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XENO-ESTROGENS IN THE AQUATIC ENVIRONMENT: DEVELOPMENT AND APPLICATION OF *IN VITRO* AND *IN VIVO* BIOASSAYS

By

Vincent J. Kramer

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ABSTRACT

XENO-ESTROGENS IN THE AQUATIC ENVIRONMENT:
DEVELOPMENT AND APPLICATION OF *IN VITRO* AND *IN VIVO*
BIOASSAYS

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A well-defined, logical framework for evaluating the estrogenic and anti-estrogenic activity of chemical substances was developed. Properties of chemical substances that determine estrogenic or anti-estrogenic potential were investigated at the molecular, cellular, and physiological levels of organization using both *in vitro* and *in vivo* bioassays. Hydroxylated polychlorinated biphenyls, (PCBs), metabolites of PCBs that have been identified in human and animal samples, were studied. Molecular modelling of the hydroxylated PCBs was used to predict estrogen receptor binding affinity and to investigate the molecular parameters that affected binding. Hydroxyl substitution in the *para* position was an important characteristic of strongly binding compounds, provided that the hydroxyl was not flanked by vicinally substituted chlorines. A functional *in vitro* bioassay utilizing a recombinant human breast cancer cell line was used to evaluate the estrogenic and anti-estrogenic potency of the hydroxylated PCBs. Hydroxylated PCBs that were previously identified as mammalian metabolites exhibited anti-estrogenicity that was readily reversed by 17ß-estradiol. However, cytotoxicity appeared to explain most of the anti-estrogenicity. A reporter gene system was designed, constructed and partially characterized for estrogen responsiveness and utility

as a screening assay. The estrogen responsive plasmid, pGudluc2.2, was not useful as a screening tool because the luciferase reporter gene was expressed at low levels and estrogen treatment caused reduction of expression, instead of induction, perhaps due to estrogen receptor repression of the promoter activity. Finally, a functional *in vivo* bioassay utilizing fathead minnows was developed and used to test the activity of the benchmark estrogen, 17ß-estradiol. Plasma alkaline-labile phosphorous, an indicator of vitellogenin expression, proved to be a useful biomarker of reproductive impairment. The biochemical and physiological responses identified in this assay will be useful in evaluating effects of waterborne xenoestrogens on fish.

To Maria, Sara Rose and Little One

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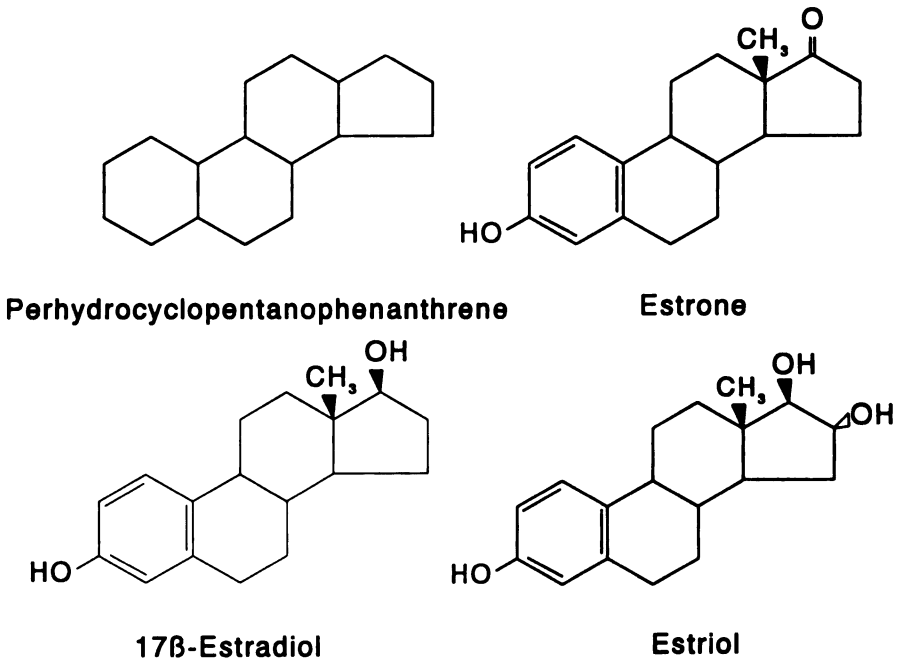
INTRODUCTION

Estrogen Agonists in the Environment

Estrogen agonists are compounds that mimic the effects of estrogen. The classic definition of an estrogen is a compound that produces cornification of the vaginal epithelium of rodents, typically mice or rats (Solmssen, 1945). Other physiological endpoints have been used to define estrogenicity including increased uterine weight and increased uterine glycogen content after dosage of an estrogenic compound (Clark and Peck, 1979). Under the classic definition, it is impossible to distinguish whether the estrogenic activity is due to the parent compound or to its metabolites. A refined definition of estrogen, recognizing the role of nuclear hormone receptors in gene expression, is a compound that binds to the estrogen receptor (ER), thus activating transcription of genes under the regulatory control of estrogen responsive elements (EREs; Evans, 1988). Therefore, the description of a compound as an estrogen is dependent upon the method of testing and can in the classical sense include compounds that require metabolic activation to an estrogenic form.

Estrogen agonists encompass a range of chemical classes and structures. The structure of the endogenous estrogens, 17ß-estradiol (E2), estrone, (E1), and estriol (E3) (Figure 1) is based upon the steroid hormone template



Perhydrocyclopentanophenanthrene Estrone

17ß-Estradiol Estriol

Figure 1. The steroid hormone "skeleton" and the endogenous estrogens

perhydrocyclopentanophenanthrene (Zubay, 1988). The exogenous estrogens can be classified into two broad categories: 1) natural products, and; 2) synthetic compounds. The chemical structural characteristics of exogenous estrogens vary but can be summarized as: 1) containing a sterically unrestricted phenol group, and; 2) a moderately hydrophobic substituent of three or more carbons bonded opposite the phenolic group (Dodds and Lawson, 1938; Mueller and Kim, 1978). The effects of phytoestrogens have been long known in the field of animal husbandry. Bennets, et al. (1946) noted infertility in sheep grazing on clover pasture containing the isoflavone formonetin which is metabolized in the sheep gut to the active estrogen equol (Shutt and Braden, 1968; Lundh et al., 1990). Phytoestrogens have been implicated in reproductive dysfunction of captive cheetah (Setchell, 1987) and in precocial

vitellogenin production in sturgeon (Pelissero, et al., 1991). Synthetic estrogens have been produced either by design: for example, diethylstilbestrol and ethynylestradiol (Solmssen, 1945; Anstead, 1990; Jordan, et al., 1986) or by accident: for example, DDT and its metabolites (Kupfer and Bulger, 1980; Nelson, et al., 1978; Jensen, et al., 1979; Heath, et al., 1969, Keith, 1966); PCB's and their metabolites (Gellert, 1978; Ecobichon and McKenzie, 1974; Korach, et al., 1988; Nelson, 1974; Bitman and Cecil, 1970); Kepone (Gellert and Wilson, 1979; Gellert, 1978; Eroschenko and Plamiter, 1980; Eroschenko, 1981); alkyl phenols (Soto, et al., 1991; Soto, et al., 1992), bisphenol A (Krishnan, et al., 1993; Brotons, et al. 1995); phthalate plasticizers and the food additive butylated hydroxyanisole (Jobling, et al. 1995). The potential for estrogen mimics to affect the reproduction and survival of fish and wildlife populations deserves careful study.

Functions of Estrogens

Estrogens, such as 17ß-estradiol (E_2), estrone (E_1), and estriol (E_3), are a group of vertebrate steroid hormones that elicit a wide range of physiological, developmental, and behavioral responses in both females and males. Estrogen plays an important role: 1) in stimulating sexual maturation, uterine growth, vaginal cornification, and mating behavior in female mammals (Selcer and Leavitt, 1991) and mating behavior in birds (Adkins, 1978); 2) via conversion by aromatase, in sexual differentiation of mammalian and avian central nervous systems (Arnold and Gorski, 1984); 3) in modulating hypothalamic

gonadotropin secretion during the primate menstrual cycle (Knobil, 1980); 4) in stimulating vitellogenesis in oviparous vertebrates (Selcer and Leavitt, 1991; Redding and Patino, 1993; Emmerson and Petersen, 1976; Wallace and Jared, 1968; deVlaming et al. 1984); 5) in differentiation of gonads and gonadal ducts in mammals (Selcer and Leavitt, 1991), birds (Fry and Toone, 1981; Johnson, 1986), fish (Adkins-Regan, 1987), amphibians (Witschi, 1971), and reptiles (Bull, et al. 1988), and; 6) in development of secondary sexual characteristics (Selcer and Leavitt, 1991).

Estrogens elicit cellular responses (Figure 2) by directly crossing cellular membranes to bind to nuclear ER (Lieberman, et al. 1986), which, in turn, bind to and activate specific regulatory segments on DNA--the EREs (Klein-Hitpass, 1986). EREs are trans-acting transcriptional enhancers (Cullen, et al. 1993). Efficient activation of the ERE occurs after binding of the ligand-bound receptor dimer (Gordon and Notides, 1986; Kumar, et al., 1987; Fawell, et al., 1990). The nucleotide sequence of the ERE in the vitellogenin A2 gene 5' flanking region of *Xenopus laevis* is a 15 base pair palindrome: 5'-AGGTCACAGTGACCT-3' (Walker, et al., 1984). However, considerable variation exists in the nucleotide sequence of EREs (Walker, et al., 1984; Chang, et al., 1992; Evans, 1988) that affects the efficiency of transcriptional enhancement (Pilat, et al., 1993). A number of accessory proteins are thought to be involved in binding of ER to the ERE. Hsp90 is released from the ER complex after E_2 binding. Dimerization of the ER complex is initiated by E_2 binding. Hsp70 remains bound to the ER complex. Tyrosine phosphorylation of ER is necessary for E_2 binding (Migliaccio, et al. 1991). Six functional

domains of the ER have been identified: A/B, the N terminal "modulator" domain confers gene transcription specificity; C, essential for activation of transcription and binding specificity to ERE; D, the "hinge" region; E, hormone binding and efficient transcriptional activation (but not essential for ERE recognition); F, the carboxyl terminal (Kumar, 1987).

Importance and Mechanisms of Anti-Estrogenicity

In the "environmental estrogen" arena, discussion of environmental anti-estrogens is under-represented. This is unfortunate because the potential effects of an anti-estrogen are just as significant to the developing or adult organism as an estrogen. As a general example, consider the role estrogen

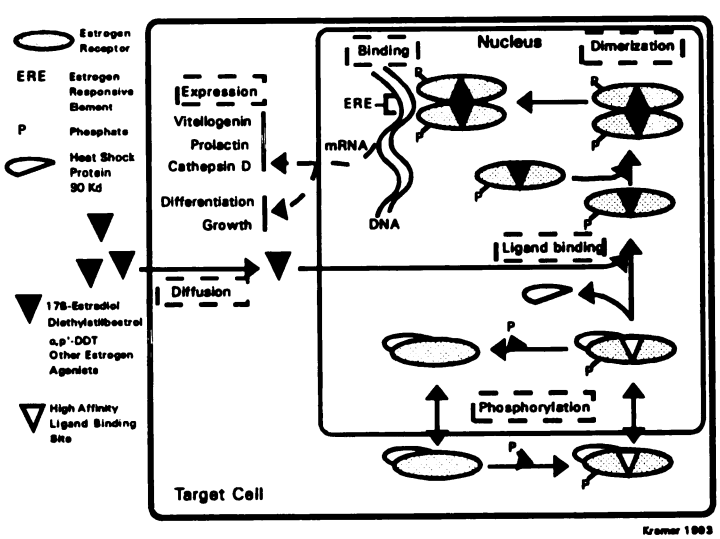


Figure 2. Mechanism of action of estrogens within cells.

plays in signalling the production of vitellogenin, necessary for egg production in all egg-laying vertebrates. There could be significant consequences for successful reproduction if reduced egg mass or egg number were caused by a blockage of estrogen action. Anti-estrogenic substances act by several mechanisms, not necessarily related to ER binding and activation. Classical estrogen antagonists such as ICI 164,384 and tamoxifen bind competitively to ER but block or reduce the effectiveness of the ligand-bound receptor to enhance gene expression (Fawell, et al. 1990; Jordan, et al. 1986). Aryl hydrocarbon receptor (AhR) agonists, such as 2,3,7,8-TCDD and coplanar PCBs, cause down-regulation of ER (Zacharewski, et al. 1991) and may also interfere with ER interactions with DNA. Aromatase inhibitors, such as aminoglutethimide, block conversion of testosterone to 17ß-estradiol and are used in the treatment of metastatic estrogen-dependent breast cancer (Farooqi, et al. 1992).

Environmental contaminants occur as mixtures that may contain both estrogenic and anti-estrogenic components. The effects of such mixtures of estrogens and anti-estrogens will be determined by the relative contributions of each type of estrogen-active compound and the nature of their interactions, either additive or non-additive. A bioassay driven approach to assess the total estrogenic or anti-estrogenic potency of the mixtures found in fish or wildlife is recommended so that the interactions of mixtures at the level of the estrogen receptor can be taken into account. Additionally, it is impossible to predict whether a compound will exhibit estrogenic or anti-estrogenic activity solely by measuring the affinity with which it binds to the estrogen receptor. Rather, the

potency of the activity, either estrogenic or anti-estrogenic, may be related to the estrogen receptor binding affinity, though anti-estrogenicity can also be elicited by mechanisms not involving binding to the estrogen receptor (as described previously).

To fully understand the toxicological action of xeno-estrogens, it is necessary to elucidate the biochemical, cellular, and physiological mechanisms of their action. To accomplish this, a framework for evaluating the estrogenicity and anti-estrogenicity of substances was developed. This framework defines the estrogenic/anti-estrogenic characteristics of a substance at several levels of toxicological organization: 1) molecular structural descriptors calculated by desktop molecular modelling; 2) an *in vitro* estrogen receptor binding affinity assay; 3) an *in vitro* estrogen-inducible luciferase reporter gene expression assay; 4) an *in vivo* fish exposure assay. The ultimate goal of such an approach is the development of quantitative structure-activity relationships that will allow the prediction of estrogenic or anti-estrogenic potential of either pure chemicals (for example, in the risk assessment process for the manufacture of new chemicals) or environmentally-derived complex mixtures (for example, in the assessment of environmental impact of contamination). The database required for such a goal is beyond the scope of this research project. However, this research project is intended to serve as a guide for the development of such a database.

General Methodological Approach

Molecular Modelling

Molecular modelling allows the estimation of molecular descriptors such as minimum energy three dimensional molecular structure and various electronic properties. These molecular descriptors can be used to quantitatively predict the biochemical properties of molecules after an appropriate number of "training" molecules have been used to establish a statistical model of the relationship. In this project, the binding of hydroxylated PCBs to the estrogen receptor was modelled using a set of molecular descriptors calculated using a desktop molecular modelling software package. It is anticipated that the molecular descriptors defined in this project may be used by others to develop models of the functional activity estrogenic and anti-estrogenic compounds in *in vitro* and *in vivo* bioassays.

In Vitro Bioassay of Estrogenicity and Anti-Estrogenicity

Aquatic organisms are exposed to complex mixtures of endogenous and exogenous estrogen agonists. Estrogen antagonists are an additional component of complex mixtures. Presently, the estrogenic or anti-estrogenic activity of a complex mixture cannot be predicted by chemical analysis alone because: 1) the synergistic effects of agonists and antagonists are unknown; 2) new examples of estrogen agonists and antagonists continue to be

discovered; 3) authentic standards for many estrogen agonists and antagonists are unavailable. The estrogenic or anti-estrogenic activity of a complex mixture can be measured using a functional *in vitro* bioassay. Two avenues of approach to the development of a functional *in vitro* bioassay have been taken. First, a well-characterized recombinant cell line, MCF-7-LUC, was obtained that was used to evaluate the estrogenicity and anti-estrogenicity of a number of pure compounds. Second, this research project also undertook an attempt to develop a genetically-engineered fish cell line, GUDLUC2.2, with the following properties: 1) sensitive--responding to a wide range of concentrations; 2) selective--responding only to estrogenic/anti-estrogenic compounds via defined biochemical pathways, and; 3) rapid--responding in a time frame to facilitate screening of large numbers of samples. The fish cell line was not developed becasue the plasmid, pGudluc2.2, was not sufficiently estrogen-responsive to be of practical use. The molecular mechanisms for the lack of success were not fully elucidated. However, designs for the construction of another cell line, GUDLUC3.0, are presented for future research. Although the screening of complex mixtures for estrogenicity/anti-estrogenicity was not the primary aim of this research project, the methods developed to assay single chemicals can be readily applied to complex mixtures.

In Vivo Bioassay of Waterborne Estrogens

Complementary to the development of the *in vitro* bioassay, an assay designed specifically to measure the effects of estrogenic compounds on the physiology, histopathology and reproduction of fathead minnows, (*Pimephales promelas*) was developed. One application of the laboratory assay will be to calibrate and validate the response of fathead minnows to effluents in caged fish studies planned by other researchers. Another application will be the development of concentration-response relationships that can be used in environmental risk assessment. Fish are the sentinels of aquatic ecosystem degradation and have signalled the presence of estrogenic compounds in affected waterways. For example, fishermen in the U.K. reported catching feminized ruffe in waters downstream of municipal wastewater treatment plants. Subsequent caged fish studies using rainbow trout and carp, confirmed the feminizing effect (induction of vitellogenin synthesis) of these waters. However, the active agent (or agents) have not been identified (UK Directorate of Fisheries Research, 1992). The proposed research will determine the relationship between induction of vitellogenin synthesis in males and females, a highly specific biomarker of estrogen exposure, and reproductive performance, an important facet in the survival of feral fish populations. Also, the effects of waterborne estrogen exposure on reproduction will be assessed. Histopathological examination will provide insights into the mechanisms of action of estrogens on male and female reproduction. However, the histopathological information is the subject of another researcher's PhD.

dissertation (Miles-Richardson, 1996)and will not be reported here. The results of the exposure of fathead minnows to 17ß-estradiol will be reported as the benchmark to which all other compounds will be compared.

Significance

Concern over environmental estrogens has risen both in the media and in regulatory agencies. There is an urgent need for contribution of scientific information to national debate that at times has focused on supposition and opinion. The aquatic environment frequently becomes a sink or site of first effects for environmental contaminants, including estrogenic contaminants. Therefore, it is important to study the effects of estrogenic contaminants in aquatic organisms. The fathead minnow is a widely used organism for aquatic toxicity testing and is an appropriate model organism for examining estrogenic endpoints. The *in vitro* assay, when fully validated, will provide a rapid, sensitive, and selective means of assessing estrogenicity and anti-estrogenicity of both pure compounds and complex mixtures. The *in vitro* assay can also be used to direct Toxicity Identification and Evaluation procedures rapidly and inexpensively. The ultimate application of the results of this proposal will be to develop an understanding of the effects of environmental estrogens/anti-estrogens on reproduction, development, and survival of environmentally-exposed fish and other aquatic vertebrates.

CHAPTER 1

HYDROXYLATED METABOLITES OF PCBs ARE ANTI-ESTROGENIC IN A HUMAN BREAST ADENOCARCINOMA CELL LINE (MCF-7)

Introduction

Although the banning of PCBs (polychlorinated biphenyls) production and use in the industrially developed world has resulted in decreasing environmental levels (Schmitt, et al. 1990), bioaccumulation and biomagnification of PCBs results in significant concentrations in upper trophic level species such as fish-eating waterbirds and mammals such as seals, and humans who eat PCB-containing seafood (Jansson, et al. 1993; DeWailly, et al. 1989, Haraguchi, et al. 1992). The relative abundance of individual chlorinated biphenyls (CBs) in environmental samples varies with the location and source of contamination, the age of the sample, and trophic level of the organism. In general, fish, wildlife, and human samples are dominated by persistent penta-CBs to hepta-CBs, due to their resistance to biodegradation relative to the more-readily metabolized mono-CBs to tetra-CBs (Sundström, et al. 1976) that possess vicinal hydrogens in the 3,4 position of one of the phenyl rings (Bergman, et al. 1992).

PCBs undergo oxidative alteration via cytochrome-requiring P450s 1A1, 1A2, 2B1, and 2B2 enzymes which catalyze the formation of intermediate arene oxides that lead to hydroxyl or methyl sulphone metabolites (Bergman, et al. 1994). Hydroxylated chlorinated biphenyls (OHCBs) and their sulfate and

glucuronide conjugates are typically excreted via feces or urine. However, organisms may retain OHCBs because these compounds are lipophilic and bind to plasma proteins, particularly transthyretin (Bergman, et al., 1994). OHCBs have been detected in excreta of wildlife (Jansson, et al. 1975) and in the blood of seals and humans (Bergman, et al. 1994). Hydroxylated metabolites of 3,3',4,4'-Cl_4-biphenyl competitively bound to human transthyretin and has been reported to cause marked reduction in plasma thyroxin levels (Brouwer, et al. 1990) and serum transport of Vitamin A (Brouwer, et al., 1986) in rodents. The metabolite was identified as 4-OH-3,3',4',5-Cl_4-BP and was shown to have a 2.5 times higher affinity for the transthyretin than thyroxine itself. This metabolite has also been indentified in mouse blood (Klasson-Wehler, et al. 1989) and in rat and mouse fetuses after maternal exposure (Morse, et al. 1995; Darnerud, et al. 1986). Similarly, 2,3,3',4,4'-Cl_5-BP forms rearranged metabolites -- 4-OH-2,3,3',4',5-Cl_5-BP and 4-OH-2',3,3',4',5-Cl_5-BP -- in mice and mink (Klasson-Wehler, et al. 1993). These metabolites have demonstrated *in vitro* a ten times higher affinity for transthyretin than thyroxin (Lans, et al. 1992), resulting in persistent retention of these metabolites in blood of both humans and seals exposed environmentally to PCBs (Bergman, et al. 1994).

The "disruption" of steroid hormone homeostasis and activity by environmental contaminants has become a cause for concern (Schmidt, 1994; Wolff, et al. 1993; Dibb, 1995). The weak estrogenic activity of commercial PCB mixtures administered to rats was recognized early in studies of PCB toxicity (Bitman and Cecil, 1970), however, only the less chlorinated Aroclor mixtures (<48% Cl by weight) exhibited estrogenicity, consistent with the

hypothesis that lesser-chlorinated OHCB metabolites were the active compounds (Nelson, 1974). 4-OH-2',4',6'-Cl_3-BP and 4-OH-2',3',4',5'-Cl_4-BP, which have not been identified as persistent, blood-retained metabolic products in humans or wildlife samples, were estrogenic in a rodent uterotrophic assay and bound competitively to estrogen receptor (Korach, et al. 1988). Also, 4-OH-2',4',6'-Cl_3-BP and 4-OH-2',3',4',5'-Cl_4-BP were estrogenic in a turtle sexual development assay (Bergeron, et al. 1994). However, the quantitatively significant OHCBs retained in wildlife and human blood have not been tested for their ability to interfere with or mimic estrogen action, and were, therefore, the subject of this research.

Estrogen agonists are compounds that mimic the effects of estrogen. The classic definition of an estrogen is a compound that produces cornification of the vaginal epithelium of rodents (Solmssen, 1945). Other physiological endpoints have been used to define estrogenicity including increased uterine weight, uterine glycogen content, protein induction (Korach, et al. 1987) and cellular proliferation (Soto, et al. 1992). It has been recognized that numerous synthetic as well as naturally-occurring compounds fit the classic definition of estrogen (McLachlan, 1993). A refined definition of estrogen, recognizing the role of nuclear hormone receptors in gene expression, is a compound that binds to the estrogen receptor, induces dimerization of the receptors that specifically bind to and activate transcription of genes under the regulatory control of trans-acting estrogen responsive elements (Kumar and Chambon, 1988). The chemical structural characteristics of estrogens vary considerably, but can be summarized as: 1) containing a sterically unhindered phenol group, and; 2) a

hydrophobic substituent of greater than 3 carbons bonded para to the phenolic hydroxyl (Dodds and Lawson, 1938, Jordan, et al. 1986).

Estrogen antagonists block the action of estrogens by interfering with the normal functioning of the estrogen receptor. Classical estrogen antagonists such as ICI 164,384 and tamoxifen bind competitively to the estrogen receptor, displacing the natural ligand 17ß-estradiol, and blocking or reducing the effectiveness of the ligand-bound receptor to enhance gene expression (Bondy and Zacharewski, 1993; Fawell, et al. 1990, Jordan, et al. 1986). The potency of classical estrogen agonists and antagonists is directly related to the binding affinity to the estrogen receptor (Chae, et al. 1991; Korach, et al. 1987; VanderKuur, et al. 1993a). Therefore, high affinity binding to the estrogen receptor can be an indication of possible anti-estrogenic activity, though receptor binding affinity alone is neither a necessary nor sufficient factor for anti-estrogenic activity. Anti-estrogenic compounds act by several mechanisms, not necessarily related to estrogen receptor binding and activation. For example, aryl hydrocarbon receptor agonists, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) and non-ortho-chlorinated PCBs, cause down regulation of estrogen receptor and may also interfere with DNA binding (Zacharewski, et al. 1991). Aromatase inhibitors, such as aminoglutethimide, block conversion of testosterone to 17ß-estradiol and are used in the treatment of metastatic estrogen-dependent brease cancer (Farooqi and Aboul-enein, 1992). Inducers of Phase I and Phase II metabolic detoxification enzymes, such as 2,3,7,8-TCDD and non-ortho-chlorinated PCBS, reduce the estrogen-dependent expression of proteins by enhancing the

enzymatic conversion of latent 17ß-estradiol, thus eliciting an anti-estrogenic response (Spink et al. 1992).

