ESTROGENIC ACTIVITY OF THE POLYBROMINATED DIPHENYL ETHER FLAME RETARDANT MIXTURE DE-71

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ABSTRACT

Minerva Mercado-Feliciano

ESTROGENIC ACTIVITY OF THE POLYBROMINATED DIPHENYL ETHER FLAME RETARDANT MIXTURE DE-71

Polybrominated diphenyl ethers (PBDEs) are widely used flame retardants suspected to act as endocrine disruptors. We tested the commercial PBDE mixture DE-71 and its in vivo metabolites for estrogenic activity. MCF-7 breast cancer cells culture, ERE-luciferase gene expression, ^{3}H-β-estradiol displacement from recombinant ERα, and ovariectomized (OVX) mice served as bioassays. Although DE-71 did not bind ERα, it was able to increase MCF-7 cell proliferation and this was prevented by the antiestrogen fulvestrant. DE-71 co-treatment reduced the effect of estradiol in MCF-7 cells. In the OVX mouse (BALB/c) 3-day assay, DE-71 administered alone had no effect on uterine or vaginal tissues but when administered subcutaneously potentiated estradiol's effect on uterine weight in a dose-dependent manner. DE-71 administered SQ to BALB/c mice for 34 days slightly increased uterine epithelial height (UEH), vaginal epithelial thickness (VET) and mammary ductal lumen area, and attenuated the estradiol-induced increase in UEH; these effects were not seen in C57BL/6 mice. DE-71 increased liver weight in BALB/c, C57BL/6 and estrogen receptor-alpha knockout (ERαKO) mice. Liver cytochrome P450 1A (CYP1A) and CYP2B activities increased 2.5-fold and 7-fold respectively when DE-71 was administered PO, but only CYP2B increased (5-fold) after SQ treatment. Six OH-PBDE metabolites were found in mice after 34-day DE-71 treatment and all were able to bind recombinant ERα. *Para*-hydroxylated metabolites

displayed a 10- to 30-fold higher affinity for ERα compared to *ortho*-hydroxylated PBDEs. *Para*-OH-PBDEs induced ERE-luciferase and produced an additive effect when coadministered with β-estradiol. DE-71 was also additive with β-estradiol. At high concentrations ($\geq 5x10^{-5}$ M), *ortho*-OH-PBDEs were antiestrogenic in the ERE-luciferase assay. In conclusion, DE-71 behaves as a weak estrogen in both MCF-7 breast cancer cells and ovariectomized adult mice. Mice strain, treatment route and duration determined if DE-71 was estrogenic. BALB/c mice are more susceptible to DE-71 effects in estrogen target tissues than C57BL/6 mice. DE-71 increased liver weight, 5%-51% depending on mouse strain and treatment regime, independently of ERα. The observations that the DE-71 mixture does not displace ^{3}H-β-estradiol from ERα while the hydroxylated metabolites do, suggest that the cellular and tissue effects were due to a metabolic activation of individual congeners.

Robert M. Bigsby, Ph.D., Chair

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List of Abbreviations

AF-1	activation function 1
AF-2	activation function 2
AhR	aryl hydrocarbon receptor
BDE-47	2,2',4,4'-tetrabrominated diphenyl ether
BDE-99	2,2',4,4', 5-pentabrominated diphenyl ether
BDE-153	2,2',4,4', 5,5'-hexabrominated diphenyl ether
BGS	bovine growth serum
BPL	branches per duct length
BM	basal media
CAR	constitutive androstane receptor
CNS	central nervous system
csBGS	charcoal-stripped bovine growth serum
CYP11A1	P450-linked side chain cleaving enzyme
CYP17A	17α-hydroxylase/17,20-lyase
CYP19	aromatase
CYP450	cytochrome P450
2,4-DBP	2,4-dibromophenol
Δ	greek letter delta, indicates change in a measurement
DEH	ductal epithelial height
DES	diethylstilbestrol
DLA	ductal lumen area

DMSO	Dimethyl sulphoxyde
E2	1,3,5[10]estratriene-3,17-β-diol; β-estradiol
EB	β-estradiol-3-benzoate
EC50	effective concentration for 50% of maximal effect
ER	estrogen receptor
ERα	estrogen receptor alpha
ERαKO	ERα-negative, ERα-knockout
ERβ	estrogen receptor beta
ERE	estrogen response element
ERK1/2	extracellular signal-regulated kinases 1 and 2, MAPK
EROD	7-ethoxyresorufin O-dealkylation
FPUF	flexible polyurethane foam
FSH	follicle-stimulating hormone
GM	growth medium
G6PD	glucose-6-phosphate dehydrogenase
IC50	inhibitory concentration for 50% inhibition
ICI	ICI182780, fulvestrant
3β-HSD	3β-hydroxtsteroid dehydrogenase
17β-HSD	17β-hydroxtsteroid dehydrogenase
LH	luteinizing hormone
MEM	minimum essential media
miRNA	micro RNA
mg/kg	miligrams per kilogram, 10^{-3} gram per 10^{3} grams

mL	milliliters, 10^{-3} liters
mM	millimolar, 10^{-3} moles per liter
MTT	3-(4,5-dimethylthiazolyl-2)2,5-diphenyltetrazolium bromide
$NADP^{+}$	β-nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced form
n	number of animals or plated wells per treatment group
nm	nanometer, 10^{-9} meters
nM	nanomolar, 10^{-9} mole per liters
4′-OH-BDE-17	4′-hydroxy-2,2′,4-tribromodiphenyl ether
2′-OH-BDE-28	2′- hydroxy-2,4,4′- tribromodiphenyl ether
4-OH-BDE-42	4- hydroxy-2,2′,3,4′-tetrabromodiphenyl ether
3-OH-BDE-47	3- hydroxy-2,2′,4,4′- tetrabromodiphenyl ether
6-OH-BDE-47	6- hydroxy-2,2′,4,4′- tetrabromodiphenyl ether
4′-OH-BDE-49	4′- hydroxy-2,2′,4,5′- tetrabromodiphenyl ether
OVX	ovariectomized
PBDE	polybrominated diphenyl ether
pM	picomolar, 10^{-12} mole per liter
PO	per oral, oral gavage
PROD	7-pentoxyresorufin O-dealkylation
PXR	pregnane X receptor
RLU	relative light units
SEM	standard error of the mean
SERM	selective estrogen receptor modulator

SQ	subcutaneous injection
2,4,5-TBP	2,4,5-tribromophenol
UEH	uterine epithelial height
µg/kg	micrograms per kilogram, 10^{-6} gram per 10^{3} grams
µL	microliters, 10^{-6} liter
µm	micrometers, 10^{-6} meter
µM	micromolar, 10^{-6} mole per liter
VDR	vitamin D receptor
VET	vaginal epithelial thickness
WT	wild type
4X	magnified 4 times its normal size
40X	magnified 40 times its normal size

Introduction

Estrogens are hormones best known for their roles in female reproductive physiology (Ganong 2007). They are partly responsible for development of secondary sex characteristics, reproductive cycle regulation, pregnancy maintenance, and lactation (Ganong 2007; Albrecht et al. 2000; Albrecht and Pepe 2003; Buhimschi 2004). But estrogens also play a major role in many non-reproductive tissues in both sexes, including bone, prostate, and the central nervous system (CNS). While normal levels of endogenous estrogens have an important physiological role in tissues, disruption of estrogen homeostasis may lead to disease. For instance, estrogens may act as tumor promoters by inducing cancer cell growth. The successful use of antiestrogens in the treatment and prevention of some breast cancers demonstrates the role of estrogens in cancer etiology.

Like other steroids, estrogens are highly lipophilic chemicals that readily cross the cell membrane. Once inside the cell, estrogens bind soluble estrogen receptors (ERα and ERβ) which mediate their effects on gene transcription. Recent work with estrogen conjugated to bovine serum albumin suggest the existence of receptors on the cell membrane that can also be activated by estrogens (Haynes et al. 2002; Bernard et al. 2006).

Over the last 30 years, the scientific community has recognized the threat of hormonally active pollutants in the environment (Kavlock et al. 1996; Cecil et al. 1971). Even before

that, Rachel Carson pointed out the possible link between increased hormone concentrations, due to liver damage by organochlorines and impaired endogenous hormone catabolism, and cancer (Carson 1962). And while the mammalian endocrine system is comprised of many different secreting organs and hormones, concern has been focused on estrogenic substances.

The term "estrogenic" describes a chemical that causes the same effects as endogenous estrogens in animals. Historically such effects have been defined mainly as proliferation of estrogen-responsive tissues like the endometrium, vagina and mammary gland, and induction of the estrous phase of the animal reproductive cycle. Such estrogenic effects have been studied at length and are still used as outcomes in assays to test the estrogenicity of a chemical. As explained in the following sections, we now know that animals can have estrogenic effects in target tissues outside the female reproductive tract, and in either gender. While "estrogenic" is an operationally defined concept, estrogenic effects are mediated by specific physiological mechanisms like receptor activation and gene expression.

General Estrogen Physiology

Estrogens promote development and maintenance of female reproductive organs (DeMayo et al. 2002; Drummond 2006), mammary glands (Clarke 2006) and secondary

sex characteristics, such as adipose tissue distribution (Bjorntorp 1997). During pregnancy, estrogen contributes to uterine growth (Hertelendy and Zakar 2004), placental development (Bazer et al. 1979), parturition (Uldbjerg and Ulmsten 1990), and preparation of the mammary gland for lactation (Neville et al. 2002).

At puberty, sex steroids induce a pubertal growth spurt. Much of the growth acceleration appears to be mediated by both direct estrogen action and estrogen-induced stimulation of other hormonal signaling (reviewed by Nilsson et al. 2005). While androgen also contributes to the pubertal growth spurt, its effects may require conversion to estrogen at the target tissues. Estrogen also acts by advancing growth plate senescence, causing proliferative exhaustion, thus stopping longitudinal bone growth. Disorders of estrogen homeostasis can therefore cause either premature end of growth (abnormally short stature) or excessive growth (abnormally tall stature).

Recent research in rodents suggests that estrogens may play a role in prostate development (reviewed by Prins et al. 2006). While the role of endogenous estrogens is not yet known, there is much evidence of the adverse effect of exogenous estrogens; exposure of rodents to estrogens early in life is associated with increased proliferation, inflammation and dysplastic epithelial changes in the prostate later in life (Harkonen and Makela 2004).

In the developing CNS, estrogens are crucial in determining gender dimorphism. The gender-specific brain is produced by the epigenetic action of gonadal hormones at critical

periods of brain development (reviewed by Carrer and Cambiasso 2002). While the female brain morphology develops in the absence of testicular secretions, irrespective of chromosomal sex, many of the masculinizing actions of androgen on the brain require conversion of testosterone to estrogen. Moreover, the diversity of estrogen effects on the adult brain implies a role beyond the control of reproductive function, since estrogen signaling is known to influence memory, motor activity, and mood (reviewed by McEwen and Alves 1999). Moreover, as the brain ages, it undergoes biochemical and structural changes regulated by estrogen (reviewed by Thakur and Sharma 2006).

In mammals, there are three primary endogenous estrogens: β-estradiol, estrone and estriol. Each has different potencies and physiological roles (reviewed by Coelingh Bennink 2004). β-Estradiol, the most potent, is the predominant estrogen in premenopausal women, secreted mainly by the ovaries. Estrone, less potent than β-estradiol, is the main estrogen in postmenopausal women, synthesized in adipose tissue from adrenal precursors. Estriol, the weakest of the three natural estrogens, is produced in large quantities by the placenta.

Estrogens Role in Female Reproductive Physiology

The blood concentration of estrogens varies throughout the life of the human female. Estrogen levels are less than 10 pM in prepubescent girls (Bay et al. 2004), and increase throughout puberty as pituitary hormones stimulate production in the ovaries (Apter 1997). In the adult premenopausal woman, plasma estrogen levels fluctuate around 0.3-

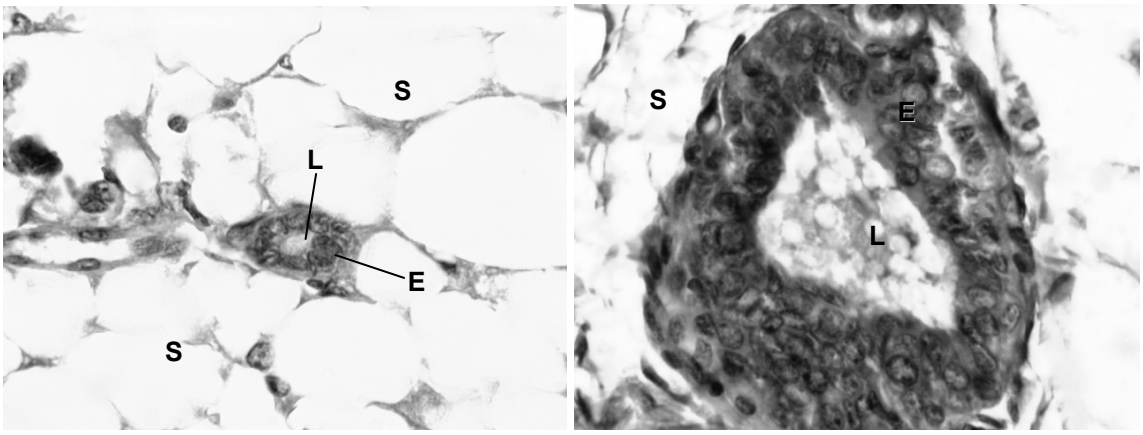
2.6 nM (Gruber et al. 2002). After menopause, estrogen levels decrease to levels below 0.1 nM (Setiawan et al. 2006).

Estrogens induce many physiological changes in the mammalian female reproductive tract and mammary glands. The effects of estrogens on mammalian female physiology have been studied extensively in the ovariectomized (OVX) adult mouse model, and such findings are outlined in this section as they relate to human physiology.

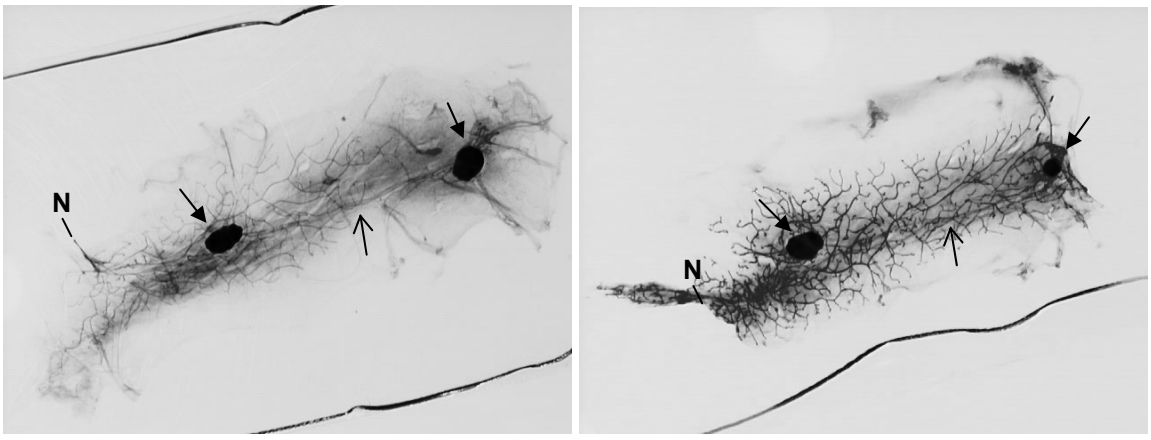
In pubertal girls, the first organ to show obvious signs of development is the mammary gland. In the OVX mouse model, β-estradiol increases mammary duct size, and branching (Raafat et al. 2001), however estrogen effects on the mammary gland may require the presence of other hormones and/or growth factors (reviewed by Sternlicht 2006 and Silberstein 2001). At the cellular level, the mammary gland ductal epithelium of the OVX mouse is composed of a single cuboidal cell layer, which becomes pseudo-stratified after estrogen treatment (Figure 1, A and B). The resulting increased thickness of mammary ducts can be observed in mammary gland whole mounts at low power (Figure 1, C and D).

Figure 1. Mammary gland changes in OVX BALB/c mouse after 34 days of β-estradiol treatment. Adult mice were ovariectomized and 3 weeks later a 34-day treatment schedule was started. Animals received treatment, vehicle or 10 μg/kg β-estradiol (sc) daily. After 34 days their inguinal mammary glands were dissected out and processed for whole mount observation and histological examination. Cross sections of inguinal mammary gland (A and B) were stained with hematoxylin and eosin and are shown at 40X magnification. Whole mounts of inguinal mammary gland (C and D) were stained with carmine alumn and are shown at a 4X magnification. A and C: Vehicle-treated. B and D: β-Estradiol-treated. N, nipple; L, lumen; E, epithelium; S, stroma. The large round structures in C and D are lymph nodes (indicated by closed arrows); they are connected by one large blood vessel (lighter shade of gray when compared with darker ducts; indicated by open arrows).

A. B.



C. D.



As estrogen levels increase gradually during puberty, they stimulate changes in uterine size and shape (Buzi et al. 1998), as well as proliferation of the blood-rich mucus membrane lining the uterus (endometrium), leading to the first menstruation (Strauss and Coutifaris 1999). Throughout a woman's adult life, the endometrium responds to cyclic hormone secretion by the ovaries (Critchley et al. 2001), triggered by the recurring process of maturation and release of an oocyte from the ovary (ovulation). During the preovulatory phase, the ovaries increase estrogen secretion (Baird and Fraser 1974), which promotes growth of the endometrium (Brenner 1994). Another hormone secreted by the ovaries after ovulation, progesterone, inhibits further growth (King et al. 1978) and causes additional changes that make the endometrium suitable for implantation and nourishment of an embryo (Brosens and Gellersen 2006).

At the cellular level, estrogen promotes cell proliferation and inhibits apoptosis in the uterus and vagina (Evans et al. 1990; Jo et al. 1993; Berman et al. 1998). Epithelial cell layers regress and/or decrease in height after ovariectomy, while estrogen treatment of OVX mice increases uterine epithelial height (UEH) and vaginal epithelial thickness (VET) after a few days of estrogen treatment (Suzuki et al. 1996). In the uterus, the columnar epithelial cells become taller with a concomitant increase in cytoplasmic volume. The epithelial cells proliferate in response to estrogen causing overcrowding and a pseudostratified appearance (Figure 2). In the vagina, the single squamous epithelial layer of the OVX mouse becomes a multi-cell layer after estrogen treatment (Figure 3).

Figure 2. Hematoxylin and eosin staining of OVX BALB/c mouse uterus cross sections. Cross sections of uterus from control vehicle or β-estradiol treated adult female OVX mice were stained with hematoxylin and eosin. A: Control, treated with vehicle for 3 days; uteri of mice treated with vehicle for 34 days had similar appearance (not shown). B: 3-day treatment, 10 μg/kg β-estradiol. C: 3-day treatment, 10 mg/kg β-estradiol; notice the fluid-filled stroma (S). D: 34-day treatment ,10 μg/kg β-estradiol treated; notice leukocyte-infiltrated stroma. S, stroma; L, lumen; E, epithelium. All photos taken at the same 40X magnification. Bars indicate epithelial thickness.

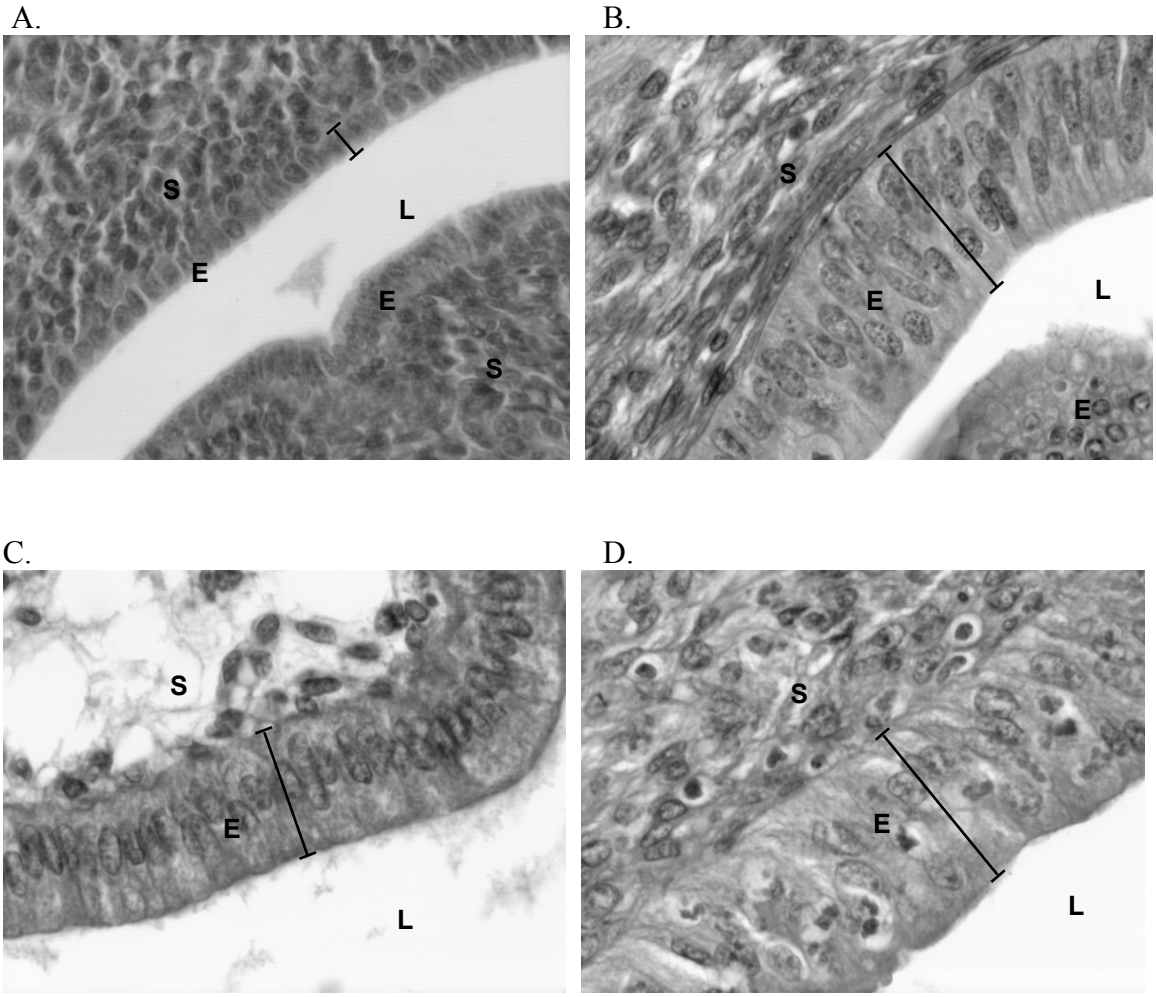
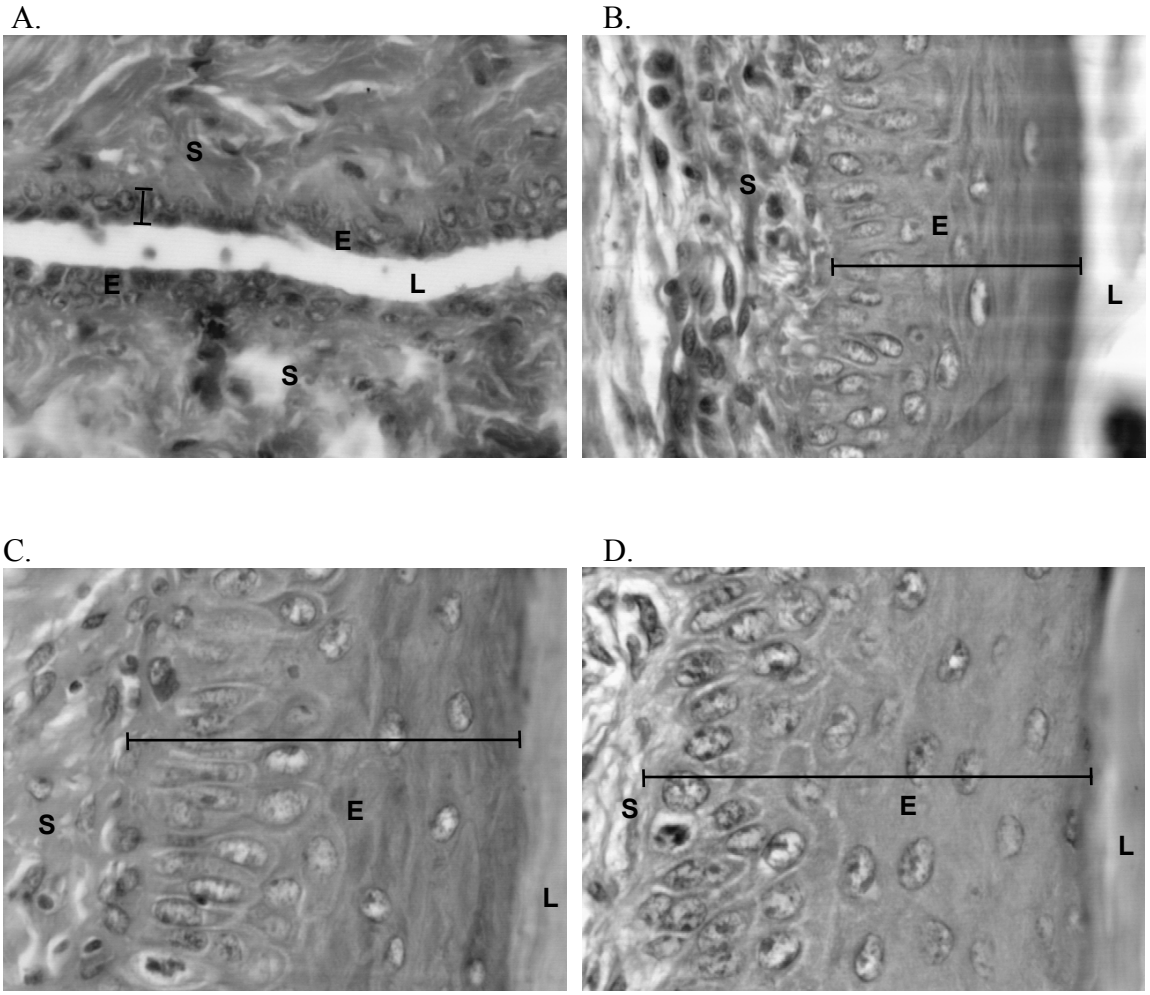


Figure 3. Hematoxylin and eosin staining of OVX mouse vagina cross sections. Cross sections of vagina from treated mice were stained with hematoxylin and eosin. A: Control, treated with vehicle for 3 days; vagina of mice treated with vehicle for 34 days had similar appearance (not shown). B: 3-day treatment, 10 μg/kg β-estradiol. C: 3-day treatment, 10 mg/kg β-estradiol. D: 34-day treatment ,10 μg/kg β-estradiol treated. Keratinized epithelial cells shed from near epithelium can be seen in lumen in B, C and D. S, stroma; L, lumen; E, epithelium. All photos taken at the same 40X magnification. Bars indicate epithelial thickness.



If there is no pregnancy, estrogen and progesterone withdrawal initiates the human endometrium into a degenerative phase, ending in menstruation (Brosens and Gellersen 2006). In the OVX mouse model, the uteri and vagina regress after ovariectomy (Suzuki et al. 1996) while estrogen treatment increases their wet weight in as few as 3 days of treatment (Evans et al. 1941; Gordon et al. 1986). This uterotrophic effect has been used as the standard bioassay for testing estrogenic action of compounds (Evans et al. 1941; Odum et al. 1997).

Maternal serum estrogen levels increase during pregnancy to peak at term (Darne et al. 1987). In the primate placenta, estrogen stimulates production of progesterone, which is required to maintain pregnancy, and enhances cortisone production, needed for maturation of the fetal hypothalamic-pituitary-adrenocortical axis (reviewed by Pepe and Albrecht 1998). Changes in the estrogen/progesterone balance at term favors cervical ripening and increased uterine activity thereby contributing to parturition (reviewed by Steer 1990).

Cessation of ovarian cycles after menopause brings about a major change in female hormonal homeostasis. The postmenopausal woman estrogen levels drop from 0.3-2.6 nM to about 0.1 nM (Setiawan et al. 2006). Among a variety of physiological changes due to the decreased estrogen levels, the most detrimental consequence is loss of bone mass and development of osteoporosis (NAMS 2006).

The proliferative and anti-apoptotic effects of estrogen in female reproductive organs also play a role in cancer etiology. Women exposed to estrogens without opposing effects of progestins show a dose and duration dependent 2- to 10-fold increase in endometrial cancer risk (reviewed by Hecht and Mutter 2006). Tamoxifen, a selective estrogen receptor modulator (SERM), has been successfully used in the treatment and prevention of breast cancer for many years (Jordan 1995). While most normal mammary epithelial cells have no estrogen receptors and depend on stromal interactions in their response to estrogen, carcinomas usually have estrogen receptors and estrogens induce their growth (Hahnel and Twaddle 1971; Welsch et al. 1981; Osborne et al. 1985). Two 4-hydroxylated metabolites of tamoxifen, 4-hydroxy-tamoxifen (4-OH-tamoxifen) and 3-hydroxy-*N*-desmethyl-tamoxifen (endoxifen) bind-to ERα and act as antagonists in mammary tissue, causing regression of ER-positive mammary tumors (Jordan 1995; Lim et al. 2005). On the other hand, 4-OH-tamoxifen behaves as an agonist in the uterus, stimulating epithelial cells and increasing the risk of cancer (Fotiou et al. 2000). The mechanism by which 4-OH-tamoxifen and other SERMs behave as estrogen agonists in some tissues and antagonists in others is not know, but a model has been proposed in which the differential expression of estrogen receptors (ERα or ERβ) and specific coregulator proteins are responsible for the differential responses in different tissues (McDonnell et al. 2001).

Metabolism and Regulation of Endogenous Estrogens

Endogenous estrogens are steroid molecules, derived from cholesterol (reviewed by Payne and Hales 2004). They are produced primarily by the ovaries, but also from conversion of adrenal androgens to estrogens in adipose and other tissues. The steroid synthetic pathway can produce three different estrogen species from progesterone or androgen precursors: β-estradiol, estrone and estriol. The cytochrome P450 (CYP450) enzymes play a major role in both the formation and deactivation of endogenous estrogens.

The first step in steroid synthesis is the removal of a six-carbon unit from cholesterol's side chain to form pregnenolone, catalyzed by P450-linked side chain cleaving enzyme (P450ssc, CYP11A1, also known as desmolase). CYP11A1 is found in the mitochondria of steroid-producing cells, but not in significant quantities in other cells; Follicle Stimulating Hormone (FSH), a peptide hormone from the anterior pituitary gland, stimulates cAMP production, which in turns signals for CYP11A1 expression and ultimately turns on the conversion of cholesterol into pregnenolone (reviewed by Payne and Hales 2004). Different pituitary hormones stimulate expression in different tissues: Adrenocorticotropic Hormone (ACTH, or corticotropin) in the adrenal gland, Luteinizing Hormone (LH) in the ovary and testis, and FSH in ovarian granulosa cells. In fact, expression of all other steroidogenic CYP450 enzymes (discussed below) and cholesterol uptake by the ovarian cells are stimulated by the the pituitary hormones FSH and LH.

Through several hydroxylating/dehydroxylating steps, pregnenolone is converted to androgens, either directly or with progesterone as an intermediate. Three different enzymes are involved in these reactions, with the same enzyme acting at different stages to produce the different androgens or progesterone: 3β-hydroxysteroid dehydrogenase (3β-HSD), 17α-hydroxylase/17,20-lyase (P450c17, CYP17A), and 17β-hydroxysteroid dehydrogenase (17β-HSD). While CYP11A1 catalysis is the rate-limiting step in steroid production, the presence of these three later enzymes in a tissue determine the production of sex steroids rather than corticoid steroids (reviewed by Miller 2002).

3β-HSD, CYP17A and 17β-HSD expression and/or activity are stimulated by increasing cAMP. 3β-HSD increases with both FSH and IGF-1 in female rats and LH in male rats, while 17β-HSD is primarily induced by FSH acting via the cAMP (reviewed by Payne and Hales 2004). The 3β-HSD and CYP17A androgenic reactions are irreversible, while those catalyzed by 17β-HSD are reversible. 3β-HSD is located in the mitochondrion; CYP17A and 17β-HSD are mostly microsomal. 3β-HSD and 17β-HSD also play a role in later estrogenic reactions, and the presence of a particular isoenzyme in different tissue determines preferential formation of either androgens or estrogens (reviewed by Simard et al. 2005, Miller 2002, and Penning 1997).

Estrogens are synthesized from androgens by loss of a methyl group at C-19 and formation of an aromatic A ring, converting testosterone to β-estradiol or androstenedione to estrone. Both these irreversible reactions are catalyzed by aromatase (CYP19). The prmoter of the CYP19 gene responds to several tissue-specific

transcription factors, including SF-1 and CRE elements in ovary, Sp1 and STAT3 in adipose tissue, and C/EBP-β in placenta (reviewed by Bulun et al. 2005). The primary site of aromatase expression in premenopausal women is the ovarian follicle, where expression is mediated primarily by FSH receptors and cAMP production. In men and postmenopausal women, the main sites of aromatase activity are extragonadal tissues such as adipose tissue, were CYP19 expression is regulated by cytokines, glucocorticoids and cAMP.

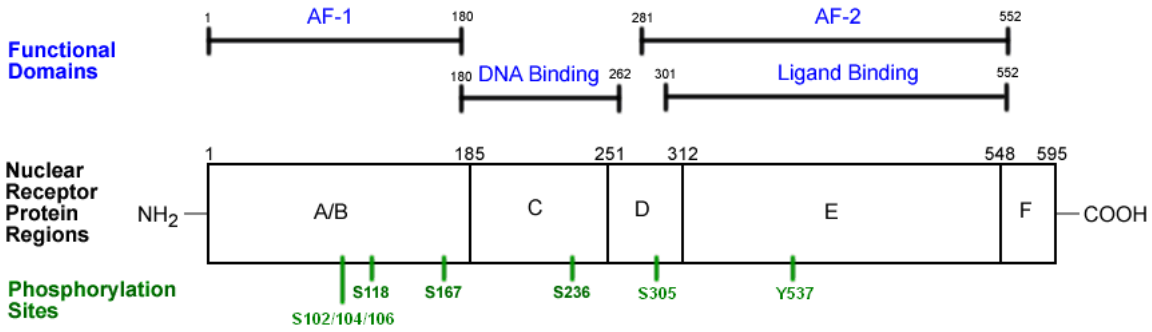
The endogenous estrogens are also deactivated by CYP450-catalyzed hydroxylation followed by conjugation by a Phase II enzyme, leading to sulfation, methylation or glucuronidation. Hydroxylation occurs most commonly at the 2- or 4-positions (reviewed by Bigsby et al. 2005). 2-hydroxylation predominates in the liver, catalyzed mostly by CYP1A2 and the CYP3A family. The inducible CYP1A1 also catalyzes 2-hydroxylation in extrahepatic tissues. CYP1B1 catalyzes estrogen 4-hydroxylation. Expression of CYP1A and 1B is regulated by the aryl hydrocarbon receptor (AhR). This nuclear receptor is activated by numerous halogenated and non-halogenated aryl hydrocarbons, and the environmental pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a particularly strong AhR ligand. TCDD induces CYP1A and CYP1B expression in various tissues, including liver, breast, and placenta. Expression of CYP3A is regulated by at least three nuclear receptors: constitutive androstane receptor (CAR), pregnane X receptor (PXR) and the vitamin D receptor (VDR). Pharmaceuticals like Clotrimazole, Phenobarbital and Rifampin (Luo et al. 2002), and environmental pollutants like methoxychlor, dieldrin, and trichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)-ethane

(DDT) isomers (Kretschmer and Baldwin 2005) are PXR ligands and induce CYP3A; CAR is activated by xenobiotics like phenobarbital and chlorpromazine (reviewed by Kretschmer and Baldwin 2005; Timsit and Negishi 2007). Both PXR and CAR are regulated by glucocorticoid signaling, and glucocorticoids are known to induce CYP3A.

Molecular Signaling by Endogenous Estrogens

At least two estrogen receptors (ERs) are known and have been well characterized: alpha and beta. Both ERα and ERβ are nuclear steroid receptors that activate transcription of target genes. They are soluble proteins found primarily in the cell nucleus. The inactive receptors are intermittently bound by chaperone proteins, including heat shock protein 90 (Hsp90), and this interaction facilitates ligand binding (Beliakoff and Whitesell 2004). The domain structure of the ERα is shown in Figure 4. Both ERα and ERβ are encoded by eight exons. The most conserved region between the two ERs is the DNA binding domain, and there is also significant sequence homology of their hormone binding domains; there is more sequence diversity in the N- and C-terminal domains as well as the hinge region between the DNA binding and hormone binding domains (reviewed by Saunders 1998).

Figure 4. ERα protein domain structure. Based on data from (Arnold et al. 1995, Ascenzi et al. 2006; Kumar et al. 1986; Meng et al. 2004; Schwabe et al. 1990; Wang et al. 2002). AF-1, Activation Function 1; AF-2, Activation Function 2. The Nuclear Receptor Protein Regions (A/B, C, D, E and F) are regions of partial homology among nuclear receptor proteins. The functional domains for ERα are labeled in blue. Kinase phosphorylation sites are indicated in green.



Two regions within ERα modify transcriptional activity: the ligand-independent activation function-1 (AF-1) located in the amino terminus, and the ligand-regulated AF-2 found within the ligand-binding domain (Beliakoff and Whitesell 2004; Smith and O'Malley 2004; Lannigan 2003). In general, ER phosphorylation controls recruitment of coactivators that enhance ER-mediated transcription, such as Steroid Receptor Coactivator 1 (SRC1) and Steroid Receptor Coactivator 3 (SRC3) (Likhite et al. 2006; Shah and Rowan 2005).

Ligand binding to ER and/or phosphorylation induces a conformational change in the receptor's hormone-binding domain, thus enhancing receptor dimerization and its ability to bind the estrogen response elements (EREs) located in the promoter region of target genes (Klein-Hitpass et al. 1989). The ERE is a palindromic sequence generally of the form GGTCAxxxTGACC with variability permitted to some extent at all positions (O'Lone et al. 2004). The transcriptional activity of ERs is modified by protein-protein interactions with a variety of cofactors (Smith and O'Malley 2004), as well as with other transcription factors, and in some instances the ERα can modify transcription by other factors, like SP-1 and AP-1, without binding DNA themselves (Tanaka et al. 2000; Denardo et al. 2007).

Two types of antagonistic ligands can inhibit the transcriptional activity of ERs. Type I antiestrogens, also known Selective Estrogen Receptor Modulators (SERMs), include pharmaceuticals like tamoxifen and raloxifene. ICI 182,780 (fulvestrant) is a Type II antiestrogen, also referred to as a "pure" antiestrogen. Although both types bind ERs with

high affinity, they interact differently with the ERs. Type I antiestrogens block transcriptional activity mediated by ERα's AF-2 only, leaving AF-1 available to initiate gene transcription (Smith and O'Malley 2004), while Type II antiestrogens block transcriptional activity mediated by either AF-1 or AF-2 (Metzger et al. 1995). In fact, studies of SERM activity led to the discovery that ER conformation is influenced by the nature of the bound ligand (Brzozowski et al. 1997; Shiau et al. 1998). This becomes important when considering the differential availability of cofactors and promoters in different tissues. As a result, Type I antiestrogens will stimulate ERα-mediated gene expression in some cell types, while blocking it in others; and they always antagonize ERβ activity (McDonnell et al. 2002). On the other hand, the pure antiestrogen fulvestrant inhibits both ERα and ERβ in all tissues. Fulvestrant also leads to rapid proteosomal degradation of ERα, which contributes to the complete inactivity of the receptor (Long and Nephew 2006).

The occurrence of rapid, non-genomic responses to estrogen suggests the existence of membrane-associated estrogen receptors signaling through kinases or other cytosolic molecules, but these ERs have not been fully characterized (Manavathi and Kumar 2006; Migliaccio et al. 2006). While some research points to ERα association with membrane proteins, at least one membrane ER, G Protein-Coupled Receptor 30 (GPR30), has been identified in breast cancer cells (Filardo et al. 2000; Thomas et al. 2005). In either case, estrogen effects originating at the membrane seem to be mediated by cross-talk with Epidermal Growth Factor Receptor (EGFR) signaling. Since several kinases are able to activate ERα by phosphorylation, it is possible that rapid estrogen signaling ultimately

induces transcription of ERE-regulated genes just as a ligand-activated nuclear receptor does. This area of research remains controversial since several researchers have not been able to show ERK1/2 activation in breast cancer cells by β-estradiol (Zheng et al. 2007; Gaben et al. 2004; Caristi et al. 2001).

Estrogen Receptor Alpha Regulation

In vivo, unliganded ERα protein has a half-life of 4-5 hours (Eckert et al. 1984), and its expression and activity are regulated by at least five different mechanisms: activation of gene promoters, gene methylation, protein phosphorylation, protein acetylation and ubiquitination. Interactions between ERα and ERβ can also modify ERα activity. Another mechanism by which ERα may be regulated, posttranscriptional silencing by micro RNAs (miRNAs) has only being recently studied.

The ERα gene is located in chromosome 6 (Gosden et al. 1986) and contains nine different promoter regions (Kos et al. 2001). The promoters are tissue specific and produce mRNA variants that differ only in their 5' untranslated region (Reid et al. 2002); still, all the possible transcripts can produce the same 66 kilodalton (kDa) protein, as well as 46 and 39 kDa variants. Hypermethylation of CpG islands within promoters can down regulate ERα expression, and this is an important mechanism by which breast cancer cells loose their sensitivity to antiestrogens therapy (Giacinti et al. 2006).

The AF-1 region contains several phosphorylation sites, and in the absence of steroid ligand, Mitogen Activated Protein Kinase (MAPK, ERK1/2) directly phosphorylates Serine 118 (and perhaps indirectly Serine 167), thereby partially activating the receptor through AF-1 (Bunone et al. 1996). Other sites in AF-1 phosphorylated by second messenger signaling pathways are S167 (a substrate of RSK and AKT) and S104/106 (a substrate of cyclinA/CDK2). Two additional phosphorylation site outside AF-1, S236 and S305, are activated by protein kinase A (PKA). Therefore, growth factors that activate kinase signaling such as Epidermal Growth Factor (EGF) and IGF-1 can induce phosphorylation of ERα and lead to transcriptional activation. While ERα phosphorylation alone can induce gene expression in cell culture systems (Bunone et al. 1996; Ince et al. 1994), the effects of phospho-ERα in the cell can be mediated by ligand binding, and the prescence of ligand seems to maintain the otherwise transient phosphorylation (Joel et al. 1998).

