SCREENING PUTATIVE ANTIANDROGENIC COMPOUNDS USING MDA-kb2 CELLS AND PHYSIOLOGICAL ASSESSMENT OF PUTATIVE ANTIANDROGENIC COMPOUNDS USING LNCaP CELLS AND HPGD EXPRESSION

Amelia K. Rinehart

Screening Putative Antiandrogenic Compounds Using MDA-kb2 Cells and Physiological Assessment of Putative Antiandrogenic Compounds Using LNCaP Cells and *HPGD* Expression

A Thesis Presented for the Master of Science Degree

Austin Peay State University, Clarksville TN

Amelia K. Rinehart

May 2014

To the Graduate Council:

I am submitting herewith a thesis written by Amelia K. Rinehart entitled "Screening Putative Antiandrogenic Compounds Using MDA-kb2 Cells and Physiological Assessment of Putative Antiandrogenic Compounds Using LNCaP Cells and *HPGD* Expression". I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biological Sciences.

Sarah Lundin-Schiller, Major Professor

We have read this thesis and recommend its acceptance:

Acceptance for the Council:

Dean of the Graduate School

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for the Master's degree at Austin Peay State University, I agree that the library shall make it available to borrowers under the rules of the library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgement of the source is made.

Permission for extensive quotation or reproduction of this thesis may be granted by my major professor, or in her absence, by the Head of the Interlibrary Services when, in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without my written permission.

Signature Amelia K. Pinkart

Date 9-9-14

TABLE OF CONTENTS

DEDICATION

This work is dedicated to my husband, Michael, whose love, encouragement and support has never wavered during this process.

I would also like to dedicate this work to my parents, who instilled in me a love of science and encouraged me to follow my dreams.

ACKNOWLEDGEMENTS

First, I would like to thank Michael for always supporting my academic endeavors. Without his patience and encouragement I would not have been able to complete my degree.

I would also like to thank my parents and grandparents for always believing in me and giving me that little push to continue on with my education.

Special thanks to Dr. Sarah Lundin-Schiller for her time, patience and expertise. Without her guidance and support I would have never been able to complete this research project. I am incredibly grateful to have had the opportunity to work with and learn from her.

I would also like to thank the numerous faculty members who spent their time offering insights. Thanks to Drs. Pitts and Meisch who provided valuable advice and for serving on my thesis committee. Thanks to Drs. Brooks and Baskauf for answering my many technical questions about lab procedures and statistics. Thanks to Dr. Joe Schiller for offering valuable suggestions on a wide array of subjects.

I would also like to thank the many graduate students who offered their friendship, support and advice. Special thanks to Veronica Mullen, Emma Gibson and Emily Towery for their help.

Special thanks to the College of Graduate Studies and the Department of Biology for funding this research.

THANK YOU!

ABSTRACT

AMELIA K. RINEHART. Screening Putative Antiandrogenic Compounds Using MDA-kb2 Cells and Physiological Assessment of Putative Antiandrogenic Compounds Using LNCaP Cells and *HPGD* Expression (Under the direction of Dr. Lundin-Schiller).

Endocrine disrupting chemicals interfere with signaling pathways of the endocrine system and are found in numerous consumer products ranging from cosmetics to water supplies. This project will screen 7 putative antiandrogenic compounds using the MDA-kb2 breast cancer cell line. Compounds displaying antiandrogenic activity will be assessed for pathophysiological effects in the LNCaP clone FGC prostate cancer cell line. MDA-kb2 cells are a stably transformed cell line with the luciferase gene downstream from the androgen receptor response element. This cell line was employed to screen tartrazine (Tart), allantoin (All), di-*n*-butyl phthalate (DBP), bisphenol A (BPA), bisphenol A glycidyl dimethacrylate (bisGMA), bisphenol A dimethacrylate (bisDMA) and bisphenol A diglycidyl ether (BADGE) for androgenic or antiandrogenic properties. Dihydrotestosterone (DHT) was used as the androgen control and hydroxyflutamide (OHF) was used as the antiandrogen control. Ethanol (EtOH) was used as the solvent for all chemicals. Compounds expressing antiandrogenic properties were then tested for physiological response in LNCaP clone FGC cell line. Physiological response was determined through quantification of *hydroxyprostaglandin dehydrogenase (HPGD)* gene using quantitative PCR (qPCR). Controls and solvent were same as those used in previous MDA-kb2 screening assay. Tartrazine, All, DBP, bisGMA, bisDMA and BADGE did not possess any androgenic or antiandrogenic properties in the MDA-kb2 cells ($p \geq 0.05$). Bisphenol A did possess antiandrogenic properties ($p \leq 0.0001$) and

was tested in LNCaP clone FGC cells. In LNCaP clone FGC cells, BPA down-regulated the androgen responsive *HPGD* gene ($p \leq 0.0002$). These studies clearly show that BPA has antiandrogenic properties that are at least in part mediated through down-regulation of *HPGD* expression.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

All – Allantoin

BADGE – Bisphenol A diglycidyl ether

BisDMA – Bisphenol A dimethacrylate

BisGMA – Bisphenol A glycidyl dimethacrylate

BPA – Bisphenol A

DBP – Di-*n*-butyl phthalate

DHT – Dihydrotestosterone

EDC – Endocrine disrupting chemical

EtOH – Ethanol

HPGD – Hydroxyprostaglandin dehydrogenase

OHF – Hydroxyflutamide

Tart – Tartrazine

CHAPTER 1. INTRODUCTION

The endocrine system controls most life processes: from regulation of embryological development and initiation of puberty to adult body functions, such as spermatogenesis. Studies have shown that some chemicals are able to mimic endogenous hormones and agonize or antagonize hormonal receptors (Pirard et al., 2012; Ye et al., 2012). These chemicals, termed endocrine disrupting chemicals (EDCs), have been found in consumer goods (Jobling et al., 1995; Plotan et al., 2013; Schlumpf et al., 2001) and the environment (Layton et al., 2001; Wang et al., 2012; Werner et al., 2010). Exposure to EDCs can have deleterious effects, especially with *in utero* exposure. In males, antiandrogens and estrogen mimicking compounds can lead to severe morphological abnormalities such as hypospadias, decreased prostate weight, or to broader issues such as testicular dysgenesis syndrome.

The Environmental Protection Agency (EPA) has stated there are over 80,000 chemicals used in the production of consumer goods and there is very little information known about their endocrine disrupting capabilities (EPA, 2012). To combat this lack of information the EPA has initiated a two-tiered screening process (EPA, 2012). Tier one consists of screening for potential endocrine disrupting capabilities, while tier two screens for effects of these compounds. This research project will contribute to that goal by screening seven putative antiandrogenic compounds with a tier one assay. Following the tier one assay, any compounds possessing antiandrogenic capabilities will be further screened with a tier two assay using LNCaP clone FGC cells. The results of this *in vitro* study will allow elucidation of possible *in vivo* effects arising from exposure to these compounds.

This research will assess if tartrazine, allantoin, di-*n*-butyl phthalate, bisphenol A, bisphenol A glycidyl dimethacrylate, bisphenol A dimethacrylate and bisphenol A diglycidyl ether possess endocrine disrupting properties that act through the androgen receptor. The MDA-kb2 breast cancer cell line (Figure 1) will be used as a tier one screening assay. MDA-kb2 is a cell line created from the MDA-MB-453 breast cancer cell line that expresses endogenous androgen receptors (Wilson et al., 2002). The MDA-kb2 cell line was transformed with an androgen responsive luciferase reporter gene construct that is activated when exposed to chemicals that act through the androgen receptor (Wilson et al., 2002). Exposure to androgens, and subsequent binding to the androgen receptor, will stimulate down-stream synthesis of luciferase. When luciferin is added, the luciferase will produce luminescence that can be detected spectrophotometrically. The greater the luminescence, the more androgen stimulation occurred. A lack of luminescence following androgen exposure indicates the androgen receptor was not stimulated due to the presence of an antagonist. Androgens such as dihydrotestosterone (DHT) bind to the androgen receptors and stimulate the production of luciferase (Wilson et al., 2002). MDA-kb2 cells will be treated with a known antiandrogen, hydroxyflutamide (OHF); a known androgen, DHT; the vehicle carrier, ethanol (EtOH); a mixture of OHF and DHT; unknowns at varying concentrations; and unknowns mixed with OHF and DHT. The antiandrogen, OHF, will competitively inhibit the androgen receptors; therefore, minimal luminescence should be measured in cells exposed to OHF.

After screening with MDA-kb2 cells, any compounds that show endocrine disrupting potential will be further tested in the LNCaP clone FGC cell line (Figure 2).

The LNCaP clone FGC cell line was derived from a metastatic site, in the left supraclavicular lymph node of a human prostatic adenocarcinoma (Horoszewicz et al., 1983). This cell line contains high affinity androgen receptors that, when stimulated, cause the up-regulation of the *HPGD* gene (Ngan et al., 2009). The *HPGD* gene produces NAD+-linked 15-hydroxyprostaglandin dehydrogenase, which is responsible for the inactivation of prostaglandins (Wolf et al., 2006). This enzyme has also been shown to possess tumor suppressing capabilities in breast (Wolf et al., 2006), colon (Myung et al., 2006), gastric (Liu et al., 2010) and lung cancers (Ding et al., 2005). To test for changes in expression of *HPGD* induced by unknown compounds, LNCaP clone FGC cells will be exposed to similar treatments as those used previously. After treatment, messenger RNA (mRNA) was extracted, complimentary DNA (cDNA) was synthesized and quantitative PCR (qPCR) was performed to determine any changes in *HPGD* gene expression.

Two unknowns are plasticizers, di-*n*-butyl phthalate (DBP) and bisphenol A (BPA), and have been shown to possess endocrine disrupting capabilities (Mylchreest et al., 2002; Murray et al., 2007) and will be screened for endocrine disrupting capabilities in this project using the MDA-kb2 cells. Exposure to DBP, found in products ranging from plastics to cosmetics (Koo and Lee, 2004), can lead to reproductive tract abnormalities such as agenesis of the epididymis and decreased sperm production (Mylchreest et al., 2002). Bisphenol A can interfere with a variety of intracellular pathways and is a known estrogen agonist (Murray et al., 2007), androgen antagonist (Bonefeld-Jørgensen et al., 2007; Lee et al., 2003) and aromatase inhibitor (Bonefeld-Jørgensen et al., 2007). Bisphenol A is commonly found in plastics and has been shown

to leech into food from packaging containing BPA (Munguia-Lopez et al., 2005). Exposure to this compound has been linked to increased susceptibility to carcinomas (Ho et al., 2006; Murray et al., 2007). Three BPA derivatives, bisphenol A glycidyl dimethacrylate (bisGMA), bisphenol A dimethacrylate (bisDMA) and bisphenol A diglycidyl ether (BADGE) are commonly used in dental sealants (Fleisch et al., 2010) and will also be screened using the MDA-kb2 cell line. An *in vivo* study performed by Al-Hiyasat and Darmani (2006) found that exposure to bisGMA led to a decrease in testicular and epididymal sperm counts in male mice. The decrease in testicular and epididymal sperm counts led to a decrease in fertility (Al-Hiyasat and Darmani, 2006). Bisphenol A dimethacrylate has been shown to possess the ability to interact via multiple cellular pathways and possesses estrogen agonistic activity, androgen antagonistic activity and can act as an aromatase inhibitor (Bonefeld-Jørgensen et al., 2007). Bisphenol A diglycidyl ether stimulated proliferation in MCF7 breast cancer cells (Olea et al., 1996) and T47D breast cancer cells (Nakazawa et al., 2002). Yang et al. (2010) found that oral exposure to BADGE led to a disruption in spermatogenesis in Sprague-Dawley rats. This disruption led to an increase in the number of immature and maturing sperm in testis (Yang et al., 2010).

Endocrine disrupting chemicals are not limited to plasticizers. Two additional chemicals, tartrazine (Tart) and allantoin (All), will be screened for endocrine disrupting capabilities. Food colorants, such as Tart, have been shown to possess xenoestrogenic capabilities in T47D cells (Datta and Lundin-Schiller, 2008). Tartrazine, commonly referred to as FD&C yellow 5, is used as a food colorant and as an ingredient in algaecide formulations (EPA, 2005). Tartrazine has also been shown to stimulate the estrogen

receptor α (ERα) and elicit a xenoestrogenic effect in MCF7 breast cancer cells (Axon et al., 2012). Allantoin is produced by plants (Jin et al., 2012) and is commonly used in multiple cosmetic formulations (Doi et al., 2009). It has been found to have anti-oxidative and anti-inflammatory effects (Jin et al., 2012).

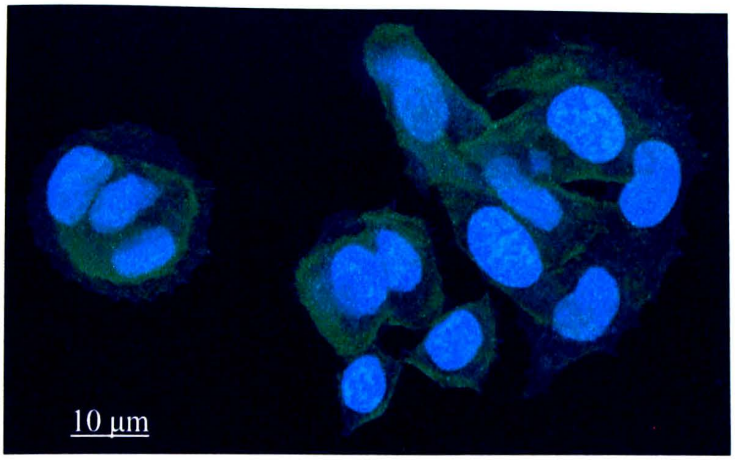

Figure 1. MDA-kb2 cells in culture stained with bisbenzamide for DNA and Bodipy ® Phallotoxin for f-actin.

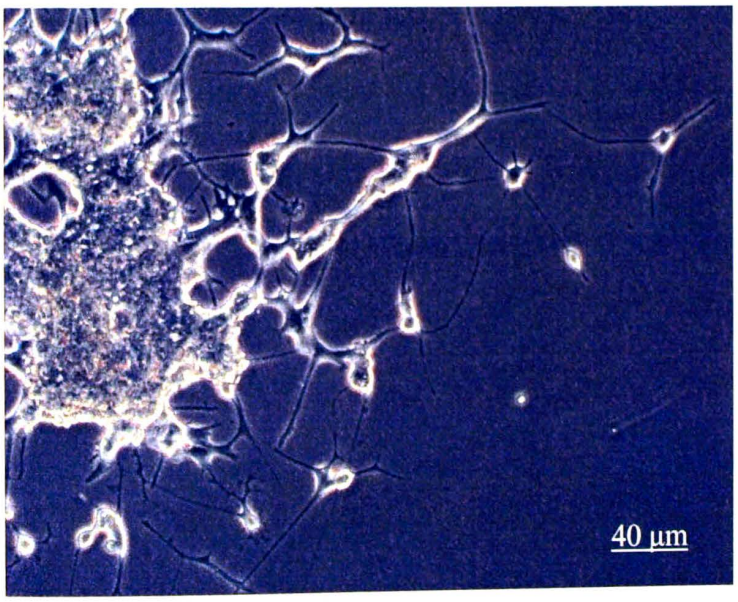

Figure 2. LNCaP clone FGC cells viewed under phase contrast microscope.

2.1 Exposure to Endocrine Disruptors

One of the most common exposure routes to EDCs is through contaminated food or water (Pirard et al., 2012; Singleton and Kahn, 2003). Numerous consumer goods contain EDCs, such as the commonly used high production volume plasticizers, BPA (Ho et al., 2006; Murray et al., 2007) and DBP (Wolf Jr. et al., 1999). Munguia-Lopez et al. (2005) showed that BPA is able to leech from the lining of metal cans into the edible contents within. Other consumer goods have also been found to contain EDCs, such as bottled mineral and flavored waters (Plotan et al., 2013), food antioxidants (Jobling et al., 1995) and cosmetics (Schlumpf et al., 2001). Consumer goods are not the only place where EDCs are being found.

Contamination by these chemicals is found in numerous water bodies around the world (Layton et al., 2011; Wang et al., 2012; Werner et al., 2010). A study by Werner et al. (2010) found that water sources near a news and kraft pulp and paper mill in Ontario, Canada, were contaminated with EDCs. This contamination resulted from mill effluent containing EDCs reaching the river waters (Werner et al., 2010). A recent survey of Tennessee waters show that they contain EDCs such as BPA (2.3µg/L), diethyl phthalate (4µg/L) and atrazine (0.857µg/L) (Layton et al., 2011). Endocrine disrupting compounds, such as BPA, were also found in waters in China (BPA, 283ng/L), (Wang et al., 2012; Ye et al., 2012a) and Greece (dissolved BPA, rivers, 15–138ng/L) (Arditsoglou and Voutsa, 2010) and mercury (Hg) was found in waters in India (Hg, 0.345mg/L) (Roy and Kalita, 2011). While this ubiquity of exposure could lead to male and female contact

with EDCs, this literature review will focus on males and subsequent effects arising from exposure.