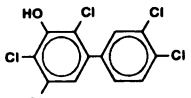
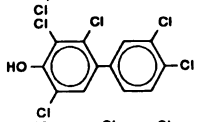
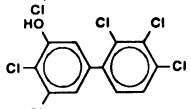
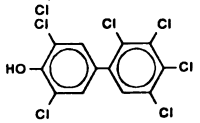
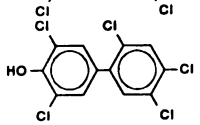
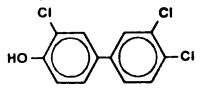
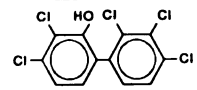
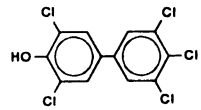
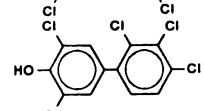
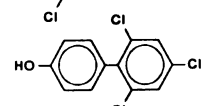
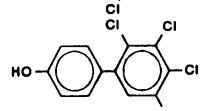
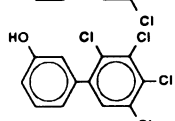
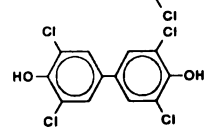
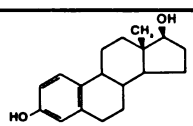
The objectives of this study were: 1) to determine the estrogenic or anti-estrogenic activity of tri-to hexa- chlorinated OHCBs (representing environmental and laboratory derived metabolites) in an *in vitro* bioassay using an estrogen-responsive luciferase reporter gene system; 2) to estimate the potency of the OHCBs in the presence of physiologically-relevant concentrations of 17ß-estradiol, and; 3) to relate the bioassay activity to molecular structure, human estrogen receptor binding affinity, and cytotoxicity.

Materials and Methods

Chemicals

A total of thirteen OHCBs were studied, nine of which were confirmed mammalian metabolites of CBs (Table 1). OHCB1 to OHCB9 were synthesized for use in this study and as analytical standards according to previously published methods (Bergman, et al. 1995, McOmie, et al. 1968). OHCB10 to OHCB13 were obtained from Ultra Scientific (North Kingston, RI). All OHCBs were authentic standards of greater than 95% purity. Stock solutions were prepared in ethanol and stored tightly sealed at -20°C.

Table 1. Environmental occurrence and structures of OHCBs and 17ß-estradiol assayed with MCF-7-LUC cells.

ID	Formula	Structure	Identified in Samples	Parent PCB	Plasma Retained
OHCB1	3-OH-2,3',4,4',5-Cl_5-BP		No published reports	PCB118?	
OHCB2	4-OH-2,3,3',4',5-Cl_5-BP		Rat, gray seal, human blood (1) Mink, mouse blood/feces (3)	PCB105 PCB118	Yes
OHCB3	3-OH-2',3',4,4',5-Cl_5-BP		Mink, mouse feces (3)	PCB105	No
OHCB4	4-OH-2',3,3',4',5, 5'-Cl_6-BP		Rat, gray seal blood (1)	PCB156	Yes
OHCB5	4-OH-2',3,4',5,5'-Cl_5-BP		Rat, gray seal blood (1)	PCB118	Yes
OHCB6	4-OH-3,3',4'-Cl_3-BP		Mouse feces (4)	PCB77	No
OHCB7	2-OH-2',3,3',4,4'-Cl_5-BP		Mink, mouse feces/blood (3)	PCB105	No
OHCB8	4-OH-3,3',4',5,5'-Cl_5-BP		No published reports	PCB126?	
OHCB9	4-OH-2',3,3',4',5-Cl_5-BP		Mink, mouse feces (3) Gray seal, human blood (1)	PCB105	Yes
OHCB10	4-OH-2',4',6'-Cl_3-BP		Rat blood(2)	PCB30	No
OHCB11	4-OH-2',3',4',5'-Cl_4-BP		No published reports	PCB61?	
OHCB12	3-OH-2',3',4',5'-Cl_4-BP		No published reports	PCB61?	
OHCB13	4,4'-$(OH)_2$-3,3',5,5'-Cl_4-BP		Mouse feces (4)	PCB77	No
E2	17ß-Estradiol		—	—	—

1: Bergman, et al. 1994; 2: Goto, et al. 1974, 1975; 3: Klasson-Wehler, et al. 1993. 4: Klasson-Wehler, et al. 1989. PCB30: 2,4,6-Cl_3-BP; PCB61: 2,3,4,5-Cl_4-BP; PCB77 3,3',4,4'-Cl_4-BP; PCB105: 2,3,3',4,4'-Cl_5-BP;PCB118: 2,3',4,4',5-Cl_5-BP; PCB126: 3,3',4,4',5-Cl_5-BP; PCB156: 2,3,3',4,4',5-Cl_6-BP.

Human Estrogen Receptor Binding Assay

Competitive binding assays were conducted using a modification of the hydroxyapatite (HAP) binding procedure of Murdoch, et al. (1990). A human adenocarcinoma cell line, MCF7 (American Type Culture Collection HTB22, Rockville, MD), was the source of human estrogen receptor (hER) for the binding assays. MCF7 cells were cultured in 75 cm^2 plates at 37 °C in a humidified atmosphere of 5% CO_2/95% air in 10% FBS medium consisting of Dulbecco's Modified Eagle's medium with Ham's F12 Nutrient Mixture without phenol red and with 15 mM HEPES buffer (Sigma Chemical, D2906, St. Louis, MO) at pH 7.4 supplemented to contain 10% (v:v) fetal bovine serum (Hyclone Laboratories, Lazan, UT), 2 mM sodium pyruvate, 1 μg/ml bovine insulin, and 10 U/ml:10μg/ml penicillin:streptomycin. Four days before harvest, the 10% FBS medium was changed to 5% DCCFBS medium in which the serum supplement was treated with dextran-coated charcoal (Hyclone Laboratories) to reduce steroid hormone concentrations. Individual lot analysis of fetal bovine serum provided by the manufacturer indicated that dextran-coated charcoal treatment reduced the 17ß-estradiol concentration from 8 pg/ml (29 pM) to 5 pg/ml (18 pM). Twenty four hours before harvest, the 5% DCCFBS medium was replaced with serum-free medium to minimize the level of 17ß-estradiol in the cells and to improve the recovery of unliganded hER. Cells at 85% confluence were gently suspended by a 30 min incubation in 1 mM EDTA in Ca-Mg free phosphate buffered saline at 37°C. Cell suspensions were combined

and centrifuged at 800g for 10 min. at room temperature to pellet cells. The cells were washed once with homogenization buffer (10 mM Tris HCl pH 7.5, 1.5 mM Na_2EDTA, 1 mM dithiothreitol, 1 mM sodium molybdate, 0.5 mM aminoethylbenzenesulfonyl fluoride (a protease inhibitor) and 10% v:v glycerol), resuspended in 2 ml ice cold homogenization buffer, disrupted with 60 passes of a Dounce homogenizer on ice, and centrifuged at 800g, at 4°C for 10 min. to pellet cellular debris. A molybdate-stabilized hER preparation was obtained by centrifugation of the supernatant at 100,000g at 4°C for 30 min. The hER preparation was stored in 200 μl aliquots at -80°C until use. Scatchard plot analysis of the hER preparation indicated a 17ß-estradiol specific binding activity of 0.22 pmol/mg protein with an apparent dissociation constant of 2.3 ± 0.4 nM (slope $\pm$ 1 SE, df = 10). Protein was determined using the Bradford dye binding assay in 1 ml homogenization buffer (Bradford, 1976).

Inhibition of 10 nM ^{3}H-17ß-estradiol (^{3}H-E2) binding to hER was measured by incubation at 4°C for 2 hr. of 40 pM hER in 1 ml Assay Buffer (10 mM Tris HCl, pH 7.5, 1.5 mM Na_2EDTA, 1 mM dithiothreitol, and 10% v:v glycerol). Duplicate analyses were conducted at four half-decadal concentrations ranging from 70 to 0.1 μM for OHCB1-9,12-13 and 3 decadal concentrations from 10 to 0.01 μM for OHCB10-11. Non-specific binding of ^{3}H-E2 was estimated by adding a 1000 fold excess of diethylstilbestrol to separate duplicate tubes and was used to define the 0% binding level. Total binding of ^{3}H-E2 was estimated in the absence of competitor and was used to define the 100% binding level. Final ethanol concentration in the assay was

1%. ^{3}H-E2 bound to hER was extracted from the assay solution by addition of 300 μl of 0.16% (w:v) HAP slurry that was gently mixed periodically over the 15 min adsorption period. The HAP-bound ^{3}H-E2/hER was filtered onto a 24 mm Whatman GF-C disk in a 10-place stainless steel vacuum manifold at 4°C and washed six times with 5 ml assay buffer to remove unbound ^{3}H-E2. The filter and HAP were placed directly into liquid scintillation vials, to which 1 ml ethanol and then 6 ml aqueous scintillation cocktail were added. ^{3}H-E2 was quantitated by 10 min counts of the full ^{3}H window and calibrated using the standard addition technique. The $\log_{10}$IC50 ($\log_{10}$ transformed concentration of inhibitor causing a 50% reduction in specific binding of ^{3}H-E2) was used as a measure of hER binding affinity and fitted to a normal distribution that was calculated using a non-linear regression technique (Bruce and Versteeg, 1992).

Luciferase Expression Assay

Functional estrogen agonism/antagonism of the OHCBs was determined using a stably-transfected MCF7 cell line (MCF7-LUC, a gift of Prof. M. Pons, INSERM, MontPelier, France) expressing an estrogen-responsive reporter gene construct--pVit-tk-luc (Pons, et al. 1990). This plasmid consists of the *Xenopus laevis* vitellogenin A2 promoter region (containing estrogen responsive elements) upstream of the *Herpes simplex* virus thymidine kinase promoter controlling expression of the firefly luciferase gene. MCF7-LUC cells maintained in 10% FBS medium were transferred to 96 well culture plates at a density of

10,000 cells/well. After 24 hr, the medium was replaced with 125 μl 5% DCCFBS medium. At the beginning of the exposure period, cells were at 85% confluence. Forty eight hours after plating, cells were exposed to the OHCBs dissolved in 5% DCCFBS medium with a final ethanol concentration of 0.5%. Four concentrations were tested in duplicate such that the concentrations were 20%, 10%, and 2% of the highest concentrations tested. The greatest concentrations tested were: OHCB6 37 μM; OHCB8, OHCB9 30 μM; OHCB1, OHCB2, OHCB3, OHCB5, OHCB7 29μM; OHCB4 27 μM; and OHCB10, OHCB11, OHCB12, OHCB13 25 μM. Control wells (four per plate) receiving no solvent and solvent control wells (four per plate) receiving 0.5% ethanol were also tested. Two plates were required to test all OHCBs. Typical *in vitro* estrogenicity assays, including the present study, have used charcoal-stripped medium to reduce background response to estrogens (Pons, et al., 1990; Soto, et al., 1991). To fully assess the agonistic/antagonistic properties of the OHCBs, the response of MCF7-LUC cells at higher E2 concentrations was also determined. Therefore, two additional complete experiments were conducted using 5% DCCFBS medium supplemented with E2 at 0.1 nM and 1.0 nM, similar to concentrations found in human plasma (Becker, 1990). The concentration of E2 in unsupplemented 5% DCCFBS medium was 0.0009 nM.

Luciferase expression of the chemically-lysed cells (Promega Corp, Madison, WI) was measured using a Dynatech ML3000 plate reading luminometer (Dynatech Laboratories, Chantilly, VA) and calibrated to standard solutions of firefly luciferase dissolved in lysis buffer supplemented with 5

mg/ml bovine serum albumin. Method detection limit was 0.014 pg luciferase with a relative standard deviation of 5.1% (20 μl lysate sample volume) Luciferase activity was normalized to soluble protein by assaying 10 μl lysate in 1 ml concentrated Bradford reagent to overcome interference by detergent components in the lysis buffer.

Cell Viability Assay

It has been reported that OHCBs are uncouplers of oxidative phosphorylation in vitro (Narasimham, et al. 1991). Therefore, the potential for cytotoxicity was considered significant and required the monitoring of cell viability. In addition to visual inspection of cell morphology by light microscopy (100x magnification), a cell viability assay kit (Molecular Probes Inc. Eugene, OR) was employed utilizing the selective uptake of fluorogenic substrate to quantify "viable" and "non-viable" cells. Viable cell activity was measured as non-specific esterase activity by the selective uptake and subsequent enzymatic cleavage of calcein AM. Production of the fluorescent product was measured using a plate scanning fluorometer (Cytofluor 2300, Perseptive Biosystems, Framingham, MA) with excitation/emission at 485/530 nm. Non-viable cells were measured simultaneously by uptake through damaged cell membranes and binding to DNA of ethidium homodimer, producing a fluorescent complex with excitation/emission at 530/645 nm. A cell viability index (Equation 1) was calculated to normalize for differences in the total number of cells in each well

and also for the effects of E2 treatment which caused increased cell proliferation:

$$\textit{Cell Viability Index} = \frac{\textit{Viable Cell Fluorescence}}{\textit{Nonviable Cell Fluorescence}} \quad (1)$$

where: fluorescence values were untransformed relative fluorescence units.

H4IIE EROD Induction Assay

Aryl hydrocarbon receptor agonists, including some non-ortho-substituted PCBs, have been reported to elicit a response resembling anti-estrogenicity in MCF-7 cells (Krishnan and Safe, 1993). Therefore, a subset of OHCBs (OHCB1, OHCB3, OHCB5, OHCB6, and OHCB8) exemplifying several structural features, was selected for assay of aryl hydrocarbon receptor agonism in the H4IIE rat hepatoma EROD (ethoxyresorufin-O-deethylase) induction assay (Sanderson, et al. 1996).

Statistical Analyses

Statistical analyses were conducted using the Statistical Analysis System Version 6.03 (SAS® Institute, 1988). Luciferase expression response curves were modeled without transformation after a normal distribution fitted by a non-

linear regression technique (Bruce and Versteeg, 1992). For inhibition of luciferase expression, a two parameter model (parameters in bold) was used (Equation 2):

$$Luc = Lucmax \times P\left[\frac{\mathbf{log(EC50)} - \log(OHCB)}{\mathbf{SLOPE}}\right] \quad (2)$$

where: Luc = luciferase expression (ng/mg protein);

log(OHCB) = $\log_{10}$ OHCB concentration, nM;

P[ ] = normal probability density function;

Lucmax = maximum luciferase expression, mean of solvent control response for each E2 concentration;

log(EC50) = $\log_{10}$ Effective Concentration causing 50% inhibition of luciferase expression, nM;

SLOPE = slope function (standard deviate) of normal probability density function.

Log(EC50) was used as a measure of potency such that greater values reflect lesser potency. SLOPE was an estimate of dispersion and described the steepness of response such that lower values describe steeper curves. To aid convergence of the nonlinear regression, SLOPE was bounded, $0.2 \leq SLOPE \leq 3.5$. To estimate the OHCB threshold for effects concentration, the log(EC20) was calculated using a modification of Equation 2 (Equation 3):

$$Luc = Lucmax \times P\left[\frac{\mathbf{log(EC20)} - \log(OHCB)}{\mathbf{SLOPE}} + Z_{20}\right] \quad (3)$$

where: **log(EC20)** $= \log_{10}$ Effective Concentration causing 20% inhibition of luciferase expression, nM;

Z_{20} = 0.8416 = normal deviate above which 20% of the standard normal distribution lies;

and all other variables are as previously described.

For induction of luciferase expression, an additional constant was added to define the basal expression level (Equation 4):

$$Luc = (Lucmax - Lucmin) \times P\left[\frac{\log(OHCB) - \log(EC50)}{SLOPE}\right] + Lucmin \qquad (4)$$

where: Lucmin = mean luciferase expression of solvent controls at 0.0009 nM E2;

and all other variables as previously described.

Log(EC20) was used to estimate the luciferase induction threshold for effects concentration of OHCB (Equation 5):

$$Luc = (Lucmax - Lucmin) \times P\left[\frac{\log(OHCB) - \log(EC20)}{SLOPE} - Z_{20}\right] + Lucmin \qquad (5)$$

where: all variables are as previously described.

Calculation of hER binding potency used an inhibition model similar to the luciferase expression model (Equation 6):

$$Bound = Bmax \times P\left[\frac{\log(IC50) - \log(OHCB)}{SLOPE}\right] \qquad (6)$$

where: Bound = ^{3}H-E2 specifically bound to hER as a percent of total specifically bound;

log(IC50) = $\log_{10}$ Inhibitory Concentration causing 50% decrease in specifically bound ^{3}H-E2;

and all other variables as previously described.

Results and Discussion

Human Estrogen Receptor Binding

All OHCBs tested in this study caused measurable reduction in specific binding of ^{3}H-E2 to hER *in vitro* (Table 2). OHCB7 and OHCB8 weakly inhibited binding of ^{3}H-E2 and, therefore, had log(IC50)s extrapolated beyond their solubility limits which were estimated empirically to be 50 μM (log(nM) = 4.7) by the formation of a visible precipitate in the assay medium. The log(IC50)s for the remaining OHCBs ranged from 4.3 to 2.5 log(nM) and when compared to E2 exhibited a relative binding inhibition (RBI) range of 0.079-5.0%. The two most important structural measures that predicted inhibition of binding to hER were: 1) substitution position of the hydroxyl group, and; 2) minimum energy torsion angle of the central biphenyl bond. Steric hindrance of the hydroxyl group did not diminish binding potency when OHCBs with similar hydroxyl positions were compared. For example, OHCB11 possesses an unhindered para-substituted hydroxyl with a binding potency of 2.5% with

Table 2. Summary of MCF-7-LUC cellular responses to OHCBs.

					Luciferase Expression									
					Log(EC20) (Log(nM) (ASE)			Log(EC50) (Log(nM) (ASE)			Slope[a] (ASE)			
		hER Binding			17ß-Estradiol (nM)			17ß-Estradiol (nM)			17ß-Estradiol (nM)			Cyto-toxicity
ID	Formula	Log(IC50) (Log(nM) (ASE[a])	Slope (ASE)	RBI_{IC50}[b] (%)	0.0009	0.1	1	0.0009	0.1	1	0.0009	0.1	1	Viability Index(SE)
OHCB1	3-OH-2,3',4,4',5-Cl_5-BP	3.3 (0.2)	1.4 (0.4)	1.0	3.2 (0.4)	3.7 (0.6)	4.5 (-)	3.9 (0.2)	4.3 (0.5)	4.6 (-)	-0.9 (0.4)	-0.7 (0.7)	-0.2 (-)	1.137 (0.024)
OHCB2	4-OH-2,3,3',4',5-Cl_5-BP	3.3 (0.2)	1.0 (0.3)	1.0	3.9 (-)	3.8 (0.3)	>4.5	>4.5	4.0 (0.2)	>4.5	-3.5 (-)	-0.2 (2.2)	NA[d]	1.072* (0.026)
OHCB3	3-OH-2',3',4,4',5-Cl_5-BP	3.0 (1.2)	2.7 (4.2)	2.0	-0.03 (1.3)	3.3 (1.3)	>4.5	1.7 (0.6)	>4.5	>4.5	-2.1 (0.9)	-2.2 (7.4)	NA	0.774** (0.025)
OHCB4	4-OH-2',3,3',4',5, 5'-Cl_6-BP	2.8 (-)	3.5 (-)	3.2	-2.7 (-)	3.6 (-)	>4.4	0.3 (-)	3.8 (-)	>4.4	-3.5 (-)	-0.2 (-)	NA	1.036** (0.029)
OHCB5	4-OH-2',3,4',5,5'-Cl_5-BP	3.4 (0.1)	0.9 (0.3)	0.8	2.0 (0.5)	4.3 (-)	>4.7	3.1 (0.2)	4.5 (0.8)	>4.7	-1.4 (0.5)	-0.2 (0.2)	NA	1.082 (0.061)
OHCB6	4-OH-3,3',4'-Cl_3-BP	3.8 (0.1)	0.7 (0.2)	0.3	1.3 (1.9)	>4.6	>4.6	2.6 (0.7)	>4.6	>4.6	-1.6 (1.4)	NA	NA	0.989** (0.026)
OHCB7	2-OH-2',3,3',4,4'-Cl_5-BP	5.7 (0.2)	3.5 (-)	0.004	-2.5 (6.6)	>4.5	4.3 (5.4)	0.4 (3.3)	>4.5	>4.5	-3.4 (3.9)	NA	-0.7 (6.5)	0.626** (0.027)
OHCB8	4-OH-3,3',4',5,5'-Cl_5-BP	5.1 (1.8)	0.9 (1.4)	0.02	-1.0 (7.6)	>4.5	>4.5	1.6 (3.1)	>4.5	>4.5	-3.1 (5.4)	NA	NA	0.913** (0.032)
OHCB9	4-OH-2',3,3',4',5-Cl_5-BP	4.2 (-)	0.2 (-)	0.1	-0.7 (-)	>4.7	>4.7	2.2 (-)	>4.7	>4.7	-3.5 (-)	NA	NA	1.133 (0.023)
OHCB10	4-OH-2',4',6'-Cl_3-BP	2.5 (1.2)	2.8 (3.8)	6.3	3.9 (0.4)	2.8 (-)	2.4 (-)	4.2 (0.2)	>4.7	>4.7	+0.4 (0.3)	-3.5 (-)	-3.5 (-)	1.106 (0.024)
OHCB11	4-OH-2',3',4',5'-Cl_4-BP	2.8 (0.6)	2.3 (1.8)	3.2	4.4 (-)	2.0 (2.3)	2.1 (0.6)	4.6 (-)	>4.7	3.8 (0.2)	+0.2 (0.03)	-3.4 (4.5	-2.1 (0.8)	1.109 (0.018)
OHCB12	3-OH-2',3',4',5'-Cl_4-BP	4.3 (0.7)	0.8 (1.4)	0.1	2.6 (0.7)	2.3 (1.5)	2.2 (-)	3.6 (0.3)	4.1 (1.2)	>4.4	-1.2 (0.9)	-2.2 (2.9)	-3.5 (-)	1.026** (0.026)
OHCB13	4,4'-$(OH)_2$-3,3',5,5'-Cl_4-BP	3.7 (0.3)	0.4 (0.5)	0.4	3.2 (0.8)	1.3 (-)	-0.03 (-)	>4.7	4.3 (-)	2.9 (-)	-2.2 (2.2)	-3.5 (-)	-3.5 (-)	1.224 (0.020)
E2	17ß-Estradiol	1.3 (0.8)	1.8 (1.5)	100		-3.9 (0.4)			-2.8 (0.3)			+1.2 (0.7)		1.156 (0.016)

[a]ASE: Asymptotic Standard Error; (-) = ASE inestimable, Jacobian matrix singular.
[b]RBI_{IC50}: Relative Binding Inhibition for IC50 = ($IC50_{E2}$/$IC50_{OHCB}$)100%.
[c]Sign of slope: + = Induction of luciferase expression, - = Inhibition of luciferase expression.
[d]NA: Not Active.
*p<0.05; **p<0.01, Student's t-test comparison with E2.

respect to E2. In comparison, OHCB4, with a structure similar to OHCB11 but with the addition of two Cl's adjacent to the hydroxyl, has a relative binding potency of 4.0% with respect to E2. The effect of minimum energy torsion angle is exemplified by comparison of OHCB4 and OHCB8. OHCB8, similar to OHCB4 except that OHCB8 lacked an ortho-substituted Cl and could assume more planar configuration than OHCB4, had a relative binding potency of 0.01%. Subsitution position of the hydroxyl group strongly affected hER

binding affinity. For example, meta-hydroxylated OHCB12 exhibited a log(IC50) of 4.3 log(nM) (RBI = 0.1%) compared to its para-hydroxylated isomer OHCB11 with a log(IC50) of 2.8 log(nM) (RBI = 3.2%).

