Published in final edited form as:

Toxicol Appl Pharmacol. 2009 June 1; 237(2): 237–245. doi:10.1016/j.taap.2009.04.001.

Cell death mechanisms in GT1-7 GnRH cells exposed to polychlorinated biphenyls PCB74, PCB118, and PCB153

Sarah M. Dickerson¹, Esperanza Guevara¹, Michael J. Woller⁴, and Andrea C. Gore^{1,2,3}

¹ Division of Pharmacology & Toxicology, University of Texas at Austin, Austin, TX 78712, USA

² Institute for Neuroscience, University of Texas at Austin, Austin, TX 78712, USA

³ Institute for Cellular & Molecular Biology, University of Texas at Austin, Austin, TX 78712, USA

⁴ Biological Sciences, University of Wisconsin-Whitewater, Whitewater, WI 53190, USA

Abstract

Exposure to endocrine-disrupting chemicals (EDCs) such as polychlorinated biphenyls (PCBs) causes functional deficits in neuroendocrine systems. We used an immortalized hypothalamic GT1-7 cell line, which synthesizes the neuroendocrine peptide gonadotropin-releasing hormone (GnRH), to examine the neurotoxic and endocrine disrupting effects of PCBs and their mechanisms of action. **Cells were treated for 1, 4, 8, or 24 h with a range of doses of a representative PCB from each of three classes:** coplanar (2,4,4',5-tetrachlorobiphenyl: PCB74), dioxin-like coplanar (2',3,4,4',5' pentachlorobiphenyl: PCB118), non-coplanar (2,2',4,4',5,5'-hexachlorobiphenyl: PCB153), or their combination. GnRH peptide concentrations, cell viability, apoptotic and necrotic cell death, and caspase activation were quantified. In general, GnRH peptide levels were suppressed by high doses and longer durations of PCBs, and elevated at low doses and shorter time points. The suppression of GnRH peptide levels was partially reversed in cultures co-treated with the estrogen receptor antagonist ICI 182,780. All PCBs reduced viability and increased both apoptotic and necrotic cell death. Although the effects for the three classes of PCBs were often similar, subtle differences in responses, together with evidence that the combination of PCBs acted slightly differently from individual PCBs, suggest that the three tested PCB compounds may act via slightly different or more than one mechanism. These results provide evidence that PCB congeners have endocrine disrupting and/or neurotoxic effects on the hypothalamic GnRH cell line, a finding that has implications for environmental endocrine disruption in animals.

Keywords

PCB; GnRH; GT1 cells; estrogen receptor; apoptosis; necrosis; endocrine disruption

Corresponding Author: Andrea C. Gore, Ph.D., Division of Pharmacology & Toxicology, The University of Texas at Austin, PHAR-Pharmacology, 1 University Station A1915, Austin, TX 78712-0125, Tel: 512-471-3669, Fax: 512-471-5002, email: E-mail: andrea.gore@mail.utexas.edu.

Conflict of Interest Statement: The authors have nothing to disclose.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Polychlorinated biphenyls (PCBs) are a family of synthetic organic pollutants that contaminate both urban and rural environments. These compounds were widely used for industrial applications between the 1930s and 1970s, due to their stability, non-flammability and high dielectric constant. The same properties that made PCBs desirable for industrial use contribute to their resistance to degradation and to their toxicity. Despite the banning of PCB manufacture in the United States in 1977, humans are still exposed via food, and have appreciable body burdens (Stellman et al., 1998).

PCBs can act as endocrine disrupting chemicals (EDCs) via their interactions with sex steroid hormone receptors, steroidogenic enzymes, and other mechanisms (reviewed in Dickerson and Gore, 2007). Indeed, PCBs have been shown to perturb endocrine and reproductive systems in a variety of species (Khan and Thomas, 2001; Salama et al., 2003; Khan & Thomas, 2004). Further, PCBs have been proposed to disrupt neuroendocrine cells in the hypothalamus, including cells that synthesize and release the neurohormone, gonadotropin-releasing hormone (GnRH) (reviewed in Gore, 2008; Dickerson & Gore, 2007). The decapeptide GnRH is released from neuroterminals in the hypothalamus into the portal capillary system leading to the anterior pituitary gland, where GnRH acts to stimulate synthesis and release of gonadotropins into the general circulation. These two hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) act at the gonads to stimulate gonadal maturation, steroidogenesis and gametogenesis. Although all three levels of the hypothalamic-pituitary-gonadal axis must function properly in order for reproductive function to occur, the hypothalamic GnRH neurons provide the driving force upon this system.

PCBs have other actions on the nervous system beside neuroendocrine GnRH cells. They target neurotransmitter receptors and biosynthetic enzymes (Mariussen and Fonnum, 2002; Seegal et al., 1991; Coccini et al., 2006; Juárez de Ku et al., 1994; Altmann et al., 2001; Gafni et al., 2004), and they act upon steroid hormone receptors in the brain (Dickerson and Gore, 2007). Importantly, GnRH neuroendocrine cells are regulated by a complex interplay of neurotransmitters and steroid hormones. Thus, PCBs may exert both direct and indirect actions upon GnRH neurons, thereby disrupting hypothalamic-pituitary-gonadal function.