ERα protein is normally down regulated by acetylation, and this modification seems to decrease ligand sensitivity but not necessarily kinase activation (Wang et al. 2001). Formation of heterodimers with ERβ decreases ERα activity in some tissues (discussed further in next section). ERα is tagged for proteosomal degradation by ubiquitin, and this process is accelerated in the presence of β-estradiol (Nirmala and Thampan 1995) or fulvestrant but not tamoxifen (Long and Nephew 2006; Fan et al. 2003).

Recent reasearch has look into miRNA regulation of ER in human breast cancer. At least one miRNA, miR-206, has been observed to be up-regulated in ER-negative breast

cancer and to decrease endogenous ERα mRNA and protein levels in human MCF-7 breast cancer cells by interacting with the 3'-untraslated region of ERα transcript (Adams et al. 2007).

Physiological Role of Estrogen Receptor Alpha

As expected from its importance in regulating female reproduction, ER expression is high in ovary, uterus, vagina and mammary gland of mammals; while ERα is found in ovary, uterus and mammary gland, ERβ is abundant in the ovary but sparse in the uterus and was not found in the mammary gland (Couse et al. 1997; Pelletier and El-Alfy 2000).

ERα mediates the majority of estrogen's effects on female reproductive tissues. The ERα knockout (ERαKO) mouse has major defects in uterine responses to estrogens, and ERβ is not required to elicit the classic effects of estrogen in the uterus: increased size, fluid uptake, protein expression, epithelial cell hypertrophy and hyperplasia, stromal cell proliferation, and increase number of glands (Harris 2007). Conversely, ERβ may dampen the uterotrophic effect of ERα (Wada-Hiraike et al. 2006).

In the male mouse, ERα is found in testis, prostate and epididymis, while ERβ is found in prostate and epididymis but not in testis. The precise role of ERs in normal prostate is not completely understood. ERα is mostly found in stromal cells, while ERβ predominates in basal epithelial cells and may exert antiproliferative and pro-antioxidant effects (Ho et al.

2006). As mentioned earlier, developmental estrogen exposure predisposes the male to prostate cancer in adulthood, probably by altering the expression of ERs (Prins et al. 2006).

The ERs also play physiological roles in the brain, lungs, cardiovascular system, liver and bone. In the brain, ERα is involved in neuroprotection and inflammatory processes (Pozzi et al. 2006), synaptic plasticity and memory (Mukai et al. 2006), and sexual differentiation (Ogawa et al. 1998; Roselli et al. 2006). In the lungs, ERα mediates alveolar maintenance and regeneration in adult female mice (Massaro et al. 2007). In the cardiovascular system, ERα plays a role in vasodilatation (Chambliss and Shaul 2002), and specific ERα polymorphisms have been implicated in the development of preeclampsia in premenopausal woman (Molvarec et al. 2007) and cardiovascular disease in men (Shearman et al. 2003) and postmenopausal women (Alevizaki et al. 2007). The majority of estrogen-mediated gene expression in bone and liver requires ERα but not ERβ; in fact, the presence of ERβ will diminish the effect of β-estradiol (Lindberg et al. 2003).

Models of Estrogen Action

In vivo uterothrophic assays (Huggins et al. 1954; Clode 2006) and in vitro receptor binding assays (Wani et al. 1975; Martucci and Fishman 1976) have been used extensively to assess the estrogenicity of chemicals. More recently, cell proliferation

assays have also been used to assess estrogenicity (Soto et al. 1995), and in vitro molecular biology techniques help explain the mechanisms by which estrogens affect target tissues (reviewed by McDonnell et al. 2002).

Immature or OVX adult rodents have served as the standard in vivo bioassay model for estrogenic activity (Evans et al. 1941; Odum et al. 1997). The animals used may be sexually immature females, that is younger than approximately 4 weeks of age for mice or 5 weeks for rats (Jackson Laboratory 1966; Lohmiller 2006), or ovariectomized adults. The absence of endogenous estrogen stimulation of target tissues allows for controlled dosing and clear correlation of effects observed with the administered dose. Several estrogenic endpoints may be measured in these assays: uterus and/or vagina wet weight; difference between uterus wet and dry weights (water imbibition); albumin content of uterine fluid (uterine vascular permeability); uterine epithelial height (hyperthrophy), stromal cell density and number of uterine glands; vaginal epithelial thickness (hyperplasia); mammary gland branching, density and epithelial thickness; and expression of estrogen target genes in different tissues (Bigsby 2007; Suzuki 2007; Shi 2004; Raafat & Hofseth 2001; Papaconstantinou 2000; Orimo 1999; Milligan 1998; Steinmetz 1998). Because the different tissues will often respond differently to the same estrogenic molecule, for example 4-OH-tamoxifen behaves as an estrogen in the uterus but as an antiestrogen in mammary gland (Jordan 1995; Fotiou et al. 2000), it is important to observe in the same assay more than one estrogenic outcome in different target tissues.

The basic technology needed to determine the ability of a chemical to bind the estrogen receptor was available since estrogens were shown to bind uterine tissue both in vivo and in vitro (Jensen et al. 1968; Glascock and Hoekstra 1959). Early assays of radioactive β-estradiol displacement from estrogen receptors were done by either dosing live animals with radioactive β-estradiol (Callantine et al. 1968) or using homogenized uterine tissue (Clark and Gorski 1969). Cloning of the human ERα (Green et al. 1986) lead to the availability of recombinant protein generated in recombinant baculovirus-infected insect cells (Elliston et al. 1990), and thus simplified receptor binding/displacement assays.

Over the last 10 years, breast cancer cell proliferation assays have provided an alternative to in vivo assays. The fact that estrogens increase proliferation of neoplastic mammary epithelium in vitro is the basis of cell proliferation assays (Weichselbaum et al. 1978). A cell line that express ERα is required for this assay, and the MCF-7 breast cancer cell line has become the standard model to study estrogen-induced cell proliferation in culture (Soto et al. 1995).

Molecular technologies allow testing for direct activation of the estrogen response element (ERE) (Seiler-Tuyns et al. 1986; Druege et al. 1986; Klein-Hitpass et al. 1986; Nagel et al. 2001), detection of phospho-proteins involved in ERα signaling (Yung et al. 1997), including ERα itself (Chen et al. 2002; Al-Dhaheri and Rowan 2006), and testing for particular characteristics of the ERα molecule— such as ligand-induced conformation and availability of its different domains for interaction with other proteins— needed for activation/deactivation by a specific ligand in a given cell context (Wu and Safe 2007).

Of these technologies, the reporter genes controlled by an ERE are widely used to test estrogenicity. These reporter genes are either transiently or stably transfected into ERα positive cells and usually confer the test cell with estrogen-inducible chloramphenicol acetyltransferase (CAT) activity or firefly luciferase activity (Arnone et al. 2004).

Estrogen Toxicology

Endogenous estrogens can become toxic when the organism is exposed to concentrations higher than what is normally needed for homeostasis. As discussed previously, relatively low plasma concentrations are associated with the normal action of endogenous estrogens in female target tissues. Aside from dose, there are two main determinants of estrogen's toxic potential: age and gender. Fetal, pre-pubertal and pubertal mammals will be more susceptible than adults, and males will be more susceptible than females to estrogen toxicity at increasing doses. Substances that antagonize estrogen action or reduce endogenous estrogen levels may also cause disease in adult females (Perry et al. 2006; Yoshida et al. 2005). In rodents, prenatal exposure to elevated estrogen levels reduces survival rates of fetus and newborn (Kirigaya et al. 2006), and decreases birth weight of males (Blaschko et al. 2006).

The toxic effects of natural estrogens in humans can be classified into three main types: disruption of the hypothalamic-pituitary-gonadal (HPG) axis, carcinogenic and teratogenic (malformation of organs). Estrogens rarely cause acute toxicity. For example,

the LD50 (lethal dose at which 50% of the experimental population dies) for ethinyl estradiol, a pharmaceutical estrogen commonly used in birth control formulations, ranges between 0.5 and 5 g/kg in rodents which die of liver or kidney failure; while in human there are no cases of serious effects after overdose except for vomiting in children (reviewed by Maier and Herman 2001). Chronic exposure to estrogens could disrupt the HPG axis in children (Halperin and Sizonenko 1983; Green 1958); although such exposures are usually detected and corrected early because of their gross effects on mammary tissues, i.e. gynecomastia in boys and premature thelarche in girls (Felner and White 2000; Henley et al. 2007; Denham et al. 2005; Larriuz-Serrano et al. 2001; Colon et al. 2000). Estrogens are recognized as tumor promoters in the liver, mammary gland, and female reproductive tract (Giannitrapani et al. 2006; Beral et al. 2007; Katalinic and Rawal 2007; Yeh 2007). While fetal exposure to estrogens is known to cause malformations of the reproductive organs in laboratory animals (reviewed by Saunders 1968), the only serious cases of teratogenicity seen in humans involved exposure to the pharmaceutical diethylstilbestrol that was given to pregnant women to prevent spontaneous abortion (Scully et al. 1974).

Estrogen toxicity occurs by at least two different mechanisms: cell cycle dysregulation and endocrine disruption. Another mechanism of estrogen action, rapid activation of cytosolic signaling molecules like endothelial nitrous oxide synthase (reviewed by Chambliss et al. 2002) has yet to be directly associated with toxicity, although the proposed rapid activation of cytosolic kinases (Manavathi and Kumar 2006; Migliaccio et al. 2006; Filardo et al. 2000) could, in theory, lead to cell cycle dysregulation.

Estrogen-target tissues proliferate in response to ERα-mediated expression of cyclin D1 (Barone et al. 2006; Altucci et al. 1996) and other proteins needed for progression of the cell cycle (reviewed by Hilakivi-Clarke et al. 2004). Interestingly, ERβ inhibits ERα-mediated expression of cyclin D1 (Liu et al. 2002). Cell cycle dysregulation is the mechanism by which estrogens act as cancer promoters or lead to benign proliferative disease in tissues like liver, mammary glands and endometrium. As discussed previously, adult pre-menopausal women have relatively high levels of endogenous estrogens, and normally this is a problem only in cancer patients or individuals genetically predisposed to cancer. Pre-menopausal women may be at risk of liver tumors from the use of oral contraceptives with high steroid formulations, although these are usually benign (Giannitrapani et al. 2006). In post-menopausal women, however, hormone replacement therapy has been associated with development of ovarian (Beral et al. 2007), breast (Katalinic and Rawal 2007), and endometrial (Yeh 2007) cancers. Accidental exposure of adult males to estrogens may lead to relatively benign proliferative conditions like gynecomastia (Bhat et al. 1990), but females using high dose oral contraceptives and males exposed to high levels of estrogens may also develop liver disease (Giannitrapani et al. 2006; Cooper et al. 2003). Gynecomastia is also a common symptom of accidental estrogen exposure in male children. While the normal human newborn may display transient gynecomastia from exposure to maternal estrogens in uterus (Gikas and Mokbel 2007), cases of young children developing gynecomastia are usually due to exposure to adult prescriptions (Felner and White 2000) and herbal oils containing estrogens (Henley et al. 2007).

Endocrine disruption refers to changes in hormonal homeostasis, and this may occur by either direct action of hormones or hormone-like chemicals, or by developmental abnormalities that lead to increased hormonal sensitivity. But it is often difficult to differentiate between these two mechanisms as they relate to a specific toxic outcome, especially in the case of estrogens, since developmental exposure to estrogens increase later tissue sensitivity to estrogens (Wadia et al. 2007).

Estrogens play a role in the etiology of adult disease after in utero exposure, an effect mediated by ERα (Couse and Korach 2004). In a classic example of this effect, young daughters of women that used the estrogenic drug diethylstilbestrol (DES) during pregnancy have a very high incidence of clear cell adenocarcinoma of the vagina, an otherwise rare condition typically found in older women (Scully et al. 1974). In rodents, prenatal estrogen exposure leads to persistent proliferation of vaginal epithelium in the adult, even without further estrogen stimulation (Kirigaya et al. 2006). Another in utero effect of DES seen in adults, both in rodents and in humans, is uterine fibroids (leiomyomata) (Baird and Newbold 2005). Fetal exposure to estrogens also sensitizes rodent mammary gland to estrogens in adulthood (Wadia et al. 2007) and induces mammary gland ductal hyperplasias and carcinoma in adults (Murray et al. 2007). In the male, developmental exposure to estrogens is associated with hyperplasia, inflammation, and dysplasia of the prostate (Prins et al. 2006). Exposure of newborn rodents to estrogens can affect bone homeostasis (Migliaccio et al. 1995), and ovarian/reproductive function in the adult (Jefferson et al. 2006; Burroughs 1995).

In female children, accidental exposure to adult estrogenic medication will lead to premature thelarche and other symptoms concomitant with premature puberty (Halperin and Sizonenko 1983; Green 1958), similar to symptoms produced by elevated levels of endogenous steroids associated with ovarian tissue tumors (Till and Schmidt 2005) or gonadal dysgenesis (Iliev et al. 2002). In several instances premature thelarche epidemics that have occurred worldwide, chronic exposure to xenoestrogens in food or in the wider environment was suspected (Colón et al. 2000; Fara et al. 1979; Larriuz-Serrano et al. 2001; Massart et al. 2005; Nizzoli et al. 1986) and remission was seen when the suspected estrogen source was removed (Saenz de Rodriguez et al. 1985). An alternative explanation for the causes of idiopathic premature thelarche is the increase of truncal fat deposits in a population due to socioeconomic changes (Zukauskaite et al. 2005) since increased fat deposits and an associated increase in leptin levels correlate with age at puberty (Novotny et al. 2003; van Lenthe et al. 1996; Anderson et al. 2003). The ability of body fat to accumulate and release environmental estrogens (Ulrich et al. 2000; Bigsby et al. 1997) may provide an alternative hypothesis linking body fat and symptoms of premature puberty, but this has not been addressed in human studies.

Other than the clinical observations described above, there is little information on the effects of human pubertal exposure to exogenous estrogens, but by reviewing animal studies it could be hypothesized that exogenous estrogens may accelerate age of menarche in girls and delay puberty in boys. Pubertal exposure to estrogenic chemicals affects behavior in juvenile and adult male rats (Della Seta et al. 2006) and in juvenile rhesus monkeys (Golub et al. 2004). Studies in prepubertal ewe lambs (Evans et al. 2004)

and juvenile rats (Rosa et al. 2003; Kim et al. 2002a; Kim et al. 2002b) showed that exogenous estrogens can disrupt lutenizing hormone secretion and thus disrupt cyclicity in females. In male rats, delayed puberty and testicular damage has been observed after treating juveniles with xenoestrogens (Tan et al. 2003), while in female rats estrogens accelerate onset of puberty (Kim et al. 2002a; Kim et al. 2002b). In contrast, prepubertal exposure to the estrogens seem to decrease the incidence of mammary tumors in rats exposed to the carcinogen 7,12-dimethylbenz[a]anthracene (Cabanes et al. 2004).

Endocrine Disruption by Xenoestrogens

The term environmental endocrine disruptor has been defined as "an exogenous agent that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (Kavlock et al. 1996). Many environmental pollutants such as the plastic monomer component bisphenol A, and the pesticides o,p-DDT, methoxychlor, and β-hexachlorocyclohexane (β-HCH) have been classified as endocrine disruptors based on limited epidemiological data and using animal bioassay, biochemical analysis, and cell culture models. The better known endocrine disruptors that have estrogenic activity or disrupt endogenous estrogen signaling are listed in Table 1. Some of these chemicals can also disrupt other hormonal systems in addition to estrogens, or may have other toxicities unrelated to estrogen signaling.

Since Kupfer and Bulger first demonstrated the metabolic activation of methoxychlor to an estrogen (Bulger et al. 1978; Kupfer and Bulger 1979), it has been discovered that several other environmental pollutants become estrogenic (Morohoshi et al. 2005; Kohno et al. 2005) or can increase in their estrogenic potency (Jansen et al. 1993; Yoshihara et al. 2004) after CYP450 metabolism. CYP450 isoenzymes catalyze redox reactions that will add a hydroxyl group to an aromatic carbon and/or deahalogenate the aromatic ring (Groves 2005; Cnubben et al. 1995). Both loss of halogen atoms and hydroxylation of the same aromatic ring (especially 4'-hydroxylation) can transform aromatic halogenated hydrocarbons into ER ligands, or increase their activity or ability to bind ER (Huggins and Jensen 1954; Vakharia and Gierthy 2000; Korach et al. 1988; Hartmann et al. 1985). CYP450 isoenzymes responsible for xenoestrogen activation include 1A2, 2A6, 2A8, 2C9, 2C18, and 2C19 (Stresser and Kupfer 1998; Hazai and Kupfer 2005; McGraw and Waller 2006; Koga et al. 1996).

As discussed previously, the CYP450 enzyme system is also responsible for deactivating endogenous estrogens. Environmental chemicals that induce CYP450, especially isoenzyme 1A1 and the 3A family (AJ Lee et al. 2003; Zhu and Lee 2005), could decrease the activity of endogenous estrogens. The cathechol estrogens formed by these enzymes may also be converted to quinones capable of forming DNA adduct or they may undergo redox cycling thus producing oxidative stress (Yager 2000; Liehr 2000).

Table 1. Examples of Endocrine Disruptors Known to Affect Estrogen Signaling

Chemical	Mechanism	Other effects	References
Bisphenol A	ER ligand	androgen disruptor	(Blair et al. 2000; Kuiper et al. 1997; HJ Lee et al. 2003)
Methoxychlor	CYP450 metabolites are ER ligand	oxidative stress, androgen disruptor	(Bulger et al. 1978; Kupfer and Bulger 1979; Blair et al. 2000; Gupta et al. 2006; Murono et al. 2006)
1,1,1-trichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl) -ethane (o,p'-DDT)	ER ligand	neurotoxic, inflamatory	(Blair et al. 2000; Bolger et al. 1998; Kim et al. 2004; Shankland 1982)
Polychlorinated Biphenyls (several congeners; Ex. 2',3,4',6'-tetrachloro-4-bisphenylol)	CYP450 metabolites are ER ligands	immunosupression, carcinogenic, neurotoxic	(Vakharia and Gierthy 2000; Carpenter 2006; Connor et al. 1997)
β-hexachloro-cyclohexane (β-HCH)	Unknown	hepatotoxic	(Ulrich et al. 2000; Bigsby et al. 1997; Schroter et al. 1987; Mills and Yang 2006; Muscat et al. 2003)
2,3,7,8-tetrachloro dibenzo-*p*-dioxin (2,3,7,8-TCDD; dioxin)	induces endogenous estrogen catabolism, cross-talk between Aryl Hydrocarbon Receptor (AhR) and ERα	dermal, vascular, hepato- and neurotoxic	(Bigsby et al. 2005; Yoshida et al. 2005; Pelclova et al. 2006; Khan et al. 2006; Wang et al. 2006)
Lead	forms complex within hormone-binding domain	Anemic; Neurotoxic	(Martin et al. 2003; Papanikolaou et al. 2005)

Wildlife studies strongly suggest that environmental endocrine disruptors should be of concern for human health. Estrogenic environmental pollutants are responsible for various detrimental effects on wildlife, including: feminization of male fish (Gross-Sorokin et al. 2006); feminization of populations (Pickford et al. 2003; Bogi et al. 2003) and hermaphroditism (Hayes et al. 2002) in amphibians; alteration of bone homeostasis (Lind et al. 2004), steroidogenesis (Crain et al. 1997) and gonadal development (Guillette et al. 1994) in reptiles; depressed sexual behavior (Halldin et al. 2005), eggshell thining (Berg et al. 2004) and masculinization of song (Quaglino et al. 2002) in birds; leiomyomas and decreased fertility in Baltic gray seals (Backlin et al. 2003). But the effects of estrogenic endocrine disruptors on humans are still controversial. While the detrimental effects of pharmacological agents like the estrogen diethylstilbestrol (DES) are well documented (Newbold et al. 2006; Veurink et al. 2005; Lauver et al. 2005), the relationship between environmental concentrations of xenoestrogens and human disease is less well defined. Xenoestrogens are suspected to play a role in geographically-confined outbreaks of premature puberty, (Massart et al. 2005) and to cause ambiguous genitalia in children exposed in utero (Paris et al. 2006). It has been speculated that exposure in utero or early in life can increase sensitivity of children to steroids (Aksglaede et al. 2006). However patient xenoestrogen exposure data is often lacking, and human case reports are too few to be certain of a direct correlation. Several epidemiological studies have looked at xenoestrogen concentrations in breast cancer patients or correlations between the disease and pesticide use (Teitelbaum et al. 2007; Rubin et al. 2006; Khanjani et al. 2006; Waliszewski et al. 2005) but most have found no correlation between adult exposure and disease. A possible complication in such

epidemiological studies is the assumption that a correlation between exposure to a specific environmental pollutant and effect will exhibit a noticeable dose-response trend. But the fact that exposure is ubiquitous in humans, not only to the particular xenoestrogens being studied but many other environmental xenoestrogens, makes it impossible to find a true "zero exposure" control group (Fürst 2006; Darnerud et al. 2006; Johnson-Restrepo et al. 2005; Erdogrul et al. 2004; Kalantzi et al. 2004; Schecter et al. 2003; Fangstrom et al. 2002; Franchi and Focardi 1991). Therefore it is difficult to discern true cause-effect relationships through epidemiological studies unless the study can be adjusted for the lack of a zero exposure group and exposures to all known xenoestrogen and their possible interactions are accounted for (Fernandez et al. 2007a and 2007b). Another issue that has yet to be addressed by epidemiological studies is the ability of environmental xenoestrogens to sensitize a developing animal to estrogen action in their adult life (Wadia et al. 2007), in other words epidemiological studies would need to find if there is a correlation between prenatal exposures to environmental xenoestrogens and adult onset diseases (Baird and Newbold 2005). This task has been taken up by the National Children's Study, which among other environmental health research it is undertaking a prospective study of developmental effects of hormonally active environmental agents (Longnecker et al. 2003).

PBDEs As Suspected Xenoestrogens

The PBDEs are a series of 209 possible brominated diphenyl ethers that differ in the number and position of bromine atoms (ATSDR 2004). Mostly the tetra, penta, hexa, octa and deca- brominated congeners were manufactured and marketed as mixtures. Commercial mixtures of PBDEs have been used extensively as flame retardants over the past 30 years in polyurethane foam-containing consumer goods (carpet padding, sofas, mattresses and other) and constitute about 5-30% by weight of foam (ATSDR 2004). The PBDEs are very stable compounds and are not chemically bonded to the material they are intended to protect from burning. Therefore it is not unexpected that PBDEs are being found more and more in environmental media (Hites 2004; Law et al. 2006) and possible exposure to them has become a public health concern. Three congeners in DE-71 are the most common PBDEs found in environmental samples: 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), 2,2',4,4',5-pentabromodiphenyl ether (BDE-99), and 2,2',4,4',5,5'-hexabromodiphenyl ether (BDE-153); it has been suggested that these three congeners are able to bioaccumulate (Hites 2004).

Based on the increase in PBDE content of mother's milk in Europe (Noren and Meironyte 2000; Lind et al. 2003), the European Union banned the use of certain PBDE congeners in 2004 (European Union 2003). Studies in the USA (California, Indiana, and Texas) and Canada have shown levels in human cord blood, peripheral blood, breast adipose tissue and milk to be 10-100 times higher than in Europe (Petreas et al. 2003; She et al. 2002; Mazdai et al. 2003; Schecter et al. 2003). California followed with a ban by

January 2008 (California Assembly 2003), and the U.S. Environmental Protection Agency (EPA) reached a voluntary agreement with the only U.S. manufacturer of penta- and octa-BDE, Great Lakes Chemical Corporation, under which the company ceased production of these chemicals at the end of 2004. A new rule effective in 2005 requires notification to EPA before commencing manufacture or import of most PBDEs to the US. The EPA would evaluate the intended use and associated activities and, if necessary, prohibit or limit that activity before it occurs (U.S.E.P.A 2006). More recent studies have found increasing PBDEs levels in environmental and human samples in Asia (Wang et al. 2007) and Australia (Toms et al. 2007), therefore PBDEs have become a pollutant of concern in the global environment.

The endocrine disrupting potential of polybrominated diphenyl ethers (PBDEs) has been studied by others, mostly regarding its effect on thyroid hormone homeostasis (Hall and Thomas 2007; Darnerud et al. 2007; Ellis-Hutchings et al. 2006; Balch et al. 2006; Fernie et al. 2005; Meerts et al. 2000). In addition to disrupting thyroid signaling, PBDEs are known to alter behavior and sexual development in rats. Viberg, Eriksson and Fredriksson found neurobehavioral alterations in rodents treated neonataly with PBDEs (Viberg et al. 2003, 2004; Eriksson et al. 2006). Others have shown PBDE treatment interferes with sexual development and behavior in rodents (Stoker et al. 2004; Kuriyama et al. 2005; Ceccatelli et al. 2006; Lilienthal et al. 2006). Neither the neurobehavioral nor developmental effects of PBDEs have been directly linked to a specific hormonal activity.

PBDEs are suspected to behave as estrogens because of the similarity of their chemical structure (Figure 5) and properties to other xenobiotics, mainly the polychlorinated biphenyls (Hooper and McDonald 2000; Pijnenburg et al. 1995; Meerts et al. 2001). Furthermore, it has been shown that hydroxylated metabolites of PCBs exert estrogenic effects (see references in Table 1), and therefore, it may be reasonable to expect that hydroxylated forms of PBDEs would also be estrogenic. Others have shown that individual PBDE congeners or certain synthetic hydroxylated congeners could exert estrogenic effects in cultured cells (Meerts et al. 2001; Hamers et al. 2006). In estrogen-responsive transcription reporter assays, BDE-28 and -100 and the 4'-hydroxy forms of BDE-30 and -119 proved to be estrogenic (Meerts et al. 2001), but these compounds have not been found as metabolites in any biological samples. In addition, several PBDE congeners were mildly antiestrogenic in the same assay. Likewise, Hamers et. al. (2006) observed weak estrogenic activity by several low-brominated BDEs, weak antiestrogenic activity for tetra- and hepta-brominated BDEs and 6-OH-BDE-47, and neither activity for the DE-71 mixture (Hamers et al. 2006). In a recent in vivo study, BDE-47 had uterotrophic effects in immature rats (Dang et al. 2007), suggesting that in vivo activation of this otherwise non-estrogenic PBDE (Meerts et al. 2001) occurs in the rat.

Here we focus on the possible estrogenicity of the penta-BDE mixture manufactured by Great Lakes Chemical Corporation (West Lafayette, Indiana, USA) known as DE-71 (see Material Safety Data Sheet in Appendix 1). DE-71 has been used as flame retardant additive in consumer products manufactured by the furniture industry, mainly in the manufacture of flexible polyurethane foam (FPUF), which is used in bed mattresses and

cushioning in upholstered products (ENVIRON 2003 cited in ATSDR 2004). Mattress FPUF contains approximately 2–3% flame retardant, cushion FPUF contains 3–5% flame retardant, and scrap materials from both industries have been used as carpet padding, resulting in carpet containing 3–5% flame retardant (ENVIRON 2003 cited in ATSDR 2004).The DE-71 mixture composition is detailed in Table 2 (Qiu et al. 2007).

Figure 5. Chemical structure of β-estradiol, several classic xenoestrogens, and DE-71 congeners (blue).

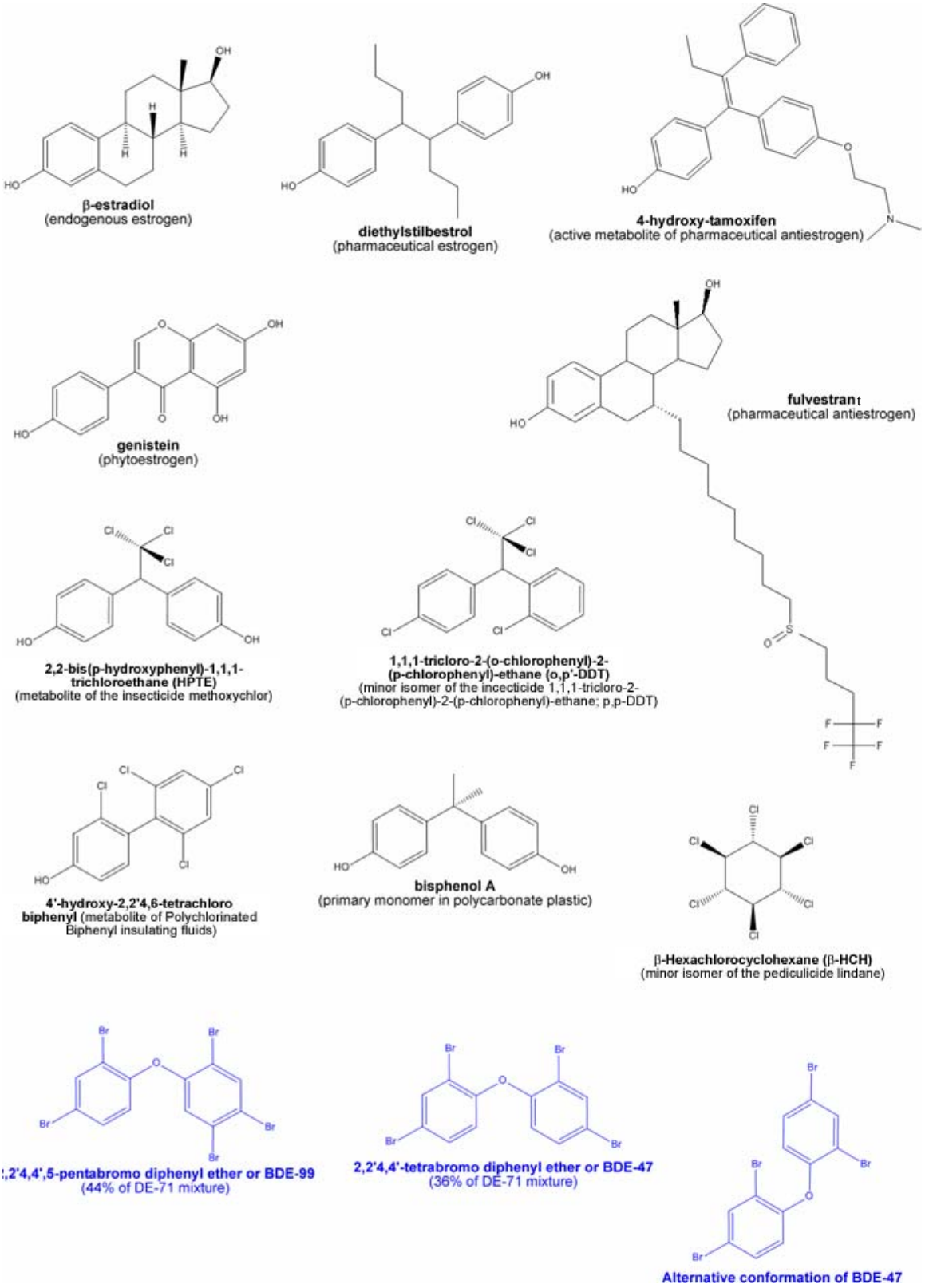


Table 2. Composition of DE-71. The composition of the DE-71 commercial mixture was determined by gas chromatographic mass spectrometry (GC/MS) analysis (from Qiu et al. 2007).

Congener Name	Chemical Name	Percent of Mixture by Weight
BDE-28	2,4,4'-tribromo diphenyl ether	0.3%
BDE-47	2,2',4,4'-tetrabromo diphenyl ether	36%
BDE-85	2,2',3,4,4'-pentabromo diphenyl ether	2.6%
BDE-99	2,2',4,4',5-pentabromo diphenyl ether	44%
BDE-100	2,2',4,4',6-pentabromo diphenyl ether	9.1%
BDE-153	2,2',4,4',5,5'-hexabromo diphenyl ether	4.3%
BDE-154	2,2',4,4',5,6'-hexabromo diphenyl ether	3.3%
Other hexa-BDEs	---	0.4%

Specific Aims of Thesis

Animal studies have clearly shown the potential for environmental estrogens to disrupt normal development and physiology and to cause disease. It is imperative that compounds with the potential to cause such problems be tested for hormonal activity so that informed public policy decisions can be made concerning their manufacture and use.

PBDEs have the potential to be estrogenic endocrine disruptors. These anthropogenic chemicals are detectable in organic and inorganic environmental media (Hites 2004; Law et al. 2006), and there is little information in the scientific literature that could be used to assess the health risks PBDEs pose to humans at the current levels found in human blood samples. Their molecular structure is similar to other environmental pollutants known to be estrogenic (Figure 5) or to be converted to estrogenic species by mammalian in vivo metabolism capable of activating ERα-mediated signaling (see references on Table 1). Therefore, we studied the estrogenic activity of the mostly penta-brominated PBDE formulation DE-71. More specifically, the main hypothesis of this thesis is that DE-71 acts as an endocrine disruptor through activation of ERα. The specific aims of this thesis are:

1- Determine if DE-71 exerts estrogenic or antiestrogenic effects in cell culture and animal models.
2- Determine the role of metabolic activation in DE-71 estrogenic or antiestrogenic effects.

3- Explore the mechanisms by which DE-71 congeners or their metabolites can activate or antagonize ERα signaling.

The results presented here will be useful in two areas: public health policy and basic estrogen signaling science. Since the few studies currently available show estrogenic activity of some PBDEs in a limited numbers of model systems, our findings will expand the urgently needed information to aid in developing science-based health risk assessments, public policies, and regulations to protect public health. At the same time, if DE-71 shows estrogenic or antiestrogenic activity, the discovery of new estrogens will allow for characterization of their interactions with ERs, and since these receptors are known to behave differently according to ligand and cellular environment, the new ligands could be used to further the study of estrogen receptor signaling pathways.

Materials and Methods

Test Chemicals

Dimethyl sulphoxyde (DMSO), 1,3,5[10]estratriene-3,17-β-diol (β-estradiol, E2), and β -estradiol-3-benzoate (EB), were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Corn oil was purchased from ICN Biomedicals Inc. (Aurora, Ohio, USA). Fulvestrant (ICI 182 780, ICI) was a gift from Astra Zeneca. The PBDE congener mixture DE-71 was a gift from the Great Lakes Chemical Corporation (West Lafayette, Indiana, USA; see Appendix 1 for chemical properties; see composition in Table 1). 4′-OH-2,2′,4-Tri-BDE (4′-OH-BDE-17); 2′-OH-2,4,4′-tri-BDE (2′-OH-BDE-28); 4-OH-2,2′,3,4′-tetra-BDE (4-OH-BDE-42); 3-OH-2,2′,4,4′-tetra-BDE (3-OH-BDE-47); 6-OH-2,2′,4,4′-tetra-BDE (6-OH-BDE-47); and 4 ′-OH-2,2′,4,5′-tetra-BDE (4′-OH-BDE-49) were gifts from Göran Marsh (Stockholm University, Stockholm, Sweden) and were synthesized as described elsewhere (Marsh et al. 2004). 2,4-Dibromophenol (2,4-DBP) and 2,4,5-tribromophenol (2,4,5-TBP) were purchased from Cambridge Isotope Laboratories (Cambridge, Massachussets, USA). DMSO was used as the primary solvent for all treatment chemicals. DMSO solutions were further diluted in tocopherol-free corn oil (Sigma-Aldrich) for animal treatments.

Cells and Culture Conditions

Several cell lines were used: MCF-7, MDA-MB-231, SKBR3 and BG1Luc4E2. MCF-7 (Soule et al. 1973), MDA-MB-231 (Cailleau et al. 1978) and SKBR3 (Fough and Trempe 1975) are all breast cancer cell lines obtained from American Type Culture Collection (ATCC, Manassas, Virginia, USA; catalog numbers HTB-22, HTB-26 and HTB-30, respectively); they were derived from pleural effusions from different female patients with metastatic disease. MCF-7, MDA-MB-231 and SKBR3 were cultured for up to 30 passages without compromising assay reproducibility. The BG1LucE2 cell line (a gift form Michael Denison, University of California at Davis, USA) are BG-1 ovarian cancer cells (Geisinger et al. 1989) stably transfected with an estrogen-responsive plasmid containing neomycin (G418) resistant marker, four concatenated ERE oligonucleotides immediately upstream of a mouse mammary tumor viral (MMTV) promoter and the firefly luciferase gene (Rogers and Denison 2000). BG1LucE2 cells were passaged no more than 9 times before using a new cryopreserved vial, since cells above the ninth passage lost their sensitivity to estrogen.

Most cell culture media and supplements were purchased from Gibco/Invitrogen (Carlsbad, California), except Bovine Growth Serum (BGS; Hyclone; Logan, Utah, USA) and geneticin (G418, Sigma). Most charcoal-stripping reagents and endotoxin-free water were purchased from Sigma-Aldrich except Dulbecco's Phosphate Buffered Saline (DPBS; Mediatech Inc., Herndon, Virginia, USA).

MCF-7, SKBR3 and MDA-MB-231 were maintained in Growth Medium (GM) with the following formulation: Minimum Essential Media (MEM) supplemented with L-glutamine (2 mM), non essential amino acids (NEAA 0.1 mM), Hepes buffer (10 mM), 0.4 pg/mL insulin and 5% v/v BGS. BG1Luc4E2 were maintained in a different medium formulation (BG1-GM): alpha-MEM supplemented with Hepes buffer (10 mM), geneticin (0.4 g/L) and 10% v/v BGS.

For assays measuring estrogenic activity, a medium formulation with minimal hormonal activity was required to decrease basal estrogenic signaling in each of the cell lines. For MCF-7, SKBR3 and MDA-MB-231 cells this Basal Medium (BM) consisted of a formulation similar to GM except phenol red free MEM and 3% charcoal-stripped BGS (csBGS) were used. For kinase phosphorylation assays, cells were incubated in serum-free BM (sfBM). Basal medium for BG1Luc4E2 (BG1-BM) consisted of phenol red-free Dulbecco's Modified Eagle Media: Nutrient Mixture F12 (DMEM:F12, Gibco/Invitrogen) supplemented with Hepes buffer (10 mM), and 10% v/v csBGS. BGS was stripped of estrogenic activity by a modification of the methods described previously (Lippman et al. 1976; Biswas and Vonderhaar 1987). Activated charcoal (0.4 g per 22 mL BGS) was washed 3 times in endotoxin-free 0.01 N HCl, then twice in endotoxin-free water. The charcoal was then dextran-coated by washing in 1 g/L dextran dissolved in DPBS. After each wash, charcoal was pelleted by centrifuging at 3500 RCF and decanting supernatant. BGS was added and rocked at 4°C for 30 min. After removing the charcoal by centrifugation as before, the BGS was decanted into another batch of unused dextran-coated charcoal and rocked again at 4°C for 30 min. The charcoal was pelleted

by centrifugation and the BGS was filtered first through 0.4 μm filter, then through 0.2 μm filter, aliquoted, and stored at -20°C. The stripped serum was tested for estrogenic activity using the cell proliferation assay described below. Serum was considered properly stripped of activity if it did not increase cell number more than double over 10 days. In comparison, the positive controls β-estradiol and GM will increase cell number about 10 times in the same time period (Figure 6).

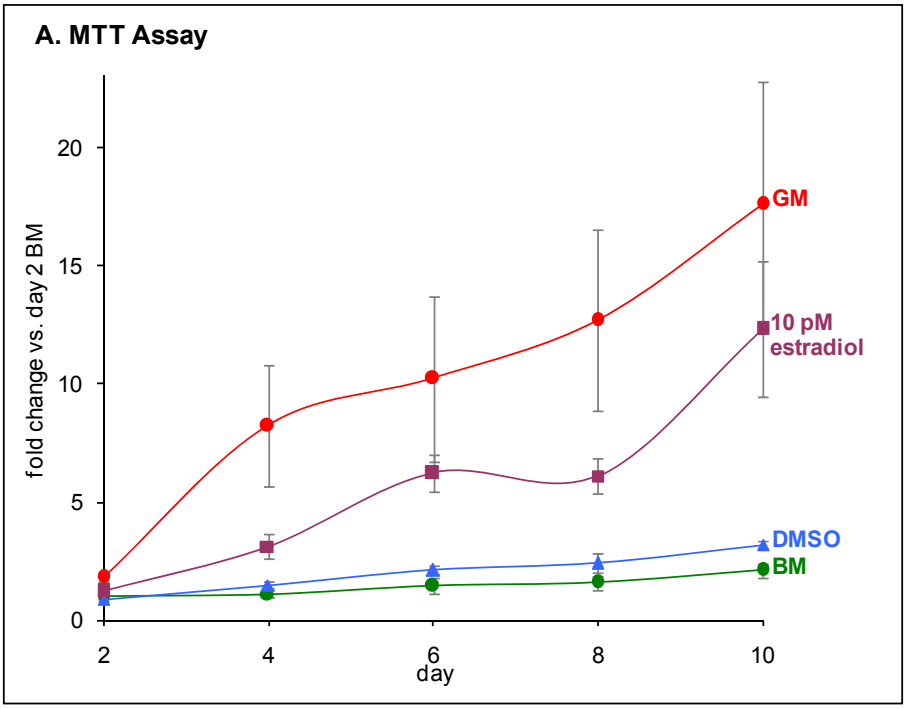
Cell Proliferation Measurements

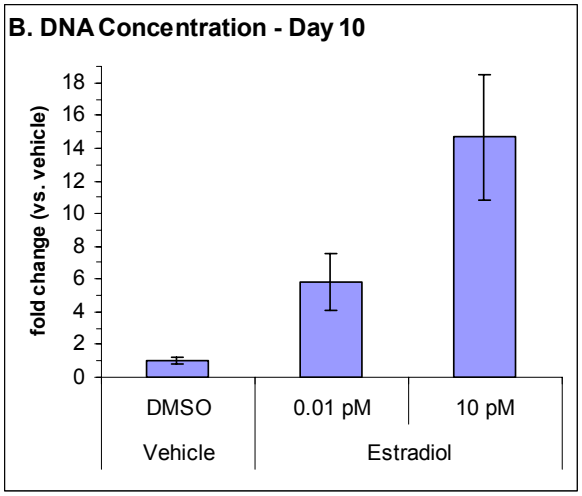
A colorimetric assay to measure cell proliferation developed by Mosmann (1983) was used with minor modifications. MCF-7 cells were plated with GM in 24-well dishes (15,000 cells/well, approximately 10% confluency). The growth of MCF-7 cells in culture is inhibited by media formulations containing charcoal-stripped mammalian serum, and this inhibition is overcome by treatment with estrogen (Soto et al. 1991; Soto et al. 1995). The day after plating, culture medium was changed to BM. Starting two days after changing to BM, cells were cultured in treatment medium (BM plus treatment), changing the treatment medium every 2 days. To determine cell number, cells were incubated in MTT (3-(4,5- dimethylthiazolyl-2)2, 5-diphenyltetrazolium bromide) for 2 hours, then lysed in acid isopropanol. Reduced (blue) MTT absorbance was read at 570 nm. Cell growth was assessed every other day during preliminary experiments and it was determined that a total incubation time of 10 day yielded an acceptable difference between vehicle treatment and 10 pM estradiol positive control (Figure 6A). The growth

of MCF-7 cells is not contact-inhibited, and cells treated with β-estradiol or GM consistently grew in layers after reaching 100% confluency.