Cell Viability

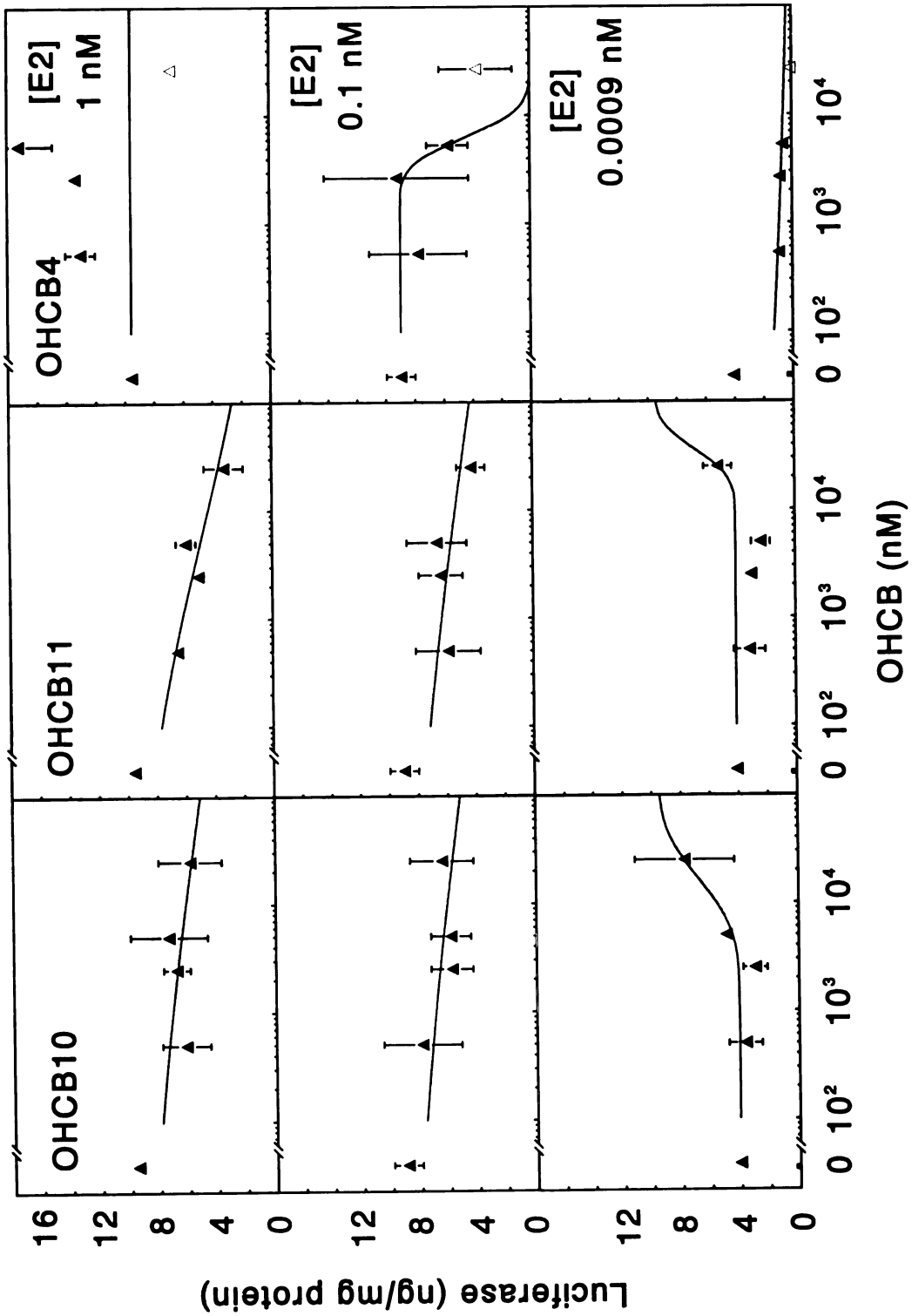
The cell viability index was significantly reduced by OHCB2, OHCB3, OHCB4, OHCB6, OHCB7, OHCB8, and OHCB11 at the highest concentrations tested (Table 2). No significant changes in the cell viability index occurred at lower OHCB concentrations (data not shown). Visible changes in cell morphology, including vacuolization, lysis and detachment were evident at cell viability indices less than 1.030. Visible, and therefore severe, cytotoxicity was caused by OHCB3, OHCB6, OHCB7, OHCB8, and OHCB12.

Effects of E2 and OHCBs on Luciferase Expression

Luciferase expression was induced 2.2 fold in MCF7-LUC cells by 0.1 nM E2 over the basal expression in the presence of 0.0009 nM E2, but only 2.3 fold by 1 nM E2 indicating near maximum induction was achieved at 1 nM (Figure 3; luciferase ng/mg protein at 0.0009, 0.1, and 1 nM E2; 4.09 (SE = 0.31, n = 32), 8.96 (SE = 1.00, n = 8), 9.55 (SE = 0.57, n = 8), respectively). EC50 for E2 induction of luciferase in the absence of OHCB was 0.0015 nM(log(EC50) = -2.81, asymptotic standard error = 0.28, n = 48).

OHCBs tested in this study were "estrogenic" (induced luciferase expression), "anti-estrogenic" (reduced luciferase expression) or inactive (did not affect luciferase expression) depending upon the concentration of E2 co-administered or present in the medium. Cytotoxicity confounded the interpretation of anti-estrogenicity, because reductions in luciferase expression could have been caused by reduced cell viability as well as inhibition of hER-mediated transcription. Therefore, OHCB concentrations that caused statistically significant reductions in the cell viability index were eliminated from the calculation of log(EC50) and log(EC20) values (Figure 3). An upper concentration limit of potential activity was defined for each OHCB as either the limit of solubility or the concentration causing statistically significant cytotoxicity. Predicted log(EC50) and log(EC20) values that fell above theupper concentration limit were labelled "Not Active" (Table 2). In general, E2 co-administration reduced the potency of the "anti-estrogenic" OHCBs, as reflected in . increasing threshold (log(EC20)) and mid-range (log(EC50)) effect concentrations (Table 2). The "anti-estrogenic" OHCBs that followed this pattern were OHCB1-9 and OHCB12 (Table 2, Figure 4A-I, L). OHCB13 was an exception in eliciting reductions in luciferase expression at lower OHCB concentrations as E2 concentration increased (Table 2, Figure 4M). OHCB10 and OHCB11 caused greater luciferase expression in the presence of 0.0009 nM E2 (Figure 3) but lesser luciferase expression at 0.1 nM E2 and 1 nM E2. Thus, OHCB10 and OHCB11 were "partial estrogens" because the inductive response at 0.0009 nM E2 was reversed at 0.1 nM E2 and 1 nM E2.

Figure 3. Representative luciferase expression of MCF-7-LUC cells exposed to OHCBs and E2 in the assay medium: OHCB4: 4-OH-2′,3,3′,4′,5,5′-Cl_6-biphenyl; OHCB10, 4-OH-2′,4′,6′-Cl_3-biphenyl; OHCB11 4-OH-2′,3′,4′,5′-Cl_4-biphenyl; E2 17ß-estradiol; (▲) ng luciferase/mg protein, n = 2; (△) ng luciferase/mg protein, points omitted from calculation of log(EC50) and log(EC20) due to cytotoxicity; symbol and bars indicate mean ± SE.


OHCB10
OHCB11
OHCB4
[E2]
1 nM
[E2]
0.1 nM
[E2]
0.0009 nM
Luciferase (ng/mg protein)
OHCB (nM)
0
4
8
12
16
0 10²
10³
10⁴

In order to estimate the potency of the OHCBs, an integrated measure of activity was calculated to take into account the effect of E2 concentration on OHCB activity. Typically, potency is estimated by the value of the EC50, or some similar effective concentration. However, because OHCB potency varied with E2 concentration, the overall potency of the OHCB was estimated by calculation of the area of activity defined as the area bordered by the highest and lowest E2 concentrations on the abscissa and on the ordinate by the curves defined by the effective concentrations (both EC20 and EC50) and the upper solubility or cytotoxicity concentration limits (Figures 4A-M). The total area of activity, defined by the log(EC20) response curve (delineating the greater than 20% effective area), and the strong activity area, defined by the log(EC50) response curve (delineating the greater than 50% effective area), were calculated (Table 3; see shaded areas on Figures 4A-M). For OHCB10 and OHCB11, the concentration of E2 at which the response changed from induction to reduction was estimated by graphical interpolation (Figures 4J-K). It was assumed that at the "cross-over" E2 concentration, the net OHCB effect on luciferase expression was nullified.

Figure 4. Effect of 17ß-estradiol (E2) co-exposure on luciferase expression response to OHCBs: (■) EC50, OHCB Effective Concentration causing a 50% reduction (or induction) of luciferase activity with respect to the solvent control response; (●) EC20, OHCB Effective Concentration causing a 20% reduction (or induction) of luciferase activity with respect to the solvent control response; (----) Relevant limits of concentration of OHCBs and E2 were determined for the calculation of the "area of activity" such that the upper limit of OHCB concentration defined as either the maximum soluble concentration (applied to OHCB1,5,9,10,11,13) or the cytotoxic concentration (OHCB2,3,4,6,7,8,12); E2 limits defined by highest (1 nM) and lowest concentrations tested (0.0009 nM); (NA) Not active, predicted EC50 or EC20 greater than upper OHCB concentration limit; (Light Gray Shading) Area of weak activity depicting an expected response of 20% to 50%; (Dark Gray Shading) Area of strong activity depicting an expected response of greater than 50%. All OHCB EC50s and EC20s depict reduction (E2 antagonism) of luciferase expression except for OHCB10 and OHCB11 that caused induction (E2 agonism) of luciferase expression at 0.0009 nM E2; (A) OHCB1, 3-OH-2,3′,4,4′,5-Cl_5-biphenyl; (B) OHCB2, 4-OH-2,3,3′,4′,5-Cl_5-biphenyl; (C) OHCB3, 3-OH-2′,3′,4,4′,5-Cl_5-biphenyl; (D) OHCB4, 4-OH-2′,3,3′,4′,5,5′-Cl_6-biphenyl; (E) OHCB5, 4-OH-2′,3,4′,5,5′-Cl_5-biphenyl; (F) OHCB6, 4-OH-3,3′4,′-Cl_3-biphenyl; (G) OHCB7, 2-OH-2′,3,3′,4,4′-Cl_5-biphenyl; (H) OHCB8, 4-OH-3,3′,4′,5,5′-Cl_5-biphenyl; (I) OHCB9, 4-OH-2′,3,3′,4′,5-Cl_5-biphenyl; (J) OHCB10, 4-OH-2′,4′,6′-Cl_3-biphenyl; (K) OHCB11, 4-OH-2′,3′, 4′,5′-Cl_5-biphenyl; (L) OHCB12, 3-OH-2′,3′,4′,5′-Cl_5-biphenyl; (M) OHCB13, 4,4′-$(OH)_2$-3,3′,5,5′-Cl_4-biphenyl.

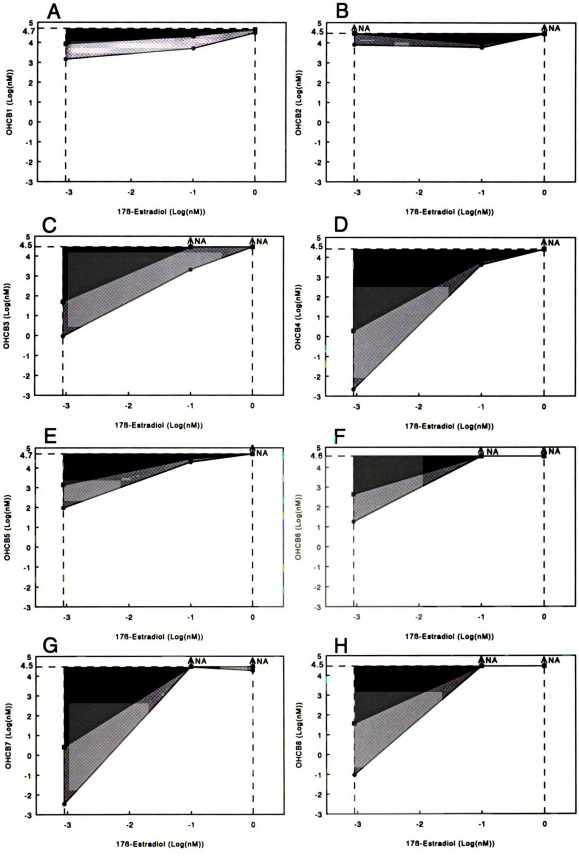
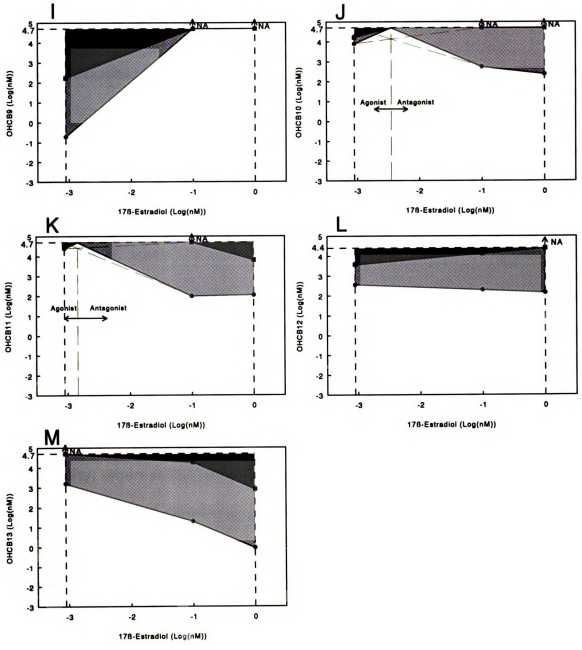

A
B
C
D
E
F
G
H
OHCB1 (Log(nM))
OHCB2 (Log(nM))
OHCB3 (Log(nM))
OHCB4 (Log(nM))
OHCB5 (Log(nM))
OHCB6 (Log(nM))
OHCB7 (Log(nM))
OHCB8 (Log(nM))
17ß-Estradiol (Log(nM))
NA

Figure 4. (Cont'd).


I
J
K
L
M
NA
Agonist
Antagonist
OHCB8 (Log(nM))
OHCB10 (Log(nM))
OHCB11 (Log(nM))
OHCB12 (Log(nM))
OHCB13 (Log(nM))
17ß-Estradiol (Log(nM))

Mechanisms of activity and relationship with chemical structure

Partial Estrogenic Activity

OHCB10 and OHCB11 exhibited partial estrogenic activity in this experiment, consistent with the observations of estrogenic activity in a rodent uterotrophic assay (Korach, et al. 1988) and turtle sexual development assay (Bergeron, et al. 1994). There appeared to be quite specific structural features that conferred partial estrogenic potential to these OHCBs. For example, a para-substituted hydroxyl was essential for partial estrogenic activity. Meta-substitution of the hydroxyl led to the anti-estrogenic activity of OHCB12 while the para-substituted structural isomer, OHCB11, exhibited partial estrogenic activity. Meta-substitution of the hydroxyl in OHCB12 also conferred cytotoxicity, whereas OHCB11 was not cytotoxic. Another important feature was the presence of chlorine atoms vicinal to the para-substituted hydroxyl. OHCB4 possessed two Cl atoms vicinal to the para-hydroxyl group and was strongly anti-estrogenic, while the structurally similar but not vicinally Cl-substituted OHCB11 exhibited partial estrogenic activity. Vicinal Cl substitution may have two effects on OHCB activity: 1) steric hindrance of a hydrogen bonding moeity on the estrogen receptor that bonds to the ligand hydroxyl; 2) reduction of the electron density on the hydroxyl oxygen by Cl -mediated electron withdrawal through the aromatic ring, with concomitant decrease in acid dissociation constant, pK_a (Waller, et al. 1995). Waller, et al. (1995) estimated pK_a of vicinal Cl substituted OHCBs in the range of 5.8 to 6.7, which

Table 3. Potency of anti-estrogenicity/estrogenicity of OHCBs estimated by the area of activity.

ID	Formula	Type of Response	Area[a] (Log(nM)^2)			Overlap Area[b] (Log(nM)^2)		Margin-of-Safety Area[c] (Log(nM)^2)
			Strong[d] Activity	Weak[e] Activity	Total[f] Activity	Strong Activity	Weak Activity	
OHCB1	3-OH-2,3′,4,4′,5-Cl_5-BP	Reduction	1.37	1.81	3.18	0	0	6.86
OHCB2	4-OH-2,3,3′,4′,5-Cl_5-BP	Reduction	0.79	0.83	1.62	0	0	7.51
OHCB3	3-OH-2′,3′,4,4′,5-Cl_5-BP	Reduction	2.84	3.53	6.37	0	0	3.76
OHCB4	4-OH-2′,3,3′,4′,5,5′-Cl_6-BP	Reduction	5.19	3.27	8.46	0	0	3.63
OHCB5	4-OH-2′,3,4′,5,5′-Cl_5-BP	Reduction	1.97	1.46	3.43	0	0	6.72
OHCB6	4-OH-3,3′,4′-Cl_3-BP	Reduction	1.98	1.41	3.39	0	0	6.46
OHCB7	2-OH-2′,3,3′,4,4′-Cl_5-BP	Reduction	4.16	3.05	7.21	0	0	4.57
OHCB8	4-OH-3,3′,4′,5,5′-Cl_5-BP	Reduction	2.97	2.65	5.62	0	0	5.12
OHCB9	4-OH-2′,3,3′,4′,5-Cl_5-BP	Reduction	2.53	3.01	5.54	0	0	5.28
OHCB10	4-OH-2′,4′,6′-Cl_3-BP	Induction	0.14	0.10	0.24	0	0	9.62
		Reduction	0.00	3.55	3.55	0	0	6.27
OHCB11	4-OH-2′,3′,4′,5′-Cl_4-BP	Induction	0.02	0.02	0.04	0	0	9.78
		Reduction	0.89	4.22	5.11	0	0	5.05
OHCB12	3-OH-2′,3′,4′,5′-Cl_4-BP	Reduction	1.27	4.94	6.21	0	0	2.74
OHCB13	4,4′-$(OH)_2$-3,3′,5,5′-Cl_4-BP	Reduction	1.56	7.51	9.07	0	0.69[g]	1.73,0[g]

[a]Area: Calculated from plot of OHCB log(EC50) and log(EC20) vs. log(E2) (See Figures 4A-2M).
[b]Overlap Area: Overlap between area of *in vitro* effects and area of possible *in vivo* concentrations of OHCBs and E2 (See Figure 6).
[c]Margin-of-Safety Area: Extent of non-overlap between area of *in vitro* effects and area of possible *in vivo* concentrations of OHCBs and E2 (See Figure 6.)
[d]Strong activity: Strong activity area defined as the area bounded by log(E2) = -3.05 to 0.00 and by log(OHCB) = log(EC50) to log(solubility) or log(cytotoxic concentration). The strong activity area represents the combinations of log(OHCB) and log(E2) that result in a response (either inhibition or induction of luciferase expression) greater than 50% of solvent control responses.
[e]Weak activity area defined as the area bounded by log(E2) = -3.05 to 0.00 and by log(OHCB) = log(EC20) to upper bound, where the upper bound was either the log(EC50) or the log(solubility)/log(cytotoxic concentration). The weak activity area represents the combinations of log(OHCB) and log(E2) that result in a response ranging from threshold (20%) to mid-range (50%).
[f]Total activity = Strong activity area + Weak activity area. The total activity area represents the combinations of log(OHCB) and log(E2) that result in a response greater than the threshold (20%).
[g]For E2 < 0.1 nM, there was no overlap area, for E2 > 0.1 the margin-of-safety area was zero.

would lead to 83.4 to 98.8% ionization of the hydroxyl group in assay medium at a pH of 7.4. In contrast, OHCBs without vicinal Cl substitution were estimated to exhibit pK_a values of 9.5 to 10, which would result in ionization

of 0.79 to 0.25% (Waller, et al. 1995). Exactly how a reduction in pK_a could result in anti-estrogenic activity is unknown. Total inductive potency (greater than 20% inductive effect) and strong inductive potency (greater than 50% inductive effect) appeared to be inversely related to hER binding log(IC50) (Figure 5), such that lower hER binding log(IC50) (hence greater hER binding affinity) was related with greater inductive potency. Such a relationship was expected for a receptor-mediated mechanism of action (VanderKuur, et al. 1993a). However, the relationship between the anti-estrogenic potency of OHCB10 and OHCB11 with hER binding log(IC50) was reversed (Table 3), possibly because the more estrogenic OHCB10 caused a less inhibitory alteration of the E2 activated receptor complex, which could take on the form of a heterodimer containing both E2 and OHCB10 ligands (Klinge, et al. 1992).

Anti-estrogenic Activity

If the activity of the solely anti-estrogenic OHCBs (all but OHCB10 and OHCB11) were mediated by competitive binding to the hER and displacement of E2, then it would have been expected that anti-estrogenic potency was related to hER binding log(IC50). In fact, there was no significant relationship between hER binding log(IC50) and either total (>20% reduction) reductive area or strong (>50% reduction) reductive area (Figures 6A and 6B, respectively). Rather, a better predictor of both total reductive potency and strong reductive potency was the mean viability index of the highest OHCB

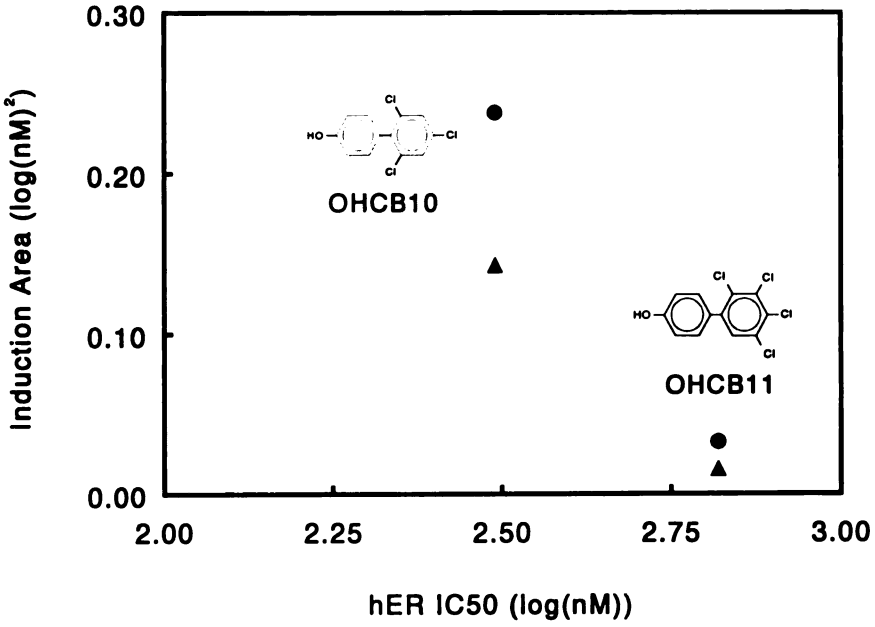


Figure 5. Relationship between OHCB inductive (E2 agonist) activity area and human estrogen receptor (hER) binding affinity represented by IC50, the OHCB Inhibitory Concentration causing a 50% decrease in specific binding of ^{3}H-E2 to hER *in vitro*: (●) Total induction area (response greater than 20% with respect to the solvent controls); (▲) Strong induction area (response greater than 50% with respect to the solvent controls). See Figure 4 for calculation of the inductive activity area.

concentrations tested (Figures 7A and 7B). The mean viability index explained 63% ($r^2 = 0.633$, $p < 0.006$) of the variation in strong reductive potency after the one outlier, OHCB4, was omitted from the regression. The mean viability index explained 46% ($r^2 = 0.456$, $p < 0.046$) of the variation in total reductive potency after the two outliers, OHCB4 and OHCB13, were omitted from the regression. The relationship between reductive potency and the viability index was observed even after the cytotoxic OHCB concentrations were omitted from the calculation of log(EC50) and log(EC20), suggesting that there were

sublethal effects caused by the OHCBs that were not detected by the cell viability assay. These sublethal effects may have also been responsible for the observed reductions in luciferase expression, though the nature of these sublethal effects remains unknown. It was observed, however, that the administration of E2 reversed the reductive effects of the OHCBs, leading to the following possibility: E2 administration reversed the sublethal effects of the OHCBs by a non-hER-mediated mechanism (since reductive potency was not related to hER binding affinity). Therefore, although it may be appropriate to describe the activity of OHCBs 1-3,5-9,12 as "anti-estrogenic", it should be noted that the "anti-estrogenicity" was not related to hER binding affinity. It appears to be unlikely that the OHCBs exhibited "anti-estrogenicity" as a result of aryl hydrocarbon receptor agonism because all OHCBs tested were inactive in the H4IIE EROD induction assay (data not shown).

