birds exposed to androgen agonists such as testosterone and methyltestosterone (Rutkowska *et al,* 2005; Selzsam *et al,* 2005; Goerlich *et al,* 2009). Marteinson *et al* (2012) demonstrated that American kestrels exposed to ß-TBECH at 30.1 ng/µL for 82 days, resulted in a number of reproductive effects. These effects included smaller clutch size and reduced egg fertility, which led to reduced hatchling success. The second study involved *in ovo* exposure of TBECH to determine embryonic and long term effects in the hatched chicks of zebra finch (Currier *et al,* 2013). Currier *et al* (2013) found that exposure to α/ß-TBECH at concentrations 1, 2 and 20 ng/µL, had no significant effects on growth and survival in both the embryos and hatched chicks. A recent study showed no adverse effects on pipping success or growth parameters in chicken embryos dosed with α-/ß-TBECH ranging from 3.4 to 54,900 ng/g (Crump et al, 2014).

TBECH has also been shown to cause developmental abnormalities in zebrafish (Pradham *et al,* 2013). Their results showed that TBECH can cause hatching delays at concentrations 1 and 10 µM, and spinal abnormalities and mortality in juveniles at 10 µM.

1.5. Molecular Receptor Targets

1.5.1. Aryl-hydrocarbon receptor

The aryl hydrocarbon receptor (AhR) is a ligand activated transcription factor that is a member of the bHLH-PAS superfamily. The members of the bHLH-PAS superfamily have two main functions: to detect and respond to environmental signals or to form heterodimers with other members of the bHLH-PAS superfamily (Swedenborg and Pongratz, 2010). The AhR is responsible for mediating cellular response to xenobiotics including environmental pollutants such as halogenated hydrocarbons and polyaromatic cyclic hydrocarbons (Denison *et al,* 2002). The AhR is known to have the highest affinity for planar hydrophobic ligands, with TCDD being the most potent synthetic AhR ligand in humans (Poland and Knutson, 1982; Stejskalova et al, 2011). Some vertebrate animals, such as aquatic birds, are less sensitive to the effects of TCDD due to their reduced binding affinity to AhR (Karchner et al, 2006). Other common AhR ligands include benzo[a]pyrene, 3-methylcholanthrene, ß-naphthoflavone, 2,3,7,8-tetrachlorodibenzofuran and 3,4,3',4,5-pentachlorobiphenyl (Safe S, 1990). In addition to mediating xenobiotic response, the AhR is involved in transcriptional cross-talk with the ER, circadian rhythm and inflammation signalling pathways (Anderson *et al,* 2013; Swedenborg and Pongratz, 2010; Vogel *et al,* 2014).

In the absence of ligand, AhR exists in the cytoplasm as a multimeric complex consisting of two molecules of heat shock proteins 90 (HSP90), the HSP90 co-chaperone p23 and a 36-kDa protein known as the hepatitis B virus X-associated protein 2 (XAP2) (Besichlag *et al,* 2008). Upon ligand binding, AhR undergoes a conformational change and dissociates from the multimeric complex. The AhR-ligand bound complex translocates from the cytoplasm to the nucleus where it heterodimerizes with ARNT, forming the aryl hydrocarbon receptor complex (AHRC). The AHRC can regulate transcription by directing coregulatory proteins (including co-activators and co-repressors) and other transcription factors to specific DNA regulatory sequences called xenobiotic or dioxin response elements (XREs/DREs) located in the promoters of AhR target genes (Beischlag *et al,* 2008). Some common AhR target genes are CYP1A1, CYP1A2 and CYP1B1, which are involved in drug metabolism (Larsen *et al,* 1998;

Quattrochi *et al,* 1994). The typical AhR-mediated responses following exposure to TCDD and other TCDD-like compounds include chloroacne, immunotoxicity, hepatotoxicity, developmental and reproductive abnormalities, and cancer (Mandal PK, 2005, Bock KW, 2013).

1.5.2. Estrogen Receptor

The ER is a member of the steroid hormone receptor subfamily of the superfamily of nuclear receptors. Nuclear receptors are ligand-inducible transcription factors that regulate gene transcription (Gronemeyer *et al,* 2004). Estrogens are steroid hormones that play vital roles in sexual and reproductive development in both males and females. The most potent endogenous ER ligand is E2. ER signalling, which is mediated by the ER, is involved in many physiological processes including reproduction, cardiovascular health, bone integrity, immune health and central nervous system (Deroo and Korach, 2006).

The structural domains that make up the ER protein are the NH_2-terminal domain (NTD), DNA-binding domain (DBD) and the COOH-terminal (C-terminal) containing the ligand binding domain (LBD) (Nilsson *et al,* 2001). Transcriptional activation is mediated by two different activation functions (AF): AF-1 and AF-2. AF-1 is constitutively active and is located at the NH_2 terminal, whereas AF-2 is ligand-dependent and is located at the C-terminal of the LBD. Both AFs function to recruit a variety of coregulatory proteins to the receptor-ligand complex when bound to target DNA. There are two subtypes of the estrogen receptor, ERα and ERß, which differs in their NTDs (Nilsson *et al,* 2001). Both subtypes can bind to E2 with similar affinities, but have tissue-specific expression patterns in humans and rodents (Gustafsson JA, 2003; Mueller and Korach, 2001). There is also a third, membrane-bound ER called the G-protein coupled estrogen receptor-1 (GPER-1), which also has a high affinity for estrogens (Thomas *et al,* 2005). The GPER-1 is coupled to G-proteins and activates various signalling cascades via second messengers including cAMP and intracellular calcium (Nilsson *et al,* 2011).

The classical genomic ER signalling pathway involves ligand activation and direct DNA binding (Heldring *et al,* 2007). Upon ligand binding, ER undergoes a

conformational change and forms a functional homodimer. This homodimer then translocates from the cytoplasm to the nucleus where it can either bind directly to specific regulatory DNA sequences called estrogen response elements (EREs) or indirectly via protein-protein interactions to the EREs within the promoter region of estrogen responsive genes. The binding of the ligand-ER complex to the EREs results in the recruitment of coregulatory proteins to the target gene, in order to activate or repress transcription (Heldring et al, 2007).

Due to the promiscuity of the ligand-binding cavity of the ER, the ER can also bind to a variety of non-steroidal compounds including phytoestrogens, selective estrogen receptor modulators (synthetic ER ligands) and environmental contaminants such as polyaromatic hydrocarbons, phthalates and pesticides (Bolger *et al,* 1998; Brzozowski *et al,* 1997; Soto *et al,* 1995). Disruptions in this signalling pathway may result in various diseases including developmental defects, osteoporosis, cardiovascular disease, neurodegenerative disease and endocrine cancers (breast, ovarian, endometrial, prostate, colorectal) (Burns and Korach, 2012; Deroo and Korach, 2006).

1.5.3. Androgen Receptor

The AR is also a member of the steroid hormone receptor subfamily of the superfamily of nuclear receptors. Androgens are male sex steroid hormones that are important for the sexual development and maintenance of reproductive structures and behaviour in males. Androgens also play a role in non-reproductive tissues including skin, bone, muscle, adipose tissue and brain (Matsumoto *et al,* 2008). The endogenous AR ligands are testosterone and its potent metabolite, DHT. Environmental pollutants that are androgen agonists and antagonists can also bind to the AR (Luccio-Camelo and Prins, 2011). Disruptions in AR signalling could lead to the development of diseases such as prostate cancer, male pattern baldness, hypogonadism and androgen insensitivity syndrome (Eder *et al,* 2001; Ellis *et al,* 2001)

The structure of the AR protein is composed of distinct functional domains: NTD, DBD, C-terminal LBD and a small hinge region (H) (Brinkman *et al,* 1989). There are two isoforms of the AR: AR-A and AR-B (**Figure 1-4**), which differs in the length of the

N-terminal sequence (Wilson and McPhaul, 1994). The NTD is responsible for most of the transcriptional activity and contains AF-1. Unlike most steroid hormone receptors like the ER, most of the transactivation function lies within the AF-1. The NTD also serves as a platform for the recruitment of co-activators, co-repressors and components of the transcriptional machinery (Lallous *et al,* 2013). The LBD is responsible for binding androgens to AR and contains the AF-2, which is important for interacting with specific motifs in the NTD in order to form the interaction between the NTD and C-terminal of LBD (N/C interaction) (He and Wilson, 2002). This N/C interaction helps to stabilize the ligand within the LBD of the receptor. The DBD recognizes and binds to target DNA sequences, in addition to mediating the dimerization of AR monomers (Shaffer *et al,* 2004). The hinge region, which is situated between the DBD and LBD, contains a ligand-dependent nuclear localization signal and a ligand-independent nuclear export signal (Zhou *et al,* 1994). These two signals are involved in nuclear translocation and DNA binding (Haelens *et al,* 2007).

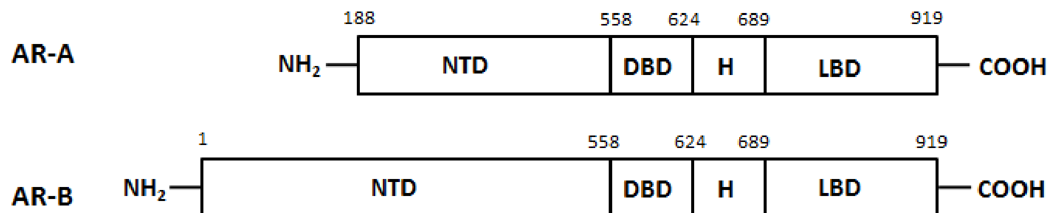


Figure 1-4. Structural domains of the two isoforms of the human androgen receptor protein.
The numbers indicate the amino acid residues.

In the absence of androgens, the inactive AR is associated with HSP 40, 70 and 90 which serve as chaperones in the cytoplasm (**Figure 1-5**) (Prescott and Coetzee, 2006). The binding of DHT to the LBD of AR causes a conformational change to AR (including the N/C interaction). This results in its dissociation from the HSPs and exposes the nuclear localization signal (He and Wilson, 2002). The interaction of the nuclear localization signal with the cytoplasmic cofactor, importin-α, allows the ligand-AR complex to translocate to the nucleus where dimerization of AR occurs (Van Royen et al, 2012). The AR-ligand homodimer then binds to the androgen response elements (AREs) within the promoter and enhancers of AR target genes (Kaku *et al,* 2008). The binding

of the ligand-AR homodimer to the AREs triggers the recruitment of co-regulatory proteins and the assembly of the transcriptional machinery (Heinlein and Chang, 2004). Un-liganded AR can either then be exported out of the nucleus and re-cycled or sent for degradation via the ubiquitin-proteasome pathway (He *et al,* 2002; Sheflin *et al,* 2000).

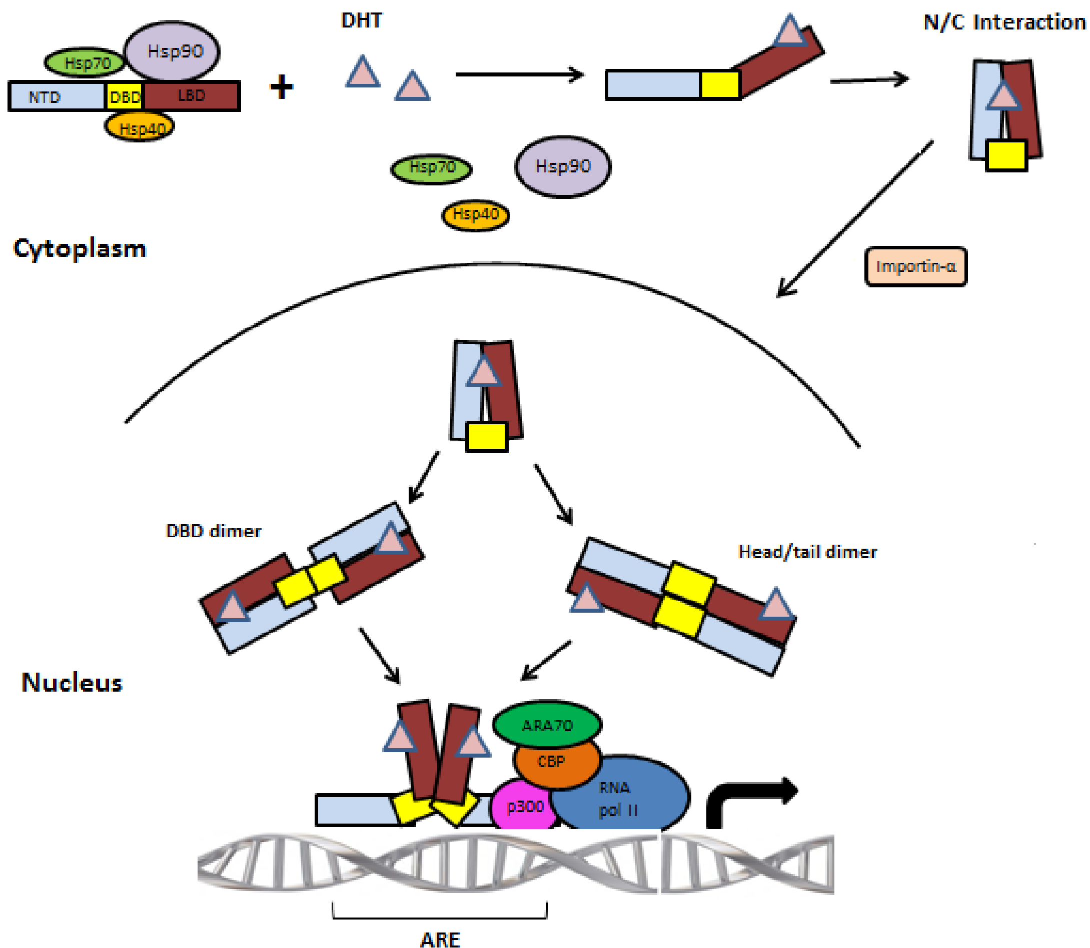


Figure 1-5. Schematic representation of the genomic androgen receptor signaling pathway (Adapted from Lallous *et al,* 2013).
AR-associated protein 70 (ARA70),CREB-binding protein (CBP), transcription activator p300 and RNA polymerase II (RNA pol II) are transcription factors and components of the transcriptional machinery. Note that the hinge region is not shown in this figure.

Furthermore, AR can also be activated via a non-genomic signalling pathway, which does not require DNA binding (Peterziel *et al,* 1999). In this non-genomic pathway, the AR can either be cytoplasmic or membrane-bound. The activated

cytoplasmic AR (bound to testosterone or DHT) can activate several kinase signalling cascades including Ras, phosphatidyl-inositol-3-kinase/Akt, protein kinase C (PKC) and the MAPK/ERK (Bennett *et al,* 2010). The activation of these kinase signalling cascades can enhance the AR genomic pathway (Bennett *et al,* 2010). The phosphorylation of AR by MAPK/ERK prevents AR from degradation and promotes the ligand-AR complex for nuclear translocation (Bennett *et al,* 2010). Moreover, activation of the membrane-bound AR with plasma membrane receptors including the G protein-coupled receptor, could result in the increase of intracellular calcium levels, which could lead to the activation of PKC (Bennett *et al,* 2010).

The androgen receptor gene is autoregulated by its own ligands, androgens at both the transcriptional and post-transcriptional level. At the transcriptional level, androgens have been shown to downregulate AR mRNA levels in rat Sertoli cells and in several cell lines, including the LNCaP and T47D cells (Blok *et al,* 1992; Krongard *et al,* 1991; Wolf *et al,* 1993). Detailed studies using nuclear run-on assays have shown that this downregulation of AR mRNA levels is due to decreased transcription of AR mRNA in the LNCaP cells (Yeap *et al,* 1999). These results suggest that downregulation of AR mRNA levels can limit the cellular response to androgens (Lee and Chang, 2003). However, it has also been shown that androgens can promote the upregulation of AR mRNA in other cell types such as human hepatocellular carcinoma and osteoblastic cell lines (Wiren *et al,* 1997; Yu *et al,* 1995). At the post-transcriptional level, androgens have been shown to mediate the upregulation of AR protein levels in LNCaP cells and the breast cancer cell line, MDA453 (Yeap *et al,* 1999). This increase in AR protein levels may be due to the rapid accumulation of AR mRNA in the polyribosomes isolated from the rat ventral prostate following testosterone treatment (Mora and Mahesh, 1999).

AR protein turnover can be regulated by the ubiquitin-proteasome pathway (Bennett et al, 2010). This pathway involves linking the targeted protein for degradation with the polypeptide co-factor, ubiquitin through a series of enzymes: ubiquitin-activating enzyme, ubiquitin-conjugating enzyme and ubiquitin-protein ligase (E3) (Lecker et al, 2006). E3 serves to recognize the specific protein targeted for degradation and adds the activated ubiquitin to it. Studies have demonstrated that AR needs to be phosphorylated at specific sites in order to be recognized by E3 (Gaughan *et al,* 2005; Lin *et al,* 2002).