In order to confirm the MTT absorbance correlated with cell growth and not increased metabolic activity, some experiments included additional plates treated in parallel for DNA concentration determination by fluorometric assay (Le Pecq and Paoletti 1966) (Figure 6B). Cells were washed twice with phosphate saline buffer and incubated for 10 seconds in cold methanol. After removing the methanol, cells were allowed to dry at room temperature and then dissolved in 0.5 M NaOH by rocking in an humidified 37°C chamber for 30 min. Samples were then transferred to microtubes and incubated at 65°C for 1 hour. 100 μL of sample was diluted in TNE buffer (10 mM Tris; 0.1 M NaCl; 1 mM EDTA; pH 7.4) containing 0.1 mg/mL Hoescht 33258 dye (Polysciences, Warrington Pennsylvania, USA), and neutralized with an equimolar amount of HCl. Fluorencense was measured in a Hoefer TKO 100 DNA Fluorometer (only measures at 460 nm) against a salmon sperm DNA standard (Invitrogen, Carlsbad CA, USA).

Figure 6. Preliminary MCF-7 cell proliferation measured by two different assays. Cells were grown in either Growth Media (GM), Basal Media (BM), BM with 0.1% DMSO (DMSO), or BM with 0.1% DMSO and β-estradiol (estradiol) at the concentrations indicated in the chart. A. Cell growth evaluated over 10 days by MTT assay, expressed as fold change compared to cell number after 2-day treatment with basal media only; each data point is the average of 4 independently plated assays on different days ± SEM. B. DNA concentration after 10 days, measured by Hoescht dye fluorescence method; each column is the average of 3 independently plated assays on different days ± SEM.

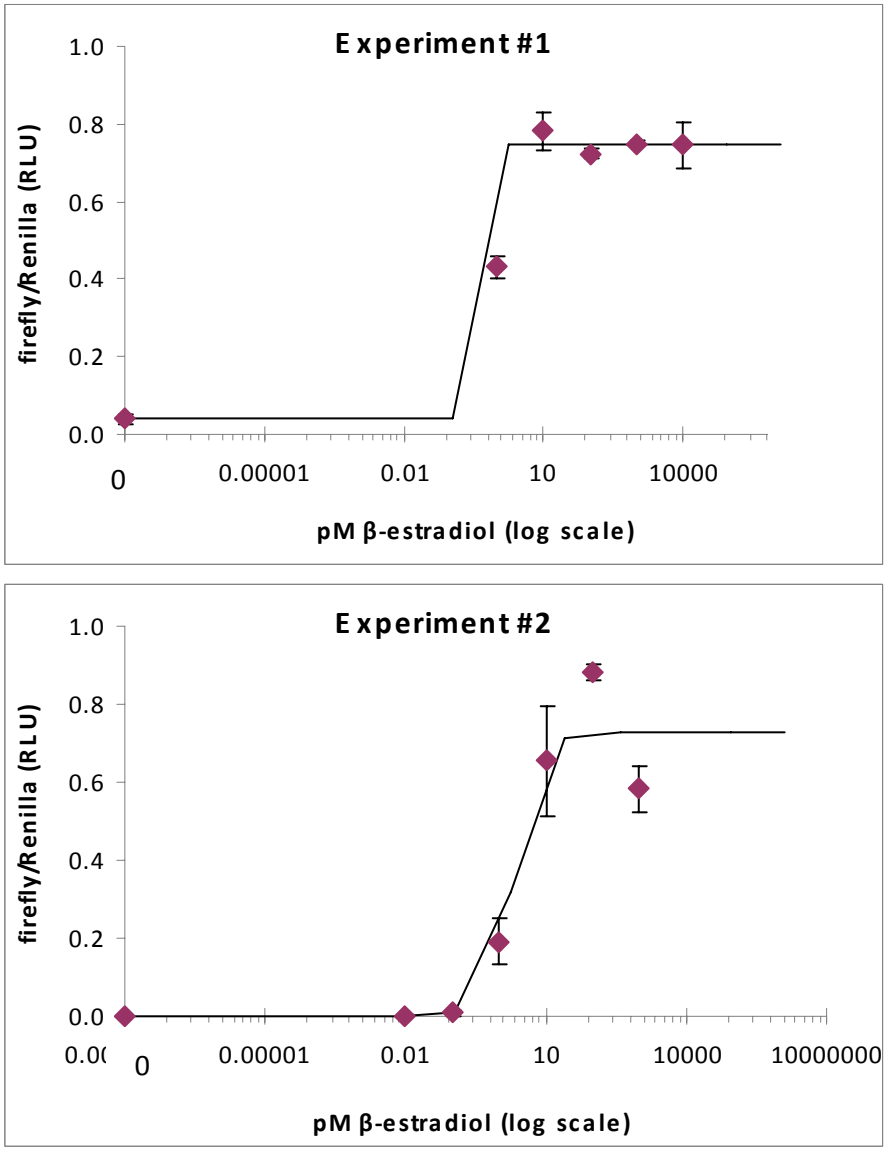




Estrogen Response Element-Luciferase Assays

Two ERE-luciferase reporter gene systems were used: one transiently transfected, the other an established stably-transfected cell line. Plasmids were replicated in *E. coli* (XL1-Blue Supercompetentcells, Stratagene) transformed by heat shock method (42°C for 50 seconds) and grown in Luria broth (LB; Gibco) with 0.1 mg/mL ampicillin; then purified using the EndoFree® Plasmid Maxi Kit (Qiagen). For the transient system, ER-negative MDA-MB-231 breast cancer cells were plated in BM at 80,000 cells/well of a 12-well plate. Two days later cells were transfected using the commercial liposome preparation Tfx-20 (3 µL/well; Promega, Madison, Wisconsin, USA) with expression vectors for ERα (7.5 ng/well; HEG0 from Dr. Pierre Chambon, Institute de Chimie Biologique, France), the estrogen-responsive firefly luciferase reporter construct ERE2pS2-luc (200 ng/well; Long et al. 2001), the control *Renilla* luciferase reporter construct pRL-TK (100 ng/well; Promega), and 692.5 ng/well of empty vector ($pcDNA_3$; Promega) for a total of 1000 ng plasmid per well. MDA-MB-231 cells do not respond to estrogen treatment in this assay unless transfected with an estrogen receptor (Bigsby, unpublished data). Cells were treated with test chemicals 1 hour after transfection, and assayed for luciferase activity after 18 hours. Results are expressed as the ratio of firefly luciferase to *Renilla* luciferase (Figure 7). β-Estradiol treatment reach a maximal 16-fold luciferase induction versus control treatment at 10 pM. Increasing concentrations of β-estradiol above 10 pM were not able to further increase luciferase induction beyond the maximum already reached at 10 pM.

Figure 7. β-Estradiol dose-response in MDA-MB-231 cells transiently transfected with ERE-luciferase and ERα. Cells were plated in basal media for 2 days then cotrasfected with plasmids expressing human ERα, the firefly luciferase with the Estrogen Response Element (ERE) in its promoter, and a constitutively active *Renilla* luciferase gene. 18 hours after transfection, cell lysates were collected for dual luciferase assay. Firefly luciferase expression was normalized to *Renilla* luciferase expression. Each data point is the average of 3 wells treated simultaneously ± SEM. RLU, Relative Light Units. Two independent preliminary experiments are shown (Experiment #1 and Experiment #2). pM, picomolar (10^{-12} moles per liter)

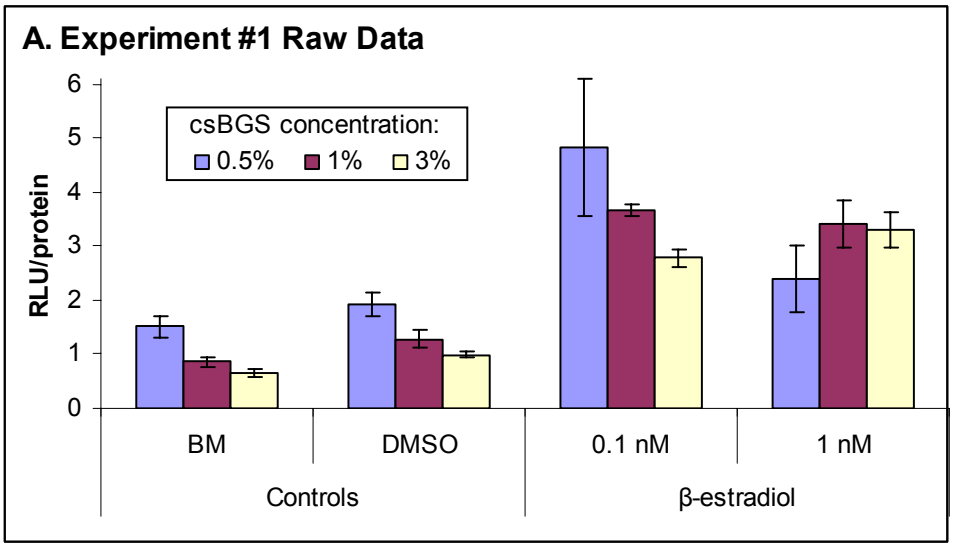


For the stable reporter system, ER-positive BG1Luc4E2 ovarian cancer cells (Rogers and Denison 2000) were plated in BG1-BM at 70,000 cells/well of a 12-well plate and grown for 5 days with change of medium before treatment. Cell were treated with vehicle, estradiol or test compound and assayed for luciferase activity after 18 hours. While the rest of our media formulations followed that described in their papers (Rogers and Denison 2000, 2002), our formulations differ in the kind of serum used. Although the optimal amount of csBGS had been determined previously for most cell lines used in our laboratory, the BG1Luc3E2 cell line was a new addition to our stock and the optimal levels of csBGS had to be determined experimentally as explained below. In addition, as part of the assay quality control for the BG1LucE2 cells we only used assays in which the maximal β-estradiol effect was more than 2.5 fold over vehicle control (Appendix 2).

BG1Luc3E2 cells were plated in BM containing different amounts of csBGS during 2 experiments. In the first experiment, cells were plated with BM containing either 0.5%, 1%, or 3% csBGS. In the second experiment, cells were plated with BM containing either 1%, 3%, 5% or 10% csBGS. After 5 days, media was changed to either the same BM it had before, or with the addition of one of three treatments: 0.1% DMSO (vehicle), 0.1 nM β-estradiol or 1 nM β-estradiol. Cells were assayed for luciferase activity after 18 hours. The results for all treatments were normalized to the BM only treatment with the same csBGS content. The intensity of the luciferase signal after β-estradiol treatment correlated with the amount of csBGS in media. BM with 10% csBGS allowed for an 18-fold increase in luciferase signal after β-estradiol treatment, which was considered a

satisfactory magnitude for the assays (Figure 8). All BG1Luc3E2 experiments thereafter were done using 10% csBGS.

Figure 8. Determination of optimal amount of csBGS in basal media for BG1Luc4E2 cell line for ERE-luciferase assay. Cells were incubated in media containing different concentrations of csBGS (by volume) during 2 different experiments. Individual wells were incubated in Basal Media alone (BM) or with 0.1% DMSO or 0.1% DMSO and the β-estradiol concentrations indicated in the chart. 18 hours after treatment, cell lysates were collected for luciferase and protein assays. Firefly luciferase expression was normalized to total protein per well. Each data point is the average of 3 wells treated simultaneously ± SEM. A and C show raw data corrected for the amount of protein in each well. B and D show data normalized to the BM treatment for each csBGS concentration. RLU, Relative Light Units.



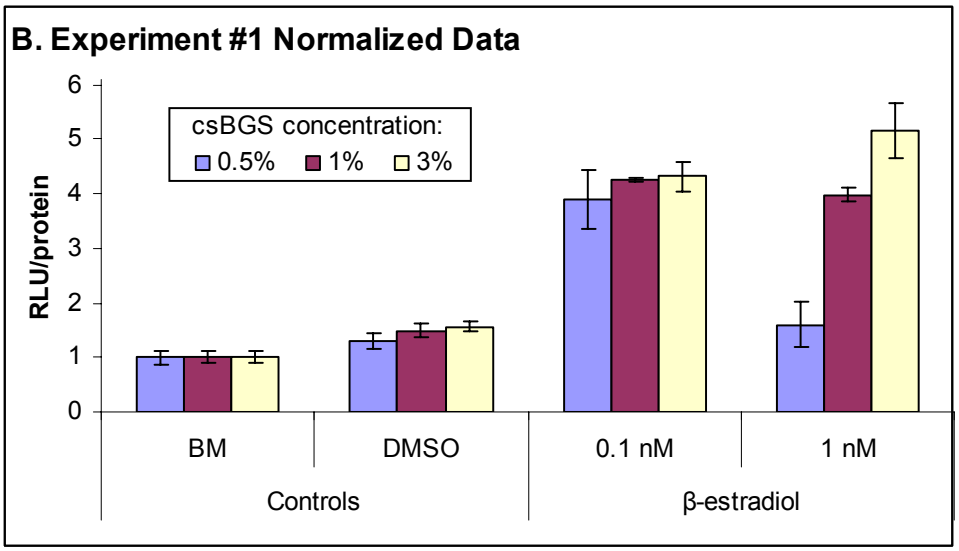


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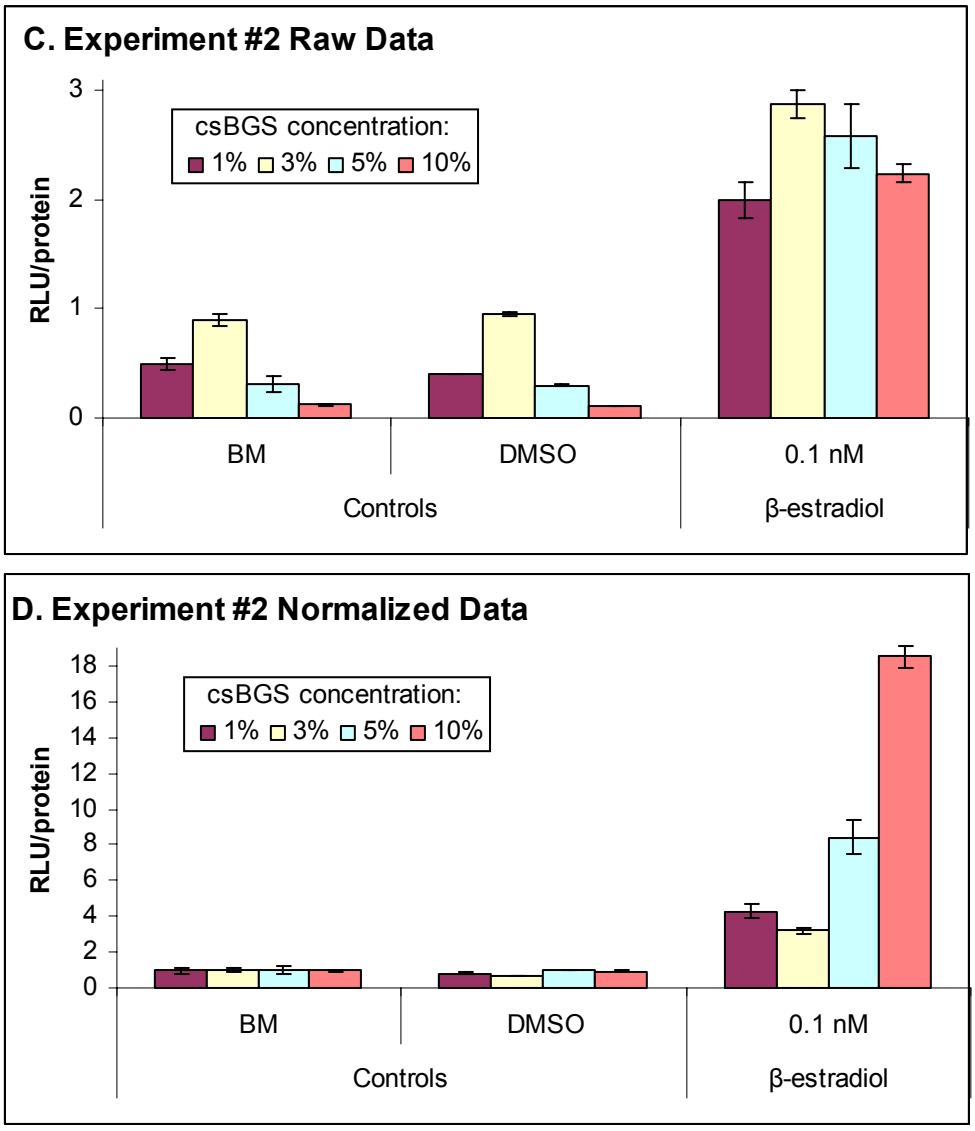
Figure 8 (cont).



In vitro Generation of Microsomal Metabolites

DE-71 or E2 were incubated with liver microsomes in order to obtain microsomal metabolites, using a procedure adapted from Bulger et al. (1978). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase (G6PD) and β-nicotinamide adenine dinucleotide phosphate (oxidized form, $NADP^+$) were purchased from Sigma-Aldrich. DE-71 (1 mM) or E2 (1 μM) were incubated with female rat liver microsomes (BD Biosciences Gentest, Woburn, Massachusetts, USA) and NADPH regenerating system (50 mm Tris buffer pH 7.5, 5 mM $MgCl_2$, 12 mM glucose-6-phosphate, 0.4 mM $NADP^+$, 2 units G6PD) in loose-capped tubes at 37°C with shaking. The incubation was allowed to continue for 24 hours in order to obtain the maximal amount of metabolite possible. The incubation mixture was then centrifuged at 105,000 x g and 4°C for 1hr to remove pelleted microsomes. The pellet was discarded.

The organic fraction was extracted from the supernatant by solid-state extraction with ethanol elution using Sep-Pak Plus C18 cartridges (Waters Corp., Milford, Massachusetts, USA), then evaporated to dryness *in vacuo* and extracts were reconstituted with DMSO at the original volume to yield 10 mM PBDE or 10 μM β-estradiol in DMSO (assuming 100% recovery). This extraction procedure was adapted from Yoshihara et al. (2004). Finally, the reconstituted extracts were tested for estrogenic activity in the ERE-luciferase assay described above. Microsomal (cytochrome P450) metabolism of β-estradiol is expected to reduce the "estrogenic" character of the steroid through hydroxylation (reviewed by Bigsby et al. 2005). Only experiments in which the

β-estradiol incubation lost estrogenic activity (positive control) were consider successful; data from experiments in which the β-estradiol incubation did not lost estrogenic activity was not used (see Appendix 4).

Recombinant Estrogen Receptor Alpha Binding Assay

Vehicle or test chemicals were incubated with 1 nM ^{3}H-estradiol (Amersham Biosciences; Buckinghamshire, England) and 0.6 nM recombinant ERα (Panvera/Invitrogen; Madison, Wisconsin, USA) in a total volume of 250 µL TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) at 4°C overnight. Hydroxylapatite (60% in TE buffer) was added, mixed well and incubated for 15 min at room temperature. The resulting slurry was washed 3 times by centrifugation at 3000 x g and buffer exchange at 4°C. Bound ligand was extracted from the receptor-hydroxylapatite complex by incubation with absolute ethanol at 30°C for 10 min. ^{3}H decay (counts per minute) was measured by liquid scintillation in a Beckman LS 5000 TD counter.

Kinase Phosphorylation Assay

MDA-MB-231, MCF-7 or SKBR-3 cells were plated in BM at a density of 10^{6} cells per 100 mm diameter cell culture plate and incubated overnight (37ºC, 5% CO_2). The next day cells were washed with DPBS and media was changed to sfBM and incubated

overnight. The next day cells were either left untreated, or treated with 1mL sfBM, positive control, DMSO, estradiol or DE-71 (concentrations as indicated in figures). Epidermal Growth Factor (EGF) or Transforming Growth Factor alpha (TGFα) were used as positive controls. Cells were incubated for either 5 or 10 minutes at 37°C and then culture plates were placed on ice. Cells were then scraped gently from plates, washed in DPBS, and lysed with Cell Lysis Buffer (Cell Signaling catalog #9803; Danvers, Massachusetts, USA). DNA and other unwanted cell debris were pelleted by centrifugation (10 minutes at 10,000 x g and 4ºC) and removed, while the proteins in the supernatant were separated by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane for immunoblotting. The PathScan® Multiplex Western Cocktail I (Cell Signaling catalog #5301), was used at a 1:200 dilutions, a mixture of primary antibodies which detects phospho-p90RSK, phospho-Akt, phospho-Erk1/2, and phospho-S6 ribosomal protein; an antibody against eIF4E is included as a loading control. Blots were also probed separately with antibodies for Erk1/2 (Cell Signaling catalog #9102) at a 1:1000 dilution or for phospho-Erk1/2 (Th202/Tyr204; Cell Signaling catalog #9101) at a 1:1000 dilution. Anti-rabbit IgG horseradish peroxidase (HRP)-linked antibody (Cell Signaling catalog #7074) was used as secondary antibody at a 1:1000 dilution. A biotinylated protein ladder was used, with its corresponding antibiotin HRP-linked antibody (Cell Signaling catalog #7727) at a 1:1000 dilution. Chemiluminescence analysis of the antibody signal was performed using SuperSignal West Femto (Pierce, Woburn Massachusetts, USA) according to the manufacturer's instructions.

Animal Treatments

All procedures performed on animals were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine. Adult BALB/c and C57BL/6 mice were purchased from Harland (Indianapolis, USA) while ERα-negative (ERα-knockout, ERαKO) mice were derived from an in-house colony of inbred mice with C57BL/6 background, maintained by the laboratory of Robert M. Bigsby at the Indiana University School of Medicine Animal Facility. Inbreed wild type mice (BALB/c and C57BL/6) were used to reduce the variability of the results. BALB/c mice are docile, easy to handle and have no known special attributes that may interfere or modify ERα signaling. The C57BL/6 mouse is the background strain of ERαKO mice and is commonly used in assays to assess estrogenic activity of chemicals. Animals were ovariectomized (as described by Olson and Bruce 1986) at 6-8 weeks old and 3 weeks later, they were treated for either 3 days or 34 days with vehicle or test compound. Animals with incomplete ovariectomies were detected at two specific steps in the procedure: at tissue harvesting, and when measuring uterine epithelial cell height (UEH). At tissue harvesting, animals with visibly intact or partial ovaries were excluded from the experiment. When measuring UEH, animals with uterus weight in the control range but UEH closer to EB-treated animals were assumed to have incomplete ovariectomies. Four animals were found to have incomplete ovariectomies (from all experiments). Data from animals with incomplete ovariectomies was not included in the final analysis.

Groups of 5-6 animals were treated daily by either subcutaneous injection (SQ) or oral gavage (PO) with either vehicle control, or DE-71 (50 mg/kg for 34 days or with 75, 150 or 300 mg/kg for 3 days). For the 34 days regime, a positive control group of 5 animals was treated SQ two times per week with 10 μg/kg EB in tocopherol-free corn oil (10 μL); EB in oil is a long acting formulation of the hormone (de Souza and Coutinho, 1975). The 34-day experiment was repeated, for a total of 7-11 animals per group. For the 3 day regime, positive control groups of 4-5 animals were treated once with either 10 μg/kg EB or 10 mg/kg EB (SQ, 10 μL). An additional group for each DE-71 treatment was also co-treated with 10 μg/kg EB. 3-day SQ treatment groups were repeated in a separate experiment (except 10 mg/kg EB), for a total of 7-10 animals per SQ treatment group, and 5 animals per PO treatment group. Doses were selected based on the maximal total dose given to mice by Staskal et al. (2005), 100 mg/kg, which resulted in no acute toxicity. The 6,200 mg/kg oral lethal dose for 50% mortality (LD50) for rats listed in the DE-71 Material Safety Data Sheet (Appendix 1) was also taken into consideration. For the 3-day assay, we used one dose lower than Staskal et. al., 75 mg/kg, and two higher doses, 150 and 300 mg/kg, once daily for 3 days, without any signs of acute toxicity (decrease body weight, anorexia, lethargy, or death). For the 34-day assay, mice were administered 50 mg/kg daily for 5 days per week, without any signs of toxicity. Chemicals were first dissolved in DMSO and then diluted in corn oil for PO treatment and administered at 0.1 mL for PO, 10-20 μL for SQ. The day after the last treatment, animals were sacrificed by decapitation. Blood was collected by exsanguination and kept at 4°C not more than 20 hours before it was centrifuged at 10,000 x *g* for 10 min at 4°C. The supernatant (serum) was collected and stored at -20°C until analysis for individual

BDE congeners and their hydroxylated metabolites (Qiu et al. 2007). The liver was perfused in situ with phosphate buffered saline by placing a cannula inside the hepatic portal vein while the superior vena cava was clamped (between heart and neck) and the inferior vena cava was cut (between liver and kidneys). The uterus and liver were weighed and expressed on a per gram of body weight basis. One uterine horn and the vagina were fixed in Bouin's Solution overnight. The liver was flash-frozen in liquid nitrogen and then stored at -70°C. The right side, #4 inguinal mammary gland was dissected whole, spread on BSA-treated slides, and fixed in Carnoy's fixative overnight. The fixed uterus and vagina were embedded in paraffin and 5 μm cross sections were stained with hematoxylin and eosin for analysis by light microscopy. Whole mounts of the mammary glands were prepared as described below. After digital micrographs were visually analyzed, the mammary tissue was removed from the whole mount and embedded in paraffin. Cross sections of the mammary tissue were stained with hematoxylin and eosin. Uterine epithelial cells height (UEH), vaginal epithelial thickness (VET), and mammary branches per duct length (BPL), ductal epithelial height (DEH) and ductal lumen area (DLA) measurements were used as estrogen-sensitive endpoints (measured using IPLab version3.5 imaging software, Scanalytics Inc., Fairfax, Virginia, USA).

Whole Mount Mammary Glands

Mammary glands were processed according to procedures described by Rasmussen et al. (2000) with minor modifications. The right inguinal mammary gland from each animal was dissected from the body in its entire fat pad and this was placed on a glass slide. After applying to slides, mammary glands were hydrated in consecutive baths of 70%, 50% and 25% ethanol (15 minutes each), and distilled water (5 minutes) before staining in carmine alumn (Sigma-Aldrich) overnight. Slides were briefly rinsed with distilled water before destaining in acid ethanol (38% 1 N HCl) for 2-4 hours or until background was minimal. Slides were then dehydrated in consecutive baths of 70%, 80%, 95%, 100% and 100% ethanol (15 minutes each). The tissue was then cleared in HistoClear (National Diagnostics; Atlanta, Gerogia, USA) twice (1 hour each) and mounted with a coverslip using Permount (Fisher; Fair Lawn, New Jersey, USA).

Cytochrome P450 Activity Assays

For each animal, about 0.2 g of frozen liver was homogenized in 1 mL high-sucrose buffer (0.15 M KCL, 0.5 M Tris, 1 mM EDTA, 0.25 M sucrose, 0.2 mM PMSF, 20 μM BHT, pH = 7.4) and centrifuged at 9000 x *g* and 4°C for 20 minutes. In order to obtain the microsomal fraction, the resulting supernatant was centrifuged at 105,000 x *g* and 4°C for 60 minutes; the pellet was washed in potassium diphosphate buffer (0.1 M $K_4P_2O_7$, 1 mM EDTA, 0.2 mM PMSF, 20 μM BHT, pH = 7.4) by resuspending it using disposable

homogenizing microtubes and pestles (Kontes Glass Company; Vineland, New Jersey, USA). Protein content of the microsomal preparation was determined using the Pierce BCA Protein Assay Kit. Each animal was assayed in duplicate.

7-Ethoxyresorufin O-dealkylation (EROD, CYP1A) activity and 7-pentoxyresorufin O-dealkylation (PROD, CYP2B) activity were measured immediately by mixing 5 μL of sample with 5 μL 250 μM NADPH and 3.4 μL of 0.6 mM 7-ethoxyresorufin or 7-pentoxyresorufin in 1.2 mL 0.1 Tris buffer at 37°C. After allowing the mixture to equilibrate for 1 min, fluorescence was measured at 530 nm excitation and 585 nm emission in a fluorometer at 1 sec intervals over the course of 1 min. Similar measurements were made with 6 different resorufin concentrations to determine a standard curve. The slope for each activity assay (Δfluorescence/second) was converted to moles of resorufin per gram per second (moles/gram*second) using the standard curve and the protein content of each sample.

Statistics

All statistics were done using GraphPad Prism version 3.0a for Macintosh (GraphPad Software; San Diego, California, USA). For each statistical analysis, it was determined if groups had unequal variances by Bartlett's test. Group averages with equal variances were compared to each other by either one-way ANOVA with Tukey post-test or unpaired T-test. Group averages with unequal variances were compared to each other by

T-test with Welch's correction. Groups treated with DE-71 alone were analyzed against vehicle controls. Groups co-treated with DE-71 and EB were analyzed against controls treated with EB alone.

Dose-response curves were also analyzed by either linear regression or sigmoidal (dose-response with variable slope) regression analysis:

Linear: $y = xm + b$

Sigmoidal: $y = B + \frac{T - B}{1 + 10^{(\log EC50 - x)H}}$.

were: y = dependent variable, x = independent variable

m = linear slope, b = y-axis intercept value

B = minimum value, T = maximum value

EC50 (or IC50) = x value at {y = (T-B)/2}

H = Hill slope, describes the steepness of the curve:

H = 1, standard sigmoid dose-response curve,

H < 1, shallower than standard curve,

H < 1, steeper than standard curve.

The regression model that best fit the data was chosen as the one that yielded the highest regression coefficient value; results are shown in chart if the regression coefficient was ≥ 0.9. All values are expressed as mean ± standard error of the mean (SEM). Except as noted in figure legends, all cell culture assays were measured 4 times per assay (i.e. four independently plated wells during the same assay, n = 4) and ERα binding assays were measured 3 times per assay (i.e. 3 independently mixed incubations during the same

assay, n = 3). Results from at least 3 different in culture or in vitro assays (i.e. assay done at different dates) were averaged for each data point, and presented as mean ± (SEM). For animal assays, the number of animals used per treatment group (n) is indicated in figure legends.

Results

Specific Aim 1: Determine if DE-71 exerts estrogenic or antiestrogenic effects using cell culture and animal models.

1.1 DE-71 acts as a weak estrogen in breast cancer cell culture model

MCF-7 breast cancer cells proliferate in culture when treated with estrogens, and this feature has been used as the basis of a simple assay to test the estrogenicity of a chemical (Soto et al. 1991; Soto et al. 1995). We used this assay to compare the capacity of DE-71 to increase MCF-7 proliferation to that of β-estradiol, the most potent endogenous estrogen in mammals (Coelingh Bennink 2004), and two known xenoestrogens studied in our laboratory: o,p'-DDT and β-HCH. DE-71 was able to significantly increase cell number as determined by MTT assay (Figure 9A), however it was 8 orders of magnitude less potent than estradiol, and 3 orders of magnitude less potent than than the xenoestrogens β-HCH and o,p'-DDT. DE-71 was also 30% less effective than either β-estradiol, β-HCH and o,p'-DDT. DNA assays confirmed this finding, indicating that the increase in reduced MTT was due to an increase in cell number and not an effect on the number of mitochondria (Figure 9B). The DNA assay was more sensitive than the MTT assay because it measures DNA already synthesized by cells in S phase before they divide into two different cells with a complete set of organelles. However, because the fluorometer measurements had a higher variability than measurements made in the spectrophotometry readings for the MTT assay, the standard error for final DNA assay

data are much larger than the standard errors for the final MTT assay data (see Methods for more information about the two instruments). In the MTT assay, DE-71 produced a biphasic dose-response curve suggesting that it may have been toxic to MCF-7 cells at concentrations above 25 μM (Figure 9A).

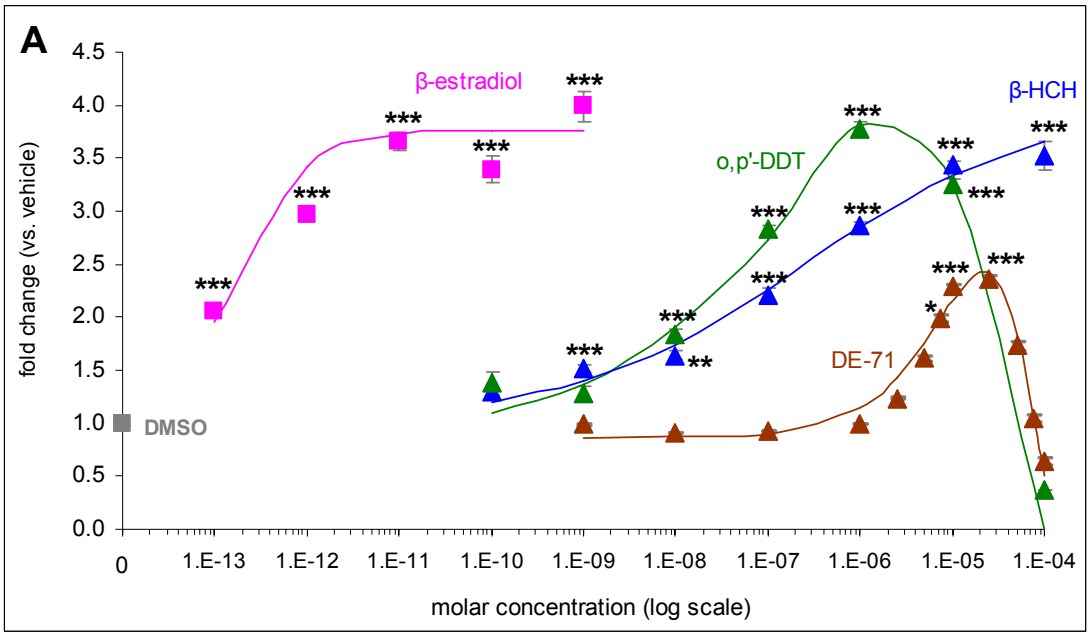
To assess if an ERα antagonist could prevent the proliferative effect of DE-71 on MCF-7 cells, some treatment groups were cotreated with 10 μM DE-71 and 10 nM ICI. A control group was cotreated with 10 pM β-estradiol and 10 μM ICI. The Type II ERα antagonist ICI blocks the effects of estrogens in all tissues, and also down-regulates the receptor by increasing its ubiquination (Long and Nephew 2006; Metzger et al. 1995). The effects of both β-estradiol and DE-71 were negated by ICI co-treatment (Figure 9C).

A ligand that cannot induce the full activity of a receptor (i.e. a "weak" ligand) can prevent other ligands from binding and fully activating the same receptor. In such a case, the "weak" ligand occupies the same binding pocket in the receptor that the "stronger" ligand would occupy, thus preventing the latter from binding. This "weak" ligand behavior is known as partial agonism, while the "strong" ligand is known as a full agonist. Because DE-71 induced a lesser cell proliferation effect on MCF-7 cells than β-estradiol, it was possible that DE-71 behaved like a partial ERα agonist and could therefore prevent the full effect of β-estradiol. To determine if DE-71 was a partial ERα agonist, MCF-7 cells were co-treated with DE-71 and β-estradiol, and cell proliferation was assessed as before. Co-treatment of cells with both β-estradiol and DE-71 resulted in a lesser increase in cell proliferation compared with β-estradiol alone, suggesting an

antagonistic effect of DE-71 on β-estradiol-induced cell proliferation (Figure 9D). The apparent antagonism was dose-dependent; the highest dose tested corresponds to that which produced maximal proliferative effect when cells were treated with DE-71 alone, without any signs of toxicity. While there were no visual signs of toxicity in the co-treated cell cultures and the concentrations of DE-71 and β-estradiol used in co-treatments produced only cell proliferation when cells were treated with each chemical individually, we did not test for a specific toxic or cytostatic effect on cells when treated with both chemicals at the same time. Therefore toxicity or cytostasis cannot be excluded as the cause of the apparent antagonistic effect of DE-71 on β-estradiol-induced cell proliferation.

In summary, DE-71 behaved as a weak estrogen in the MCF-7 cell culture assay, and may be a partial ER agonist.

Figure 9. Effects of DE-71 on MCF-7 cell proliferation in culture. Cells were cultured for 10 days in basal media with either 0.1% DMSO (vehicle control), β-estradiol, 1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)-ethane (o,p'-DDT), β-hexachlorocyclohexane (β-HCH) or DE-71 at the concentrations indicated in the chart. A: Dose-response measured by MTT assay. B: Dose-Response measured by DNA assay. Data points and columns are averages of at least 2 independent assays with 4 replicated each ± SEM. *. $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; versus vehicle control (DMSO).



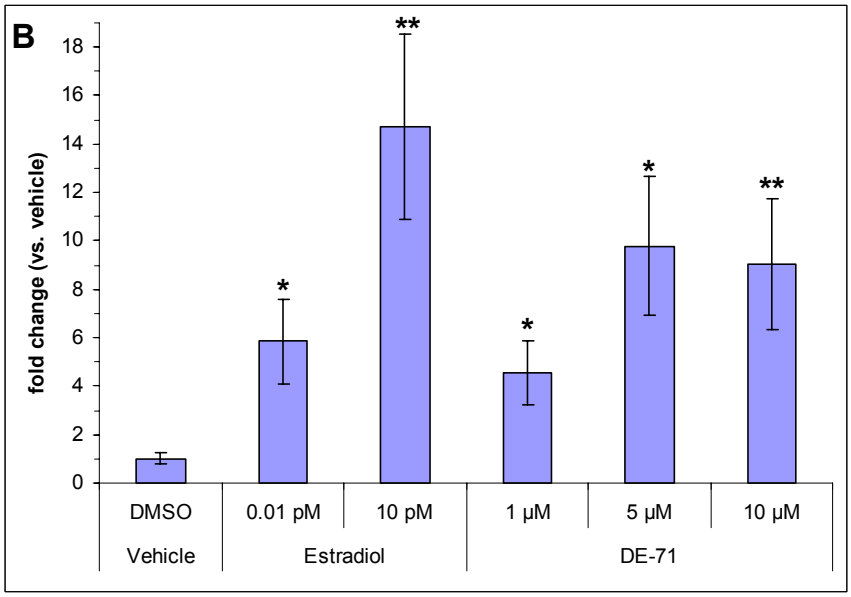


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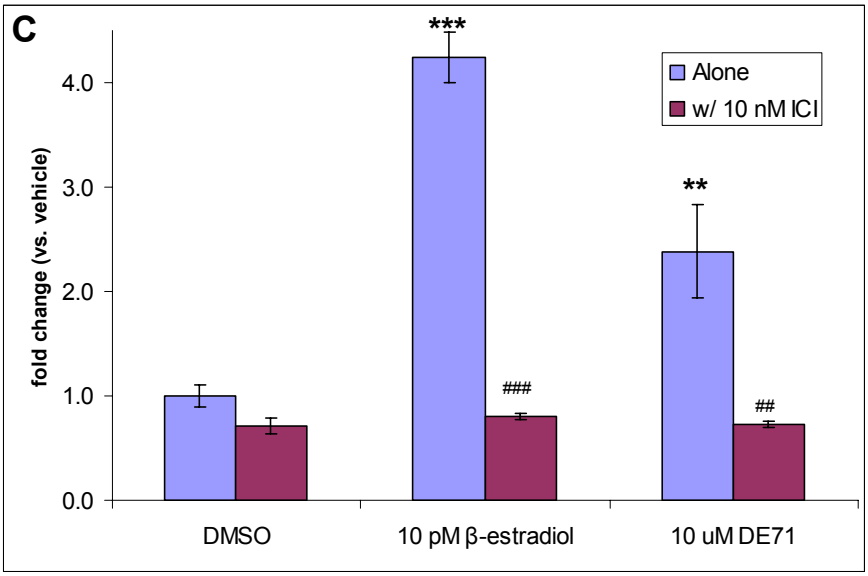
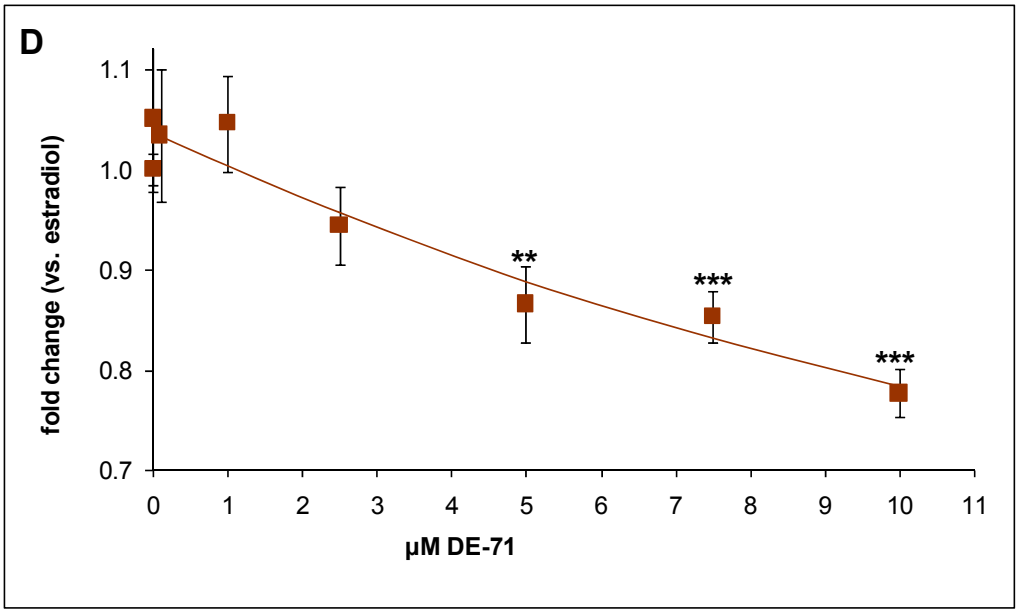
Figure 9 (cont.). Effects of DE-71 on MCF-7 Cell proliferation in culture. Cells were cultured for 10 days in basal media with either 0.1% DMSO (vehicle control), β-estradiol, fulvestran (ICI), or DE-71, or with DE-71 in combination with β-estradiol or ICI at the concentrations indicated in the chart. C: Antagonism of DE-71 effect by the pure antiestrogen ICI measured by MTT assay; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; vs. vehicle control (DMSO). $^{\#\#}$, $p < 0.01$; $^{\#\#\#}$, $p < 0.01$; vs. same treatment without ICI. D: Antagonism of 10 pM β-estradiol effect by DE-71 measured by MTT assay; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; vs. 10 pM β-estradiol treatment alone. Trend line determined by linear regression model. Data points and columns are averages of at least 2 independent assays ± SEM.