There were two OHCBs that caused reductions in luciferase expression that were not explained by reductions in the cell viability index. OHCB4 exhibited strong (>50% reduction) reductive potency at a level much greater than predicted by its slight reduction in cell viability (Figure 7A). And, both OHCB4 and OHCB13 exhibited total (>20% reduction) reductive potency at levels greater than predicted from the cell viability index. These observations suggest that OHCB4 and OHCB13 exerted a reductive effect on luciferase expression by a mechanism other than sublethal cytotoxic inhibition of luciferase expression. OHCB4 bound to hER with the same relatively strong affinity as OHCB11, a partial estrogen. Also, OHCB4 and OHCB11 are

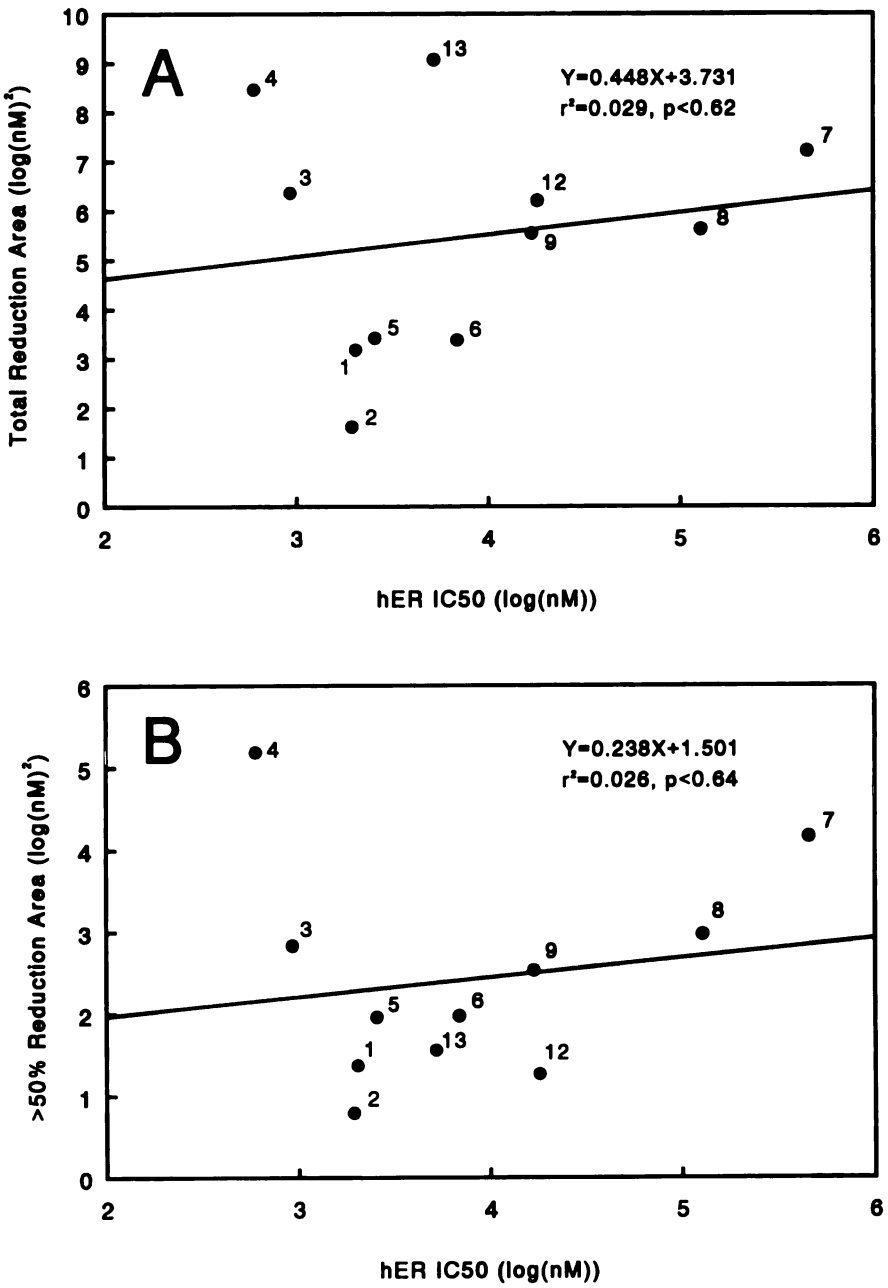


Figure 6. Relationship between OHCB reductive (E2 antagonist) activity area and human estrogen receptor (hER) binding affinity represented by IC50, the OHCB Inhibitory Concentration causing a 50% decrease in specific binding of ^{3}H-E2 to hER *in vitro*: (Number label) Indicates OHCB identification number from Table 1; (A) Total reductive activity area (response greater than 20% with respect to the solvent controls); (B) Strong reductive activity area (response greater than 50% with respect to solvent controls). See Figure 4 for calculation of reductive activity area. p = probability that the slope of the regression line is equal to zero, $\alpha = 0.05$.

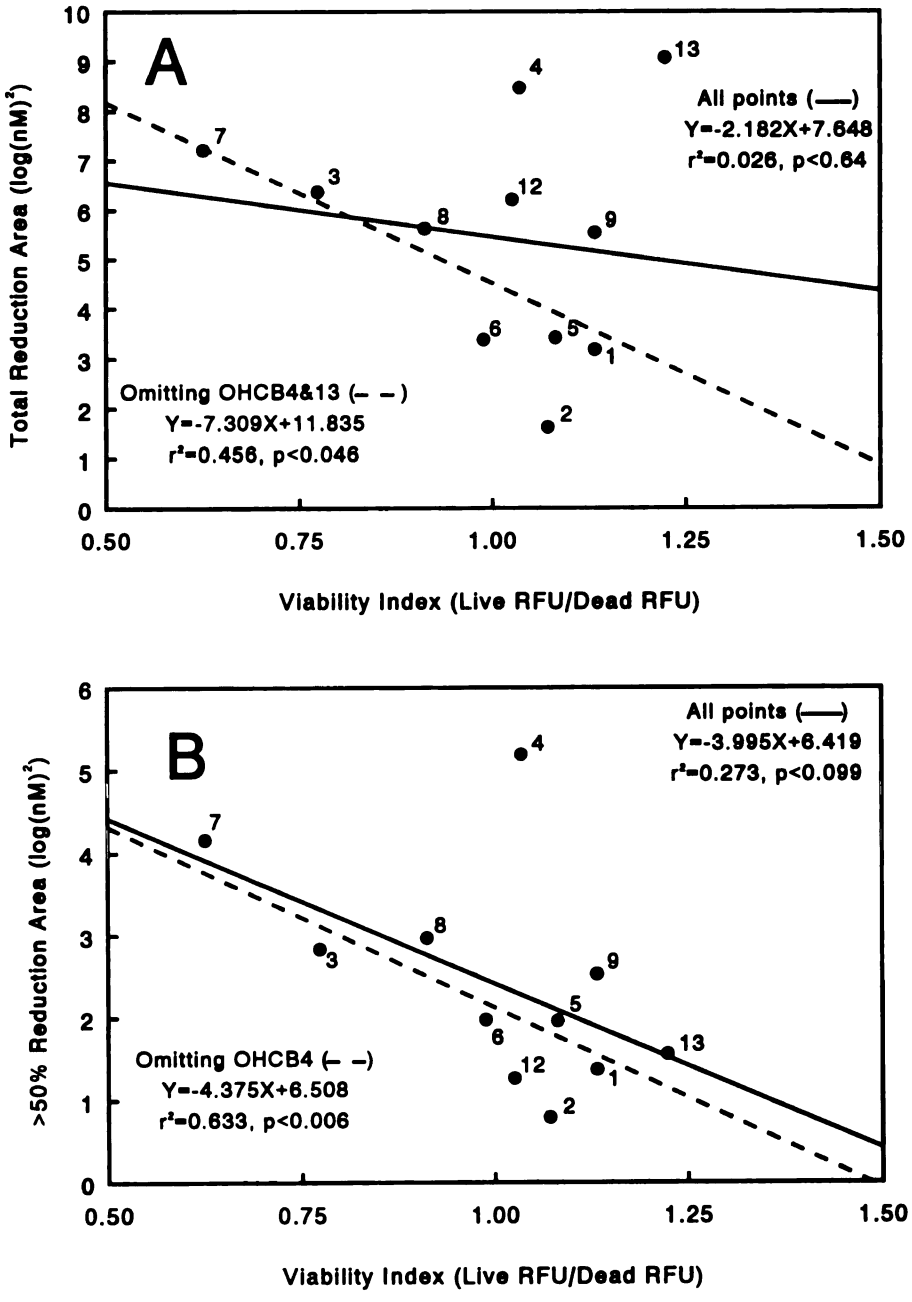


Figure 7. Relationship between OHCB reductive (E2 antagonist) activity area and cell viability index for all OHCBs except OHCB10 and OHCB11 that exhibited partial estrogen agonist activity: (—) All points included in regression; (---) Regression omitting outliers as noted; (A) Total reductive activity area (response greater than 20% with respect to solvent controls); (B) Strong reductive activity area (response greater than 50% with respect to solvent controls). p = probability that the slope of the regression line is equal to zero, $\alpha = 0.05$.

structurally very similar. Taken together, these two observations suggest that OHCB4 may be acting to inhibit luciferase expression by an hER-mediated mechanism. OHCB13 may also be acting via an hER-mediated mechanism, though its reductive activity was enhanced by E2 administration--a response that cannot be explained by a simple competitive inhibition model of E2 binding and activiation of hER. The mechanism of action of OHCB13 and OHCB4 remain to be fully elucidated. However, it may be appropriate to describe their activity as "anti-estrogenic" by an hER-mediated mechanism.

Implications for human risk assessment

The potential for OHCBs to exhibit estrogenic or anti-estrogenic activity in humans can be estimated by comparison of the thresholds for effect with the concentrations of OHCBs observed in human tissues. Because E2 affects the activity of OHCBs, the E2 concentration in the tissue is also a necessary factor to consider. In children of 1 mo to 4 yr of age, plasma E2 concentrations normally range from 0.04 to 0.11 nM. In adult human females, the E2 concentrations vary between 0.18 to 1.5 nM and in males from 0.04 to 0.22 nM (Becker, 1990). Only two of the OHCBs tested in this experiment have been positively identified in human plasma--OHCB2 (4-OH-2,3,3',4',5-Cl_5-BP) and OHCB9 (4-OH-2',3,3',4',5-Cl_5-BP). And, OHCB2 is the only OHCB presently quantitated (Bergman, et al. 1994) in human plasma--5.3 ± 2.9 nmol/L plasma (mean ± SD). Comparing the observed concentration of OHCB2, 5.3 nM, with the threshold effect concentrations--EC20 = 7900 nM at 0.0009 nM

E2, EC20 = 6300 nM at 0.1 nM E2, and EC20 > 32,000 nM at 1 nM E2-- reveals that the observed concentration of OHCB2 is 3 orders of magnitude lower than the threshold for effects concentration at 0.0009 nM E2 and 0.1 nM E2. Thus, OHCB2 can reasonably be expected to not elicit a detectible anti-estrogenic (or estrogenic) effect in humans, provided that the responses *in vivo* can be adequately predicted by the responses *in vitro*. The concentration of OHCB9 in human plasma has not been quantitated. However, a range can be estimated as 10 to 50% of the total PCB concentration (Bergman, et al. 1994). Using the concentration of total PCBs reported by Bergman, et al. (1994), $3.6 \pm 1.6\ \mu g/g$ plasma lipid and assuming a 0.5% lipid fraction in plasma, the concentration of OHCB9 is estimated to range from 5.3 to 26.5 nM. The lowest threshold for anti-estrogenic effects for OHCB9 was observed at 0.0009 nM E2--EC20 = 0.2 nM. Thus, the predicted range of OHCB9 concentrations exceeds the threshold for effects concentration at 0.0009 nM E2. However, at E2 concentrations observed in human adults and children, the anti-estrogenic activity of OHCB9 was completely reversed, suggesting that OHCB9 may be expected to be inactive in adults and children (Figure 8). Using this same range estimate for OHCB plasma concentration, it may be concluded that all OHCBs except OHCB13 would not exhibit detectible anti-estrogenic activity within the range of E2 concentrations observed in children and adults. OHCB13 did exhibit weak anti-estrogenic activity at 1 nM E2. However, OHCB13 has not been identified in human plasma.

Conclusions

As a tool for monitoring the estrogenic/anti-estrogenic activity of environmental contaminants, the luciferase expression assay described in this study holds promise in that the assay: 1) detects estrogenic compounds by a specific receptor-mediated pathway; 2) detects anti-estrogenic compounds by antagonism of co-administered E2; 3) accounts for the effects of cytotoxicity that may confound anti-estrogenic responses, and; 4) utilizes a high-throughput 96 well plate format coupled with high-sensitivity plate-scanning instrumentation. Other assays of estrogenicity have been reported (Soto, et al. 1992, Bergeron, et al. 1994) that were not designed to incorporate all of these attributes. Of particular note, any *in vitro* estrogenicity assay that only utilizes medium stripped of available steroid hormones, while maximizing the sensitivity for detection of weak estrogens, does not adequately assess the effect of physiologically-relevant concentrations of E2 on the activity of those weak estrogens. For example, OHCB10 and OHCB11 were estrogenic when DCCFBS medium (0.0009 nM E2) was used, but were weakly anti-estrogenic at higher E2 concentrations. Thus, the label "estrogen" or "anti-estrogen" should not be applied without testing at multiple levels of E2. The methods of the present assay were based upon the assay developed by Pons, et al. (1990) for detection of estrogenic and anti-estrogenic compounds.

The risk of "hormone disruption" (Schmidt, 1994) presented by environmentally-relevant OHCBs (i.e. those OHCBs detected in human and wildlife samples) appears to be negligible, particularly when the important

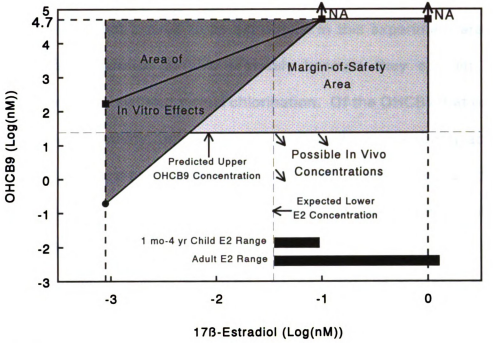


Figure 8. Comparison of the area for *in vitro* reductive (E2 antagonism) effects and the area for possible *in vivo* concentrations defined by the lower limit of average E2 plasma concentrations (nM) in humans (Becker, 1990) and the upper limit of the predicted OHCB concentration (nM) in an adult human plasma sample (OHCB9 for example, Bergman, et al. 1994). Determination of the areas for reductive *in vitro* effects depicted in Figure 4. The predicted OHCB concentration is the estimated upper limit of the range of concentrations of OHCBs in human plasma (10-50% of the total plasma PCB concentration, Bergman, et al. 1994). Overlap of the *in vitro* reductive effects area with the *in vivo* possible concentrations area indicates a condition in which the OHCB could potentially inhibit E2 activity *in vivo*. On the other hand, the extent of non-overlap between the two areas indicates the "margin-of-safety" area (⣿) between the threshold for *in vitro* effects (EC20) line and the possible *in vivo* concentrations area.

effects of physiologically-relevant E2 concentrations are included in the analysis. Hydroxylated PCB metabolites that are selectively retained in plasma do not pose a risk of estrogenic activity because the chemical structural characteristics that favor plasma retention (chlorination vicinal to a para-substituted hydroxyl and a total of 5-7 Cl's per molecule) do not favor

estrogenic activity (para-substituted hydroxyl without vicinal chlorination). Also, the OHCBs that proved to be estrogenic in this experiment are rapidly conjugated and excreted *in vivo* precisely because they contain a para-substituted hydroxyl without vicinal chlorination. Of the OHCBs that exhibited "anti-estrogenic" activity, only two could be described as probably acting via an estrogen receptor mediated pathway, and these two have not been identified in human plasma. The remaining OHCBs exhibited "anti-estrogenicity" that was related to their effect on cell viability, and therefore, cannot be described as exhibiting "hormone disruption" solely by an estrogen receptor mediated mechanism. Furthermore, the "anti-estrogenic" activity of the remaining OHCBs was eliminated by E2 concentrations normally found in both adults with higher E2 concentrations and children with relatively lower E2 concentrations. When the low potential for estrogenic or anti-estrogenic activity present by OHCBs is compared with the relatively strong potential for interfence with thyroxin and vitamin A transport (Brouwer, et al. 1990, Bergman, et al. 1994), further research into the "hormone disruption" caused by OHCBs should probably be focused on the latter.

CHAPTER 2

SPECIFIC BINDING OF HYDROXYLATED PCBs AND OTHER COMPOUNDS TO THE ESTROGEN RECEPTOR: STRUCTURE-ACTIVITY RELATIONSHIPS

Introduction

Estrogen Receptor Agonists and Antagonists

There are four broad classifications of estrogens and anti-estrogens: 1) direct-acting agonists; 2) direct-acting antagonists; 3) indirect-acting agonists, and; 4) indirect-acting antagonists. Direct-acting estrogen agonists competitively bind to the estrogen receptor, displace 17ß-estradiol, activate estrogen receptor dimerization, enhance estrogen-inducible gene expression in target tissues or cell lines (Kumar and Chambon, 1988), and cause characteristic physiological changes in whole animals (Knobil, 1980; Figure 2). Examples of direct-acting estrogens include diethylstilbestrol, coumestrol, and *o,p'*-DDT. In contrast, direct-acting estrogen antagonists also competitively bind to the estrogen receptor, displace 17ß-estradiol, but block receptor function causing down-regulation of estrogen-inducible gene expression (Bondy and Zacharewski, 1993) and inhibition of characteristic physiological changes in whole animals (Korach, et al. 1985). Examples of direct-acting estrogen antagonists include tamoxifen and ICI 164,384. It is important to note that a necessary condition of direct action is the competitive binding of the agonist or antagonist to the estrogen receptor. Also, metabolic activation (the enzymatic

conversion of an inactive "parent" compound to an active "metabolite") may produce direct-acting metabolites (that bind with greater affinity to the estrogen receptor) from parent compounds that do not bind with great affinity to estrogen receptor. For example, methoxychlor does not appreciably bind to the estrogen receptor, Yet, the dihydroxy metabolite of methoxychlor binds with great affinity to the estrogen receptor and is a potent estrogen (Bulger, 1978a). Metabolic activation may be responsible for the estrogenic activity of a diphenylsiloxane (Levier and Jankowiak, 1972). Substances may also exert agonist or antagonist activity through mechanisms that do not include competitive binding to the estrogen receptor. Indirect-acting estrogen agonists do not bind to the estrogen receptor but may mimic estrogen action by blocking androgen action (for example, the anti-androgen *p,p'*-DDE, (Kelce, et al. 1995)) or inhibiting estrogen metabolizing enzymes (for example, chronic ethanol-induced liver damage promotes gynecomastia in alcoholic males (Becker, 1990). Finally, indirect-acting estrogen antagonists do not bind appreciably to the estrogen receptor but may induce estrogen metabolizing enzymes (Standeven, et al. 1994; Spink, et al. 1992; Johnson, et al. 1993; Singh, 1989; Dieringer, et al. 1979) or may block normal estrogen receptor function at the DNA. For example, activated aryl hydrocarbon receptor interferes with ER binding to DNA and down-regulates nuclear ER content in cells (Zacharewski, et al. 1991; Krishnan and Safe, 1993).

Uses and Limitations of ER Binding Assay

Ligand binding is the first step in the chain of events leading to expression of cellular and physiological responses controlled by steroid hormone receptors (Evans, 1988; Kumar and Chambon, 1988). The ability of the estrogen receptor to specifically bind a large variety of molecules is well known and has been extensively studied, including: chlorinated organic compounds (Welch, et al. 1969; Nelson, 1974; Kupfer, 1975; Kupfer and Bulger, 1976; Bulger, et al. 1978a,b; Nelson, et al. 1978; Hammond, et al. 1979; Robison and Stancel, 1982; Korach, et al. 1988; Thomas and Smith, 1993; Chae, et al. 1991, Waller, et al. 1995), polycyclic aromatic hydrocarbons (Mankowitz and Rydstrom, 1982; Chang and Liao, 1987; Pasqualini, et al. 1990; Hwang, et al. 1992), steroid hormone metabolites and derivatives (van Aswegen, et al. 1989; Schutze, et al. 1993; Teutsch, et al. 1988, vanderKur, et al. 1993), non-steroidal pharmaceuticals (Korach, et al. 1985; Miquel and Gilbert, 1988; Korach, et al. 1987; McCague, et al. 1988; Anstead, et al. 1990), natural products of plant, fungal and bacterial origin (Katzenellenbogen, et al. 1979; Whitten, et al. 1992), alkyl phenols (Mueller and Kim, 1978) and other organic compounds (Krishnan, et al. 1993). Although ER binding is essential for direct estrogen agonism or antagonism, receptor binding affinity alone is not sufficient to predict whether a substance will elicit estrogenic or anti-estrogenic activity. Rather, specific molecular structural characteristics of the substance determine the agonistic or antagonistic activity. Although broad classes of compounds have been empirically identified as "anti-estrogenic" or "estrogenic", the mode

of activity (agonist or antagonist) and potency cannot presently be predicted based solely upon molecular structure. Receptor binding affinity, however, is directly related to potency when comparing compounds with similar modes of activity (Chae, et al. 1991; VanderKuur, et al. 1993a). Thus, measurement of receptor binding affinity is useful for: 1) assessing the potential for direct action, either as an agonist or antagonist, and; 2) predicting potency when the mode of action is known. A limitation of assessing the potential for direct action by estrogen receptor binging affinity is that the substance may require metabolic activation for estrogen agonism or antagonism. Metabolites can be qualitatively assayed by performing an enzymatic conversion of the parent compound prior to the receptor binding assay. The quantitative measurement of receptor binding affinity is limited in this case because metabolites may not be readily identified or quantitated.

Objectives

The objectives of this research project were to: 1) survey a wide range of pure compounds representing primarily chlorinated aromatic environmental contaminants, and; 2) develop structure-binding relationships for a single class of chlorinated aromatic compounds, the hydroxylated polychlorinated biphenyls (OHCBs).

Materials and Methods

Chemicals

Most hydroxylated PCBs (OHCBs) were obtained from Ultra Scientific (North Kingston, RI). However, some OHCBs were obtained by collaboration with Prof. Å. Bergman (University of Stockholm, Sweden). Chlorinated by-products of pulp and paper production were obtained by collaboration with Dr. J. Koistinen (University of Jyvåskylå, Finland). Other compounds were obtained from Sigma Chemical (St. Louis, MO) and Aldrich Fine Chemicals (Milwaukee, WI). Most compounds were dissolved in ethanol and stored at -20 °C. However, some highly insoluble compounds (generally those that contained fused ring systems or were co-planar biphenyls) were dissolved in 1-propanol and stored at -20 °C. 2,4,6,7-[^{3}H]-17ß-estradiol was obtained from Dupont/NEN at a specific activity of 90-110 Ci/mmol. All buffers and salts were of reagent grade and obtained from Sigma Chemical.

Calf Uterine Estrogen Receptor Competitive Binding Assay

Estrogen receptor was obtained from calf uterus because this tissue contains abundant receptor with low concentrations of bound estrogen. One calf uterus (approximately 500 g fresh weight) was sufficient to supply receptor for the entire research project (and another unrelated research project conducted by another student) with a majority of the tissue left unused. The

isolation of calf uterine estrogen receptor and competitive binding assays followed a standard operating procedure (Appendix 1) that was based upon methods adapted from Murdoch, et al. (1990) and Clark and Peck (1979). Competitive binding curves were modelled after a normal distribution (Equation 6, Bruce and Versteeg, 1992).

