QUANTITATION OF MCF-7 BREAST CANCER CELL PROLIFERATION, *IN VITRO*, IN RESPONSE TO POLYCHLORINATED BIPHENYL MIXTURES EXTRACTED AND ANALYZED FROM HUMAN BREAST TISSUE

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Introduction

Breast cancer is a complex and deadly disease. Although there have been many breakthroughs in characterizing malignancies and in treatments such as hormone therapy, there are still numerous questions about neoplastic origins and mechanisms of action. Several factors have been implicated as contributors to the disease process. These include genetics, diet, and occupational exposures. Other factors clearly related to lifetime estrogenic exposure, such as parity, length of lactation, age of menarche and late menopause have also been implicated in increased risk. These etiologic factors, however, do not fully account for the increases observed in breast cancer occurrence and deaths. Therefore, a potential link between environmental contaminants and breast cancer has been suggested. Some studies show that certain exogenous chemicals induce or promote mammary carcinoma in animal models (Dunnick et al., 1995; Huff et al, 1996; Davis et al, 1993) wheresas others tend to refute these findings (Swanson et al, 1995)

Many of the chemicals of concern are those persistent lipophilic compounds sequestered in adipose tissue after exposure and released slowly over time. One broad class of chemicals known as organochlorines (OCs) are ubiquitous contaminants that are highly lipophilic and can be stored in the breast at increased concentrations. Polychlorinated biphenyls (PCBs) in particular have been found worldwide in serum, milk, and adipose tissues of men and women worldwide. In some cases, OC concentrations have been correlated with increased breast cancer risk; specifically among women with elevated (Dewailly et al, 1994; Bradlow et al., 1995). In addition to carcinogenicity, there is also the threat of harmful exposures nursing children may receive by consuming milk contaminated with these compounds (Fürst et al., 1994; Larsen, et al., 1994; González, et al., 1995; Schlaud et al, 1995).

One of the underlying mechanistic links between the many and seemingly unrelated risk factors for breast cancer may relate to increased estrogenic responsiveness and exposure. Increasing estrogenic activity within tissues has been highly correlated with increasing breast cancer rates in several studies (Davis et al., 1993; Pike et al, 1993; Aldercreutz et al., 1994). These effects may be mediated by one or several mechanisms. Some exogenous compounds, for example, may elicit their effects by specifically binding to the arylhydrocarbon receptor (AhR), the estrogen receptor (ER) or the progesterone receptor (PR), resulting in changes in gene expression. Other OC compounds show more indirect effects, by altering estrogen metabolism that may affect breast cancer growth (positively and negatively) and/or growth of existing tumors. PCBs may be important in the carcinogenic process via oxidative damage or DNA adduction. Still other evidence points to metabolically activated, hydroxylated PCBs (biphenylols) that bind to the ER, thereby acting as a promoter of breast carcinogenesis.

In this study, PCB mixtures were extracted and analyzed from normal breast tissue provided by 10 healthy adult human females. These mixtures were then introduced to cultures of estrogen-receptor postitive (ER+) breast epithelial cell lines (MCF-7) to characterize possible effects on normal and estradiol-induced cellular proliferation. Since PCBs were normally present in combination rather than as individual congeners, possible synergistic/antagonistic effects among congeners could be characterized.

Materials and Methods

Tissue Samples- Breast tissue samples were obtained from reductive mammoplasties courtesy of the Greenville Hospital Systems (GHS; South Carolina), for analysis of PCB concentrations. The PCB congeners isolated from breast tissue were then compared with those found at a Pickens County, S.C. wastesite where Aroclor 1254 was the predominant species. Correlation of these two studies provides a practical risk-exposure scenario, which warrants closer study of these and other congeners' effect on carcinogenesis.