1.2 DE-71 acts as a weak estrogen in OVX mice model after 3 days of treatment

The rodent uterothrophic assay is the oldest and most frequently used model to assess the estrogenicity of a chemical (Clode 2006; Huggins et al. 1954). Animals are ovariectomized to minimize endogeous estrogen production before challenge with a chemical suspected to have estrogenic activity. After treatment, animals are sacrificed and their estrogen responsive tissues (uterus, vagina, mammary gland) are examined for signs of stimulation. Most notably, an estrogen will increase the size of the uterus and vagina, increase the height or thickness of epithelial cell layers surrounding the lumen of the uterus, vagina, and mammary ducts, and increase water uptake and accumulation in some estrogen target tissues (Bigsby 2007; Suzuki 2007; Shi 2004; Raafat and Hofseth 2001; Papaconstantinou 2000; Orimo 1999; Milligan 1998; Steinmetz 1998). Therefore, we used ovariectomized (OVX) mice to assess the estrogenicity of DE-71. Two dosing regimes were used: 3 days and 34 days.

A 3-day treatment regimen was used in ovariectomized adult BALB/c mice to assess the estrogenic effects of short-term exposure to DE-71 at 3 doses: 75, 150 and 300 mg/kg; groups of animals were also treated with 10 μg/kg EB plus DE-71 at each of the three doses (as described in Methods). There was no significant decreases in body weight after treatment (Table 3), nor any other clinical signs that would indicated an acute toxic effect of the DE-71 doses used (loss of appetite, lethargy, bleeding, convulsions). Uterine wet weight, UEH, and VET were used as estrogenic endpoints. Oral DE-71 administration

had no effect on any of the estrogenic parameters measured (Figures 10-12). SQ DE-71 administration alone had no statistically significant effect (Figures 10A, 11A and 12A). However, the 6-fold increase in uterine wet weight induced by 10 μg/kg EB was enhanced an additional 36%-50% by SQ DE-71 in a dose-dependent manner and this enhanced response was equivalent to the estrogen effect produced by 10 mg/kg EB (Figure 10B).

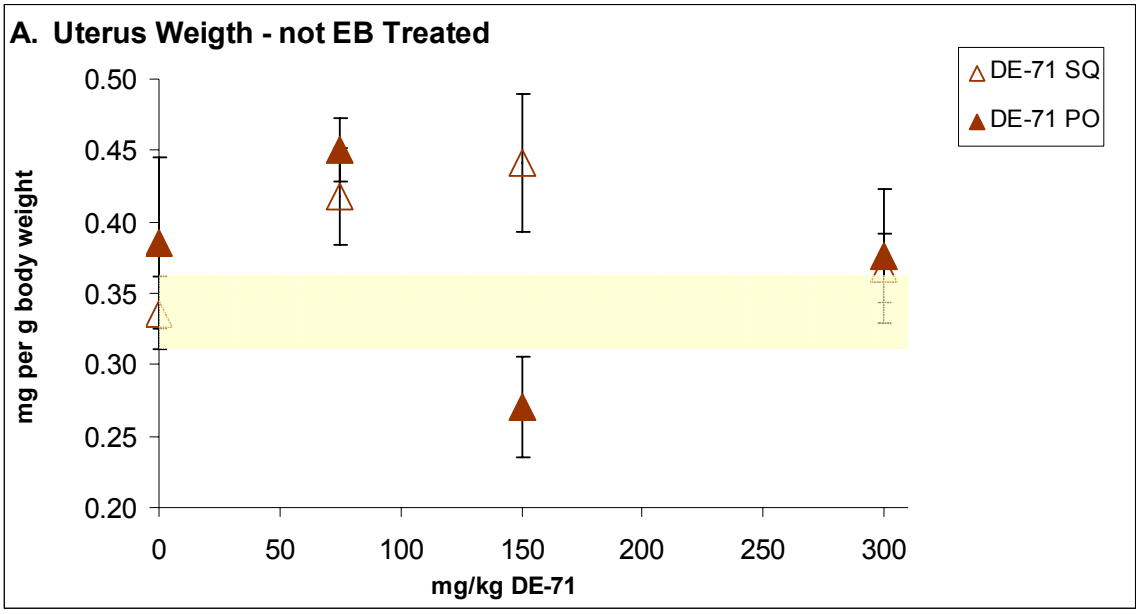
DE-71 treatment alone had no effect on UEH or VET. However, regression analysis indicated that DE-71 increased UEH 4%-8% ($R^2 = 0.999$) and VET 4%-17% ($R^2 = 0.995$) in SQ EB-treated mice (Figures 11B and 12B). It should be noted that these enhanced responses in the uterine and vaginal epithelia were above the maximal effects produced by 10 mg/kg EB.

The results of 3-day in vivo treatment show that DE-71 had no estrogenic effects when BALB/c mice are treated short-term with DE-71 alone, but that DE-71 can potentiate the effects of EB in estrogen target tissues.

Table 3. Average body weight (mg/kg) of BALB/c mice treated for 3-days by SQ or PO routes with vehicle, DE-71, EB, or co-treated with DE-71 and EB. Animals were weighed after the corresponding treatment, immediately before necropsy. Values are presented as mean ± SEM. *, $p < 0.05$ versus vehicle control. n, number of animals per group.

Route	Treatment	Dose	Average ± SEM	n
SQ	DMSO	10 uL	20.3 ±0.44	10
	DE-71	75 mg/kg	22.3 ±0.37 *	10
		150 mg/kg	21.0 ±0.38	10
		300 mg/kg	19.8 ±0.41	10
	EB	10 ug/kg	21.6 ±0.43	10
		10 mg/kg	22.8 ±0.43	4
	DE-71 + 10 ug/kg EB	75 mg/kg	22.3 ± 0.53 *	10
		150 mg/kg	22.2 ±0.48	10
		300 mg/kg	22.5 ±0.57 *	10
PO	Corn Oil	100 uL	20.7 ±0.64	5
	DE-71	75 mg/kg	22.8 ±0.41	5
		150 mg/kg	19.7 ±0.39	5
		300 mg/kg	19.6 ±0.65	5
	DE-71 + 10 ug/kg EB	75 mg/kg	21.1 ±0.85	5
		150 mg/kg	23.1 ±0.78	5
		300 mg/kg	21.2 ±0.86	5

Figure 10. Relative uterus weight of OVX BALB/c mice after 3 days of treatment. A: Dose-Response for mice treated with DE-71 alone; yellow box indicates average ± SEM for SQ vehicle control treated mice. B: Dose-Response for mice treated with DE-71 and EB in combination; line represents curve modeling of SQ-treated mouse data with $R^2 \geq 0.9$; yellow box indicates average ± SEM for mice treated with 10 µg/kg EB only; pink box indicates average ± SEM of mice treated with 10 mg/kg µg/kg EB only. Each data point is the average of 4 - 10 mice ± SEM. ** $p < 0.01$ and * $p < 0.05$ vs. 10 mg/kg µg/kg EB on. PO, oral treatment; SQ, subcutaneous treatment; EB, β-estradiol benzoate.



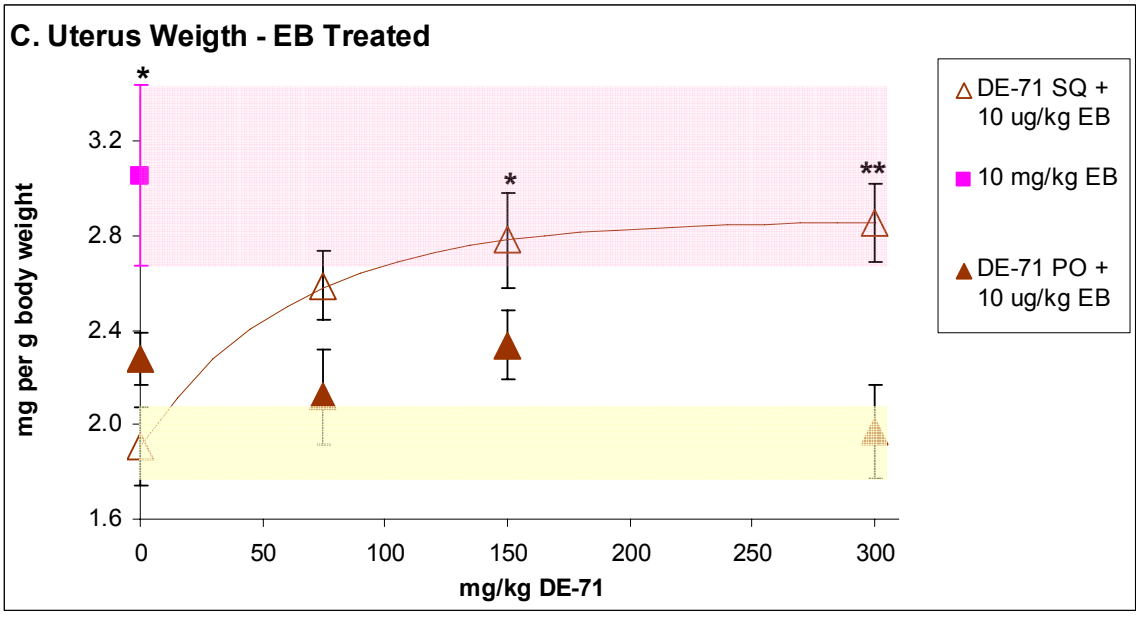
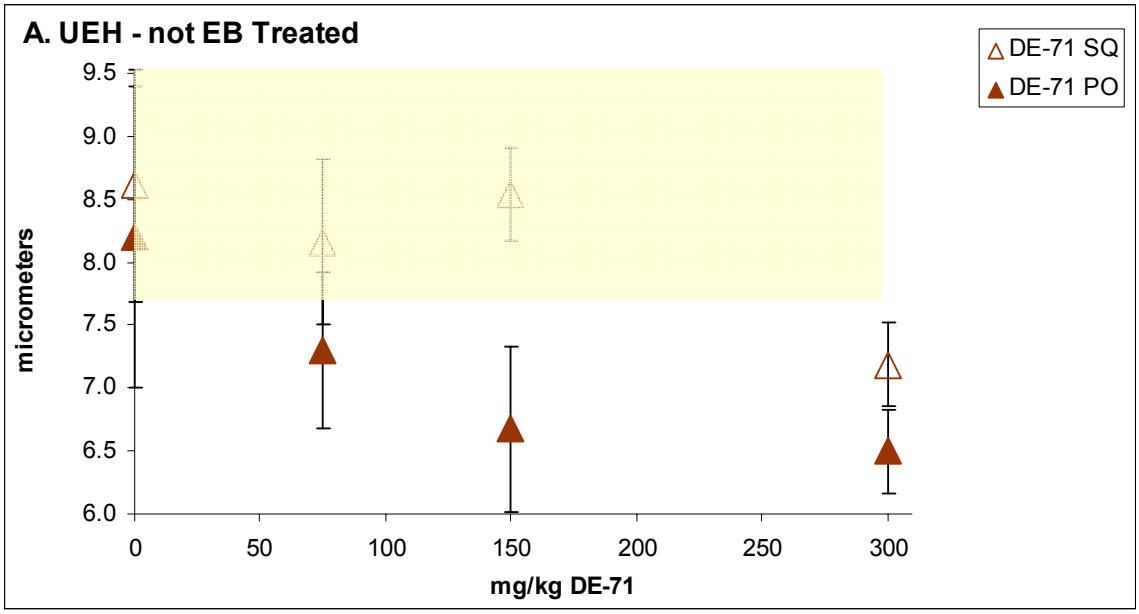


Figure 11. Uterine epithelial height of OVX BALB/c mice after 3 days of treatment. A: Dose-response for mice treated with DE-71 alone; yellow box indicates average ± SEM for SQ vehicle control treated mice. B: Dose-response for mice treated with DE-71 and EB in combination; yellow box indicates average ± SEM for mice treated with10 µg/kg EB only; line represents curve modeling of SQ-treated mouse data with $R^2 \geq 0.9$; pink box indicates average ± SEM of mice treated with 10 mg/kg EB only. Each data point is the average of 5 - 10 mice ± SEM. PO, oral treatment; SQ, subcutaneous treatment; EB, β-estradiol benzoate.



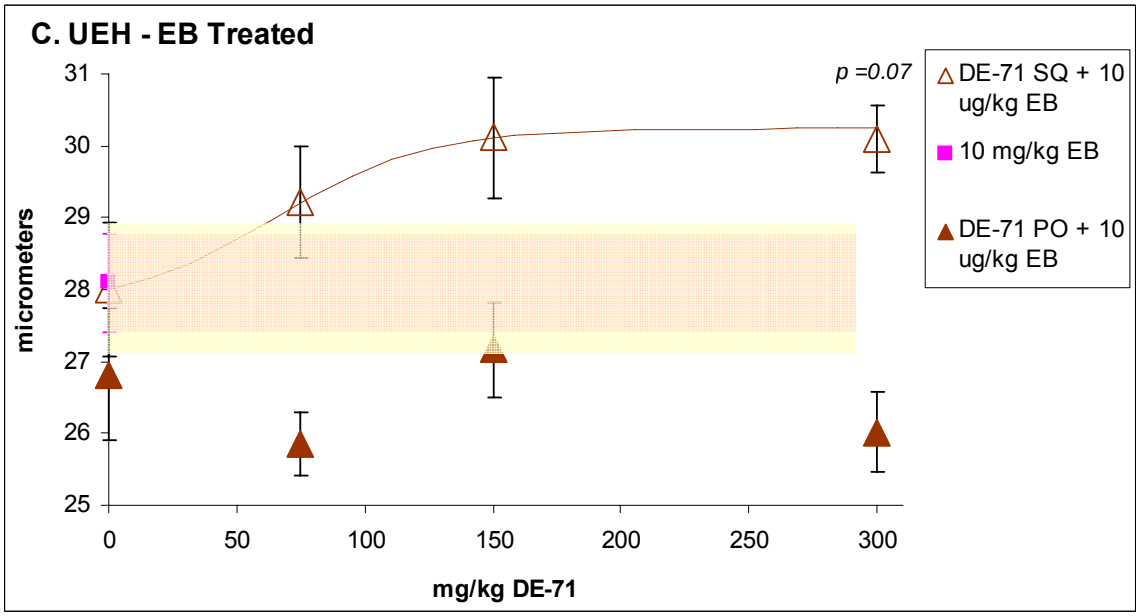
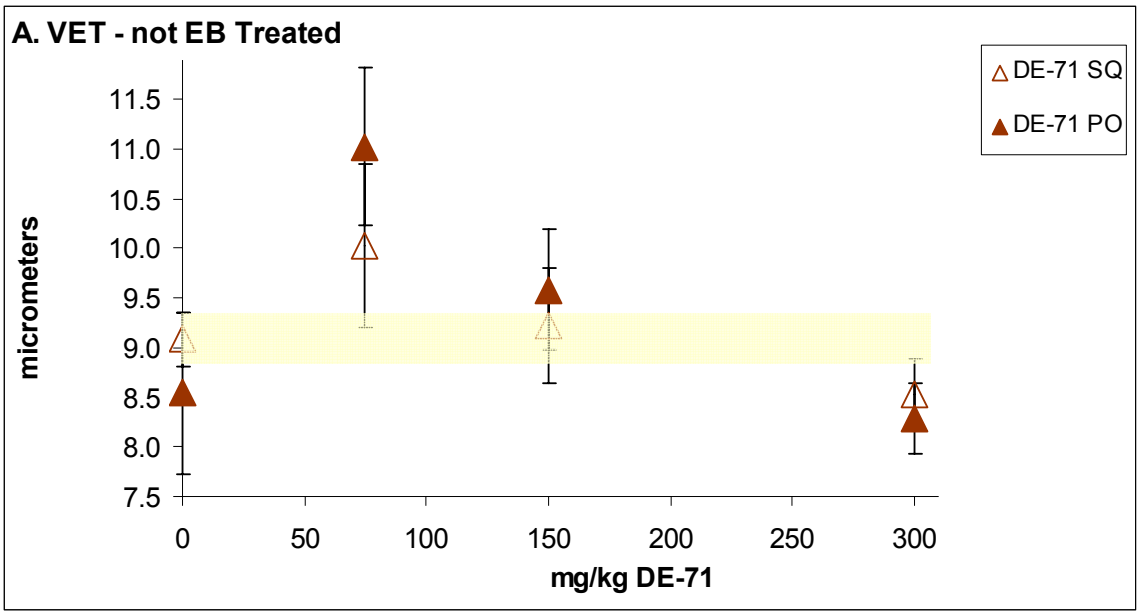
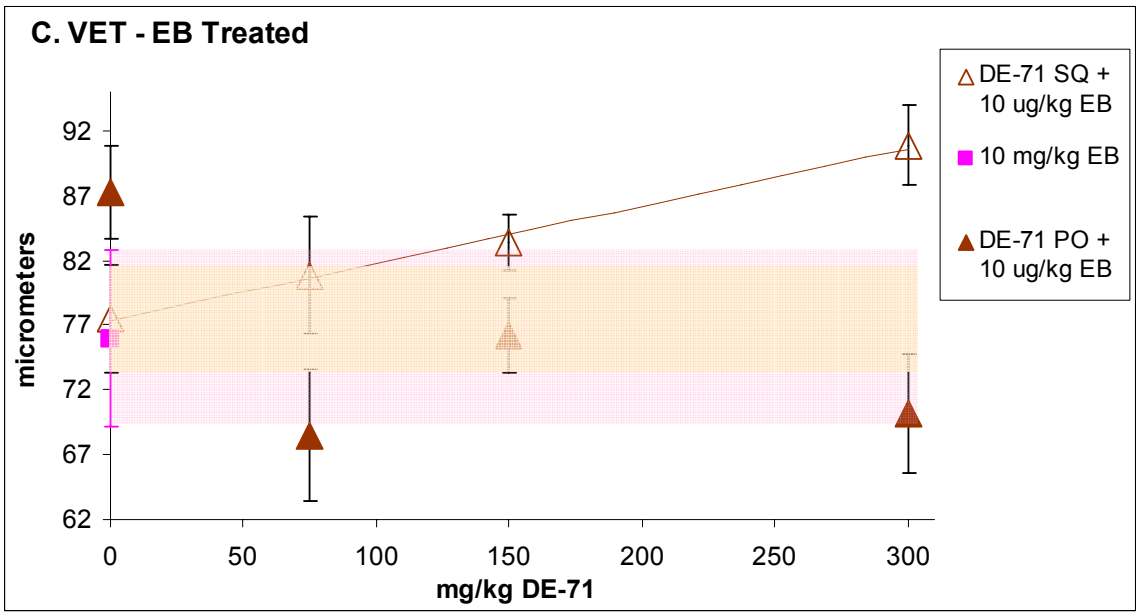


Figure 12. Vaginal epithelial height of OVX BALB/c mice after 3 days of treatment. A: Dose-response for mice treated with DE-71 alone; yellow box indicates average ± SEM for SQ vehicle control treated mice. B: Dose-Response for mice treated with DE-71 and EB in combination; yellow box indicates average ± SEM for mice treated with 10 µg/kg EB only; line represents curve modeling of SQ-treated mouse data with $R^2 \geq 0.9$; pink box indicates average ± SEM of mice treated with 10 mg/kg only. Each data point is the average of 5 - 10 mice ± SEM. PO, oral treatment; SQ, subcutaneous treatment; EB, β-estradiol benzoate.





1.3 DE-71 acts as a weak estrogen in OVX mice model after 34 days of treatment

To assess the chronic estrogenic effect of DE-71 exposure in vivo, ovariectomized BALB/c, C57BL/6 wild type and ERαKO mice were treated for 34 days (as described in Methods). DE-71 was administered daily at 50 mg/kg by either oral gavage (PO) or by subcutaneous injection (SQ). Uterine and vaginal parameters were examined as in the 3-day assay. In addition, mammary duct branching, mammary ductal epithelial height (DEH), and mammary ductal lumen area (DLA) were assessed as a measure of mammotrophic effects. Data from two experiments with BALB/c mice are shown separately because the baseline values for uterine weights differed between experiments. Mammary parameters were examined only in the second experiment.

There was no significant decreases in body weight after treatment (Table 4), nor any other clinical signs that would indicated an acute toxic effect of the DE-71 doses used (loss of appetite, lethargy, bleeding, convulsions). As with the 3-day assay, oral DE-71 administration had no effect on any of the estrogenic parameters measured in BALB/c mice (Figures 13, 15 and 16); C57BL/6 mice were not treated orally.

There was a large increase (8- to 12-fold; $p < 0.001$) in uterine weight of wild type mice after estradiol treatment (Figures 13B and 14B); the BALB/c uterine weight response to 10 ug/kg EB at 34 days was similar to the increase produced by 10 mg/kg EB for 3 days,

therefore both the 10 mg/kg 3-day treatment and the 10 μg/kg 34-day EB treatments may have produced the maximal uterotrophic effect.

SQ treatment for 34 days with DE-71 alone produced no significant change in uterine weight (Figures 13A and 14A). EB-induced uterine weight was unaffected by co-treatment with DE-71 (Figures 13B and 14B).

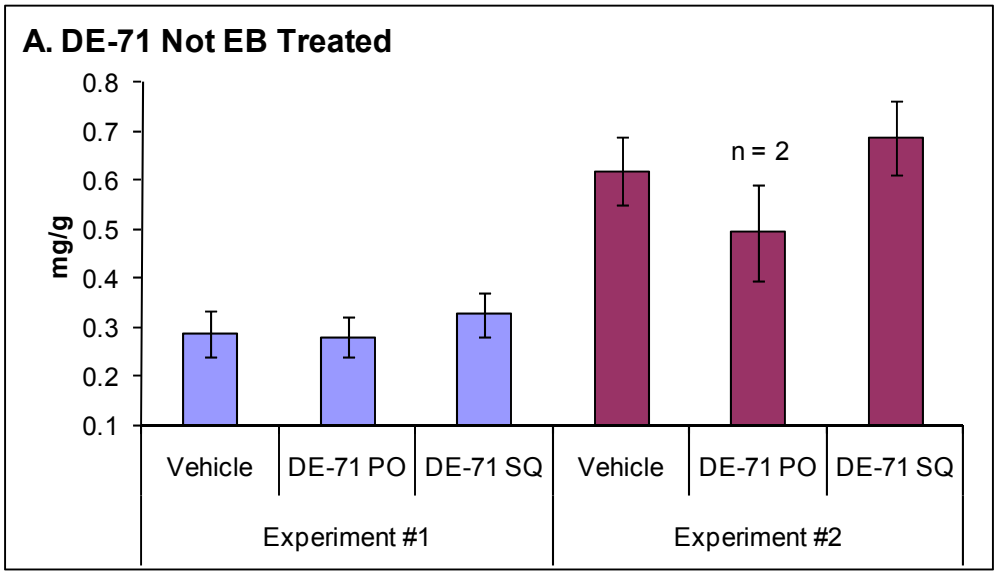
In BALB/c mice, DE-71 administered subcutaneously for 34 days caused a 20% increase in UEH (Figure 15A) and about a 35% increase in VET (Figure 16A). On the other hand, there was a 19% decrease in the estrogen-induced UEH increase in BALB/c mice co-treated with DE-71 subcutaneously that was statistically significant in the first experiment but not in the repeat experiment (Figure 15B). The slight decrease in UEH of co-treated C57BL/6 mice was not statistically significant (Figure 17B). Co-treatment did not alter the EB-induced increase in VET (Figures 16B and 18B).

Since we observed weak estrogenic effects of DE-71 in BALB/c mice treated SQ for 34 days, C57BL/6 wild type and ERαKO mice were also treated with 50 mg/kg DE-71 SQ for 34 days in order to determine if the in vivo effects of DE-71 were mediated by ERα. As expected, estradiol treatment had no effect on ERαKO uterine weights, UEH or VET. However, the experiment was not informative because DE-71 did not have an effect on any of the uterine or vaginal parameters in either wild type C56BL/6 mice or ERαKO animals (Figures 14, 17 and 18).

Table 4. Average body weight (mg/kg) of BALB/c and C57BL/6 mice treated for 34 days by SQ or PO routes with vehicle, DE-71, EB, or co-treated with DE-71 and EB. Animals were weighed after the corresponding treatment, immediately before necropsy. Values are presented as mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; versus vehicle control. n, number of animals per group.

Experiment	Treatment	Dose	Average ± SEM	n
BALB/c #1	Corn Oil PO	100 µL	18.7 ± 0.53	5
	DE-71 PO	50 mg/kg	20.0 ± 0.39	6
	DE-71 SQ	50 mg/kg	21.8 ± 0.36 **	5
	EB	10µg/kg	21.2 ± 0.36 **	5
	DE-71 PO + 10 µg/kg EB	50 mg/kg	20.8 ± 0.36 *	6
	DE-71 SQ + 10 µg/kg EB	50 mg/kg	24.2 ± 0.44 **	5
BALB/c #2	Corn Oil PO	100 uL	20.4 ± 0.51	5
	DE-71 PO	50 mg/kg	20.0 ± 1.00	2
	DE-71 SQ	50 mg/kg	21.0 ± 0.71	5
	EB	10µg/kg	22.2 ± 0.58	5
	DE-71 PO + 10 µg/kg EB	50 mg/kg	21.8 ± 0.80	5
	DE-71 SQ + 10 µg/kg EB	50 mg/kg	22.4 ± 0.93	5
C57BL/6 WT	DMSO SQ	10 µL	23.9 ± 0.73	9
	EB	10µg/kg	21.7 ± 1.17	6
	DE-71 SQ	50 mg/kg	23.4 ± 0.94	7
	DE-71 PO + 10 µg/kg EB	50 mg/kg	24.4 ± 0.95	7
C57BL/6 ERαKO	DMSO SQ	10 µL	22.7 ± 0.74	7
	EB	10µg/kg	21.7 ± 0.67	6
	DE-71 SQ	50 mg/kg	20.1 ± 0.60	9

Figure 13. Relative uterus weight (mg per gram body weight) of OVX BALB/c mice after 34 days of treatment. A: Groups treated with 50 mg/kg DE-71 alone. B: Groups treated with 10µg/kg EB alone or in combination with 50 mg/kg DE-71. Mice were treated during two independent experiments: #1 and #2; Each column is the average of 5 - 6 mice ± SEM except as noted in chart. PO, oral treatment; SQ, subcutaneous treatment; EB, β-estradiol benzoate.



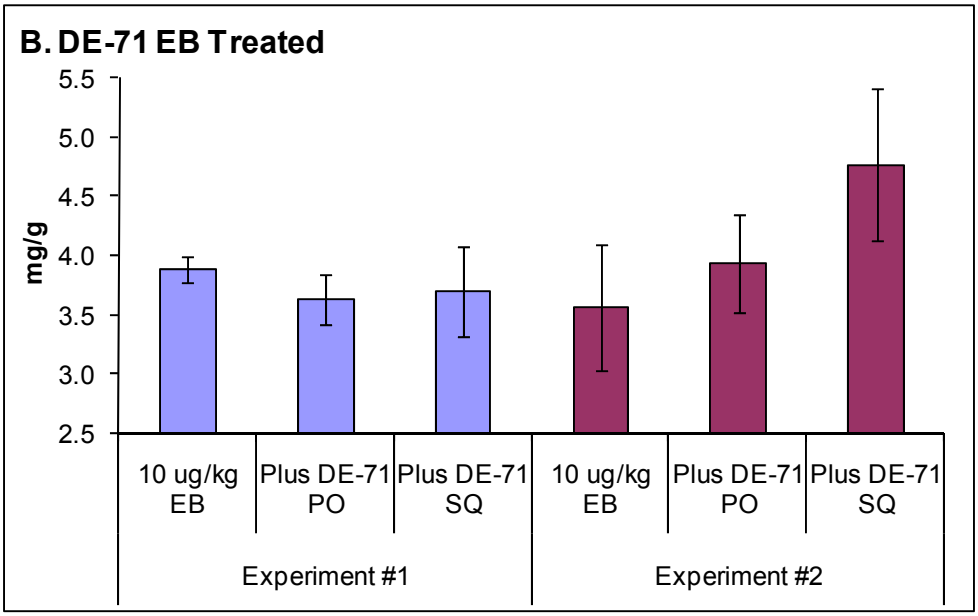


Figure 14. Relative uterus weight of OVX C57BL/6 wild type (WT) and ERαKO mice after 34 days of treatment. A: Groups treated with 50 mg/kg DE-71 alone. B: Groups treated with 10µg/kg EB alone or in combination with 50 mg/kg DE-71. Each column is the average of 6-9 mice ± SEM. SQ, subcutaneous treatment; EB, β-estradiol benzoate.

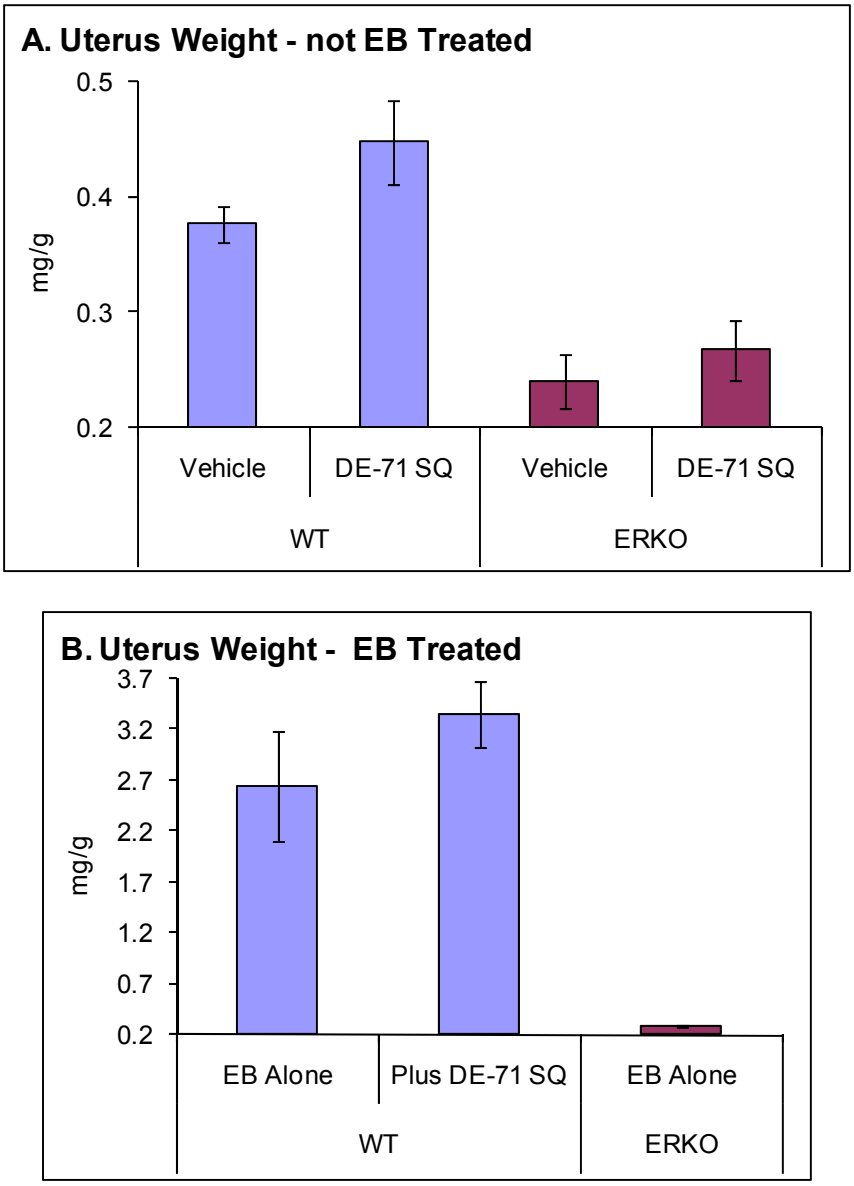
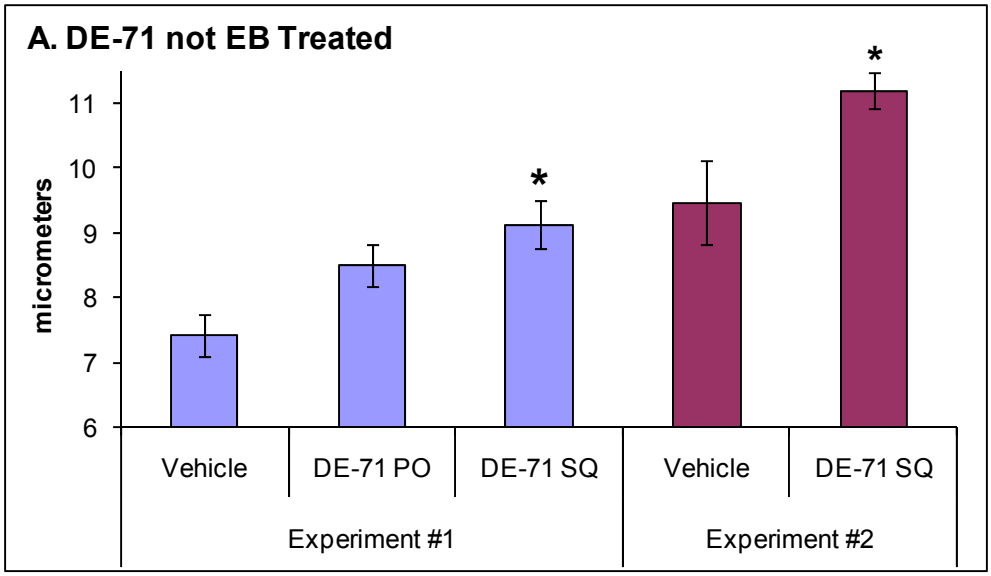


Figure 15. Uterine epithelial height of OVX BALB/c mice after 34 days of treatment. A: Groups treated with 50 mg/kg DE-71 alone; tissue from PO treated mice during the second experiment was not available for measurement. B: Groups treated with 10µg/kg EB alone or in combination with 50 mg/kg DE-71. Mice were treated during two independent experiments: #1 and #2. Each column is the average of 5-6 mice ± SEM except as noted in chart. *, $p < 0.05$ vs. vehicle. #, $p < 0.05$ vs. 10µg/kg EB. PO, oral treatment; SQ, subcutaneous treatment; EB, β-estradiol benzoate.



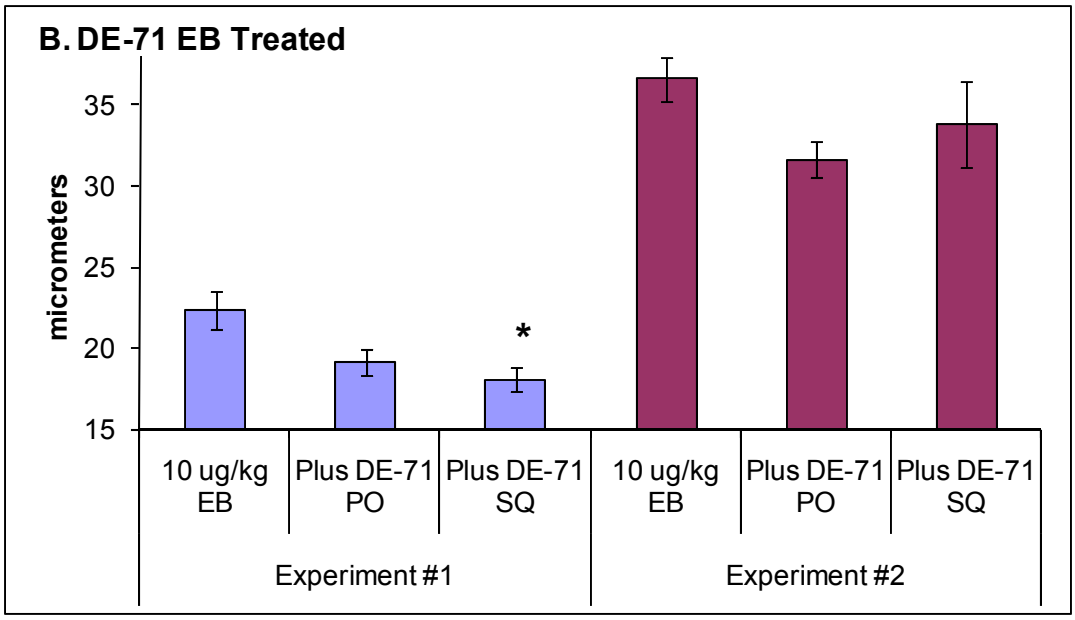


Figure 16. Vaginal epithelial height of OVX BALB/c mice after 34 days of treatment. A: Groups treated with 50 mg/kg DE-71 alone. B: Groups treated with 10µg/kg EB alone or in combination with 50 mg/kg DE-71. Mice were treated during two independent experiments: #1 and #2. Each column is the average of 5-6 mice ± SEM except as noted in chart. *, $p < 0.05$ vs. vehicle. PO, oral treatment; SQ, subcutaneous treatment; EB, β-estradiol benzoate.

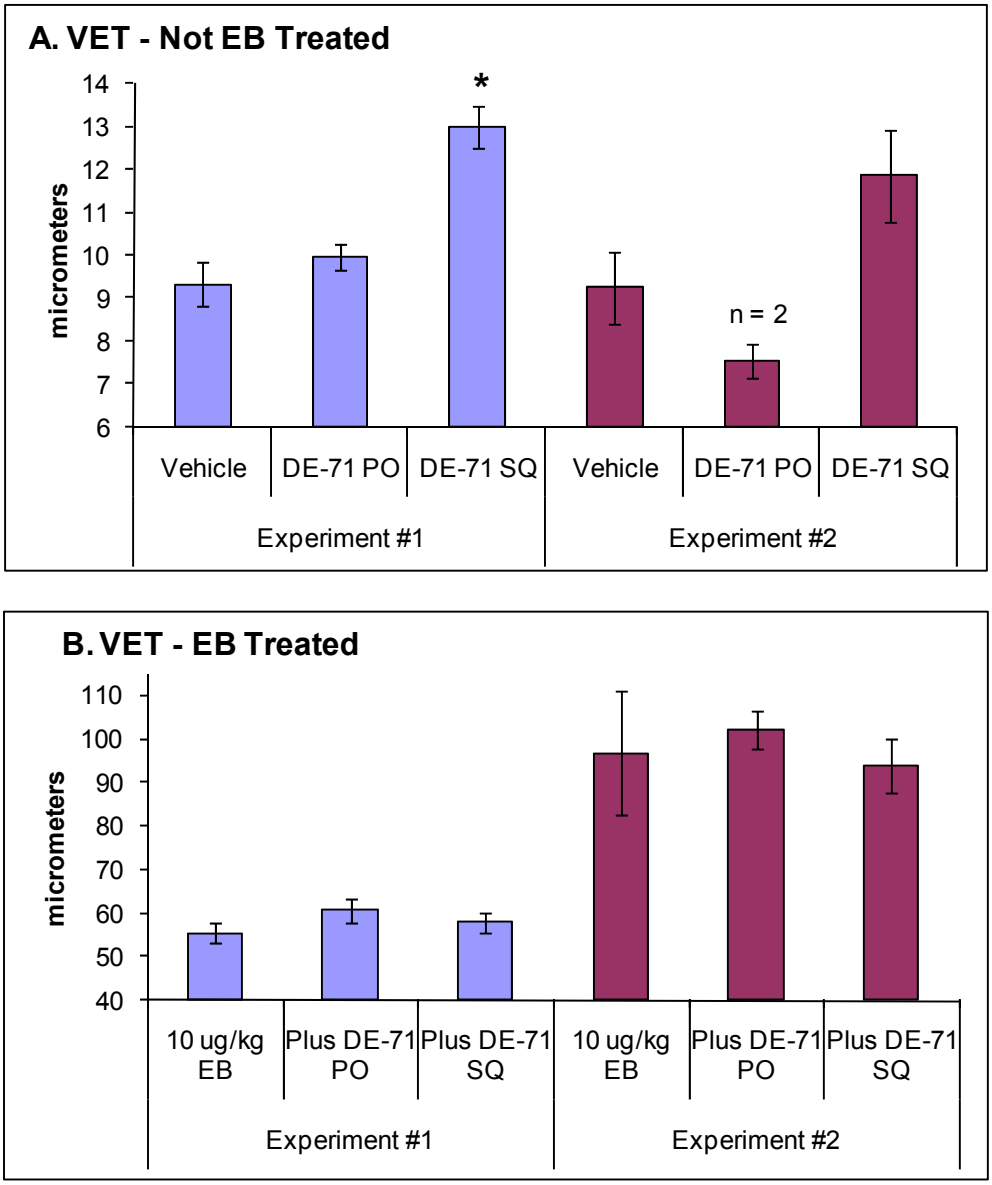


Figure 17. Uterine epithelial height of OVX C57BL/6 (WT and ERαKO) mice after 34 days of treatment. A: Groups treated with 50 mg/kg DE-71 alone. B: Groups treated with 10µg/kg EB alone or in combination with 50 mg/kg DE-71. Each column is the average of 6-9 mice ± SEM. SQ, subcutaneous treatment; EB, β-estradiol benzoate.