2.2 Endocrine Disruptor Presence in the Body

It has been shown that EDCs are able to enter the body. Pirard et al. (2012) tested the urine concentrations of BPA in Belgian young adults, around 29 years of age and the results show that 97% of the tested subjects had BPA present in their urine (mean, 11.17nM). Young adults are not the only age group exposed to these compounds. A study performed by Ye et al. (2012b) tested sera of children 3-11 years of age for endocrine disruptors, such as benzophenone-3 and results showed that 60% of the tested subjects had known endocrine disruptors present. This is of vital concern as children have increased sensitivity to endocrine disruptors. Once EDCs are present in the body, they interfere with the endocrine system via a variety of mechanisms.

2.3 Mechanism of Action

The endocrine system secretes chemical messengers, called hormones, directly into the blood where they circulate until they reach their target cells. Target cells have high affinity receptors that bind the hormones (Li and Al-azzawi, 2009). When the hormone binds to its receptor, it induces a change within the cell (Li and Al-azzawi, 2009). These hormones are necessary to maintain homeostasis.

Numerous hormones are essential to sexual differentiation, development and maintenance of proper sexual functions. Two primary reproductive hormones are testosterone and estradiol. For example, testosterone is necessary for fetal development of the male reproductive tract (Wilson et al., 1981). Estradiol is necessary for

development of the female reproductive tract. Any disruption of these hormones could lead to a disruption of the processes they regulate.

Endocrine disrupting compounds interfere with the reception or signaling pathways of endogenous hormones in a variety of ways. These compounds can act as agonists by directly binding the receptor and stimulating a response similar to the one that would be elicited by the endogenous hormone (Danzo, 1997). Alternatively, EDCs can act as antagonists, occupying the receptor but not eliciting down-stream events (Danzo, 1997). In which case, the cell does not receive the signal from the endogenous hormone and does not initiate the appropriate cellular process.

Two indirect mechanisms of action are inhibition of steroid hormone synthesis and epigenetic transgenerational inheritance. Inhibition of steroid hormone synthesis can be accomplished by inhibiting enzymes necessary for production of hormones. Brodie et al. (1999) found that that the production of estrogen can be reduced by the presence of an aromatase-inhibiting 4-hydroxyandrostenedione. Kinneberg et al. (2006) found that the fungicide, prochloraz, inhibits production of cytochrome P450 enzymes, such as aromatase, in zebrafish which led to a decrease in the conversion of testosterone to estradiol. The subsequent decrease in estradiol and increase in testosterone led to masculinization in zebrafish. Male zebrafish had increased spermatozoa numbers in their testes which is also characteristic of increased testosterone.

Epigenetic transgenerational inheritance occurs when heritable changes are made to the molecular processes that regulate gene expression thus altering phenotype without altering DNA sequence. These changes are made in such a way that they will be

inherited by subsequent generations and frequently involve alterations of DNA methylation where increased methylation is associated with gene silencing and decreased methylation is associated with gene activation (Crews and McLachlan, 2006). Guerrero-Bosagna et al. (2012) found that vinclozolin, a fungicide with known antiandrogenic properties (Ostby et al, 1999), acted in this manner and the effects of this endocrine disruptor were expressed through four generations of mice. During embryonic development, mouse germ cell genomes are demethylated upon colonization of the gonads and undergo remethylation in a sex dependent manner. Vinclozolin exposure in mice during this period of embryonic development altered normal methylation patterns. Pregnant mice were injected with vinclozolin to allow embryonic exposure between days 7-13 of development. This exposure led to a permanent change in the methylation of the primordial germ cells. Similar to embryonic demethylation, there is also a period of demethylation in a sex dependent manner during fertilization, and any changes in methylation patterns caused by vinclozolin will not undergo demethylation during this period. The altered genome is then passed to subsequent generations via sperm. Cells and tissues developing from altered sperm genome will also contain an altered epigenome. This altered genome will increase susceptibility to adult onset diseases, such as spermatogenic cell defects and testicular, prostate and kidney abnormalities (Guerrero-Bosagna et al., 2012). Guerrero-Bosagna et al. (2012) found that the fourth generation of mice had 28 increased DNA methylation sites and 12 decreased DNA methylation sites.

2.4 Exposure in Males

Although the detailed mechanisms of action are, in many cases, still under investigation, deleterious health effects correlated with EDC exposure continue to be

documented (Diamanti-Kandarakis et al., 2009). Over the past 50 years, there has been an increase in reproductive abnormalities in males, such as a decline in sperm quality (Anderson et al., 2000; Carlsen et al., 1992; Rolland et al., 2013). Skakkebæk et al. (2001) proposed that reproductive abnormalities, such as declining semen quality, increased incidences of testicular cancer, cryptorchidism, and hypospadias, are part of a larger issue termed testicular dysgenesis syndrome (TDS). To clarify environmental contaminants could be one cause of TDS, Swan (2006) studied the relationship between environmental pesticide exposure and sperm quality. The results indicate that there is a correlation between pesticide exposure, as measured by urine metabolites, with semen quality, measured by sperm concentration, semen volume and sperm motility. Results show that men with higher levels of urine pesticide metabolites for compounds such as alachlor, atrazine and diazinon were more likely to suffer from reduced semen quality than the controls ($p = 0.0007$, $p = 0.012$ and $p = 0.0004$ respectively; Swan, 2006). While this study shows a correlation between EDCs and environmental exposure, this study does not illustrate cause and effect. Additional studies are needed not only to further elucidate cause and effect relationship, but also to determine if these EDCs act in an estrogenic or antiandrogenic manner.

Exposure to estrogen mimicking compounds may be one cause of TDS. A well-documented example is the exposure of the male fetus to diethylstilbestrol (DES). Diethylstilbestrol was prescribed to pregnant females to prevent miscarriage. This chemical acted as a synthetic non-steroidal estrogen during male fetal development and caused numerous reproductive abnormalities such as hypospadias, microphallus and non-cancerous epididymal cysts (Sultan et al., 2001). The detrimental effects of synthetic

estrogen exposure were further corroborated in a study performed by Ho et al. (2006), who found that fetal exposure to BPA led to increased susceptibility to precancerous prostate lesions. The presence of these precancerous lesions indicated that *in utero* exposure to estrogen mimicking compounds may promote prostate cancer (Ho et al., 2006).

Antiandrogens disrupt the development and function of the male reproductive system by interfering with the androgen signaling system. In a study performed on Sprague-Dawley rats, Ostby et al. (1999) found that exposure to vinclozolin during gestation caused feminization of male fetuses as indicated by decreased anogenital distance, retained nipples, cleft phallus with hypospadias, small to absent accessory sex glands, such as the prostate gland, and cryptorchidism. These effects were also replicated in a study performed by Parks et al. (2000) in which diethylhexyl phthalate, an antiandrogen, was administered to rats during gestation. The offspring in this study were also feminized, recreating the abnormalities induced by exposure to vinclozolin (Ostby et al., 1999). It is clear that *in utero* exposure to estrogen mimicking or antiandrogenic chemicals can cause abnormalities of the male fetus.

CHAPTER 3. MATERIALS AND METHODS

3.1 Chemicals

L-15 Leibovitz media + 2.05mM L-Glutamine (L-15; SH30525.01) was purchased from HyClone Laboratories, Inc., Logan, UT. RPMI media + L- Glutamine – Phenol Red (RPMI; 11835-030), antibiotic/antimycotic (AbAm), phosphate buffered saline pH 7.4 (PBS), SYBR Select Mastermix (4472908) and Optical 96-Well Reaction Plates (4306737) were purchased from Life Technologies, Grand Island, NY. Charcoal stripped fetal bovine serum (CS FBS) was purchased from Atlanta Biologicals, Lawrenceville, GA. Trypsin-EDTA solution (T4174), hydroxyflutamide (H4166), di-*n*-butyl phthalate (48559), tartrazine (T0388), allantoin (93791), bisphenol A (133027), bisphenol A dimethacrylate (156329), bisphenol A glycidyl dimethacrylate (494356), bisphenol A diglycidyl ether (15138) were purchased from Sigma-Aldrich, St. Louis, MO. Luciferase assay systems (E1500) were purchased from Promega, Madison, WI. PrimePCR SYBR Green Assay: *HPGD* primer (qHsaCID0037880), PrimePCR SYBR Green Assay: *ACTB* primer (qHsaCED0036269) and iScript Reverse Transcription Supermix for RT-qPCR (170-8841) were purchased from Bio-Rad, Hercules, CA. RNeasy Mini Kit (74104) was purchased from Qiagen, Valencia, CA.

3.2 Culture of MDA-kb2 Cells

MDA-kb2 cells (CRL-2713) were purchased from American Type Culture Collection, Manassas, VA. MDA-kb2 cells were cultured in L-15 media supplemented with 10% heat-inactivated CS FBS (HI CS FBS) and 2% AbAm (complete L-15 media) in a tissue-treated 75 cm^2 flask (Falcon, 3023). The complete L-15 media were changed every 48 hours. MDA-kb2 cells were incubated at 37°C without CO_2 at 89% humidity.

At 100% confluence, flask was split. Cells were loosened from the seed flask using trypsin (0.25% w/v)-EDTA (0.03% w/v) solution, resuspended in complete L-15 media and subsequently plated at $1x10^4$ cells/ml in a fresh 75 cm^2 flask or in 96-well plates (Costar, 3610) prior to experimentation.

3.3 Culture of LNCaP clone FGC Cells

LNCaP clone FGC cells (CRL-1740) were purchased from American Type Culture Collection, Manassas, VA. LNCaP clone FGC cells were cultured in RPMI media supplemented with 10% HI CS FBS and 2% AbAm (complete RPMI media) in a tissue treated 75 cm^2 flask (Falcon, 3023). The complete RPMI media were renewed twice weekly. LNCaP clone FGC cells were incubated at 37°C with 5% CO_2 with 90% humidity. At 70% confluence, flask was split. Cells were loosened from the seed flask using trypsin (0.25% w/v)-EDTA (0.03% w/v) solution, resuspended in complete RPMI media and subsequently plated at $1x10^4$ cells/ml in a fresh 75 cm^2 flask or in 6-well plates (Costar, 3516) prior to experimentation.

3.4 Heat-Inactivated Charcoal Stripped Fetal Bovine Serum

All CS FBS was heat-inactivated for use during cell culturing experimentation. Charcoal stripped FBS (50ml) was removed from -20°C and thawed in a water bath at 37°C. Serum was mixed by inversion during thawing. After complete thawing, CS FBS remained in 37°C water bath for an additional 15 minutes to allow serum to equilibrate with the water bath. Next, the water bath temperature was raised to 56°C. It took 35 minutes for water bath and serum to reach 56°C; during this step serum was mixed by inversion every 10 minutes. Once water bath and serum reached 56°C, the serum was incubated for an additional 30 minutes while mixing via inversion every 10 minutes.

After 30 minutes, CS FBS was heat-inactivated and removed from water bath. Serum was allowed to cool to room temperature for a minimum of 30 minutes. The HI CS FBS was then stored at 4°C until needed.

3.5 Removal of Steroid Contamination from Glassware and Utensils

All glassware and utensils were manually washed using Alconox lab detergent (Alconox, 1104) following manufacturer's instructions. Glassware and utensils were then rinsed four times using tap water followed by an additional five rinses using deionized water. After rinsing with deionized water, all were rinsed three times using 95% ethanol, paying close attention to any interior surfaces and hard to reach areas. All glassware and utensils were then inverted and air dried. After air drying all glassware and utensils were wrapped in aluminum foil and autoclaved.

3.6 Creating Working Solutions

With the exception of Tart and All, all chemicals were dissolved in 95% EtOH and stored at 4°C for later use. The compounds, DHT, OHF, DBP and BPA, were dissolved in 95% EtOH and vortexed to create a 100mM stock solution. In order to obtain a workable consistency, bisGMA was warmed to 56°C in a water bath. After equilibrating to 56°C and obtaining a working consistency, bisGMA was maintained at 37°C in water bath. An appropriate amount of bisGMA was then added to 95% EtOH and vortexed to create a 100mM stock solution. During creation of subsequent working dilutions, and before experimental treatments, bisGMA was warmed to 37°C to ensure that the compound was completely dissolved. Bisphenol A dimethacrylate and BADGE were added in the appropriate quantities to 95% EtOH to create 100mM stock solutions. Stock solutions were then warmed to 56°C to allow crystals to dissolve. After all crystals

were dissolved, stock solutions were maintained at 37°C during creation of subsequent dilutions. Prior to treatments, bisDMA and BADGE solutions were warmed to 37°C to ensure compounds were completely dissolved. Tartrazine and All were dissolved in L-15 media to create 10mM stock and stored at 4°C. During each subsequent dilution of Tart and All, the appropriate volume of EtOH was added to maintain 0.01% EtOH concentrations across all treatments.

3.7 Transactivation Assay Using MDA-kb2 Cells

MDA-kb2 cell transactivation assay was performed following protocol determined by Wilson et al. (2002). MDA-kb2 cells were cultured in 96-well plates (Costar, 3610). Briefly, cells were treated with either no hormone (negative control); DHT (positive control); OHF (antiandrogen); DHT + OHF; unknown chemical; unknown chemical + DHT; unknown chemical + OHF. Each chemical or chemical combination was tested in replicates of 4 and the entire screening assay was conducted twice for each compound; an additional triplicate was conducted for BPA and DBP. For negative control, cells were treated with 0.01% and 0.02% EtOH. These EtOH concentrations (0.01% and 0.02%) represented working concentrations that cells were exposed to across all treatments. A dose response test of the positive control (DHT) was performed using 0.01nM, 0.1nM, 1nM, 10nM and 100nM DHT. The antagonist, OHF, was tested at a concentration of 1μM. A check of OHF was performed by creating a treatment of 1μM OHF + 1nM DHT. Unknowns were tested at concentrations of 10nM, 100nM, 1μM and 10μM. Unknowns were tested for androgenic activity via treatment of 1μM OHF + 10μM unknown. Unknowns were tested for antiandrogenic activity via treatment of 1nM DHT + 10μM unknown. During experimentation, complete L-15 media were removed

from each well and each well subsequently received 100µl of appropriate treatment. After 24 hours, treatment media were removed and 100µl of treatment media were re-administered. After 24 hours, second treatment media were removed and cell lysates were created.

To create cell lysates, all treatment media were removed from each well. Cells were then washed once with 50µl of room temperature PBS. All PBS was removed and plates were incubated on lab bench for an additional minute at 45° to allow any remaining PBS to pool at the bottom. Any residual PBS was removed and 25µl of room temperature cell culture lysis buffer (CCLB) 1X (Promega, E1500) was added. Cell culture lysis buffer (1X) was created according to manufacturer instructions. After addition of CCLB, plates were incubated on lab bench for 30 minutes and visually inspected to ensure complete lysis. After complete lysis, plates were sealed with Parafilm and stored at -80°C until luminescence could be quantified.

To quantify luminescence, plates and reagents were brought to room temperature. Luciferase assay reagent was prepared by adding luciferase assay buffer to luciferase assay reagent and vortexing briefly. After mixing, 100µl of luciferase reagent was then added to each well. Luminescence was quantified in relative light units using Biotek Synergy HT spectrophotometer following manufacturer instructions. Luminescence was measured from the bottom of each well using a 2 second delay before the start of each measurement. Luciferin was added to 6 wells simultaneously and luminescence was quantified within one minute after addition of substrate to ensure maximal luminescence was measured.

To ensure that antiandrogenic activity was not due to cell death, a trypan blue exclusion cytotoxicity test was performed. Cells were grown in 35mm dishes and then treated for 48 hours with 0.01% EtOH, DHT and BPA in triplicate. Following incubation, 100µl of 0.4% trypan blue was added directly to media (1.5ml). Cells with intact cell membranes (viable cells) will exclude trypan blue and those without intact cell membranes (nonviable cells), will take up trypan blue. Cells incubated for 2 minutes. Viable and nonviable (blue) cells were counted in three randomly selected fields of view for each 35mm plate using the 40X objective on an inverted phase contrast microscope.