Molecular Modelling

Molecular descriptors were calculated using Hyperchem® desktop modelling software (Hypercube Software, Waterloo, Ontario) for the OHCBs only. The global minimum energy conformation was identified by an initial search of the central torsion bond angle at restrained 30 degree increments using the AM1 semi-empirical method with an energy gradient criterion of 0.01 rms (roor mean square). Because the conformational energy is symmetrical about 180 degrees, the initial minimum energy torsion angle was selected from the 0-180 degree range for final unrestrained optimization to a gradient cutoff of 0.001 rms. The final minimum energy torsion angle was estimated using the fully optimized, relaxed molecule. The sine tranformed torsion angle (to achieve linearization) was used in subsequent statistical analysis. Fourteen molecular descriptors were calculated and included in a multiple regression analysis (Table 4; SAS Institute, 1988). The minimum energy structure of 17ß-estradiol was also modelled but its molecular descriptors were not included in subsequent statistical analysis. A minimum set of molecular descriptors was selected that optimally predicted the log(IC50) for specific binding to the calf uterine estrogen

Table 4. Molecular descriptors of hydroxylated PCBs calculated using Hyperchem®

Descriptor	Definition	Units
	Electronic Variables	
EGAP	Energy gap: Energy difference between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO), a measure of the reactivity of the molecule.	electron volts
OCHARGE	Oxygen partial charge: Partial charge on the oxygen of the hydroxyl group, affects strength of hydrogen bonding and pKa.	electronic charge e^-
DIPOLE	Molecular dipole moment: Dipole moment calculated on entire molecule.	Debyes
ZDIPOLE	Dipole moment along Z axis: Dipole calculated on long axis of molecule, affected by position of hydroxyl group.	Debyes
	Spatial Variables	
SASA	Solvent accessible surface area: Surface area accessible to solvent molecule with mean radius of 1.4 Å.	Å^2 Angstrom^2
SAVOL	Solvent accessible volume: Volume accessible to solvent molecule with mean radius of 1.4 Å.	Å^3
VDWSA	Van der Waals surface area: Surface area at the van der Waals radius for each atom in molecule.	Å^2
VDWVOL	Van der Waals volume: Volume at the van der Waals radius for each atom in molecule.	Å^3
XYZVOL	XYZ volume: Volume of the box defined by the dimensions of the molecule in the X,Y, and Z directions.	Å^3
STERIC	Steric volume: Van der Waals volume of substituents vicinal to the hydroxyl group, measures steric hindrance affecting hydroxyl group.	Å^3
BULK	Bulk volume: Van der Waals volume of non-hydroxylated ring, measures volume of "bulky" constituent of molecule.	Å^3
BULKPER	Bulk volume percent: Bulk volume expressed as a percentage of total van der Waals volume of molecule.	%
	Structural Variables	
SINTORS	Sine of the torsion angle: Torsion angle of central biphenyl bond, sine transformed for linearization, affected by degree of *ortho* substituted chlorination.	Sin(degrees)
OHDIST	OH distance: Distance of OH group from molecular center of mass, measures "extremity" of OH group, related to substitution position, *para* > *meta* > *ortho*.	Å

receptor. The optimum set of descriptors was selected by maximizing the coefficient of determination (r^2) for each subset of descriptors, such that the best one variable model, best two variable model, best three variable model, etc. was selected. The progress of this selection process was plotted and thepoint at which no appreciable additional increase (less than 10% of the total) in predictive power with increasing descriptors was selected as the optimum model. Two models were evaluated in this way, an unweighted model and a weighted model. The weighted model incorporated information on the reliability of the log(IC50) measurements. In general, the accuracy of the log(IC50) determination decreased as the absolute value of the log(IC50) increased, because greater log(IC50)'s were estimated using increasingly incomplete competitive binding curves. Therefore, the weight given to log(IC50) observations was proportional to the inverse of the log(IC50) (Equation 7).

$$Weight = \frac{1}{\log(IC50)} \tag{7}$$

Results and Discussion

A total of 53 chemicals were tested for the ability to bind specifically to calf uterine ER (Tables 5-7). All but one hydroxylated PCB, (OHCB8) bound specifically to calf uterine ER with measurable affinity (Table 5). Three DDT isomers and derivatives were assayed, two of which bound weakly to the ER (Table 6). Structure affected binding of the DDT compounds such that binding affinity log(IC50) followed the pattern: *o,p'*-DDT<*o,p'*-DDE<*o,p'*-DDD (Not

Table 5. Prediction of log(IC50), (log(nM)) using multiple linear regression of the optimum five molecular descriptors.

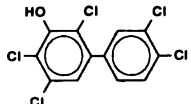
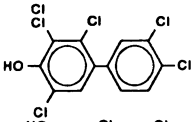
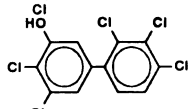
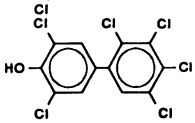
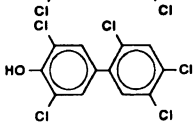
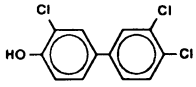
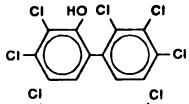
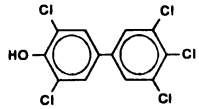
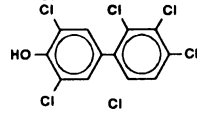
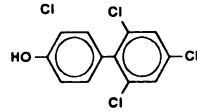
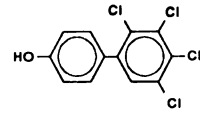
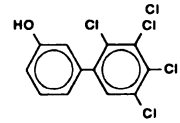
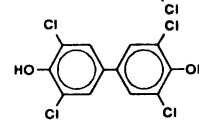
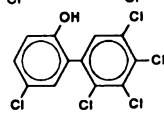
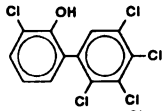
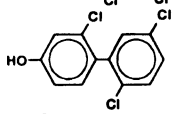
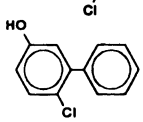
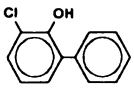
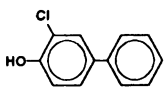
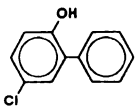
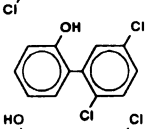
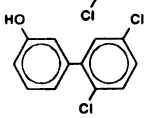
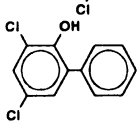
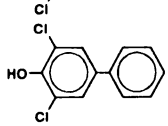
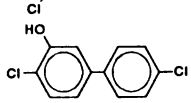
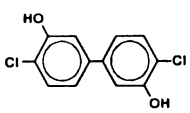
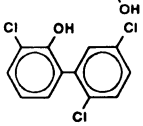
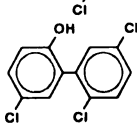
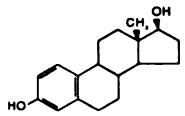
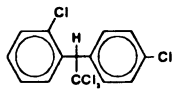
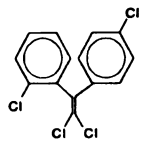
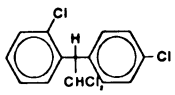
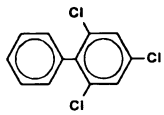
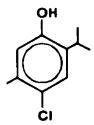
ID	Formula	Structure	Observed Log(IC50) (ASE)*	Predicted Log(IC50) (SE) Weighted Model	Predicted Log(IC50) (SE) Unweighted Model
OHCB1	3-OH-2,3',4,4',5-Cl_5-BP		4.26 (--)	4.81 (0.39)	5.20 (0.42)
OHCB2	4-OH-2,3,3',4',5-Cl_5-BP		4.13 (0.10)	4.84 (0.44)	5.26 (0.48)
OHCB3	3-OH-2',3',4,4',5-Cl_5-BP		3.86 (0.34)	5.11 (0.42)	5.59 (0.46)
OHCB4	4-OH-2',3,3',4',5, 5'-Cl_6-BP		7.43 (--)	4.95 (0.36)	5.34 (0.38)
OHCB5	4-OH-2',3,4',5,5'-Cl_5-BP		5.50 (--)	5.04 (0.33)	5.29 (0.36)
OHCB6	4-OH-3,3',4'-Cl_3-BP		3.85 (0.08)	4.20 (0.42)	4.22 (0.48)
OHCB7	2-OH-2',3,3',4,4'-Cl_5-BP		4.60 (0.18)	5.51 (0.43)	5.45 (0.48)
OHCB8	4-OH-3,3',4',5,5'-Cl_5-BP		NA	5.67 (0.43)	6.02 (0.47)
OHCB9	4-OH-2',3,3',4',5-Cl_5-BP		8.44 (--)	5.78 (0.46)	6.27 (0.48)
OHCB10	4-OH-2',4',6'-Cl_3-BP		2.84 (0.14)	2.78 (0.39)	2.85 (0.50)
OHCB11	4-OH-2',3',4',5'-Cl_4-BP		2.15 (0.15)	2.70 (0.40)	2.86 (0.53)
OHCB12	3-OH-2',3',4',5'-Cl_4-BP		3.41 (0.21)	2.78 (0.34)	2.85 (0.46)
OHCB13	4,4'-$(OH)_2$-3,3',5,5'-Cl_4-BP		4.94 (0.22)	5.29 (0.44)	5.53 (0.48)
OHCB14	2-OH-2',3',4',5,5'-Cl_5-BP		5.11 (0.90)	4.57 (0.44)	4.68 (0.49)

Table 5. (Cont'd).

ID	Formula	Structure	Observed Log(IC50) (ASE)	Predicted Log(IC50) (SE) Weighted Model	Predicted Log(IC50) (SE) Unweighted Model
OHCB15	2-OH-2',3,3',4',5'-Cl_5-BP		4.89 (0.60)	4.24 (0.57)	5.20 (0.42)
OHCB16	4-OH-2,2',5'-Cl_3-BP		3.63 (0.08)	3.59 (0.58)	5.26 (0.48)
OHCB17	3-OH-6-Cl-BP		4.42 (0.16)	4.08 (0.40)	5.59 (0.46)
OHCB18	2-OH-3-Cl-BP		6.73 (--)	6.78 (0.60)	5.34 (0.38)
OHCB19	4-OH-3-Cl-BP		4.20 (0.13)	4.56 (0.29)	5.29 (0.36)
OHCB20	2-OH-5-Cl-BP		5.86 (0.31)	4.88 (0.28)	4.22 (0.48)
OHCB21	2-OH-2',5'-Cl_2-BP		5.12 (0.25)	4.61 (0.35)	5.45 (0.48)
OHCB22	3-OH-2',5'-Cl_2-BP		4.26 (0.04)	4.20 (0.41)	6.02 (0.47)
OHC23	2-OH-3,5-Cl_2-BP		4.94 (0.27)	5.47 (0.26)	6.27 (0.48)
OHCB24	4-OH-3,5-Cl_2-BP		5.08 (0.24)	4.77 (0.44)	2.85 (0.50)
OHCB25	3-OH-4,4'-Cl_2-BP		4.44 (0.13)	4.87 (0.37)	2.86 (0.53)
OHCB26	3,3'-$(OH)_2$-4,4'-Cl_2-BP		6.11 (0.82)	5.17 (0.45)	2.85 (0.46)
OHCB27	2-OH-2',3,5'-Cl_3-BP		4.69 (0.14)	5.38 (0.37)	5.53 (0.48)
OHCB28	2-OH-2',5,5'-Cl_3-BP		4.25 (0.05)	4.57 (0.33)	4.68 (0.49)
r^2				0.616	0.549
SSE				3.60	20.51

a: Asymptotic standard error, -- indicates ASE inestimable by nonlinear regression.

Table 6. Estimates of calf uterine estrogen receptor binding log(IC50), (log(nM))for miscellaneous compounds.

ID	Formula	Structure	Log(IC50) (ASE)[a]	Reason for Analysis
E2	17ß-Estradiol		0.837 (0.069)	Native ligand
D1	*o,p'*-dichloro-diphenyl-1,1,1-trichloroethane		4.93 (0.38)	Reported estrogenic (1)
D2	*o,p'*-dichloro-diphenyl-1,1-dichloroethene		5.77 (--)	Metabolite of D1
D3	*o,p'*-dichloro-diphenyl-1,1-dichloroethane		NA[b]	Metabolite of D1
PCB30	2,4,6-dichloro-biphenyl		6.77 (--)	Parent PCB of OHCB10
A2	4-chloro-2-isopropyl-5-methyl-phenol		6.99 (--)	Structure

a: Asymptotic standard error, -- indicates ASE inestimable by nonlinear regression.
b: Not Active.
1: Welch, et al. 1969.

Table 7. Compounds of environmental concern that did not bind to calf uterine estrogen receptor.

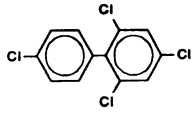
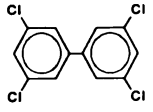
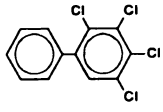
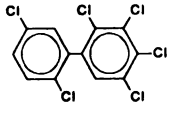
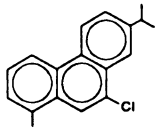
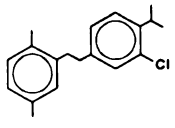
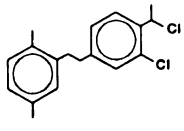
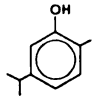
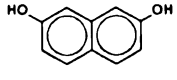
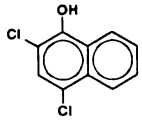
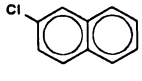
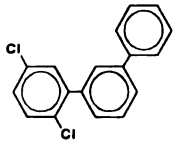
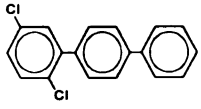
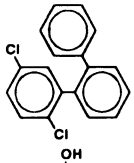
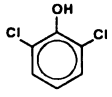
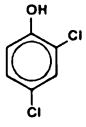
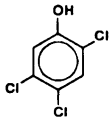
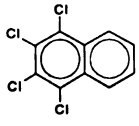
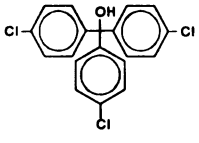
ID	Formula	Structure	Reason for Analysis
PCB75	2,4,4',6-tetrachloro-biphenyl		Similar to OHCB10
PCB80	3,3',5,5'-tetrachloro-biphenyl		Similar to OHCB13
PCB61	2,3,4,5-tetrachloro-biphenyl		Parent PCB of OHCB11
PCB141	2,2',3,4,5,5'-hexachloro-biphenyl		Similar to OHCB14
J1	9-chloro-retene		Identified in bleached kraft mill effluent, BKME (1)
J2	1-(2,5-dimethylphenyl)-2-(3-chloro-4-isopropylphenyl)-ethane		Identified in BKME (1)
J3	1-(2,5-dimethylphenyl)-2-(3-chloro-4-ß-chloro isopropylphenyl)-ethane		Identified in BKME (1)
A1	5-isopropyl-2-methyl-phenol		Structure
A3	2,7-naphthalenediol		Structure
A4	2,4-dichloro-1-naphthol		Structure
A5	2-chloro-naphthalene		Structure

Table 7. (Cont'd.)

ID	Formula	Structure	Reason for Analysis
A6	2,5-dichloro-*m*-terphenyl		Structure
A7	2,5-dichloro-*p*-terphenyl		Structure
A8	2,5-dichloro-*o*-terphenyl		Structure
A9	2,6-dichloro-phenol		Identified in BKME (2)
A10	2,4-dichloro-phenol		Identified in BKME (2)
A11	2,4,5-trichloro-phenol		Identified in BKME (2)
A12	1,2,3,4-tetrachloro-naphthalene		Identified in BKME (2)
A13	tris(4-chlorophenyl)-methanol		Identified in marine mammals and birds (3)

1: Koistinen, et al. 1992; 2: Paasivirta, et al. 1985; 3: Jarman, et al. 1992.

active). Also exhibiting measurable affinity were 17ß-estradiol, (E2) as a positive control and reference for the other compounds, 2,4,6-trichlorobiphenyl, (PCB30) and 4-chloro-2-isopropyl-5-methyl-phenol (A2). Compounds that did not bind to ER (Table 7) comprised individual PCB congeners, chlorinated polyaromatic hydrocarbons, chlorinated bibenzyls, chlorinated phenols and other compounds found in the aquatic environment.

Quantitative structure-binding relationships were developed using multiple linear regression of either weighted or unweighted observations. The fourteen molecular descriptors for the OHCBs (and E2 for comparison) were calculated (Table 8) and subjected to multiple linear regression using the maximum r^2 selection method for inclusion of regressors. A five regressor model was selected that optimally explained the variation in the dependent variable, log(IC50), (Figure 9). The maximum variation in log(IC50) explained by the full 14 variable weighted model was 72.66% ($r^2 = 0.7266$), and by the full 14 variable unweighted model was 70.63% ($r^2 = 0.7063$). The five variables that were selected in both the weighted and unweighted models were: EGAP, XYZVOL, STERIC, OCHARGE, and ZDIPOLE (Table 9). Each variable described different aspects of the OHCBs molecular structure. EGAP is a "global" electronic parameter that describes the overall reactivity of the molecule. Inspection of the coefficient for this variable indicated that EGAP varied directly with log(IC50), larger values of EGAP were related with larger values of log(IC50), suggesting that more reactive molecules bind less well to ER. However, the coefficient for EGAP was only marginally significantly different from zero with $p < 0.12$. All other variable coefficients were

Table 8. Results of molecular modelling of hydroxylated PCBs.

ID	EGAP (eV)	OCHARGE (e^-)	DIPOLE (Debye)	ZDIPOLE (Debye)	SASA ($Å^2$)	SAVOL ($Å^3$)	VDWSA ($Å^2$)
OHCB1	8.5821	-0.2268	1.315	0.826	468.87	761.72	272.03
OHCB2	8.5863	-0.2248	1.435	0.918	470.69	764.14	273.65
OHCB3	8.5342	-0.2323	2.130	1.499	469.90	762.26	274.55
OHCB4	8.4466	-0.2258	1.080	0.502	492.80	801.09	288.48
OHCB5	8.4714	-0.2265	1.131	-0.096	477.76	766.53	275.60
OHCB6	8.3717	-0.2409	1.444	-0.895	732.70	684.90	242.97
OHCB7	8.5571	-0.2325	2.241	-1.189	462.72	757.33	272.81
OHCB8	8.3020	-0.2256	1.389	0.115	471.86	766.06	274.36
OHCB9	8.5409	-0.2267	1.473	1.404	472.96	762.36	273.65
OHCB10	8.7786	-0.2493	1.166	0.384	428.99	689.13	245.35
OHCB11	8.4336	-0.2475	2.024	0.105	446.34	720.40	256.58
OHCB12	8.5599	-0.2495	2.853	-0.031	445.89	720.67	256.40
OHCB13	8.2920	-0.2267	1.970	-0.455	462.35	746.55	267.42
OHCB14	8.4883	-0.2435	3.146	-0.002	461.99	759.43	272.22
OHCB15	8.3832	-0.2331	1.681	-1.052	463.59	756.41	271.90
OHCB16	9.0237	-0.2393	1.785	-0.677	421.67	685.97	244.18
OHCB17	8.8422	-0.2479	1.149	-0.157	384.64	603.43	211.26
OHCB18	8.7977	-0.2409	2.673	0.617	388.57	601.80	209.85
OHCB19	8.5731	-0.2433	1.023	-0.064	389.98	602.30	210.06
OHCB20	8.5836	-0.2443	1.532	-0.342	381.45	602.40	211.62
OHCB21	8.7715	-0.2471	1.709	-0.126	404.26	642.08	227.96
OHCB22	8.7837	-0.2508	1.474	0.977	409.82	646.65	228.50
OHCB23	8.5487	-0.2369	0.616	0.035	399.28	642.81	226.84
OHCB24	8.5292	-0.2287	1.975	-1.021	412.96	645.09	226.32
OHCB25	8.4694	-0.2441	1.082	0.302	405.66	646.75	228.43
OHCB26	8.4176	-0.2440	0.549	0.480	415.61	664.91	235.95
OHCB27	8.7795	-0.2395	1.095	-0.304	425.09	681.95	243.56
OHCB28	8.5309	-0.2456	1.511	-0.216	425.24	685.59	243.61
E2	9.2362	-0.2524	1.214	-0.073	470.30	806.80	296.25

Table 8. (Cont'd.)

ID	VDWVOL (Å^3)	XYZVOL (Å^3)	STERIC (Å^3)	BULK (Å^3)	BULKPER %	OHDIST (Å)	SINTORS
OHCB1	237.95	214.23	30.66	113.52	47.7	4.10	0.8699
OHCB2	237.82	224.46	30.94	113.44	47.7	4.62	0.8730
OHCB3	237.95	177.34	17.01	127.75	53.7	4.06	0.8772
OHCB4	251.78	181.35	31.18	141.42	56.2	4.60	0.8797
OHCB5	238.24	155.58	31.30	127.73	53.6	4.60	0.8595
OHCB6	209.87	101.90	17.02	113.57	54.1	4.59	0.6550
OHCB7	237.65	157.93	133.01	127.56	53.7	2.47	0.9171
OHCB8	238.15	102.84	31.18	127.67	53.6	4.66	0.6626
OHCB9	237.81	164.28	31.21	127.41	53.6	4.60	0.8779
OHCB10	210.06	245.61	2.99	142.41	67.8	4.36	1.0000
OHCB11	223.73	206.96	3.01	141.73	63.3	3.44	0.8599
OHCB12	223.82	204.26	3.01	141.71	63.3	3.05	0.8691
OHCB13	230.57	103.46	31.19	120.12	52.1	4.60	0.6663
OHCB14	237.64	209.20	133.13	141.51	59.5	2.96	0.8475
OHCB15	237.74	236.80	142.27	141.63	59.6	3.23	0.9208
OHCB16	209.70	237.20	17.00	99.45	47.4	5.98	0.9997
OHCB17	181.56	152.96	3.02	85.22	46.9	4.36	0.8526
OHCB18	181.31	148.60	155.32	85.27	47.0	3.44	0.7970
OHCB19	181.29	121.48	17.03	85.19	47.0	4.60	0.6530
OHCB20	181.50	138.13	76.89	85.19	46.9	3.05	0.6542
OHCB21	195.63	199.46	105.48	113.80	58.1	2.94	0.8270
OHCB22	195.65	164.57	3.00	113.67	58.0	3.15	0.8489
OHCB23	195.31	148.11	90.74	85.12	43.6	3.08	0.6717
OHCB24	195.57	140.07	31.16	85.24	43.6	4.62	0.6570
OHCB25	195.93	96.49	17.02	99.48	50.8	5.14	0.6469
OHCB26	201.95	76.86	17.01	105.76	52.4	3.34	0.6435
OHCB27	209.79	184.76	119.39	113.73	54.2	2.55	0.8343
OHCB28	209.51	174.94	105.05	113.61	54.2	2.43	0.9021
E2	267.87	246.54	3.02	213.67	82.9	7.89	--

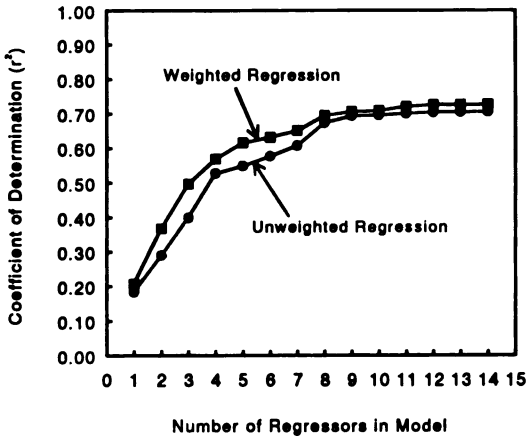


Figure 9. Progress of multiple regression relating calf uterine binding log(IC50) to molecular descriptors of OHCBs in which the best combination of descriptors that maximized r^2 was selected for each number of descriptors.

significantly different from zero, $\alpha = 0.05$. ZDIPOLE described the dipole moment of the molecule along the Z axis, which was defined as the axis aligned with the central biphenyl bond. ZDIPOLE was affected by the substitution position of Cl and OH groups and varied directly with log(IC50) such that more positive values of ZDIPOLE were associated with greater values of log(IC50). OCHARGE described the partial charge on the oxygen of the hydroxyl group and was strongly affected by the substitution of vicinal chlorines . Vicinal chlorines caused electron withdrawal through the aromatic ring system from the hydroxyl oxygen resulting in a more positive partial charge. Electron withdrawal from the oxygen would result in greater propensity to ionize in aqueous solution (because the proton is less tightly bound) resulting in reduced pK_a (Waller, et al. 1995). Also, reduced partial charge on the oxygen would also reduce the hydrogen bond acceptor strength (hydrogen bond basicity) of the oxygen.

Though not known, the hydrogen bonding attributable to the phenolic hydroxyl group of estrogens may contribute significantly to the ER binding energy. The remaining two variables describe spatial characteristics of the OHCBs. STERIC

Table 9. Optimum five variable weighted and unweighted models for the prediction of log(IC50), (log(nM)) for binding to calf uterine estrogen receptor.