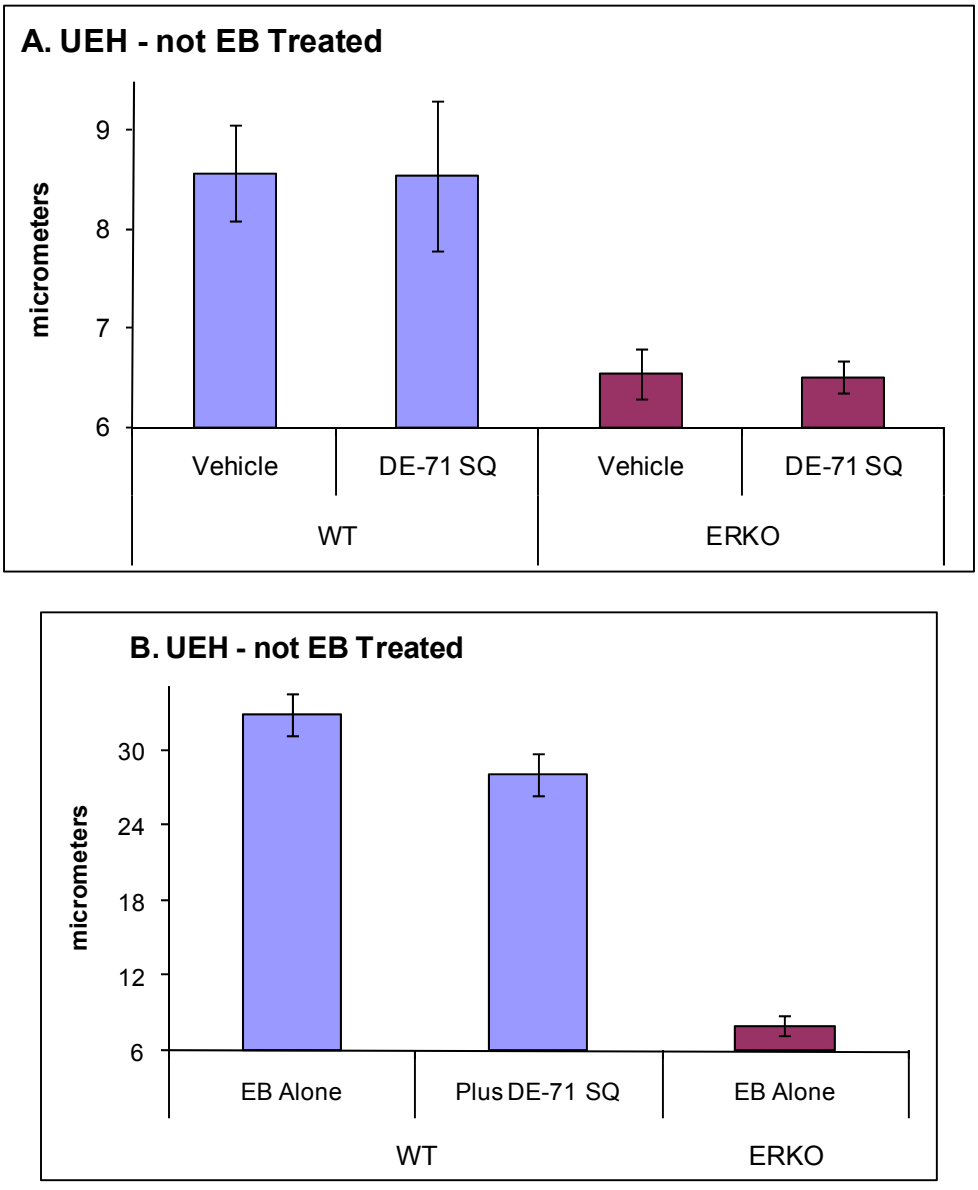
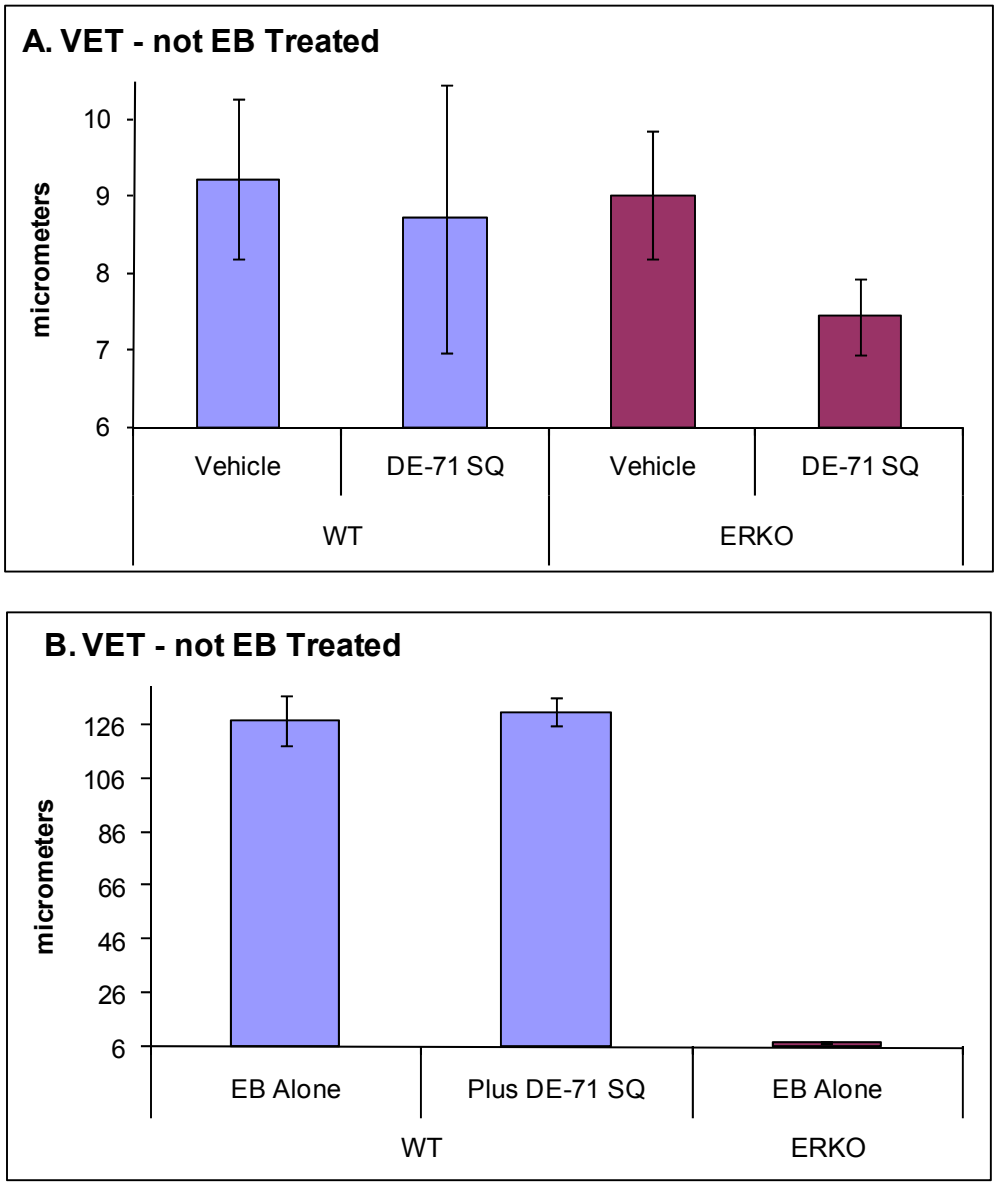


Figure 18. Vaginal epithelial height of OVX C57BL/6 (WT and ERαKO) mice after 34 days of treatment. A: Groups treated with 50 mg/kg DE-71 alone. B: Groups treated with 10µg/kg EB alone or in combination with 50 mg/kg DE-71. Each column is the average of 6-9 mice ± SEM. SQ, subcutaneous treatment; EB, β-estradiol benzoate.



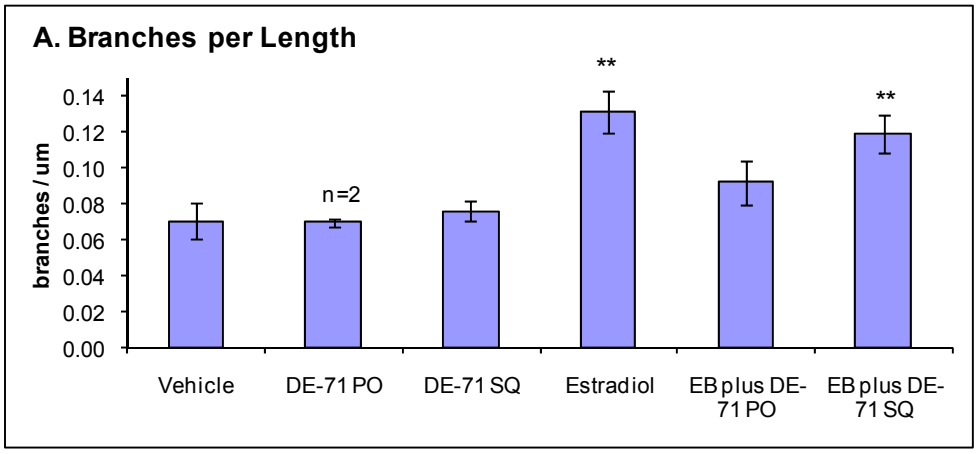
As discussed previously, estrogen treatment will increase the size of epithelial cell layers and increase water uptake and accumulation in estrogen target tissues (Bigsby 2007; Suzuki 2007; Shi 2004; Raafat & Hofseth 2001; Papaconstantinou 2000; Orimo 1999; Milligan 1998; Steinmetz 1998). In the mammary gland, estrogens also increase branching of the mammary ducts. Therefore mammary glands of BALB/c mice treated for 34 days (see Methods) were analyzed as whole mounts to determine branching per duct length (BPL), and then re-processed and cut transversely to measure ductal epithelial height (DEH) and ductal lumen area (DLA).

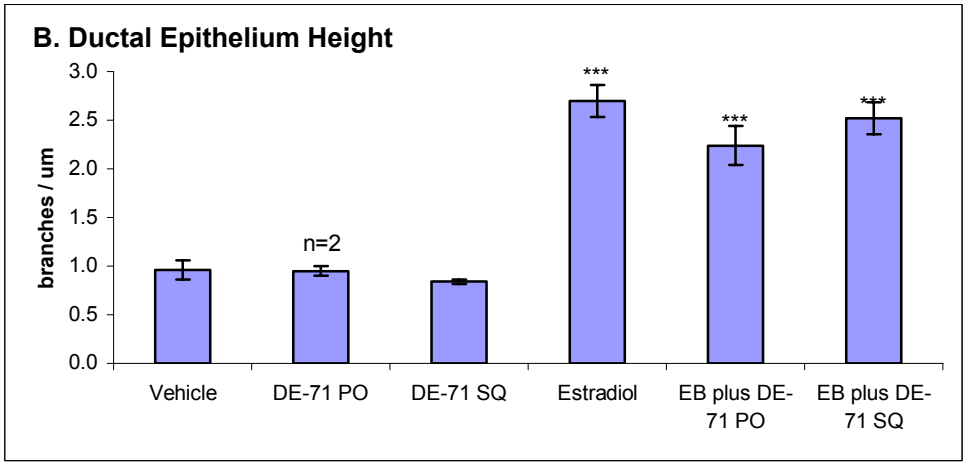
EB treatment increased all mammary parameters over vehicle controls (Figure 19). While DE-71 SQ treatment alone had no effect on BPL or DEH, there was a small (2-fold) but statistically significant increase in lumen area over vehicle controls (Figure 19C). DE-71 did not alter the effects of EB on any of the mammary gland parameters; a decrease in DLA in mice co-treated with DE-71 PO was not statistically significant.

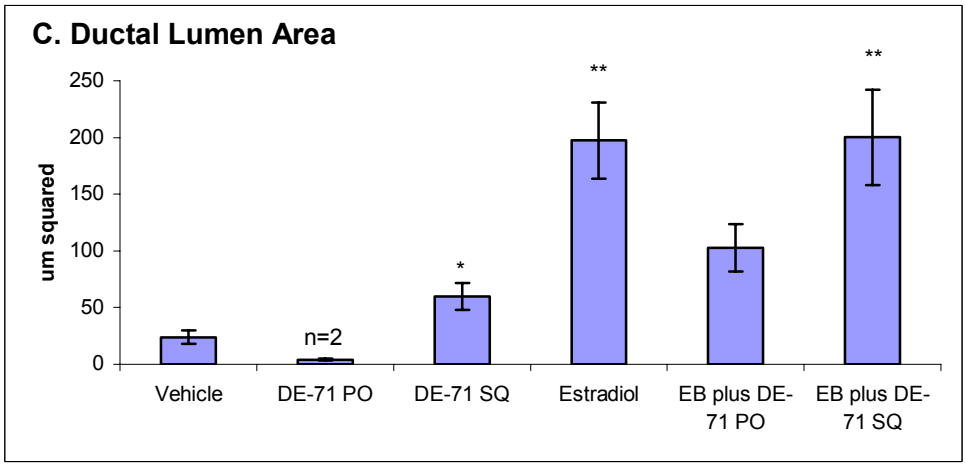
The results of 34-day in vivo treatment show that DE-71 may act as a weak estrogen when BALB/c mice are treated long-term with DE-71. By itself, DE-71 increased UEH, VET, and DLA but when administered in combination with EB it decreased the EB-induced increase in UEH. C57BL/6 mice seem to be less sensitive than BALB/c mice to the effects of DE-71. These effects suggest the behavior of a partial agonist and are very different from the potentiating of EB's effects seen in the 3-day in vivo assay. From the in vivo experiments it is still unclear if the PBDE congeners are responsible for the observed estrogenic activity or if they were metabolically activated. The next sections

will address the formation of hydroxylated metabolites in vivo and in vitro, and compare the estrogenic activity of metabolites to that of the original DE-71 mixture.

Figure 19. Mammary gland parameters for OVX BALB/c mice after 34 days of treatment (second experiment only). Mice were treated with either 10 µg/kg EB or 50 mg/kg DE-71 alone or in combination. A: Branches per length. B: Ductal Epithelial Height. C. Ductal Lumen Area. *, $p < 0.05$ vs. vehicle; **, $p < 0.01$ vs. vehicle; ***, $p < 0.001$ vs. vehicle. Each column is the average of 5 mice ± SEM except as noted. PO, oral treatment; SQ, subcutaneous treatment; EB, β-estradiol benzoate.







Specific Aim 2: Determine the role of metabolic activation in DE-71 estrogenic or antiestrogenic effects.

2.1 Phenolic DE-71 metabolites were found in plasma of mice after 34 days of treatment

Environmental pollutants like methoxychlor, bisphenol A and polychlorinated biphenyls become estrogenic (Morohoshi et al. 2005; Kohno et al. 2005) or can increase in their estrogenic potency (Jansen et al. 1993; Yoshihara et al. 2004) after CYP450 metabolism. Phenolic aromatic halogens are usually more estrogenic than non-hydroxylated aromatic halogens (Arcaro 1997; Vakharia and Gierthy 2000). Therefore to determine if phenolic PBDE metabolites had formed in mice, serum from the above described 34-day treated BALB/c mice was analyzed by gas chromatographic mass spectrometry to detect the PBDE congeners in DE-71 and possible phenolic metabolites (Qiu et al. 2007). The average total DE-71 concentration ranged from 1150 ng/g serum in SQ treated mice to 2150 ng/g in PO treated mice. These amounts are equivalent to μM levels in serum given an approximate molecular weight of 543 g/mol for DE-71. Eight phenolic metabolites, six hydroxylated PBDEs (OH-PBDEs) and two bromophenols, were identified and quantified (Table 5). All the OH-PBDEs found were mono-hydroxylated, two at *ortho*-positions (2'-OH-BDE-28 and 6-OH-BDE-47), one at the *meta*- position (3-OH-BDE-47), and three at the *para*- position (4'OH-BDE-17, 4-OH-BDE-42, 4'-OH-BDE-49). The position of the added hydroxyl group is of notice because aromatic xenobiotics often become estrogenic after being hydroxylated at the *para*- position (Arcaro 1997; Blair et

al. 2000; Cnubben et al. 1995; Huggins and Jensen 1954; Koda et al. 2005; Stresser and Kupfer 1998; Vakharia and Gierthy 2000). The total PBDE serum concentration (including OH-PBDEs) was 67% higher in PO-treated than SQ-treated mice, but the amount of *para*-PBDEs relative to total PBDE in serum was slightly higher in SQ-treated than PO-treated mice (11% and 9%, respectively). These findings raise the possibility that the estrogenic effects seen in vivo and in culture are due to the metabolites and not necessarily the original DE-71 congeners.

Table 5. Average concentrations (μM) of individual phenolic DE-71 metabolites and total non-phenolic DE-71 congeners found in blood plasma of 34-day treated BALB/c Mice (modified from Qiu et al. 2007). Mice were treated with 50 mg/kg/day DE-71 for 34 days. Concentrations were determined by gas chromatographic mass spectrometry (GC/MS) analysis.

Compound	Vehicle Controls and Blank Samples	PO	SQ
2,4-DBP	0.01 ± 0.02	0.29 ± 0.09	0.25 ± 0.10
2,4,5-TBP	0.001 ± 0.002	0.24 ± 0.09	0.26 ± 0.12
4'-OH-BDE-17	Not detected	0.04 ± 0.02	0.03 ± 0.02
2'- OH -BDE-28	0.0002 ± 0.0005	0.03 ± 0.01	0.01 ± 0.01
4- OH -BDE-42	0.002 ± 0.01	0.36 ± 0.24	0.24 ± 0.18
3- OH -BDE-47	Not detected	0.11 ± 0.05	0.07 ± 0.03
6- OH -BDE-47	Not detected	0.04 ± 0.02	0.02 ± 0.01
4'- OH -BDE-49	0.001 ± 0.002	0.08 ± 0.04	0.07 ± 0.04
DE-71 (all non-OH congeners)	< 0.02	3.9 ± 0.8	2.1 ± 0.2

2.2 In vitro microsomal metabolism increases estrogenic character of DE-71

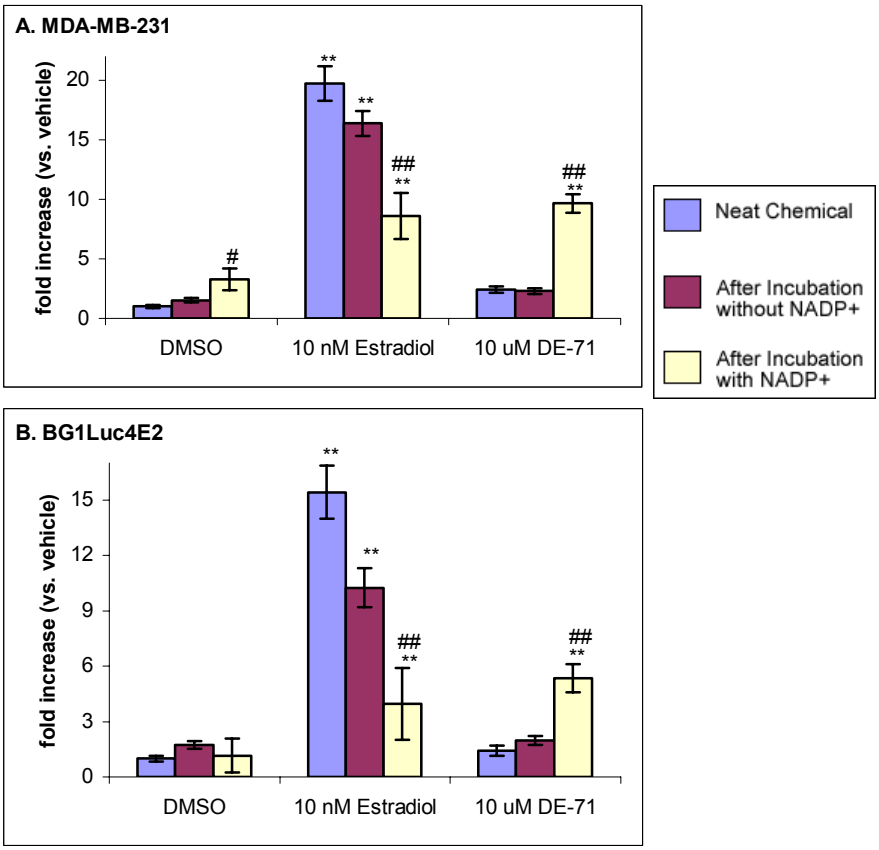
Cytochrome P450 (CYP450) isoenzymes can increase the estrogenicity of environmental pollutants by adding a hydroxyl group to an aromatic ring (Stresser and Kupfer 1998). Such a hydroxylated molecule may interact with ERα in a manner similar to endogenous estrogens like estradiol, which has a phenolic ring known to form hydrogen bonds with specific protein residues in the ERα (Brzozowski et al. 1997). Compared to other tissues, the mammalian liver contains a very high concentration of CYP450 isoenzymes (reviewed by Seliskar and Rozman 2007). The majority of CYP450 hydroxylating activity in the mammalian liver resides in the endoplasmic reticulum of hepatocytes. Extraction of the endoplasmic reticulum from cells yields small vesicle known as microsomes (Palade and Siekevitz 1956), which are often use as in vitro models to study CYP450 metabolism (Koga et al. 1996; Kohno et al. 2005; Kupfer and Bulger 1979; Vakharia and Gierthy 2000). In order to determine if microsomal metabolism could increase its estrogenic activity, DE-71 was incubated with female rat microsomes and a complete NADPH generating system for 24 hours (see Methods). The incubation product was then tested in the ERE-luciferase assays. Since β-estradiol is deactivated by CYP450 enzymes (Lee et al. 2003), similar incubations of β-estradiol and female rat liver microsomes (with or without complete NADPH generating system) were run in parallel to the DE-71 incubations as positive controls. The use of microsomes rather than a more complete cellular preparation prevents the conjugation of hydroxylated metabolites by cytosolic enzymes (Bock et al. 1987). Conjugation of larger functional groups at the

hydroxyl would prevent the metabolite from interacting with ERα, therefore decreasing its estrogenic activity.

After extraction by solid state procedure (see Methods), incubations were reconstituted to their original volume in DMSO. If 100% recovery had been achieved, the estrogenic activity of the reconstituted estradiol control incubations (lacking NADPH) should be the same as that of a "fresh" DMSO solution made from the original chemical at an equal molarity. Figure 20 shows that, in the case of β-estradiol, recovery was approximately 67% to 75%.

As expected, microsomal incubation decreased the activity of β-estradiol by 50% (from 16.4 fold over vehicle to 8.6 fold over vehicle in MDA-MB-231 cells, and from 10.2 fold over vehicle to 4.0 fold over vehicle in BG1Luc4E2 cells). The extracts from DE-71 microsomal incubates produced an ERE-luciferase response that was 4-fold that of the "fresh" DE-71 solution (Figure 20). The microsomes were ineffective when the NADPH generating system was omitted from the incubation.

Figure 20. ERE-luciferase induction by microsomal metabolites of DE-71. DE-71 or E2 were incubated for 24 hours with liver microsomes and an NADPH generating system (see Methods). Similar incubations but with an incomplete the NADPH generating system (lacking $NADP^+$) were run in parallel and served as negative control. After solid phase extraction, metabolites were reconstituted in BM to a nominal concentration of 10 nM β-estradiol or 10 µM DE-71. A: Incubates were tested in transiently transfected MDA-MB-231 cells; cells were transfected with an ERE-luciferase reporter gene, and constitutively active plasmids expressing ERα and a *Renilla* luciferase control gene; 2 independent microsomal incubations were each tested in separate transient transfection assays, the results are presented as the means ± SEM of these 2 assays. B: Tested in stably transfected BG1Luc4E2 cells; 1 microsomal incubation tested in the reporter assay (mean ± SEM, n = 4). Error bars indicate ± SEM. **, $p < 0.01$ vs. vehicle control; #, $p < 0.05$, ##, $p < 0.01$ vs. same treatment without complete NADPH generating system.



2.3 DE-71 and its phenolic metabolites activate the estrogen response element

β-Estradiol induces ERE-luciferase at concentrations above 0.1 pM in both the transiently transfected MDA-MB-231 cells (Figures 7 and 20A) and the stably transfected BG1Luc3E2 (Figure 20B) reporter cell culture systems. In the BG1Luc3E2 cells, β-estradiol had an EC50 in the pM range (Table 6). In the previous section, we showed that pre-incubation of DE-71 with rat liver microsomes increase its estrogenic activity in the ERE-luciferase reporter gene assay, and we speculated that this effect was due to hydroxylation of PBDE congeners. In this section, DE-71 and the hydroxylated metabolites found in mice were tested in BG1Luc3E2 cells to determine if they were able to activate ERE-mediated gene transcription. The potencies and effectiveness of tested chemicals were compared to that of β-estradiol using several parameters: the effective concentration for 50% of maximal effect (EC50), calculated from the chemical's own maximal observed luciferase activation; estrogen equivalent potency (EEP), the concentration of chemical required to produce an effect equivalent to estradiol's EC50; relative estrogen potency, ratio of the EC50 for β-estradiol to the chemical's EEP; and relative effect, the chemical's maximum luciferase induction divided by the maximal β-estradiol luciferase induction. The first 3 parameters – EC50, EEP, and relative estrogen potency – describe the chemicals potency and how it compares to the potency of β-estradiol. The last parameter, relative effect, compares the effectiveness of the chemical to that of β-estradiol. Results for DE-71 and metabolites are listed in Table 6; the data used for calculations are graphed in Figure 21.

DE-71 was able to induce ERE-luciferase at concentrations above 5 μM, reaching 100% estrogenic effectiveness at 10^{-4} M (Figure 21B). The EC50 for DE-71 was 3.7 x 10^{-5} M. Because the maximal effect of DE-71 was very close to the maximal effect of β-estradiol, its EC50 (3.7 x 10^{-5} M) and its estrogen equivalency potency (EEP; 3.9 x 10^{-5} M) were similar.

Two metabolites, 4'-OH-BDE-17 and 4-OH-BDE-42 were more potent than DE-71, with EC50 in the μM range (Table 6). 4'-OH-BDE-17 had a relative estrogenic potency that was approximately 10-fold that of DE-71 and it was also more effective increasing the luciferase signal than both DE-71 and β-estradiol, reaching an estimated maximal effect 30% higher than β-estradiol (Figure 21C). Conversely, 4-HO-BDE-42 was 36% less effective than DE-71, reaching an estimated maximal effect about 70% of β-estradiol's (Figure 21D). 4'-OH-BDE49 had an EC50 similar to DE-71 but a much lower efficacy; its maximal effect did not even reach 50% of β-estradiol's (Figure 21E).

Two hydroxylated BDE-47 congeners, 3-OH-BDE-47 and 2'-OH-BDE-28, had very little effect in the ERE-luciferase assay (Figure 21, G and H). Two bromophenol DE-71 metabolites found in mice, 2,4-DBP and 2,3,5-TBP, did not induce significant ERE signaling (Figure 21, I and J).

In summary, of the eight hydroxylated metabolites found in serum of mice treated with DE-71, only the three *para*-PBDEs were able to induce ERE-luciferase with a potency

equal or greater than DE-71. However, all the PBDEs able to induce ERE-luciferase were less potent than β-estradiol by at least six orders of magnitude.

Table 6. Estimated Effective Concentrations for 50-percent ERE-luciferase induction (EC50), Estrogen Equivalent Potency, Relative Estrogen Potency and Relative Effect of β-estradiol, DE-71 and OH-BDEs found in mice. BG1Luc4E2 cells were incubated in basal media for 5 days, and then treated with a range of concentrations for each test chemical (see Figure 21). ERE-luciferase induction was measured 18 hours after treatment.

	Estimated EC50[1] (moles/L)	**Estrogen Equivalent Potency [2] (moles/L)**	**Relative Estrogen Potency [3] (ratio)**	**Luciferase Induction at EC50 (fold-increase)**	**Relative Effect[4] (ratio)**
β-estradiol	1.2×10^{-12}	---	1.00	3.3	1.00
DE-71	3.7×10^{-5}	3.9×10^{-5}	3.1×10^{-8}	3.4	1.03
2,4-DBP	No effect	---	---	---	---
2,4,5-TBP	No effect	---	---	---	---
4'-OH-BDE-17	4.7×10^{-6}	3.5×10^{-6}	3.4×10^{-7}	4.3	1.30
2'-OH-BDE-28	NA	NR	---	---	---
4-OH-BDE-42	5.3×10^{-6}	8.2×10^{-5}	1.5×10^{-8}	2.3	0.70
3-OH-BDE-47	NA	NR	---	NA	---
6-OH-BDE-47	No effect	---	---	---	---
4'-OH-BDE-49	1.3×10^{-5}	NR	---	1.2	0.36

[1] Determined using the chemical's own maximum effect set at 100%. [2] EEP, concentration inducing the same luciferase activity as β-estradiol's EC50. [3] Ratio of β-estradiol EC50 to EEP. [4] test chemical-to-β-estradiol ratio of maximum luciferase induction. NA, not available because effect was insufficient to calculate an EC50; NR, β-estradiol EC50 not reached.

Figure 21. ERE -luciferase induction by β-estradiol, DE-71 and OH-BDEs found in mice. BG1Luc4E2 cells were incubated in basal media for 5 days, and then treated with a range of concentrations for each test chemical. ERE-luciferase induction was measured 18 hours after treatment. Each point is the average of at least 2 independent assays ± SEM, each normalized to vehicle control (0.1% DMSO = 1). Modeled data for β-estradiol (dashed pink line) and DE-71 (dotted brown line) shown in all OH-BDE charts for comparison. Modeled data for each OH-BDE is shown as a solid blue line. All concentrations are moles/liter.

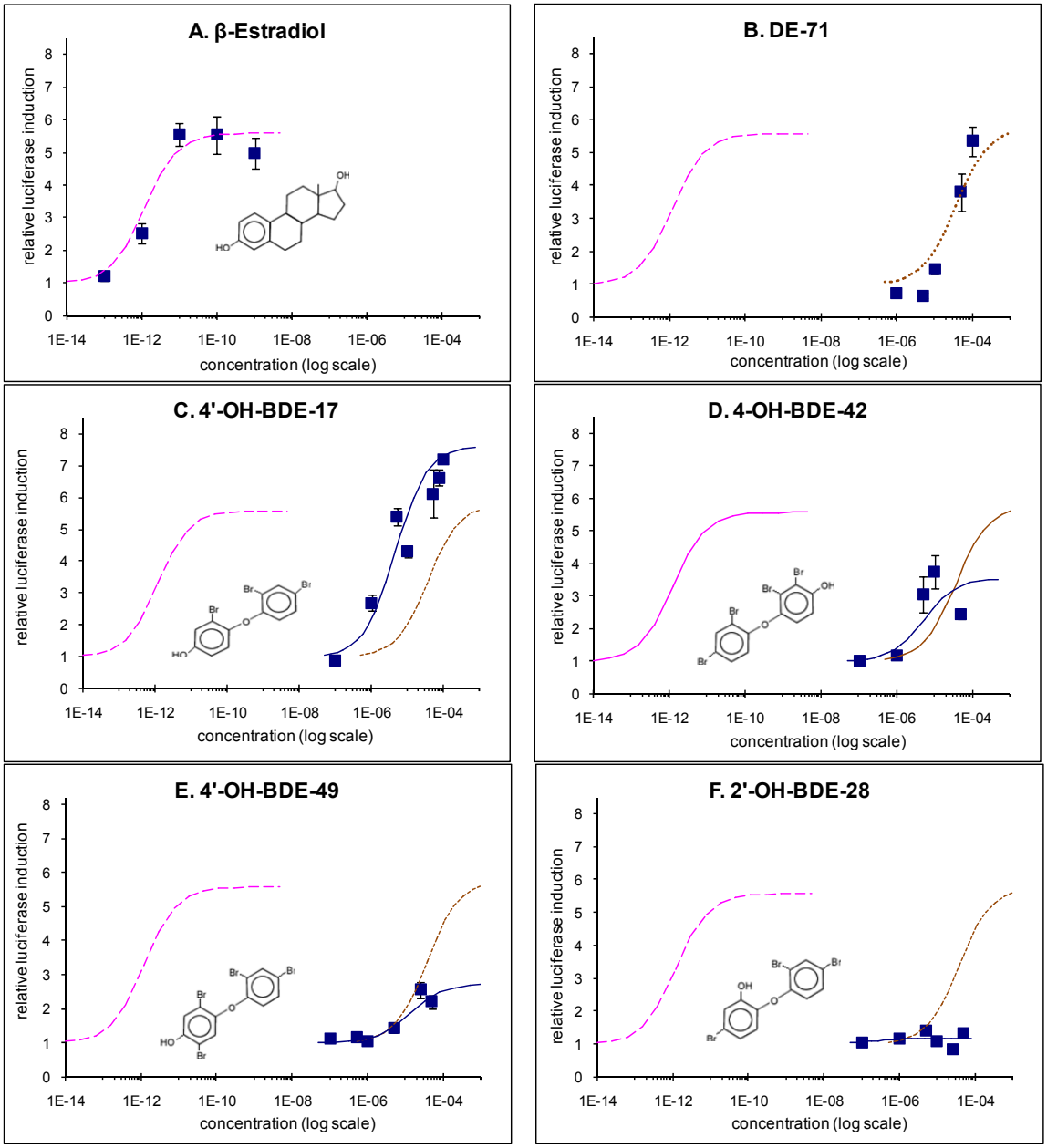


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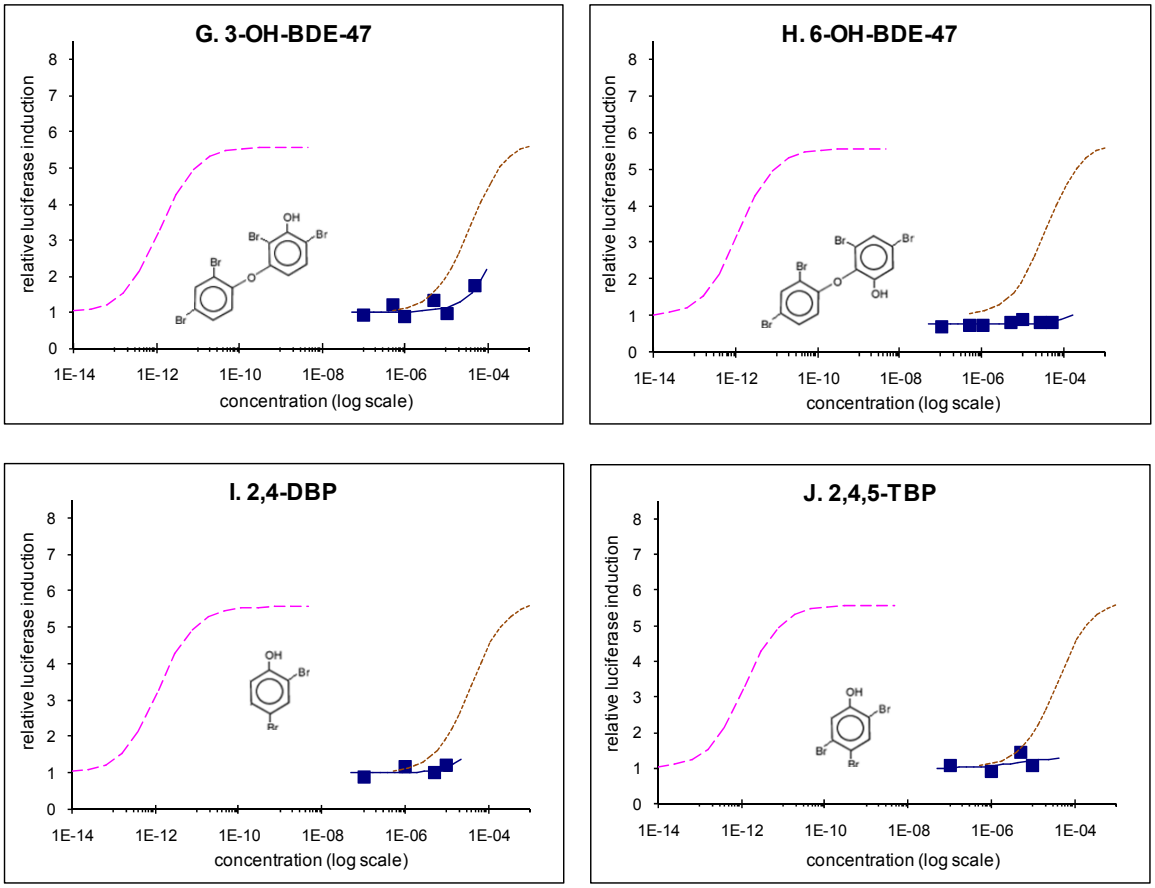
Figure 21 (cont.). ERE -luciferase induction by β-estradiol, DE-71 and OH-BDEs found in mice.



Specific Aim 3: Explore the mechanisms by which DE-71 congeners or their metabolites can activate or antagonize ERα signaling.

3.1 DE-71 phenolic metabolites found in mice displace beta-estradiol from Estrogen Receptor Alpha

Since DE-71 and several OH-PBDEs metabolites found in serum of DE-71 treated mice are able to induce ERE-luciferase, we wanted to determine if these chemicals are ERα ligands. We also wanted to determine if metabolites that did not induce ERE-luciferase were ERα ligands, because they could be receptor antagonists or otherwise interact with ERα. To assess the ability of DE-71 and the hydroxylated metabolites to bind ERα as ligands, we examined their abilities to displace radioactive H^3-β-estradiol from recombinant ERα in vitro as described in Methods (Recombinant ERα Binding Assay). The potencies of tested chemicals were compared to that of β-estradiol using two parameters: the inhibitory concentration for 50% displacement of ^{3}H-β-estradiol from receptor (IC50), calculated based on data shown in Figure 22. 2; and the Relative Affinity of the chemical, calculated as the ratio of ^{3}H-β-estradiol IC50 to test chemical IC50 (expressed as percent of β-estradiol). Results are summarized in Table 7 and Figure 22.

Neither the DE-71 mixture nor the bromophenols, 2,4-DBP and 2,4,5-TBP, were able to displace H^3-β-estradiol from ERα (Figure 22; B, I and J). The hydroxylated PBDEs bind ERα but with a much lower affinity than β-estradiol. Of the OH-PBDEs tested, the *para*-

hydroxylated congeners (at either #4 position) have a higher affinity for the estrogen receptor than 2-, 3-, or 6-OH-PBDEs (Table 7).

4'-OH-BDE-17, 4'-OH-BDE-49 and 4-OH-BDE-42 were the most potent, with IC50 in the μM range (Table 5; Figure 22; C, D and E). 6-OH-BDE-47, 3-OH-BDE-47 and 2'OH-BDE-28 had IC50 one order of magnitude higher than the *para*-OH-BDEs (Table 7; Figure 22; F, G and H). In general, the potency of each OH-BDE displacing ^{3}H-β-estradiol from ERα corresponds with their ability to activate ERE-luciferase, and the congeners with the highest IC50 values induce very little (3-OH-BDE-47) or no significant ERE-luciferase activity (2'OH-BDE-28 and 6-OH-BDE-47).

In summary, while neither DE-71 nor its bromophenol metabolites (2,4-DBP and 2,4,5-TBP) were able to displace H^{3}-β-estradiol from recombinant ERα, all the OH-PBDE metabolites found in serum of mice treated with DE-71did displace H^{3}-β-estradiol from recombinant ERα.

Table 7. Effective concentration for displacement of 50-percent (IC50)[1] of 1 nM ^{3}H-β-estradiol from recombinant ERα in vitro and relative affinity of β-estradiol, DE-71 and OH-BDEs found in mice (by ERα binding assay).

	IC50[1]	**Relative Affinity[2]**
β-estradiol	7.7×10^{-10}	100
DE-71	No effect	---
2,4-DBP	No effect	---
2,4,5-TBP	No effect	---
4'-OH-BDE-17	2.3×10^{-6}	0.03
2'-OH-BDE-28	6.2×10^{-5}	0.001
4-OH-BDE-42	3.4×10^{-6}	0.02
3-OH-BDE-47	2.9×10^{-5}	0.003
6-OH-BDE-47	2.8×10^{-5}	0.003
4'-OH-BDE-49	2.7×10^{-6}	0.03

1 The concentration of test compound to yield 50% displacement of ^{3}H-β-estradiol from receptor, calculated based on data shown in Figure 22. [2] Relative affinity was calculated as ^{3}H-β-estradiol IC50 ÷ test chemical IC50 × 100.

Figure 22. Displacement of 1 nM ^{3}H-β-estradiol from recombinant ERα in vitro by β-estradiol, DE-71 and OH-BDEs found in mice. Increasing concentrations of test compound were incubated overnight with 1 nM ^{3}H-β-estradiol and 0.6 nM recombinant ERα. Excess ^{3}H-β-estradiol was then washed by hydroxylapatite method and remaining radioactivity (bound to receptor) was measured by liquid scintillation. Each data point is the average of at least 2 independent assays with 3 replicates per assay ± SEM, except 3-OH-BDE47 and 6-OH-BDE47 (only one representative assay shown; multiple assays could not be averaged because of large scale differences). Modeled data for β-estradiol (pink line) and DE-71 (brown line) shown in all OH-BDE charts for comparison. Modeled data for each OH-BDE is shown as a blue line. All concentrations are moles/liter.

