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Detection of Estrogen Receptor Endocrine Disruptor Potency of Commonly Used Organochlorine Pesticides Using The LUMI-CELL ä Bioassay

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Introduction

Organochlorine pesticides are found in many ecosystems worldwide as result of agricultural and industrial activities and exist as complex mixtures. The use of these organochlorine pesticides has resulted in the contamination of lakes and streams, and eventually the animal and human food chain. Many of these pesticides, such as pp'-DDT, pp'-DDE, Kepone, Vinclozolin, and Methoxychlor (a substitute for the banned DDT), have been described as putative estrogenic endocrine disruptors, and act by mimicking endogenous estrogen[1-3].

Estrogenic compounds can have a significant detrimental effect on the endocrine and reproductive systems of both human and other animal populations[4]. Studies by Jefferson et al, (2002) have shown a strong association between several EDCs (17ß-Estradiol, DES, Zeralanol, Zeralenone, Coumestrol, Genistein, Biochanin A, Diadzein, Naringenin, Tamoxifin) and estrogenic activity via uterotropic assay, cell height, gland number, increased lactoferrin, and a transcriptional activity assay using BG1Luc4E2 cells (provided by Xenobiotic Detection Systems International Inc. (XDS), Durham, NC)[4]. Some other examples of the effects of these EDCs are: decreased reproductive success and feminization of males in several wildlife species; increased hypospadias along with reductions in sperm counts in men; increase in the incidence of human breast and prostate cancers; and endometriosis[3-5]. Because these chemicals are ubiquitous, highly lipophilic, and often chlorinated, it ensures their persistent presence in the environment (i.e. water supply, soil, river sediment) resulting in their bioaccumulation in the food chain[3,5,6]. Taken together, these data show a pattern of food chain contamination and the detrimental effects of EDCs, therefore understanding the estrogenic potency of these and other potential EDCs, is extremely important.

The association between the exposure and bioaccumulation of endocrine (hormone) disruptor chemicals (EDCs) and their adverse effects on human and wild life populations has raised concern worldwide. These concerns over the effects of environmental EDCs, lead to the passage of U.S. Congressional legislation (Food Quality Act of 1996 and Safe Water Reauthorization Act Amendments of 1996), which mandated the EPA to investigate the exposure to environmental EDCs[7,8]. Based on this mandate, the EPA established the Endocrine Disruptor Steering and Testing Advisory Committee (EDSTAC), a committee charged with defining the course of action to accomplish this goal. EDSTAC submitted their report to the U.S. Congress in August of 2000. Subsequent to this report, the EPA established the Endocrine Disruptor Screening Program (EDSP) within the agency. This EDSTAC report proposed that the EPA pursue the standardization and validation of Tier I (screening) and Tier II (testing) assays specific and sensitive for EDCs, which may act as agonists and/or antagonists. Therefore, there is a growing

need for a fast reliable high-throughput system for the screening of known and potential environmental contaminants, which act to disrupt normal endocrine homeostasis [9].

In order to detect the endocrine disrupting potency of organochlorine pesticides and other compounds using a bioassay system, Xenobiotic Detection System (XDS) in collaboration with Dr. Michael S. Denison (Univ. California-Davis), have developed the LUMI-CELL™ ER bioassay. BG-1 (human ovarian carcinoma) cells were stably transfected with an estrogen-responsive luciferase reporter gene plasmid (pGudLuc7ere) containing the estrogen responsive element (ERE) and luciferase reporter gene[6]. The resulting cell line (BG1Luc4E2), used in XDS's LUMI-CELL™ ER estrogenic cell bioassay system, responds in a time-, dose dependent- and chemical-specific manner with the induction of luciferase gene expression in response to exposure to estrogen and estrogenic chemicals in a high-throughput screening (HTPS) format[6]. In initial studies, BG1Luc4E2 cells were able to detect as little as 0.1pM of 17ß-estradiol[6]. In initial studies, treatment of BG1Luc4E$_2$ resulted in significant induction of luciferase activity for estrogen, diethylstilbesterol, 17ß-estradiol, o,p'-DTT, Bisphenol A, Nonylphenol, Genistein, and Diadzein. Luciferase activity was not increased by any other steroid tested (i.e. progesterone, testosterone, all-trans retinoic acid, thyroid hormone, and dihydrotestosterone)[6]. These results are consistent with previously published data indicating the lack of estrogenic activity of these chemicals [10-12], and clearly demonstrates the specificity of this system as a bioassay to detect estrogenic chemicals.