3.8 Quantification of *HPGD* Gene Expression Using LNCaP clone FGC Cells

Determination of *HPGD* gene expression using LNCaP clone FGC cells was based on procedures developed by Ngan et al. (2009) with modifications. LNCaP clone FGC cells possess numerous high affinity androgen receptors (Horoszewicz et al., 1983) making this a choice cell line for testing androgenic or antiandrogenic compounds. LNCaP clone FGC cell line was previously profiled via microarray by Stronach et al. (2009) where *HPGD* was shown to be strongly up-regulated when exposed to DHT. Prior to experimentation, LNCaP clone FGC cells were cultured in 6-well plates (Costar, 3516). Similar to the assay described above, cells were either treated with no hormone (EtOH; negative control); DHT (positive control); OHF (antiandrogen); DHT + OHF; BPA or BPA + DHT. For negative control, cells were exposed to 0.01% EtOH. For agonist and antagonist treatments, cells were exposed to 1nM DHT or 1µM OHF. A check of OHF was performed via treatment of 1µM OHF + 1nM DHT. Bisphenol A was tested at a concentration of 10µM BPA. Antiandrogenic activity of BPA was tested via treatment of 10µM BPA + 1nM DHT. Cells were exposed to various treatments for 24

hours after which media were removed and mRNA was extracted. The entire experiment was performed three times.

Messenger RNA was extracted using RNeasy Mini Kit (Qiagen, 74104) following protocol supplied by manufacturer. Quantification of mRNA was performed using Nanodrop ND1000 spectrophotometer. After isolation, mRNA was reverse transcribed using iScript Reverse Transcription Supermix for RT-qPCR (Biorad, 170-8841). Reverse transcription reactions contained 4µl 5x iScript reverse transcription supermix and a variable volume of mRNA template so that total amount of template mRNA was the same in each reaction. Each reaction was brought to a total volume of 20µl with nuclease-free water. Reverse transcription was performed using a Thermo Electron Corporation PXE 0.2 thermal cycler under the following conditions: 25°C for 5 minutes, 42°C for 30 minutes and then 85°C for 5 minutes.

After performing reverse transcription reactions, cDNA was amplified using an ABI 1500 Sequence Detection System using SYBR Select Mastermix (Life Technologies, 4472908). The gene of interest was *HPGD,* and *β-actin* gene was used as endogenous control. Both primers, *HPGD* primer (Biorad, qHsaCID0037880) and *β-actin* primer (Biorad, qHsaCED0036269), were validated by Biorad. Each 96-well qPCR plate (4306737) contained three technical replicates of each treatment for both *HPGD* and *β-actin*. Each reaction contained a total volume of 20µl and consisted of: 1µl 20x PrimePCR assay (*HPGD* or *β-actin*), 10µl SYBR Select Master Mix, 2µl cDNA and 7µl of nuclease-free water. Thermocycler conditions consisted of: 1 cycle at 95°C for 2 minutes, 40 cycles at 95°C for 5 seconds and then 60°C for 30 seconds and a holding stage at 4°C or dissociation stage.

Cycle threshold (Ct) values were obtained and analyzed using the comparative Ct method ($\Delta\Delta$Ct) as described by Bookout and Mangelsdorf (2003). To determine relative efficiencies, log of RNA concentration versus ΔCt were graphed. The slope of the line was close to zero ($m < 0.1$) indicating the amplification efficiency of *HPGD* and *β-actin* are similar; therefore, the $\Delta\Delta$Ct method may be applied (Bookout and Mangelsdorf, 2003). Before determining fold induction, average Ct and standard deviation (std) were first calculated for each triplicate. ΔCt was calculated as: average Ct_{HPGD} – average $Ct_{\beta\text{-}actin}$. Then $\Delta\Delta$Ct was calculated as: $\Delta Ct_{treatment} - \Delta Ct_{control}$. Fold induction over control was then calculated as: $2^{(-\Delta\Delta Ct)}$.

3.9 Statistical Analysis for MDA-kb2 Assay

Each unknown compound was tested twice; one additional experimental replicate was conducted for BPA and DBP. Each 96-well plate is considered to be one replicate and within each plate there were four technical replicates (wells) of each treatment. Data were analyzed using a two-way analysis of variance (ANOVA) with treatment and replicates (plates) as main effects. All data were analyzed using JMP Pro 10 software (SAS Institute, Inc., Cary, NC). Log of relative light units was used during analysis. A $p \leq 0.05$ indicated statistically significant effects. Data were compared using LSMEANS Tukey Kramer HSD connecting letters report. To determine androgenic activity, 1μM OHF + 10μM unknown treatment was compared to 1μM OHF treatment. To determine antiandrogenic activity, 1nM DHT + 10μM unknown was compared to 1nM DHT treatment. For cytotoxicity test, data were analyzed using one-way ANOVA where $p \leq 0.05$ represented statistically significant effects.

3.10 Statistical Analysis for *HPGD* Gene Expression

Data for *HPGD* gene expression were analyzed using a one-way ANOVA. A p value ≤ 0.05 was considered to represent statistically significant effects of treatment on *HPGD* expression. Data were compared using LSMEANS Tukey Kramer HSD connecting letters report. All data were analyzed using JMP Pro 10 software (SAS Institute, Inc., Cary, NC). Expression of *HPGD* from cells exposed to 10μM BPA + 1nM DHT was compared to expression in cells exposed to 1nM DHT treatment to see if exposure to BPA elicited antiandrogenic effects on gene expression.

4.1 Luciferase Activity Measured in Transactivation Assay Using MDA-kb2 Cells

MDA-kb2 cells respond in a dose dependent manner to increasing concentrations of the agonist, DHT. At 0.1nM DHT, there was a significant increase in luminescence when compared to the negative control, EtOH. At 1nM DHT, there was a significant difference when compared to the 0.1nM DHT and the control. At this concentration there was a plateau in response and no further significant increases were noted at higher concentrations of DHT (Figure 3).

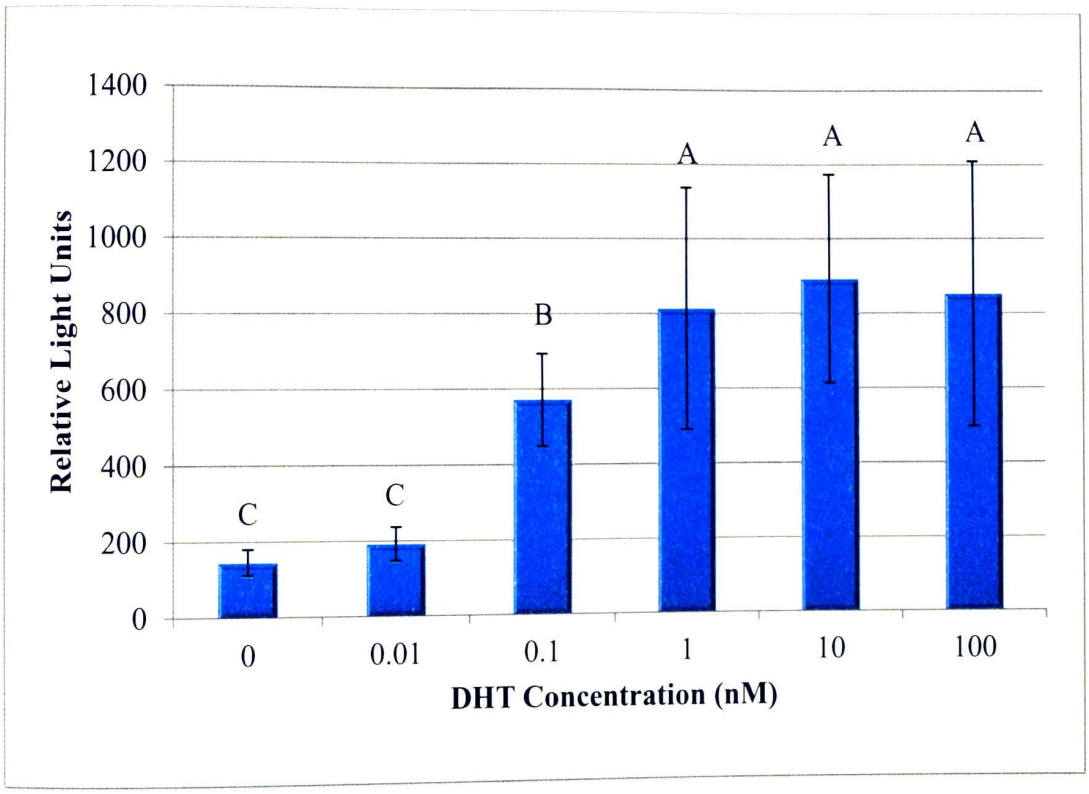

Figure 3. The effect of dihydrotestosterone (DHT) on MDA-kb2 luciferase expression in Relative Light Units (mean ± std, n=8). Treatments marked with different letters are statistically different ($p \leq 0.05$).

The androgen agonist, 1nM DHT, induced a significant increase in luminescence when compared to the negative control (EtOH). The androgen antagonist, 1μM OHF, did not induce luciferase expression, but significantly inhibited DHT (1nM) induced expression (Figure 4). These four controls were repeated in every assay to establish the validity of the assay.

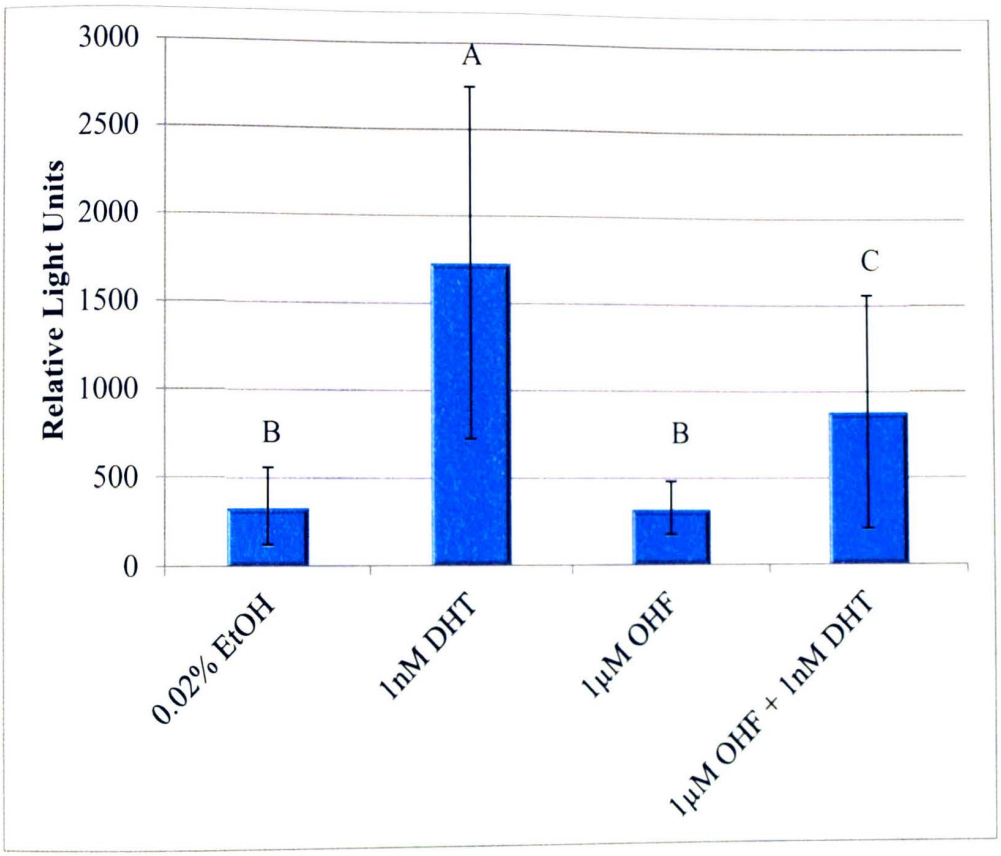

Figure 4. The effect of EtOH, DHT, OHF and DHT + OHF on luciferase activity of the MDA-kb2 cells measured in Relative Light Units (mean ± std, n=8). Treatments marked with different letters are statistically different ($p \leq 0.05$).

Comparison of treatments 1nM DHT and 1nM DHT + 10μM BPA show statistically significant difference in luminescence; therefore, BPA possesses antiandrogenic properties in this assay. Bisphenol A did not possess androgenic activity in this assay as BPA treatment did not induce a significant increase in luminescence over negative control (EtOH) (Figure 5).

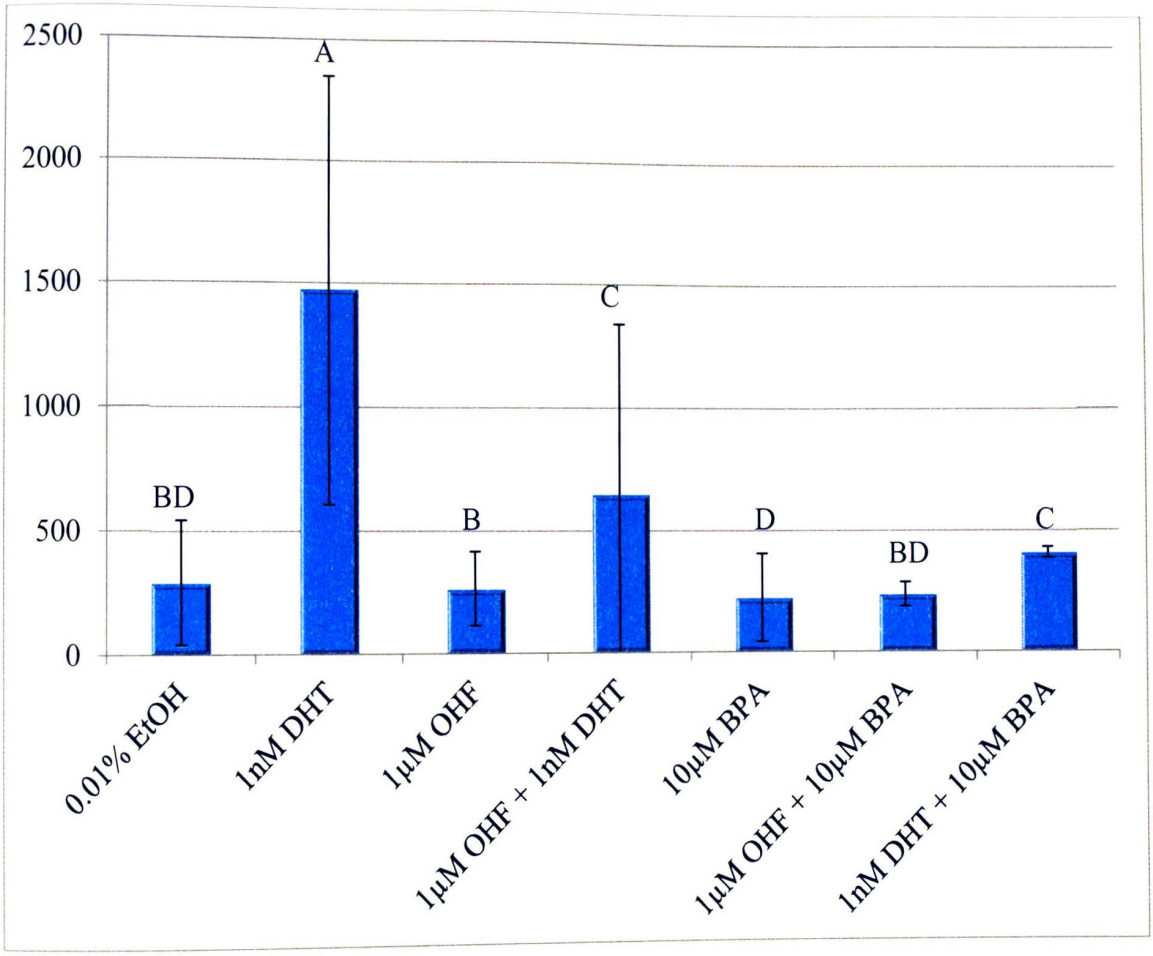

Figure 5. The effects of bisphenol A on luciferase activity of MDA-kb2 cells measured in Relative Light Units (mean ± std, n=12). Treatments marked with different letters are statistically different ($p \leq 0.0001$).

A cytotoxicity test using BPA was performed to ensure that antiandrogenic properties seen in MDA-kb2 assay were not due to cell death (Table 1).

Treatment	Mean % Dead	Std % Dead
0.01% EtOH	4%	1.40%
1nM DHT	3.5%	1.5%
10μM BPA	4.60%	2%

Table 1. Viability test on MDA-kb2 cells treated with BPA as compared to positive and negative controls. There was no effect of cytotoxicity ($p = 0.61$).

Comparison of treatments 1nM DHT and 1nM DHT + 10μM Tart show that there was no statistically significant difference; therefore, Tart did not possess any antiandrogenic properties in this assay. Tartrazine did not possess androgenic activity in this assay as Tart treatment did not induce a significant increase in luminescence over negative control (EtOH) (Figure 6).