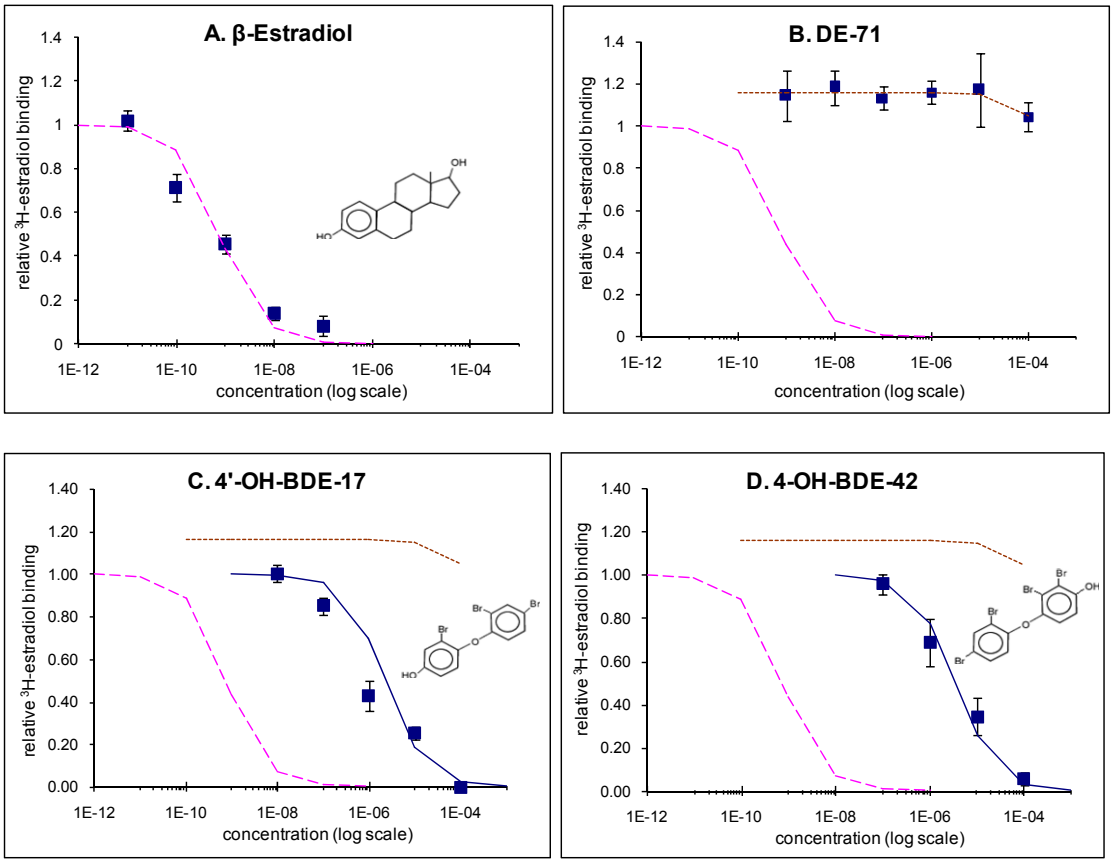


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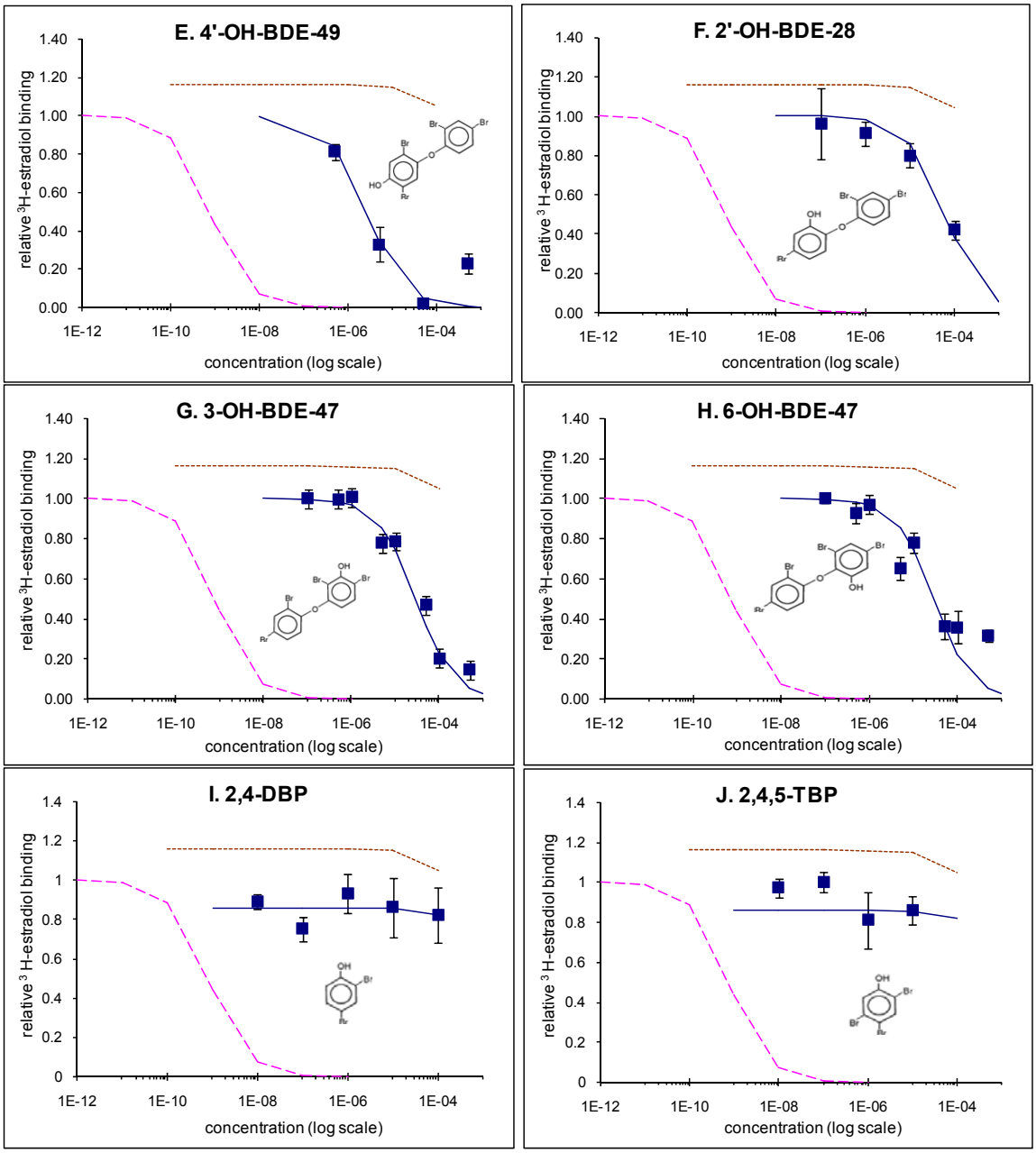
Figure 22 (cont). Displacement of 1 nM ^{3}H-β-estradiol from recombinant ERα in vitro by β-estradiol, DE-71 and OH-BDEs found in mice.



3.2 DE-71 phenolic metabolites modify activation of estrogen response element signaling by beta-estradiol.

DE-71 and its OH-PBDE metabolites found in mouse serum were able to activate ERE-luciferase signaling and/or displace ^{3}H-β-estradiol from ERα. The fact that *meta*- and *ortho*-OH-PBDEs bind ERα without inducing ERE activity suggests that these chemicals may act as ERα antagonists. Since the DE-71 formulation induced ERE signaling without binding ERα, it is possible that DE-71 can modify ERα activity without binding the receptor. In order to determine if DE-71 or the OH-PBDEs were able to modify β-estradiol-induced ERE-luciferase activity, a cell line stably transfected with an ERE-luciferase reporter gene, BG1Luc3E2, was co-treated with 10 pM β-estradiol and either DE-71 or one of the OH-PBDE metabolites found in mouse serum. The two bromophenol metabolites found in mice, 2,4-DBP and 2,4,5-TBP were not tested because they had no significant effect in either the ERE-induction or the ^{3}H-β-estradiol displacement assays.

Cotreatment with β-estradiol and DE-71 was able to increase ERE-luciferase induction beyond the maximal effect of β-estradiol alone. The same was true about 4'-OH-BDE-17, 4'-OH-BDE-49 and 2'-OH-BDE-28 (Figure 23; C, E and F). Another PBDE tested, 4-OH-BDE-42 induced ERE-luciferase above the β-estradiol maximum, but it was not statistically significant (Figure 23D). At high concentrations, two of the hydroxylated PBDEs tested, 2'-OH-BDE-28 and 6-OH-BDE-47, were able to antagonize the effect of β-estradiol.

6-OH-BDE47 was the more potent antagonist, showing an effect at 5 μM, while antagonism by 2'-OH-BDE-28 was observed only at 50 μM (Figure 23, F and H). Both PBDEs also seem to potentiate the effect of 10 pM β-estradiol at lower concentrations in a manner similar to other PBDEs tested, although this effect was statistically significant only for 2'-OH-BDE-28. However, because of the limited availability of some of the hydroxylated congeners only DE-71 and 2'-OH-BDE-28 were tested at a concentration of 50 μM, therefore it is possible that other hydroxylated PBDEs have the same biphasic behavior in the ERE-luciferase assay. Cell protein content per well indicated that there was no toxic effect produced by the high concentrations of 6-OH-BDE47 or 2'-OH-BDE-28 that produced the antiestrogenic effects. While protein per well varied 15% between treatment groups, there was no dose-response decrease in protein level that would indicate toxicity. Interestingly, both OH-PBDEs found to be estrogen antagonist were able to displace ^{3}H-β-estradiol from ERα (Figures 22, F and H and 23, F and H) but did not induce significant ERE-luciferase (Figure 21, F and H).

In summary, co-incubation of estrogen-responsive cells with β-estradiol and either DE-71 or one of its estrogenic in vivo metabolites results in a larger estrogenic effect than β-estradiol treatment alone. In the same cells, the non-estrogenic *meta*- metabolite 3-OH-BDE-47 can potentiate the effect of β-estradiol, while non-estrogenic *ortho*- metabolites are antiestrogenic at high concentrations.

Figure 23. ERE -luciferase induction after β-estradiol treatment or coteatment with β-estradiol and DE-71 or OH-BDEs found in mice. BG1Luc4E2 cells were incubated in basal media for 5 days, and then treated with a range of concentrations for each test chemical. ERE-luciferase induction was measured 18 hours after treatment. Data for co-treatment with 10 pM β-estradiol and the specified chemical shown as blue squares. Each point is the average of at least 2 independent assays ± SEM, each normalized to vehicle control (0.1% DMSO = 1). Modeled data for β-estradiol only dose-response (pink dashed line) shown in all charts for comparison. *, $p > 0.5$; **, $p > 0.01$; ***, $p > 0.001$ vs. maximal effect of β-estradiol alone. All concentrations are moles/liter.

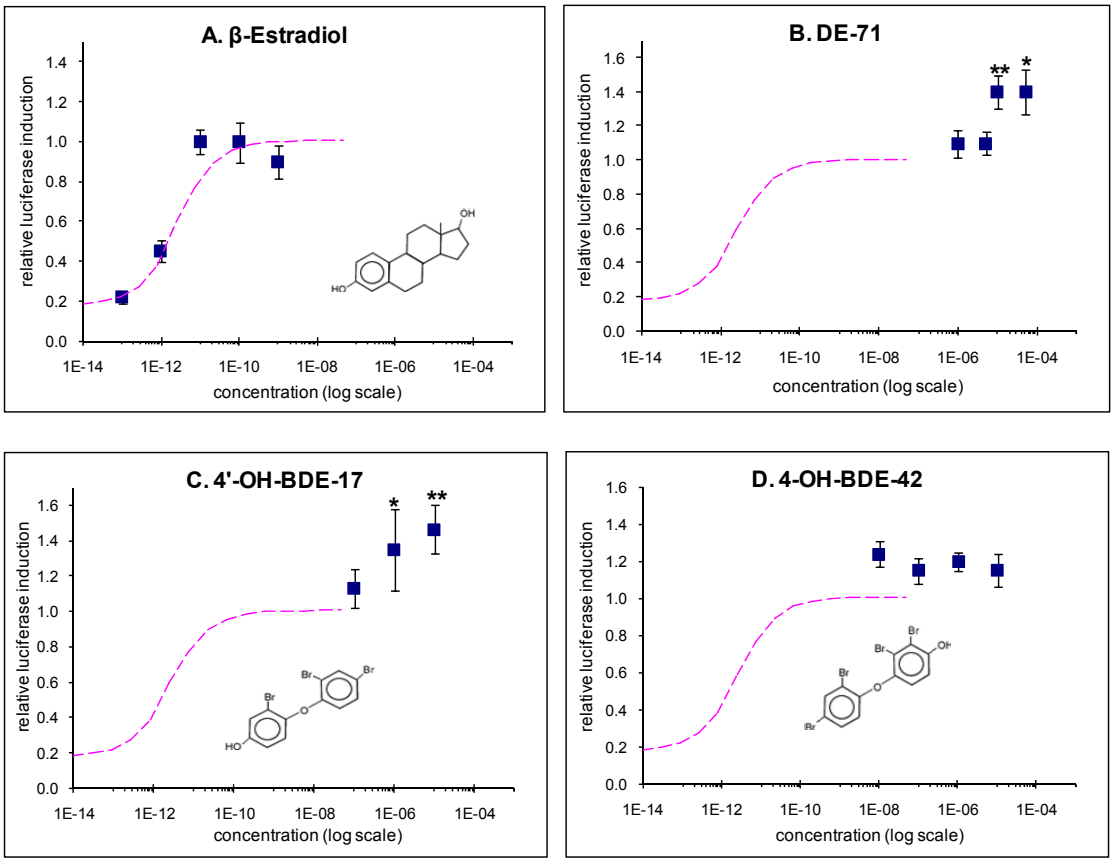


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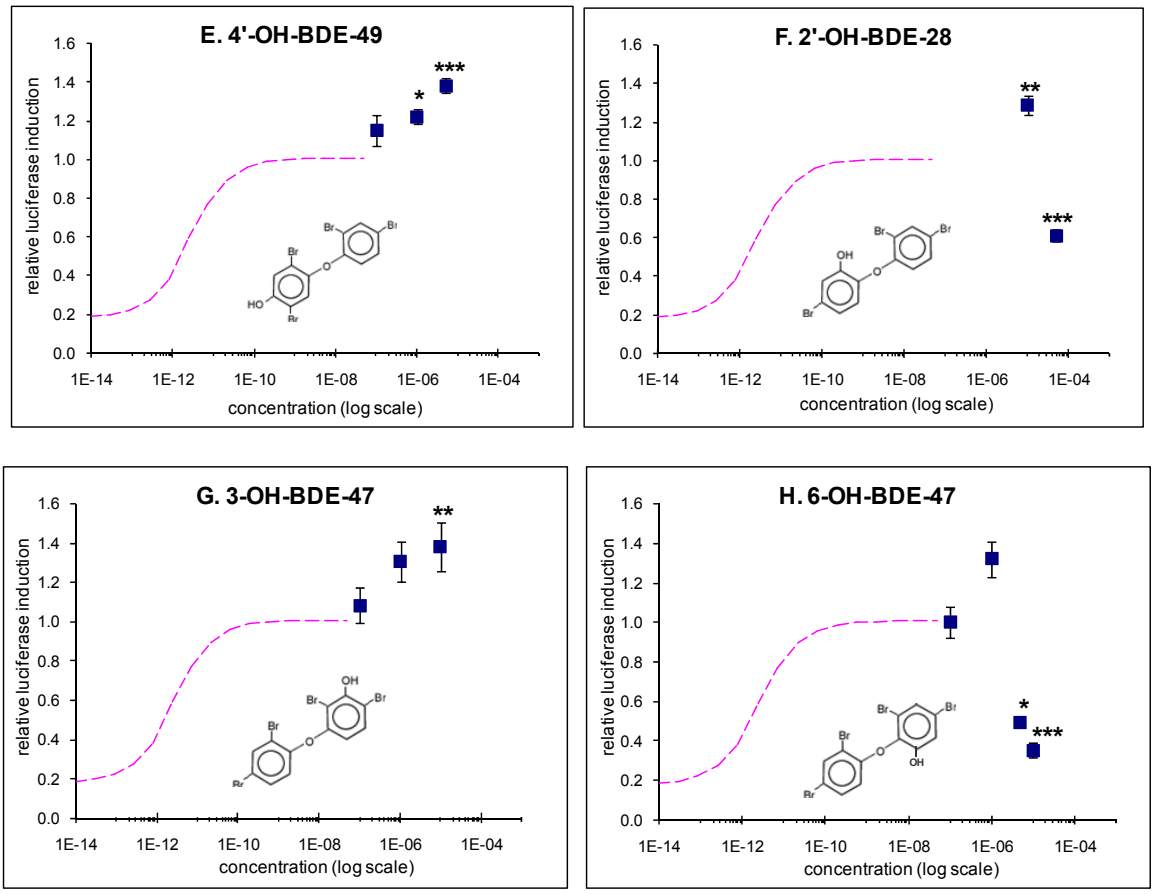
Figure 23 (cont.). ERE -luciferase induction after β-estradiol treatment or coteatment with β-estradiol and DE-71 or OH-BDEs found in mice.



3.3 DE-71 does not produce rapid activation of Extracellular-Signal Regulated Kinases.

The results of in vivo and in culture assays described above suggest DE-71 may have a proliferative effect on cells in vivo and in culture. Furthermore, the activities of DE-71 and some of its hydroxylated metabolites are additive with β-estradiol. One in vivo metabolite, 3-OH-BDE-47, cannot induce ERE-mediated gene expression by itself but is able to potentiate the effect of β-estradiol, suggesting a mechanism of ERE activation distinct from direct ligand activation of ERα. Therefore, we investigated the Epidermal Growth Factor (EGF) / Extracellular-Signal Regulated Kinases (ERK1/2) pathway as an alternative mode for the estrogenic activity of DE-71.

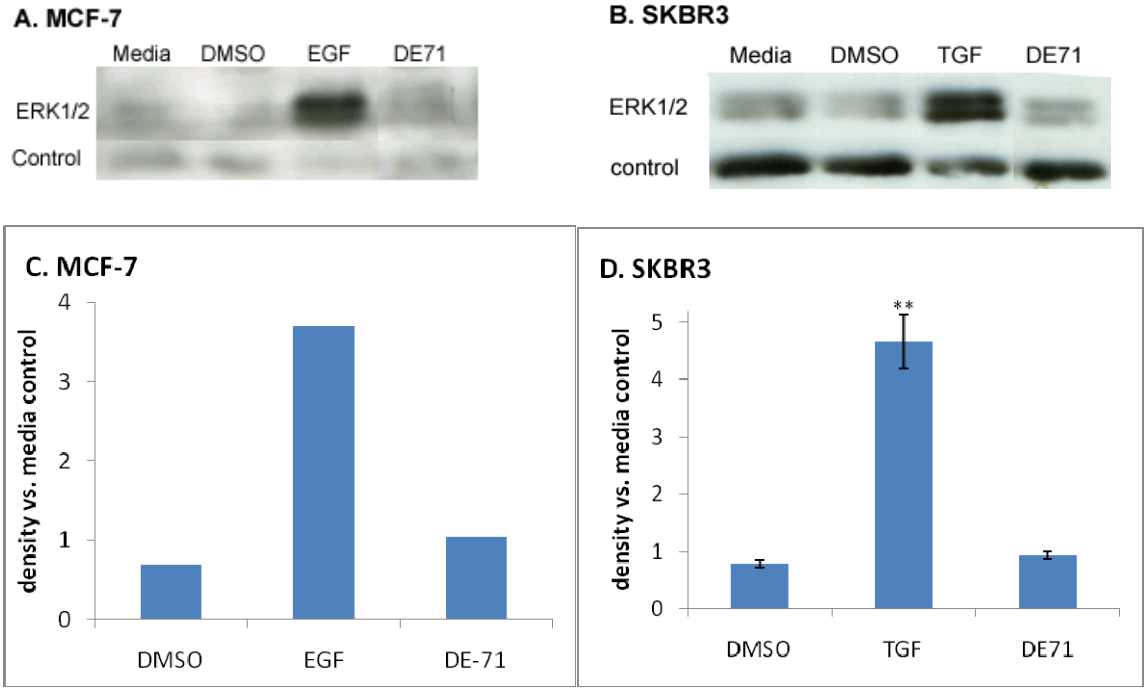
ERK1/2 play a central role in cell proliferation control, and can be activated by a rapid cytosolic signaling cascade that starts when EGF or transforming growth factor alpha (TGFα) activate the plasma membrane-bound Epidermal Growth Factor Receptor (EGFR) (reviewed by Meloche and Pouysségur, 2007). EGF has estrogen-like effects in the reproductive tract and may be an important mediator of estrogen action in vivo (Migliaccio et al. 2006; Nelson et al. 1991). Furthermore, several reports suggest that estrogens produce rapid cellular responses through non-classical estrogen receptors (reviewed by Filardo and Thomas 2005, and by Hammes and Levin 2007). These rapid responses involve activation of ERK1/2 though phosphorylation within 5-15 minutes of treatment. In one proposed pathway, estrogens interact directly with a G-protein coupled receptor, GPR30, setting off a series of events that leads to proteolytic activation of EGF

and subsequent induction of a kinase cascade mediated by EGF receptor. Therefore it is possible that PBDEs have estrogenic effects on target tissue by activating the EGFR/ERK1/2 signaling cascade independently from ERα ligand-induced activation.

To determine if DE-71 could activate ERK1/2, breast cancer cells were incubated for 5 minutes with DE-71 and lysates were analyzed by immunobloting. A rapid activation (5 minutes after treatment) was chosen as the endpoint because, while EGFR ligands can rapidly activate the EGFR/ERK1/2 signaling cascade, estrogen can increase transcription of the EGFR gene 6 hours after treatment (Das et al. 1994) and of the EGF gene 1 hour after treatment (Wang et al. 1994). This could lead to an indirect increase in ERK1/2 activity after β-estradiol treatment (Kim-Schulze et al. 1998). Therefore the short treatment time ensured that any increase in ERK1/2 activation was not due to an indirect activation of EGFRs. DE-71 treatment of breast cancer cells was not able to induce phosphorylation of ERK-1 or ERK-2 in either of the breast cancer cell lines tested, MCF-7 and SKBR3 (Figure 24). However, since we did not test if the OH-PBDE metabolites found in mice could rapidly activate ERK1/2, DE-71 inability to activate ERK1/2 does not preclude activation of the same protein by DE-71 metabolites.

In our hands, treatment of breast cancer cells with β-estradiol did not produce an increase in ERK1/2 phosphorylation (see Appendix 4). As discussed in the Appendix, several reports have indicated that the rapid ERK1/2 response to β-estradiol is inconsistent. Regardless of an inability to demonstrate any rapid effect of estradiol, our results suggest that DE-71 does not activate ERK1/2 and therefore this is unlikely to be the mechanism though which it enhances estrogen action.

Figure 24. ERK1/2 phosphorylation status after 5 minutes treatment of breast cancer cells with growth factor or DE-71. MCF-7 or SKBR3 breast cancer cells were treated with 10 µM DE-71. Epidermal Growth Factor (EGF) or Transforming Growth Factor (TGF) treatments (10 ug/ml) were included in each experiment as a positive control. After 5 minutes, media containing treatments was removed and cells were washed in cold (4ºC) phosphate buffered saline, then lysed and proteins were separated from other cell debris and determined by immunoblot as described in Methods. Phospho-ERK1/2 was detected with antibody against phospho-Th202/Tyr204 (Cell Signaling catalog #9101), and eIF4E was detected as loading control (Cell Signaling catalog #9742). Representative immunoblots: A: MCF-7; B: SKBR3. Total phospho-ERK densitometry (corrected for loading control): C: MCF-7, one representative experiment; D. SKBR3; average of 2 independent assays ± SEM. **, p > 0.01 vs. DMSO control.



3.4 DE-71 increases liver weight and Cytochrome P450 activity in mice liver.

PBDEs are known to induce CYP2B and CYP3A activities in rat liver, and the commercial DE-71 mixture may contain small amounts of dioxin and furans which are known inducers of CYP1A (Sanders et al. 2005; Zhou et al. 2002). Since these three CYP450 isoenzymes can deactivate β-estradiol (Lee et al. 2003; Zhu and Lee 2005), it is possible that the antiestrogenic effect seen in UEH of 34-day treated mice is due to increased EB metabolism rather than ligand binding to ERα. In order to asses the possibility that increase metabolic activity in the liver was decreasing the activity of administered EB, liver weights and activities of CYP1A (7-ethoxyresorufin O-dealkylation, EROD) and CYP2B (7-pentoxyresorufin O-dealkylation, PROD) enzyme activities were determined (CYP3A activity was not determined).

There was a 20%-51% increase in liver weight in BALB/c mice treated with DE-71 for 34-days compared with vehicle control, while livers of 3-day treated mice increased in weight up to 39% in a dose-dependent manner (Figure 25). Estradiol treatment did not increase liver weight in BALB/c mice. However, estradiol potentiated the effect of DE-71 administered orally but had no effect on SQ DE-71 treatments (Figure 25B). As with BALB/c mice, the livers of DE-71 treated C57BL/6 wild type mice were 27-29% bigger than vehicle treated controls. Estradiol administered alone or in combination with DE71 had no effect on liver weights of C57BL/6 WT mice. The size of ERαKO mouse livers increased 30% after DE-71 treatment.

There was a large increase in PROD activity over controls in DE-71 treated BALB/c mice, about 7-fold in the PO treated group and 5-fold in the SQ treated group (Figure 26A). Still, PROD activity in DE-71-treated animals was much lower than EROD activity in either DE-71 or vehicle-treated animals, i.e. the maximal PROD was about one third the minimal EROD activity. Liver microsomal EROD activity also increased (2.5-fold) but only for PO treated mice (Figure 26B). Estradiol treatment had no effect on either EROD or PROD activity (data not shown).

In summary, DE-71 increased the mouse liver activity of two CYP450 isoenzymes that play an important role in β-estradiol catabolism: CYP1A and CYP2B. Another CYP450 known to play in β-estradiol metabolism, CYP3A, was not tested. Enzyme induction relative to vehicle-treated mice was larger in PO-treated than SQ-treated animals.

Figure 25. Effects of DE-71 on liver weight. A: BALB/c mice were treated orally for either 3 days with 75, 150 or 300 mg/kg DE-71, or with β-estradiol (10 µg/kg or 10 mg.kg), or with DE-71 and β-estradiol in combination. The same subcutaneous treatment had no effect (data not shown). All weights are expressed as percentage of total body weight. * = $p < 0.05$, ** = $p < 0.01$, vs. vehicle. # = $p < 0.05$ vs. DE-71 same dose without estradiol. n = 5-10 mice per SQ group, n = 4-5 mice per PO group, except as noted. PO, oral treatment; SQ, subcutaneous treatment.

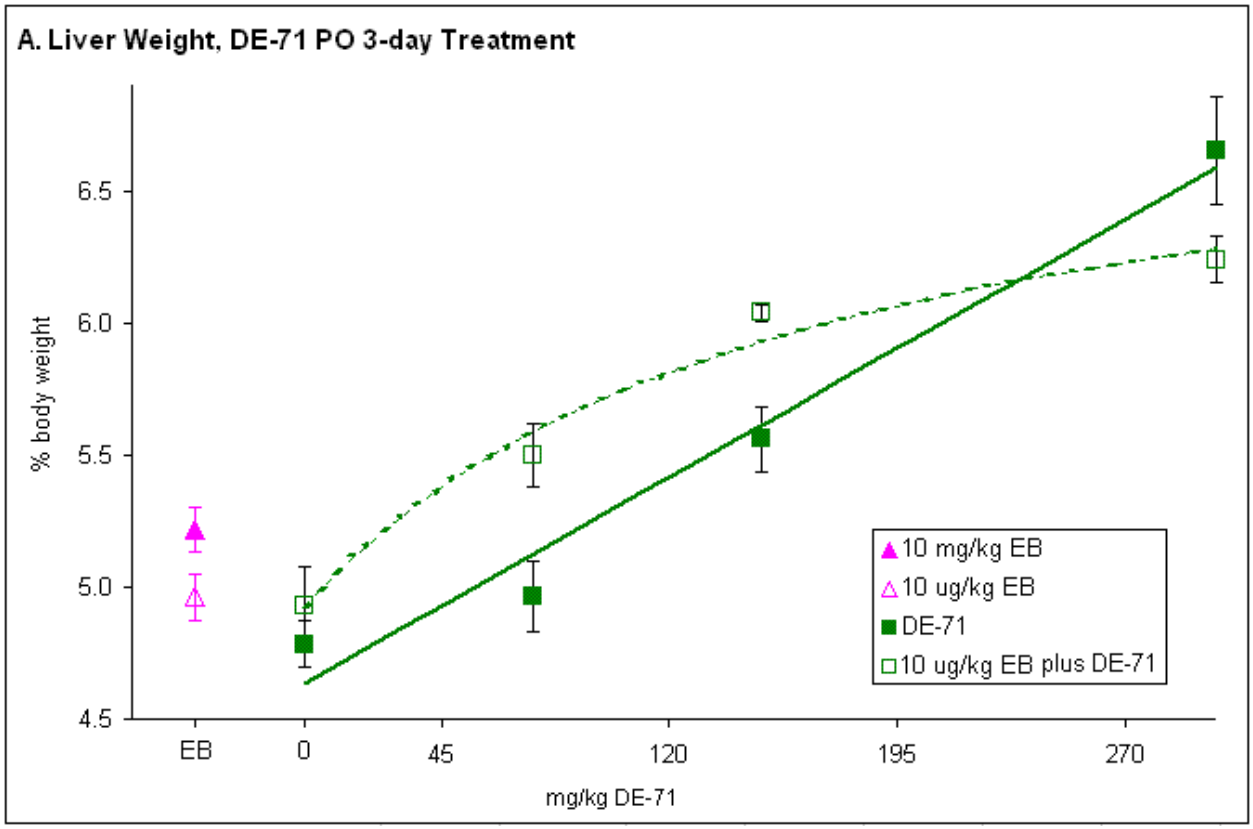


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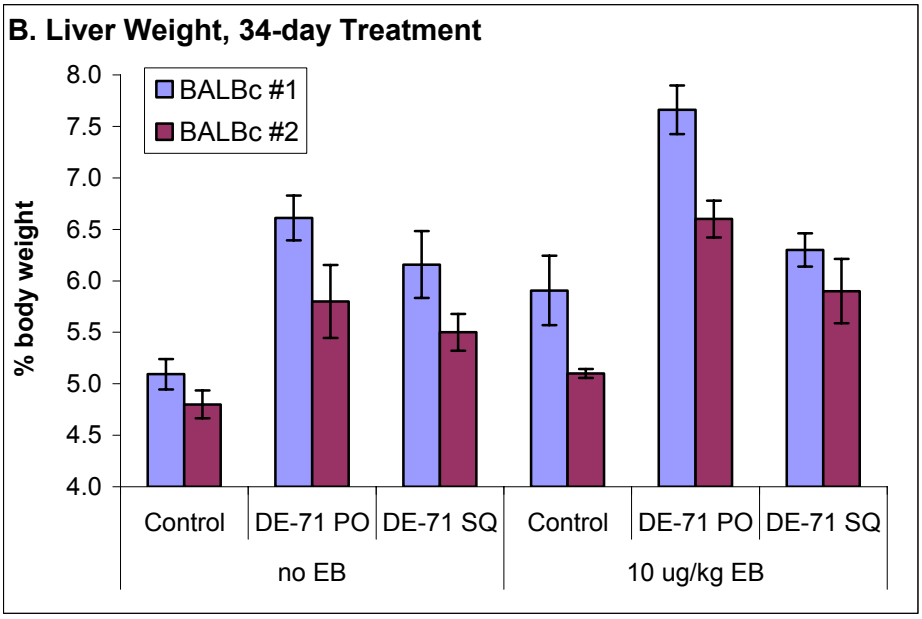
Figure 25 (cont). Effects of DE-71 on liver weight. B: BALB/c mice were treated for 34 days with 50 mg/kg DE-7, or 10 µg/kg β-estradiol, or with DE-71 and EB in combination during two experiments (BALB/c #1 and BALB/c #2). C: C57BL/6 mice were treated for 34 days with 50 mg/kg DE-7, or 10 µg/kg β-estradiol, or with DE-71 and EB. All weights are expressed as percentage of total body weight. * = $p < 0.05$, ** = $p < 0.01$, vs. vehicle. # = $p < 0.05$ vs. DE-71 same dose without estradiol. n = 5-10 mice per SQ group, n = 4-5 mice per PO group, except as noted. EB, β-estradiol benzoate; PO, oral treatment; SQ, subcutaneous treatment; WT, wild-type mice; KO, ERα knockout mice.



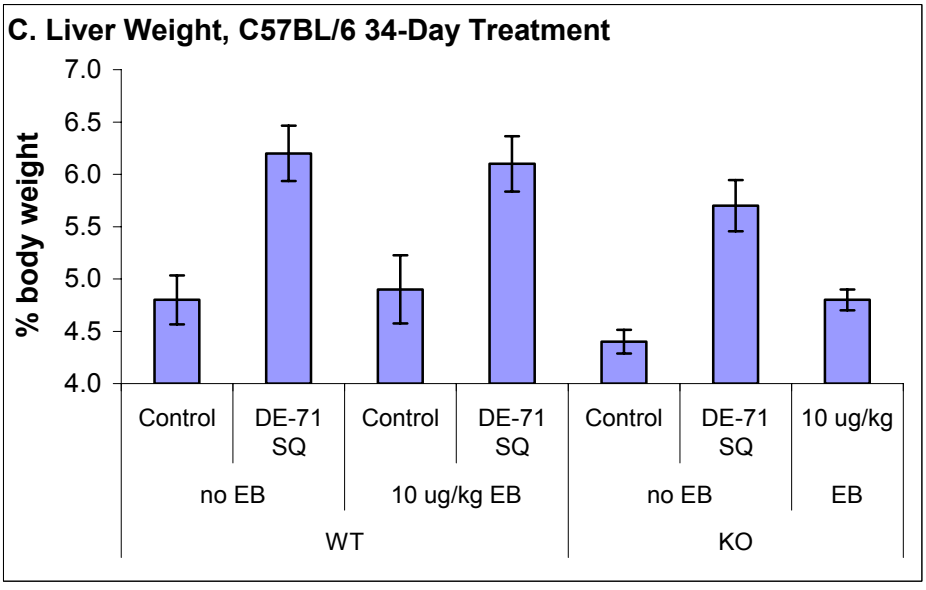
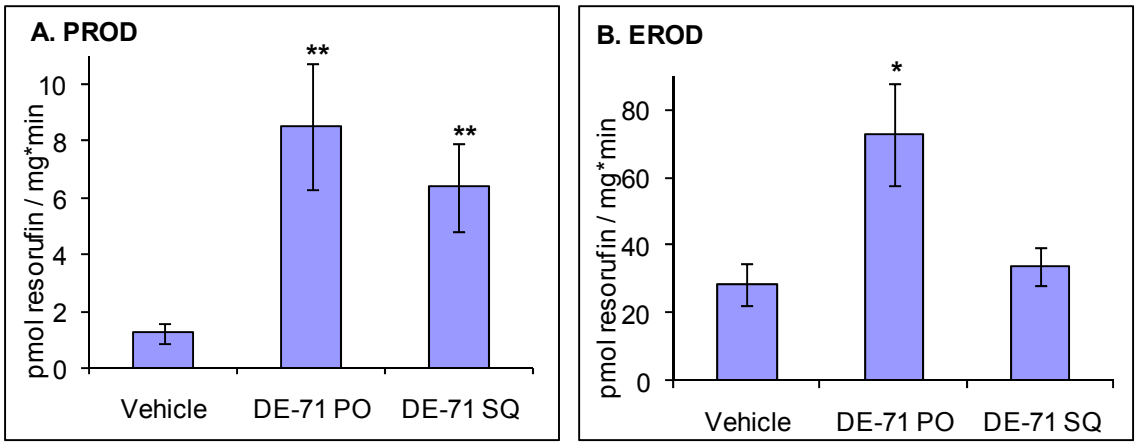


Figure 26. Effects of DE-71 on liver microsomal cytochrome P450 activity. BALB/c mice were treated for 34 days with 50 mg/kg DE-71 alone or with DE-7110 and µg/kg estradiol. A: 7-pentoxyresorufin O-dealkylation (PROD, CYP2B) activity. B: 7-ethoxyresorufin O-dealkylation (EROD, CYP1A) activity. n = 8 mice per group. * = $p < 0.05$, ** = $p < 0.01$, vs. vehicle.



Discussion

PBDEs are suspected to behave as estrogens because of the similarity of their chemical structure to other estrogenic xenobiotics, mainly the polychlorinated biphenyls (reviewed by Ulbrich and Stahlmann 2004; Winneke et al. 2002; Crews et al. 1995). Furthermore, it has been shown that hydroxylated metabolites of PCBs exert estrogenic effects (Blair et al. 2000; Kuiper et al. 1997) and therefore it may be reasonable to expect that hydroxylated forms of PBDEs would also be estrogenic. Both the PCBs and PBDEs are very hydrophobic rectangular molecules less than 450 Å in size. Based on the characteristics of the ERα binding domain found by crystallographic analysis (Brzozowski et al. 1997; Shiau et al. 1998) hydrophobic chemicals less than 450 Å in size can interact with the ligand binding pocket of ERα. The presence of *para*- hydroxyl groups at the ends of rectangular molecules seems to enhance the interaction between ligand and protein. Crystalographic analysis of the ERα ligand binding domain shows that *para*-hydroxyl groups in β-estradiol, DES, OH-tamoxifen and raloxifene form hydrogen bonds with Glu 353 and Arg 394, at the same time that hydroxyl groups at the opposite end of the molecule could form hydrogen bonds with His 524 (Brzozowski et al. 1997; Shiau et al. 1998). Our findings indicate that the PBDE mixture DE-71 is estrogenic in culture and in vitro, although much less potent than β-estradiol. Since DE-71 fails to displace ^{3}H-β-estradiol from ERα, the activity and mechanisms of these estrogenic effects of DE-71 seem to be mediated by formation of hydroxylated metabolites, and were explored through three specific aims, each of which is discussed below.

Specific Aim 1: Determine if DE-71 exerts estrogenic or antiestrogenic effects using cell culture and animal models.

The DE-71 mixture is a weak estrogen

a. Estrogenic Effects

Meerts et. al. (2001) tested 17 PBDE congeners for estrogenic activity in an ERE-luciferase assay (ER-CALUX; Legler et al. 1999). Two of the congeners in DE-71, BDE-28 and -100, were mildly estrogenic, compared to β-estradiol. BDE-100 was the most potent of the PBDEs tested by Meerts et al. (2001), although it was not the most effective. Using the same ER-CALUX bioassay, Hamers et. al. (2006) showed weak estrogenic activity for several DE-71 congeners, BDE-28,-47, and -100, but not for the DE-71 mixture. Results from Meerts et al. and Hamers et al. agree in the relative potency of these chemicals, and both groups agree the EC50 for BDE-100 to be in the micromolar range.

Although Hamers et al. were unable to see an estrogenic effect of DE-71 in the ER-CALUX assay, here we showed estrogenic effects of DE-71 in three different models: the MCF-7 cell proliferation assay, the in vivo adult mouse model, and the ERE-luciferase assay. We observed DE-71-induced ERE-luciferase in the BG1Luc3E2 cells (Table 6 and Figure 21B) with potency similar to BDE-28 and -100 in the ER-CALUX assay as shown

by Meerts et al. and Hamers et al. The difference between our ERE-luciferase results for the DE-71 mixture and those from Hammers et al. may be accounted by the fact that we tested higher concentrations (1 nM to 500 μM) than Hammers et al. (up to 10 μM).

The MCF-7 cell proliferation assay used here to test DE-71 (Figure 9) is probably more sensitive than the ER-CALUX and BG1Luc3E2 gene expression systems due to the longer time of incubation with the chemical (10 days vs. 24 hours) thereby allowing accumulation of the estrogenic effect. Alternatively, MCF-7 cells are known to express cytochrome P450 enzymes CYP 1A1, CYP 1A2 and CYP 1B1 (Peters et al. 2004; Barber et al. 2006) and it may be that during the 10-day incubation they metabolically convert BDE congeners to more active hydroxylated forms (see Specific Aim 2, below). The fact that the antiestrogen fulvestrant prevented DE-71 from increasing cell number (Figure 9C) suggests the involvement of an estrogen receptor. There was no indication of toxicity from fulvestrant treatment since cell numbers were not significantly different from DMSO control treatment. Barber et al. (2006) also saw increased cell number after treating MCF-7 with individual BDE congeners, including BDE-47, -99 and -153.

Other researchers have shown that both BDE-99 and DE-71 interfere with rodent sexual development after prenatal exposure (Kuriyama et al. 2005; Ceccatelli et al. 2006; Lilienthal et al. 2006) although the specific hormonal activity involved was not demonstrated. As noted by Ceccatelli et al. (2006), PBDE remains in the offspring for months after birth, making it impossible to determine if increased expression of estrogen target genes is due to non-specific developmental defects, to hormonally sensitive

developmental effects, or to adult hormone-like effect (or a combination of these effects). While such research is suitable to assess the sensitivity of the prenatal and perinatal animal to low doses of chemicals, the pubertal development protocol used by Stoker et al. (2004) and the adult gonadectomized rodent model used here are more suitable to assess estrogenic activity by looking at well-known responses to estrogen after chemical challenge. Classic estrogenic responses like increased uterine weight, UEH and VET in the adult OVX mouse are a strong indication of the involvement of estrogen signaling pathways and are standard end points used to assess estrogenicity of a chemical (Suzuki et al. 1996; Hayashi et al. 1988; Kimura et al. 1976).

Our results show that in the OVX mouse DE-71 administered subcutaneously produced estrogenic effects in SQ-treated mice (Figures 10, 11, 12, 13, 15, 16, and 19). However, the magnitude of the effects was small (4-50% increase over control) compared to the effect of EB (3-11 fold), therefore they may be biologically insignificant. DE-71 administered orally had no effect on any of the measured parameters. Dang et al. (2007) showed small uterotrophic effects of BDE-47 in the rat after SQ treatment, among them a 40% increase in uterus weight 24 hours after one dose of 200 mg/kg. Since, in our experiments, DE-71 had estrogenic effects in vitro and in SQ treated mice, the lack of effect in PO treated mice receiving the same dose as SQ treated mice suggest rapid clearance via liver metabolism and/or urinary excretion. In other words, the 50 mg/kg SQ dose was enough to allow concentrations large enough at the target tissues to have an effect, but the 50 mg/kg PO was not enough for the target tissues to reach an effective concentration.

Staskal et al. (2005 and 2006) showed that PBDEs are rapidly metabolized and excreted in the mouse. In our mice, the rapid clearance of administered DE-71 may account for the lack of effects in PO-treated mice, suggesting first-pass liver metabolism plays an important role in PBDE clearance. However, analysis of serum samples from 34-day treated mice (Qiu et al. 2007) found similar amounts of parent compounds and metabolites in the blood of either SQ or PO treated animals, with the exception of BDE-153 which was 5-times higher in the PO group. The concentration of total congeners was approximately 2000 ng/mL or similar to what would be achieved with 5 μM treatment in vitro. In addition, the serum concentrations of hydroxylated metabolites were similar in SQ- and PO-treated mice. Therefore, if either the parent PBDEs or their hydroxylated metabolites were responsible for the estrogenic responses seen in SQ-treated mice at the concentrations found in serum, we would expect the same responses in PO-treated mice. Thus the concentrations of parent compound and metabolites found in blood do not explain the difference in biological responses between SQ and PO treated mice.

However, by avoiding the high activity of conjugating (Phase II) enzymes in the liver and gut (Cassidy and Houston 1984, Li et al. 2004), it is possible that SQ administered PBDEs reached higher levels in peripheral tissue compared to PO administered PBDEs. It is also possible that PBDEs are activated by CYP450 in peripheral tissues such as adipose or at the target tissues (Shimada et al. 2003, Yoshinari et al. 2006). Others have also seen higher efficacy of SQ dosing over PO dosing of estrogens, as is the case of the xenoestrogen bisphenol A (Berger et al. 2007) and steroidal estrogen (Savvas et al. 1992).

Further work of tissue levels of PBDEs and their metabolites will be required to define the pharmacokinetics that regulate the effects of the route of exposure.

We found differences in the estrogenic responses to DE-71 between tissues, dosing regimens and mouse strains. The differences may involve the ability of DE-71 to cause either of the main estrogen effects on target tissues: edema, hypertrophy or hyperplasia. The overall increase in uterus size is a combination of these three effects. Rodents show maximal extravasation of intravenous markers in the uterus at estrus, and SQ estrogen can induce significant water uptake in rat uterus just two hours after injection (reviewed by Spaziani 1975). By the sixth hour after estrogen treatment, there are measurable increases in not only water uptake but also accumulation of amino acids, glycogen and ribonucleic acid precursors; these events and an initial increase in RNA and protein biosynthesis contribute to increased uterine wet weight (reviewed by Segal and Scher 1967). Proliferation (hyperplasia) and transformation of epithelial cells to large columnar secretory cells (hypertrophy) are part of a late response to β-estradiol that occurs 18-30 hours after estrogen peak levels (Hewitt et al. 2003).