Regressor	Weighted Model			Unweighted Model		
	Coefficient	SE	p	Coefficient	SE	p
EGAP	1.8156	1.1391	0.126	1.3841	1.3788	0.327
XYZVOL	-0.0148	0.0041	0.002	-0.0144	0.0048	0.007
STERIC	0.0125	0.0037	0.003	0.0121	0.0041	0.008
OCHARGE	60.030	21.443	0.011	68.155	24.813	0.012
ZDIPOLE	0.5075	0.2700	0.074	0.6832	0.2954	0.031
INTERCEPT	5.2121	9.2562	0.579	10.9236	10.5179	0.311

described the volume of the substituents bonded vicinally to the hydroxyl group and varied directly with log(IC50). Thus, steric hindrance of the hydroxyl group appeared to inhibit binding to the ER. XYZVOL described the volume of the box that just enclosed the OHCB. XYZVOL was a volume parameter that was affected by Cl and OH substitution pattern, torsion angle, and total number of Cls. Thus, XYZVOL incorporated information on several aspects of the structure of the OHCB and was most significantly related to log(IC50) in the multiple regressions (weighted $p<0.002$; unweighted $p<0.007$). The performance of the multiple regression models was of limited success as

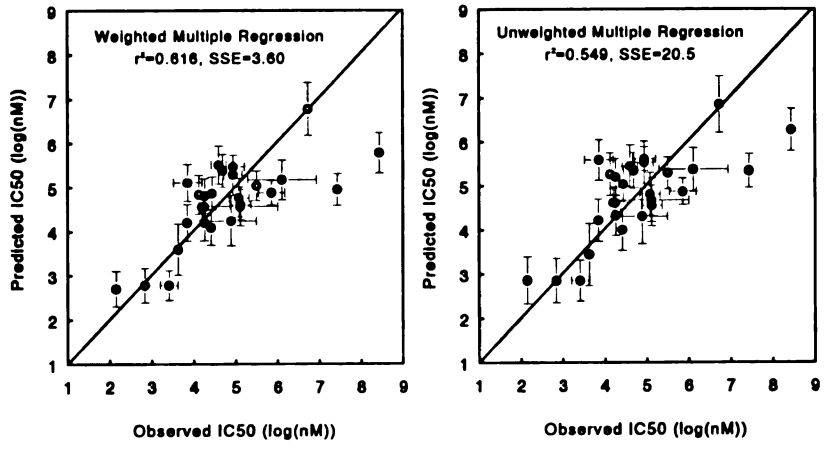


Figure 10. Comparison of predicted vs. observed log(IC50) for binding of OHCBs to calf uterine estrogen receptor using both weighted and unweighted five variable multiple regression models; * = asymptotic standard error of observed log(IC50) inestimable by nonlinear regression and therfore not shown; other error bars not shown because the bar was smaller than the symbol; vertical error bars are standard error of the individual predicted log(IC50).

indicated by the relatively low coefficients of determination. However, the model did predict log(IC50) to within 1 log unit for 24/28 OHCBs studied. This level of resolution is useful for prediction of the general range of binding affinity and would be useful for identifying those OHCBs that have the potential for strong binding. Some of the error in the predictions was also related to the error associated with the experimental estimation of the log(IC50)'s (Figure 10). Nearly all, 26/28, of the OHCBs fell within two experimental estimate standard errors of the multiple regression prediction. Those OHCBs with relatively large log(IC50)'s, (greater than 7 log(nM)) were poorly predicted by the models. However, the experimental estimate of these log(IC50)'s was also the most uncertain because of extrapolation well beyond the highest concentrations tested (50 μM). Despite this limitation, the model did predict the log(IC50)'s

of the very weakly binding OHCBs to be greater than 5 log(nM), beyond the solubility of these compounds (observed at 4.7 log(nM)). The use of the weighted regression did improve the predictive power of the model by about 7%. The effect of the weighting was to de-emphasize the highly uncertain observations greater than 7 log(nM) while emphasizing the more accurate observations below 3 log(nM). The weighted multiple regression model provided the best framework for predicting ER binding by OHCBs and may prove useful in predicting the binding affinity of OHCBs that have not yet been assayed, including those recently identified as human and wildlife metabolites of PCBs (Bergman, 1994).

CHAPTER 3

DEVELOPMENT AND CHARACTERIZATION OF AN ESTROGEN RESPONSIVE LUCIFERASE REPORTER PLASMID, pGUDLUC2.2

Introduction

A wide variety of synthetic and natural chemical products possess estrogenic (and anti-estrogenic) activity (McLachlan, 1993; Safe, 1994). The role of estrogen mimics in human diseases such as breast cancer and testicular abnormalities remains uncertain and controversial (Colborne and Clement, 1992; Colborne, et al. 1996; Safe, 1995). The comprehensive screening of synthetic chemicals for estrogenic activity has been suggested (ESCREEN, Soto, et al. 1992) utilizing a rapid *in vitro* bioassay based upon the proliferative response of MCF-7 cells (a human breast adenocarcinoma cell line) to 17ß-estradiol and other estrogens. A functional *in vitro* bioassay approach to screening for estrogenic compounds provides a rapid, high-throughput technique that can surpass a simple estrogen receptor binding assay in detecting active compounds by incorporating all of the biochemical processes involved in the cellular response to estrogens. A weakness of the ESCREEN bioassay is that the precise genetic elements controlling the proliferative response of the MCF-7 cells has not been characterized, leading to the possibility that other non-estrogen pathways can cause proliferative responses. The proliferative effects of insulin and tumor growth factor *α* on MCF-7 cells are just two examples of non-estrogen related proliferative responses (Safe, et al. 1992; Liu, et al. 1992).

To improve the specificity of response to estrogen exposure, estrogen-inducible reporter genes have been used in a number of cell lines (White, et al. 1994; Routledge and Sumpter, 1996; VanderKuur, et al. 1993b). In this research project, a plasmid containing a luciferase reporter gene under the transcriptional control of tandem consensus-sequence estrogen responsive elements (EREs) was designed and constructed to take advantage of the specificity of reporter gene constructs and the superior sensitivity of the luciferase light-emitting reaction. The plasmid, pGudluc2.2, was designed to resemble a luciferase reporter gene containing-plasmid (pGudluc1.1) under the transcriptional control of dioxin responsive elements (DREs) was successfully constructed and utilized by others in the Aquatic Toxicology Laboratory at Michigan State University (El-Fouly, et al. 1994). The objective of the research project was to characterize the response of the plasmid, pGudluc2.2, in MCF-7 cells after transient and stable transfections and to evaluate the potential for use of the stably transfected cell line for use as an estrogen and anti-estrogen screening bioassay.

Materials and Methods

Plasmids for the construction of pGudluc2.2 and reagents for the assay of luciferase activity were obtained from Promega Corp. (Madison, WI). The tandem ERE sequence was synthesized in the Macromolecular Structure Facility at Michigan State University.

Construction of pGudluc2.2

The plasmid pGudluc2.2 was based upon the DRE-containing plasmid pGudluc1.1 (El-Fouly, et al. 1994). The construction of pGudluc2.2 proceeded by insertion at the HindIII site of the mouse mammary tumor virus promoter (MMTV promoter, 1810 bp) upstream of the coding region of the firefly luciferase gene found in pGL2Basic (Figure 11). For pGudluc1.1, a 482 bp fragment of the regulatory region of the mouse cytochrome P4501A gene containing four DRE sequences was inserted 100 bp upstream of the MMTV promoter start site (El-Fouly, et al. 1994). For pGudluc2.2 (5858 bp), a 1090 bp fragment between the distal BglII site (a second BglII site lies 29 bp upstream of the HindIII site) and the HindIII site within the MMTV promoter sequence, (preserving the MMTV promoter start site) was excised and replaced by a 54 bp BglII/HindIII oligonucleotide containing two consensus EREs (Figure 12).

Transfection of pGudluc2.2 into MCF-7 cells

MCF-7 cells were chosen as the benchmark cell line for evaluation of the effectiveness of pGudluc2.2 in conferring estrogen-inducible luciferase expression because MCF-7 cells have been extensively characterized and contain abundant functional ER (Eckert and Katzenellenbogen, 1982). Transient tranfections were conducted to assess the estrogen responsiveness of the plasmid. Initially, Polybrene® (Gibco BRL, Gaithersburg, MD) mediated

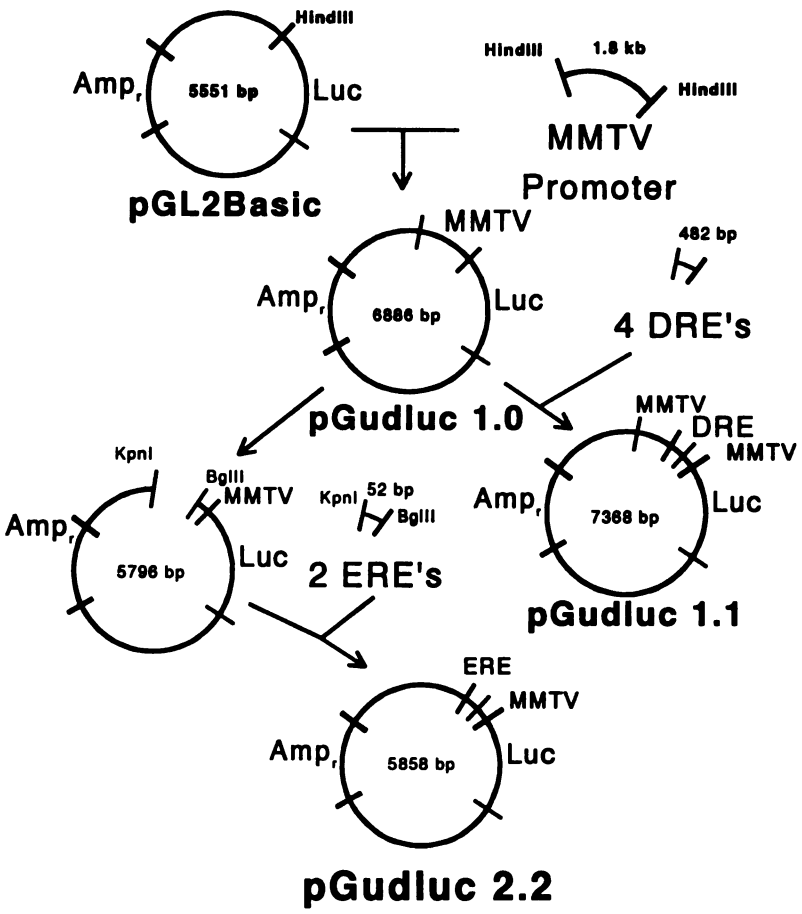


Figure 11. Construction history of pGudluc2.2.

CTT CCT GGT CAG CGT GAC CGG AGT CAC ATG TTC CTG GTC AGC GTG ACC GGA GTA
C ATG GAA GGA CCA GTC GCA CTG GCC TCA GTG TAC AAG GAC CAG TCG CAC TGG CCT CAT CTA G

KpnI ERE ERE BglII

Figure 12. Oligonucleotide containing two EREs used in pGudluc2.2.

introduction of pGudluc2.2 was not successful. Therefore, Lipofectin® (Gibco BRL) reagent was used instead with success according to the manufacturers protocol. Luciferase expression of transiently-transfected MCF-7 cells was elevated 48 hours after transfection by treatment with 10 nM 17ß-estradiol (Figure 13) prompting initiation of the stable transfections. Lipofectin® was also

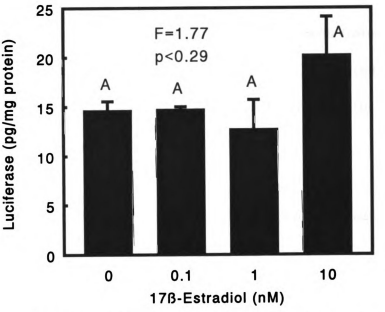


Figure 13. Response to 17ß-estradiol by MCF-7 cells transiently transfected with pGudluc2.2; N = 2 at each concentration; bars indicate mean ± 1 SE; letters indicate means are not significantly different by Fisher's LSD, $\alpha = 0.05$; F statistic produced by ANOVA.

used in the stable transfection to deliver pGudluc2.2 and a selective resistance marker, pSV2neo (a neomycin resistance gene; Southern and Berg, 1982) at a molar concentration ratio of 20:1. A large marker to reporter plasmid ratio was used to insure that resistant clones likely incorporated both plasmids. Selection of antibiotic resistant clones was achieved by subcloning of

transfectants three days after plasmid incorporation at a 1:3 dilution in culture medium (10% FBS Medium) containing 900 mg/ml G418 (a neomycin analog under the trade name Geneticin®, Gibco BRL). Bacterial contamination of 100 mm Petri plates was encountered initially and eliminated by the use of 75 cm^2 flasks. Resistant clones were visually identified, subcultured and grown to confluence in 48 well culture plates. The time from transfection to confluent cultures of clones was approximately three months. Seven clones (GLUC1-7) were isolated from approximately 1 million MCF-7 cells transfected initially. Four of the seven clones lost their resistance to G418 during subculturing, leaving three clones, GLUC 1, GLUC5 and GLUC6, available for characterization of estrogen inducibility of luciferase expression.

Characterization of Stably Transfected Estrogen Responsive Luciferase Reporter Gene

Estrogen responsiveness of the three stably transfected clones was evaluated in the absence of selective pressure by G418, though clones were continuously subcultured in G418-containing medium to maintain their phenotype. It was observed that G418 treatment completely inhibited luciferase expression, possibly by monopolizing the protein synthesis machinery of the cells to produce the antibiotic resistance necessary for the survival of the cells. On Day 1, clones were subcultured into G418-free 10% FBS medium in 48 well tissue culture plates at a concentration of 10,000 cells per well. Vigorous growth and attachment was promoted by incubation overnight in 10%

FBS medium. On Day 2, the medium was replaced by fresh 10% FBS medium to reduce the concentration of residual G418. On Day 3, the medium was supplemented with 17ß-estradiol at concentrations ranging from 0.1 to 100 nM. A concentration of 10 nM E2 was sufficient to maximally induce MCF7LUC cells, so the range of E2 was selected to include that concentration. In one experiment, the time course of induction of luciferase expression was investigated over a period of 9 days following initiation of exposure to E2. The optimal exposure duration was selected from this experiment and applied in further experiments that characterized the response of GLUC1 to E2, DES (a potent non-steroidal estrogen), tamoxifen (a direct-acting anti-estrogen). GLUC5 and GLUC6 were assayed for their response to E2 only because their pattern of response to E2 mirrored that of GLUC1. Luciferase expression was measured as previously described (Chapter 1, Materials and Methods).

Results and Discussion

The plasmid, pGudluc2.2, transiently transfected in MCF-7 cells, exhibited a 1.3 fold induction of luciferase activity at a concentration of 10 nM E2 (Figure 13). It was thought that stable transfection would improve the induction potential by improving the efficiency of gene transcription of genomically incorporated DNA. The stable transfection produced three successful clones that stably expressed both luciferase activity and G418 resistance. GLUC1 was more thoroughly characterized than GLUC5 and GLUC6 after it was realized that GLUC5 and GLUC6 responded similarly to E2,

suggesting that a complete repeat of the characterization used for GLUC1 was unnecessary. The time course of luciferase expression in response to E2 indicated that the maximal response was achieved by at least 72 hours after treatment with E2 began (Figure 14). Most notably, E2 caused a concentration dependent reduction in luciferase expression that was not expected. This

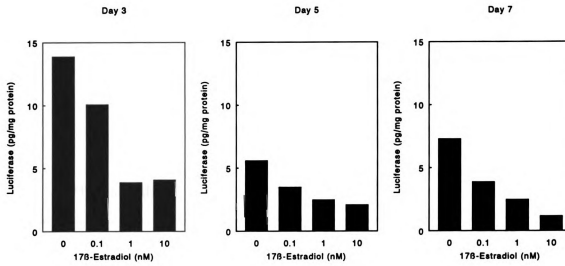


Figure 14. Time course of luciferase expression response to 17ß-estradiol by MCF-7 cells stably transfected with pGudluc2.2, clone GLUC1; N = 1 at each concentration.

reduction pattern persisted over 7 days of treatment of E2. Also notable was the relatively low level (5-15 pg luciferase/ml protein) of luciferase expression in the absence of E2. By comparison, MCF7LUC cells express 4000 pg luciferase/mg protein in the absence of added E2 and can be induced to express up to 10,000 pg luciferase/mg protein. A second experiment was conducted on GLUC1 that examined the response to E2, DES, and tamoxifen after 48 hours exposure. Again, luciferase expression appeared to be reduced by E2

and DES, though only E2 produced statistically significant reductions in luciferase expression (Figure 15). Thus, ER agonists caused concentration related inhibition of luciferase expression by stably-tranfected pGudluc2.2. Further evidence that the response was mediated by ER was provided by the treatment with tamoxifen and dexamethasone. Tamoxifen treatment caused a slight increase in luciferase expression at concentrations greater than or equal to 82 nM, suggesting that antagonism of ER improved expression of luciferase

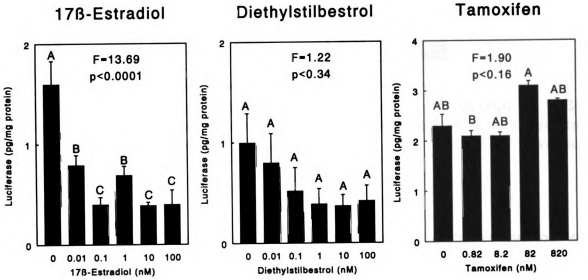


Figure 15. Luciferase expression response of GLUC1 to 17ß-estradiol, diethylstilbestrol and tamoxifen; N = 4 at each concentration; bars indicate mean ± 1SE; letters indicate means are not significantly different by Fisher's LSD, $\alpha = 0.05$; F statistic produced by ANOVA.

(Figure 15). GLUC5 and GLUC6 responded similarly to GLUC1 with mean luciferase expression levels tending toward lower values as exposure to E2 increased (Figure 16).

The mechanism explaining reduction of luciferase expression by ER agonists appears to directly involve ER. ER mediation of the reduction in luciferase expression is supported by the responses to E2 and DES, both ER

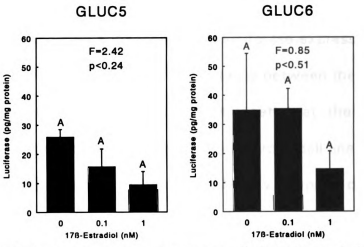


Figure 16. Luciferase expression response of GLUC5 and GLUC6 to 17ß-estradiol; N = 2 at each concentration; bars indicate mean ± 1 SE; letters indicate means are not significantly different by Fisher's LSD, $\alpha = 0.05$; F statistic produced by ANOVA.

agonists. Blockage of the expression of luciferase could be caused by binding of the E2 or DES liganded ER to the ERE sequences, consequently resulting in a blockage of the function of the MMTV promoter. Enhancer sequences normally work to induce promoter function by forming a looped DNA structure that stabilizes the RNA polymerase and other elongation factors at the promoter start site (Cullen, et al. 1993). It would appear that the proper formation of an active RNA polymerase complex at the MMTV promoter did not

occur in MCF-7 cells stably transfected with pGudluc2.2 and that an estrogen-activated ER mediated this blockage. Tamoxifen reversed the ER mediated blockage of luciferase expression. Tamoxifen binds competitively to ER but inhibits the proper formation of an active ER dimer complex, thus inhibiting effective binding of ER to the ERE. Therefore, ERE binding by ER appears to be related to the blockage of luciferase expression. This evidence suggests that the specific construction of ERE and MMTV promoter in pGudluc2.2 was not conducive to ER mediated enhancement of luciferase expression when stably transfected in MCF-7 cells. Perhaps the distance between the ERE and MMTV promoter was not optimal, though DREs located at the same distance effectively enhanced luciferase expression in a trout cell line (El-Fouly, et al. 1994). Another explanation may be that the MMTV promoter does not function optimally in MCF-7 cells (as evidenced by the low background level of expression) and that binding of ER to the EREs blocks binding of the DNA polymerase complex to the MMTV promoter start site. Further mechanistic studies are required to elucidate the mechanism of action of pGudluc2.2 in MCF-7 cells.

Whatever the explanation for the unexpected activity of pGudluc2.2 in MCF-7 cells, the practical conclusion is that the GLUC cell lines probably can not be used for rapid, large-scale screening of estrogenic or anti-estrogenic compounds because the absolute level of expression of luciferase was too low to allow adaptation of the assay to a 96 well format. The reversed response of the GLUC cell lines to E2 and other estrogens could be useful if the background expression level could be raised by two to three orders of

magnitude, thus providing a wider dynamic range. A reversed response curve would also allow improved reproducibility because variablility in luciferase measurements generally increases with increasing luciferase concentration. Also, cytotoxicity would not confound the response to anti-estrogens as found in MCF7LUC cells because anti-estrogens in GLUC cells produce increased luciferase expression whereas cytotoxicity reduces luciferase expression.

A new plasmid, pGudluc3, has been designed that incorporates the strong and widely effective SV40 promoter and a modified firefly luciferase gene that promotes transcription of the gene in vertebrate cell lines, based upon the pGL3 luciferase reporter vectors (Promega Corp.). The ERE sequences will be tested for their ability to enhance luciferase expression from two sites in the plasmid, both upstream and downstream of the luciferase gene. In the pGL3Control vector, the SV40 enhancer sequence is normally located downstream of the luciferase gene. Therefore, in one version of pGudluc3, the ERE sequence will be placed downstream of the luciferase gene replacing the SV40 enhancer normally located there. Also, the number of EREs in the sequence will be varied by concatemerization to identify the optimal number of EREs needed for enhancement of luciferase gene transcription. These experiments will be conducted by other researchers in the Aquatic Toxicology Laboratory at Michigan State University.

CHAPTER 4

REPRODUCTIVE IMPAIRMENT AND INDUCTION OF PLASMA ALKALINE-LABILE PHOSPHATE, A BIOMARKER OF ESTROGEN EXPOSURE, IN FATHEAD MINNOWS (*PIMEPHALES PROMELAS*) EXPOSED TO WATERBORNE 17ß-ESTRADIOL

Introduction

The aquatic environment frequently becomes a sink or site of first effects for environmental contaminants, including estrogenic contaminants. Therefore, it is important to study the effects of estrogenic contaminants in aquatic organisms. The fathead minnow (*Pimephales promelas*) is a widely used organism for aquatic toxicity testing and is an appropriate model organism for examining estrogenic endpoints, though work to date has fosuced on larger species, including rainbow trout (Purdom, et al. 1994), carp (U.K. Directorate, 1992), channel catfish (Nimrod and Benson, 1996), goldfish (Wiegand and Peter, 1980), Siberian sturgeon (Pelissero, et al. 1991) and flounder (Emmersen and Petersen, 1976). An advantage to using the fathead minnow as a model organism is that it can be readily induced to breed in captivity, facilitating the development of assays that monitor the effect of waterborne substances on reproduction (Donaldson, 1990; Donaldson and Scherer, 1983). Successful reproduction is critically important to the long term survival of fish species. Therefore, importance should be attached to the determination of the effects of a waterborne contaminant on fish reproduction, just as signficant efforts

have been made to assess the acute and chronic lethality of contaminants. Also, because estrogens play a critical role in modulating reproductive activity of fish (Donaldson and Hunter, 1983; Stacey, et al. 1987), it is logical, indeed imperative, to study the effects of waterborne xenoestrogens on reproduction.

The objectives of this research were: 1) to assess the effects of waterborne 17ß-estradiol on the reproductive function of fathead minnows as a benchmark to which other xenoestrogens can be compared, and 2) to correlate the effects on reproductive function with a biomarker of estrogen exposure, plasma vitellogenin (Vtg) expression, measured as plasma alkaline-labile phosphorous (plasma ALP). (A third objective of the overall research project was to relate impairment in reproductive function to histopathological changes in the gonads. However, histopathology was analyzed and reported in another Ph.D. dissertation (Miles-Richardson, 1996) and will not be reported here.)

Materials and Methods

Chemicals

Crystalline 17ß-estradiol was obtained from Sigma Chemical (St. Louis, MO) and stored in the dark under nitrogen. A stock solution (10 mM) in ethanol was stored at -20 °C for use in making the aqueous working solution. All chemicals were of reagent grade.

Experimental Design

Fathead minnows reproduce under specific conditions that can be manipulated to induce reproduction. Therefore, the experimental design was optimized to account for these conditions. Reproduction was defined in this assay as the number of eggs laid per female per tank during the experimental period. Because fathead minnows require the presence of more than one male and female per tank to optimally induce egg laying, the reproductive performance of individual females could not be determined. Also, reproductive output is strongly affected by the actions of the male. Specifically, males entice females to enter a breeding area, usually a recessed area under some structure, and by vigorous lateral rubbing induces the female to lay adherant eggs on the underside of the structure, simultaneously fertilizing the eggs. The male then expels the female from the breeding area and takes up the task of cleaning and protecting the eggs from predation until hatching. It has been observed in the Aquatic Toxicology Laboratory that the presence of more than one male in the breeding tank enhances the development of the secondary sexual characteristics of the males, though crowding leads to excessive mortality. Also, placing more than one female in the breeding tank improves the probability that at least one of the females will have developed mature eggs in time for the experimental breeding period. A population of three males and three females per tank was selected as optimum based upon the considerations mentioned previously and the observations made during pilot studies. Each male was provided a breeding area constructed from a clay flower pot (7.5 cm

diameter), cut in half, and placed on the bottom of the tank to provide a shelter (hereafter referred to as breeding tiles). Three concentrations of 17ß-estradiol were tested, 27.24 ng/L, 272.4 ng/L and 2724 ng/L (0.1 nM, 1 nM, and 10 nM, respectively), in addition to a solvent control and control receiving no solvent. Treatments were duplicated and reproduction was reported as the tank average over the entire period in which reproduction was monitored. Statistical analyses included ANOVA (SAS PROC GLM, SAS Institute, 1988) with the nominal concentrations as treatments and regression analysis (SAS PROC REG, SAS Institute, 1988) using the measured concentrations to compare the precision of the analyses.