Here we describe studies in which XDS's LUMI-CELL™ ER estrogenic cell bioassay system was used for high throughput screening (HTPS) analysis of the estrogenic disrupting potency of several commonly used pesticides and organochlorines: p,p'DDT; p,p'-DDE; DDD; α-chlordane; ψ-chlordane; Kepone; Methoxychlor; Vinclozolin; Fenarimol; 2,4,5-Trichlorophenoxyacetic Acid; and Dieldrin. Our results demonstrate the utility of XDS's LUMI-CELL™ ER bioassay HTPS system for screening chemicals for estrogenic activity.

Methods and Materials

The majority of chemicals were purchase from the Aldrich Chemical Co., Sigma Chemical Corporation, and Chem Service Inc.

LUMI-CELLä ER Bioassay. The BG1Luc4E2 cell line was constructed as previously described by Rodgers and Dennison (2000) [6]. Briefly, BG1 cells were stably transfected with an estrogen-responsive luciferase reporter gene plasmid (pGudLuc7ere) and selected for using G418 resistance.

Cell Culture and Bioassay Plates. BG1Luc4E2 cells were grown in RPMI 1640. The cells were transferred into flasks containing DMEM media (supplemented with 5% carbon stripped fetal calf serum and G418 sulfate solution), and incubated for four days before harvesting for BG1Luc4E2 bioassay plates. The cells were then plated in 96 well plates and incubated at 37^o C for 24-48 hours prior to dosing.

Bioassay Dosing Process. Once the assay plate completed its incubation, the media solution in each well was removed and two hundred microliters of DMEM containing the indicated

concentration of the desired chemical to be tested (dissolved in DMSO) was added to each well. The plate was then incubated for 24 hours before analysis of luciferase activity.

Bioassay Analysis by Berthold Luminometer. After lysing the cells (Promega lysis buffer), the luciferase activity was measured in a Berthold Orion Microplate Luminometer, with automatic injection of 50 microliters of luciferase enzyme reagent (Promega) to each well. The relative light units (RLUs) measured were compared to that induced by the 17β-estradiol standard after subtraction of the background activity. Each compound was tested at least three times on three different sets of plates and the EC50 value in mmol/ml was determined using the Microsoft Excel Forecast function.

Discussion and Results

There is a growing concern for a need for a system fast, reliable, inexpensive method to detect estrogenic EDCs in the environment. This concern arises from the detrimental effects of EDCs on human and wildlife populations resulting from its bioaccumulation in the food chain. Here we report a fast, reliable, relatively inexpensive high throughput cell based recombinant bioassay screening method (LUMI-CELL™ ER bioassay) for xenoestrogenic EDCs.

Thirteen organochlorine pesticides suspected of possessing xenoestrogenic endocrine disrupting potential were tested using XDS's LUMI-CELL™ ER estrogenic cell bioassay system for this study. Four of these pesticides (p,p'-DDE, p.p'-DDT, Kepone, and Methoxychlor) were

Table 1: Compounds tested with XDS's LUMI-CELLTM bioassay, CAS numbers and their relative EC 50 values.