The different dosing regimens of DE-71 determined which estrogenic effect was seen in the uteri. While the continuous dosing for 34-days had no significant effect on uterine weight (Figure 13), DE-71 given during the 3-day experiment potentiated the effect of EB on uterine weight in a dose-responsive manner (Figure 10). On the other hand, DE-71 by itself was able to induce hypertrophy in the uterine epithelium (UEH) and hyperplasia in the vaginal epithelium (VET) after 34-days (Figures 15 and 16) but not in the 3-day

treatment scheme (Figures 11 and 12). This suggests that the 3-day dosing regimen may have been more effective in causing edema while 34-day dosing may have been more effective in causing cellular hypertrophy and hyperplasia. Another water uptake-related effect was seen in the mammary glands of 34-day treated mice: there was an increase in ductal lumen area, an indicator of fluid retention (Figure 19). The toxicokinetics of parent compounds and metabolites are likely to play a role in these different tissue responses. Still, it is impossible to determine the causes or the role of edema versus hyperthrophy in such differences from the data presented here. Keeping in mind that DE-71 is a mixture of several different PBDEs, and that at least 8 metabolites were identified in blood, we could speculate that different distributions and availability of individual PBDEs or metabolites at target tissues may be the reason for the tissue and dosing differences.

We found a difference in response between mouse strains: the uterine and vaginal epithelium seems to be more sensitive to the effects of DE-71 in BALB/c (Figures 15 and 16) than C57BL/6 mice (Figures 17 and 18). Using uterine weight and estrous activity as endpoints, Morozova (1991) found that the CBA mouse was more sensitive to estrogen than the C57BL/6 strain. On the other hand there is literature regarding the higher sensitivity to estrogen of C57BL/6 compared to other strains. Silberberg and Silberberg (1951) found that the vagina and uterus glands of ovariectomized C57BL/6 mice were more susceptible to estrogen stimulation than Dba or A strains. Spearow et al. (1999 and 2001) showed that male C57BL/6 mice were more susceptible to inhibition of testes weight, vesicular gland weight and spermatogenesis than CD-1 mice. Very little information could be found regarding the BALB/c strain compared to others. Calderon et

al. (2003) did find that BALB/c, C57BL/6 and other inbreed mouse strains were more susceptible to the estrogen-sensitization to vaginal candidiasis than CD-1 mice. Strain effects on the response of the rat uterus and vagina to BPA and tamoxifen has also been reported (Bailey and Nephew 2002; Long et al. 2000). Others have traced strain differences in estrogenic response to specific gene regions by quantitative trait locus analysis (Shull et al. 2007; Tachibana et al. 2006) but the differences are likely to be tissue and response specific.

While our findings may illustrate a real difference in estrogen sensitivity between the BALB/c and C57BL/6 strains, an alternative explanation is a change in assay conditions between the BALB/c and the C57BL/6 experiments. The experiments described herein were performed over the course of 2 years therefore animals may have been exposed to different sets of environmental variables. The literature on the effects of the weak estrogen bisphenol A illustrates this difficulty. Although several laboratories have reported that bisphenol A displays uterothrophic activity (Steinmetz et al. 1998; Long et al. 2000; Markey et al. 2001; Papaconstantinou et al. 2000; Kitamura et al. 2005), when Tinwell et al. (2000) and Ashby et al. (2004) compared results of several assays within one laboratory they found that small effects were only detected whn control uterine weight were at a minimum. Tinwell et al. (2002) also concluded that it may be difficult for an investigator to confirm small increases in uterine weight by repeating the same assay. Low environmental exposures to hormone receptor ligands may decrease the sensitivity of in vivo assays by increasing baseline values measured in vehicle-treated control animals. Xenoestrogen and phytoestrogen perturbation of hormone homeostasis

may be a common occurrence when testing weak endocrine disruptors and may be due to differences in animal housing/bedding (Howdeshell et al. 2003, Markaverich et al. 2005), feed (Thigpen et al. 2002, Kato et al. 2004, Ciana et al. 2005), or other environmental variables. In our own experiments, we found that there was a period of 5 months during which control uterine weights were nearly double the average of all controls from other experiments performed over the course of 2 years (see Appendix 6). In fact, the experiment designated as Experiment #2 in Figures 13, 15, 16 and 25 was among those with the higher control values. Therefore we cannot acertain if the difference in BALB/c and C57BL/6 mice to DE-71 are due to differences in strain responses or to unknown environmental variables.

b. Antiestrogenic effects

There is little information in the scientific literature about antiestrogenic effects of DE-71 or its hydroxylated metabolites. The DE-71 congener BDE-153 is mildly antiestrogenic in the ER-CALUX assay according to Meerts et. al. (2001), but Hamers et. al. (2006) did not observe antiestrogenic activity from BDE-153, nor from the DE-71 mixture. We did not see an antiestrogenic effect of DE-71 in the BG11Luc3E2 system either, but rather a synergistic increase of the maximal effect of β-estradiol. However, we observed antiestrogenic effects of DE-71 in the MCF-7 cell proliferation assay, and a possible antiestrogenic effect in the vivo adult mouse model, behaviors expected from a weak estrogen receptor agonist. Early research on estrogen action demonstrated that the

uterotrophic effect of potent estrogens like estradiol and estrone can be antagonized by co-treatment with weaker estrogens such as estriol, 16-epiestriol, and some phytoestrogens (Hisaw et al. 1954, Velardo and Sturgis 1955, Lerner et al. 1963, Collins et al. 1997). Here we saw a similar activity of DE-71 in both MCF-7 cells and mice, comparable to that of the weaker phytoestrogens: a small estrogenic effect when administered alone, and a small antiestrogenic effect when co-administered with a strong estrogen like β-estradiol. Environmental pollutants like methoxychlor (Eroschenko et al. 2000), bisphenol A (Schmidt et al. 2006) and tetramethrin (Kim et al. 2005) are also know to have the same weak estrogen/antiestrogen effects.

In the BALB/c mouse, we observed both estrogenic and antiestrogenic effects on uterus, while in the vagina we only observed estrogenic effects. UEH and VET were increased in BALB/c mice treated for 34 days with SQ DE-71 alone (Figures 15A and 16A). Co-treatment of the same strain with EB and DE-71 showed a decrease of the EB effect in UEH but not VET (Figures 15B and 16B). However, in the 3 day dose response studies, DE-71 potentiated the effect of RB on UEH, suggesting that pharmacokinetics play a role in the type of response seen. It is possible that the in vivo estrogen activity of DE-71 has a different effect on hypertrophy (UEH) than hyperplasia (VET), i.e. it is a stronger hyperplasic than hypertrophic agent. On the other hand, the differences may be due to tissue-specific sensitivity to a weak estrogen. Furthermore, regarding the uterine epithelial hypertrophy, the increase by DE-71 alone and the decrease (compared to EB treatment) when co-administered with EB suggests two possible mechanisms: (1) that DE-71 (or its metabolites) behave as a weak estrogen agonist, i.e. acting as an antagonist

in the presence of the strong estrogen EB; (2) the antagonist effects may have been due to accumulation of a metabolite, such as 6-OH-BDE-47, that behaves as an antagonist at high concentrations (Figure 23). These results are in agreement with in vitro results discussed previously.

Specific Aim 2: Determine the role of metabolic activation in DE-71 estrogenic or antiestrogenic effects

DE-71 was metabolized into active species in vivo and in vitro

As discussed in the *Introduction*, some environmental pollutants can become estrogenic or increase their estrogenic potency after CYP450 metabolism. In the case of halogenated aromatic compounds like the PBDEs, CYP450 can both remove halogen atoms from the molecule and/or add a hydroxyl group. Both modifications could increase the molecule's affinity for estrogen receptors.

We observed metabolic activation of DE-71 into active species both in vivo and in vitro. In the in vitro experiments, we pre-incubated DE-71 with rat liver microsomes, imitating the classical experiments by which Kupfer and Bulger demonstrated the metabolic activation of the pro-estrogen methoxychlor (Bulger et al. 1978; Kupfer and Bulger 1979). Pre-incubation of DE-71 with microsomes under enzyme activating conditions increased its estrogenic activity.

Mammalian liver microsomes are rich in Cytochrome P450, a group of isoenzymes responsible for metabolism of many endogenous and exogenous chemicals. As discussed previously, estrogens in general are targets for both activation and deactivation by CYP450 (see *Introduction* for detailed explanation and CYP isoforms). While some specific CYP450 isoenzymes are responsible for either the anabolism or catabolism of endogenous estrogens, others can interconvert one endogenous estrogen into another. The biological activities of environmental chemicals have been found to be either increased or decreased by specific CYP450 enzymes(Goldstein and Faletto 1993), and some of these chemicals are known to have estrogenic activity or be converted into estrogens by CYP450 metabolism (Bulger et al. 1978; Kupfer and Bulger 1979; Morohoshi et al. 2005; Kohno et al. 2005). Aromatic ring hydroxylation is the most common mechanism by which CYP450 enzymes increase the estrogenic activity of a chemical, and several isoenzymes show preference for hydroxylating a common class of anthropogenic environmental pollutants, the halogenated polyaromatic hydrocarbons (halogenated PAHs), sometimes converting them into estrogens. These CYP450 isoenzymes include 1A1/2, 2A6, 2C9, 2C18, and 2C19 (Stresser and Kupfer 1998; Niwa et al. 2001; Yoshihara et al. 2004; McGraw and Waller 2006; White et al. 2000). Suitable substrates are usually PAH with two to four aromatic rings, sometimes fused, rectangular in shape and very hydrophobic (Lewis 1997, 2000).

Based on our results and those of Qiu et al. (2007), some of the PBDE congeners in DE-71 are also likely candidates for CYP450 activation as estrogens. We show here that all

the OH-PBDEs found in mice after DE-71 treatment interact with ERα in vitro, as evidenced by the results of ^{3}H-β-estradiol displacement assays (Table 7 and Figure 22). Individual OH-PBDEs had different abilities in the ERE-luciferase assay (Table 6 and Figure 21), which was used to assess their estrogenic potency (amount needed to induce a response), and efficacy (size of maximal response).

Qiu et al. (2007) found six hydroxylated-PBDEs metabolites in serum collected from the mice that had been treated for 34-days with DE-71 orally and subcutaneously (Tables 5 and 6; Figure 21). Those with *para*- hydroxylation (4'-OH-BDE-17, 4-OH-BDE-42 and 4'-OH-BDE-49) had ERE-luciferase activities higher than or similar to DE-71, and two out of three were able to synergize with β-estradiol. *Ortho*-and *meta*- hydroxylated congeners (2'-OH-BDE-28, 3-OH-BDE-47 and 6-OH-BDE-47) had little or no estrogenic activity but some (2'-OH-BDE-28 and 6-OH-BDE-47) behaved as antiestrogens, whereas DE-71 was not antiestrogenic in the ERE-luciferase assay. Others have found these same hydroxylated PBDE metabolites in animals exposed to PBDEs in the laboratory (Malmberg et al. 2005; Marsh et al. 2006; Kierkegaard et al. 2001) and in wildlife (Kelly 2006; Verreault et al. 2005; Valters et al. 2005), especially marine animals. But this is the first time these chemicals have been shown to have a specific hormonal activity.

Based on findings by Qiu et al. (2007) in mice and others in rats (Malmberg et al. 2005; Marsh et al. 2006), the DE-71 congener BDE-47 seems to be activated to estrogenic OH-PBDEs in laboratory rodents; BDE-47 itself has been found to have little estrogenic

activity (Hamers et al. 2006, Meerts et al. 2000). Others have also found OH-PBDEs in wild marine animals that could be BDE-47 metabolites (Kelly 2006; Verreault et al. 2005). However, the source of OH-BDEs in the marine environment can be both natural and anthropogenic, since some marine organisms produce natural brominated compounds (Vetter 2006).

PBDEs were originally suspected to be estrogenic because of their similarity to three other classes of estrogenic pollutants: the polychlorinated biphenyls (PCBs), polychlorobisphenyl ethanes (o,p'-DDT, methoxychlor, and others) and diphenyl propanes (bisphenol A and its metabolites). Most of these chemicals are already *para*-hydroxylated or require hydroxylation at the *para*- position to bind and activate ER (Stresser and Kupfer 1998; Arulmozhiraja et al. 2005), but some like o,p'-DDT have estrogenic activity without having a hydroxyl group. Our results show that *para*-hydroxylation increases the estrogenic activity of BDE-47, and that para-hydoxylated metabolites are more potent and effective estrogens than PBDEs with *ortho*- or *meta*-hydroxylation. In fact, the only *meta*-OH-PBDE we tested had activity not by itself but only by potentiating the effect of β-estradiol, and the two *ortho*-OH-PBDEs tested were antiestrogenic.

Below we review each of the metabolites found in mice blood serum, including an overview of our results and those of other researchers.

a. 4′-OH-BDE-17

In the ERE-luciferase assay, 4′-HO-BDE-17 was more potent and more effective than DE-71 (Table 6 and Figure 21C). When cells were co-treated with β-estradiol, 4′-HO-BDE-17 acted synergistically to increase ERE-luciferase beyond the maximal β-estradiol effect (Figure 23C). This dose-dependent increase was statistically significant at concentration of 1 uM (35% increase) and above.

Qiu et al. (2007) deduced that 4′-HO-BDE-17 was formed in mice from oxidative debromination of BDE-47, a minor metabolic pathway that yielded a small amount of product compared with concentrations of other metabolites (11-17 ng/g of serum, or 3-4% of the metabolites found). There is little information available about this metabolite; it was found in rat feces after BDE-47 administration (Marsh et al. 2006) and in East Hudson Bay beluga whale blubber (females, 0.012 ng/g lipid) but not in their milk or blood (Kelly 2006).

b. 2'-OH-BDE-28

2'-OH-BDE-28 induced very little ERE-luciferase (Table 6 and Figure 21F) and had a low potency in the ^{3}H-β-estradiol/ERα displacement assay (Table 7 and Figure 22F). Interestingly, it seems to potentiate the effect of 10 pM β-estradiol until a threshold is reached, then at higher concentrations it acts as an antagonist (Figure 23F). Potentiation

of the β-estradiol effect was observed at 10 uM (about 30% increase), then antagonism at 50 uM (about 40% decrease).

As with 4′-HO-BDE-17, this metabolite seems to originate from oxidative dehalogenation of BDE-47 (Qiu et al. 2007) and was found in mice at 5-11 ng/g serum (about 2% total OH-PBDEs found in mice). Marsh et al. (2006) also found 2'-OH-BDE-28 in laboratory rats exposed to BDE-47.

c. 4-OH-BDE-42

When considered against its own maximal effect, 4-HO-BDE-42 was more potent but less effective than DE-71, and less potent and effective than 4′-HO-BDE-17, but its relative estrogen potency was lower than DE-71 (Tables 6 and 7; Figures 21D and 22D). It was the most abundant metabolite found in DE-71 treated mice (120-180 ng/g of serum, or 32-38% of the metabolites found), presumably formed by hydroxylation of BDE-47, a major metabolic pathway for PBDEs in animals (Qiu et al. 2007). Others have detected this chemical in laboratory rats after exposure to BDE-47 (Marsh et al. 2006) or to a mixture of PBDEs similar to DE-71 (Malmberg et al. 2005). 4-HO-BDE-42 has also been found in the wild, in polar bear plasma (up to 0.22 ng/g; Verreault et al. 2005), East Hudson Bay beluga whales (Kelly 2006), and Detroit River fish (up to 1.2 pg/g; Valters et al. 2005).

d. 4′-OH-BDE-49

The potency of 4′-HO-BDE-49 was similar to that of DE-71, but it was much less effective (Tables 6 and 7; Figures 21E and 22E). It was able to synergize with10 pM β-estradiol, increasing ERE-luciferase induction by 20-40% (Figure 23E). As was the case for 4-HO-BDE-42, hydroxylation of BDE-47 is the most likely pathway of 4′-HO-BDE-49 formation; Qiu et al. (2007) found 34-42 ng/g of serum (about 9% of total metabolites) in DE-71 treated mice. Others have detected this chemical in laboratory rats after exposure to BDE-47 (Marsh et al. 2006) or to a mixture of PBDEs similar to DE-71 (Malmberg et al. 2005). 4′-HO-BDE-49 has also been found in rainfall and surface waters from southern Ontario, Canada (concentration not reported; Ueno et al. 2005), Detroit River fish (up to 170.5 pg/g; Valters et al. 2005), artic glaucous gull plasma and polar bear plasma (up to 0.54 and 0.32 ng/g, respectivetly; Verreault et al. 2005).

e. 3-OH-BDE-47

Although 3-OH-BDE-47 displaced ^{3}H-β-estradiol from recombinant ERα (Table 7 and Figure 22G), it had very little effect inducing ERE-luciferase (Figure 21G). It did enhance the effect of 10 pM β-estradiol but with less potency that 4′-HO-BDE-17 or 4′-HO-BDE-49, since the increase over β-estradiol became significant only at a concentration of 10 μM (Figure 23G).

3-OH-BDE-47 was also most likely formed by hydroxylation of BDE-47 (Marsh et al. 2006; Qiu et al. 2007), therefore this OH-PBDE may be less potent and effective than the parent compound. In the wild, 3-OH-BDE-47 has been found in male and female beluga whale blubber (0.012 and 0.017 ng/g lipid, respectively) as well as in beluga calf blubber (0.027 ng/g lipid; (Kelly 2006), and in glaucous gull plasma (up to 0.5 ng/g; Verreault et al. 2005).

f. 6-OH-BDE-47

6-OH-BDE-47 is another product of BDE-47 hydroxylation (Marsh et al. 2006; Qiu et al. 2007). This chemical has been found in laboratory rats after BDE-47 exposure (Marsh et al. 2006), female beluga whale blood (9.91 ng/g lipid; Kelly 2006), Detroit River fish (3.1 – 20.5 pg/g; Valters et al. 2005), and in glaucous gulls plasma (0.26 ng/g; Verreault et al. 2005).

6-OH-BDE-47 did not induce ERE-luciferase (Figure 21H) but was a more potent antiestrogen than 2'OH-BDE-28 , decreasing the ERE-luciferase induced by 10 pM β-estradiol by 50% at a concentration of 5 μM (Figure 23H). At a concentration of 1 μM, 6-OH-BDE-47 seemed to potentiate the effect of estradiol β-estradiol but the effect was not statistically significant. Hammers et al. (2006) also found 6-OH-BDE-47 to be antiestrogenic, and while others found it to be cytotoxic (Harju et al. 2007), we observed no toxic effects at concentrations as high as 50 μM.

Specific Aim 3: Explore the mechanisms by which DE-71 congeners or their metabolites can activate or antagonize ERα signaling.

OH-PBDEs but not DE-71 bind Estrogen Receptor Alpha

The inability of DE-71 to bind ERα (Figure 22B) was surprising since it was fully estrogenic in the cell culture models and slightly estrogenic in the ovariectomized mouse. Finding DE-71 metabolites that did bind ERα provided a possible explanation for the discrepancy between DE-71 effects and its inability to directly interact with ERα. Both the 34-day in vivo mouse experiments and the 10-day in culture MCF-7 cell proliferation experiments provide ample time for both the formation of more active metabolites and the accumulation of the estrogenic effect. In those experiments, concentrations of 5-10 μM produced estrogenic effects. In other experiments, DE-71 was able to weakly induce ERE-luciferase starting at 10 μM, suggesting that estrogenic metabolites may have formed even over a much shorter incubation period (18 hrs). The fact that pre-incubation with rat liver microsomes increased the ERE-luciferase induction by DE-71 suggested that metabolic activation of individual DE-71 congeners played a role in the estrogenic effect. Therefore, the results of three different kinds of studies, 34-day in vivo, MCF-7 cell culture, and ERE-luciferase induction with microsomal preincubation, support the hypothesis that DE-71 congeners need to be metabolically activated in order to have a significant estrogenic effect.

DE-71 and para- *and* meta-*hydroxylated metabolites induce ERE-luciferase beyond β-estradiol maximal effect*

In general, stimulation of estrogen responsive tissues and cell lines by endogenous estrogens will reach a maximal effect, after which increasing concentrations of estrogens will not increase the response. As with other receptor-dependent biological responses, an increase in tissue response to increasing concentrations can be observed as long as there are estrogen receptors available to bind the added estrogen. Once maximal receptor occupancy has been reached, additional amounts of ligand cannot produce additional effect. But here we show effects beyond the β-estradiol maximum when coadministered with DE-71 or some of its metabolites, and in the ERE-luciferase assay when cells were treated with 4'-OH-BDE-17 alone. In the 3-day mouse experiments, SQ DE-71 cotreatment potentiated the effect of β-estradiol on UEH and VET in a dose-dependent manner (Figures 11B and 12B). DE-71 and several of its metabolites were able to synergize the effect of β-estradiol on ERE-luciferase induction (Figure 23). Therefore, at least the effects on two different assays, epithelial parameters in the 3-day in vivo assay and ERE-luciferase assay, suggest an additional mechanism of action, other than ERα occupancy. DE-71 also potentiated the effect of β-estradiol on uterine weight of 3-day treated mice (Figure 10B), but this effect reached a plateau at the β-estradiol maximum, and therefore could be explained by additional ERα occupation.

The induction of estrogenic activity by xenoestrogens beyond the maximal effect of β-estradiol has been observed previously in our laboratory and by others. We have observed

o,p'-DDT induce ERE-luciferase beyond β-estradiol maximal effect (Bigsby, unpublished data). Meerts et al. (2001) observed a similar response from 4-phenoxyphenol and 4'-OH-BDE-30 (4'-OH-2,4,6-tribromodiphenyl ether). Our laboratory has also observed induction of classical ERα-mediated estrogenic effects by β-hexachlorohexane (β-HCH), a chemical that does not bind ERα (Steinmetz et al. 1996). As for the synergistic or potentiating effect seen here from DE-71 and its *para*- and *meta*-hydroxylated metabolites, we are the first to report such an effect from PBDE congeners. Connor et al. (1997) observed similar effects for estrogenic *para*-OH-PCBs. Taken together, these findings point to an additional mechanism of ERE activation by the test chemicals other than acting as an ERα ligand.

Ortho-*hydroxylated metabolites are antiestrogens in the ERE-luciferase assay*

In addition to the estrogenic DE-71 metabolites described above, we found two antiestrogenic DE-71 metabolites in the ERE-luciferase assay: 2'-OH-BDE-28 and 6-OH-BDE-47. Both chemicals are *ortho*-hydroxylated and neither had a significant effect on ERE-luciferase induction by themselves. Hamers et. al. (2006) also showed weak antiestrogenic activity for 6-OH-BDE-47 in the ERE-CALUX assay, but we are the first to report the antiestrogenic activity of 2'-OH-BDE-28. A similar pattern of antagonism and mixed agonism/antagonism was observed by Connor et al. (1997) in *para*-

hydroxylated PCBs. They showed antiestrogenic activity for several congeners in an ERE-luciferase assay, and agonist/antagonist behavior for others.

ERα antagonists work by inducing a conformational change in the receptor that does not allow binding of coregulatory proteins to the ER. This conformational change involves the position of a helical structure in the ER ligand binding domain, known as Helix 12, which allows the formation of a competent AF-2 region capable of interacting with coactivators when the receptor is bound to β-estradiol but not to when it is bound to antagonists (Brzozowski et al. 1997). Since nuclear ERs operate as transcription factors, a variety of coregulatory proteins modify their transcriptional activity (reviewed by Glass and Rosenfeld 2000), and failure to recruit coregulators will significantly reduced ligand dependent transcriptional activation (Danielian et al. 1992).

The synergistic and potentiating effects described in previous sections may seem to contradict the antiestrogenic effect of DE-71 cotreatment on MCF-7 cells. While antiestrogenic metabolites may form in culture, the proliferative effect on MCF-7 cells after DE-71 treatment by itself suggests that if any antiestrogenic metabolites are formed, their effect was not strong enough to interfere with the effects of estrogenic DE-71 congeners and/or metabolites. A more likely cause for the antiestrogenic effect seen in culture is that DE-71 or its metabolites are weak estrogens that prevent a strong estrogen like β-estradiol from interacting with ERα. Since we showed the activity of DE-71 to be very weak in vivo and in culture, this is a possible explanation. Another possible explanation for the induction of MCF-7 cell proliferation by DE-71 is that it acts on

another signaling pathway, such as those that induce ERK1/2 activation, but we have shown this does not occur (Figure 24).

DE-71 increases liver weight and Cytochrome P450 activity in mouse liver

Another alternative cause for the antiestrogenic effects of DE-71 seen in the MCF-7 cell proliferation assay and in one of the 34-day mouse experiments is induction of CYP450 enzymes capable of deactivating β-estradiol. Since DE-71 treatment increased liver weight (Figure 25) while at the same time diminishing the action of administered EB on uterine epithelia (Figure 15), it is possible DE-71 can alter liver metabolic pathways that regulate systemic estrogen activity. A major pathway responsible for estradiol deactivation is catalyzed by cytochrome P450 (CYP450) enzymes (Lee et al. 2003). In order to evaluate DE-71's capacity to induce estradiol catabolism in the liver, the activity of a major estradiol deactivating CYP450, isoenzyme 1A1, was assessed by measuring liver EROD activity. Another CYP450 family known to be induced by PBDEs, 2B (Sanders et al. 2005; Stoker et al. 2004; Zhou et al. 2002), was also assessed by measuring PROD activity. A third important enzyme in estrogen catabolism also induced by PBDEs, CYP3A (Sanders et al. 2005; Pacyniak et al. 2007), was not tested. DE-71 administered orally increased PROD activity in BALB/c mice (about 10-fold; Figure 26A), an effect in accordance with Sanders et al. (2005) findings of increased CYP2B gene expression in rats after treatment with DE-71 or some of its BDE congeners. We also found a statistically significant increase in EROD activity in the PO-treated mice

(Figure 26B). Sanders et al. (2005) also found that DE-71, but not its three main component congeners (BDE-47, 99 and 153), strongly upregulated CYP1A1 and suggested that such an increase might be due to dioxin and/or furan contamination of the DE-71 mixture.

If the antiestrogenic effect of DE-71 was due to an increase in CYP450 enzyme activity, the larger effect should have occurred in the PO-treated animals, i.e., in the animals with the largest increases in EROD and PROD activities. But this was not the case; instead the larger and statistically significant decrease in EB effect occurred in animals treated SQ (Figure 15). Therefore, at least in our experiment, the antiestrogenic effect of DE-71 (or its metabolites) seems more likely due to interference of receptor activation and not to a metabolic deactivation of administered EB activity.

Conclusions and Further Studies

The main hypothesis of this thesis is that the PBDE mixture DE-71 acts as an endocrine disruptor through activation of ERα. The experiments presented here show that DE-71 behaves as a weak estrogen in four models of estrogenic activity: MCF-7 breast cancer cell proliferation, ERE-luciferase reporter gene induction, ERα binding assay and estrogen target tissue responses in the ovariectomized adult mouse. We showed that DE-71 causes classic ERα-mediated effects in the uterus, vagina and breast and that hydroxylated metabolites of DE-71 are able to bind ERα and induce ERE-mediated

transcription. Since the congeners in DE-71 did not themselves displace estradiol from its receptor, it is likely that estrogenic effects of the PBDE mixture were also mediated by hydroxylated metabolites capable of binding ERα. Likewise, the antiestrogenic activity observed in the MCF-7 cell proliferation assay is most likely due to interaction of hydroxylated DE-71 metabolites with estrogen receptors and not to metabolic depletion of co-administered estrogens. These findings show that DE-71 could act as an endocrine disruptor; however its congeners and metabolites are weak activators of ERα when compared with a strong endogenous estrogen like β-estradiol.

In mice, treatment route and duration determined if DE-71 was estrogenic or not, and also if the main effect was edema, hypertrophy or hyperplasia. BALB/c mice are more susceptible to DE-71 effects in estrogen target tissues and in liver than C57BL/6 mice. DE-71 also increased liver weight in both mouse strains tested, and this effect was not dependent on ERα.

Several OH-PBDE metabolites were found in mouse serum after DE-71 treatment. While all *para*-OH-PBDEs were more estrogenic than DE-71, all of the metabolites found were much less potent than β-estradiol. The only *meta*-OH-PBDE found in mouse blood was not estrogenic by itself but was able to potentiate the ERE-luciferase induction of β-estradiol. Several of the estrogenic *para*-OH-PBDEs also behaved synergistically when cells were cotreated with β-estradiol. The two *ortho*-OH-PBDEs found in mouse blood were antiestrogenic at high concentrations.

The levels of DE-71 needed to have estrogenic effects in our studies (μM range) are much higher than the highest concentrations found so far in human blood serum (0.1 – 5 nM; Mazdai et al. 2003). But there is no current information on the levels of OH-PBDEs in human serum, or the role that human enzymes (especially CYP450) may play in the formation of DE-71 metabolites. Mouse tissues do not have the same CYP450 activities as human tissues (Bogaards et al. 2000); therefore the metabolites we found in mouse serum may not be representative of metabolites formed in humans. To find out if human CYP450 enzymes can convert DE-71 congeners to more estrogenic forms, DE-71 could be incubated with recombinant human CYP450 protein, especially CYP3A which accounts for the largest amount of CYP450 activity in the human liver (Guengerich 2005). To determine if humans produce hydroxylated PBDEs in vivo, human blood samples can be chemically analyzed for metabolites.

Moreover, since we found estrogenic effects only in SQ treated animals and proposed that the majority of PO-administered DE-71 metabolites may be cleared from the body before reaching peripheral tissues, it would be informative to determine which metabolites are formed at estrogen target tissues. This could be accomplished by in vitro incubations of DE-71 with microsomal and/or cytosolic fractions from specific estrogen target tissues, and/or chemical analysis of estrogen target tissues from treated mice for the presence of OH-PBDEs.

The observation that some of the hydroxylated PBDEs generated supramaximal estrogenic effects suggests that these compounds also work through mechanisms other

than direct activation of ERα. Further research is needed to determine if these mechanisms involve activation of ERβ and its interaction with EREs, and/or nongenomic estrogen signaling, such as activation of ERK1/2 by DE-71 metabolites. Although DE-71 did not rapidly activate ERK1/2 in a manner similar to EGF, we did not test the hydroxylated compounds. Another area of further research would be to explore if either DE-71 congeners or OH-PBDEs can interact with a secondary binding site on ERα and thus achieve an effect of larger magnitude than when occupying only the traditional ligand binding domain. Further in vitro and in vivo studies detecting an endogenous estrogen-inducible gene would confirm that OH-PBDEs are able to induce ERE-mediated gene expression.

The key findings of this thesis, that DE-71 is mildly estrogenic in mammals and can be converted to more active metabolites in vivo, support the current public health policy in the USA and Europe that human exposure to DE-71 must be decreased or eliminated. Further studies are needed to determine permissible levels in the environment given the most likely routes of exposure for humans and the sensitivity of specific subpopulations to estrogenic effects (developing fetus, very young children, and patients with estrogen-sensitive cancers). Risk-based permissible exposure levels are needed in order to accurately monitor environmental media (food, surface dust, air, water and soil), guide contaminated site cleanups, and issue advisories and warnings regarding the consumption of wild caught fish and game.

Appendix 1. DE-71 Technical Information and Materials Safety Data Sheet

Great Lakes Chemical Corporation

PA

Business Unit or Division: Polymer Additives
One Great Lakes Boulevard, P.O. Box 2200, West Lafayette, IN 47996-2200

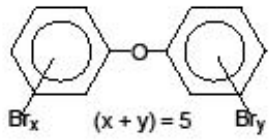
Document Type: **Technical Information**

Product Name: **Great Lakes DE-71™**

Halogenated Flame Retardant

Great Lakes DE-71 is a high viscosity liquid flame retardant containing 71% aromatic bromine.

Pentabromodiphenyl Oxide
CAS Reg. Number [32534-81-9]



Typical Properties

Appearance	Viscous Amber Liquid
Bromine Content, %	71
Specific Gravity @ 25 °C, g/ml	2.3

Viscosity

Temperature, °C	25	50	60	70
Viscosity, cps	> 200,000	11,270	1,955	497

Thermogravimetric Analysis (10 mg @ 10 °C/minute under N_2)

Weight Loss, %	5	10	50	95
Temperature, °C	214	228	271	298

Solubility (g/ 100 g Solvent)

Water	< 0.1	Toluene	Complete
Methylene Chloride	Complete	Methyl Ethyl Ketone	Complete
Methanol	2	Polyol	Complete
Styrene	Complete	Triethyl Phosphate	Complete

Great Lakes DE-71 is recommended as an additive flame retardant for unsaturated polyester, rigid and flexible polyurethane foam, epoxies, laminates, adhesives, and coatings. **Great Lakes DE-71** is readily soluble in styrene, polyols, isocyanates, methylene chloride, and phosphates.

The use of proper protective equipment is recommended. Excess exposure to the product should be avoided. Wash thoroughly after handling. Store the product in a cool, dry, well-ventilated area away from incompatible materials. Unless stated, proper storage will permit usage of the product for 6 to 12 months from the date of receipt. For additional handling and toxicological information, consult the GLCC Material Safety Data Sheet.

MATERIAL SAFETY DATA SHEET

MSDS Number: 00043 **Effective Date:** 01/12/2006
Product Name: Great Lakes DE-71™ **Page:** 1 of 7

SECTION I - CHEMICAL PRODUCT AND COMPANY IDENTIFICATION

Product Name:	**Great Lakes DE-71™**		
Supplier:	Chemtura USA Corporation		
Address:	199 Benson Road	**City:**	Middlebury
State:	Connecticut	**Zip:**	06749
Emergency Telephone Number:	**1-800-949-5167**		
Information Telephone Number:	1-765-497-6100	**Fax:**	1-765-497-6123
Chemtrec Phone:	**1-800-424-9300; Internationally call 703-527-3887**		
Effective Date:	01/12/2006	**Supercede Date:**	01/21/2005
MSDS Prepared By:	Chemtura Product Safety Group		
Synonyms:	Pentabromodiphenyl ether; Pentabromodiphenyl oxide; PBDPE		
Product Use:	Flame Retardant; See Additional Information.		
Chemical Name:	Benzene, 1,1'-oxybis, pentabromo derivative		
Chemical Family:	Brominated diphenyl oxide		

Additional Information

Manufacturer:
Great Lakes Chemical Corporation, A Chemtura Company
P.O. Box 2200
West Lafayette, Indiana 47996-2200

This material is regulated by a SNUR which can be located in the Federal Register, December 6, 2004 (Volume 69, Number 233) p.70404-70412.

SECTION II - COMPOSITION/INFORMATION ON INGREDIENTS

INGREDIENT NAME	CAS NO.	%	EXPOSURE LIMITS
Hexabromodiphenyl oxide	36483600	TS	Y (Hazardous) 15 mg/m3 (PNOR) (OSHA PEL TWA) Not established (OSHA PEL STEL) Not established (OSHA PEL CEIL) 10 mg/m3 (PNOS) (ACGIH TLV TWA) Not established (ACGIH TLV STEL) Not established (ACGIH TLV CEIL)
Pentabromodiphenyl oxide	32534819	TS	Y (Hazardous) 15 mg/m3 (PNOR) (OSHA PEL TWA) Not established (OSHA PEL STEL) Not established (OSHA PEL CEIL) 10 mg/m3 (PNOS) (ACGIH TLV TWA) Not established (ACGIH TLV STEL) Not established (ACGIH TLV CEIL)
Tetrabromodiphenyl oxide	40088479	TS	Y (Hazardous) Not established (OSHA PEL TWA) Not established (OSHA PEL STEL) Not established (OSHA PEL CEIL) Not established (ACGIH TLV TWA) Not established (ACGIH TLV STEL) Not established (ACGIH TLV CEIL)

Tribromodiphenyl oxide	49690940	TS	Y (Hazardous) Not established (OSHA PEL TWA) Not established (OSHA PEL STEL) Not established (OSHA PEL CEIL) Not established (ACGIH TLV TWA) Not established (ACGIH TLV STEL) Not established (ACGIH TLV CEIL)

*Indented chemicals are components of previous ingredient.

Additional Information

No information available

SECTION III - HAZARDS IDENTIFICATION

Emergency Overview: Clear, amber, dense, viscous liquid
Not acutely hazardous to health by ingestion, inhalation or skin absorption.
Causes eye irritation.
Persistent in the environment.

Relevant Routes of Exposure: Inhalation and ingestion.

Signs and Symptoms of Overexposure:
General reddening and irritation to the eyes.

Medical Conditions Generally Aggravated By Exposure: None reported

Potential Health Effects: See Section XI for additional information.

Eyes: Causes eye irritation.
Skin: Not expected to be a hazard in normal industrial use.
Ingestion: Not expected to be a hazard in normal industrial use.
Inhalation: Not expected to be a hazard in normal industrial use.
Chronic Health Effects: Long term overexposure may cause liver effects.
Long term overexposure may cause thyroid effects.

Carcinogenicity:

NTP:	No	ACGIH:	No
IARC:	No	OTHER:	No
OSHA:	No		

Additional Information

No information available

SECTION IV - FIRST AID MEASURES

Eyes: Flush with large volumes of water for at least 15 minutes. Get medical attention.
Skin: Wash with large volumes of soap and water for at least 15 minutes. If irritation develops, get medical attention.
Ingestion: If conscious, give person 1 to 2 glasses of water. Get medical attention immediately.
Inhalation: Remove person to fresh air. Get medical attention.
Antidotes: No information available
Notes to Physicians and/or Protection for First-Aiders: No information available

Additional Information

No information available

SECTION V - FIRE FIGHTING MEASURES

Flammable Limits in Air (% by Volume): Not available
Flash Point: None
Autoignition Temperature: Not available
Extinguishing Media: All conventional media are suitable.

SECTION V - FIRE FIGHTING MEASURES

Fire Fighting Instructions:	Wear a self-contained breathing apparatus and protective clothing to prevent skin and eye contact in fire situations.
Unusual Fire and Explosion Hazards:	Under fire conditions, toxic and irritating fumes may be emitted.
Flammability Classification:	Non-flammable liquid
Known or Anticipated Hazardous Products of Combustion:	Hydrogen bromide and/or bromine Carbon monoxide and carbon dioxide

Additional Information

No information available

SECTION VI - ACCIDENTAL RELEASE MEASURES

Accidental Release Measures:	Wearing appropriate personal protective equipment, collect spill with the aid of an inert absorbent and place in suitable labeled containers for disposal. Wash spill area after pick-up is complete, collecting all clean up water for appropriate disposal.
Personal Precautions:	See Section VIII.
Environmental Precautions:	Persistent in the environment. Avoid releasing to the environment. Do not allow spills to enter surface waters (streams, rivers, ponds, lakes, etc.).

Additional Information

No information available

SECTION VII - HANDLING AND STORAGE

Handling:	Use appropriate personal protection equipment. Avoid eye, skin and clothing contact. Do not breathe mist or vapor. Avoid repeated and prolonged contact. Avoid overheating.
Storage:	Store in a cool, dry, well-ventilated area away from incompatible materials. Keep container tightly closed.
Other Precautions:	No information available

Additional Information

No information available

SECTION VIII - EXPOSURE CONTROLS/PERSONAL PROTECTION

Engineering Controls:	Adequate general ventilation is recommended when handling to control airborne levels.
Ventilation Requirements:	Use local exhaust to minimize misting and vapor. Use mechanical ventilation for general area control.
Personal Protective Equipment:	
Eye/Face Protection:	Chemical safety glasses with side shields or chemical safety goggles
Skin Protection:	Rubber or plastic gloves Clothing designed to minimize skin contact
Respiratory Protection:	Wear a NIOSH/MSHA approved organic cartridge respirator if misting or vapor occurs. Consult the OSHA respiratory protection information located at 29CFR 1910.134 and the American National Standard Institute's Practices of Respiratory Protection Z88.2.
Other Protective Clothing or Equipment:	No information available
Exposure Guidelines:	See Section II.

SECTION VIII - EXPOSURE CONTROLS/PERSONAL PROTECTION

Work Hygienic Practices: Wash thoroughly after handling.
Wash contaminated clothing before reuse.

Additional Information

No information available

SECTION IX - PHYSICAL & CHEMICAL PROPERTIES

Appearance:	Clear, amber, dense, very viscous liquid	**Percent Volatile:**	Not available
Boiling Point:	Not available	**pH Value:**	Not available
Bulk Density:	Not available	**pH Concentration:**	Not available
Color:	Amber	**Physical State:**	Liquid
Decomposition Temperature:	Not available	**Reactivity in Water:**	Not water reactive
Evaporation Rate:	Not available	**Saturated Vapor Concentration:**	Not available
Freezing Point:	Not available	**Softening Point:**	Not available
Heat Value:	Not available	**Solubility in Water:**	13.3 ug/L at 25 degrees C
Melting Point:	Not available	**Specific Gravity or Density (Water=1):**	2.3 at 25 degrees C
Molecular/Chemical Formula:	Mixture	**Vapor Density:**	Not available
Molecular Weight:	564.7	**Vapor Pressure:**	4.69E-5 Pa at 21 degrees C
Octanol/Water Partition Coefficient:	LOG KOW = 6.568	**Viscosity:**	>200,000 cps at 25 degrees C
Odor:	No odor	**Volatile Organic Compounds:**	Not available
Odor Threshold:	Not available	**Water/Oil Distribution Coefficient:**	Not available
Particle Size:	Not available	**Weight Per Gallon:**	Not available

Additional Information

No information available

SECTION X - STABILITY AND REACTIVITY

Stability: Stable under normal conditions of handling and use.
Conditions to Avoid: None
Incompatibility With Other Materials:
None known
Hazardous Decomposition Products:
Thermal decomposition may produce the following:
Hydrogen bromide and/or bromine
Carbon monoxide and carbon dioxide
Hazardous Polymerization: Will not occur
Conditions to Avoid: None

Additional Information

No information available

SECTION XI - TOXICOLOGICAL INFORMATION

VALUE (LD50 OR LC50)	ANIMAL	ROUTES	COMPONENTS
>200 mg/L	Rat	Acute Inhalation	DE-71
>2,000 mg/kg	Rabbit	Acute Dermal	DE-71
6,200 mg/kg	Rat	Acute Oral	DE-71

Toxicological Information:
This material has been determined not to be a primary skin irritant in rabbits.
This material has been found to be a slight eye irritant in rabbits.

This material did not cause sensitization in a guinea pig maximization study.