Once a chain of ubiquitin molecules are added to the targeted protein, it is immediately recognized by the 26S proteasome, which is a multi-catalytic proteasome complex (Lecker et al, 2006). The ubiquitin-tagged protein is then linearized and inserted into the 26S proteasome where it is degraded into peptides (Lecker *et al,* 2006). These peptides are then further degraded into amino acids by peptidases in the cytoplasm (Lecker *et al,* 2006).

The synthetic pathway of androgens involves the steroid precursor, cholesterol (Figure 1-6). Cholesterol is first converted to pregnenolone which can then be converted to either progesterone or the adrenal androgen, dehydroepiandrosterone (DHEA) to androstenedione (Handa et al, 2008). Androstenedione is then converted to testosterone via the enzyme, 17ß-hydroxysteroid dehydrogenase. Testosterone can be converted to either estradiol via aromatase or to its more potent androgen metabolite, DHT via 5α-reductase. DHT can then be further converted to the androgen metabolites, 5α androstane-3α,17ß-diol (3α-Diol) and 5α androstane-3ß,17ß-diol (3ß-Diol), which can bind weakly to the AR (Handa *et al,* 2008). The conversion of DHT to 3α-Diol is reversible but not for DHT to 3ß-Diol which suggests that DHT can also be produced from 3α-Diol (Bauman *et al,* 2006). Androgen synthesis is regulated by the hypothalamic-pituitary-gonadal axis (Gommela L, 2009). Regulation starts at the hypothalamus, in which neurosecretory cells produce and release the gonadotropin-releasing hormone which is also known as the luteninzing hormone-releasing hormone (LHRH) (Tammela TLJ, 2012). The gonadotropes in the anterior pituitary responds by synthesizing and releasing the gonadotropins, follicle-stimulating hormone (FSH) and lutenizing hormone (LH), in a pulsatile manner (Tammela TLJ, 2012). In males, the FSH and LH are responsible for gonadal function. It is the LH that controls the synthesis of androgens in the testes (Gomella L, 2009). Normal serum levels of testosterone are between 10.4-34.7 nM ± 0.728-2.429 in men aged 17 years or older (Gomella L, 2009).

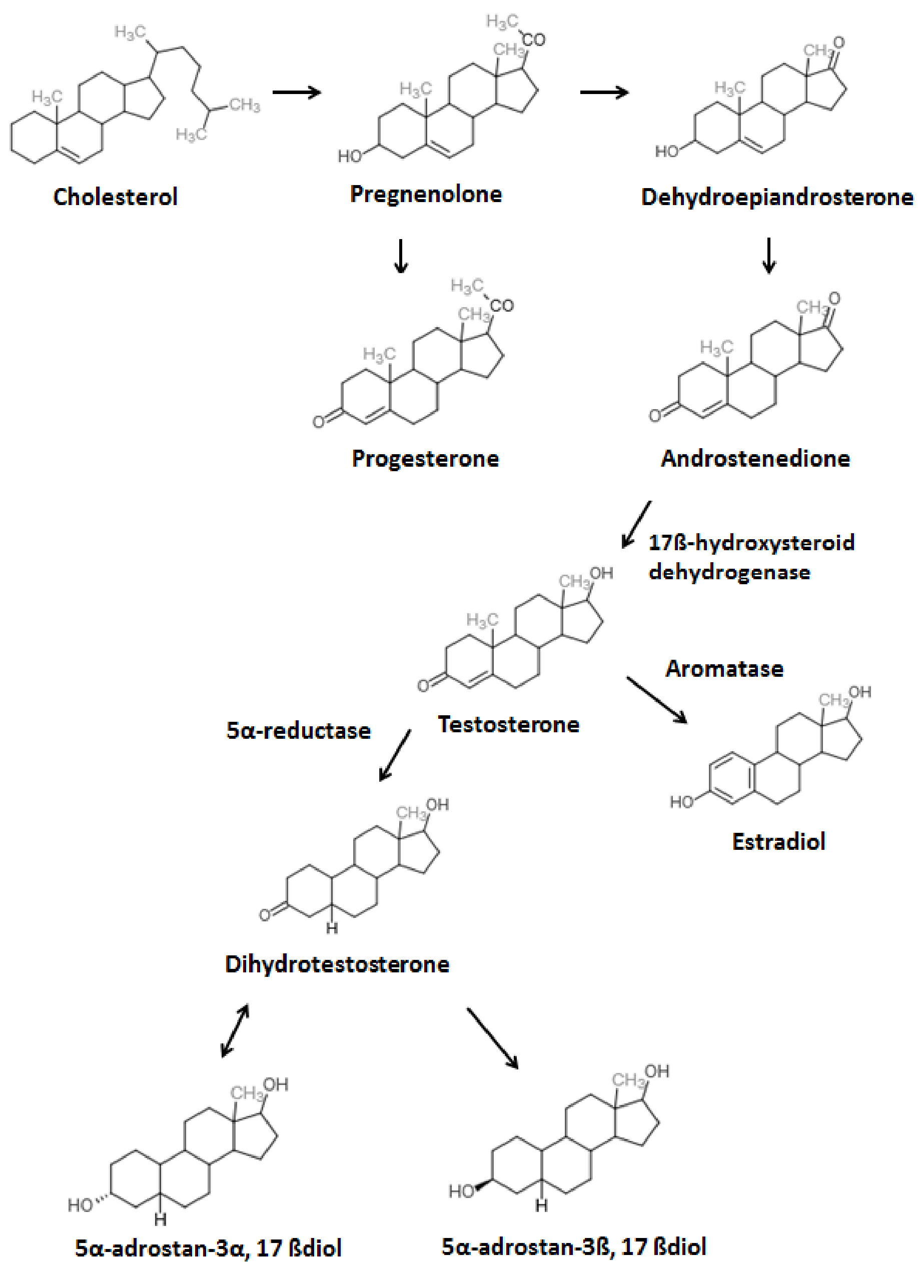


Figure 1-6. Pathway of androgen synthesis from the precursor, cholesterol.

1.6. Prostate Cancer and PSA

1.6.1. Etiology

Prostate cancer is one of the most diagnosed male cancers and leading cause of cancer deaths in men in Western countries (American Cancer Society, 2008). It is estimated to be the third leading cause of cancer deaths in Canada, ranking behind lung and colorectal cancers, and second leading cause of cancer deaths in the US (American Cancer Society, 2013; Canadian Cancer Statistics, 2013). The function of a normal prostate is to produce a slightly acidic fluid containing citric acid, enzymes, metal ions and lipids to help nourish the sperm (Kumar and Majumder, 1995). The functioning of a normal prostate is dependent on the actions of androgens regulating the balance between cellular proliferation and apoptosis; however, this balance is lost in prostate cancer (Denmeade *et al,* 1996). Prostate cancer tumours are dependent on androgens for their development, growth and survival in the early stages and can be treated with androgen ablation therapy or radiation (Feldman and Feldman, 2001). Common androgen ablation therapies involve blocking the AR signalling pathway using anti-androgens or reducing the circulating levels of androgens by surgical castration, estrogens and LHRH agonists or antagonists (Tammela TLJ, 2012). However, in many instances, the tumor eventually returns and becomes androgen-independent. This type of prostate cancer is known as androgen-independent prostate cancer or castration-resistant prostate cancer (CRPC). CRPC is a lethal type of prostate cancer as tumors are unresponsive to androgen ablation therapies and become metastatic. The survival of the tumor in CRPC/CRPC is still dependent on the AR signalling pathway, as the AR in these tumors are constitutively active and continue to express AR regulated genes (Decker *et al,* 2012).

1.6.2. Relationship between Prostate Cancer and PSA

The androgen-regulated gene, PSA is a protein produced in healthy prostate tissues, benign prostatic hyperplasia and in all stages of prostate cancer (Lilja *et al,* 2008). PSA is a serine protease with chymotrypsin-like activity and is a member of the family of glandular kallikrein-related peptidases (Balk *et al,* 2003). Its function in the normal prostate is to cleave semenogelin I and II, proteins that are involved in liquefying the seminal fluid (Balk *et al,* 2003). The prostate gland is composed of a layer of secretory epithelial cells which are enclosed by a layer of basal cells and followed by the basement membrane. PSA is normally produced by the prostatic epithelial cells and

secreted into the glandular lumen with some leaking into the serum. However, the amount of PSA leaking into the serum is approximately one million times lower than in the lumen (Williams *et al,* 2007). In prostate cancer, cancerous cells disrupt the basal cell layer and the basement membrane, allowing more PSA to leak into the bloodstream (**Figure 1-7**) (Balk *et al,* 2003). PSA levels in blood can range from <0.1 to 10^4 ng/mL with levels above 10^2 ng/mL detected in men with advanced prostate cancer (Lilja *et al,* 2008). Furthermore, rising PSA levels is also due to aberrant AR activity in CRPC (Ryan *et al,* 2006). Therefore, PSA has been chosen to serve as a serum biomarker for prostate cancer detection, responses to therapy, recurrence and even for the early detection of prostate cancer risk (Lilja *et al,* 2008).

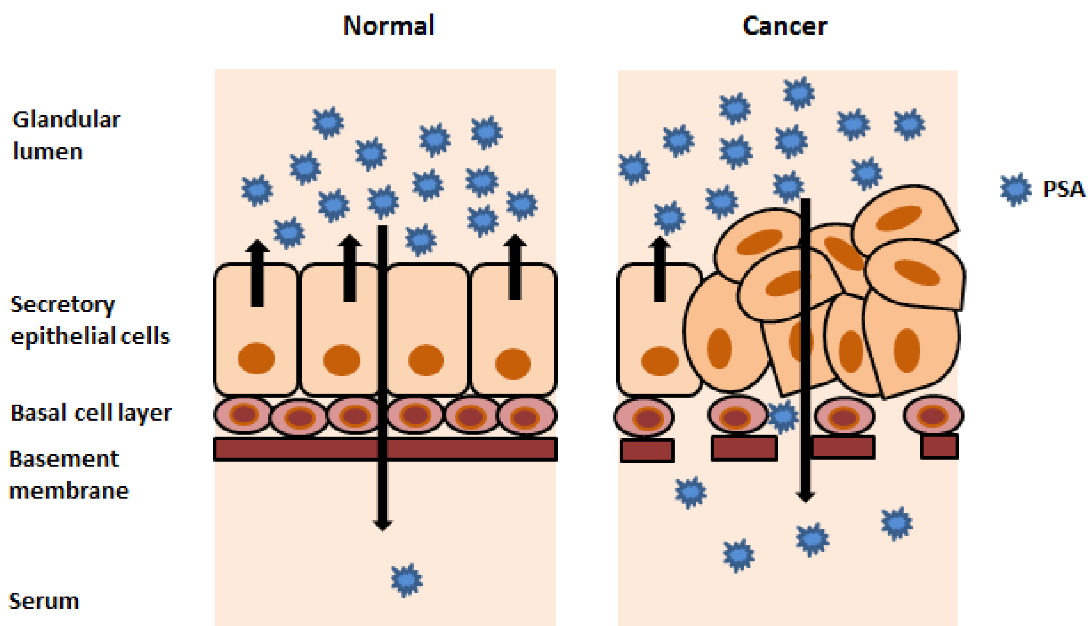


Figure 1-7. Schematic diagram showing how PSA leaks into the serum in prostate cancer compared to in a healthy prostate.

1.6.3. PSA Testing

In 1986, PSA testing was approved by the US Food and Drug Administration as a biomarker for prostate cancer monitoring (National Cancer Institute, 2012). In 1994, it was approved as a screening tool for prostate cancer in combination with the digital rectal exam (National Cancer Institute, 2012). Since then, PSA has been widely used as a serum biomarker for detecting and monitoring prostate cancer. However, its use as a

screening tool remains controversial as it has resulted in numerous cases of under diagnosis and over diagnosis (National Cancer Institute, 2012). Based on several large cohort studies in the past, it has concluded that PSA levels of 4.0 ng/mL or higher was an early indicator of prostate cancer (Fitzpatrick *et al,* 2009). However, studies later showed that some men with prostate cancer had PSA levels below 4.0 ng/mL and some men with no prostate cancer had PSA levels above 4.0 ng/mL (Thompson *et al,* 2004). It is concluded that there is no specific value for a normal or abnormal PSA level in the blood but generally, the higher the PSA level, the higher the chance of having prostate cancer (National Cancer Institute, 2012). Based on two new large randomized, controlled studies: the US Prostate, Lung, Colorectal and Ovarian and the European Randomized Study of Screening for Prostate Cancer trials and other studies in the past, the US Preventive Services Task Force concluded that for every 1000 men between ages 55-69 who underwent screening, 100-120 men got false-positive results and 110 men would be diagnosed with prostate cancer (Andriole *et al,* 2012; Moyer VA, 2012; Schröder *et al,* 2012). Despite the controversies about the practicality of PSA as a screening biomarker, the PSA test is still widely used as a clinical screen for prostate cancer (Flahavan *et al,* 2014).

1.7. Research Objectives and Hypothesis

The increasing incidence of reproductive and developmental defects in wildlife and hormone-responsive cancers in humans, have been linked to exposures to endocrine disrupting chemicals including DDT, DDE and TBECH. The combined effects of these endocrine disrupting chemicals and endogenous hormones on the effect of estrogen-, androgen- and AhR-responsive mRNA and protein expression has yet to be fully investigated. Less attention has been directed to the weakly estrogenic isomer, *p,p'*-DDT. However, given *p,p'*-DDT's greater composition in the technical DDT mixture, I decided to further investigate the effects of this isomer. Furthermore, much of the focus of this project was later directed to the effects of *p,p'*-DDT, *p,p'*-DDE and TBECH on the androgen-regulated genes, PSA and prostate-specific membrane antigen (PSMA).

The first objective of this project is to examine the effects of *p,p'*-DDT and *p,p'*-DDE on the mRNA and protein levels of ER, AR and AhR target genes in various mammalian and human cancer cell lines. The second objective is to examine the effects of TBECH on mRNA and proteins levels of AR target genes in the human prostate cancer cell line, LNCaP. The third objective is to determine the potential molecular mechanism by which *p,p'*-DDT and *p,p'*-DDE exert its anti-androgenic effects on the AR. Finally, the last objective is to investigate if *p,p'*-DDT and *p,p'*-DDE can inhibit the 26S proteasome. The results of the last objective led to the serendipitous discovery of the potential role that the 26S proteasome may play in CRPC.

Given the extensive evidence of endocrine disruption by DDT and DDE, I hypothesized that these EDCs could negatively impact ER, AR and AhR-target mRNA and protein expression. I also hypothesized that TBECH can negatively impact AR-target mRNA and protein expression. Furthermore, given the anti-androgenic properties of *p,p'*-DDT and *p,p'*-DDE and the androgenic properties of TBECH, I hypothesized that *p,p'*-DDT and *p,p'*-DDE can repress PSA, while TBECH can activate PSA. These results could potentially be implicated in producing false-negative PSA tests in men who have prostate cancer and who have been exposed to these EDCs. Given the efforts to reduce screening to reduce over-treatment burden, these EDCs could set up an environment in individuals with prostate cancer that would positively select for transformed androgen-insensitive cells that are more refractile to conventional therapies worsening the prognosis.

Chapter 2. Materials and Methods

2.1. Chemicals and Cell Culture

2.1.1. Chemicals

The chemicals, *p,p'*-DDT, *p,p'*-DDE (Supelc) and TBECH (Wellington Laboratories) were prepared in dimethyl sulfoxide (DMSO). The synthetic androgen, methyltrienolone (R1881) was generously supplied by the Vancouver Prostate Centre (Vancouver Coastal Health Research Institute). Stock solutions of E2, R1881 and TCDD (Sigma Aldrich) were prepared in DMSO.

2.1.2. Cell Culture

The human prostate cancer cell line, LNCaP and the human prostatic carcinoma cell line, 22RV1, were both maintained in RPMI-1640 media with L-glutamine (BioWhittaker, Lonza), 5% (v/v) fetal bovine serum (FBS; HyClone, PerBio, Thermo Fisher Scientific Inc.) and supplemented with 100 units/mL potassium penicillin-100 μg/mL streptomycin sulphate (1% (v/v) P/S; BioWhittaker, Lonza).

The human breast cancer cell line, MCF-7, human endometrial carcinoma cell line, ECC-1 and the mouse hepatoma, Hepa1c1c7, were all maintained in Dulbecco's Modified Eagle's Medium with 4.5 g/L glucose and 4.5 g/L L-glutamine (DMEM; BioWhittaker, Lonza) with 10% (v/v) FBS (HyClone, PerBio, Thermo Fisher Scientific Inc) and supplemented with 1% P/S (BioWhittaker, Lonza). The DMEM for MCF-7 was additionally supplemented with 0.01 μg/mL bovine pancreas insulin solution (Sigma Aldrich).