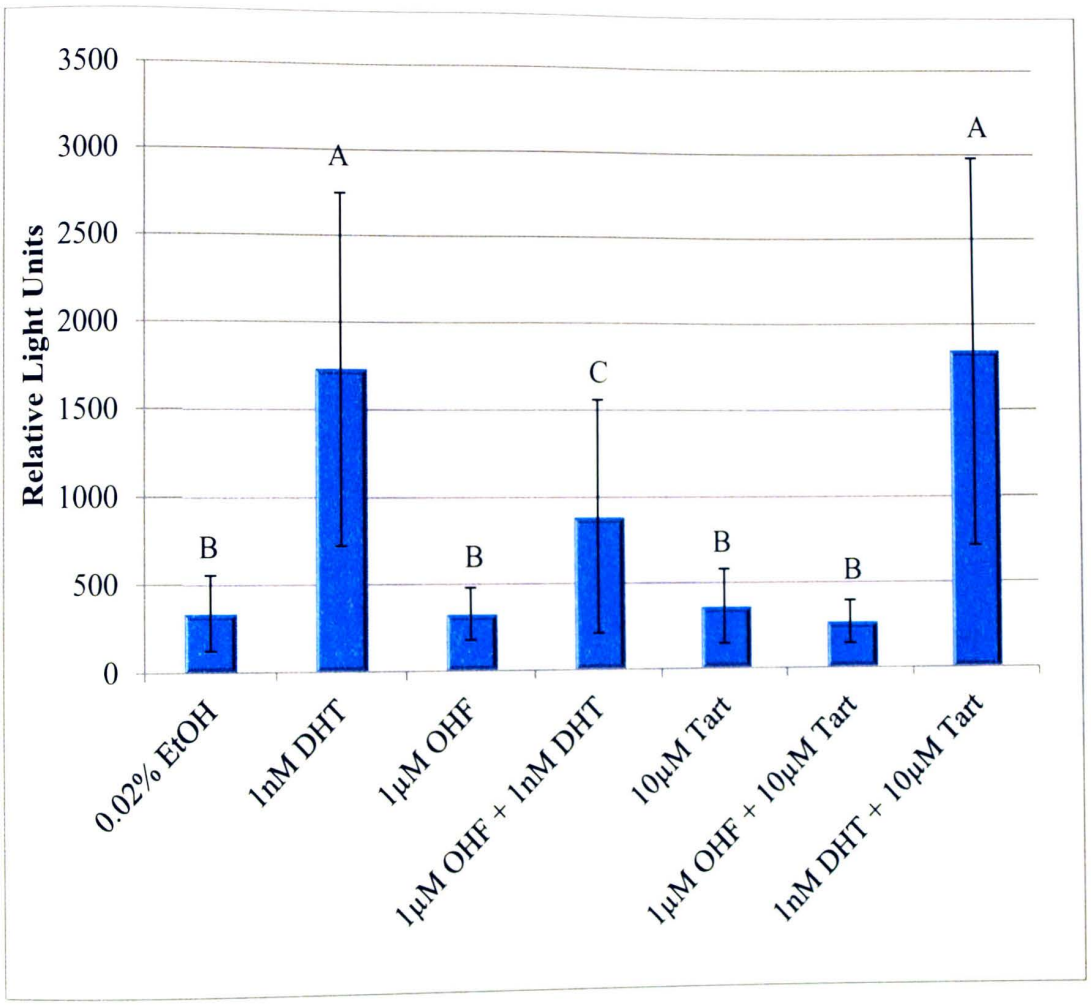

Figure 6. The effects of tartrazine on luciferase activity of MDA-kb2 cells measured in Relative Light Units (mean ± std, n=8). Treatments marked with different letters are statistically significantly different ($p \leq 0.0001$).

Comparison of treatments 1nM DHT and 1nM DHT + 10μM All show that there was no statistically significant difference; therefore, All did not possess any antiandrogenic properties in this assay. Allantoin did not possess androgenic activity in this assay as All treatment did not induce a significant increase in luminescence over negative control (EtOH) (Figure 7).

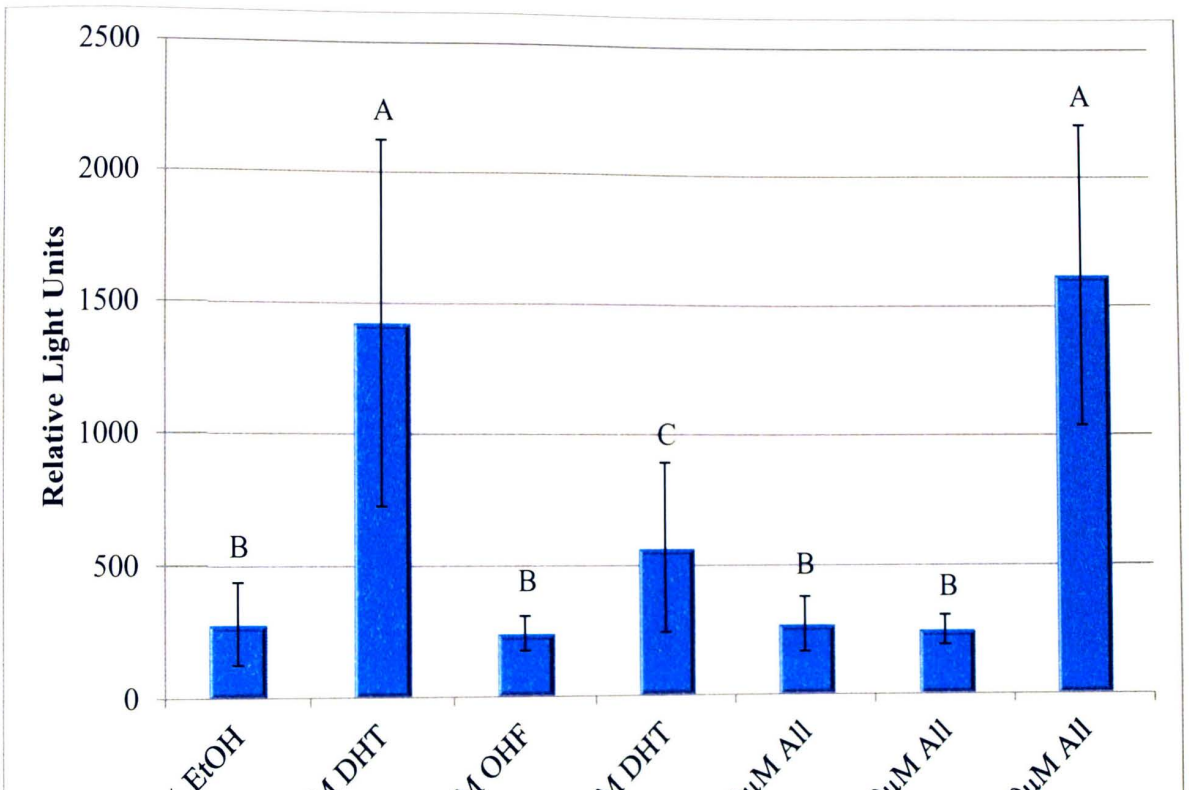

Figure 7. The effects of allantoin on luciferase activity of MDA-kb2 cells measured in Relative Light Units (mean ± std, n=8). Treatments marked with different letters are statistically significantly different ($p \leq 0.0001$).

Comparison of treatments 1nM DHT and 1nM DHT + 10μM BisGMA show no statistically significant difference; therefore, bisGMA did not possess any antiandrogenic properties in this assay. Bisphenol A glycidyl dimethacrylate did not possess androgenic activity in this assay as bisGMA treatment did not induce a significant increase in luminescence over negative control (EtOH) (Figure 8).

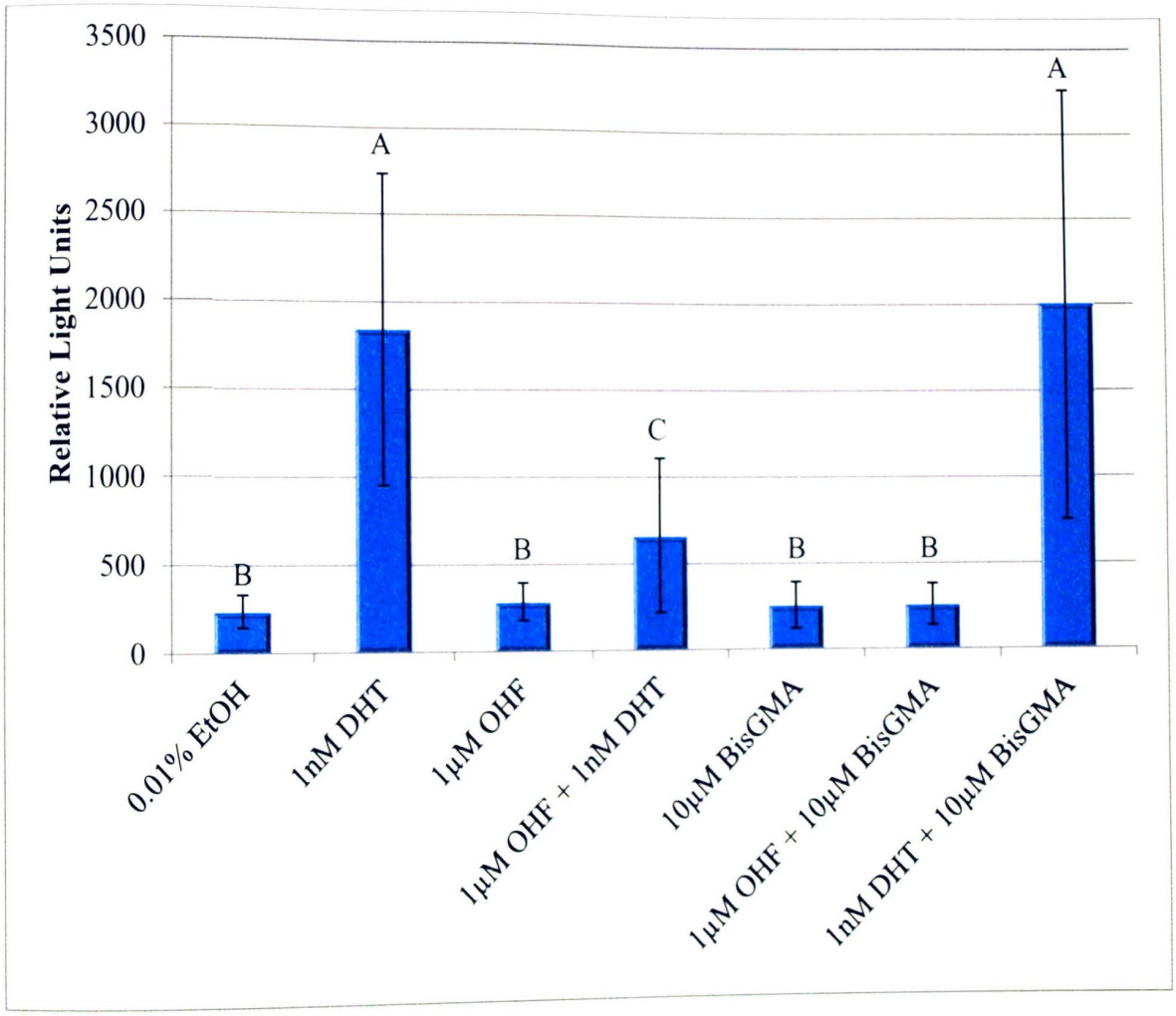

Figure 8. The effects of bisphenol A glycidyl dimethacrylate on luciferase activity of MDA-kb2 cells measured in Relative Light Units (mean ± std, n=8). Treatments marked with different letters are statistically significantly different ($p \leq 0.0001$).

Comparison of treatments 1nM DHT and 1nM DHT + 10μM BisDMA show no statistically significant difference; therefore, bisDMA did not possess any antiandrogenic properties in this assay. Bisphenol A dimethacrylate did not possess androgenic activity in this assay as bisDMA treatment did not induce a significant increase in luminescence over negative control (EtOH) (Figure 9).

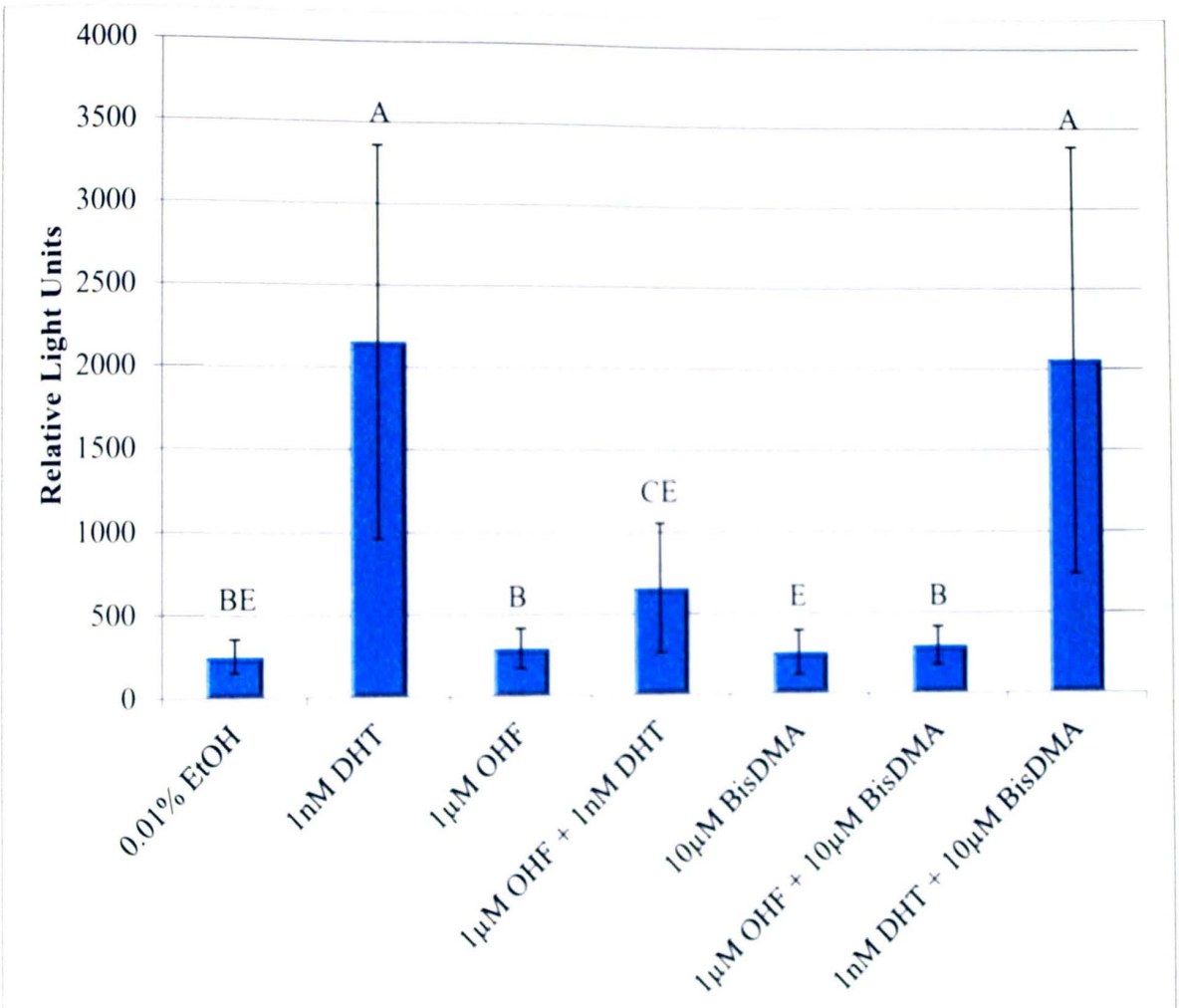

Figure 9. The effects of bisphenol A dimethacrylate on luciferase activity of MDA-kb2 cells measured in Relative Light Units (mean ± std, n=8). Treatments marked with different letters are statistically significantly different ($p \leq 0.0001$).

Comparison of treatments 1nM DHT and 1nM DHT + 10μM BADGE show no statistically significant difference; therefore, BADGE did not possess any antiandrogenic properties in this assay. Bisphenol A diglycidyl ether did not possess androgenic activity in this assay as BADGE treatment did not induce a significant increase in luminescence over negative control (Figure 10).