Cell Culture Media- Cells were cultured in Dulbeco's Modified Eagle's Medium (without phenol red or sodium bicarbonate) supplemented with the following: 5% fetal calf serum (Hyclone), 0.05% Pen/Strep (Gibco/BRL), 0.05% Gentamicin (Gibco/BRL), 2.62 $x10^{-2}$ M sodium bicarbonate (Sigma), $4.36x10^{-7}$ M insulin (Gibco/BRL), $1x10^{-2}$ M HEPES (Gibco/BRL), and 1% non-essential amino acids (Gibco/BRL). The experimental media was the same with the exception that 5% charcoal-dextran filtered fetal calf serum (FCS; Hyclone) replaced non-treated FCS to prevent confounding effects from endogenous steroids in the serum. In the preparation of both types of media, all the ingredients except FCS were brought to 950 mL in MilliQ® Water and the pH was adjusted to 7.6. This solution was then vacuum pumped through a 0.2 µm pore Acrocap filter (Gelman) into a roller bottle and the appropriate FCS was then added using sterile technique and the media stored at 4°C± 2°C. Trypsin-EDTA was prepared by thawing a 100 mL bottle of 10x Trypsin-EDTA (Sigma) and then adding to 900 mL MilliQ® water. The pH was then adjusted to 7.4, the solution sterile filtered as mentioned above, and stored in 12 ml aliqouts at -20°C ± 1°C.

Maintenance of Stock Cultures- The MCF-7 breast adenocarcinoma cell line was generously provided by Drs. Michael Kilgore and Patricia Tate from the GHS/Clemson Univ. (S.Carolina) biomedical cooperative [ATCC cell line, HTB-22]. All cells were housed in a humidified incubator at a temperature of 37°C with 5% carbon dioxide. Upon reaching 80-90% confluency (approximately $1x10^{6}$ cells/mL), as determined by heamocytometer counts and inverted microscopic examination, cells were loosened from the flask surface by treating one minute with one milliliter of 1x trypsin-EDTA. The trypsin-EDTA was then removed and the cells incubated for 4 minutes in the aforementioned incubator. Cells were then split and resuspended in an appropriate volume of media. If the cells were to be frozen, they were trypsinized as above and suspended in medium composed of 10% DMSO, 80% FBS, and 10% of the prepared media described above. They were stored in sterile cryogenic vials housed in a Cryo 1°C Freezing Container (Nalgene) at -80°C ± 1°C for 24 hours and then stored in the vapor phase of a liquid nitrogen tank until needed.

Plating and Dosing of Cells- Cells were maintained in 75cm^2 flasks and the media changed approximately every day or every 2 days, depending upon confluency. Upon reaching 80-90%

confluency, one flask of cells was split 1:10 by trypsinization to seed new flasks or an entire flask was used to seed five, 6-well plates for dosing. The dosing regimen consisted of seeding each well of a 6-well plate at an approximate concentration of 30,000 cells/well, and allowing cell anchorage over a 24 hour period in DMEM's non-phenol red media with 5% charcoal-dextran treated fetal calf serum, as previously mentioned. Dosing solutions for E_2 and TCDD were prepared 1,000-fold concentrated so that when they were added at a concentration of 1 uL solution/1mL media, the cells were exposed to the appropriate concentration of each treatment. Due to the restricted amount of PCB extracts available, and to prevent cytotoxicity from excessive vehicle concentrations, the extracts were blown down with a stream of N_2 gas and concentrated ten-fold. The subsequent thousand-fold dilution upon addition to the wells in the assay brought the final PCB extract concentration to one-tenth of its actual concentration found in the breast tissues.