All cell lines were maintained at 37°C, 20% O2 and 5% CO2. Information regarding the different cancer cell lines is presented in **Table 1.**

Table 1. Information on the different cancer cell lines used.

Cell line	Origin and disease	Receptors expressed
LNCaP	Human prostate adenocarcinoma	Mutated AR*, AhR
22Rv1	Human prostatic carcinoma xenograft, CWR22R**	Constitutively expressed AR
ECC-1	Human endometrial carcinoma	ER, PR, wild-type AR
MCF-7	Human breast adenocarcinoma	ER, PR, AhR
Hepa-1c1c7	Mouse hepatocellular carcinoma	AhR

* Point mutation in the ligand-binding domain and therefore, can also bind to estrogens and progesterones
** Androgen insensitive cell line (Sramkoski *et al,* 1999)

2.2. Reverse transcriptase and Real-time PCR

2.2.1. Chemical Treatment

LNCaP cells were serum-starved in 10% charcoal stripped FBS (Gibco®) and 1% P/S in RPMI-1640 media in Multiwell™ 6-well cell culture plates for 24 h prior to chemical treatment. MCF-7, ECC-1 and Hepa1c1c7 cells were serum-starved in phenol-free DMEM with 4 g/L glucose, 1% (v/v) L-glutamine and 1% P/S in Multiwell™ 6-well cell culture plates for 24 h prior to chemical treatment.

LNCaP cells were treated with vehicle (DMSO), R1881 at 1 nM, *p,p'*-DDE and *p,p'*-DDT alone at 1 and 10 μM and in combination with R1881 for 24 h. For the dose response curve, LNCaP cells were treated with vehicle, R1881 at 1 nM, and in combination with *p,p'*-DDE or *p,p'*-DDT in increasing concentrations from 10 nM to 50 μM for 24 h. Another set of LNCaP cells were treated with vehicle, R1881 at 1 nM, α/ß-TBECH or γ/δ-TBECH alone at 1 and 10 μM and in combination with R1881 for 24 h.

ECC-1 cells were treated with vehicle, E2 at 10 nM, *p,p'*-DDE and *p,p'*-DDT alone at 1 and 10 μM and in combination with E2 for 24 h. MCF-7 and Hepa-1c1c7 cells were treated with vehicle, TCDD at 2.5 nM, *p,p'*-DDE and *p,p'*-DDT alone at 1 and 10 μM and in combination with TCDD for 24 h. For the MCF-7, ECC-1 and Hepa-1c1c7

cells, 200 μL of 30% bovine serum albumin (BSA; EMD Millipore) was added to each well of the Multiwell™ 6-well cell culture plates immediately prior to chemical treatment.

2.2.2. RNA extraction

Twenty-four h after chemical treatment, cells were washed with 1x phosphate buffered saline solution twice and harvested in 750 μL of TRIzol (Invitrogen) and 200 μL of chloroform (Caledon Laboratories) in 1.5 mL microcentrifuge tubes. Tubes were shaken vigorously for 15 seconds and allowed to sit for ~5-10 minutes on ice. This was followed by a 10 minutes centrifugation at 11,000 *x g* at 4°C. After centrifugation, ~300 μL was pipetted from the top aqueous layer into a new set of microcentrifuge tubes. Next, 500 μL of isopropanol was added into each tube, inverted and was allowed to sit on ice for ~5 minutes. Tubes were then centrifuged at 11,000 *x g* for 10 minutes at 4°C again. Following this, the supernatant was removed from the tubes and 750 μL of cold 75% ethanol was added to resuspend the RNA pellet. After addition of ethanol, tubes were inverted and subjected to a third centrifugation at 11,000 *x g* for 10 minutes at 4°C. Following this, ethanol was removed from the tubes and tubes were allowed to air dry for a few minutes before resuspending the RNA pellet in 20 μL of cold RNase-free water.

2.2.3. Reverse transcriptase PCR

RNA concentrations in each sample were measured on the NanoVue spectrophotometer. A total of 2 μg of RNA was subjected to reverse transcription using a high capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer's protocols (per reaction: 1x RT buffer, 1x RT random primers, 4 mM dNTPs (deoxyribonucleotide triphosphates), 50 units MultiScribe™ Reverse transcriptase). The reverse transcription reaction was carried out in the following conditions: 25°C for 5 minutes, 37°C for 120 minutes, 85°C for 5 minutes and held at 4°C until use (Veriti® 96-Well Thermal Cycler, Applied Biosystems).

2.2.4. Quantitative Real-time PCR

cDNAs were amplified by real-time PCR using a Power SYBR Green PCR kit (Applied Biosystems) according to the manufacturer's protocols (per reaction: 1x SYBR® green, 1x Rox reference dye, 3.0 pM primer pair). For each sample, 4 μL cDNA was used and diluted 1:15. Standards were made using the positive control sample with the following dilutions: 1:10, 1:100, 1:1,000, 1:10,000 and 1:100,000. DNA was amplified under the following conditions: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute and ended off 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). Olignonucleotide pairs used to amplify cDNA sequences for CATD, CYP1A1, IGF-1, PSA, PSMA and 36B4 (Integrated DNA Technologies) are shown in **Table 2**. Triplicate reactions were performed 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 every assay.

Table 2. List of oligonucleotide primer pairs for quantitative real-time PCR.

Gene	Gene Name	Primer Pairs (forward and reverse)
CATD	Cathepsin D	(F) 5'-CCGTGCCGCTGATTCAG-3' (R) 5'-GGGACAGCTAGCCTTTGC-3'
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	(F) 5'-CACTCTTCCTTCGTCCCCCT-3' (R) 5'-TGGTTGATCTGCCACTGGTT-3'
IGF-1	Insulin growth factor-1	(F) 5'-GACAGGCATCGTGGATGAG-3' (R) 5'-GACAGAGCGAGCTGACTTG-3'
PSA	Prostate specific antigen	(F) 5'-GACCACCTGCTACGCCTCA-3' (R) 5'-GGAGGTCCACACTGAAGTTTC-3'
PSMA	Prostate specific membrane antigen	(F) 5'-AACTGGACCCCAGGTCTGGA-3' (R) 5'GAGGATTTTATAAACCACCCGAA-3'
36B4	Acidic ribosomal phosphoprotein P0	(F) 5'-CCACGGTGCTGAACATGCT-3' (R) 5'TCGAACACCTGCTGGATGAC-3'

2.3. Protein Extractions and Western Blotting

2.3.1. Chemical Treatment

LNCaP and 22RV1 cells were serum-starved in 10% charcoal stripped FBS and 1% P/S in RPMI-1640 media in 100 mm plates for 24 h prior to chemical treatment. ECC-1 cells were serum-starved in phenol-free DMEM with 4 g/L glucose, 1% (v/v) L-glutamine and 1% P/S for 24 h prior to chemical treatment.

LNCaP cells were treated with vehicle, R1881 at 1 nM, *p,p'*-DDE and *p,p'*-DDT alone at 1 and 10 μM and in combination with R1881 at 1 nM for 24 h. Another set of LNCaP cells were treated with vehicle, R1881 at 1 nM, α/ß-TBECH or γ/δ-TBECH alone at 1 and 10 μM and in combination with R1881 at 1 nM for 24 h.

ECC-1 cells were treated with vehicle, E2 at 10 nM, *p,p'*-DDE and *p,p'*-DDT alone at 1 and 10 μM and in combination with E2 at 10 nM for 24 h. For ECC-1 cells, 1 mL of 30% BSA was added to each 100 mm plate immediately prior to chemical treatment.

To determine whether *p,p'*-DDT or *p,p'*-DDE can inhibit the 26S proteasome or affect AR turnover, two sets of LNCaP cells were treated with vehicle, R1881 at 1 nM, *p,p'*-DDE and *p,p'*-DDT at 1 and 10 μM in combination with R1881 at 1 nM for 24 h. For the second set of treated LNCaP cells, 5 μM of MG132, a 26S proteasome inhibitor was also added. Next, to determine AR turnover, a 7 h MG132 treatment was performed. LNCaP cells were treated with 5 μM of MG132 at 1 min, 15 min, 30 min, 45 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h and at 7 h. Next, to determine if the AR proteome was affected, LNCaP cells were treated with 5 μM of MG132 at 1 min and 7 h, and compared with the untreated 22RV1 cell lysates that express a shorter androgen-insensitive splice variant that lacks the carboxy-terminal ligand binding domain (Dehm *et al,* 2008). Finally, to determine whether the shorter AR isoform is a product of protein degradation, LNCaP cells were treated with DMSO, either proteasome inhibitor (P.I.) or MG132 (5μM) alone or in combination (P.I. + MG132) for 24 h. To determine whether the shorter AR isoform is a product of *de novo* transcription of a splice variant, LNCaP cells were treated with

DMSO, either actinomycin-D or MG132 (5μM) alone or in combination (MG132 + actinomycin-D) with or without R1881 (10nM) for 24 h.

2.3.2. Protein Extraction and Determination Assay

Twenty-four h after addition of chemicals, whole cells lysates were first washed with cold 1x PBS twice. Each sample was then harvested with 500 mL of cell lysis buffer mix (1 sample per 1mL: 20 μL of 100x protease inhibitor, 0.34 μL of ß-mercaptoethanol and 0.98 mL of cell 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)) and collected in 1.5 mL microcentrifuge tubes on ice. Samples were vortexed every 5 minutes for 20 minutes and kept on ice in between, followed by a centrifugation at 14,000 RPM for 15 minutes at 4°C. After centrifugation, aliquots of the supernatant were then collected in 1.5 mL microcentrifuge tubes.

Protein concentrations were determined using the RC DC™ protein assay (Bio-Rad) according to the manufacturer's protocol. A 2 mg/mL stock solution of BSA (Bio-Rad) was used to make a set of protein standards for the assay. The six standards were 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. Each sample was diluted in 1/3, using the lysis buffer mix as the diluent and blank. To each sample, 125 μL of RC Reagent I was added and the sample was briefly vortexed before incubating at room temperature for 1 minute. Next, 125 μL of RC Reagent II was added, vortexed and centrifuged at 15,000 *x g* for 5 minutes at room temperature. After centrifugation, supernatant from each tube was discarded followed by addition of 127 μL of Reagent A (per sample: 5 μL DC reagent S and 250 μL DC reagent A) to each sample. Samples were incubated for 5 minutes at room temperature and vortexed again after. Finally, 1 mL of Reagent B was added and samples were vortexed immediately and incubated at room temperature for 15 minutes before the absorbance readings. A SmartSpec™ Plus Spectrophotometer (Bio-Rad) was used to measure the absorbance of the BSA standards and the protein samples at wavelength 750 nm.

2.3.3. Nuclear and Cytoplasmic Protein Extraction

LNCaP cells were treated with vehicle, R1881 at 1nM, *p,p'*-DDE, *p,p'*-DDT or R1881 in combination with *p,p'*-DDE (10 μM), *p,p'*-DDT (10 μM) for 1 h. One h after addition of chemicals, nuclear and cytoplasmic proteins were extracted using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) as per manufacturer's protocol. Briefly, cells were harvested with trypsin-EDTA and centrifuged at 500 *x g* for 5 minutes. After centrifugation, the cell pellet was suspended by washing the cells with 1x PBS and subjected to a second centrifugation at 500 *x g* for 3 minutes. Next, the supernatant was carefully removed and 100 μL of cold CER1 reagent was added to the pellet and vortexed vigorously for 15 seconds. Sample tubes were then placed on ice for 10 minutes. After that, 55 μL of cold CER II was added to each tube, vortexed vigorously for 5 seconds and placed on ice for 1 minute. Sample tubes were vortexed for another 5 seconds followed by a third centrifugation at maximum speed for 5 minutes. Immediately after centrifugation, supernatant (cytoplasmic extract) was transferred to a pre-chilled tube and stored at -80°C until use. For the insoluble pellet fraction (produced after third centrifugation) containing the nuclei, 500 μL of cold NER reagent was added to each sample tube and vortexed vigorously for 15 seconds. Sample tubes were then vortexed for 15 seconds for every 10 minutes, for the next 40 minutes and kept on ice in between. Tubes were then centrifuged at maximum speed and the supernatant (nuclear extract) was immediately transferred to a pre-chilled tube. Samples were stored at -80°C until use.

2.3.4. Western Blotting

Western blotting was performed as described previously (Labrecque *et al,* 2012) with a few minor changes. Equal amounts of protein were resolved on a SDS-polyacrylamide gel (8% or 10% acrylamide/bis, 1 M Tris, 5 mM SDS, 0.1% ammonium persulfate, 0.01% tetramethylethylenediamine) in 1x Running buffer (1 M Tricine, 1 M Tris-Cl, 50 mM SDS) which was then transferred to a polyvinylidene fluoride (PVDF) membrane in transfer buffer (25mM Tris-Cl, 250 mM Glycine, 0.1% SDS, methanol). After the transfer, the membrane was activated with 100% methanol for 1 min and was blocked with 1x TBST in 5% milk (2.5 mM Tris, 140 mM NaCl, 2.5 mM KCl, 0.5%

Tween20, 5% non-fat milk powder) for 1 h at room temperature. The membranes were probed separately with the following primary antibodies: anti-CATD (1/1500; rabbit polyclonal IgG; Santa Cruz Biotechnology), anti-PSA C-19 (goat polyclonal IgG; Santa Cruz Biotechnology, Inc.) anti-PSMA (1/1667; mouse monoclonal IgG; BC Cancer Agency), anti-AR PG-21 (1/1500; rabbit polyclonal IgG; Millipore), anti-AR-N20 (1/500; rabbit polyclonal IgG; Santa Cruz Biotechnology, Inc.), anti-AR-C19 (1/500; rabbit polyclonal IgG; Santa Cruz Biotechnology, Inc.), anti-Histone H1 FL-219 (1/1000; rabbit polyclonal IgG; Santa Cruz Biotechnology, Inc.) and anti-α-tubulin (1/1500; mouse monoclonal IgG; Santa Cruz Biotechnology, Inc.) in 1x TBST in 5% milk. Blots were then probed with horseradish peroxidise conjugated anti-mouse or anti-rabbit IgG in 1x TBST in 5% milk for 1 h at room temperature. The blots were washed three times with 1x TBST for 5 min each and the proteins were detected using the ECL prime detection kit (GE Healthcare). Blots were then visualized using the Syngene Dyversity blot imaging system. Densitometry analysis was performed to measure the amount of AR and PSMA protein induction or repression. Data from three separate observations were normalized to α-tubulin and analyzed using GeneTools 4.01.2 software (Syngene).

2.4. PSA Protein Determinations

PSA levels in conditioned media were determined using an ultrasensitive COBAS CORE II immuno-detection system at the Vancouver Prostate Centre. Briefly, LNCaP cells were cultured to approximately 50% confluency before chemical treatment. LNCaP cells were treated with vehicle, R1881 at 1 nM, *p,p'*-DDT or *p,p'*-DDE or α/ß-TBECH or γ/δ-TBECH alone at 10 nM, 50 nM, 100 nM, 500 nM, 1 µM, 5 µM, 10 µM and 50 µM and in combination with R1881 at 1 and 10 µM for 24 h. Twenty-four hours later, cells were harvested with TRIzol (Invitrogen) and stored at -80°C until delivered to the Vancouver Prostate Centre.

2.5. Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assay was performed as described previously (Labrecque *et al,* 2012) with a few minor changes. LNCaP cells were seeded

onto 150 mm plates and serum-starved for 24 h prior to chemical treatment. Cells were treated with vehicle, R1881 at 1 nM, p'p'-DDE at 10 μM, p'p'-DDT at 10 μM, and a combination of R1881 at 1 nM and either p'p'-DDE 10 μM or p'p-DDT 10 μM for 45 min. After treatments, chromatin complexes were chemically cross-linked using a 1% formaldehyde/0.7 M HEPES solution (final concentration), pH 7.8 and complexes were sonicated to yield DNA fragments of 200 to 500 bp size. Complexes were pre-cleared with protein A agarose beads (CalBiochem) and pre-cleared samples were incubated overnight at 4ºC with AR rabbit polyclonal antibody (Santa Cruz). Immuno-adsorbed complexes were captured on protein A agarose beads and washed with 0.5 x RIPA, TSE II (20 mM Tris-HCl pH 8.1, 500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100) and TSE III (10 mM Tris-HCl pH 8.1, 0.25 LiCl M, 1 mM EDTA, 1% NP-40, 1% deoxycholate) and then followed by three washes with TE8 (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). Samples were eluted off of the beads using 100 mM $NaHCO_3$ and 1% SDS, and cross-links were reversed at 65°C overnight. Samples were extracted with phenol-chloroform and DNA was then precipitated with 2 μL pellet paint (Novagen), 1/10 volume Na-acetate and 2 volumes of 100% ethanol. Immuno-adsorbed DNA was analyzed by PCR. Primers for the PSA promoter regions are shown in **Table 3**.

Table 3. Primer sequences for PSA promoter regions.