Maintenance and Acclimation of Fish

Fathead minnows were reared and maintained in the Aquatic Toxicology Laboratory under a 16:8 light:dark cycle in continuosly flowing, dechlorinated tap water at 17-19 °C and fed a 1:1 (v:v) mixture of Tetramin (Tetrawerke, Germany) and Purina Trout Chow (2-4 mm pellet size) once daily at a rate of approximately 0.5% of fresh body weight. The dry food was supplemented with *Artemia* (brine shrimp) nauplii. Freeze-dried *Spirulina* algae was fed to fry up to 2.5 cm length (as this was necessary to avoid vitamin deficiency during development leading to scoliosis and lordosis in adults).

A three week acclimation period preceded the exposure period. Three males and three females, reproductively mature and of 12 to 18 months of age were visually sexed and transferred to randomly assigned 19L glass aquaria

held at 19 °C in a circulating water-jacketed bath (Frigid Units Living Stream, Toledo OH) receiving 3 L/hr dechlorinated tap water through a solenoid-controlled proportional diluter apparatus (Ace Glass, Vineland, NJ). Each tank received vigorous aeration, though escape of aerosols from the tanks was prevented by acrylic lids. Light was delivered at the rate of 16:8 light:dark from two 40 watt fluorescent bulbs fitted with a cellulose acetate sheet that blocked wavelengths of light below 290 nm to minimize UV-induced degradation of 17ß-estradiol. The temperature of the water jacket was raised 2.3 °C per week to a final temperature of 26 °C, the optimal temperature for breeding. The determination of the sex of the fish was confounded by the fact that some female-appearing fish later developed male characteristics by the end of the acclimation period. The identification of male individuals was unambiguos due to the appearance of large breeding tubercles on the snout, dark vertical banding, a prominent dorsal fat pad and stiffening and whitening of the first ray of the pectoral, pelvic and dorsal fins. It was necessary to recheck the identity of the female fish each week. Any "female" fish that developed male characteristics was removed and replaced with a female fish taken from a separate tank held at the temperature of the acclimation tanks. The pattern of sexual development observed in this laboratory resembles protogyny ("first female") reported to occur in other fish (Matty, 1985), though protogyny has not been reported to occur in fathead minnows. Fish were not allowed to breed during the acclimation period by not placing the breeding tiles in the tanks. The feeding regimen used during normal maintenance of the fish was continued throughout the acclimation and exposure periods.

Exposure of Fish to 17ß-Estradiol

A three week exposure period commenced immediately after the acclimation period. 17ß-estradiol (E2) was delivered at concentrations of 27.24 ng/L, 272.4 ng/L and 2724 ng/L via the proportional flow diluter accompanied by a final ethanol solvent concentration of 0.0001% (1 ppm ethanol by volume). The solvent control treatment received 0.0001% ethanol also. A control treatment received no ethanol. Fish were exposed to 17ß-estradiol for 6 days before the commencement of breeding. Breeding was initiated by placement of the breeding tiles in the tanks and monitored for two periods of 6 days each with one day of rest in between. The experiment was ended two days early (19 days of exposure in total) due to the development of morbidity in fish exposed to 2724 ng/L E2. Twice daily, breeding tiles were checked for eggs, and when discovered, were counted and photographed. A fresh tile was used to replace the tile containing eggs. Tanks were siphon-cleaned of accumulated food and fecal material weekly. Wastewater was treated by trickle filtration through 100 kg activated charcoal.

Collection of Tissues

At the end of the exposure period tissues were collected for histopathology and analysis of plasma vitellogenin. Fish that died before the end of the experiment were not used in the analysis of histopathology or vitellogenin because autolysis occurred rapidly after death. After anaesthesis

with a lethal concentration of tricaine methanesulfonate (1 g/L), the caudal peduncle was rapidly severed with a scalpel dipped in heparin solution (10 mg/ml) and blood collected from the caudal vein with a heparinized hematocrit tube. Blood volumes collected ranged from 20-250 μl. The abdomen was injected with Bouin's preservative solution to speed preservation of internal organs. Then, the entire fish was immersed in Bouin's solution for 24 hr and transferred to ethanol for storage until histopathological analysis. A subset of male gonads were dissected out before treatment with Bouin's solution for ultrastructural analysis by transmission electron microscopy (Miles-Richardson, 1996).

Vitellogenin Analysis

Blood, collected in heparinized hematocrit tubes, was centrifuged at 1300 g for 10 min at 4°C to separate red blood cells from the plasma. The plasma was stored at -80°C until analysis of alkaline-labile phosphorous. Plasma alkaline-labile phosphorous has been used as a non-specific indicator of Vtg expression (Wallace and Jared, 1968; Parker and McKeown, 1987) because Vtg is highly phosphorylated and comprises a large majority of the phosphorylated plasma proteins when it is induced. Vitellogenin is a high molecular weight (130-180 kd) protein found in all egg-laying vertebrates, synthesized and secreted by the liver, transported by the blood to developing oocytes in the ovary where it is actively transported into oocytes and cleaved into the two primary components of the egg yolk, phosvitin and vitellin (Chen,

1983; Silversand and Haux, 1989; Tyler, et al. 1988a; Tyler, et al. 1988b; Babin and Vernier, 1989; deVlaming, et al. 1984; Ho, 1991). A number of highly specific immunoassays have been developed for measuring Vtg, though none have been developed for use with fathead minnow vtg (So, et al. 1985; Copeland, et al. 1986; Giorgi, et al. 1982; Benfey, et al. 1989). Alkaline-labile phosphorous, as *ortho*-phosphate released from Vtg and other plasma proteins, was extracted from 10 µl plasma according to the method of Wallace and Jared (1968) using 1/2 the recommended volumes. *Ortho*-phosphate was determined by colorimetric assay of the acidified phosphomolybdate complex using a commercially available kit (Sigma Cat. No. 670-A).

Water Quality

Four general water quality parameters were measured throughout the exposure period, dissolved oxygen and water temperature on every other day and pH and alkalinity weekly. Alkalinity was determined by titration of bromcresol green indicator to a pH of 4.3 according to APHA Method 403 (APHA, 1985). 17ß-estradiol was measured on every other day of the exposure period. 5.0 ml of water was volumetrically sampled from each tank and stored in 13 x 100 mm test tubes capped with Teflon® lined lids at -80°C until analysis. Extraction of E2 from the water was accomplished by two 2 ml extractions with diethyl ether. After combining the ether extracts, the solvent was evaporated by a gentle stream of nitrogen at room temperature. E2 was redissolved in 0.5 ml buffer and quantified by ELISA (Cayman Chemical, Ann

Arbor, MI).

Statistical Analysis

Quality control parameters for the measurement of E2 included relative standard deviation, limit of detection and recovery (Figure 17). Recovery could not be quantified for Vtg analysis because characterized standards for fathead minnow Vtg are unavailable. However, recovery of phosphate from proteinaceous samples was estimated by Martin and Doty (1949) at approximately 75% using the phosphmolybdate colorimetric assay.

The effect of waterborne E2 on egg production was analyzed by ANOVA and nonlinear regression. One-way ANOVA tested the hypothesis that egg production was not affected by E2 treatment. The Lowest Observed Effect Level (LOEL) was determined by Dunnett's t tests ($\alpha = 0.05$) as the lowest treatment level that caused a statistically significant difference in egg production compared to the control and solvent control treatments, since there was no effect of solvent on egg production. The No Observed Effect Level was determined as the highest treatment level that did not cause a statistically significant difference in egg production. The Maximum Allowable Toxicant Concentration (MATC) was calculated as the geometric mean of the LOEL and NOEL and represents the expected threshold for effects. Because ANOVA utilizes nominal concentrations to define levels, information on the actual concentration-response curve is lost. Therefore, a nonlinear regression technique was used to model the concentration-response after a normal

distribution function (Equation 8, Bruce and Versteeg, 1992) utilizing the measured values of E2 in one case and the nominal values in a second case. Because the nominal values of E2 for the control and solvent control

$$Eggs = Eggs_{max} \times P\left[\frac{\log(ECX) - \log(E2)}{SLOPE} + Z_X\right] \quad (8)$$

where: Eggs = Egg production, eggs laid/female;

log(E2) = $\log_{10}$ E2 concentration, ng/L;

P[ ] = normal probability density function;

$Eggs_{max}$ = maximum egg production;

log(ECX) = $\log_{10}$ Effective Concentration causing X% inhibition of luciferase expression, ng/L;

SLOPE = slope function (standard deviate) of normal probability density function.

Z_X = normal deviate above which X% of the standard normal distribution lies, for X = 5, 20, 50, Z_X = 1.6449, 0.8416, 0.

treatments were 0, they could not be defined on a log-concentration scale. Therefore, the nonlinear regression model using nominal concentrations was modified to include the response information from the controls (Equation 9). Using the nonlinear regression model, the NOEL and LOEL were estimated, respectively, as the EC5 and EC20 (after back-transformation from the log(EC5) and log(EC20) estimates produced by the model). The EC50 was also estimated. Induction of vitellogenin, as indicated by plasma alkaline-labile phosphorous (ALP), was also modelled by nonlinear

$$If\ E2 > 0\ then\ Eggs = Eggs_{max} \times P\left[\frac{\log(ECX) - \log(E2)}{SLOPE} + Z_X\right]$$

$$If\ E2 = 0\ then\ Eggs = Eggs_{max} \qquad (9)$$

where: all variables are as previously described.

regression after a normal distribution function (Equation 10). However, this model was applicable only to the responses of the male fish because female fish did not exhibit a plateau of ALP with increasing E2 concentration. For female fish, a log-linear model was fitted to the linear portion of the response curve, from which the threshold for effects concentration could be estimated.

$$ALP = (ALP_{max} - ALP_{min}) \times P\left[\frac{\log(E2) - \log(ECX)}{SLOPE} - Z_X\right] + ALP_{min} \qquad (10)$$

where: ALP = alkaline-labile phosphorous in plasma, mg/ml as P;

ALP_{max} = maximum plasma ALP, mg/ml;

ALP_{min} = minimum plasma ALP, mg/ml;

and all other variables as defined above.

The relationship between ALP and egg production was analyzed by log-linear regression. These models related a biomarker of estrogen exposure, ALP, with a reproduction parameter, egg production, as a means of establishing a predictive relationship that may be useful in monitoring environmental exposure.

Results and Discussion

Mortality

A total of 9 fish died during the exposure period (Table 10). Most of the mortalities occurred in the last week of the exposure period, though one female from the solvent control treatment died on the first day of exposure. Four males of 12 exposed to 272.4 and 27.24 ng/L nominal E2 died during the exposure period, apparently due to the effects of the treatment, while only two females of 12 in this group died, which suggests a sex related difference in

Table 10. Fathead minnow mortality occurring during the exposure period.

Tank	Nominal [17ß-Estradiol] (ng/L)	Day of Exposure	Sex	Possible reason for death
1	2724	9	Male	Toxicosis
1	2724	12	Male	Toxicosis
1	2724	14	Female	Toxicosis
2	2724	9	Male	Toxicosis
2	2724	13	Female	Low DO, aeration interrupted
3	272.4	No deaths	No deaths	
4	272.4	16	Male	Toxicosis
5	27.24	No deaths	No deaths	
6	27.24	6	Female	Male aggression, crowding
7	Solvent Control	No deaths	No deaths	
8	Solvent Control	1	Female	Male aggression, crowding
9	Control	No deaths	No deaths	
10	Control	16	Female	Male aggression, crowding

mortality caused by exposure to E2. However, because this study was not designed to assess acute toxicity, the sample sizes were small and cannot be used to definitively determine acute toxicity of E2.

Water Quality

General water quality parameters were uniform over all tanks throughout the exposure period (Table 11). Dissolved 17ß-estradiol was detected in all tanks including control and solvent control tanks (Figure 17). Two possible sources of E2 in the control and solvent control tanks was: 1) excretion of E2 and E2 metabolites by females, and; 2) dissolution of E2 from fish meal in the food. The measured concentrations of the E2-treated tanks were 79 ± 25% (mean ± SE) of the nominal concentrations.

Egg Production

Fathead minnow egg production was reduced in a concentration dependant manner by exposure to waterborne 17ß-estradiol over a 13 day period of breeding (Figure 18). The reduction was statistically significant when analyzed by ANOVA using pooled control and solvent control responses ($F = 4.46$, $p < 0.056$). Dunnett's t tests revealed that only the egg production at the highest nominal concentration of E2, 2724 ng/L, was significantly reduced with respect to the pooled control egg production. The estimate of MATC using the ANOVA results was much greater than the MATC estimated

Table 11. Water quality during the exposure period, mean(SE).

Tank	pH	Alkalinity (mg $CaCO_3$/L)	Dissolved Oxygen (mg/L)	Temperature (°C)	17ß-Estradiol (ng/L)	
	N=3	N=3	N=19	N=19	Measured N=10	Nominal
1	8.17 (0.38)	310 (3.3)	7.2 (0.2)	26.1 (0.04)	1470 (652)	2724
2	8.19 (0.38)	302 (6.2)	7.3 (0.04)	26.1 (0.04)	870 (281)	2724
3	8.15 (0.35)	318 (6.7)	7.3 (0.04)	26.1 (0.04)	517 (426)	272.4
4	8.16 (0.36)	325 (17.6)	7.3 (0.05)	26.1 (0.03)	105 (15)	272.4
5	8.19 (0.38)	302 (8.5)	7.3 (0.05)	26.1 (0.04)	13.0 (1.7)	27.24
6	8.13 (0.35)	328 (11.7)	7.3 (0.06)	26.1 (0.04)	30.6 (9.9)	27.24
7	8.19 (0.38)	327 (4.4)	7.3 (0.05)	26.0 (0.04)	6.05 (2.50)	0
8	8.20 (0.38)	318 (4.3)	7.3 (0.04)	26.1 (0.04)	3.54 (0.70)	0
9	8.15 (0.33)	322 (3.3)	7.4 (0.04)	26.0 (0.04)	14.8 (4.5)	0
10	8.05 (0.29)	325 (2.9)	7.4 (0.08)	26.0 (0.07)	9.67 (3.80)	0

by nonlinear regression (Table 12). However, the use of nominal versus measured E2 concentrations in the nonlinear models produced nearly identical regression curves and estimates of EC50, EC20 and EC5 that were not statistically different (Figure 18). In fact, the standard error of the estimates was greater using the measured E2 concentrations, probably because the estimation of the maximum egg production level was less certain than that derived from the nominal E2 concentration model. The two methods of calculating the MATC, ANOVA vs. regression, produced widely differing values because ANOVA is a very conservative estimation method and regression is a generally less conservative method. Because ANOVA is strongly affected by

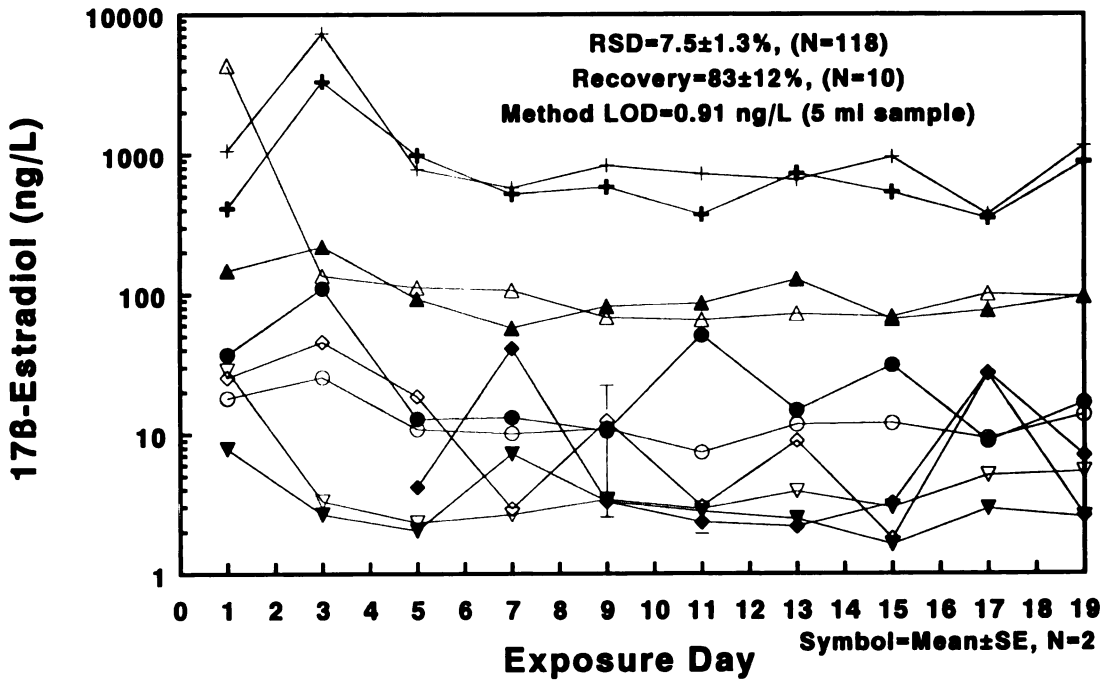


Figure 17. 17ß-Estradiol measured by ELISA during exposure period, RSD = Relative standard deviation of duplicate measurements, Method LOD = Method limit of detection, Recovery = recovery of spiked samples containing 2724 ng/L E2; + = Tank 1, + (bold) = Tank 2, △ = Tank 3, ▲ = Tank 4, ○ = Tank 5, ● = Tank 6, ▽ = Tank 7, ▼ = Tank 8, ◇ = Tank 9, ◆ = Tank 10.

variation at each level, significant differences in levels are difficult to detect, especially with highly variable data such as egg production. The regression model incorporates the variation over the entire response curve, thereby reducing the effect of individual variability on the estimate of MATC. It is important to note that there is no general consensus on what level of effect

should be defined as NOEL and LOEL when using a regression model. EC5 and EC20 were selected to estimate NOEL and LOEL based upon the suggestions of Bruce and Versteeg (1992). A 5% change (EC5) probably represents the threshold below which effects can reasonably be expected to be biologically unimportant and therefore was selected as the NOEL. A 20% change (EC20) may be considered a reasonable level of effect beyond which detrimental biological consequences could be expected and was chosen as the LOEL. Because of the significant variability in egg production within each level, the use of ANOVA for determining an MATC was probably the better method.

Vitellogenin Induction as a Biomarker of Reproductive Impairment

Vitellogenin expression, as indicated by plasma ALP, was significantly induced by exposure to waterborne 17ß-estradiol in both female and male fathead minnows (Figure 19). The threshold concentration of E2 for induction of vitellogenin was similar in females (46 ng/L E2) and males (51 ng/L E2) though females exhibited a baseline ALP level 6 times that of males (0.4726 mg/ml P females vs. 0.078 mg/ml P males). As a biomarker of reproductive impairment by waterborne 17ß-estradiol, log(ALP) was significantly correlated with egg production. Male log(ALP) was slightly less predictive of egg production (Figure 20B), explaining 50% of the variation ($r^2 = 0.50$), than female log(ALP) which explained 62% of the variation in egg production (Figure 20A). The observation that the trend in reduction of egg production continued into the lowest concentrations of ALP suggests that induced plasma vitellogenin could

Table 12. Calculation of No Observed Effect Level (NOEL) or EC5, Lowest Observed Effect Level (LOEL) or EC20, Maximum Allowable Toxicant Concentration (MATC), and EC50 using ANOVA and nonlinear regression of nominal and measured 17ß-estradiol concentrations for reduction of egg production of fathead minnows.

Method	NOEL / EC5 (ng/L)	LOEL / EC20 (ng/L)	MATC (ng/L)	EC50 (ng/L)
ANOVA	272.4	2724	861	--
Nonlinear/Nominal	7.4	33.6	15.7	164
Nonlinear/Measured	3.0	18.2	7.3	122

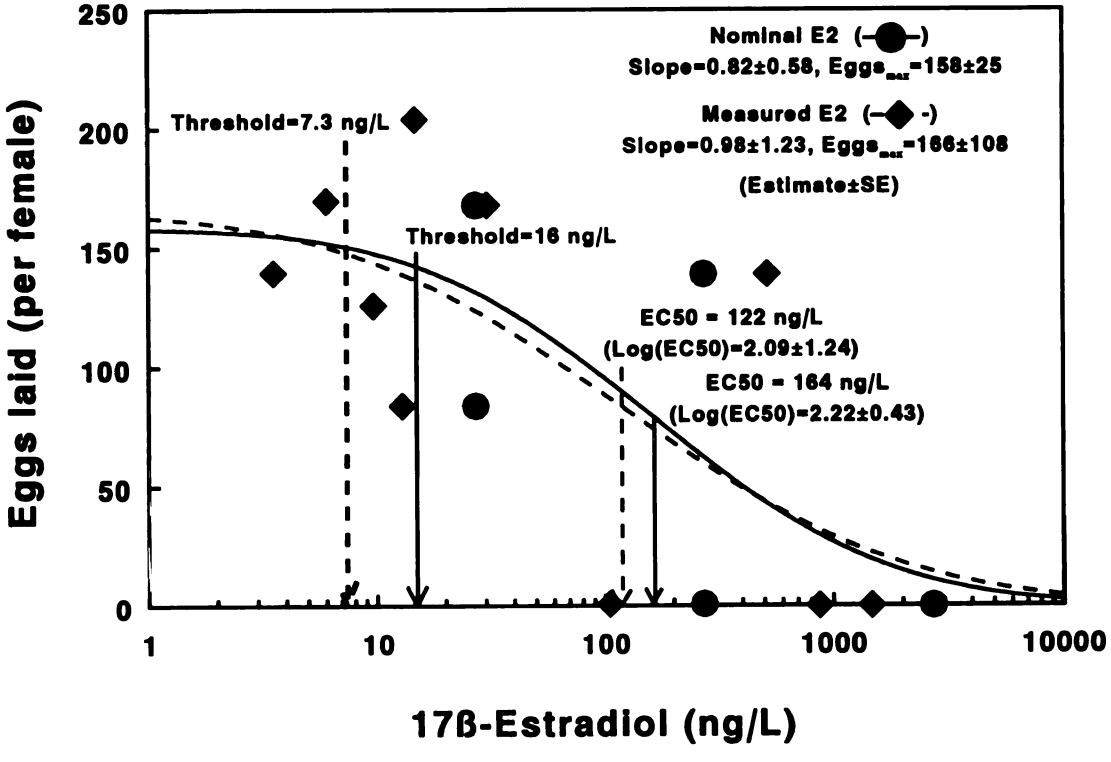


Figure 18. Reduction of egg production by exposure of fathead minnows to waterborne 17ß-estradiol (E2) modeled by nonlinear regression after a normal distribution function; ◆ = E2 measured in each tank by ELISA, ● = E2 nominal concentration.

prove to be a sensitive indicator of potential reproductive impairment in fish.