This material was not found to be mutagenic in in vitro microbial assays and negative results were obtained for the induction of structural and numerical chromosome aberrations in human peripheral blood lymphocytes.

Liver cell enlargement and thyroid hyperplasia were observed in a 90-day oral study in rats. Upon compound withdrawal, thyroid hyperplasia was reversible; necrosis was observed in some enlarged liver cells.

In a 30-day oral study in rats, no compound related liver or thyroid effects were observed at 1 mg/kg.

In a 28-day oral diet study in rats, hepatic centrilobular hypertrophy was observed at 100 and 1,000 ppm. Thyroid hyperplasia and increased liver weights were observed at 1,000 ppm. A NOEL was established to be <100 ppm (<~10 mg/kg/day).

In a 28-day gavage study in rats, hepatic hypertrophy and increased liver weight was observed at 250 mg/kg/day.

In a rat developmental study, no developmental effects were seen at the highest dose tested of 200 mg/kg/day. A maternal NOEL was established at 10 mg/kg/day and a fetal NOEL was established at 100 mg/kg/day.

Other acute and chronic health hazards, as well as target organs, are unknown.

Additional Information

No information available

SECTION XII - ECOLOGICAL INFORMATION

Ecological Information:

EC50 in Daphnia magna (48H) = 19 ug/L (nominal); 14 ug/L mean measure concentration; NOEL = 6.5 ug/L

LC50 in rainbow trout (96H) >26 ug/L (nominal); >21 ug/L mean measured concentration; NOEL = 26 ug/L

EC10, EC50, EC90 in freshwater algae (96H) >26 ug/L (nominal); >14 ug/L mean measured concentration

LC50 in earthworms (14 Days) >500 mg/kg of dry soil

In a Carbon Dioxide Evolution Test, minimal degradation of the test substance was observed over a 93 day test period. This material evolved an average of approximately 0.0 and 2.4% TCO on days 29 and 93 respectively.

In a Daphnia magna life cycle study, a NOEC of 5.3 ug/L, LOEC of 9.8 ug/L and a MATC of 7.2 ug/L were established.

In an early life-stage toxicity study in rainbow trout, a NOEC of 8.9 ug/L, LOEC of 16 ug/L and a MATC of 12 ug/L were established.

In a bioaccumulation study in carp, this material was found to bioaccumulate.

In prolonged sediment toxicity tests utilizing Chironomus riparius, Lumbriculus variegatus, and Hyalella azteca, the EC 50 (28 Day) for all test organisms was >50 mg/kg.

In seedling emergence tests, the 21 day NOEC in corn, tomato, onion, soybean, ryegrass and cucumber was established to be 16 mg/kg, 125 mg/kg, 1000 mg/kg, 1000 mg/kg, 1000 mg/kg, and 1000 mg/kg, respectively.

Persistent in the environment.

SECTION XII - ECOLOGICAL INFORMATION

Do not allow spills to enter surface waters (streams, rivers, ponds, lakes, etc.). Avoid releasing to the environment.

Additional Information

No information available

SECTION XIII - DISPOSAL CONSIDERATIONS

Disposal Considerations: Dispose of waste at an approved chemical disposal facility in compliance with all current Local, State/Province, Federal/Canadian laws and regulations.

Additional Information

No information available

SECTION XIV - TRANSPORT INFORMATION

U.S. DOT

Proper Shipping Name:	Not regulated		
Hazard Class:	N/A	**ID Number:**	N/A
Packing Group:	N/A	**Labels:**	N/A
Special Provisions:	N/A	**Packaging Exceptions:**	N/A
Non-Bulk Packaging:	N/A	**Bulk Packaging:**	N/A
Passenger Air/Rail Limit:	N/A	**Air Cargo Limit:**	N/A
Vessel Stowage:	N/A	**Other Stowage:**	N/A
Reportable Quantity:	N/A		

AIR - ICAO OR IATA

Proper Shipping Name:	Not regulated		
Hazard Class:	N/A	**ID Number:**	N/A
Subsidiary Risk:	N/A	**Packing Group:**	N/A
Hazard Labels:	N/A	**Packing Instructions:**	N/A
Air Passenger Limit Per Package:	N/A	**Packing Instruction - Cargo:**	N/A
Air Cargo Limit Per Package:	N/A	**Special Provisions Code:**	N/A

WATER - IMDG

Proper Shipping Name:	Not regulated		
Hazard Class:	N/A	**ID Number:**	N/A
Packing Group:	N/A	**Subsidiary Risk:**	N/A
Medical First Aid Guide Code:	N/A		

Additional Information

No information available

SECTION XV - REGULATORY INFORMATION

U.S. Federal Regulations:

The components of this product are either on the TSCA Inventory or exempt (i.e. impurities, a polymer complying with the exemption rule at 40 CFR 723.250) from the Inventory.

This material is regulated by a SNUR which can be located in the Federal Register, December 6, 2004 (Volume 69, Number 233) p.70404-70412.

The following materials are subject to TSCA Section 12(b) (Section 4) one-time export notification:
Pentabromodiphenyl oxide

The following materials are subject to TSCA Section 12(b) (Section 5) annual export notification:
Tetrabromodiphenyl oxide
Pentabromodiphenyl oxide
Hexabromodiphenyl oxide

SECTION XV - REGULATORY INFORMATION

State Regulations:
None known

International Regulations:
This material (or each component) is listed on the following inventories:
Canada - DSL
EU - EINECS
Australia - AICS
Japan - ENCS
Korea - ECL
Philippines - PICCS
China - List I

Canadian WHMIS Hazard Class and Division = D.2.b

SARA Hazards:

Acute:	Yes	**Chronic:**	Yes
Reactive:	No	**Fire:**	No
Pressure:	No		

Additional Information

The above regulatory information represents only selected regulations and is not meant to be a complete list.

SECTION XVI - OTHER INFORMATION

NFPA Codes:

Health:	NR	**Flammability:**	NR
Reactivity:	NR	**Other:**	NR

HMIS Codes: * indicates chronic health hazard.

Health:	1*	**Flammability:**	0
Reactivity:	0	**Protection:**	X

Label Statements: Not available

Other Information:
Abbreviations:
(L) = Loose bulk density in g/ml
LOEC = Lowest observed effect concentration
MATC = Maximum acceptable toxicant concentration
NA = Not available
N/A = Not applicable
NL = Not limited
NOAEL = No observable adverse effect level
NOEC = No observed effect concentration
NOEL = No observable effect level
NR = Not rated
(P) = Packed bulk density in g/ml
PNOR = Particulates Not Otherwise Regulated
PNOS = Particulates Not Otherwise Specified
REL = Recommended exposure limit
TS = Trade secret

Additional Information

Information on this form is furnished solely for the purpose of compliance with OSHA's Hazard Communication Standard, 29CFR 1910.1200 and the Canadian Hazardous Products Act and associated Controlled Products Regulations and shall not be used for any other purpose.

Appendix 2. Problems optimizing reporter assay based on BG1LucE2 cells.

As described in *Methods*, ER-positive BG1Luc4E2 ovarian cancer cells are stably transfected with an ERE-luciferase reporter gene (Rogers and Denison 2000). In our laboratory these cells displayed high variability of results when grown in the same low estrogen conditions successfully used for other cell lines (weekly 1:10 passage, plating in 3%csBGS media and waiting 2 days before treatment). The variability was due to increased luciferase signal from control-treated (DMSO) cells (Figure A1A), therefore the difference between control and maximal estrogen effect changed from one experiment to another, and in some experiments was minimal. The result was a high variability of estrogen efficacy (Figure A1B). Several measures were taken to improve the reliability of the assay (see *Methods* for detailed media formulation): (1) increased pre-incubation in basal medium (BM); (2) addition of the antibiotic geneticin (G418) to growth medium (GM) to continue selective pressure on the cells; (3) optimization of the amount of csBGS in the estrogen-depleted BM; and (4) passaging cells in very dilute suspension (suggested by M. Denison laboratory). Increased incubation time in BM should decrease the background estrogenic activity in cultured cells; and increasing amounts of serum has been reported to decrease basal estrogenic signaling in culture (Soto and Sonnenschein, 1985). In order to minimize the population of cells with constitutively active luciferase gene, cells were passed in a very dilute suspension by drawing a trypsinized cell suspention into a pipette, expelling the cells and then washing the same pipette again in the fresh GM of the new flask. In the end we concluded that

several conditions were required to minimize assay variability: very dilute passage (by "pipette wash") into GM containing 0.4 mg/mL G418, and 4 days pre-incubation in BM containing 10% csBGS. Cells were not used beyond passage #11, and passages before #7 yielded better results. Using this techniques the basal control luciferase levels were maintained at a minimum (see Figure A1A, last nine data points) and the difference between maximum induced luciferase and control luciferase was maintained at acceptable levels (maximal β-estradiol effect above 2.5-fold; Figure A1B, last nine data points).

Figure A1: Influence of cell passage, growing and plating conditions on reproducibility of ERE-luciferase assay using BG1LucE2 cells. A. Variability of response to vehicle (control) treatment; before finding the optimal assay conditions, activity after control treatments fluctuated widely. B. Variability of maximum estrogen effect in each assay as fold increase over vehicle treatment; each point represents an independent experiment. *, New vial of cells from original frozen stock was used starting at this assay. **, Cells were frozen before holiday break and same cells were thawed and used starting at this assay. Shaded box indicates experiments not used in this thesis (maximum β-estradiol effect < 2.5 fold).

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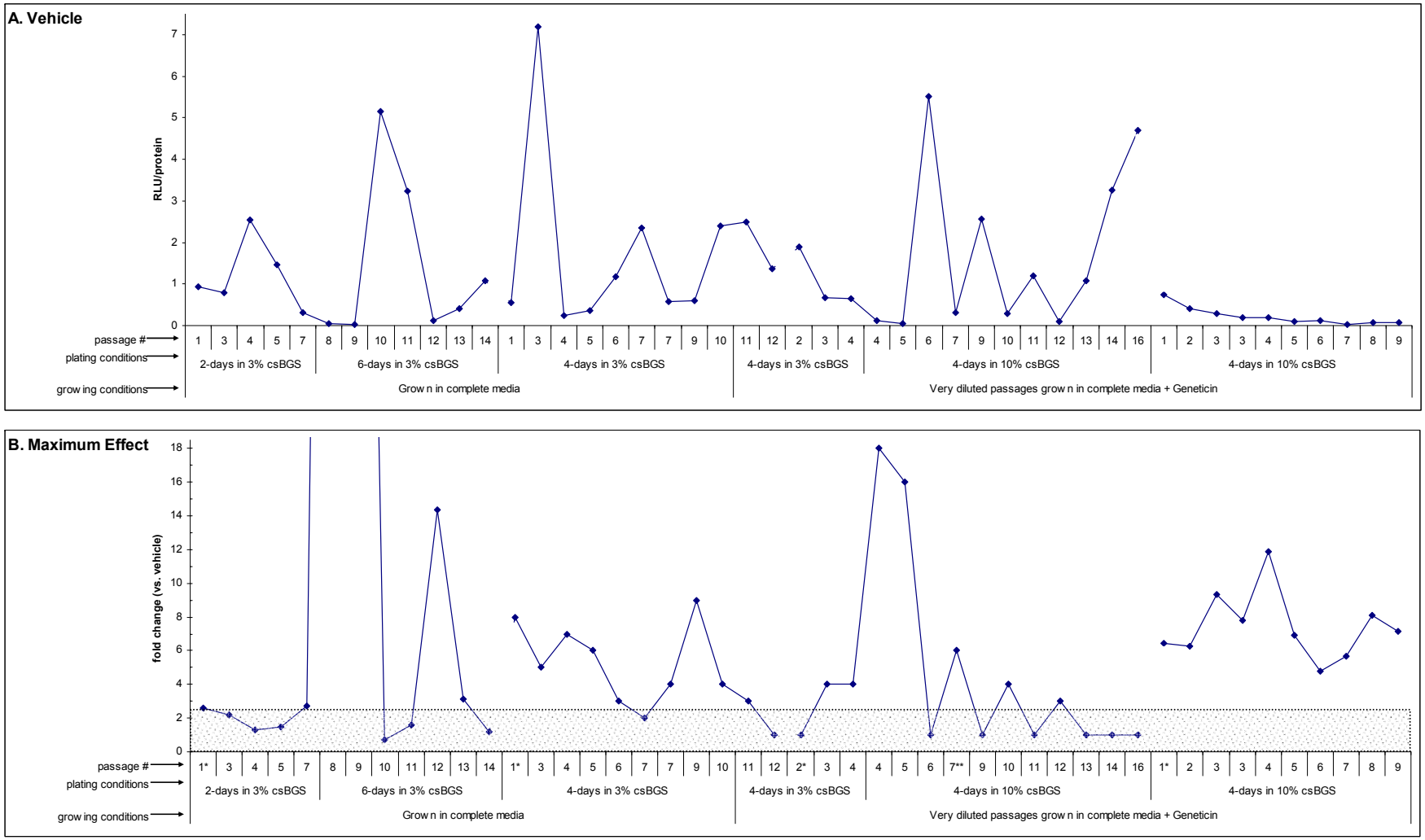
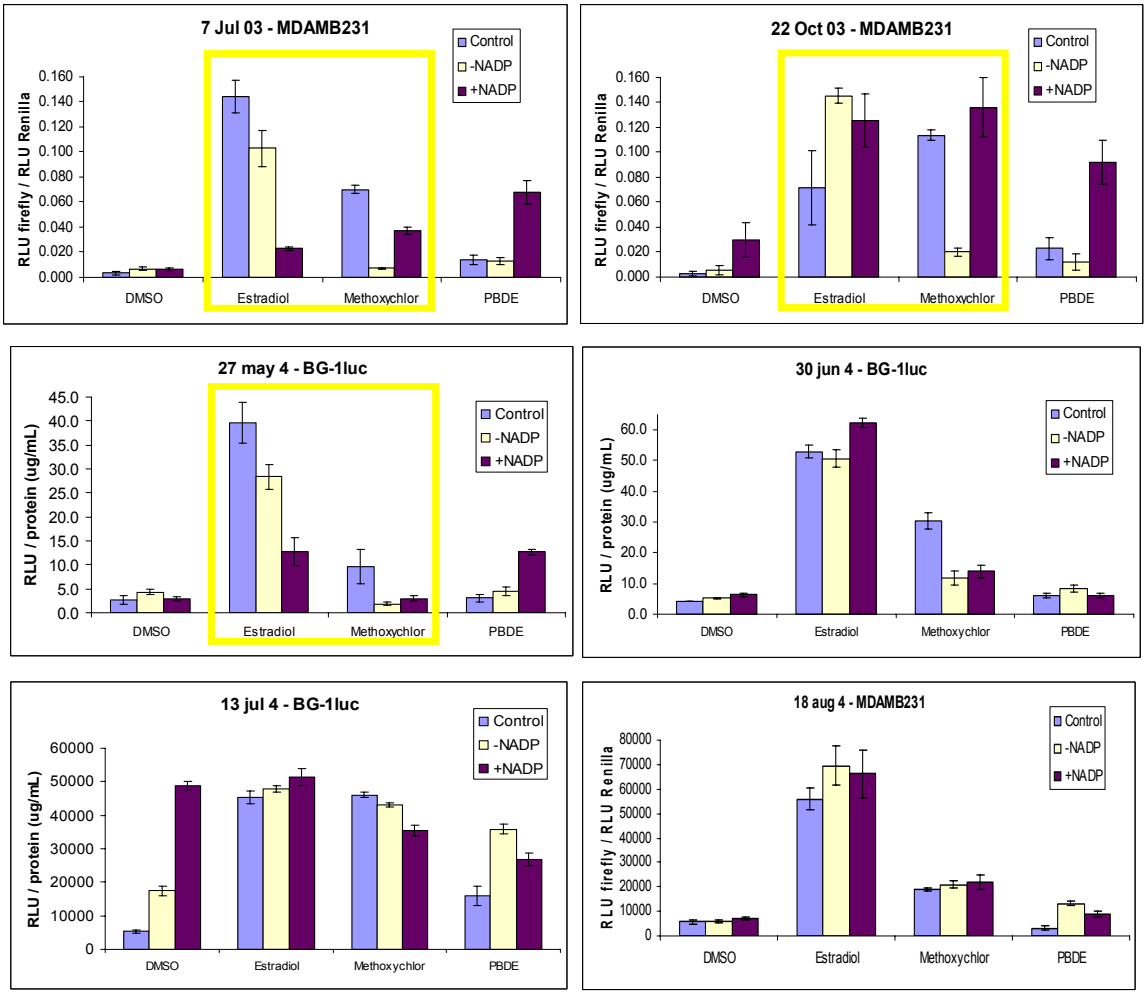
Figure A1 (cont): Influence of cell passage, growing and plating conditions on reproducibility of ERE-luciferase assay using BG1LucE2 cells.



Appendix 3: Selection of successful microsomal incubations.

DE-71 was incubated with female rat liver microsomes and an NADPH generating system (as described in *Methods*) to determine if such treatment would increase the estrogenicity of DE-71. The incubation product was then tested for estrogenicity by ERE-luciferase assay. Although several incubations were performed (Figure A3), only a few showed the expected CYP450 activity on the positive controls β-estradiol and methoxychlor. CYP450 are known to decrease the estrogenic activity of β-estradiol (Lee et al. 2003) and increase that of methoxychlor (Bulger et al. 1978; Kupfer 1979). In the thesis *Results* section we only used experiments in which the controls worked, i.e., there was a decreased activity for β-estradiol and increased activity for methoxychlor in the ERE-luciferase assay.

Figure A2. Luciferase assays after incubating β-estradiol (estradiol), methoxychlor or DE-71 with female rat liver microsomes for 24 hours. Each chart represents an independent assay (3-4 wells per treatment). Control treatments consisted of "fresh" chemical solution (i.e. not incubated with microsomes) added to media. –NADPH treatments consisted of chemical incubated with microsomes but without NADPH generating system (see *Methods*). +NADPH treatments consisted of chemicals incubated with microsomes and NADPH generating system. Final treatments were: 0.1% DMSO, 10 nM β-estradiol, 10 µM methoxychlor or DE-71 (PBDE). Only the experiments with positive controls marked with a yellow rectangle were included in this thesis. Three other experiments known to have procedural errors were not included in this thesis either (data not shown).

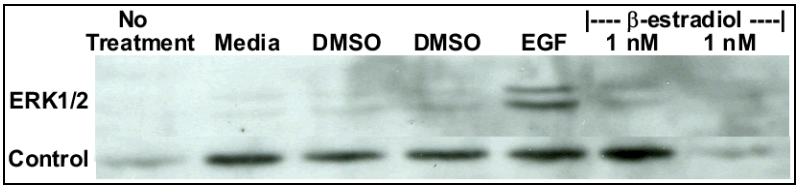


Appendix 4. Inability of β-estradiol treatment to activate ERK1/2 in culture.

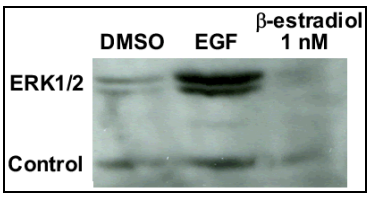
Others have seen rapid activation (5-10 minutes after treatment) of ERK1/2 after exposing cultured cells to β-estradiol (Manavathi and Kumar 2006; Migliaccio et al. 2006; Filardo et al. 2000; Thomas et al. 2005). On the other hand, Lobenhofer and Marks 2000, Bonapace et al. 1996 and Gaben et al. 2004 showed that this effect of estrogen was inconsistent and the authors questioned the validity of the hypothesis that ERK1/2 mediate the rapid effects of the hormone. Nonetheless, a clear interaction between growth factor pathways that exert their activity through ERK1/2 and enhanced estrogen receptor action has been shown (Ignar-Trowbridge et al. 1995; Smith 1998). Since some xenoestrogens do not bind the estrogen receptor or exert estrogenic effects beyond the maximal effect of β-estradiol (Meerts et al. 2001; Steinmetz et al. 1996; Bigsby, unpublished data), we explored ERK1/2 activation as a possible indicator of DE-71 estrogenic activity not mediated directly by nuclear ERs. We were unable to detect rapid activation of ERK1/2 after β-estradiol treatment of several cell lines, including the breast cancer cell lines MCF-7 and SKBR3. Representative results are included in Figure A1.

Figure A3. ERK1/2 activity in breast cancer cells 5 minutes after treatment with β-estradiol, except as indicated. Each blot represents and independent assay. Phospho-ERK1/2 was detected with antibody against phospho-Th202/Tyr204 (Cell Signaling #9101), and eIF4E was detected as loading control (Cell Signalilng # #9742). EGF treatment (10 ug/ml) was included in each experiment as a positive control.

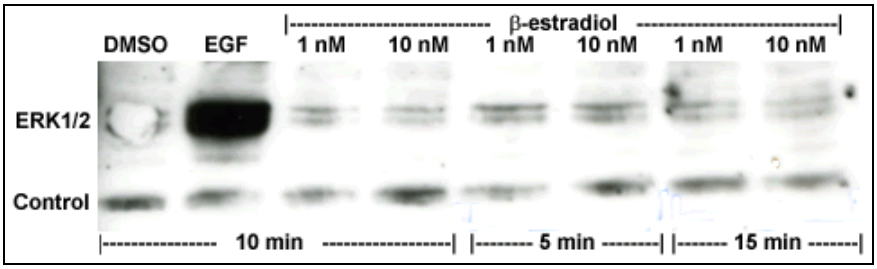
A. SKBR3, 9/23/2004 assay (DMSO and β-estradiol were plated and treated in duplicate)



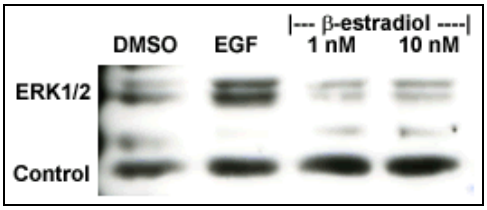
B. MCF-7, 10/29/04 assay



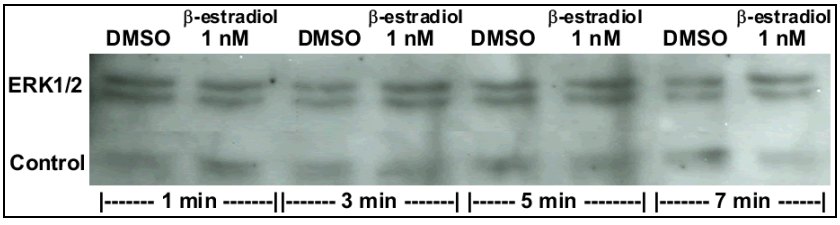
C. MCF-7, 11/12/2004 assay



D. SKBR3, 11/12/2004 assay



E. MCF-7, 11/22/2004 assay



Appendix 5. Effect of CYP450 inhibitors on DE-71 induced MCF-7 cell proliferation.

Metabolism by cytochrome P450 (CYP450) isoenzymes can increase the estrogenic activity of environmental pollutants (Bulger et al. 1978; Kupfer 1979; Morohoshi et al. 2005; Kohno et al. 2005; Jansen et al. 1993; Yoshihara et al. 2004) and here we show that the estrogenicity of DE-71 is also increased by in vivo metabolism. We also showed that DE-71 increases MCF-7 cell proliferation in culture. Therefore, we attempted to determine if MCF-7 cell proliferation could be hampered by CYP450 antagonists. MCF-7 cells were co-treated with 10 μM DE-71 and various concentrations of either alpha-naphtoflavone (αNF) or *N*, *N*-di-ethylaminoethyl-2,2-diphenylvalerate hydrochloride (SKF 525a; SKF). However, we concluded these experiments were not informative due to the secondary activities of both SKF and αNF.

SKF seem to decrease the induction of cell growth by DE-71 (Figure A5A). However, SKF is also a weak estrogen (Kupfer and Bulger 1982). In our assays, although an 11% decrease in β-estradiol effect after co-incubation with 1 μM SKF was not statistically significant, in one assay 10 μM SKF co-incubation diminished the effect of β-estradiol by 59%. Therefore we cannot rule out that the decrease in DE-71 effect is due to competition for estrogen receptors rather than CYP450 inhibition.

αNF was able to decrease the DE-71 induced MCF-7 cell proliferation only in some assays, therefore the effect was lost in the overall statistical analysis of the grouped assay data (Figure A5B). While αNF is a well known inhibitor of the CYP1A1 isoenzyme, it

can increase CYP3A4 activity by selectively binding and activating an otherwise inactive subpopulation of the enzyme (Koley et al. 1997). Since CYP450 metabolism can decrease the estrogenicity of β-estradiol (Lee et al. 2003), the interaction between αNF and CYP3A4 could explain the sharp decrease in cell proliferation we observed when cells were co-treated with 10 pM β-estradiol and 10 μM αNF.

Figure A4. Effects of CYP450 inhibitors on MCF-7 cell proliferation assay. A: SKF. B: αNF. Cells were treated as described in Methods and Figure 9, except they were cotreated with either 10 pM β-estradiol or 5-10 μM DE-71 and a CYP450 inhibitor at the concentrations indicated in the chart. Each column is the average of at least 2 independent assays, except as noted on chart. Different from control (0 M SKF = 0.1% DMSO): *, $p < 0.05$; ***, $p < 0.001$. Different from 10 μM DE-71: $^{\#\#\#}$, $p < 0.001$. Different from 10 pM β-estradiol: $^{+++}$, $p < 0.001$.

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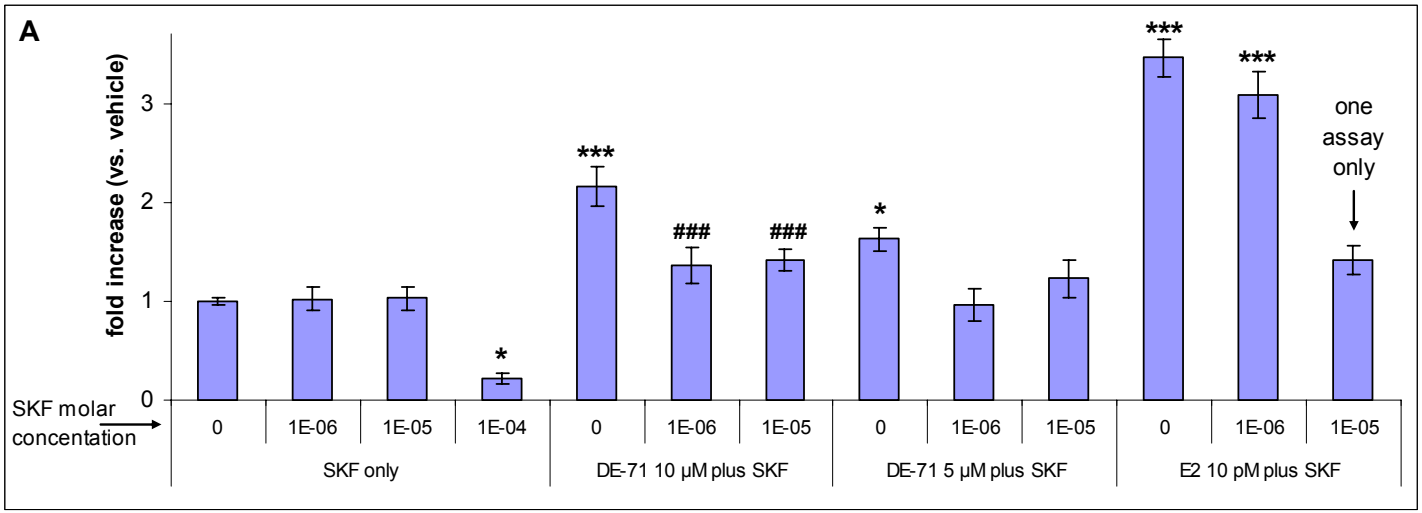
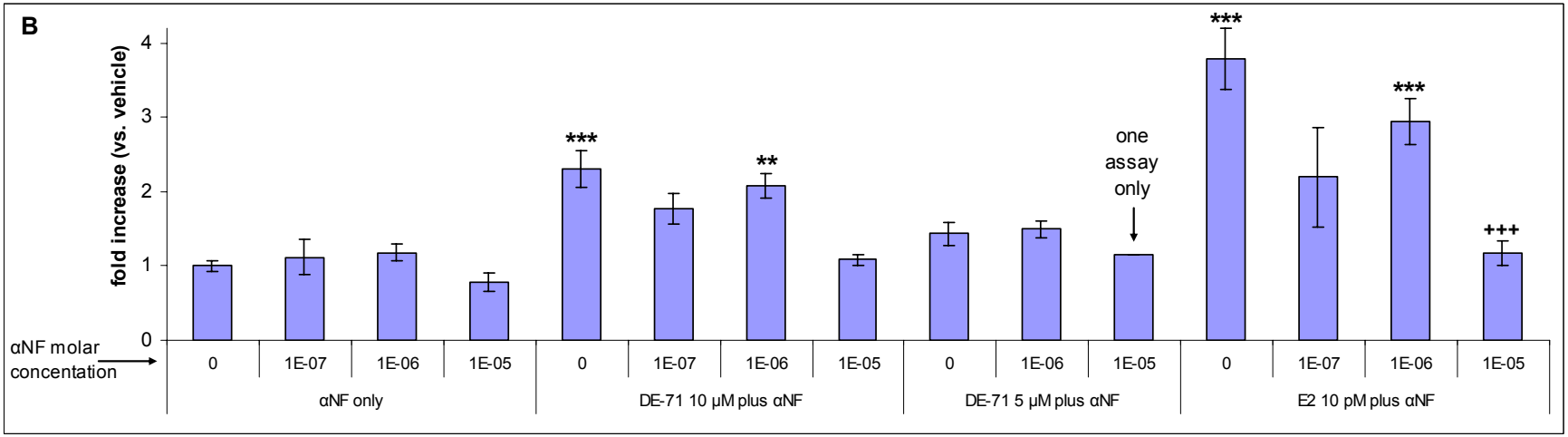
Figure A4 (cont). Effects of CYP450 inhibitors on MCF-7 cell proliferation assay.





Appendix 6. Variability in Relative Uterine Weight of Control Ovariectomized Wild Type Mice.

The table below lists average relative uterine weights (RUW; uterus weight in mg divided by body weight in grams) for control treatment groups of mouse experiments in our laboratory between March 2005 and January 2007. All in vivo experiments described in this thesis were done within that period of time. In most instances, average RUWs were less than 0.39 mg/g for control-treated animals except for two experiments on BALB/c mice performed between August and December 2005. The first of these experiments (RUW = 0.62 mg/g) had a 34-day treatment regime and its results are labeled "Experiment #2" throughout this document, and shown in Figures 1, 13, 15, 16, 19 and 25. Because of the difference in control RUW from the first 34-day treatment regime experiment (labeled "Experiment #1"; RUW = 0.29 mg/kg), data from each 34-day experiment were analyzed separately. The second experiment with a high RUW had a 3-day treatment regime in BALB/c mice, and was discarded because of dosing errors in EB treated groups. Other 3-day experiments in BALB/c mice had similar average RUWs values and their results were combined into one data set for statistical analysis. All C57BL/6 experiments had similar average RUWs and were combined into one data set for statistical analysis.

Table A1. Dates and Relative Uterine Weights of Control Ovariectomized Wild Type Mice from DE-71 Assays. Mice were treated with either 100 μL corn oil by oral gavage, or 10 μL DMSO by subcutaneous injection. Within experiments, there was no statistically significant difference between groups treated orally or subcutaneously (data not shown), therefore combined data is shown as mean ± SEM.

Year	Dates	Strain	mg/g	Comments
2005	March 6 - May 11	BALB/c	0.29 ± 0.05	BALB/c Experiment #1
	May 26 - June 29	C57BL/6	0.38 ± 0.02	
	August 3 - September 8	BALB/c	0.62 ± 0.07	BALB/c Experiment #2
	December 5 - 13	BALB/c	0.57 ± 0.03	Not used due to EB dose error
2006	April 17 - 21	BALB/c	0.32 ± 0.03	
	May 17 - June 21	C57BL/6	0.35 ± 0.06	
	September 27 - November 1	C57BL/6	0.39 (n = 1)	
	December 4 - 8	BALB/c	0.37 ± 0.03	
2007	January 19 - 22	BALB/c	0.35 ± 0.06	

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CURRICULUM VITAE

Minerva Mercado Feliciano

EDUCATION:

Ph.D. in Toxicology, February 2008, Indiana University. Advisor: Dr. Robert M. Bigsby. Dissertation: "Estrogenic Activity of the Polybrominated Diphenyl Ether Flame Retardant Mixture DE-71"

M.S. in Environmental Sciences, Hazardous Waste Management Mayor, February 1997, Indiana University, Bloomington IN 47405

B.S. in Environmental Sciences, Cum Laude, June 1992, University of Puerto Rico, Rio Piedras, PR 00931

RESEARCH EXPERIENCE:

Indiana University School of Medicine
Van Nuys Medical Science Building, 635 Barnhill Dr., Indianapolis Indiana 46202

- **Dr. Robert M. Bigsby**, Dept. Obstetrics and Gynecology. November 2007 to present. **Research Project: Estrogenic activity of DE-71 Through Estrogen Receptor Beta.** Responsibilites: Determine if DE-71 or its in vivo hydroxylated metabolites activate ERβ in vitro. Mentor undergraduate students.

- **Dr. Robert M. Bigsby,** Dept. Obstetrics and Gynecology. June 2003 to November 2007. **Thesis Project: Estrogenic Activity of the Polybrominated Diphenyl Ethers.** Skills learned: Cytochrome P450 metabolic activity assays; breast cancer cell culture; luciferase transient and stable transfection assays; receptor binding competition assays; rodent ovariectomy, dosing and tissue harvest; uterine histology and light microscopy analysis; protein immunoblotting.

- **Dr. James E. Klaunig**, Dept. Pharmacology and Toxicology, November 2002 to May 2003. **Research Project: Role of the Kupffer Cell in 2-butoxyethanol Induced Endothelial Cell Proliferation in Male Mice.** Skills learned: rodent dosing, minor surgery, tail vein injection and tissue harvesting; hematocrit; liver histochemistry; RNA isolation and reverse transcriptase PCR.

- **Dr. James E. Klaunig**, Dept. Pharmacology and Toxicology, June to October 2002. **Research Rotation.** Skills learned: liver cell proliferation counts; comet assay; endothelial cell culture.

- **Dr. William J. Sullivan Jr.**, Dept. Pharmacology and Toxicology March to May 2002. **Research Rotation.** Skills learned: SDS-PAGE and agarose electrophoresis, general cell culture techniques, enzyme kinetic assays, cell transformation, protein and DNA purification techniques.

- **Dr. Joseph A. DiMicco**, Dept. Pharmacology and Toxicology, December 2001 to February 2002. **Research Rotation.** Skills learned: handling of live rodents, minor stereotaxic brain surgery and microinjection, arterial cannulation and blood pressure monitoring.

PROFESSIONAL EXPERIENCE

Indiana Department of Environmental Management
100 North Senate Street, Indianapolis IN 46206

- **Environmental Manager – Risk Assessment**, February 2001 to July 2001
  Maintained default table of allowed contaminant concentrations in soil and groundwater for risk-based cleanup guidance document. Assisted development of web-based software to calculate non-default allowed contaminant concentrations according to risk-based guidance. Maintained public information web site.

- **Solid Waste Data Analyst/Planner**, May 1994 to May 1996 and January 1998 to February 2001
  Administered reporting programs and databases. Performed customized data analysis, data modeling and geographical maps regarding solid waste data by request from state and local officials and the general public. Used data analysis to evaluate and recommended revisions to State law, regulations, procedures, forms, and guidance. Published annual report and made public presentations.

- **Environmental Planner - Temporary Assignment**, May 1996 to January 1998
  Lead inter-office environmental indicators development team. Coordinated department-wide performance measures for Indiana State of the Environment report. Assisted in development of Agreement document between IDEM and the U.S. EPA, including: assistance to senior managers developing strategic plan and indicators; negotiations with U.S. EPA regarding indicators and data management; and public and staff involvement. Trained staff on environmental indicators and performance measures. Represented Indiana in regional and national measurement/indicators workgroups.

Servicios Científicos y Técnicos, Environmental Consultants
607 El Centro, Avenida Muñoz Rivera, Hato Rey PR 00918.

- **Field Trip Guide**, Summer 1992
  Guide to High and Middle School students from low-income communities, visiting various forest reserves, water treatment facilities, and other sites of environmental significance

U.S. Environmental Protection Agency, Caribbean Division
Centro Europa Bldg., Suite 417, 1492 Ponce de Leon Ave., San Juan, Puerto Rico 00907-4127

- **Howard Hudges Research Fellow**, Summer 1991
  Research Project: Acid deposition research at Puerto Nuevo Industrial Area

Fernando Feliciano-Reyes, Consulting Mechanical Engineer
20 Minerva St. Levitville, Levittown, PR 00951

- **Engineer's Assistant** (part time), March 1988 to September 1990
  Office administration. Calculation of global thermic transfer values for the envelope of new buildings as part of the Certification for the Energy Code of Puerto Rico

VOLUNTARY WORK

Lead Risk Assessor, Indiana Home Lead Assessment Program, 1999-2000
Performed over 20 lead risk assessments, mostly for Spanish speaking-families Northern and Central Indiana

ADDITIONAL TRAINING:

- **Indiana Risk Integrated System of Closure Training**, January 2001
- **OSHA 40 Hour Safety Training**, July 8, 2000 (last refresher)
- **USEPA Introduction to Groundwater Investigations**, October 1999
- **Lead Inspector/Risk Assessor Training**, February 1999. Certified as assessor/inspector in Indiana 1999-2001.
- **GPS Mapping for GIS with GeoExplorer II**, October 1998
- **Introduction to ArcView**, February 1997
- **Resource Conservation and Recovery Act (RCRA) Overview**, May 1998
- **Overview of Risk Assessment**, August 1996
- **Landfill Specialist Course**, August 1994

PROFESSIONAL SOCIETY MEMBERSHIP

Society of Toxicology – Student Member since 2004

Endocrine Society – Student Member since 2005

PUBLICATIONS

Papers and Abstracts:

Minerva Mercado-Feliciano and Robert M. Bigsby (2007) **The Polybrominated Diphenyl Ether Mixture DE-71 is Mildly Estrogenic.** Environmental Health Perspectives, submitted.

Xinghua Qiu, Minerva Mercado-Feliciano, Robert M. Bigsby, and Ronald A. Hites (2007) **Measurement of Polybrominated Diphenyl Ethers and Metabolites in Mouse Plasma after Exposure to a Commercial Pentabromo Diphenyl Ether Mixture.** Environmental Health Perspectives,115(7):1052-8.

Minerva Mercado Feliciano and Robert M. Bigsby (2006) **In Vivo Estrogenic and Antiestrogenic Effects of the PBDE Mixture DE-71.** Poster presented at the 45th Annual Meeting of the Society of Toxicology (March 2006, San Diego CA), 5th Gordon Research Conference on Environmental Endocrine Disruptors (June 2006, Il Ciocco, Italy), and 88th Annual meeting of the Endocrine Society (June 2006, Boston MA).

Minerva Mercado Feliciano and Robert M. Bigsby (2005) **Estrogenic Activity of the Polybrominated Biphenyl Ethers.** Poster presented at the 44th Annual Meeting of the Society of Toxicology (March 2005, New Orleans LA) and 87th Annual Meeting of the Endocrine Society (June 2005, San Diego CA).

Minerva Mercado Feliciano and Robert M. Bigsby (2004) **Estrogenic Activity of Xenobiotics.** Poster presented at the Midwest Regional Molecular Endocrinology Conference (May 2004, Indianapolis IN).

Book Chapters:

Robert M. Bigsby, Minerva Mercado-Feliciano and Josephine Mubiru (2005) **Molecular Mechanisms of Estrogen Disruption in Estrogen Dependent Processes**; in "Endocrine Disruptors: Effects on Male & Female Reproductive Systems", Pages 217-247. 2nd Edition. Taylor & Francis Books, Inc. (ISBN: 0-8493-2281-2)

Minerva Mercado-Feliciano. **Chemicals in Water: Combined Effect on Public Health** (May 2003). Water Science and Issues; published by Macmillan Reference USA (ISBN: 0-02-865611-3).

Richard B. Worth and Minerva Mercado-Feliciano (January 2002). **Solid Waste Measuring**. Macmillan Science Library – Mathematics; edited by Max Brandenberger, published by Macmillan Reference USA (ISBN: 0-02-865561-3)

Government Publications:

1998 and 1997 Indiana Solid Waste Facility Annual Report, IDEM, April 1999 and April 1998.

1998 and 1997 Environmental Performance Partnership Agreement between IDEM and the U.S. EPA Region 5, October 1997 and September 1996 (co-authored with IDEM EnPPA Workgroup)

Indicators of the Environment Fact Sheet, IDEM, September 1996

Indiana Municipal Waste Landfills, IDEM January 1995 (co-authored with Richard B. Worth)

Summary of Indiana Solid Waste Facility Data 1991-1994, IDEM, August 1995 (co-authored with Elizabeth San Miguel and Cindy Clendenon)

FELLOWSHIPS AND AWARDS

Academic:

Endocrine Society Forum on Endocrine Disruptors Student Award. June 2005. Poster: Estrogenic Activity of the Polybrominated Biphenyl Ethers. 87th Annual Meeting of the Endocrine Society

Society of Toxicology Student Travel Award. March 2005. 44th Annual Meeting of the Society of Toxicology

Ruth L. Kirschstein National Research Service Award - Predoctoral Fellowship for Minority Students. National Institute of Environmental Health Sciences. July 2004 to May 2007.

Public Service:

IDEM Key Staff Member Award, July 18, 2001. For playing a key role in IDEM's 1999-2001 Agency-Wide Priority – Reducing Toxic Exposures. Lori Kaplan, Commissioner.

IDEM Exceptional Service Award, September 20, 2000. For exceptional work above and beyond their assigned duties. These volunteers participated in a program to improve public health through technical inspections for the presence of lead in more than 1000 homes and day care centers around the state. Lori F. Kaplan, Commissioner.

IDEM Exceptional Service Award, September 20, 2000. For acting as interpreters and translators during a recent enforcement action, to communicate with a non-English speaking public. These staff bridge the language gap that resulted in re-certification or initial training of an ever-increasing number of Hispanics in the environmental field of asbestos abatement. Lori F. Kaplan, Commissioner

IDEM Special Commissioner's Award, November 22, 1996. For contribution to exceptional team effort which lead to the fiscal year 1997 Environmental Performance Partnership Agreement between the U.S. Environmental Protection Agency Region 5 and the Indiana Department of Environmental Management. Michael O'Connor, Commissioner.