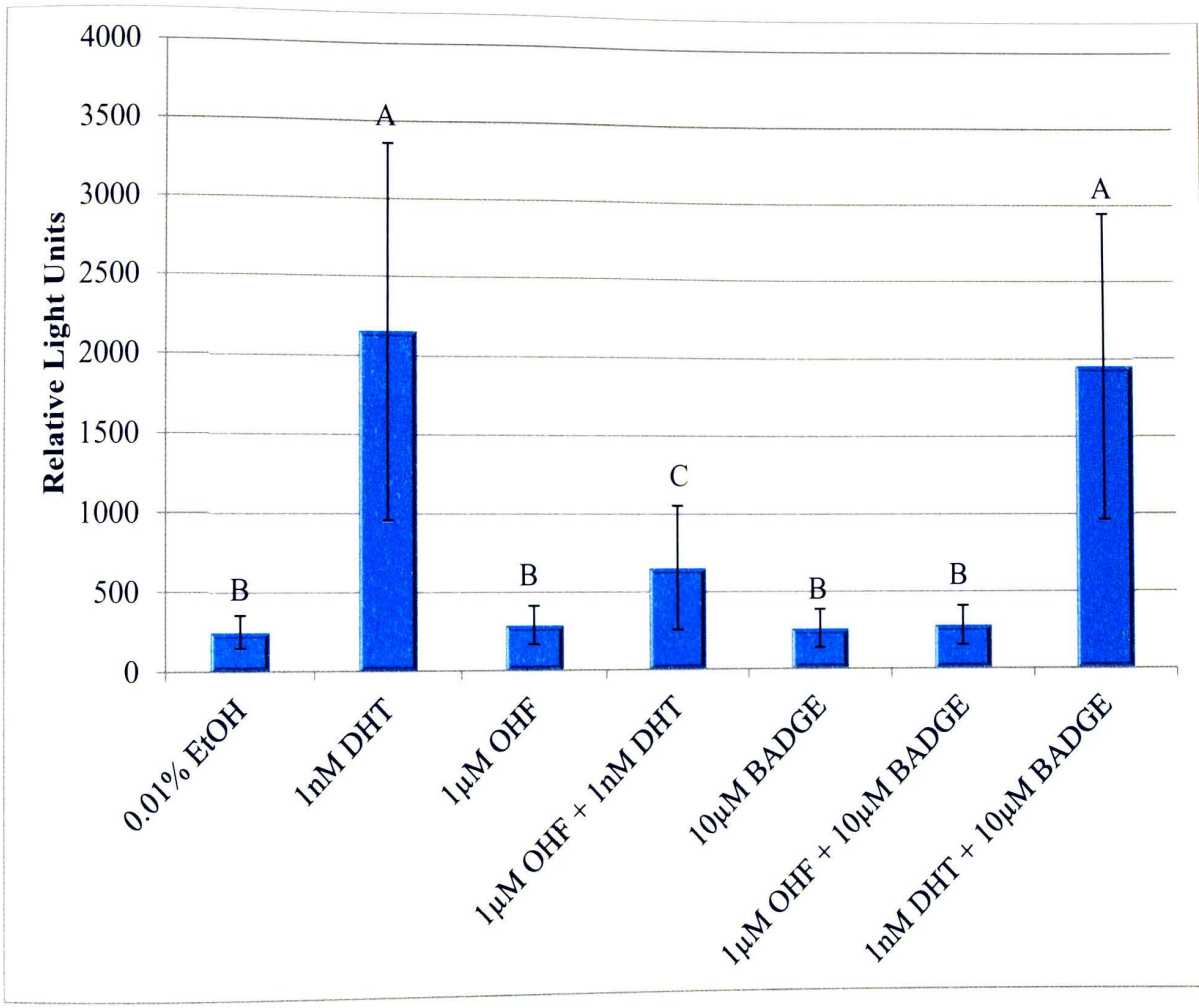

Figure 10. The effects of bisphenol A diglycidyl ether on luciferase activity of MDA-kb2 cells measured in Relative Light Units (mean ± std, n=8). Treatments marked with different letters are statistically significantly different ($p \leq 0.0001$).

Comparison of treatments 1nM DHT and 1nM DHT + 10μM DBP show no statistically significant difference; therefore, DBP did not possess any antiandrogenic properties in this assay. Di-*n*-butyl phthalate did not possess androgenic activity in this assay as DBP treatment did not induce a significant increase in luminescence over negative control (Figure 11).

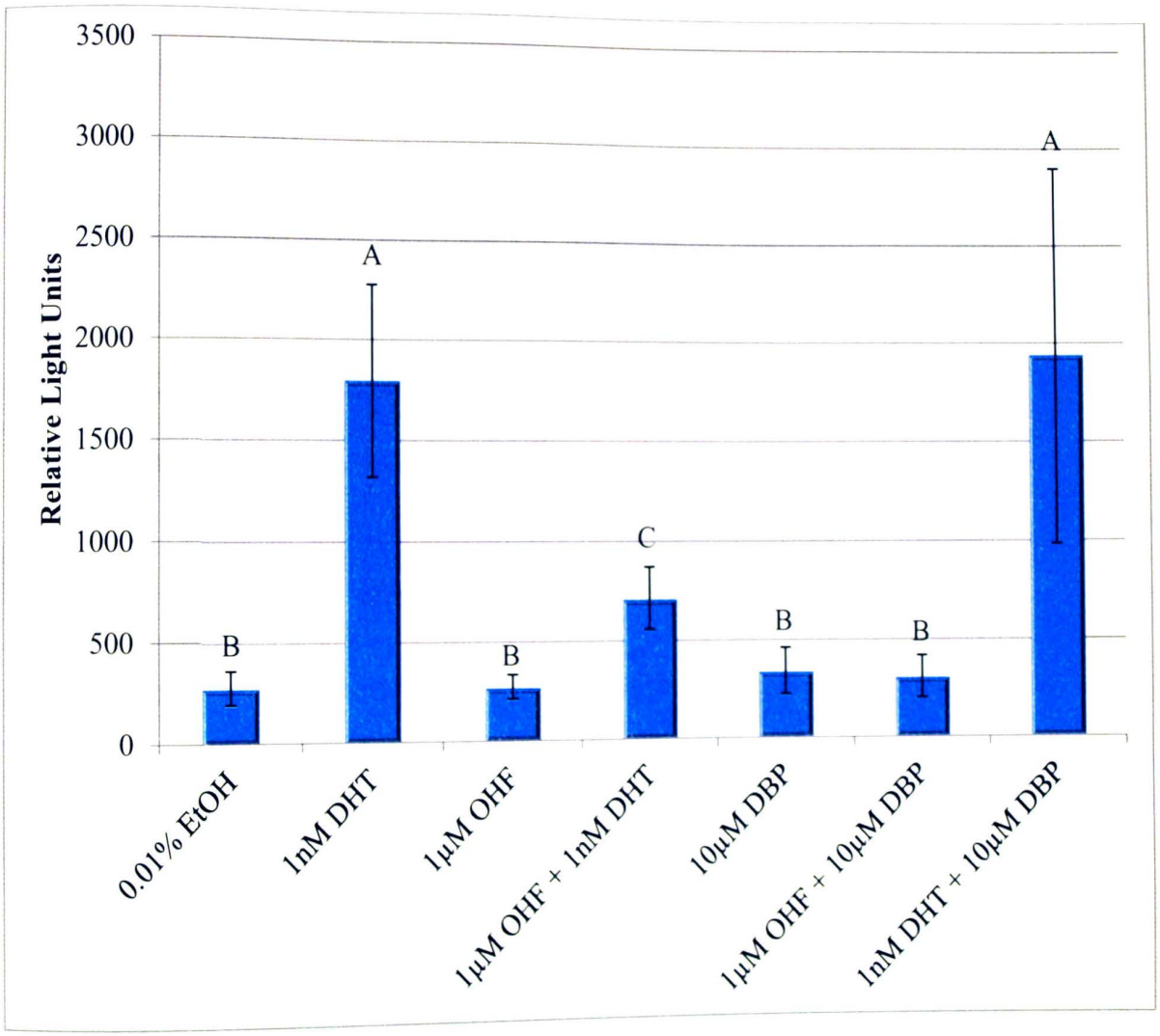

Figure 11. The effects of di-*n*-butyl phthalate on luciferase activity of MDA-kb2 cells measured in Relative Light Units (mean ± std, n=12). Treatments marked with different letters are statistically significantly different ($p \leq 0.0001$).

4.2 Quantification of *HPGD* Gene Expression Using LNCaP clone FGC Cells

LNCaP clone FGC cells contain endogenous androgen receptors and have been shown to up-regulate *HPGD* gene expression when exposed to DHT (Ngan et al., 2009). Therefore, this cell line was chosen to determine a pathophysiological response of an androgen responsive gene when exposed to BPA. Unlike the previous cell line used, MDA-kb2, which is a transformed cell line used within a Tier 1 screening assay, assessment using LNCaP clone FGC cells will allow Tier 2 screening for effects elicited through exposure to BPA.

Slope of the line, m = 0.03 (Figure 12), shows that the amplification efficiencies of genes are similar ($m < 0.1$) and Comparative Ct Method can be used (Bookout and Mangelsdorf, 2003). ΔCt was calculated from three technical replicates. Calculations used to determine ΔCt = ave. Ct_{HPGD} – ave. $Ct_{\beta\text{-}actin}$.

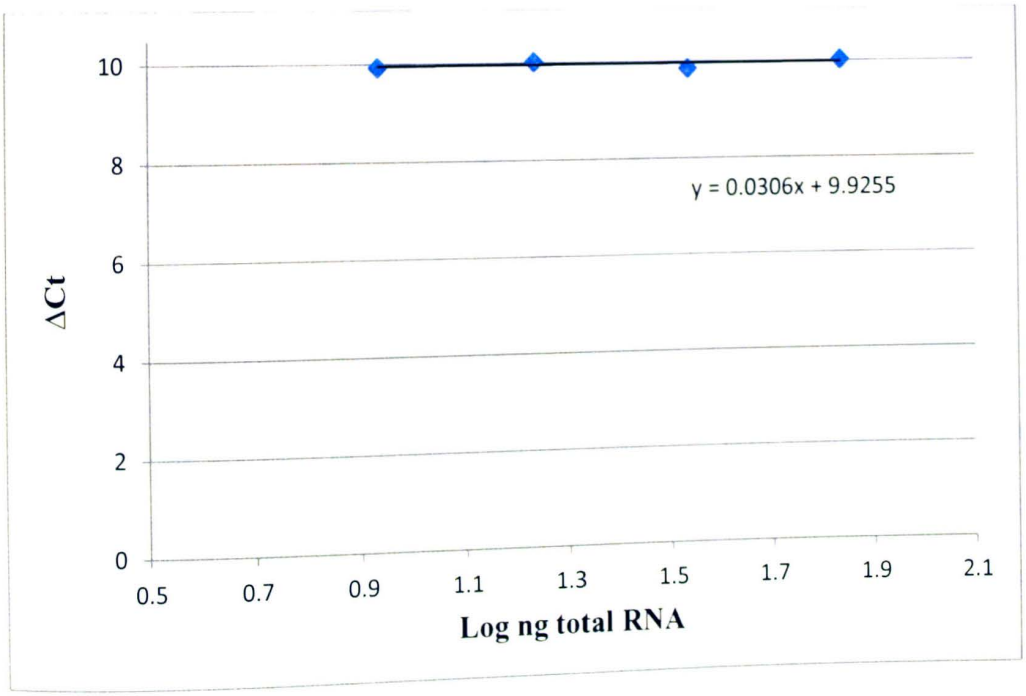

Figure 12. Amplification efficiencies of *HPGD* and *β-actin* in LNCaP clone FGC cells.

Dihydrotestosterone (1nM) induced a significant increase HPGD expression compared to negative control (Figure 13). Both OHF (10μM) and BPA (10μM) significantly inhibited the ability of DHT to induce HPGD expression in the LNCaP cells (Figure 13).

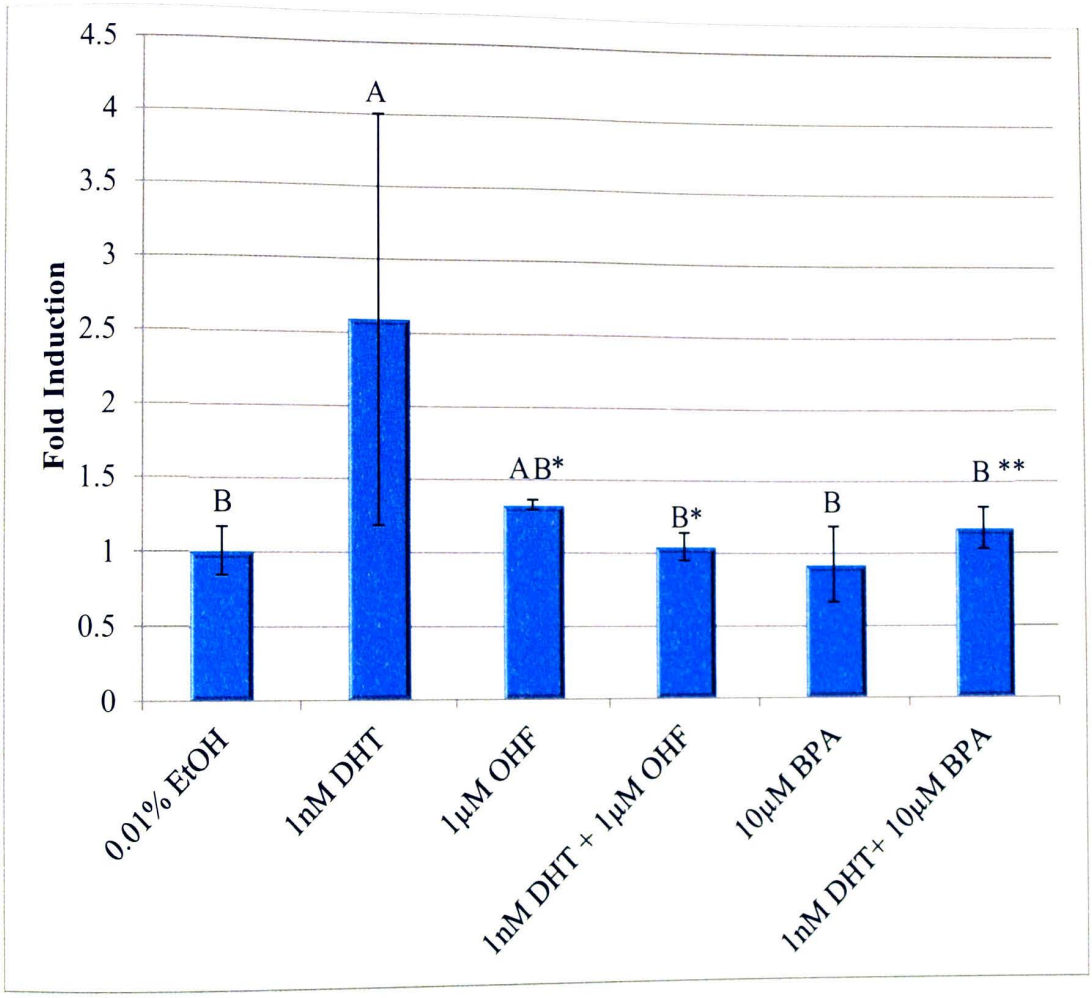

Figure 13. Fold induction of *HPGD* in LNCaP clone FGC cells when exposed to various treatments (mean ± std, n = 9). Treatments marked with different letters are statistically significantly different ($p \leq 0.0002$). Bars with '*' represent the mean of 3 replicates. Six replicates were excluded due to contamination and subsequent loss of functionality of

OHF after first experiment. Bar with '**' represents the mean of 6 replicates as three replicates had values too low to be detected.

The goal of this research project was to evaluate commonly used chemicals for androgenic or antiandrogenic properties. Tartrazine, allantoin, di-*n*-butyl phthalate, bisphenol A, bisphenol A glycidyl dimethacrylate, bisphenol A dimethacrylate and bisphenol A diglycidyl ether were screened with a stably transfected breast cancer cell line, MDA-kb2. Compounds with androgenic or antiandrogenic properties were further evaluated for physiological response via quantification of the androgen responsive *HPGD* gene in LNCaP clone FGC cells. The results of this study show that Tart, All, DBP, bisGMA, bisDMA and BADGE do not possess androgenic or antiandrogenic properties in the MDA-kb2 assay. Bisphenol A does show statistically significant antiandrogenic properties in the MDA-kb2 assay and in LNCaP clone FGC cells.

The MDA-kb2 assay can be used as a tier one screening assay for androgenic or antiandrogenic compounds through quantification of luciferase production. Wilson et al. (2002) transformed the MDA-MB-453 breast cancer cell line that contained endogenous androgen receptors (AR) and glucocorticoid receptors (GR) using the androgen responsive mouse mammary tumor virus promoter upstream from androgen responsive luciferase gene (MMTV-LUC reporter plasmid) creating the MDA-kb2 cell line. MDA-kb2 cells do not contain progesterone receptor (PR) or ERα, however, very low levels of estrogen receptor beta (ERβ) are expressed. Androgen receptors are ligand-activated transcription factors (Li and Al-azzawi, 2009). When androgen binds to the AR, it induces a conformational change that allows the AR complex to bind the androgen response elements located in the regulatory region of target genes which causes it to assemble the transcriptional machinery required to express androgen-regulated genes (Li

and Al-azzawi, 2009). In MDA-kb2 cells, the androgen-regulated luciferase gene will begin to produce the enzyme luciferase when the AR is stimulated (Wilson et al., 2002). Luciferase production can be quantified by adding luciferin, the substrate, and measuring relative light units produced. As MDA-kb2 cells do not contain PR or ERα and very low levels of ERβ, putative endocrine disrupting compounds will bind to AR or GR. Distinction between stimulated receptors can be determined with the AR antagonist, OHF (Wilson et al., 2002). Compounds that bind to the AR and act as agonists will cause a subsequent increase in luminescence when compared to the antagonist control, OHF. Compounds that bind to AR as antagonists will cause a subsequent decrease in luminescence when compared to agonist control, DHT.