Compound	CAS Number	EC 50 Value	Relative Induction to b-Estradiol	Relative Induction to highest Pesticide
ß-Estradiol	50-28-2	1.55E-11 ± 6.93E-13	1.00E+00	-
a-Chlordane	12789-03-6	1.32E-06 ± 2.01E-07	1.17E-05	1.00
Kepone	143-50-0	1.38E-06 ± 3.97E-07	1.13E-05	0.96
DDD	72-54-8	1.71E-06 ± 5.10E-07	9.06E-06	0.77
pp' DDT	789-02-6	2.94E-06 ± 3.78E-07	5.28E-06	0.45
Metoxychlor	72-43-5	3.46E-06 ± 6.10E-07	4.48E-06	0.38
ψ-Chlordane	57-74-9	4.86E-06 ± 1.67E-06	3.19E-06	0.27
pp' DDE	72-55-9	6.74E-06 ± 2.58E-06	2.30E-06	0.20
Fenarimol	60168-88-9	8.11E-06 ± 1.26E-06	1.92E-06	0.16
2,4,5-Trichlorophenoxyacetic Acid	93-76-5	1.21E-05 ± 2.60E-06	1.29E-06	0.11
Dieldrin	60-57-1	1.39E-05 ± 8.51E-07	1.12E-06	0.10
Linuron	330-55-2	1.45E-05 ± 6.25E-06	1.07E-06	0.09
Mirex	2385-85-5	Non-active	N/A	N/A
Vinclozolin	50471-44-8	Non-active	N/A	N/A
Progesterone	57-83-0	Non-active	N/A	N/A