Tritiated Thymidine Incorporation Assay- At exactly 20 hours after treatment, each well received 1.89 $x10^{-2}$ M hydroxyurea (Sigma) and 1.0 uCi/well of tritium-labelled (methyl position) thymidine, 70-86 Ci/mmol specific activity at 1.0 mCi/ml radioactive concentration in aqueous solution (Amersham). All plates were then incubated for 4 hours (37°C; 5% carbon dioxide). Plates were removed from the incubator and all the media was aspirated with a pasteur pipet, followed by the addition 1 mL of 1x PBS and then aspirating again. This last PBS rinse was repeated three times to ensure complete removal of unincorporated nucleotides. The cells were then lysed by adding 500 ul of 1x trypsin-EDTA and 500 uL of a lysing solution composed of the following: 2.0 M sodium hydroxide (Sigma), 6.82 $x10^{-3}$ M, N-lauroyl sarcosine (ICN Biomedicals), and 1.03 $x10^{-2}$ M, EDTA (Sigma), in 100 mL of deionized, distilled water. Plates were allowed to sit overnight before proceeding to the next step. Cell lysates were then individually collected into 15mL centrifuge tubes (Fisher). Following removal of the lysate, each well was rinsed three times with 1x PBS and each rinse was added to the collected lysate. To each centrifuge tube, 2 drops of 0.5% Phenol red (Sigma) and 3 drops of formaldehyde, 37% w/w (Fisher) were added. Samples were then adjusted to a pH of 7.4 by addition of 3N NaOH (Sigma-Aldrich) and/or 3N HCL (Sigma-Aldrich), using the phenol red as a visual indicator. Samples were then filtered through a Millipore filtration membrane using 0.45 um poresize, 25 mm diameter nitrocellulose membranes (Whatman/Fisher) to capture the single-stranded DNA. Each sample was emptied into a filtration well and then the empty centrifuge tube was rinsed with 1 mL of 1x PBS three times to completely collect the sample. Filters were then air dried on an aluminum foil grid and placed into 20 mL Borosilicate scintillation vials with foil-lined caps (Fisher). Vials were filled with 8mL of Scintiverse scintillation fluid (Fisher). The caps were placed on the vials and each vial was wiped with an anti-static dryer sheet (Bi-Lo) to prevent static interference with the scintillation counts. Scintillation counts were recorded on a Beckman LS6500 Multi-purpose Scintillation Counter using a count time of 10 minutes, and ^{14}C at < 1.0 uCi as a calibration standard prior to each use. The bioassay was optimized for the physiologic maximum proliferation level of $1x10^{-8}$ M estradiol and antiestrogenicity resultant from $1x10^{-8}$ M TCDD.

Analytical Chemistry: The breast tissue was warmed to room temperature and homogenized with a glass stirring rod. One to two grams of tissue were weighed and mixed with 15 to 30 g sodium sulfate (Na_2SO_4). Samples were then soxhlet extracted for 16 hours with acetone:hexane (1:1) refluxing at 6 cycles per hour. Following extraction, the samples were concentrated on a rotovap under vacuum in a 40C waterbath, transferred to 10 ml volumetric flasks and brought to volume.

The samples were then split into two, 5 ml portions. One ml of each portion was processed through two Waters Envirogel gel permeation chromatography (GPC) cleanup columns (19 x 150 mm and 19 x 300 mm) arranged in series. The column packing was a styrene divinylbenzene copolymer. Methylene chloride (CH_2Cl_2) was the carrier solvent. The remaining sample was placed back into frozen storage. The fraction of the GPC eluant containing the methoxychlor to the perylene peaks was collected for each sample. Eluants of the same sample were combined. For instance, after soxhlet extraction, sample A was concentrated on a rotovap and brought up to 10 mls in a volumetric flask. Sample A was then split into two portions, A1 and A2. One ml from A1 and 1 ml from A2 were processed separately through the GPC. The methoxychlor to perylene fractions were collected for each A1 and A2. These fractions were then recombined back into a single sample A. The sample was then exchanged back into hexane and brought to 2 mls for residue determination, as follows. Individual PCB congeners were quantified by capillary column gas chromatography using an HP 5890 Series II equipped with a 60m DB-5 capillary column and an electron capture detector. Samples were injected via an HP autosampler. Residues were quantified based on a standard curve generated by external standards. Samples were run in sets of 1 to 10 samples. Each set also contained one spike (clean sample spiked with known standard concentration) and one blank. All samples, including the spike and blank, were also treated with an OC surrogate containing TCm-xylene and DCBP in order to insure recoveries. Solvents were of pesticide grade or greater quality and the Na_2SO_4 was baked for 24 hours at 130° C prior to use.

Experimental Design and Statistical Analysis: Due to a limited amount of available extract, the proliferation assay was run only twice. The data was analyzed by grouping the results in a randomized block design, using the two test days as replicates, and then comparing the t-tests at $p<0.100$.