Bisphenol A did show strong antiandrogenic properties in MDA-kb2 cells. Bisphenol A is a known endocrine disruptor and can act agonistically through the estrogen receptor (Murray et al., 2077). Our results show that BPA will bind to the AR and act as an AR antagonist. These results are supported by studies performed by Bonefeld-Jørgensen et al. (2007) where BPA bound to the AR in the CHO cell line and further supported by Lee et al. (2003) where BPA bound to the AR in a yeast two-hybrid system and in a transfected HeLa cell line. Bisphenol A can competitively bind to the AR and thus inhibits binding of endogenous hormones and expression of androgen regulated genes (Lee et al., 2003).

In our study Tart did not possess any androgenic or antiandrogenic properties in MDA-kb2 assay. While Tart does possess endocrine disrupting capabilities (Datta and Lundin-Schiller, 2008), it appears to act primarily as a xenoestrogen in T47D and MCF7 breast cancer cell lines (Axon et al., 2012; Datta and Lundin-Schiller, 2008). In this

assay Tart did not bind to the AR and did not stimulate the production of luciferase. Therefore, it is unlikely that Tart will possess endocrine disrupting capabilities via binding to the AR.

Allantoin also did not appear to possess any androgenic or antiandrogenic properties in the MDA-kb2 assay. To our knowledge, there are no known studies looking at All as a potential endocrine disruptor. Our results indicate that this compound does not elicit androgenic or antiandrogenic effects through the AR.

Di-*n*-butyl phthalate is a known endocrine disruptor that possesses properties similar to those seen with androgen receptor antagonists (Mylchreest et al., 2002). Our results show that it does not possess androgenic or antiandrogenic capabilities when screened with MDA-kb2 cells. This indicates that DBP does not bind to AR and exert endocrine disrupting properties through this mechanism. This result is supported by Foster et al. (2001) who also found that DBP does not interact with AR. Thompson et al. (2004) found that DBP exerts its endocrine disruption through interference of the steroidogenesis pathway via reduction of proteins necessary for testosterone production in fetal testis. This reduction of testosterone leads to effects similar to those seen when exposed to androgen receptor antagonists (Thompson et al., 2004).

Bisphenol A glycidyl dimethacrylate did not stimulate the AR in our study; therefore, it does not appear to possess any androgenic or antiandrogenic properties in MDA-kb2 cells. To our knowledge, there are few studies that have elucidated the ability of bisGMA to act as an endocrine disruptor. It is worth noting in a previous *in vivo* study, exposure to bisGMA led to a decrease in fertility in male mice (Al-Hiyasat and

Darmani, 2005). However, this could be caused through contamination with the parent compound, BPA (Al-Hiyasat and Darmani, 2005).

Bisphenol A dimethacrylate has been shown to act as an endocrine disruptor through multiple pathways and exert estrogenic activity, androgen antagonist activity and act as an aromatase inhibitor (Bonefeld-Jørgensen et al., 2007). Bonefeld-Jørgensen et al. (2007) tested for agonist and antagonist activity in a Chinese hamster ovary cell line that had been stably transfected with human AR and the MMTV-LUC reporter plasmid (Bonefeld-Jørgensen et al., 2007). Their results found the lowest effect concentration was 2.5×10^{-6}M and maximum effect concentration was 2×10^{-5}M (Bonefeld-Jørgensen et al., 2007). While our results do not show bisDMA as possessing androgenic or antiandrogenic properties, this result could be due to differences in transfected cell lines or due to differences in concentrations tested. Our tested concentration was 1×10^{-5}M; therefore, it is unlikely that the difference is due to concentration. Future studies dedicated to discovering possible causes of this discrepancy are warranted.

Our results show that BADGE did not bind to AR and thus stimulate luciferase production. This leads to the conclusion that BADGE does not possess androgenic or antiandrogenic properties in the MDA-kb2 assay. This result is supported by Satoh et al. (2004) where BADGE did not bind to AR in a stably transfected Chinese hamster ovary cell line.

In order to further explore physiological function of BPA, we quantified expression of *HPGD* gene in LNCaP clone FGC cells after exposure to BPA. We chose LNCaP clone FGC cells due to the fact that they contain numerous endogenous cytosol

and nuclear ARs (Horoszewicz et al., 1983) and express *HPGD*, which is an androgen responsive gene that is up-regulated when exposed to DHT (Ngan et al., 2009). Additionally, *HPGD* expression has been shown to be associated with cancer suppression in several cell lines (discussed below) making it an interesting pathophysiological target for endocrine disruptors. As our MDA-kb2 assay showed that BPA bound to the androgen receptor and acted antagonistically to reduce luciferase production, our hypothesis was that when LNCaP cells are exposed to BPA, it would bind to the AR and therefore decrease production of this androgen responsive gene. To our knowledge, there have been no other studies of *HPGD* expression when exposed to BPA. Our results do show down-regulation of *HPGD* when exposed to BPA; therefore, we believe BPA is acting via the AR to inhibit stimulation of *HPGD* gene.

Before the results of *HPGD* suppression in this study can be fully understood, a brief overview of the arachidonic acid and prostaglandin pathway is necessary. Briefly, prostaglandin endoperoxide (PGH_2) will be synthesized from arachidonic acid catalyzed by cyclooxygenase 1 (COX-1) and 2 (COX-2) (Smith et al., 2000). Cyclooxygenase 1is a housekeeping gene that maintains homeostatic balances (Harris et al., 2002); however, COX-2 is an inducible gene that is up-regulated with inflammatory agents (Smith et al., 2000). Prostaglandin endoperoxide can be converted to various products depending on which synthases are present (Tai et al., 2006). One such product, prostaglandin E_2 (PGE_2) is produced by microsomal PGE synthase (mPGES) found in epithelial cells (Jakobsson et al., 1999). Hydroxyprostaglandin dehydrogenase oxidizes prostaglandins via the 2-step model where prostaglandins are first taken up into the cell and

subsequently cytoplasmically oxidized to metabolites that exhibit greatly reduced biological function (Nomura et al., 2004; Tai et al., 2006).

It has been found that many cancers express high levels of COX-2 and PGE_2 (Bakhle, 2001). PGE_2 stimulates cell proliferation (Sheng et al., 2001), causes local immunosuppression and has been found to promote cancer (Harris et al., 2002). Therefore, catabolism of PGE_2 should decrease tumor growth (Tai et al., 2006). Indeed, numerous cancers often have reduced levels of *HPGD* and it has been discovered that down-regulation of this gene is associated with progression of cancers such as bladder (Celis et al., 1996), lung cancer (Heighway et al., 2002) and colorectal cancer (Myung et al., 2006).

However, a different trend is noted in prostate cancer. Currently, removal of androgens is one of the main treatments of prostate cancers (Vainio et al., 2011). As *HPGD* is an AR regulated gene, it would appear that androgen starvation would have a detrimental effect on gene expression, and thus, negatively impact the treatment. However, Vainio et al. (2011) found that increased *HPGD* levels are associated with advancement of metastatic prostate cancer and *HPGD* expression is necessary for survival and proliferation of these cancer cells. It is even suggested that decreasing levels of *HPGD* might provide alternative means of treatment of prostate cancer (Vainio et al., 2011). Therefore, while in some cancers down-regulation of *HPGD* is associated with progression of the cancer and negative effects, interestingly, in LNCaP clone FGC cells suppression of *HPGD* by BPA may be seen as a positive effect. It is important to note that there are a plethora of genes in cells and this study strictly focuses on one.

Therefore, future studies are necessary to determine broad impacts of BPA exposure on prostate cancer genes.

Endocrine disrupting compounds are found in consumer goods (Jobling et al., 1995; Plotan et al., 2013; Schlumpf et al., 2001) and in the environment (Jobling et al., 1995; Wang et al., 2012; Werner et al., 2010). It has been shown in numerous studies that these compounds can enter the body and cause detrimental effects (Diamanti-Kandarakis et al., 2009). The results of this research show that BPA is able to bind to the androgen receptor and successfully inhibit downstream effects. This capability was also verified by the results of the LNCaP study, where BPA was able to successfully inhibit expression of *HPGD* gene. The results of these *in vitro* studies may be used to determine possible *in vivo* effects of exposure to these compounds.

References

REFERENCES

Al-Hiyasat AS, and Darmani H. 2006. *In vivo* effects of BISGMA – a component of dental composite – on male mouse reproduction and fertility. *Journal of Biomedical Materials Research Part A* 78: 66-72.

Andersen AG, Jensen TK, Carlsen E, Jørgensen N, Andersson AM, Krarup T, Keiding N, and Skakkebæk NE. 2000. High frequency of sub-optimal semen quality in an unselected population of young men. *Human Reproduction* 15: 366-372.

Arditsoglou A and Voutsa D. 2010. Partitioning of endocrine disrupting compounds in inland waters and wastewaters discharged into the coastal are of Thessaloniki, Northern Greece. *Environmental Science and Pollution Research International* 17: 529-538.

Axon A, May F, Gaughan LE, Williams FM, Blain PG, and Wright MC. 2012. Tartrazine and sunset yellow are xenoestrogens in a new screening assay to identify modulators of human oestrogen receptor transcriptional activity. *Toxicology* 298: 40-51.

Bakhle YS. 2001. COX-2 and cancer: a new approach to an old problem. *British Journal of Pharmacology* 134: 1137-1150.

Bonefeld-Jørgensen EC, Long M, Hofmeister MV, and Vinggaard AM. 2007. Endocrine-disruption potential of bisphenol A, bisphenol A dimethacrylate, 4-*n*-nonylphenol, and 4-*n*-octylphenol *in vitro*: new data and a brief review. *Environmental Health Perspectives* 115: 69-76.

Bookout AL, and Mangelsdorf DJ. 2003. Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. *Nuclear Receptor Signaling* 1: e012.

Brodie A, Lu Q, and Long B. 1999. Aromatase and its inhibitors. *Journal of Steroid Biochemistry and Molecular Biology* 69: 205-210.

Carlsen E, Giwercman A, Keiding N, and Skakkebæk NE. 1992. Evidence for decreasing quality of semen during past 50 years. *British Medical Journal* 305: 609-613.

Celis JE, Østergaard M, Basse B, Celis A, Lauridsen JB, Ratz GP, Anderson I, Hein B, Wolf H, Ørntoft TF, and Rasmussen HH. 1996. Loss of adipocyte-type fatty acid binding protein and other protein biomarkers is associated with progression of human bladder transitional cell carcinomas. *Cancer Research* 56: 4782-4790.

Crews, D and McLachlan JA. 2006. Epigenetics, endocrine disruption, health and disease. *Endocrinology* 147: S4-S10.

Cummings AM, Metcalf JL, and Birnbaum L. 1996. Promotion of endometriosis by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in rats and mice: time-dose dependence and species comparison. *Toxicology and Applied Pharmacology* 138: 131-139.

Danzo BJ. 1997. Environmental xenobiotics may disrupt normal endocrine function by interfering with the binding of physiological ligands to steroid receptors and binding proteins. *Environmental Health Perspectives* 105: 294-301.

Datta P and Lundin-Schiller S. 2008. Estrogenicity of the synthetic food colorants tartrazine, erythrosin B, and sudan I in an estrogen-responsive human breast cancer cell line. *Journal of Tennessee Academy of Science* 83: 45-51.

Dhami MSI, Vernon H, Kilagannon R, and Manolis A. 1984. Risks of prenatal exposure to diethylstilbestrol amongst males and females. *Journal of the Canadian Chiropractic Association* 28: 379-383.

Diamanti-Kandarakis E, Bourguignon J-P, Giudici LC, Hauser R, Prins GS, Soto AM, Zoeller RT, and Gore AC. 2009. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocrine Review* 30: 293-342.

Ding Y, Tong M, Liu S, Moscow JA, and Tai H. 2005. NAD+-linked 15-hydroxyprostaglandin dehydrogenase (15-PGDH) behaves as a tumor suppressor in lung cancer. *Carcinogenesis* 26: 65-72.

Doi T, Kajimura K, Takatori S, Fukui N, Taguchi S, and Iwagami S. 2009. Simultaneous measurement of diazolidinyl urea, urea and allantoin in cosmetic samples by hydrophilic interaction chromatography. *Journal of Chromatography B* 877: 1005-1010.

EPA, 2012. Endocrine Disruptor Screening Program (EDSP). http://www.epa.gov/endo/index.htm. Accessed December 2012.

EPA, 2005. Reregistration Eligibility Decision (RED) Document for Erioglaucine and Tartrazine (Aquashade*). http://www.epa.gov/oppsrrd1/REDs/aquashade_red.pdf. Accessed February 2014.

Faqi AS and Chahoud I. 1998. Antiestrogenic effects of low doses of 2,3,7,8-TCDD in offspring of female rats exposed throughout pregnancy and lactation. *Bulletin of Environmental Contamination and Toxicology* 61: 462-469.

Fleisch AF, Sheffield PE, Chinn C, Edelstein BL, and Landrigan PJ. 2010. Bisphenol A and related compounds in dental materials. *Pediatrics* 126: 760-768.

Foster PMD, Cattley RC, and Mylchreest E. 2000. Effects of di-*n*-butyl phthalate (DBP) on male reproductive development in the rat: implications for human risk assessment. *Food and Chemical Toxicology* 38: S97-S99.

Gray Jr. LE and Ostby JS. 1995. *In utero* 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) alters reproductive morphology and function in female rat offspring. *Toxicology and Pharmacology* 133: 285-294.

Guerrero-Bosagna C, Covert TR, Haque M, Settles M, Nilsson EE, Anyway MD, and Skinner MK. 2012. Epigenetic transgenerational inheritance of vinclozolin induced mouse adult onset disease and associated sperm epigenome biomarkers. *Reproductive Physiology* 34: 694-707.

Harris SG, Padilla J, Koumas L, Ray D, and Phipps RP. 2002. Prostaglandins as modulators of immunity. *TRENDS in Immunology* 23: 144-150.

Heighway J, Knapp T, Boyce L, Brennand S, Field JK, Betticher DC, Ratschiller D, Gugger M, Donovan M, Lasek A, and Rickert P. 2002. Expression profiling of primary non-small lung cancer for target identification. *Oncogene* 21: 7749-7763.

Ho S, Tang W, Belmont de Frausto J, and Prins GS. 2006. Developmental exposure to estradiol and bisphenol A increases susceptibility to prostate carcinogenesis and epigenetically regulates phosphodiesterase type 4 variant 4. *Cancer Research* 66: 5624-5632.

Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, Mirand EA, and Murphy GP. 1983. LNCaP model of human prostatic carcinoma. *Cancer Research* 43: 1809-1818.

Hughes Jr. CL. 1988. Phytochemical mimicry of reproductive hormones and modulation of herbivore fertility and phytoestrogens. *Environmental Health Perspectives* 78: 171-175.

Jakobsson P-J, Thorén S, Morgenstern R, and Samuelsson B. 1999. Identification of human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. *Proceedings of the National Academy of Sciences of the United States of America* 96: 7220-7225.

Jin SE, Son YK, Min B, Jung HA and Choi JS. 2012. Anti-inflammatory and antioxidant activities of constituents isolated for Puerarialobata roots. *Archives of Pharmacal Research* 35: 823-837.

Jobling S, Reynolds T, White R, Parker MG, and Sumpter JP. 1995. A variety of environmentally persistent chemicals including some phthalate plasticizers, are weakly estrogenic. *Environmental Health Perspectives* 103: 582-587.

Kinneberg K, Holbech H, Petersen GI, and Bjerregaard P. 2006. Effects of the fungicide prochloraz on the sexual development of zebrafish (Danio rerio). *Comparative Biochemistry and Physiology* 145: 165-170.

Koo HJ and Lee BM. 2004. Estimated exposure to phthalates in cosmetics and risk assessment. *Journal of Toxicology and Environmental Health* 67: 1901-1914.

Layton A, Menn F, and Eldridge M. 2011. Survey of Pharmaceutical and Personal Care Products in Untreated Drinking Water. *Tennessee Department of Environment and Conservation*. 1-37.