Mechanism of Reproductive Impairment Caused by 17ß-Estradiol

17ß-estradiol is the most potent of the natural occurring estrogens. Therefore, this study provides a valuable benchmark for determining the type of effects expected to be caused by substances acting through the estrogen receptor. 17ß-estradiol significantly affected the physiology of both male and female fish, as evidenced by elevated plasma ALP and reduced egg production. Because this study was not designed to fully investigate the biochemical and physiolgical actions of E2 underlying the reduction of egg production, the mechanism of this action remains to be fully elucidated. However, it seems likely that the reduction in egg production was related to effects in both males and females. Estrogen injection of male fish caused feminization of secondary sexual characteristics, testicular degeneration and inhibition of spermatogenesis in several fish species (Matty, 1985). In this experiment, waterborne exposure to 17ß-estradiol caused reduction of male secondary sexual characteristics and testicular degeneration(Miles-Richardson, 1996). Although the changes in testicular histology probably reduced spermatogenesis and male fertility, it was not determined whether the changes in secondary sexual characteristics could have significantly altered male sexual behavior, which is critical for succesful laying of eggs. Reduction in the male "libido" by estrogen exposure remains a possible mechanism of reduction of egg production. Overt toxicity was suggested by the mortality affecting males at the highest E2 exposure

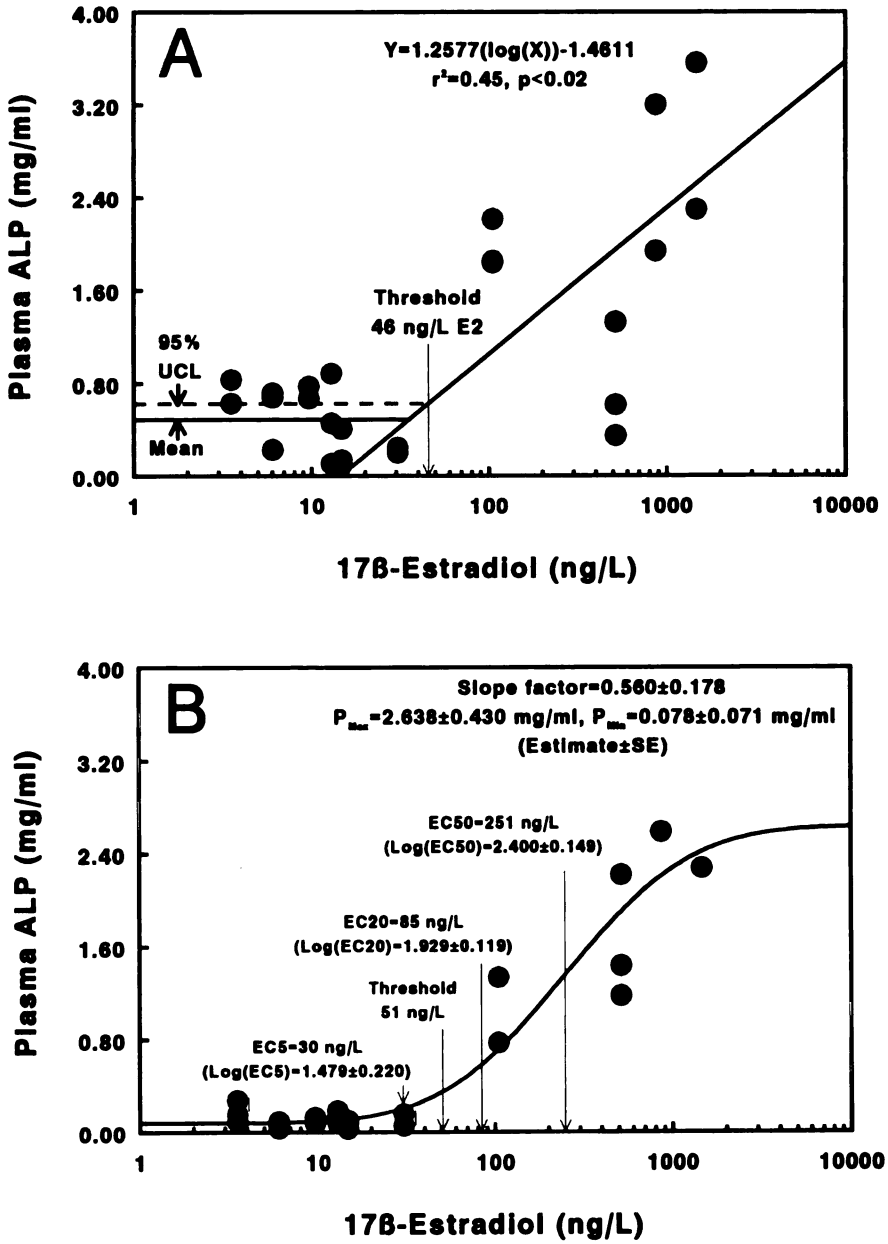


Figure 19. Induction of plasma alkaline-labile phosphorous (ALP), an indication of vitellogenin expression, in female (A) and male (B) fathead minnows exposed to waterborne 17ß-estradiol (ELISA measured concentrations of E2); in A, o = measurements not included in log-linear regression, sub-threshold mean = 0.473 mg/ml P, 95% UCL = 95% upper confidence limit on the sub-threshold mean, threshold = intersection of regression line with 95% UCL; in B, threshold = geometric mean of log(EC5) and log(EC20), nonlinear regression used to model apparent plateau.

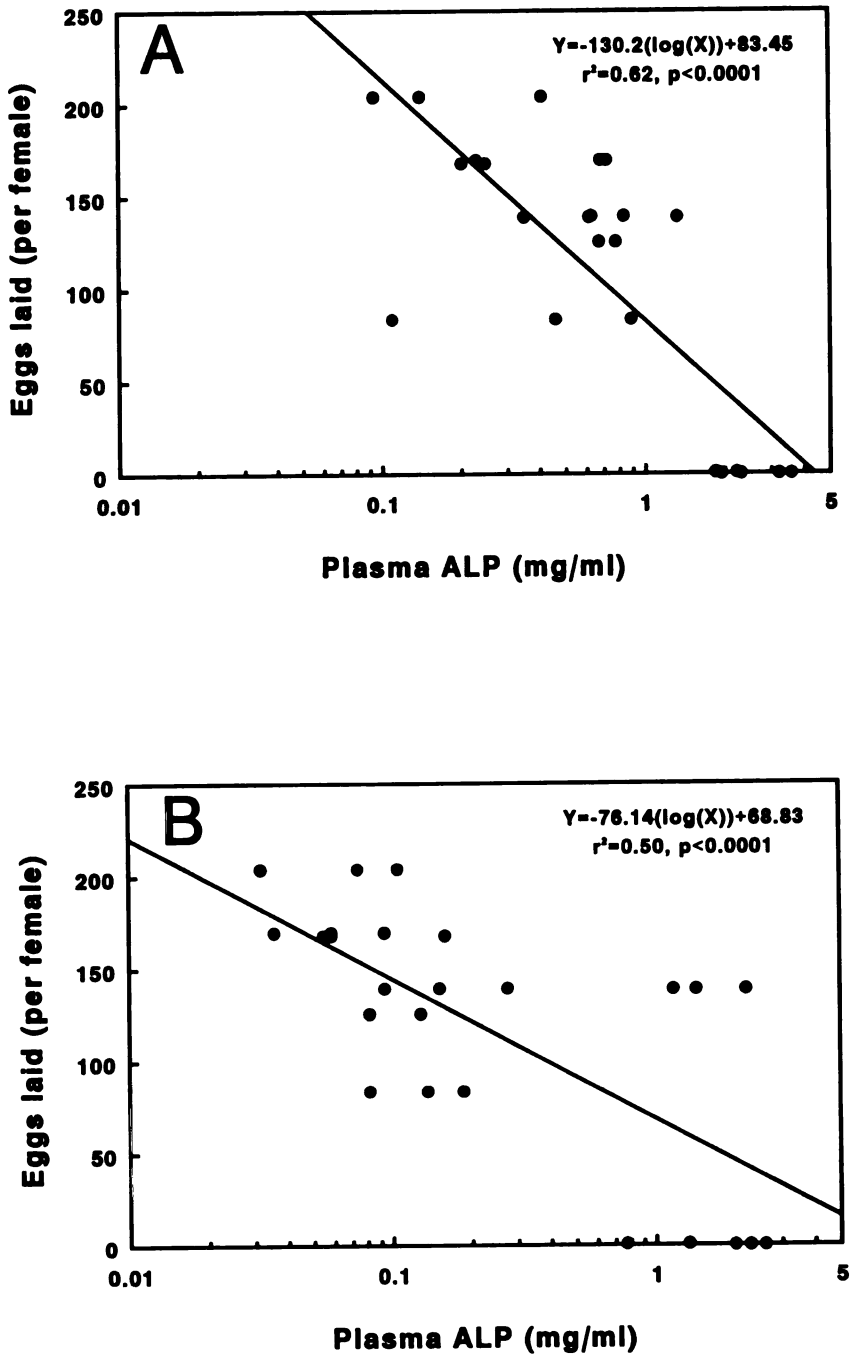


Figure 20. Correlation between egg production and plasma alkaline-labile phosphorous in female (A) and male (B) fathead minnows exposed to waterborne 17ß-estradiol.

concentrations. The relatively great induction of vitellogenin in males may have been related to this mortality (Herman and Kincaid, 1988; Carragher and Sumpter, 1991).

The female gonads were also affected by 17ß-estradiol exposure. Increased follicular atresia (breakdown of mature follicles), an increased prevalence of primary follicles and reduced mature follicles was related to E2 exposure (Miles-Richardson, 1996). The reduction of mature follicles in E2-exposed fish is directly related to a reduction in egg laying simply because fewer mature follicles were available for laying. In normally developing and spawning fish, a surge of plasma 17ß-estradiol initiates vitellogenesis and egg development well in advance of the spawning event, such that E2 levels return to background levels during spawning (Scott and Sumpter, 1983). In this experiment, E2 was administered after the female fish had been in the process of developing oocytes during the acclimation period. The effect of E2 adminstration appears to have been to "reset" the egg development "clock" by triggering atresia of mature follicles and initiating another sequence of vitellogenesis and egg development. Mortality of females appeared to have been unrelated to E2 exposure. The toxicity of vitellogenin postulated to occur in male fish was probably avoided in females because the developing eggs provided a location for the deposition of the vitellogenin, a process unavailable to male fish. Vitellogenesis was greatly induced in females exposed to E2 but was not at similar levels in unexposed females, even though unexposed females laid large numbers of eggs. Thus, unexposed females were probably not actively synthesizing vitellogenin and developing eggs at the end of the

exposure period. The primary effect of E2 exposure on female fathead minnows was to alter the timing of egg production, thereby blocking spawning during the exposure period of the experiment. In an environmental setting, the exposure of gravid female fish to an estrogenic substance could reset the reproductive cycle and cause the female to miss critical windows of opportunity for successful spawning, especially in seasonally reproducing species. Also, vitellogenin induction in male fathead minnows was correlated with E2 exposure and may have contributed to the excessive mortality of males. The results of this experiment firmly establish critical morphological, histological, and biochemical indicators of waterborne estrogen exposure and establish a relationship between these indicators with reproductive performance, an important parameter affecting fish populations in the environment.

SUMMARY AND CONCLUSIONS

Estrogen mimics are a ubiquitous component of the environment occurring in foods, plants, and fungi and in chemicals of human origin. Because estrogens play an important role in sexual development, behavior and reproduction, there is valid cause for concern over "disruption" of the normal activity of estrogens by both natural and synthetic estrogen mimics. However, this concern should be focused by issues of the dose and potency of the particular compounds thought to be estrogen mimics. This research has sought to develop a framework for evaluating the potential for substances to affect estrogen action. The molecular properties governing the affinity with which hydroxylated PCBs bind to the estrogen receptor was investigated, revealing that moderate precision in predicting binding affinity can be achieved with a relatively small set of general molecular parameters. However, it is important to note that receptor binding affinity is only one aspect of estrogen mimickry. Therefore, a functional bioassay was necessary to define the activity of the hydroxylated. The MCF-7-LUC cell line provided a rapid and specific assay for both estrogenic and anti-estrogenic activity. The results of the MCF-7-LUC assays demonstrated that receptor binding, while important in indicating which compounds had the potential to specifically interact with the estrogen receptor, could not predict whether a particular compound exhibited estrogenic or anti-estrogenic activity. Rather, it appears that very specific structural characteristics differentiate the mode of activity. Although the MCF-7-LUC cell line was useful in screening hydroxylated PCBs, alternative cell lines were

desired for testing on a more species-specific basis, such as the rainbow trout cell line RTH-149. Therefore, development of an estrogen-inducible reporter gene system was attempted for introduction in fish cell lines. Unfortunately, the plasmid system adopted in this research proved to be incompatible with the estrogen responsive elements that were introduced into the system, even though a similar arrangement was successful using dioxin responsive elements. The specific mechanism by which the estrogen responsive elements caused reduction in luciferase expression was not elucidated because it was beyond the scope of this work. However, it may prove to be an interesting example of estrogen receptor induced repression of gene expression. Finally, a functional bioassay using whole animals was developed. Fathead minnows were selected because of their favorable breeding characterstics to serve as a model organism for testing the effects of waterborne xeno-estrogen exposure on reproduction. The research project completed study on the benchmark estrogen, 17ß-estradiol, and identified a suite of responses at the biochemical, physiological, and histopathological levels that will be useful in characterizing exposure to estrogenic compounds in the aquatic environment.

APPENDIX

APPENDIX

Standard Operating Procedure

Estrogen Receptor Preparation and Competitive Binding Assay

Version 2.1 February 23, 1995

Prepared by: Vincent J. Kramer

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Department of Fisheries and Wildlife
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East Lansing, Michigan 48824-1222

A. Preparation of estrogen receptor from tissue or cell culture

1. Remove tissue (for example, calf uterus, fish liver etc.) from animal and quickly freeze in liquid nitrogen unless tissue is to be processed immediately. For cell culture, see Preparation of ER from cells in culture.

2. All procedures should be performed at 4 °C. Mince tissue to blocks about .5 cm in width in an equal volume of homogenization buffer.

Homogenization buffer
10 mM Tris HCl pH 7.5
1.5 mM Ethylenediaminetetraacetic acid, disodium salt (EDTA)
1 mM Dithiothreitol (DTT)
10 % glycerol, v:v
1 mM Sodium Molybdate
0.1 mM Phenylmethylsulfonyl fluoride (PMSF) or
0.5 mM Aminoethylbenzenesulfonyl fluoride (AEBSF)

Note: AEBSF is more stable and soluble than PMSF.

3. Homogenize thoroughly on ice with mechanical homogenizer (for example Tekmar Tissuemizer) , 5 min. pulsed on high setting to avoid heating the sample.

4. Centrifuge first at 4 °C, 800 g, 20 min. to pellet debris. Centrifuge supernatant at 4 °C, 100000 g (42000 rpm Beckman Ti70 rotor) 30 min

to produce "cytosolic" fraction.

5. If it is suspected that the receptor preparation contains endogenous 17ß-estradiol (as in tissue of adult organisms) the endogenous E_2 must be removed by charcoal stripping.

5a. Prepare Dextran Coated Charcoal (DCC):
0.5 % Norit A charcoal 50 mesh w:v
0.05 % Dextran, 70 Kd w:v
in TEDG buffer.

5b. Add 1 ml DCC to 10 ml supernatant and warm to room temp and hold at room temperature 5 min. to release E_2 from receptor.

Note: Estrogen receptor is heat labile.

5c. Centrifuge at 5000 g, 10 min, 4 °C.

6. Aliquot supernatant 1 ml each into 1.5 ml microcentrifuge tube, freeze at -80°C until used.

7. Perform Scatchard analysis (See Part B) and protein concentration measurement characterize preparation.

B. Competitive binding assay using radio-labelled 17ß-estradiol

This procedure is adapted from Murdoch et al. 1990. It measures the inhibition of binding of the native ligand, 17ß-estradiol, to the estrogen receptor by a competitor or mixture of competitors. The ligand is radio-labelled so that the amount of radioactivity bound to the estrogen receptor is related to the extent of inhibition of binding by the competitor. For example, a high affinity competitor will displace most of the radio-labelled ligand resulting low radioactivity bound to the receptor. The amount of radioactivity bound to the receptor is determined by adsorption of the protein (and protein-bound radioactivity) in the assay mixture to hydroxyapatite which is then quantitated by scintillation counting. Since the radio-labelled ligand also binds non-specifically to other proteins in the assay mixture, a separate experiment must be performed to quantify the non-specific binding. This is accomplished by displacing 100% of the radio-labelled ligand from the estrogen receptor with a high concentration of the non-steroidal estrogen diethylstilbestrol which binds specifically to the estrogen receptor. Total binding must also be assessed by measuring the binding of radio-labelled ligand in the absence of competitor.

Note: All steps in the following procedure must be carried out at 4 °C. Therefore, use of a cold room is highly recommended since cooling the

filter apparatus and solutions is cumbersome on the bench top.

1. Prepare/label 10 glass test tubes (15 X 85 mm borosilicate culture tubes) as follows:

1a. Tube 1, "T", total binding measurement;

1b. Tubes 2-9, "Competitor concentration, nM", concentration of competitor in assay mixture;

1c. Tube 10, "NS", Non-specific binding measurement.

2. Prepare buffer and working solutions.

2a. ^{3}H-17ß-estradiol working solution: Make 500 μl 250 nM ^{3}H-E_2. Stock solution is approximately 10 μM ^{3}H-E_2 (DuPont-NEN, 2,4,6,7-^{3}H-17ß- estradiol, 100 Ci/mmol, in ethanol). The exact volume of stock solution used will vary as determined by the specific activity and concentration of the stock solution which is unique to each lot of ^{3}H- E_2. The final ethanol concentration is ~2.4%.

2b. Diethylstilbestrol working solution: 1 mM in ethanol.

2c. Competitor working solutions: Highest concentration-10 mM or limit of solubility. Dilute subsequent solutions serially by 1/10th such that a minimum of four concentrations are tested spanning four orders of magnitude (for example 10 mM, 1 mM, 100 μM, 10 μM). All solutions should be dissolved in 100% ethanol. When diluted in assay test tube, the concentrations will be 1/100th of the working solution concentration.

Note: If the competitor is insoluble in ethanol, 1-propanol has been used successfully (for example, to dissolve coplanar PCB's and chlorinated naphthalenes).

2d. TEDG assay buffer-Make 1 L for an assay of 10 test tubes. Sterile filter and store at 4 °C.

TEDG Assay Buffer

10 mM Tris HCl, pH 7.5
1.5 mM Ethylenediaminetetraacetic acid, Na_2
1 mM Dithiothreitol
10% glycerol v:v

2e. Hydroxyapatite (HAP) slurry: 160 mg dry HAP/ml TEDG. Store at 4 °C.

3. Add to each test tube in order:

3a. 900 µl TEDG assay buffer

3b. 10 µl competitor (Tubes 2-9), solvent (Total binding measurement, Tube 1), or DES (Non-specific binding measurement, Tube 10).

3c. 40 µl 250 nM ^{3}H-E_2

3d. 50 µl ER preparation (Final ER concentration should be about 1 nM or less. This is dependant upon specific activity of the ER preparation).

Note: The final solvent concentration in the assay is 1%.

4. Mix well by gentle swirling, incubate 4 °C, 2 hours, in dark.

5. Add 300 µl HAP slurry to adsorb proteins.
Mix well by hand and incubate 15 min, 4 °C, mixing every 5 min.

Note: Do not vortex solutions containing radioactive materials because aerosolization will occur posing the risk of inhalation of radioactivity.

6. Filter HAP out of solution using a 10 place filter manifold (Hoefer Scientific) with stainless steel holders, 24 mm Whatman GF-C glass fiber filters.

Note: Do not use Gelman glass fiber filters because they are too brittle.

Note: Install an aerosol trap between the filter manifold and the vacuum source to prevent radioactive contamination of vacuum source.

7. Wash HAP 6 times with 5 ml TEDG.

Note: This is a critical step in the procedure. All unbound radio-label must be rinsed from HAP to insure reproducibility.

8. After final wash, rinse down sides of filter holder with 1 ml TEDG and allow vacuum to dry filter slightly. Add 1 drop TEDG to the center of

each filter to ease removal of filter disc from apparatus. Add any HAP adhering to the filter holder to the scintillation vial.

9. Place filter with HAP in scintillation vial. Add 6 ml scintillation cocktail (Safety-Solve for aqueous samples). Shake vigorously. Allow sample to stand at room temperature overnight before counting to allow scintillation fluid to completely extract ^3H-E_2 from HAP. Count tritium using scintillation counter.

10. Prepare blanks and standards with HAP and filter in vial to mimic quenching of sample. A typical standard consists of 1 μl 250 nM ^3H-E_2 Working Solution (expected disintegrations per minute (dpm) is about 62,000 depending upon specific activity of stock solution which is different for every lot). Counting efficiency is typically approximately 0.35 but must be determined for every experiment.

C. Data analysis

Data is reduced and summarized using a spreadsheet program The probit method for calculating IC50 (Bruce and Versteeg, 1992) is executed using the statistical software package SAS (Appendix 2).

1. Subtract blank cpm from all samples.

2. Convert to pmol ^3H-E_2 bound for all samples.

3. Subtract non-specifically bound ^3H-E_2 (the reading from the "NS" tube) from all samples to determine specifically bound ^3H-E_2.

4. Calculate IC-50 (concentration of competitor causing 50% reduction in specific binding of ^3H-E_2) using probit method for continuous data (Bruce and Versteeg, 1992).

5. Scatchard analysis is performed to determine Kd and Bmax for the receptor preparation only. The typical analysis consists of measuring specific binding at several concentrations of radio-labelled estradiol, typically an order of magnitude above and below the expected Kd (approximately .1 nM). The data is plotted according to the equation:

$$B/F = Bmax/Kd - 1/Kd * B$$

where B = specifically bound ^3H-E_2, pM; F = free (unbound) ^3H-E_2, pM; Bmax = maximum specifically bound ^3H-E_2; Kd = the dissociation constant, pM (Clark and Peck, 1979).

SAS Program for Analysis of Competitive Binding Curves

```
/* Non-linear regression model for continuous */
/* toxicity data. Bruce and Versteeg. 1992. ET&C 10: 1485-1494*/
/* Models a cumulative normal distribution */
/* This version is for analysis of estrogen receptor competitive */
/* binding curves. */
/* c# = tritiated estradiol bound specifically, pM, for competitor c*/
/* non-specific binding already subtracted from total bound */
/* bmax = maximum tritiated estradiol bound, pM */
/* specifically, also upper limit of displacement curve */
/* log(icXX) = log10 of the icXX, usually XX = 50% */
/* sigma = slope function of inhibition curve */
/* NOTE: Original code used the Gauss searching algorithm. */
/* This version uses DUD to avoid problems associated with */
/* small N and erratic der.sigma */
/* ALSO NOTE: The "nohalve" option was removed, this improved */
/* stability of search. */
data a;
 input i c1  date$;
/* date = date data collected */
/* i = inhibitor concentration, nM */
 if i = 0 then log_i = .;
  else log_i = log10(i);
cards;
 0.0  100.0   6/12
 1.0  99.76   6/12
 10   93.08   6/12
 100  66.32   6/12
 500  44.79   6/12
1000  28.99   6/12
5000  37.74   6/12
10000 10.29   6/12
50000  3.20   6/12
0.0   104.31  6/22
1.0   115.12  6/22
10.0   97.94  6/22
100    61.66  6/22
500    55.08  6/22
1000   57.75  6/22
5000   42.29  6/22
10000  25.31  6/22
50000  20.41  6/22


title 'Competitive binding curve calf uterine estrogen receptor';
```

```
data b;
 set a end=last;
 output;
if last then do;
 c1=.;  i=.;
 do log_i=-1 to 5 by .25;
 output;
end;
end;
proc print data=b;
 var i log_i c1 ;

proc nlin data=b method=dud maxiter=300 smethod=cubic converge=1e-10
;

 title2 'model:c1=bmax*P((logIC50-log_i)/sigma)';
 title3 'weighted regression er=calf uterine';
 parms log_ic50=2.5 sigma=1.4 bmax=100;
 bounds sigma>1e-10, bmax>0;
 if i ne 0 then do;
  z=(log_ic50-log_i)/sigma;
  ex=z;
  px=probnorm(ex);
  model c1=bmax*px;
 end;
 if i=0 then do;
  model c1=bmax;
 end;
 output out=d predicted=pred;
proc plot data=d;
 plot c1*log_i='0'
      pred*log_i='.'/overlay;
      title2;
proc print data=d;
 var i log_i c1 pred;
 run;
```

Preparation of ER from cells in culture

1. Four days before harvesting ER from cultured cells (for example, MCF-7 cells), change medium from 10% FBS Medium (the normal culture medium) to 5% DCCFBS Medium. Grow up at least twenty (20) 100 mm diameter culture plates (75 cm^2) to make about 10 ml of ER preparation.

2. One day before harvesting, switch medium to Serum-free Medium. This causes the cells to depurate 17β-estradiol via metabolism.

10% FBS Medium
Dulbecco's Modified Eagle's Medium/
Ham's F12 Nutrient Mixture
without Phenol Red and sodium bicarbonate
with 15 mM HEPES buffer and L-glutamine
(Sigma D2906)
10% Fetal bovine serum (Hyclone)
2 mM Sodium pyruvate
1 μg/ml Bovine insulin
10 U/ml:10 μg/ml Penicillin:Streptomycin

5% DCCFBS Medium
Same as 10% FBS Medium except replace
10% Fetal Bovine Serum with
5% Dextran-coated charcoal stripped FBS
(available from Hyclone Laboratories)

Serum free Medium
Same as 10% FBS Medium except omit
10% Fetal Bovine Serum

3. Remove cells from plate surface by incubating cells in 1 mM EDTA in Ca-Mg free phosphate buffered saline for 30 min at 37 °C. Cells should lift easily off plate without scraping. Transfer cells to 15 ml conical centrifuge tubes at room temperature. Combine cells from 20 plates.

4. Centrifuge at 800 g at room temperature 10 min. to pellet cells.

5. Discard supernatant, wash cells once with Homogenization Buffer as in Step 4.

6. Resuspend cells in 2 ml ice-cold Homogenization Buffer and transfer to either a Potter-Elvejhem homogenizer with Teflon Pestle or a Dounce Homogenizer (whichever is available).

7. Disrupt cells with 60 passes of the pestle on ice. Rinse pestle when completed with 1.5 ml Homogenization buffer into the homogenate.

8. Transfer homogenate to 15 ml conical centrifuge tube on ice. Rinse homogenizer with 1.5 ml buffer and combine with homogenate.

9. Centrifuge 10 min. 800 g at 4 °C to pellet nuclei and debris.

10. Transfer supernatant ("cytosol") to 30 ml polycarbonate ultracentrifuge tube. Wash pellet with 1 ml Homogenization buffer and centrifuge as in Step 9. Combine supernatant with homogenate in ultracentrifuge tube.

11. Centrifuge "cytosolic" fraction at 30 min. 42000 g at 4 °C in Beckman L7 Ultracentrifuge or appropriate substitute.

12. Transfer supernatant to 15 ml conical centrifuge tube or an appropriate number of 1 ml centrifuge tubes for storage at -80 °C (or in liquid nitrogen if available).

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