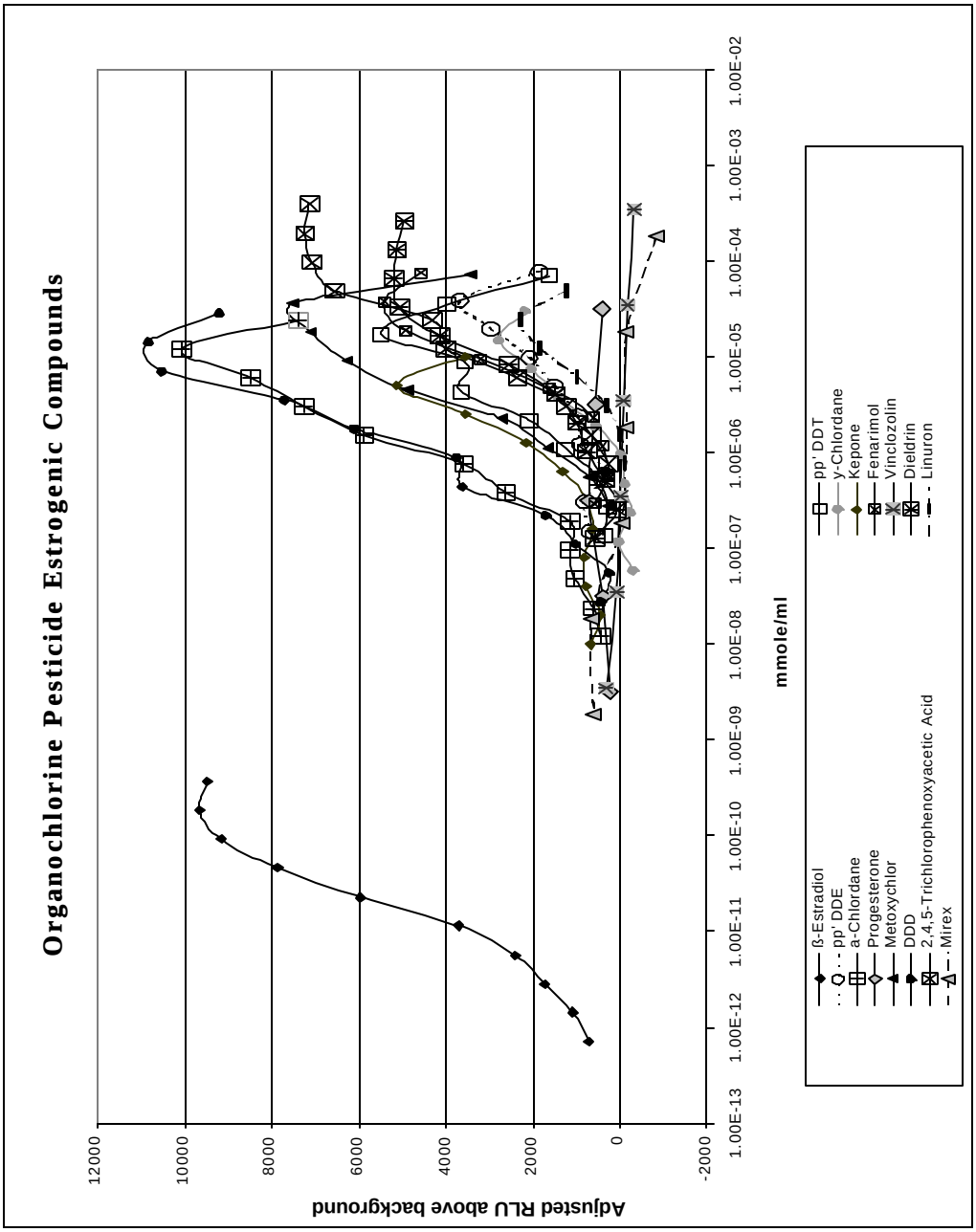

Figure 1. Organochlorine Pesticide compounds tested to demonstrate dose dependent response relationships using XDS's LUMI-CELL™ ER HTPS bioassay system for known and potential estrogenic EDCs.

recommended by ICVAM for validation of ER binding and transcriptional activation assays and are thought to posses estrogenic activity, thereby making them potential endocrine disruptors[2]. An additional six pesticides, not included in the ICVAM requirements for validation, which are found as environmental contaminants, were tested to determine their potential estrogenic activity[3,13-18].

In this study, all of the compounds with historical data suggesting that they may poses estrogenic activity were shown to poses estrogenic activity (Figure 1 and Table 1). Vinclozlin and Mirex were the only organochlorine pesticides not to demonstrate any estrogenic activity. This is not surprising given the results of previous studies showing Vinclozlin not to possess endocrine disruptive potency at concentration below 600 ppm[19].

When comparing the estrogenic potency of the organochlorine pesticides (Table 2), the order of induction of activity with respect to their EC50 values is α-Chlordane > Kepone > DDD > pp' DDT > Methoxychlor > ψ-Chlordane > pp' DDE > Fenarimol > 2,4,5-Trichlorophenoxyacetic Acid > Dieldrin > Linuron > Mirex = Vinclozolin.

For the most part organochlorines pesticides start to show significant induction of luciferase reporter gene activity at or below the 1 µM (or ~1ppm) (Fig. 1). This is lower than several other reports showing affect in animal studies. Okoumassoun et al. (2002), found a significant increase in vitellogenin, the serum phospholipoglycoprotein precursor to egg yolk, in rainbow trout hepatocytes and male rainbow trout when exposed to as little as 10 µM DDT[13]. Laug et al. looked at rat liver and found effects at doses as low as 5 ppm DDT[20]. Khasawinah et al., in two separate studies, found the same lower effect level (5 ppm) in ICR mice and Fischer 344 rat's livers when dosed with Chlordane[21]. Davis et al., found when C3HeB/Fe mice and C3H mice were administered as little as 10 ppm Dieldrin there was a significant increase in benign hepatomas and hepatic carcinomas[22]. When Tomatis et al. fed CF-1 mice 250 ppm DDD for 150 weeks or 250 ppm DDE for 130 weeks, they noted a significant increase in lung tumors and the in incidence of hepatomas, respectively[23]. A significant increase in sperm-head abnormalities was observed when Swiss-Webster mice were administered as little as 25 ppm Trichloroacetic acid[24]. When dogs were dosed with Vinclozolin, the lowest significant effect was seen 600 ppm with in increase in adrenal gland weight[19]. This is consistent with our data for Vinclozolin, showing no activity at lower levels.

According to the previously published data, the average minimal effective dose for pesticides / organochlorines in animals appears to be 5 ppm or greater[13,20-24]. XDS's LUMI-CELL™ ER bioassay is capable detecting pesticides and organochlorines at < 1ppm (with a lower limit of detection of < 1ppt). If limits are to be imposed on the food, feed and pharmaceutical industries as to the content of estrogenic EDCs in consumable products, the limit should reflect the lower average minimal effective dose of 5 ppm or lower. The LUMI-CELL™ ER bioassay has an EC50 detection of 1.99×10^{-11} for 17β-estradiol. This level of detection is far lower than any limit likely to be imposed by any regulatory agency. This data clearly demonstrates that XDS's LUMI-CELL™ ER high-throughput bioassay system is a fast, reliable, and relatively inexpensive method for detection of environmental EDCs, meeting requirements mandated by the EPA and ICCVAMs Tier I (screening) requirements for EDC detection assays.

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