Primer pair	Direction	Primer sequence*
ARE I	Forward	TCTGCCTTTGTCCCCTAGAT
	Reverse	AACCTTCATTCCCCAGGACT
ARE II	Forward	AGGGATCAGGGAGTCTCACA
	Reverse	GCTAGCACTTGCTGTTCTGC

*Shang *et al,* 2002

2.6. Conversion of Human Levels of DDT and DDE Data

We performed a data analysis of the global human tissue levels of *p,p'*-DDT and *p,p'*-DDE compiled by Jaga and Dharmani (Jaga and Dharmani, 2003) from literature spanning from 1989-2000. In their study, the mean concentrations were presented in ng/g of lipid for adipose tissue and ng/g for serum. In order to make a comparison to our concentrations, we converted the mean concentrations in ng/g to mol/L by multiplying the density of the specific human tissue (g/L) followed by converting ng/L to g/L and

dividing this number by the molecular weight of either DDT (354.99 g/mol) or DDE (318.02 g/mol). Finally, this concentration in mol/L was converted to μM. This same conversion was applied for the human adipose tissue and serum levels that we have compiled for the 2000-2012 period, except for two studies (Waliszewski *et al,* 2010; Waliszewski *et al,* 2012) in which adipose tissue concentrations presented in mg/kg of lipid were converted to ng/g. The density of human adipose tissue is 0.905g/mL or 905g/L (Martin, et al. 1994) and for human blood serum is 1.024 g/mL or 1024 g/L.

2.7. Statistical Analysis

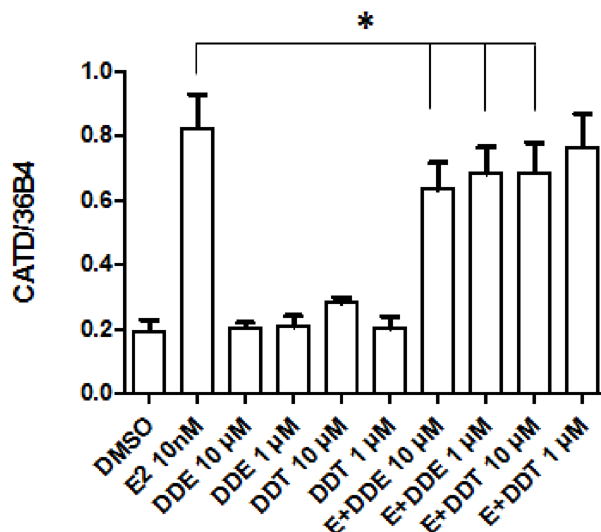
Statistical analyses were performed using one-way ANOVA with Bonferroni's Multiple Comparison Test. Values are presented as means ± standard deviation (SD). A p value <0.05 was considered to be significant.

Chapter 3. Results

3.1. DDT and DDE

3.1.1. Messenger RNA accumulation of CATD and CYP1A1 and protein accumulation of CATD

Numerous studies have clearly established the estrogenic effects of both DDT and DDE isomers. Nevertheless, only a few studies have investigated the effect of the less estrogenic isomer, *p,p'*-DDT and the potent anti-androgen, *p,p'*-DDE in the presence of E2. ECC-1 cells were treated with E2, *p,p'*-DDT and *p,p'*-DDE at 1 and 10 μM and in combination with E2. Although our results showed significant E2-inducible repression with *p,p'*-DDT at 10 μM and *p,p'*-DDE at both concentrations, at the mRNA level, Western blot results failed to show any significant changes at the protein level (**Figure 3-1**). The CATD protein levels of the combined E2 and *p,p'*-DDT or *p,p'*-DDE treatment appears similar to E2 treatment alone (**Figure 3-1**). Therefore, I decided not to pursue the effects of DDT and DDE on estrogen-response genes.



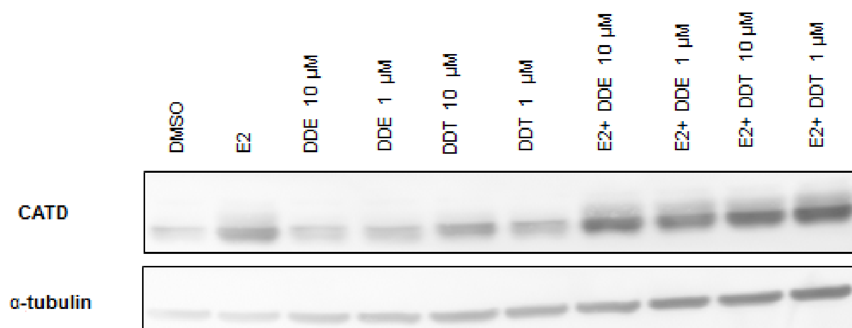


Figure 3-1. Effects of *p,p'*-DDT and *p,p'*-DDE on mRNA and protein accumulation of CATD.
Cells were treated with vehicle, E2 at 10 nM, *p,p'*-DDE and *p,p'*-DDT alone at 1 µM and 10 µM and in combination with E2 for 24 h. (A) The mRNA levels for CATD and 36B4 were determined by real-time PCR and normalized to constitutively expressed 36B4 gene. Data represents the average of two experiments run in triplicate and values are expressed as mean values ± SD. *$p<0.05$, one-way ANOVA. E=E2. (B) Western blotting was performed using antibodies directed against CATD, PSMA and α-tubulin. Representative blot of three separate experiments.

Since Jeong et al (2002) demonstrated that *o,p'*-DDT can downregulate TCDD-inducible CYP1A1 activity in Hepa-1c1c7 cells and Wojtowicz et al (2011) demonstrated that both DDT isomers and *p,p'*-DDE can downregulate TCDD-inducible CYP1A1 protein

expression in human placental cells, we decided to confirm these results in the Hepa-1c1c7 and MCF-7 cell lines before investigating further. Hepa-1c1c7 cells were treated with TCDD, *p,p'*-DDT and *p,p'*-DDE at 1 and 10 µM and in combination with TCDD. MCF-7 cells were treated with E2, *p,p'*-DDT or *p,p'*-DDE at 1 and 10 µM and in combination with E2. However, our results showed that *p,p'*-DDT and *p,p'*-DDE had no significant effects on TCDD-inducible CYP1A1 mRNA accumulation in both cell lines (**Figure 3-2**). Therefore, I decided not to pursue the effects of DDT and DDE on AhR function.

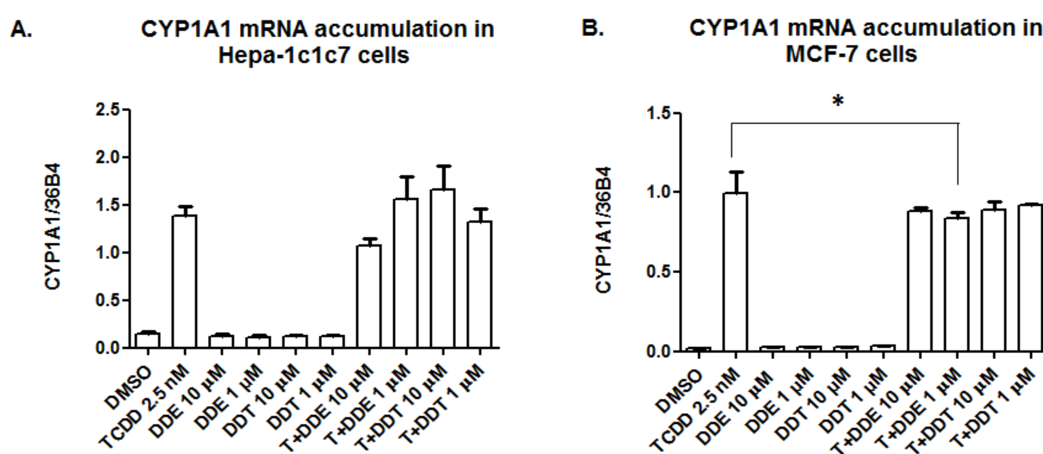


Figure 3-2. Effects of *p,p'*-DDT and p,p' CYP1A1 mRNA accumulation in Hepa-1c1c7 and MCF-7 cells.
Cells were treated with vehicle, TCDD at 2.5 nM, *p,p'*-DDE and *p,p'*-DDT alone at 1 µM and 10 µM and in combination with TCDD for 24 h. The mRNA levels for CYP1A1 and 36B4 were determined by real-time PCR and normalized to constitutively expressed 36B4 gene. Data represents the average of two experiments run in triplicate and values are expressed as mean values ± SD. *$p<0.05$, one-way ANOVA. T=TCDD.

3.1.2. Messenger RNA accumulation of PSA and PSMA

The ability of *p,p'*-DDT and *p,p'*-DDE to displace endogenous and synthetic androgens from AR and repress androgen-inducible transcription have been documented (Kelce, et al. 1995; Kelce et al. 1997; Lemaire, et al. 2004; Maness, et al. 1998). However, the roles of these chemicals with regards to the expression of certain clinically relevant AR target genes, namely PSA (among others) is unknown. In an effort to better understand how these androgen-disrupting chemicals might interfere with the accurate determination of PSA levels, I treated LNCaP cells with R1881 alone or in

combination with different concentrations of *p,p'*-DDT and *p,p'*-DDE. I determined that both *p,p'*-DDT and *p,p'*-DDE repressed R1881-inducible PSA mRNA levels in a dose-dependent manner (**Figure 3-3A and B**). The IC_{50} was calculated to be 167 nM and 358 nM for *p,p'*-DDT and *p,p'*-DDE, respectively, using the GraphPad Prism software. In addition, nearly 100% repression was achieved at concentrations between 5 and 10 μM with both chemicals. Both *p,p'*-DDT and *p,p'*-DDE at 10 μM, significantly repressed PSA basal mRNA levels ($p<0.05$) compared to vehicle (**Figure 3-3C**). I have demonstrated for the first time that *p,p'*-DDT and *p,p'*-DDE represses R1881-inducible PSA expression in LNCaP cells.

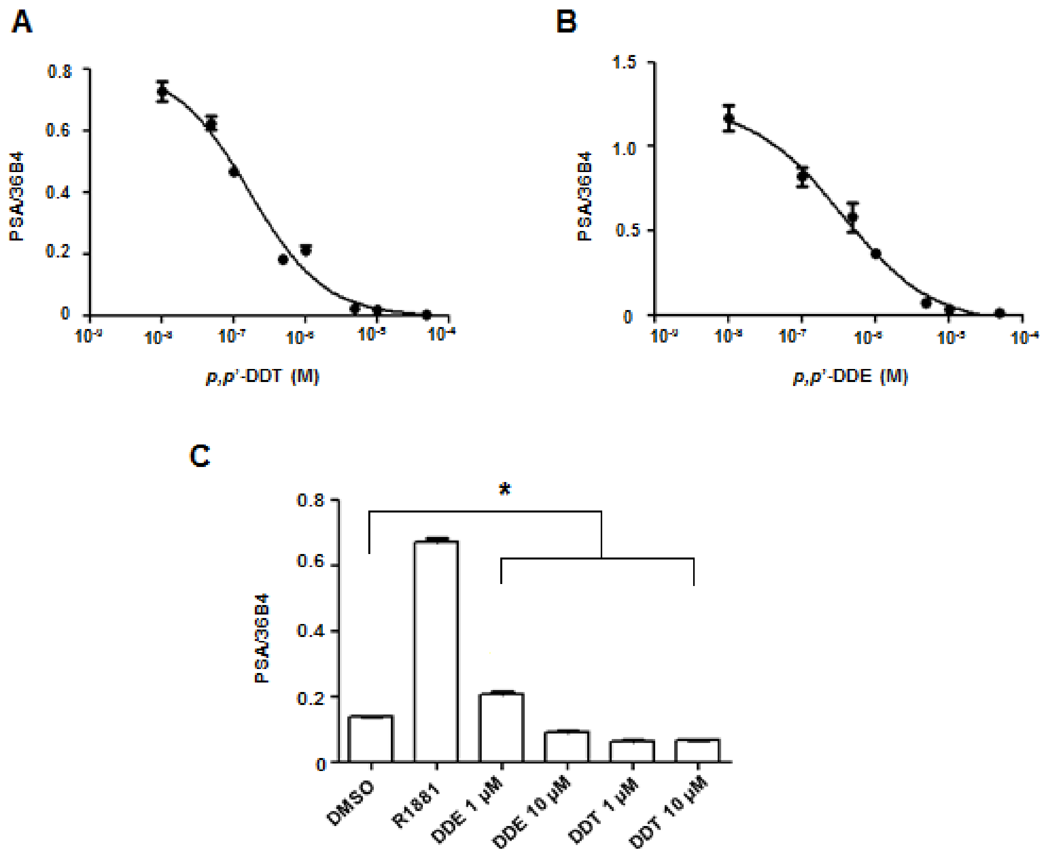


Figure 3-3. Effects of *p,p'*-DDT and *p,p'*-DDE on R1881-inducible and basal PSA mRNA accumulation.
(A,B) LNCaP cells were treated with vehicle (DMSO), R1881 at 1 nM, and combinations of R1881 with *p,p'*-DDT or *p,p'*-DDE at 10 nM, 50 nM, 100 nM, 500 nM, 1 μM, 5 μM, 10 μM and 50 μM for 24 h. (C) Cells were treated with vehicle, R1881 at 1 nM, *p,p'*-DDE and *p,p'*-DDT alone at 1 μM and 10 μM for 24 h. The mRNA levels for PSA, PSMA and 36B4 were determined by real-time PCR and normalized to constitutively expressed 36B4 gene. Data represents the average of three experiments run in triplicate and values are expressed as mean values ± SD. *p<0.05, one-way ANOVA.

In order to verify that this was a bona fide effect mediated via AR, I examined the effect of *p,p'*-DDT and *p,p'*-DDE on other AR target genes. PSMA expression is repressed by androgen-activated AR in LNCaP cells (Evans, et al. 2011; Noss, et al. 2002). Consistent with the notion that *p,p'*-DDT and *p,p'*-DDE are AR antagonists, *p,p'*-DDT and *p,p'*-DDE were able to significantly relieve the R1881-inducible repression of PSMA mRNA accumulation ($p<0.05$) compared to R1881 alone (**Figure 3-4A**). This

anti-androgenic effect was also observed by examining the R1881-inducible expression of IGF-1 (**Figure 3-4B**). Both *p,p'*-DDT and *p,p'*-DDE at 10 μM significantly repressed R1881-inducible IGF-1 expression ($p<0.05$) compared to R1881 alone. Thus, it appears that *p,p'*-DDT and *p,p'*-DDE exhibit their anti-androgenic effects directly via the AR.

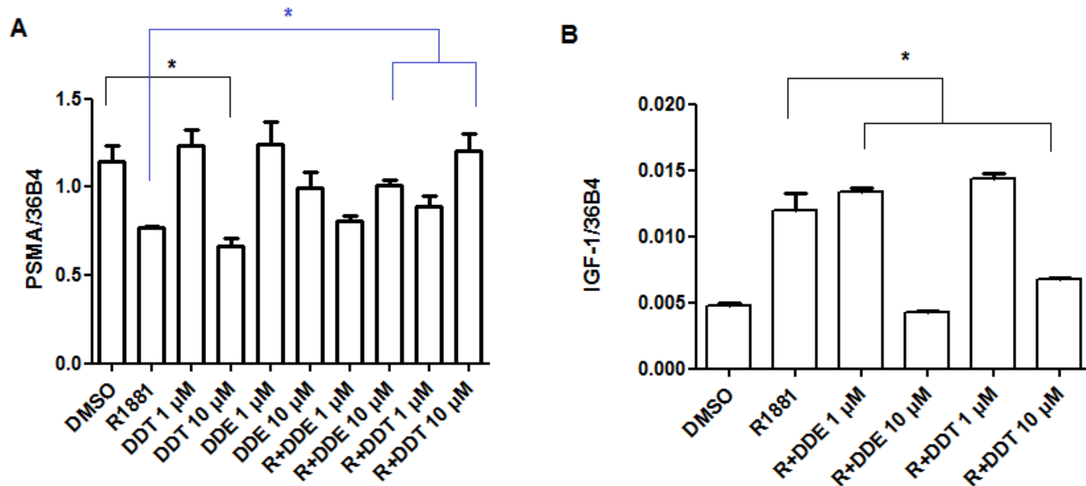


Figure 3-4. Effects of *p,p'*-DDT and *p,p'*-DDE on PSMA and IGF-1 mRNA accumulation.
(A) LNCaP cells were treated with vehicle, R1881 at 1 nM, *p,p'*-DDE and *p,p'*-DDT alone at 1 μM and 10 μM and in combination with R1881 for 24 h. (B) Cells were treated with vehicle, R1881 at 1 nM and in combination with *p,p'*-DDE and *p,p'*-DDT at 1 μM and 10 for 24 h. The mRNA levels for PSA, PSMA and IGF-1 and 36B4 were determined by real-time PCR and normalized to constitutively expressed 36B4 gene. Data represents the average of two experiments run in triplicate and values are expressed as mean values ± SD. *$p<0.05$, one-way ANOVA. R=R1881.