Lee HJ, Chattopadhyay S, Gong EY, Ahn RS, and Lee K. 2003. Antiandrogenic effects of bisphenol A and nonylphenol on the function of the androgen receptor. *Toxicological Sciences* 75: 40-46.

Li J and Al-Azzawi F. 2009. Mechanism of androgen receptor action. *Maturitas* 63: 142-148.

Liu Z, Wang X, Lu Y, Du R, Luo G, Wang J, Zhai H, Zhang F, Wen Q, Wu K, and Fan D. 2010. 15-hydroxyprostaglandin dehydrogenase is a tumor suppressor of human gastric cancer. *Cancer Biology & Therapy* 10: 780-787.

Munguia-Lopez EM, Gerardo-Lugo S, Peralta E, Bolumen S, and Soto-Valdez H. 2005. Migration of bisphenol A (BPA) from can coatings into a fatty-food simulant and tuna fish. *Food Additives and Contaminants* 22: 892-898.

Murray TJ, Maffini MV, Ucci AA, Sonnenschein C, and Soto AM. 2007. Induction of mammary gland ductal hyperplasias and carcinoma *in situ* following fetal bisphenol A exposure. *Reproductive Toxicology* 23: 383-390.

Mylchreest E, Sar M, Wallace D, and Foster P. 2002. Fetal Testosterone insufficiency and abnormal proliferation of Leydig cells and gonocytes in rats exposed to di(n-butyl) phthalate. *Reproductive Toxicology* 16: 19-28.

Myung S, Rerko RM, Yan M, Platzer P, Guda K, Dotson A, Lawrence E, Dannenberg AJ, Lovegren AK, Luo G, Pretlow TP, Newman RA, Willis J, Dawson D, and Markowitz SD. 2006. 15-Hydrogxyprostaglandin dehydrogenase is an *in vivo* suppressor of colon tumorigenesis. *Proceedings of the National Academy of Sciences of the United States of America* 103: 12098-12102.

Nakazawa H, Yamaguchi A, Inoue K, Yamazaki T, Kato K, Yoshimura Y, and Makino T. 2002. *In vitro* assay of hydrolysis and chlorohydroxy derivatives of bisphenol A diglycidyl ether for estrogenic activity. *Food and Chemical Toxicology* 40: 1827-1832.

Ngan S, Stronach EA, Photiou A, Waxman J, Ali S, and Buluwela L. 2009. Microarray coupled to quantitative RT-PCR analysis of androgen-regulated genes in human LNCaP prostate cancer cells. *Oncogene* 28: 2051-2063.

Nomura T, Lu R, Pucci ML, and Schuster VL. 2004. The two-step model of prostaglandin signal termination: *in vitro* reconstitution with the prostaglandin transporter and prostaglandin 15 dehydrogenase. *Molecular Pharmacology* 65: 973-978.

Olea N, Pulgar R, Pérez P, Olea-Serrano F, Rivas A, Novillo-Fertrell A, Pedraza V, Soto AM and Sonnenschein C. 1996. Estrogenicity of resin-based composites and sealants used in dentistry. *Environmental Health Perspectives* 104: 298-305.

Ostby JE, Monosson W, Kelce R, and Gray Jr. LE. 1999. Environmental antiandrogens: low doses of the fungicide vinclozolin alter sexual differentiation. *Toxicology and Industrial Health* 15: 48-64.

Parks L, Ostby J, Lambright C, Abbott B, Klinefelter G, Barlow N, and Gray Jr. LE. 2000. The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicological Sciences* 58: 339-349.

Pirard C, Sagot C, Deville M, Dubois N, and Charlier C. 2012. Urinary levels of bisphenol A, triclosan and 4-nonlyphenyl in a general Belgian population. *Environmental International* 48: 78-83.

Plotan M, Frizzell C, Robinson V, Elliot CT, and Connolly L. 2013. Endocrine disruptor activity in bottled mineral and flavoured water. *Food Chemistry* 15: 1590-1596.

Rolland M, Moal JL, Wagner V, Royère D, and Mouzon JD. 2013. Decline in semen concentration and morphology in a sample of 26,609 men close to general population between 1989 and 2005 in France. *Human Reproduction* 28: 462-470.

Roy S, and Kalita JC. 2010. Determination of endocrine disrupting compounds in water bodies around Guwahati City, Assam, India through gas chromatography/mass spectrophotometry. *International Journal of ChemTech Research* 3: 1840-1844.

Satoh K, Ohyama K, Aoki N, Iidia M, and Nagai F. 2004. Study on anti-androgenic effects of bisphenol a diglycidyl ether (BADGE), bisphenol F diglycidyl ether (BFDGE) and their derivatives using cells stably transfected with human androgen receptor, AR-EcoScreen. *Food and Chemical Toxicology* 42: 983-993.

Schlumpf M, Cotton B, Conscience M, Haller V, Steinmann B, and Lichtensteiger W. 2001. *In vitro* and *in vivo* estrogenicity of UV screens. *Environmental Health Perspectives* 109: 239-244.

Sharpe RM, and Skakkebæk NE. 2008. Testicular dysgenesis syndrome: mechanistic insights and potential new downstream effects. *Fertility and Sterility* 89: 33-38.

Sheng H, Shao J, Washington MK, and DuBois RN. 2001. Prostaglandin E_2 increases growth and motility of colorectal carcinoma cells. *The Journal of Biological Chemistry* 276: 18075-18081.

Singleton D, and Kahn SA. 2003. Xenoestrogen exposure and mechanisms of endocrine disruption. *Frontiers in Bioscience* 8: 110-118.

Skakkebæk NE, Rajpert-De Meyts E, and Main KM. 2001. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Human Reproduction* 16: 972-978.

Smith WL, DeWitt DL, and Garavito RM. 2000. Cyclooxygenases: structural, cellular and molecular biology. *Annual Review of Biochemistry* 69: 145-162.

Sultan C, Balaguer P, Terouanne B, Georget V, Paris F, Jeandel C, Lumbroso S, and Nicolas J. 2001. Environmental xenoestrogens, antiandrogens and disorders of male sexual differentiation. *Molecular and Cellular Endocrinology* 178: 99-105.

Swan SH. 2006. Semen quality in fertile US men in relation to geographical area and pesticide exposure. *International Journal of Andrology* 29: 62-68.

Tai H-H, Cho H, Tong M, and Ding Y. 2006. NAD^+- hydroxyprostaglandin dehydrogenase: structure and biological functions. *Current Pharmaceutical Design* 12: 955-962.

Thompson CJ, Ross SM, and Gaido KW. 2004. Di(*n*-butyl) phthalate impairs cholesterol transport and steroidogenesis in the fetal rat testis through a rapid and reversible mechanism. *Endocrinology* 145: 1227-1237.

Vanio P, Gupta S, Ketola K, Mirtti T, Mpindi J-P, Kohonen P, Fey V, Perälä M, Smit F, Verhaegh G, Schalken J, Alanen KA, Kallioniemi, and Iljin K. 2011. Arachidonic acid pathway members *PLA2G7*, *HPGD*, *EPHX2*, and *CYP4F8* identified as putative novel therapeutic targets in prostate cancer. *The American Journal of Pathology* 178: 525-536.

Wang B, Huang B, Jin W, Zhao S, Li F, Hu P, and Pan X. 2012. Occurrence, distribution, and sources of six phenolic endocrine disrupting chemicals in the 22 river estuaries around Dianchi Lake in China. *Environmental Science and Pollution Research International* epub. ahead of print.

Werner JJ, Ouellet D, Cheng CS, Ju YJ, and Law RD. 2010. Pulp and paper mill effluents induce distinct gene expression changes linked to androgenic and estrogenic responses in the fathead minnow (Pimephales promelas). *Environmental Toxicology and Chemistry* 29: 430-439.

Wilson JD, Griffin JE, Leshin F, and George W. 1981. Role of gonadal hormones in the development of the sexual phenotypes. *Human Genetics* 58: 78-84.

Wilson VS, Bobseine K, Lambright CR, and Gray, Jr. LE. 2002. A novel cell line, MDA-kb2, that stably expresses an androgen- and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists. *Toxicological Sciences* 66: 69-81.

Wolf I, O'Kelly J, Rubinek T, Tong M, Nguyen A, Lin BT, Tai H, Karlan BY, and Koeffler HP. 2006. 15-Hydroxyprostoglandin dehydrogenase is a tumor suppressor of human breast cancer. *Cancer Research* 66: 7818-7823.

Wolf Jr. C, Lambright C, Mann P, Price M, Cooper RL, Ostby J, and Gray Jr. LE. 1999. Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethanesulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicology and Industrial Health* 15: 94-118.

Yang Y, Lee S, Kim K, and Hong Y. 2010. Actute testis toxicity of bisphenol A diglycidyl ether in Sprague-Dawley rats. *Journal of Preventive Medicine and*

Ye X, Guo X, Cui X, Zhang X, Zhang H, Wang MK, Qiu L, and Chen S. 2012a. Occurrence and removal of endocrine-disrupting chemicals in wastewater treatment plants in the Three Gorges Reservoir area, Chongqing, China. *Journal of Environmental Monitoring* 14: 2204-2211.

Ye X, Zhou X, L. Wong, and Calafat AM. 2012b. Concentrations of bisphenol A and seven other phenols in pooled sera from 3-11 year old children: 2001-2002 National Health and Nutrition Examination Survey. *Environmental Science and Technology* 46: 12664-12671.

Appendices

Appendix I

Raw Data for All and Tart

Raw Data Allantoin and Tartrazine

	1	2	3	4	5	6	7	8	9	10	11
A	117 0.01% EtOH	188 0.01% EtOH	158 0.01% EtOH	121 0.01% EtOH	192 OHF	161 10 nM All	154 10 nM All	188 10 nM All	121 10 nM All	214 OHF + All	172 OHF + All
B	136 0.02% EtOH	150 0.02% EtOH	148 0.02% EtOH	124 0.02% EtOH	165 OHF	156 100 nM All	121 100 nM All	133 100 nM All	159 100 nM All	207 OHF + All	206 OHF + All
C	179 0.01 nM DHT	143 0.01 nM DHT	122 0.01 nM DHT	146 0.01 nM DHT	190 OHF	159 1 uM All	173 1 uM All	185 1 uM All	158 1 uM All	1061 DHT + All	1037 DHT + All
D	357 0.1 nM DHT	306 0.1 nM DHT	360 0.1 nM DHT	298 0.1 nM DHT	204 OHF	171 10 uM All	162 10 uM All	198 10 uM All	165 10 uM All	1070 DHT + All	1200 DHT + All
E	117 1 nM DHT	1201 1 nM DHT	1090 1 nM DHT	1074 1 nM DHT	344 OHF + DHT	148 10 nM Tart	156 10 nM Tart	150 10 nM Tart	150 10 nM Tart	175 OHF + Tart	163 OHF + Tart
F	1076 10 nM DHT	934 10 nM DHT	1212 10 nM DHT	1285 10 nM DHT	256 OHF + DHT	125 100 nM Tart	138 100 nM Tart	156 100 nM Tart	160 100 nM Tart	123 OHF + Tart	176 OHF + Tart
G	1238 100 nM DHT	1257 100 nM DHT	1031 100 nM DHT	1194 100 nM DHT	247 OHF + DHT	161 1 uM Tart	147 1 uM Tart	179 1 uM Tart	134 1 uM Tart	929 DHT + Tart	817 DHT + Tart
H	176 Blank Well	241 Blank Well	233 Blank Well	277 Blank Well	260 OHF + DHT	157 10 uM Tart	172 10 uM Tart	140 10 uM Tart	163 10 uM Tart	846 DHT + Tart	656 DHT + Tart

Appendix II

Raw Data for Tart and BPA

Raw Data for Tart and BPA

	1	2	3	4	5	6	7	8	9	10	11
A	514 0.01% EtOH	660 0.01% EtOH	674 0.01% EtOH	611 0.01% EtOH	516 OHF	756 10 nM Tart	731 10 nM Tart	475 OHF + Tart	805 10 nM BPA	739 10 nM BPA	275 OHF + BPA
B	615 0.01 nM DHT	889 0.01 nM DHT	1080 0.01 nM DHT	962 0.01 nM DHT	427 OHF	676 100 nM Tart	549 100 nM Tart	312 OHF + Tart	568 100 nM BPA	585 100 nM BPA	184 OHF + BPA
C	1721 0.1 nM DHT	2039 0.1 nM DHT	2479 0.1 nM DHT	2211 0.1 nM DHT	453 OHF	592 1 uM Tart	574 1 uM Tart	344 OHF + Tart	487 1 uM BPA	489 1 uM BPA	250 OHF + BPA
D	2007 1 nM DHT	2664 1 nM DHT	2898 1 nM DHT	2814 1 nM DHT	459 OHF	569 10 uM Tart	541 10 uM Tart	370 OHF + Tart	396 10 uM BPA	353 10 uM BPA	268 OHF + BPA
E	2111 10 nM DHT	2609 10 nM DHT	2670 10 nM DHT	2524 10 nM DHT	1777 OHF + DHT	572 10 nM Tart	508 10 nM Tart	2315 DHT + Tart	549 10 nM BPA	426 10 nM BPA	446 DHT + BPA
F	1967 100 nM DHT	2074 100 nM DHT	2369 100 nM DHT	2301 100 nM DHT	1580 OHF + DHT	556 100 nM Tart	555 100 nM Tart	3005 DHT + Tart	509 100 nM BPA	628 100 nM BPA	433 DHT + BPA
G	446 0.02% EtOH	537 0.02% EtOH	600 0.02% EtOH	558 0.02% EtOH	1497 OHF + DHT	525 1 uM Tart	597 1 uM Tart	2984 DHT + Tart	561 1 uM BPA	436 1 uM BPA	446 DHT + BPA
H	589 Blank Well	770 Blank Well	616 Blank Well	689 Blank Well	1102 OHF + DHT	544 10 uM Tart	594 10 uM Tart	3206 DHT + Tart	530 10 uM BPA	510 10 uM BPA	443 DHT + BPA

Appendix III

Raw Data for All and DBP

Raw Data Allantoin and DBP

	1	2	3	4	5	6	7	8	9	10	11
A	269 0.01% EtOH	353 0.01% EtOH	331 0.01% EtOH	333 0.01% EtOH	257 OHF	330 10 nM All	271 10 nM All	343 10 nM All	594 10 nM All	493 OHF + All	178 OHF + All
B	285 0.02% EtOH	354 0.02% EtOH	354 0.02% EtOH	272 0.02% EtOH	363 OHF	493 100 nM All	356 100 nM All	292 100 nM All	516 100 nM All	531 OHF + All	127 OHF + All
C	825 0.01 nM DHT	557 0.01 nM DHT	495 0.01 nM DHT	436 0.01 nM DHT	286 OHF	426 1 uM All	480 1 uM All	268 1 uM All	513 1 uM All	483 DHT + All	137 DHT + All
D	1310 0.1 nM DHT	1251 0.1 nM DHT	1158 0.1 nM DHT	1367 0.1 nM DHT	276 OHF	354 10 uM All	362 10 uM All	288 10 uM All	641 10 uM All	406 DHT + All	223 DHT + All
E	1936 1 nM DHT	2360 1 nM DHT	1900 1 nM DHT	1714 1 nM DHT	977 OHF + DHT	460 10 nM Di-n	440 10 nM Di-n	2446 10 nM Di-n	529 10 nM Di-n	523 OHF + Di-n	1785 OHF + Di-n
F	3381 10 nM DHT	2368 10 nM DHT	1777 10 nM DHT	2752 10 nM DHT	970 OHF + DHT	483 100 nM Di-n	573 100 nM Di-n	1913 100 nM Di-n	599 100 nM Di-n	448 OHF + Di-n	1268 OHF + Di-n
G	2755 100 nM DHT	2569 100 nM DHT	1776 100 nM DHT	2612 100 nM DHT	698 OHF + DHT	329 1 uM Di-n	370 1 uM Di-n	2028 1 uM Di-n	347 1 uM Di-n	369 DHT + Di-n	999 DHT + Di-n
H	788 Blank Well	979 Blank Well	568 Blank Well	521 Blank Well	772 OHF + DHT	398 10 uM Di-n	363 10 uM Di-n	2195 10 uM Di-n	635 10 uM Di-n	463 DHT + Di-n	1835 DHT + Di-n