Results and Discussion

Extracts from ten individual breast samples were analyzed for PCBs and then tested for their effect on cellular proliferation at 1/10 final concentration found in breast.

Analysis of extracts: Individual PCB congeners ranged from 5 to 24 congeners per sample. The values for total PCBs in the samples ranged from 0.011-0.533 ug PCBs/g breast tissue (Table 1). In a recent report, congeners 153, 138 and 180 were the most prevalent in humans (50% total PCBs) and foxes (Corsolini et al. 1995). In our study as well, these three congeners were found in eight of the ten breast tissue samples examined. Of the remaining samples, one sample contained only 153 and 138 (TP) while the other (VME) contained none of the three congeners. Therefore, in most cases, these three congeners represented a major part of the PCB total. Congener 180 levels ranged from 8.3 - 79.0 %, congener 153 levels ranged from 3.8-24.1 %, and congener 138 levels ranged from 2.0-11.9% (data not shown).
Due to the impact of certain substitution groups on stereochemistry and possible receptor binding abilities, the dominant substitution patterns may offer an explanation of certain toxicological effects. As shown in Table 2, the majority of congener substitution patterns found were the di-*ortho* substitution type at 68.3 - 99.98 % of the total. There were virtually no coplanar congeners present in most samples. A few congeners could not be distinguished (referred to as 'undistinguished' hereafter) from one another in the quantitation methods, so they are grouped together under a combined heading,. For example, congeners 105 & 168 could not be

distinguished and are thus grouped under the heading mono-*ortho*/di-*ortho*. However, in all cases, these represent a small proportion of the total yield (0.96-2.25%).

Table 1. Congener number and total PCB concentrations (ug/g) in extracts from 10 normal human breast samples.

Sample ID	Number of Congeners Detected	Total PCBs (ug/g)
JW	5	0.011
MC	11	0.053
HW	14	0.081
GCT	24	0.533
TYT	13	0.072
AGC	21	0.136
VME	11	0.109
FH	23	0.186
TP	13	0.0922
PF	15	0.290

Table 2. Substitution pattern percentages per sample.

Sample ID	Mono-ortho/coplanar	Mono-ortho	Mono-ortho/Di-ortho	Di-ortho
JW	0	27.48	0	72.52
MC	0	13.18	0	86.82
HW	0	14.98	0	85.02
GCT	0	15.14	0.96	83.90
TYT	0	6.16	0	93.84
AGC	0	19.01	2.25	78.74
VME	0	17.73	0	82.27
FH	2.80	16.83	0	80.37
TP	21.40	10.31	0	68.30
PF	0	0.02	0	99.98

Cell Proliferation Effects in Response to Cell Extracts: In the cell proliferation experiments, eight of the ten samples had increased cellular proliferation either alone, in combination with E_2 or both. Two of the ten smples (AGC and FH) had no significant increases compared to controls. Two samples (PF and HW) increased cell proliferation both alone and in combination with E_2 . Two samples (GCT and TYT) showed an increase in cellular proliferation only in the presence of estradiol. Four extracts (VME, JW, MC, and TP) caused increased cellular proliferation when introduced alone, but not in combination with E_2 . Finally, inhibition of estradiol-induced proliferation was evident for six samples (VME, JW, MC, AGC, FH, and TP).

The breast sample extracts had a range of effects on basal proliferation and estradiol-induced proliferation. When treatment-specific increases in thymidine incorporation were relatively low (10-20%) compared to controls, then estradiol generally acted as an agonist, increasing the thymidine counts incorporated compared to treatment alone. However, when treatment-specific increases were highest (JW, TP), then the addition of estradiol revealed an antagonistic interaction. Therefore, a biphasic response is suggested. Further investigation is required to develop the dose-response relationships for total PCBs and/or specific PCB congeners. Since estradiol concentrations were relatively high to elicit a maximal response, toxicity should be a consideration with the addition of active compounds. It is not clear whether toxicity played any role in decreasing estradiol-induced proliferation in this study. However, in future studies, a range of concentrations should be examined to characterize possible agonist/antagonist properties of tissue-derived contaminants.

Acknowledgements
This study was funded by EcoRisk, Inc.

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