3.1.3. Protein accumulation of PSA, PSMA and AR

Elevated circulating PSA protein is the standard detection method for prostate cancer. Because I saw a clear dose-dependent repression of androgen-inducible PSA mRNA levels by *p,p'*-DDT and *p,p'*-DDE, I was interested in determining whether there was a concomitant change in excreted PSA protein by these cells. Thus, I determined the levels of PSA in conditioned media from LNCaP cells following treatment with either *p,p'*-DDT and *p,p'*-DDE in combination with R1881 using an ultrasensitive COBAS CORE II immuno-detection system. I determined that there was a significant concomitant decrease in R1881-inducible levels of extracellular PSA in the media of *p,p'*-DDT- and *p,p'*-DDE-treated LNCaP cells ($p<0.05$) compared to R1881 alone

(**Figure 3-5A and B**). The raw values for PSA protein concentrations following *p,p'*-DDT and *p,p'*-DDE treatments are shown in **Table 4.** Moreover, I examined intracellular PSA levels and found that intracellular PSA was also repressed when cells were treated with R1881 in combination with either p,p-DDT and *p,p'*-DDE at 10 µM, but *p,p'*-DDT appears to be more effective than *p,p'*-DDE (**Figure 3-5C**). These results confirm my observations at the transcriptional level and highlight the potential clinical relevance.

Table 4. Raw values of PSA protein concentrations (ng/mL) in LNCaP measured with the COBAS PSA detection system*.

Concentration of chemical	*p,p'*-DDT[a]	*p,p'*-DDE[a]
DMSO	1.10 ± 0.04	1.62 ± 0.14
R1881 1 nM	13.31 ± 1.46	23.06 ± 1.04
10 nM	1.31 ±0.38	0.46 ± 0.17
50 nM	0.87 ± 0.11	0.31 ± 0.08
100 nM	0.27 ± 0.11	1.21 ± 0.11
500 nM	0.32 ± 0.11	0.99 ± 0.05
1 µM	0.61 ± 0.014	1.48 ± 0.05
5 µM	0.56 ± 0.13	0.90 ± 0.15
10 µM	0.62 ± 0.08	1.15 ± 0.37
50 µM	0.49 ± 0.33	0.78 ± 0.18
R1881 + chemical 1 µM	6.19 ± 0.54	5.17 ± 0.95
R1881 + chemical 10 µM	1.56 ± 0.29	3.19 ± 0.36

* PSA was excreted from the cell and measured in conditioned media.

[a] Mean values ± SD (n=3 per treatment group)

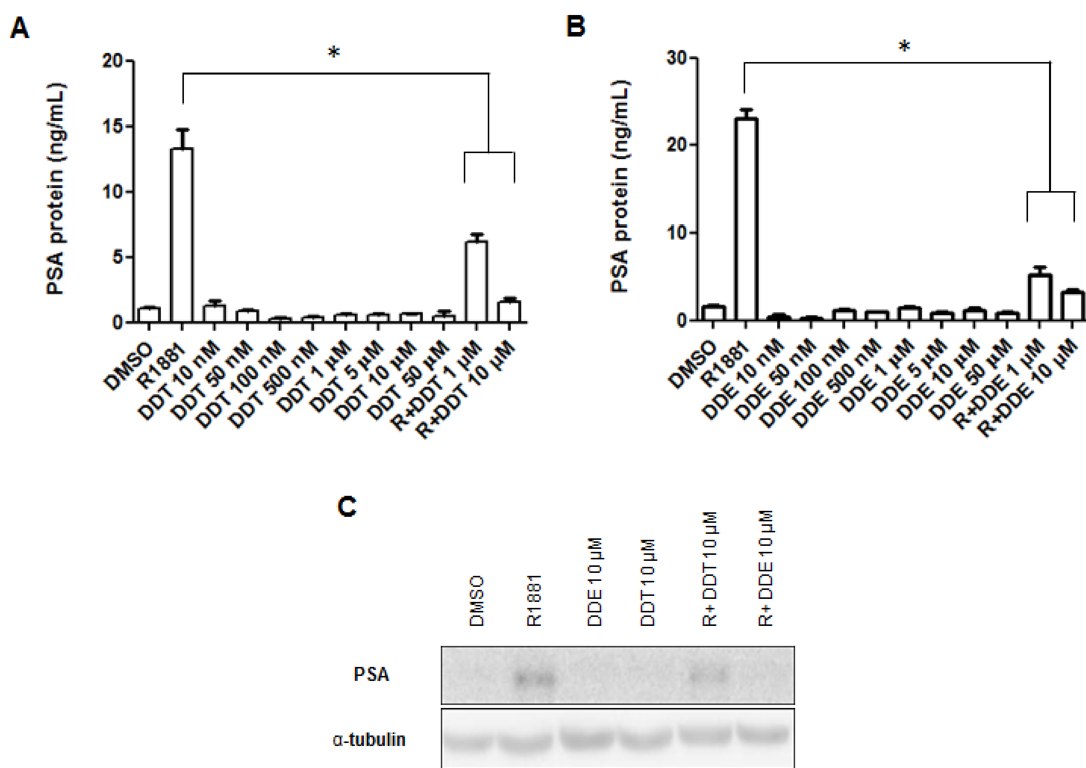


Figure 3-5. Repression of R1881-inducible PSA protein levels by *p,p'*-DDT and *p,p'*-DDE.
(A, B) LNCaP cells were treated with vehicle, R1881 at 1 nM, and *p,p'*-DDT or *p,p'*-DDE at 10 nM, 50 nM, 100 nM, 500 nM, 1 µM, 5 µM, 10 µM and 50 µM for 24 h. PSA was excreted from the cell and measured in conditioned media using the COBAS PSA detection system. Extracellular PSA protein levels are present in absolute values (in ng/mL). Data represents three observations for each experiment and values are expressed as mean values ± SD. *p<0.05, ANOVA. R=R1881 (C) Western blot analysis of intracellular levels of PSA and α-tubulin after chemical treatment for 24 h. Cells were treated with vehicle, R1881 (1 nM), *p,p'*-DDE and *p,p'*-DDT alone at 1 µM and 10 µM and in combination with R1881. Data represents one observation from one experiment.

I also examined the effect of *p,p'*-DDT and *p,p'*-DDE on AR protein levels. While it has been reported that AR mRNA accumulation is repressed by exposure to androgens, AR protein levels are stabilized in LNCaP cells (Blok, et al. 1992; Wolf, et al. 1993). Both *p,p'*-DDT and *p,p'*-DDE alone were unable to mimic R1881-inducible expression of AR. Furthermore, when LNCaP cells were treated with 10 µM *p,p'*-DDT or *p,p'*-DDE in combination with R1881, I observed significantly lower AR protein levels ($p<0.05$) compared to R1881 alone **(Figure 3-6A and B)**. For PSMA protein levels, *p,p'*-DDT at both concentrations were able to significantly relieve the R1881-inducible

PSMA repression ($p<0.05$) compared to R1881 alone (**Figure 3-6A and C**). *P,p'*-DDT and *p,p'*-DDE alone at both concentrations appear to further upregulate basal PSMA protein levels, but this upregulation was not found to be statistically significant (**Figure 3-6A and C**). Indeed, PSMA levels appeared to be rather dramatically induced by both chemicals in the absence of androgen. These results again confirm the anti-androgenic properties of *p,p'*-DDT and *p,p'*-DDE.

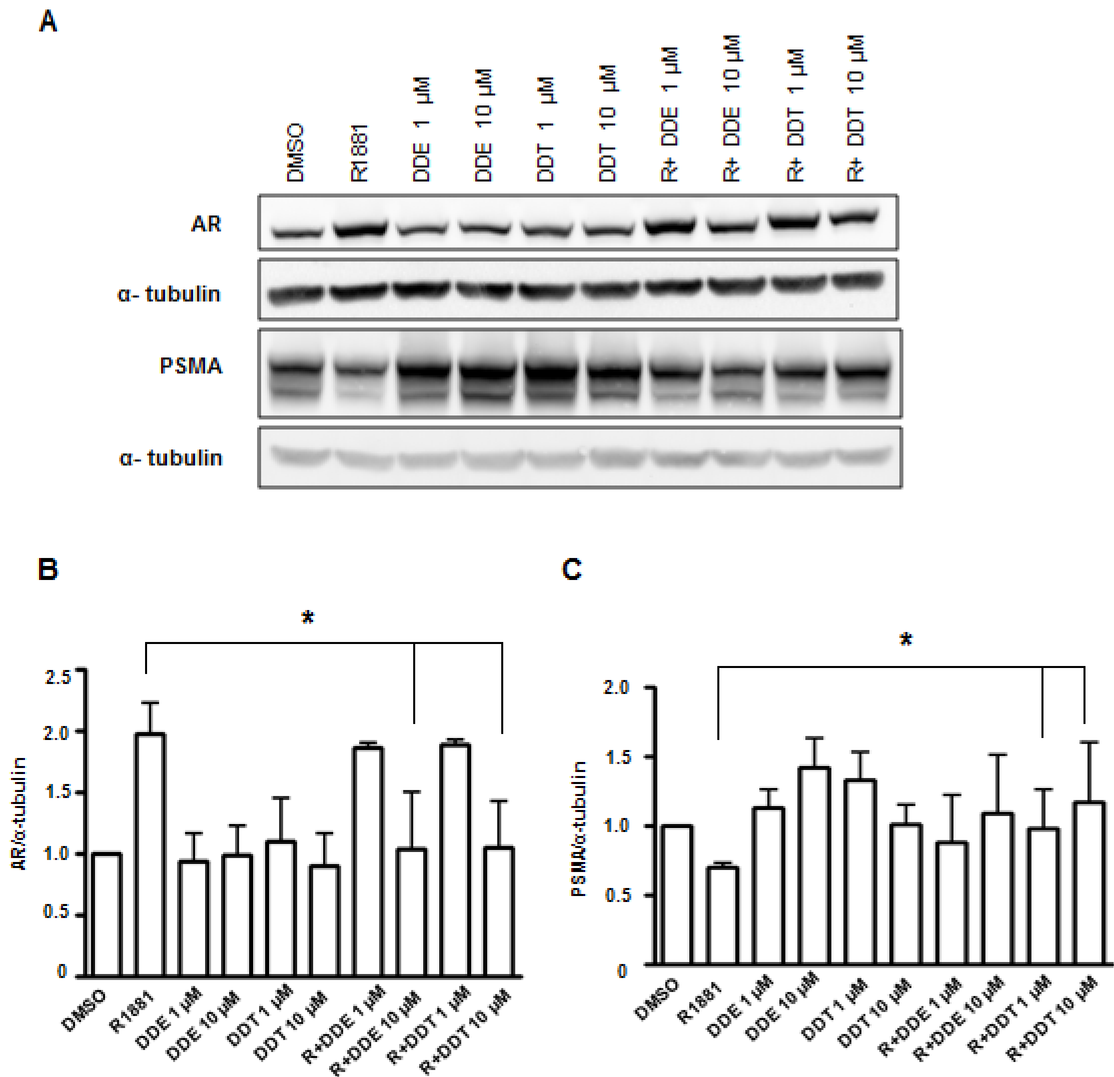


Figure 3-6. **Effects of *p,p'*-DDT and *p,p'*-DDE on AR and PSMA protein levels.** (A) Western blot of AR, PSMA and α-tubulin protein levels after chemical treatments for 24 h. LNCaP cells were treated with vehicle, R1881 at 1 nM, *p,p'*-DDE and *p,p'*-DDT alone at 1 µM and 10 µM and in combination with R1881 for 24 h. Western blotting was performed using antibodies directed against AR, PSMA and α-tubulin. (B, C) Densitometry analysis was performed on the AR and PSMA Western blots (normalized to α-tubulin). Data represents three observations for each experiment and values are expressed as mean values ± SD. *p<0.05, one-way ANOVA. R=R1881.

To determine if the concentrations used in this study are environmentally and physiologically relevant, I converted the mean values of global human tissue levels of *p,p'*-DDT and *p,p'*-DDE compiled by Jaga and Dharmani (2003) and from more recent literature spanning from 2000-2012, to molar equivalents for comparison and these results are presented in **Table 5.** The highest concentrations of *p,p'*-DDT and *p,p'*-DDE in adipose tissue were approximately 79 μM and 176 μM, respectively from the Mexican cohort of malaria control workers (Rivero-Rodriguez, et al. 1997). The highest concentrations of *p,p'*-DDT and *p,p'*-DDE in serum were approximately 1 μM and 10 μM, respectively, also in Mexico during the period of 1990-1996 (Lopez-Carrillo, et al. 2001; Romieu, et al. 2000). The concentration range of *p,p'*-DDT and *p,p'*-DDE in the adipose tissue and serum ranged from 0.051- 1.823 μM and 0.036-1.56 μM, respectively in Canada (Aronson et al. 2000). For the U.S. during the period of 1994-1997, the concentration range of *p,p'*-DDT and *p,p'*-DDE in adipose tissue was 0.031-2.24 μM (Bagga, et al. 2000; Zheng, et al. 1999) and the mean concentration of *p,p'*-DDE in serum was 5.84 μM (Hoppin, et al. 2000). Finally, in a more recent examination of levels in human serum , the highest *p,p'*-DDT concentration was 2.23 μM in China (Nakata, et al. 2005), while the highest *p,p'*-DDE concentration was 15.42 μM in Bolivia during the period of 2010-2011 (Mercado, et al. 2013). The highest concentration of *p,p'*-DDE in adipose tissue was 13.58 μM, also in China (Nakata et al. 2005) followed by 6.69-7.08 μM in Japan (Kunisue, et al. 2007), Singapore (Li, et al. 2006) and Mexico (Waliszewski et al. 2010) (**Table 6**). These values suggest that worldwide, many individuals have levels of these pesticides that are capable of repressing the cellular production of PSA.

Table 5. Global mean concentrations of *p,p'*-DDT and *p,p'*-DDE in human adipose tissue and serum during the periods of 1989-2000.*

Countries and sampling year(s)	Adipose tissue		Serum	
	p,p'-DDT (μM)	*p,p'*-DDE (μM)	*p,p'*-DDT (μM)	*p,p'*-DDE (μM)
Canada 1994-1997	-	-	0.036	1.56
Canada 1999	0.051	1.82	-	-
US 1994-1997	0.14	2.24	-	-
US 1994-1996	0.031	1.12	-	-
US 1996-1997	-	-	-	5.84
Mexico 1990-1995	-	-	0.55	10.06
Mexico 1991	10.19	28.30	-	-
Mexico 1994-1996	-	-	1.03	1.72
Mexico 1996	78.59	175.57	-	-
Mexico 1997-1998	3.10	12.27	-	-
Poland 1989-1992	1.36	16.26	-	-
Tanzania 1992	7.69	7.21	-	-
Greenland 1990-1994	0.36	9.04	-	-
Jordan 1996	1.78	7.01	-	-
Japan 1999	0.16	4.53	-	-
Germany 1990-1991	0.19	2.56	-	-
Finland 2000	0.28	1.60	-	-
Spain 1990-1991	-	8.05	-	-
Germany 1990-1991	-	3.85	-	-
Holland 1990-1991	-	3.82	-	-
Switzerland 1990-1991	-	3.52	-	-
New Ireland 1990-1991	-	2.86	-	-
Sweden 1996-1997	-	-	0.059	2.14
Egypt 1996	-	-	0.011	0.12
Brazil 1995-1996	-	-	-	2.23
Brazil 1999	-	-	-	1.15
Nicaragua 2000	-	-	-	22.93

*Adapted from Jaga and Dharmani, 2003

Table 6. Recent global mean concentrations of *p,p'*-DDT and *p,p'*-DDE in human adipose tissue and serum during the periods of 2000-2012.