Appendix IV

Raw Data for BisDMA and BADGE

Raw Data BisDMA and BADGE

	1	2	3	4	5	6	7	8	9	10	11
A	349 0.01% EtOH	367 0.01% EtOH	335 0.01% EtOH	328 0.01% EtOH	337 OHF	332 10 nM BisDMA	331 10 nM BisDMA	288 10 nM BisDMA	283 10 nM BisDMA	395 OHF + BisDMA	367 OHF + BisDMA
B	299 0.02% EtOH	364 0.02% EtOH	317 0.02% EtOH	272 0.02% EtOH	397 OHF	322 100 nM BisDMA	335 100 nM BisDMA	320 100 nM BisDMA	312 100 nM BisDMA	428 OHF + BisDMA	418 OHF + BisDMA
C	376 0.01 nM DHT	397 0.01 nM DHT	446 0.01 nM DHT	413 0.01 nM DHT	410 OHF	304 1 uM BisDMA	332 1 uM BisDMA	315 1 uM BisDMA	345 1 uM BisDMA	3390 DHT + BisDMA	3125 DHT + BisDMA
D	1307 0.1 nM DHT	1397 0.1 nM DHT	1529 0.1 nM DHT	1441 0.1 nM DHT	454 OHF	376 10 uM BisDMA	392 10 uM BisDMA	345 10 uM BisDMA	410 10 uM BisDMA	3467 DHT + BisDMA	3185 DHT + BisDMA
E	2838 1 nM DHT	3202 1 nM DHT	3537 1 nM DHT	3481 1 nM DHT	1065 OHF + DHT	358 10 nM BADGE	346 10 nM BADGE	376 10 nM BADGE	403 10 nM BADGE	464 OHF + BADGE	358 OHF + BADGE
F	2800 10 nM DHT	3185 10 nM DHT	3418 10 nM DHT	3893 10 nM DHT	1025 OHF + DHT	350 100 nM BADGE	377 100 nM BADGE	334 100 nM BADGE	369 100 nM BADGE	413 OHF + BADGE	341 OHF + BADGE
G	2231 100 nM DHT	3239 100 nM DHT	3149 100 nM DHT	3007 100 nM DHT	1008 OHF + DHT	352 1 uM BADGE	354 1 uM BADGE	339 1 uM BADGE	365 1 uM BADGE	2981 DHT + BADGE	2571 DHT + BADGE
H	673 Blank Well	630 Blank Well	578 Blank Well	650 Blank Well	985 OHF + DHT	361 10 uM BADGE	373 10 uM BADGE	389 10 uM BADGE	379 10 uM BADGE	3122 DHT + BADGE	2771 DHT + BADGE

Appendix V

Raw Data for BisDMA and BADGE

Raw Data for BisDMA and BADGE

	1	2	3	4	5	6	7	8	9	10	11
A	180 0.01% EtOH	127 0.01% EtOH	169 0.01% EtOH	141 0.01% EtOH	175 OHF	126 10 nM BisDMA	121 10 nM BisDMA	150 10 nM BisDMA	125 10 nM BisDMA	218 OHF + BisDMA	182 OHF + BisDMA
B	146 0.02% EtOH	164 0.02% EtOH	125 0.02% EtOH	149 0.02% EtOH	186 OHF	157 100 nM BisDMA	120 100 nM BisDMA	122 100 nM BisDMA	126 100 nM BisDMA	141 OHF + BisDMA	198 OHF + BisDMA
C	99 0.01 nM DHT	155 0.01 nM DHT	146 0.01 nM DHT	171 0.01 nM DHT	196 OHF	159 1 uM BisDMA	153 1 uM BisDMA	146 1 uM BisDMA	152 1 uM BisDMA	957 DHT + BisDMA	966 DHT + BisDMA
D	247 0.1 nM DHT	244 0.1 nM DHT	308 0.1 nM DHT	260 0.1 nM DHT	163 OHF	132 10 uM BisDMA	119 10 uM BisDMA	107 10 uM BisDMA	154 10 uM BisDMA	734 DHT + BisDMA	662 DHT + BisDMA
E	1018 1 nM DHT	1068 1 nM DHT	979 1 nM DHT	1131 1 nM DHT	270 OHF + DHT	164 10 nM BADGE	117 10 nM BADGE	115 10 nM BADGE	142 10 nM BADGE	172 OHF + BADGE	189 OHF + BADGE
F	1186 10 nM DHT	1150 10 nM DHT	1264 10 nM DHT	1342 10 nM DHT	240 OHF + DHT	125 100 nM BADGE	126 100 nM BADGE	141 100 nM BADGE	143 100 nM BADGE	154 OHF + BADGE	158 OHF + BADGE
G	1161 100 nM DHT	1519 100 nM DHT	985 100 nM DHT	1234 100 nM DHT	405 OHF + DHT	183 1 uM BADGE	145 1 uM BADGE	118 1 uM BADGE	136 1 uM BADGE	810 DHT + BADGE	1317 DHT + BADGE
H	58 Blank Well	162 Blank Well	92 Blank Well	141 Blank Well	230 OHF + DHT	131 10 uM BADGE	148 10 uM BADGE	162 10 uM BADGE	144 10 uM BADGE	1043 DHT + BADGE	1017 DHT + BADGE

Appendix VI

Raw Data for DBP and BisGMA

Raw Data DBP and BisGMA

	1	2	3	4	5	6	7	8	9	10	11
A	269 0.01% EtOH	353 0.01% EtOH	331 0.01% EtOH	333 0.01% EtOH	341 OHF	320 10 nM DBP	306 10 nM DBP	324 10 nM DBP	288 10 nM DBP	325 OHF + DBP	321 OHF + DBP
B	285 0.02% EtOH	354 0.02% EtOH	354 0.02% EtOH	272 0.02% EtOH	378 OHF	341 100 nM DBP	301 100 nM DBP	357 100 nM DBP	305 100 nM DBP	406 OHF + DBP	371 OHF + DBP
C	422 0.01 nM DHT	487 0.01 nM DHT	402 0.01 nM DHT	446 0.01 nM DHT	373 OHF	417 1 uM DBP	373 1 uM DBP	370 1 uM DBP	361 1 uM DBP	3241 DHT + DBP	3314 DHT + DBP
D	1552 0.1 nM DHT	1430 0.1 nM DHT	1370 0.1 nM DHT	1531 0.1 nM DHT	437 OHF	368 10 uM DBP	342 10 uM DBP	370 10 uM DBP	420 10 uM DBP	3309 DHT + DBP	2819 DHT + DBP
E	2164 1 nM DHT	2508 1 nM DHT	2676 1 nM DHT	3176 1 nM DHT	1025 OHF + DHT	389 10 nM BisGMA	453 10 nM BisGMA	385 10 nM BisGMA	359 10 nM BisGMA	402 OHF + BisGMA	320 OHF + BisGMA
F	2559 10 nM DHT	2656 10 nM DHT	2742 10 nM DHT	3623 10 nM DHT	1189 OHF + DHT	392 100 nM BisGMA	386 100 nM BisGMA	367 100 nM BisGMA	381 100 nM BisGMA	388 OHF + BisGMA	364 OHF + BisGMA
G	2430 100 nM DHT	2218 100 nM DHT	2388 100 nM DHT	2966 100 nM DHT	1037 OHF + DHT	425 1 uM BisGMA	394 1 uM BisGMA	359 1 uM BisGMA	341 1 uM BisGMA	3353 DHT + BisGMA	3280 DHT + BisGMA
H	542 Blank Well	554 Blank Well	571 Blank Well	555 Blank Well	1033 OHF + DHT	411 10 uM BisGMA	387 10 uM BisGMA	330 10 uM BisGMA	383 10 uM BisGMA	2702 DHT + BisGMA	3293 DHT + BisGMA

Appendix VII

Raw Data for BPA and BisGMA

Raw Data for BPA and BisGMA

	1	2	3	4	5	6	7	8	9	10	11
A	145 0.01% EtOH	185 0.01% EtOH	167 0.01% EtOH	118 0.01% EtOH	171 OHF	120 10 nM BPA	174 10 nM BPA	180 10 nM BPA	157 10 nM BPA	208 OHF + BPA	224 OHF + BPA
B	132 0.02% EtOH	196 0.02% EtOH	117 0.02% EtOH	146 0.02% EtOH	208 OHF	154 100 nM BPA	129 100 nM BPA	130 100 nM BPA	134 100 nM BPA	119 OHF + BPA	215 OHF + BPA
C	106 0.01 nM DHT	144 0.01 nM DHT	147 0.01 nM DHT	143 0.01 nM DHT	174 OHF	141 1 uM BPA	176 1 uM BPA	121 1 uM BPA	149 1 uM BPA	401 DHT + BPA	415 DHT + BPA
D	337 0.1 nM DHT	377 0.1 nM DHT	287 0.1 nM DHT	360 0.1 nM DHT	192 OHF	142 10 uM BPA	130 10 uM BPA	113 10 uM BPA	122 10 uM BPA	457 DHT + BPA	396 DHT + BPA
E	1030 1 nM DHT	1004 1 nM DHT	907 1 nM DHT	1262 1 nM DHT	251 OHF + DHT	129 10 nM BisGMA	186 10 nM BisGMA	123 10 nM BisGMA	145 10 nM BisGMA	160 OHF + BisGMA	163 OHF + BisGMA
F	1119 10 nM DHT	1233 10 nM DHT	1306 10 nM DHT	1181 10 nM DHT	249 OHF + DHT	130 100 nM BisGMA	110 100 nM BisGMA	129 100 nM BisGMA	111 100 nM BisGMA	142 OHF + BisGMA	126 OHF + BisGMA
G	1543 100 nM DHT	1347 100 nM DHT	1116 100 nM DHT	1189 100 nM DHT	265 OHF + DHT	128 1 uM BisGMA	129 1 uM BisGMA	115 1 uM BisGMA	130 1 uM BisGMA	655 DHT + BisGMA	754 DHT + BisGMA
H	135 Blank Well	240 Blank Well	208 Blank Well	133 Blank Well	228 OHF + DHT	179 10 uM BisGMA	114 10 uM BisGMA	152 10 uM BisGMA	89 10 uM BisGMA	872 DHT + BisGMA	1130 DHT + BisGMA

Appendix VIII

Raw Data for DBP and BPA

Raw Data DBP and BPA

	1	2	3	4	5	6	7	8	9	10	11
A	114 0.01% EtOH	109 0.01% EtOH	103 0.01% EtOH	86 0.01% EtOH	134 OHF	106 10 nM DBP	100 10 nM DBP	91 10 nM DBP	111 10 nM DBP	128 OHF + DBP	121 OHF + DBP
B	127 0.02% EtOH	109 0.02% EtOH	106 0.02% EtOH	113 0.02% EtOH	122 OHF	89 100 nM DBP	101 100 nM DBP	117 100 nM DBP	1437 100 nM DBP	632 OHF + DBP	726 OHF + DBP
C	125 0.01 nM DHT	136 0.01 nM DHT	144 0.01 nM DHT	111 0.01 nM DHT	139 OHF	114 1 uM DBP	122 1 uM DBP	103 1 uM DBP	675 1 uM DBP	1013 DHT + DBP	1262 DHT + DBP
D	316 0.1 nM DHT	277 0.1 nM DHT	284 0.1 nM DHT	341 0.1 nM DHT	156 OHF	100 10 uM DBP	95 10 uM DBP	103 10 uM DBP	133 10 uM DBP	1419 DHT + DBP	1020 DHT + DBP
E	423 1 nM DHT	1065 1 nM DHT	956 1 nM DHT	688 1 nM DHT	212 OHF + DHT	111 10 nM BPA	97 10 nM BPA	93 10 nM BPA	102 10 nM BPA	155 OHF + BPA	155 OHF + BPA
F	1320 10 nM DHT	1254 10 nM DHT	1305 10 nM DHT	1428 10 nM DHT	267 OHF + DHT	122 100 nM BPA	81 100 nM BPA	108 100 nM BPA	107 100 nM BPA	123 OHF + BPA	111 OHF + BPA
G	1450 100 nM DHT	1002 100 nM DHT	1088 100 nM DHT	743 100 nM DHT	147 OHF + DHT	143 1 uM BPA	106 1 uM BPA	106 1 uM BPA	108 1 uM BPA	407 DHT + BPA	335 DHT + BPA
H	179 Blank Well	152 Blank Well	172 Blank Well	147 Blank Well	210 OHF + DHT	118 10 uM BPA	97 10 uM BPA	79 10 uM BPA	83 10 uM BPA	408 DHT + BPA	300 DHT + BPA

Appendix IX

Raw Data for Viability Test on MDA-kb2 Cells

Treatment	Dead			Alive					Mean	STD
	1	2	3	1	2	3	Total	%Dead	% Dead	% Dead
0.01% EtOH	4	8	3	102	68	58	243	2.590674		
	9	15	7	224	225	252	732	5.354059		
	11	4	10	138	220	161	544	4.317789	4.087507	1.396011
1nM DHT	3	4	4	200	96	54	361	1.899827		
	8	13	7	245	261	243	777	4.835924		
	11	4	6	224	228	242	715	3.626943	3.454231	1.475648
10µM BPA	5	7	10	206	190	161	579	3.799655		
	6	10	2	185	248	100	551	3.108808		
	8	8	24	170	175	262	647	6.908463	4.605642	2.023995
10µM DBP	3	6	3	143	195	178	528	2.072539		
	8	9	5	161	151	145	479	3.799655		
	7	10	19	215	185	286	722	6.217617	4.029937	2.082112

Appendix X

Raw Data for *HPGD* Expression - Replicate 1

1

	Ave Beta Actin Ct	HPGD Ct	ΔCt	Analysis: ΔCt control average	ΔΔCt	Fold induction
EtOH	22.14333	32.19	10.04667	10.01333	0	0.977159968
		32.08	9.936667		0	1.05457863
		32.2	10.05667		0	0.970410231
DHT	24.22	33.34	9.12		-0.89333	1.85746282
		32.8	8.58		-1.43333	2.700699892
		33	8.78		-1.23333	2.351095813
OHF	22.43667	32.02	9.583333		-0.43	1.347233577
		32.03	9.593333		-0.42	1.337927555
	-	32.09	9.653333		-0.36	1.283425898
DHT+OHF	22.65667	32.53	9.873333		-0.14	1.101905116
		32.77	10.11333		0.1	0.933032992
		32.55	9.893333		-0.12	1.086734863
BPA	22.27667	32.09	9.813333		-0.2	1.148698355
		32.3	10.02333		0.01	0.993092495
		32.07	9.793333		-0.22	1.164733586
BPA + DHT	23.05333	33.06	10.00667		-0.00667	1.004631674
		32.58	9.526667		-0.48667	1.401203665
		32.81	9.756667		-0.25667	1.194715135

Appendix XI

Raw Data for *HPGD* Expression - Replicate 2

2

	Ave Beta Actin Ct	HPGD Ct	ΔCt	Analysis: ΔCt control average	ΔΔCt	Fold Induction
EtOH	26.29333	36.71	10.41667	10.28333	0	1.09682498
		36.07	9.776667		0	0.703846792
		36.95	10.65667		0	1.295342252
DHT	24.56333	34.24	9.676667		-0.60667	1.522736872
		34.55	9.986667		-0.29667	1.228303149
		35.3	10.73667		0.453333	0.730353422
OHF	23.55667	34.57	11.01333		0.73	0.602903914
		33.92	10.36333		0.08	0.946057647
		34.53	10.97333		0.69	0.61985385
DHT + OHF	27.08	37.13	10.05		-0.23333	1.175547906
		36.03	8.95		-1.33333	2.5198421
		0	0		-10.2833	1246.211598
BPA	24.35667	35.29	10.93333		0.65	0.637280314
		35.77	11.41333		1.13	0.456915725
		35.11	10.75333		0.47	0.721964598
BPA + DHT	26.71333	missing	missing		missing	missing
		missing	missing		missing	missing
		missing	missing		missing	missing

Appendix XII

Raw Data for *HPGD* Expression - Replicate 3

3

	Ave Beta Actin Ct	HPGD Ct	ΔCt	Analysis: ΔCt control average	ΔΔCt	Fold Induction
EtOH	21.34667	32.89	11.54333	11.49	0	0.963707118
		32.67	11.32333		0	0.890898718
		32.95	11.60333		0	1.081724666
DHT	19.82	29.15	9.33		-2.16	4.469148552
		29.28	9.46		-2.03	4.084048503
		29.18	9.36		-2.13	4.377174805
OHF	20.13667	28.15	8.013333		-3.47667	11.13219875
		28.12	7.983333		-3.50667	11.36610991
		28.11	7.973333		-3.51667	11.44516746
DHT + OHF	20.40667	28	7.593333		-3.89667	14.89407546
		27.97	7.563333		-3.92667	15.20703164
		28.02	7.613333		-3.87667	14.68902432
BPA	20.28333	31.8	11.51667		0.026667	0.981685855
		31.52	11.23667		-0.25333	1.191957944
		31.77	11.48667		-0.00333	1.002313162
BPA + DHT	20.37	31.82	11.45		-0.04	1.028113827
		31.57	11.2		-0.29	1.222640278
		31.62	11.25		-0.24	1.180992661