Countries and sampling year(s)	Adipose tissue		Serum		References
	p,p'-DDT (μM)	*p,p'*-DDE (μM)	*p,p'*-DDT (μM)	*p,p'*-DDE (μM)	
China 2002	2.23	13.58	-	-	Nakata *et al,* 2005
Japan 2003-2004	0.14	7.08	-	-	Kunisue *et al,* 2007
Spain 2003-2004	-	0.26[a]	-	0.57[a]	Arrebola *et al,* 2013
Singapore 2003-2005	0.48	6.93	-	-	Li *et al,* 2006
Italy 2006	-	0.57[b]	-	0.36[b]	Bergonzi *et al,* 2009
Hong Kong 2006-2007	0.26	5.54	-	-	Qin YY, 2010
Czech Republic 2007	0.063	1.65	-	-	Pulkrabova *et al,* 2009
Mexico 2008	0.49[a]	6.69[a]	-	-	Walisewski *et al,* 2009
Bolivia 2010	0.10[b]	1.09[b]	-	-	Arrebola *et al,* 2012
Italy 2010	0.025	5.70	-	-	Schiavone *et al,* 2010
Belgium 2009-2012	0.058	1.04	-	-	Malarvannan *et al,* 2013
Mexico 2011-2012	0.29[a]	2.93[a]	-	-	Waliszewski *et al,* 2012
Greenland 2000	-	-	0.097[b]	3.83[b]	Cote *et al,* 2006
Hong Kong 2005	-	-	0.38	5.80	Tsang *et al,* 2011
Bolivia 2010-2011	-	-	-	15.42[a]	Mercado *et al,* 2013

[a]median, [b]geometric mean

3.1.4. Recruitment of AR to PSA promoter

Little is known concerning the molecular mechanism of the anti-androgenic actions of *p,p'*-DDT and *p,p'*-DDE, therefore we decided to employ a ChIP assay to determine whether *p,p'*-DDT and *p,p'*-DDE represses AR target genes by blocking the recruitment of AR to the PSA promoter region. There are two well characterized proximal androgen response elements (AREs) in the PSA promoter, ARE I and ARE II. The oligonucleotides that I used to amplify chromatin are directed to ARE I and ARE II (Shang, et al. 2002). LNCaP cells were treated with DMSO, 1 nM R1881, and 10 μM *p,p'*-DDE, or *p,p'*-DDT alone or in combination with R1881 for 45 min. The 10 μM concentration was chosen for *p,p'*-DDT and *p,p'*-DDE treatments as this was the

concentration that was shown to significantly repress AR protein levels. We observed an enrichment of both ARE I and ARE II promoter chromatin in samples treated with R1881 alone, however little or no signal was observed in isolates treated with *p,p'*-DDT and *p,p'*-DDE alone or in combination with R1881 suggesting that R1881-inducible recruitment of AR to the PSA promoter is blocked in the presence of *p,p'*-DDT and *p,p'*-DDE (**Figure 3-7A**). Next, subcellular fractionation was performed to determine if these chemicals could interfere with AR recruitment to the PSA 5' regulatory regions by blocking translocation of AR to the nucleus. Following an 1 h treatment, *p,p'*-DDT and *p,p'*-DDE at 10 μM were unable to block the nuclear translocation of AR (**Figure 3-7B**). However, co-treatment with *p,p'*-DDT and *p,p'*-DDE did appear to partially block the R1881-inducible depletion of cytoplasmic AR. Thus, it is unlikely that the failure of AR to bind ARE's in the PSA promoter is due to lack of nuclear accumulation of AR. Nevertheless, it seems clear that *p,p'*-DDT and *p,p'*-DDE prevents AR recruitment at the promoter regions of the PSA gene in LNCaP cells. Furthermore, this strengthens the hypothesis that *p,p'*-DDT and *p,p'*-DDE mediate their anti-androgenic effects through AR.

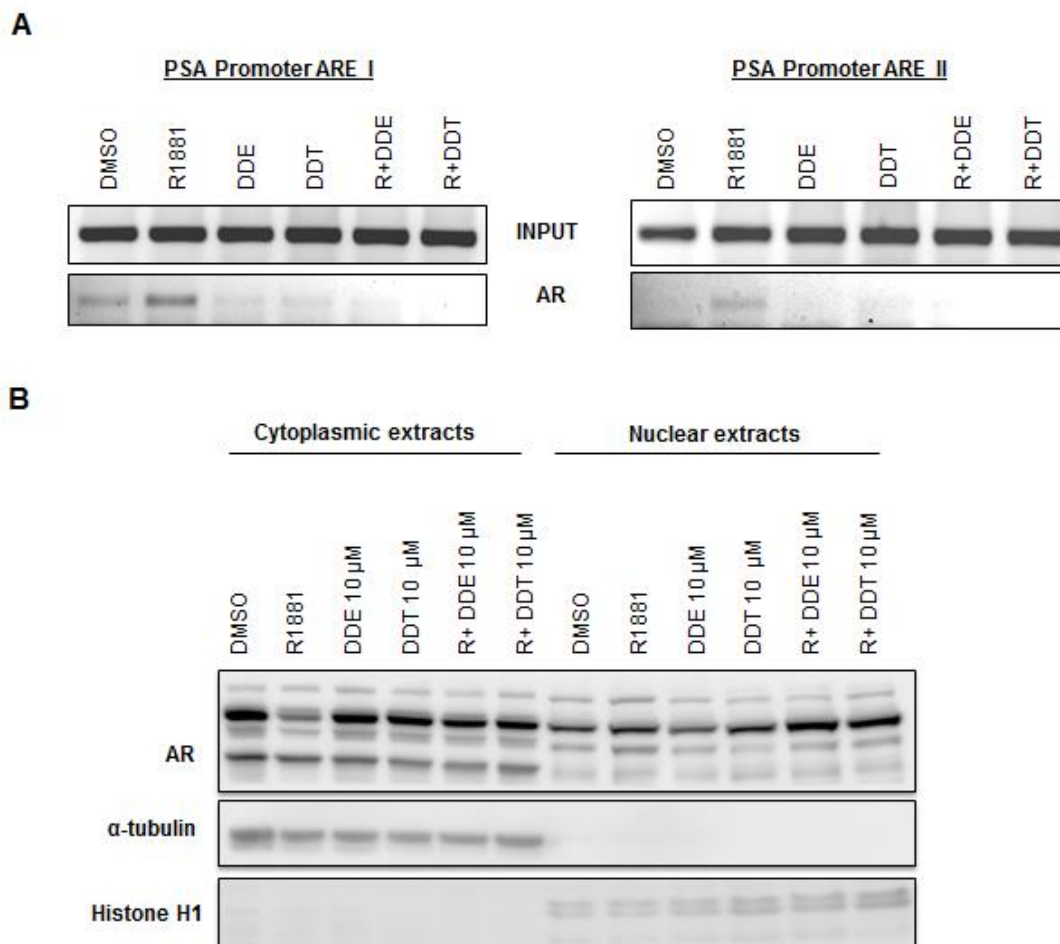


Figure 3-7. Effects of *p,p'*-DDT and *p,p'*-DDE on the R1881-inducible recruitment of AR at the PSA gene.
(A) Chromatin immunoprecipitation assays of PSA promoter regions in LNCaP cells using antibodies targeting AR. Cells were treated with vehicle, R1881 at 1 nM, *p,p'*-DDE and *p,p'*-DDT alone at 1 µM and 10 µM and in combination with R1881 for 45 min. (B) Western blotting of fractionated cytoplasmic and nuclear LNCaP cell lysates following treatment with vehicle, R1881 at 1 nM, *p,p'*-DDE and *p,p'*-DDT alone at 1 µM and 10 µM and in combination with R1881 for 1 h was performed using antibodies directed against AR, α-tubulin (cytoplasmic marker) and histone H1 (nuclear marker). Data represents one observation for each experiment.

3.1.5. Effect of MG132 on AR and PSA protein levels

Next, I decided to investigate whether proteasome-mediated protein degradation can serve as another potential target for EDCs. In this system, proteins are tagged with

ubiquitin in order to be recognized by the 26S proteasome. MG132, which is a 26S proteasome inhibitor, has been shown to increase endogenous AR protein levels in both LNCaP and HepG2 cell lines (Sheflin *et al,* 2000). Moreover, MG132 was shown to repress AR transcription (in an androgen-dependent manner) by either blocking the nuclear translocation of AR or by blocking the binding of AR to its transcriptional coregulators, in LNCaP cells (Lin *et al,* 2002). Lin et al (2002) also showed that MG132 can suppress the expression of the androgen-regulated PSA gene. Based on this evidence, I decided to determine whether *p,p'*-DDT and/or *p,p'*-DDE can affect 26S proteasome activity and AR turnover. Following MG132 treatment for 24 h, I observed an accumulation of two AR protein products formed prior to 26S proteasome processing, in the absence of androgens (**Figure 3-8**). The first band is at 110kDa, which is the molecular weight of the full length AR and the second band is approximately between 80-90kDa (**Figure 3-8,** molecular weight markers are not shown). Interestingly, in the presence of MG132 and R1881, there is less accumulation of the smaller AR protein product (short AR isoform) (**Figure 3-8**). In the presence of R1881, *p,p'*-DDT and/or *p,p'*-DDE do not appear to affect 26S proteasome activity as there is no accumulation of the smaller AR protein product (**Figure 3-8**). I also wanted to determine whether stabilization of the 80-90 kDa AR isoform is linked to increased AR transcriptional activity by probing the membrane for PSA. However, the Western blot indicated that MG132 prevented R1881-inducible PSA expression (**Figure 3-8**). Collectively, these results led us to investigate the potential role of this short AR isoform in CRPC and whether this AR isoform could potentially be an AR splice variant. AR splice variants, which are truncated forms of the AR, have been shown to be the one of the underlying causes of sustained AR signalling in CRPC (Gillis *et al,* 2013).

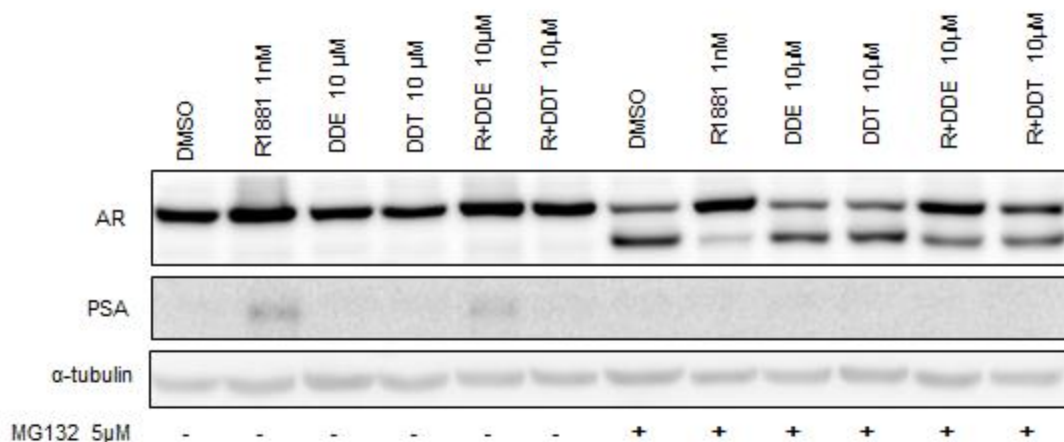


Figure 3-8. Effects of MG132 on AR and PSA protein levels following *p,p'*-DDT and *p,p'*-DDE treatment.
LNCaP cells were treated with vehicle, R1881 at 1 nM, *p,p'*-DDE and *p,p'*-DDT alone at 1 µM and 10 µM and in combination with R1881 in the absence and presence of 5 µM MG132. Western blotting was performed using primary antibodies directed against AR, PSA and α-tubulin.

3.1.6. Determination of AR turnover using MG132

Because MG132 led to the accumulation of a smaller ~80-90 kDa AR isoform, I was interested in determining when this short AR isoform will appear in LNCaP cells. This was accomplished by performing a 7 h MG132 time course treatment to determine AR turnover. The Western blot shows that the short AR isoform starts to accumulate at 3 h, which indicates a 3 h period for AR to turnover (**Figure 3-9**). I also observed that the short AR isoform gradually accumulated between 3 to 7 h (**Figure 3-9**).

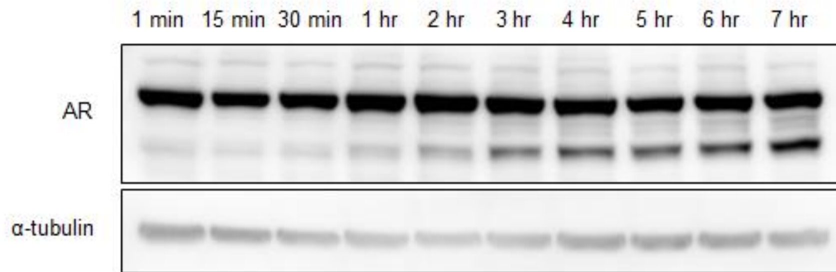


Figure 3-9. Determination of AR turnover in a 7 h MG132 time course treatment. LNCaP cells were treated with 5 μM at 7 h, 6 h, 5 h, 4 h, 3 h, 2 h, 1 h, 30 min, 15 min and 1 min. Cells were harvested for protein immediately after the 1 min treatment. Western blotting was performed using antibodies directed against AR and α-tubulin.

3.1.7. Structural determination of the enriched AR isoform upon 26S proteasome inhibition

Lastly, I wanted to determine the structure of the enriched short AR isoform following 26S proteasome inhibition. LNCaP cells were treated with 5 μM of MG132 at 7 h and 1 min and compared to the 22Rv1 cells, which is a cell line derived from CRPC. Western blotting was performed by probing the membrane with primary antibodies directed to the full length of AR, C-terminus of AR (AR C-19) and N-terminus of AR (AR N-20). The Western blot showed that following MG132 treatment for 7 h, there is an accumulation of the short AR isoform in LNCaP cells. These results appear to be a phenocopy of the 22Rv1 cells as these two short AR isoforms are also present in this cell line (**Figure 3-10 left lane**). Furthermore, when probing for the C-terminus of AR, the short AR isoform is absent (highlighted by the box), which indicates that the short AR isoform does not contain the C-terminus (**Figure 3-10 middle lane**). When probing for the N-terminus of AR, the short AR isoform is present (highlighted by the box), which indicates that this short AR isoform contains the N-terminus (**Figure 3-10 right lane**). However, the N-terminus of the short AR isoform appears to be less pronounced compared to the short AR isoform in 22Rv1 cells. Collectively, these results show that this short AR isoform contains the NTD but lacks the LBD.

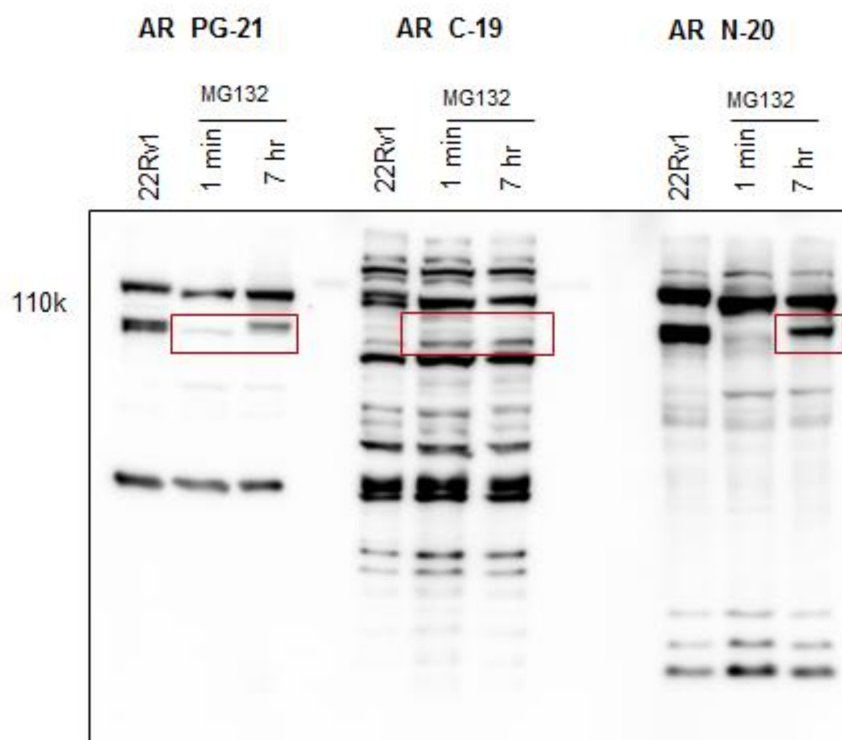


Figure 3-10. Structural determination of the AR isoform upon 26S proteasome inhibition.
LNCaP cells were treated with 5 µM of MG132 at 7 h and 1 min. Cells were immediately harvested for proteins after the 1 min treatment. Untreated 22Rv1 cell lysates were harvested for proteins. Western blotting was performed using primary antibodies directed against AR PG-21 (full length), AR C-19 (C-terminus) and AR N-20 (N-terminus).

3.1.8. Determination of whether the enriched AR isoform is cleaved or is a product of *de novo* transcription

In collaboration with Mark Labrecque from the Beischlag Lab, we investigated to determine whether this enriched short AR isoform is cleaved or is a product of *de novo* transcription. First, we wanted to determine whether this short AR isoform is cleaved as a result of protein degradation. LNCaP cells were treated with vehicle or MG132 with or without the protease inhibitor for 24 h. Western blotting was performed by probing the membrane with primary antibody directed to the full length AR (AR PG-21). The results show that the addition of protease inhibitor (P.I.) with MG132, failed to prevent the accumulation of the short AR isoform (abbreviated as AR_s) (**Figure 3-11A**). This indicates that this short AR isoform is not the result of protein degradation of the full

length AR (abbreviated as AR_L). Next, we wanted to determine whether this short AR isoform is a product of *de novo* transcription of a splice variant using actinomycin-D, a transcription inhibitor. LNCaP cells were treated with vehicle or MG132 with or without actinomycin-D in the absence and presence of R1881 for 24 h. Again, Western blotting was performed by probing the membrane with primary antibody directed to the full length AR (AR PG-21). The results show that co-treatment of actinomycin-D with MG132 prevents the accumulation of the short AR isoform and this was not overcome by the addition of R1881. Collectively, these results clearly demonstrate that the short AR isoform is a product of *de novo* transcription of a splice variant.

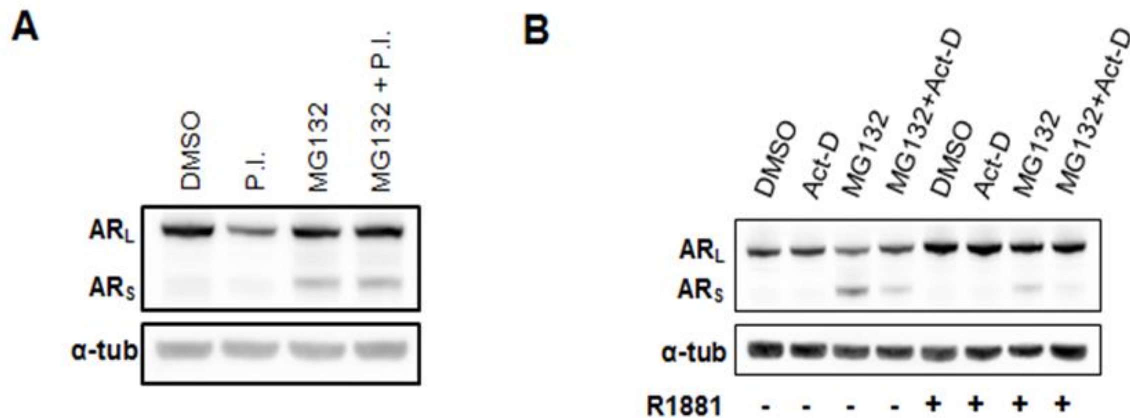


Figure 3-11. Appearance of the short AR isoform is the result of the accumulation of protein resulting from *de novo* transcription.
(A) LNCaP cells were treated with vehicle or MG132 (5 μM) with or without protease inhibitor (P.I.) and harvested after 24 h. (B) LNCaP cells were treated with vehicle or MG132 (5 μM) with or without actinomycin-D or with R1881 (10 nM) and harvested after 24 h. Whole cell lysates were examined by Western blot analysis using AR PG-21 and α-tubulin.

3.2. TBECH

3.2.1. Messenger RNA accumulation of PSA and PSMA

Two studies have demonstrated that TBECH is a potent hAR agonist, with the δ/γ-TBECH mixture being the more potent agonist (Larsson *et al,* 2006; Khalaf *et al,* 2009). However, the combination effects of androgens and TBECH on AR target genes have yet to be investigated. Here, I have shown that δ/γ-TBECH alone at 1 and 10 μM,

significantly induced PSA basal mRNA levels ($p<0.05$) (**Figure 3-11A**). Furthermore, in the presence of R1881, both TBECH diastereomers at 10 μM, significantly induced PSA mRNA levels ($p<0.05$) (**Figure 3-11A**). However, in the presence of R1881, α/ß- and δ/γ-TBECH at 1 μM significantly reduced R1881-inducible PSA mRNA levels ($p<0.05$) (**Figure 3-11A**). These results suggest that δ/γ-TBECH alone is a more potent hAR agonist compared to α/ß-TBECH alone in LNCaP cells. Furthermore, the results suggest that α/ß-TBECH at 10 μM, appears to be the more potent hAR agonist in R1881-inducible PSA mRNA expression in LNCaP cells. In terms of another AR target gene, α/ß-TBECH alone at 1 μM significantly induced PSMA basal mRNA levels ($p<0.05$) whereas δ/γ-TBECH alone at 10 μM significantly repressed PSMA basal mRNA levels ($p<0.05$) (**Figure 3-12B**). In the presence of R1881, α/ß-TBECH at 1 μM and δ/γ-TBECH at 10 μM significantly relieved the R1881-inducible repression of PSMA mRNA levels ($p<0.05$) (**Figure 3-12B**). On the other hand, in the presence of R1881, δ/γ-TBECH at 1 μM continued to significantly repress R1881-inducible repression of PSMA mRNA levels ($p<0.05$) (**Figure 3-12B**). Collectively, these results suggest that TBECH diastereomers can act as partial agonists to the hAR in LNCaP cells.

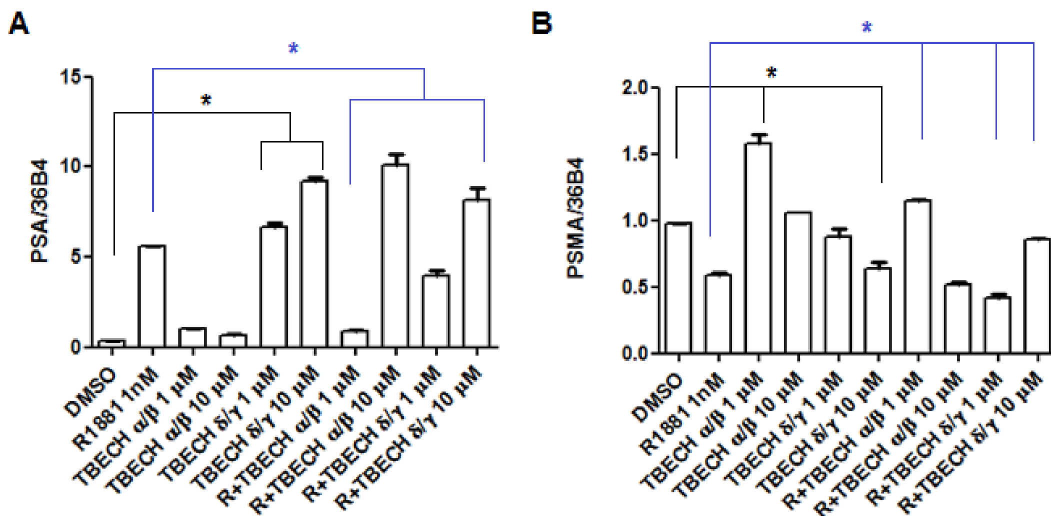


Figure 3-12. Effects of TBECH on PSA and PSMA mRNA accumulation.
(A, B) LNCaP cells were treated with vehicle, R1881 at 1 nM, α/ß -TBECH and δ/γ -TBECH alone at 1 μM and 10 μM and in combination with R1881 for 24 h. The mRNA levels for PSA, PSMA and 36B4 were determined by real-time PCR and normalized to constitutively expressed 36B4 gene. Data represents the average of three experiments run in triplicate and values are expressed as mean values ± SD. *$p<0.05$, one-way ANOVA. R=R1881.

3.2.2. Protein accumulation of PSA, PSMA and AR

A previous study has demonstrated that TBECH diastereomers activated AR in a dose-dependent manner in HepG2 cells (Khalaf *et al,* 2009). Furthermore, the same study showed that both α/ß- and δ/γ-TBECH at 100 nM and 1 μM, alone significantly induced basal PSA protein levels in LNCaP cells. Because I observed that TBECH can induce PSA mRNA levels, I was interested in determining whether there was a concomitant change in excreted PSA protein by these cells. Thus, I determined the levels of PSA in conditioned media from LNCaP cells following treatment with either α/ß-TBECH and δ/γ-TBECH in combination with R1881 using an ultrasensitive COBAS CORE II immuno-detection system. In the presence of R1881, α/ß-TBECH at 1 and 10 μM significantly repressed R1881-inducible extracellular PSA protein levels ($p<0.05$) (**Figure 3-12A**). On the other hand, δ/γ-TBECH alone at 10 and 50 μM significantly induced extracellular basal PSA protein levels ($p<0.05$) (**Figure 3-12B**). These results confirm that the δ/γ-TBECH mixtures alone, are more potent hAR agonists compared to the α/ß-TBECH mixtures alone. Moreover, in the presence of R1881, δ/γ-TBECH at 1 μM significantly repressed R1881-inducible extracellular PSA protein levels ($p<0.05$) (**Figure 3-12B**). The raw values for extracellular PSA protein concentrations following α/ß-TBECH and δ/γ-TBECH treatments are shown in **Table 7.** These results suggest that TBECH may act as an antagonist in the presence of androgens.

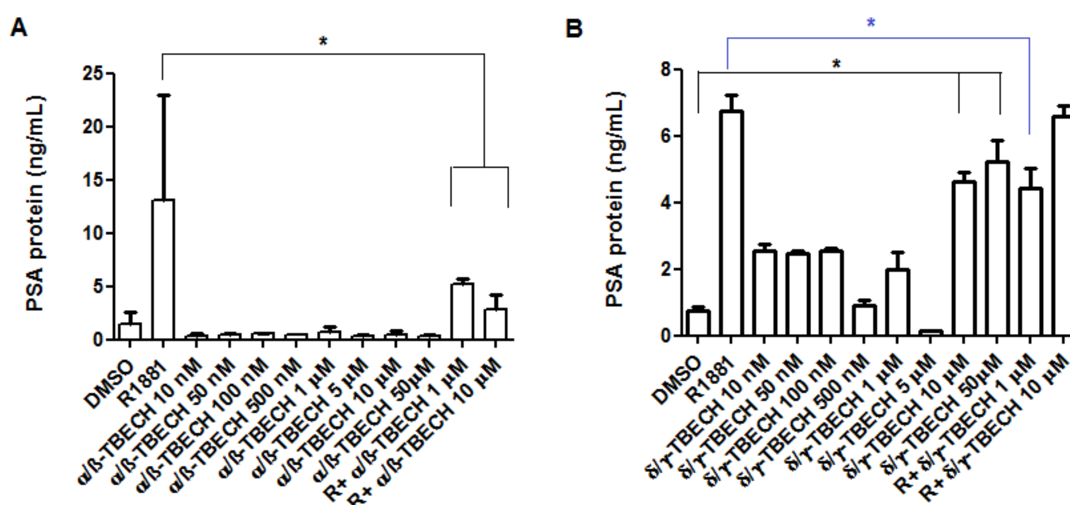


Figure 3-13. Effects of TBECH on R1881-inducible PSA protein levels.
(A, B) LNCaP cells were treated with vehicle, R1881 at 1 nM, and α/ß -TBECH or δ/γ –TBECH at 10 nM, 50 nM, 100 nM, 500 nM, 1 µM, 5 µM, 10 µM and 50 µM for 24 h. PSA was excreted from the cell and measured in conditioned media using the COBAS PSA detection system. Extracellular PSA protein levels are present in absolute values (in ng/mL). Data represents three observations for each experiment, except for the DMSO and R1881 treatment groups in α/ß -TBECH experiment, which represents only two observations. Values are expressed as mean values ± SD. *p<=0.05, one-way ANOVA. R=R1881.

Table 7. Raw values of PSA protein concentrations (ng/mL) in LNCaP measured with the COBAS PSA detection system*.

Concentration of chemical	α/ß-TBECH[a]	δ/γ-TBECH[a]
DMSO	1.46 ± 1.12[b]	0.75 ± 0.21
R1881 1 nM	13.12 ± 9.90[b]	6.75 ± 0.86
10 nM	0.31 ± 0.24	2.56 ± 0.35
50 nM	0.50 ± 0.08	2.47 ± 0.12
100 nM	0.58 ± 0.02	2.55 ± 0.10
500 nM	0.41 ± 0.005	0.92 ± 0.26
1 µM	0.72 ± 0.47	1.97 ± 0.90
5 µM	0.38 ± 0.04	0.15 ± 0.007
10 µM	0.52 ± 0.26	4.63 ± 0.51
50 µM	0.38 ± 0.06	5.21 ± 1.13
R1881 + chemical 1 µM	5.16 ± 0.51	4.44 ± 1.03
R1881 + chemical 10 µM	2.89 ± 1.37	6.60 ± 0.56

* PSA was excreted from the cell and measured in conditioned media.
[a] Mean values ± SD (n=3 per treatment group)

[b] Mean values ± SD (n=2 per treatment group)

Furthermore, I have demonstrated that TBECH also has an effect on the intracellular protein levels of two AR target genes: AR and PSMA. The δ/γ-TBECH alone at 1 and 10 μM induced basal AR protein levels whereas the α/ß-TBECH did not (**Figure 3-13**). In the presence of R1881, it appears that both TBECH mixtures (except for α/ß-TBECH at 1 μM) slightly repressed R1881-inducible AR protein levels (**Figure 3-13**). In terms of PSMA, I wanted to determine whether the changes observed at the mRNA level were concomitant at the protein level. Both TBECH mixtures alone at 1 and 10 μM appear to repress basal protein levels (**Figure 3-13**). In the presence of R1881, it appears that α/ß-TBECH at 1 μM slightly relieved the R1881-inducible repression of PSMA protein levels (**Figure 3-13**).

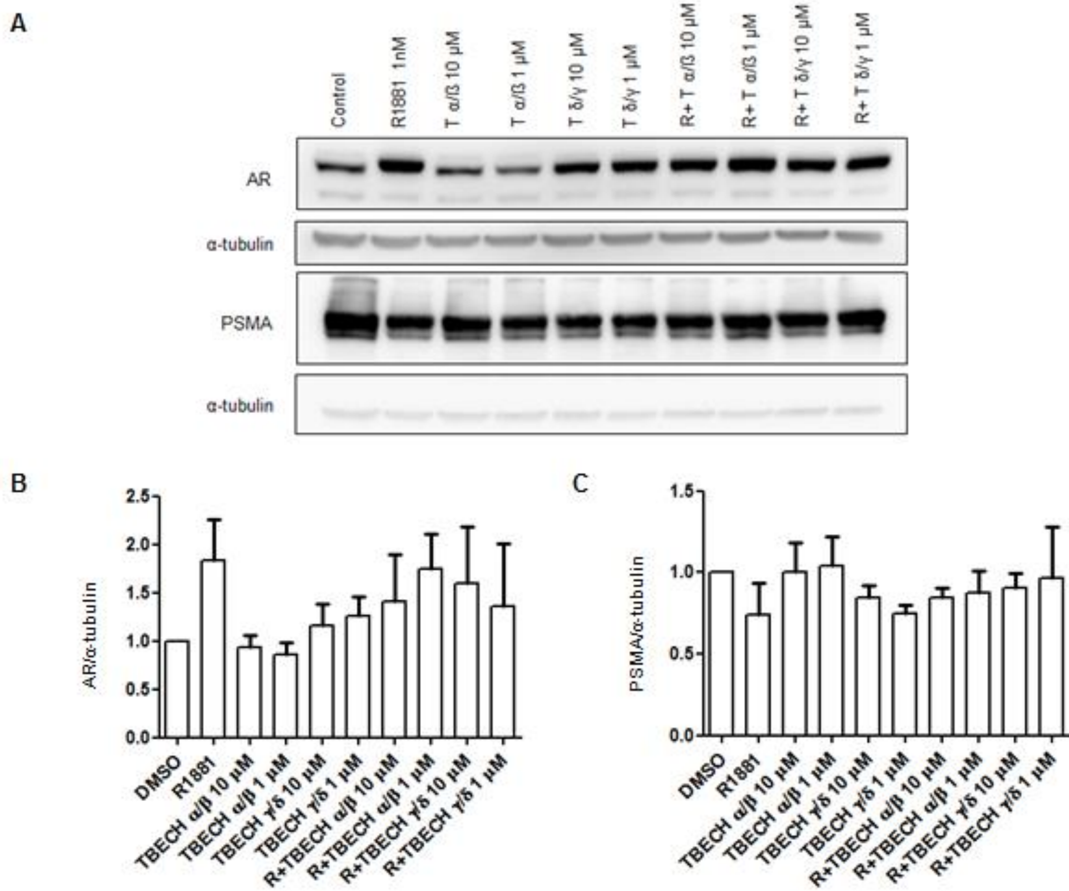


Figure 3-14. Effects of TBECH on AR and PSMA protein levels.
(A) Western blot of AR, PSMA and α-tubulin protein levels after chemical treatments for 24 h. LNCaP cells were treated with vehicle, R1881 at 1 nM, α/ß-TBECH or δ/γ-TBECH alone at 1 μM and 10 μM and in combination with R1881 for 24 h. Western blotting was performed using antibodies directed against AR, PSMA and α-tubulin. (B, C) Densitometry analysis was performed on the AR and PSMA Western blots (normalized to α-tubulin). AR data represents two observations for each experiment and PSMA data represents three observations for each experiment. Values are expressed as mean values ± SD. Treatment groups were not found to be statistically significant.

Chapter 4. Discussion

4.1. New insight on the molecular mechanism by which *p,p'*-DDT and *p,p'*-DDE exert its anti-androgenic effects

Due to the extensive global use of DDT in the past and its tendency to persist in the environment and bioaccumulate in biota, it is expected that almost every human has been exposed to DDT in their lifetime (Turusov *et al,* 2002). Even though this insecticide was subsequently restricted in many countries from the 1970s to 2000, humans are still continually exposed to DDT primarily through diet. The use of DDT was heavily restricted in the U.S. and Canada during the early 1970s. In this study, we were interested in determining the effects of the persistent organic pollutants, *p,p'*-DDT and *p,p'*-DDE on AR target gene and protein expression in the human LNCaP prostate cancer cell line. My experimental evidence demonstrates that *p,p'*-DDT and *p,p'*-DDE can repress R1881-inducible PSA mRNA and protein levels in these cells. Previous studies by Kelce and colleagues have shown that *p,p'*-DDE is a potent AR antagonist using a competitive ligand binding assay that can repress AR gene transcription in monkey kidney CV-1 cells transiently co-transfected with the human AR expression vector and a mouse mammary tumor virus promoter-luciferase reporter vector (Kelce et al. 1997). In a subsequent study, Kelce et al (1997) again showed that *p,p'*-DDE can alter two AR target genes, prostate specific binding protein and testosterone-repressed prostatic message-2 in rat ventral prostates. They also demonstrated via immuno-histochemical analysis that *p,p'*-DDE can reduce the nuclear localization of AR in rat epididymal cells, but suggested that this reduction of AR in the nucleus is not due to the blocking of the translocation of the AR-ligand bound complex to the nucleus but may be due to the increased degradation of the complex in the cytoplasm (Kelce et al. 1997). However, I have demonstrated that *p,p'*-DDT and *p,p'*-DDE at 10 µM can partially block the R1881-inducible depletion of cytoplasmic AR, but can still lead to the accumulation of nuclear AR (**Figure 3-7B**). Overall, my results suggest that *p,p'*-DDT and *p,p'*-DDE likely exert their transcriptional anti-androgenic actions by blocking the binding of AR to

the PSA promoter. This is evidenced by repression of R1881-inducible enrichment of PSA promoter chromatin after immuno-precipitation with antibodies directed against AR (**Figure 3-7A**). These chemicals may exert their actions by inducing a conformational change to the AR-ligand complex. Such a change could result in the redirection of AR away from chromatin or by inhibiting interactions between AR and its co-regulators, thereby altering the affinity of AR for its cognate response elements in the PSA gene resulting in decreased transcription.

The LNCaP cell line was derived from a patient with metastatic prostate cancer. This cell line harbors a point mutation in the LBD of AR, located at amino acid 877. This mutation results in alanine being substituted for threonine (T877A) (Veldscholte *et al,* 1992). In addition to binding androgens, this mutated AR can also bind to estrogens, progesterones and anti-androgens (Veldscholte *et al,* 1992). The lack of estrogenic activity in *p,p'*-DDT and *p,p'*-DDE was confirmed in the ECC-1 cell line, which harbors ER and wild-type AR.

PSMA is a type II transmembrane glycoprotein that is expressed in all types of prostate tissue including normal and benign, and is highly expressed in advanced and metastatic prostate cancers (Silver *et al,* 1997; Jemaa *et al,* 2010). Its function as a glutamate carboxypeptidase in prostate cancer still remains unclear. PSMA expression has been correlated with recurrent prostate cancer (Ross *et al,* 2003) and is expressed in tumor vasculature in solid tumors (Silver *et al,* 1997). Therefore, PSMA can serve as a useful imaging biomarker for monitoring prostate cancer progression and recurrence of the disease (Mease *et al,* 2013). Even though PSMA is a bona fide AR-target gene and is normally up-regulated by androgens in prostate cancer, PSMA has been shown to be an androgen-repressed gene in several prostate cancer cell lines, including the LNCaP cells (Evans *et al,* 2011; Noss *et al,* 2002). My results show that in the absence of R1881 (vehicle treatment), PSMA protein expression is upregulated compared to in the presence of R1881, which is consistent with the findings from Wright et al (1996). Moreover, *p,p'*-DDT and *p,p'*-DDE treatments alone appears to further up-regulate basal PSMA protein expression in LNCaP cells. My results also show that *p,p'*-DDT and *p,p'*-DDE can relieve the R1881-inducible PSMA repression, which is consistent with reports that anti-androgens can up-regulate PSMA expression (Evans et al. 2011) and support

the hypothesis that these chemicals act as anti-androgens directly targeting AR. Several studies have shown that PSMA expression is up-regulated following androgen-ablation therapy in prostate cancer patients (Wright *et al,* 1996; Kawakami *et al,* 1997). Following androgen-ablation therapy, there is very low circulating levels of testosterone in the patient (Gomella L, 2009). As PSMA is used as a imaging biomarker for prostate cancer, anti-androgens like *p,p'*-DDT and *p,p'*-DDE could potentially result in false-positive imaging results.

4.2. Global concentrations of *p,p'*-DDT and *p,p'*-DDE in human tissues and its effect on prostate cancer diagnosis

The mean concentrations of *p,p'*-DDT and *p,p'*-DDE measured in the majority of human adipose tissue samples and serum collected from different countries around the world, were found to be several orders of magnitude higher than the concentrations that we have shown to repress androgen-inducible PSA mRNA and protein expression (**Tables 5 and 6**). Serum contains approximately 1% fat (Smith AG, 2001) and may serve as a biomarker for previous exposure as these chemicals are highly lipid soluble. The highest concentrations in Jaga and Dharmani (2003) were found in the adipose tissue as human adipose tissue contains 65% fat (Smith AG, 2001). Compared to our estimated IC_{50}, the mean adipose concentrations in the global population are 1.12 to 470 times greater for *p,p'*-DDT (excluding Canada, U.S. and Japan) and 4.5 to 482 times higher for *p,p'*-DDE. For the recent compilation of human adipose tissue and serum levels, we can see a trend for higher levels of *p,p'*-DDE and lower levels of *p,p'*-DDT in human tissues (**Table 6**). Although levels of DDT in humans are gradually declining globally, the levels of *p,p'*-DDT and *p,p'*-DDE in the majority of adipose tissue from both the global study and the recent compilation of exposure data were in the 1-15 μM range. These body burden levels show that *p,p'*-DDT and *p,p'*-DDE still persist in the environment a decade later. Given the long half-life of these chemicals and the fact that DDE accumulates in adipose tissue with age (Smith AG, 2001), levels in exposed individuals could still likely be well within a concentration range that could repress PSA levels.

Prostate cancer is one of the leading causes of cancer deaths in men in Western countries. The ability of these chemicals to repress PSA at both the mRNA and protein level could have a profound effect on prostate cancer diagnosis. Exposure to *p,p'*-DDT and *p,p'*-DDE in combination with R1881 can result in a 2-8.5 times further decrease in PSA protein levels compared with just R1881 treatment alone (**Table 4**). These PSA protein levels were measured with the COBAS PSA detection system; the same system that is used to measure PSA protein levels in patients across North America. Thus, significant repression of PSA levels by these chemicals could mask the existence of a pathological state resulting in a false negative prostate cancer screen.

Furthermore, global repression of androgen signalling would mimic androgen ablation and, therefore could create a favourable selective pressure for the transformation to an androgen-resistant and more lethal cancer. Indeed, our results highlight the need for an alternative testing method for prostate cancer. Pesticide exposure has been linked to higher risk of prostate cancer in agricultural workers or farmers (Alavanja, et al. 2003; Band, et al. 2011; Koutros, et al. 2013; Meyer, et al. 2007; Settimi, et al. 2003). Some studies have found a positive association between DDT exposure and increased risk of prostate cancer in agricultural workers based on case-control studies in British Columbia, Canada (OR=1.68; 95% CI: 1.04-2.70 for high exposure) (Band et al. 2011); in Italy (OR=2.1, 95% CI=1.2-3.8) (Settimi et al. 2003), and in an Agricultural Health study cohort in Iowa and California in the US (combined OR=1.14-1.38, CI=0.59-2.21 for four different exposure scenarios) (Alavanja et al. 2003). Furthermore, another study found a positive association between an increased risk of prostate cancer and *p,p'*-DDE levels in adipose tissues of prostate cancer patients in Sweden (OR=2.30, CI=0.77-6.85 with a mean *p,p'*-DDE concentration of 654 ng/g of lipid) (Hardell et al. 2006). Our results indicate it is likely that some of this increased risk may be due to lack of early detection associated with physiologically relevant concentrations of *p,p'*-DDT and *p,p'*-DDE that would mask the presence of prostate cancer in a clinical screen by repressing PSA production. More worrisome is that given the efforts to reduce screening to relieve overtreatment burden, we speculate that as anti-androgens, these agents force cancers to progress to a therapeutically refractile disease state. Thus, these data provide yet another facet to the already complex issue of ongoing use of DDT for public health, particularly malaria control (Attaran, et al. 2000).

It also argues for the need for continued monitoring of DDT exposure in areas of ongoing use, particularly given that use for malaria occurs indoors. Also evident is the need for a more sensitive and sophisticated method for prostate cancer detection.

4.3. Loss of 26S proteasome function, putative AR splice variant, and its role in castration-resistant prostate cancer

CRPC is characterized by constitutively active AR signalling in the absence of androgens (Decker *et al,* 2012). The underlying causes of CRPC development involves several mechanisms including AR gene amplification and protein overexpression, AR increased sensitivity, AR gene mutations resulting in promiscuous ligand binding and ligand-independent AR activation (Feldman and Feldman, 2001). Recently, a novel mechanism underlying constitutively active AR signalling in CRPC was discovered. This new mechanism involves AR splice variants, which are truncated forms of the AR that are partially or completely lacking the LBD (Dehm *et al,* 2008; Dehm and Tindall, 2011). The loss of the LBD results in the constitutive activation of AR in the absence of androgens (Dehm *et al,* 2008). Studies have shown that the NTD of the AR gene has potent transcriptional activity by itself (Dehm *et al,* 2007). The formation of these AR splice variants are due to abberant alternative RNA splicing or structural rearrangements of the hAR gene (Sun *et al,* 2010; Li *et al,* 2011).

The 22Rv1 cells were first discovered to express two AR proteins with molecular weights of 110kDa and a 75-80kDa (Tepper *et al,* 2002). The lower molecular weight protein was initially thought to be a proteolytic cleavage product of the full-length AR protein, but RNA interference studies determined that this smaller AR protein product was a separate protein species arising from alternative RNA splicing (Dehm *et al,* 2008). Cloning and sequencing studies revealed that this smaller AR protein product consist of a novel 17-bp sequence (known as exon 2b) spliced to either exon 2 or 3 in its mRNA sequence (Dehm *et al,* 2008). Antibody mapping studies showed that this smaller AR protein species consists of a truncated carboxy-terminal extension, which is encoded by exon 2b (Dehm *et al,* 2008). Further studies showed that this second AR protein species was constitutively active and was responsible for the androgen-independent

cellular proliferation in 22Rv1 cells (Dehm *et al,* 2008). My results showed that in the absence of R1881, a short AR isoform appears when the 26S proteasome is blocked by MG132 in LNCaP cells (**Figure 3-8 and 3-10 left lane**). This appears to be similar to the two AR protein species in 22Rv1 cells (**Figure 3-10 left lane**). It appears that R1881 blocks the accumulation of this short AR isoform which is consistent with AR's sequestration in the nuclei. The Western blot results also showed that the AR isoform contains the NTD, but lacks the LBD which matches the description of AR splice variants (**Figure 3-10 middle and right lanes**). This result suggests that loss of 26S proteasome function can lead to the accumulation of the AR isoform which mimics CRPC. To confirm whether this AR isoform is a splice variant, we showed that co-treatment with actinomycin-D and MG132 prevented the accumulation of the AR isoform. This result demonstrated that the AR isoform is a product of *de novo* transcription of a splice variant (**Figure 3-11**). Independently, a group of investigators had already demonstrated that MG132 treatment results in the accumulation of a ~90kDa AR protein in LNCaP cells and identified this ~90kDa protein as a C-terminal truncated AR (Harada *et al,* 2012). They showed that in the absence of androgens, there is an accumulation of the C-terminal truncated AR in both androgen-independent and androgen-dependent LNCaP cell lines (Harada *et al,* 2012). Moreover, their evidence suggests that some of this C-terminal truncated AR is a product of proteolysis cleavage of the full-length AR and can function as a ligand-independent transcription factor in the prostate cancer cell line, PC-3. They suggested that this C-terminal truncated AR is produced under conditions similar to hormone therapy (absence of androgens) (Harada *et al,* 2012). However, Harada et al (2012) used an extremely high dose of protease inhibitor (500 μM) and was only able to partially block the accumulation of this smaller AR product. More importantly, all these results suggest that the loss of 26S proteasome function could be linked to the development of metastatic CRPC.

4.4. TBECH as a partial agonist of the human AR

Due to our limited knowledge on the environmental fate and toxicity of TBECH, there are increasing concerns as these diastereomers can leach out from consumer products and into the environment. All four TBECH diastereomers have been detected

in the eggs of herring gulls at concentrations between 0.11-0.54 ng/g w.w. from the Great Lakes of North America region (Gauthier *et al,* 2009). The ß-TBECH has been detected in the blubbers of beluga whales at concentrations between 1.1-9.3 ng/g l.w. (Tomy *et al,* 2008). Furthermore, there is evidence that TBECH is capable of long range atmospheric transport as they have been detected in the biota in the sub-Arctic and the Arctic (de Wit *et al,* 2010; Newton *et al,* 2014). However, based on several *in vivo* bird studies, the TBECH diastereomers do not appear to be bioaccumulative as they can be metabolized or eliminated rapidly (Marteinson *et al,* 2012; Currier *et al,* 2013). TBECH has been shown to be a potent hAR agonist and is able to induce androgen-regulated expression of AR and basal PSA protein levels in HepG2 and LNCaP cells, respectively (Larsson *et al,* 2006; Khalaf *et al,* 2009). As the incidence of hormone-responsive cancers in humans such as prostate cancer has significantly increased, these molecular studies raise concerns that TBECH may also have adverse effects on human health. My results show that both α/ß- and δ/γ-TBECH mixtures are partial agonists of hAR in LNCaP cells. I have shown that both TBECH mixtures can repress extracellular R1881-inducible PSA protein levels, but relieve R1881-inducible repression of PSMA protein levels. These results demonstrate the antagonistic and agonistic properties of the TBECH diastereomers. My results also demonstrated that δ/γ-TBECH mixture is the more potent hAR agonist, which is similar to the results of the Khalaf et al (2009) study. Given that TBECH is capable of repressing R1881-inducible PSA protein levels, it is possible that exposure to TBECH can produce false-negatives in a clinical PSA screen in men with prostate cancer. However, the δ/γ-TBECH mixture alone appear to induce basal PSA and AR protein levels (in the absence of androgens), which raises concerns that these diastereomers can be disruptive in healthy individuals.

Chapter 5. Conclusion

The global levels of *p,p'*-DDT and *p,p'*-DDE found in human adipose tissue are all in the low to mid-micromolar range which is comparable to the concentrations that I have shown to repress PSA levels. I have shown that physiologically relevant, low levels of *p,p'*-DDT and *p,p'*-DDE repress androgen-inducible PSA mRNA and protein levels in LNCaP cells. Furthermore, I have shown that the α/ß- and δ/γ-TBECH mixtures are partial agonists of the human androgen receptor in LNCaP cells. I have demonstrated that the TBECH diastereomers can repress R1881-inducible PSA protein levels at physiologically relevant, low levels, in LNCaP cells. Due to the environmental persistence and the endocrine disrupting actions of DDT and DDE, the ongoing concerns have always been focused on the toxicological endpoints or the adverse effects on the environment or human health. However, I have now shown the indirect adverse health impacts due to exposure extend to other clinical outcomes as DDT and DDE can put individuals with prostate cancer at an increased risk for a false negative PSA screen. Furthermore, DDT and DDE can also put individuals with undiagnosed prostate cancer risk for developing metastatic CRPC.

Limitations in this study include using a synthetic androgen as opposed to a natural one such as DHT. A second limitation is using only two concentrations for the R1881-inducible target gene and protein expression studies. A third limitation is using only one human prostate cancer cell line. Lastly, one of the major limitations is drawing clinical conclusions that exceed these *in vitro* data. Future directions could extend this work to clinical studies in order to confirm clinical relevance.

On the other note, I have also shown that a short AR isoform is produced in the absence of androgens following MG132 treatment, which blocks the 26S proteasome of the ubiquitin-proteasome degradation pathway. Although this work has been published by another group of investigators, we have demonstrated that this AR isoform is a

product of *de novo* transcription of a splice variant, and not a product of protein degradation of the full length AR. Collectively, these results show that loss of the 26S proteasome and the short AR isoform may play a role in the development of CRPC. Future directions could include cloning and sequencing the short AR isoform in the LNCaP cell line, to determine whether this isoform is the same as the AR splice variant in the 22Rv1 cell line.

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