The mechanisms of action and the degree of toxicity of PCBs are dependent upon degree of chlorination, three-dimensional structure, timing and duration of exposure, dosage, and cell type (Ruiz et al., 2008; Kodavanti, 2006). PCBs are grouped into three general categories: coplanar, dioxin-like coplanar, and non-coplanar, with differential effects on cell death, including apoptosis and necrosis as shown in neuronal hippocampal or cortical cell cultures or immortalized neuronal cell lines (Sanchez-Alonso et al., 2003, 2004; Inglefield et al. 2001; Howard et al., 2003; Inglefield et al., 2001; Costa et al., 2007; Lee et al., 2004; Kang et al., 2001; Hwang et al., 2001). However, the neurotoxicity of PCBs has not been addressed for neuroendocrine cells. Although previous work (Gore et al., 2002) has shown that PCBs can alter release of GnRH and change GnRH gene expression in the GT1-7 immortalized hypothalamic cell line (Mellon et al., 1990), the mechanisms of neurotoxicity have never been studied or quantified. Here, we hypothesized that the three different classes of PCBs (coplanar, dioxin-like coplanar, non-coplanar) would cause a differential endocrine disruption of GnRH release, and induce differing levels of apoptosis or necrosis in GT1-7 hypothalamic cells. We also evaluated effects of a mixture of the three PCBs in order to assess whether there were additive or synergistic effects on these endpoints. Finally, we examined the role of the nuclear estrogen receptor in mediating effects of PCBs on GnRH release by co-administering PCBs with an estrogen receptor antagonist, ICI 182,780.

Methods

Chemicals

We purchased 2,4,4',5-tetrachlorobiphenyl (CAS RN: 32690-93-0; PCB74- 99.9% purity; lot no. 102293), 2',3,4,4',5' pentachlorobiphenyl (CAS RN: 31508-00-6; PCB118- 99.9% purity; lot no. 961002LB-AC), and 2,2',4,4',5,5'-Hexachlorobiphenyl (CAS RN: 35065-27-1; PCB153- 99.9% purity; lot no. 062703JR-AC), from Accustandard (New Haven, CT, USA). Dimethylsulfoxide (DMSO), and staurosporine (positive control for apoptotic cell death) were purchased from Sigma (St. Louis, MO, USA) and 4',6-diamidino-2-phenylindole (DAPI) was purchased from Calbiochem (La Jolla, CA, USA). ICI 182,780 was purchased from Tocris Bioscience (Ellisville, MO, USA). We purchased phenol red-free Dulbecco's Modification of Eagle's Medium (DMEM), fetal calf serum, L-glutamine, penicillin, and streptomycin from CellGro (MediaTech, Inc., Herndon, VA, USA). The CellTiter-Blue™ Cell Viability and APO-One Homogeneous Caspase-3/7 Activation assay was purchased from Promega Corporation (Madison, WI, USA). Anti- mouse cleaved caspase-3 and anti-mouse cleaved caspase-9 antibodies were from Cell Signaling Technology, Inc. (catalog no. 9664 and 9509, respectively; Beverly, MA, USA), while anti-mouse cleaved caspase-8 antibody was obtained from R&D Systems, Inc. (catalog no. AF1650; Minneapolis, MN, USA).

GT1-7 cell culture and maintenance

GT1-7 cells (kindly provided by Dr. Pamela Mellon; Mellon et al., 1990) were maintained in phenol red-free DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics (100 U/ml, penicillin, 100 mg/ml streptomycin). Cells were grown at 37°C with 5% CO₂ as described previously (Gore, 2002; Gore et al., 2002), and used between passages 15 – 20. Cells were sub-cultured into six-well or 96-well tissue culture dishes 2–3 days before experiments in Dulbecco's minimum essential medium (DMEM) plus FCS and antibiotics, and grown to approximately 60–70% confluency. To avoid growth hormone or steroid effects of FCS, we switched to serum-free medium four hours before experiments. All experiments were performed on triplicate monolayers of either approximately 1×10⁶ cells for six-well plates or 50,000 cells for 96-well plates, and were repeated three to four times on separate culture dishes.

PCB congener treatments

GT1-7 cells were treated with either vehicle (DMSO), individual PCBs [PCB74, PCB118 or PCB153 (each at 0.1, 1, 10, 100 μM)] or a PCB Mix [1:1:1 mixture of PCB74, PCB118, and PCB153 (0.1, 1, 10, 100 μM final additive molar equivalent of individual PCBs)]. For some experiments, staurosporine (200 nM; Sigma) was used as a control for apoptotic cell death (Koh et al., 1995). We diluted all drugs in DMSO and used them at a final dilution of 1:1000 in medium. The vehicle was applied at a 1:1000 dilution. In separate experiments, cells were co-administered 1000 nM ICI 182,780. We maintained cultures at 37°C with 5% CO₂ after application of drugs. Following 1, 4, 8, or 24 h of treatment, cells were viewed on an inverted phase contrast microscope at a magnification of ×250. Photomicrographs were imaged using a Nikon digital camera mounted on the microscope.

GnRH Peptide Assay

GnRH peptide levels in the medium were measured in two double-antibody radioimmunoassays with duplicate samples of 100 μl. For each experiment, media from triplicate cultures were pooled into a single sample. Three pooled samples per experiment were analyzed, and the experiment was repeated four times. The antibody to GnRH was provided by Dr T. Nett (R1245). Synthetic GnRH used as trace and standard was purchased from Richelieu Laboratories (Montreal, Canada). Assay sensitivity was 0.1 pg/tube at 95% binding.

The intra-assay coefficients of variation were 3.4% and 3.9%, and the inter-assay coefficient of variation was 3.1%.

Determination of Cell Viability

We used the CellTiter-Blue® Cell Viability Assay (Promega Corporation) to measure the viability of cells after PCB treatment. This fluorometric assay is based on the metabolic capacity of viable cells to reduce resazurin into resorufin, which is highly fluorescent at 590 nm. Nonviable cells lose metabolic capacity to reduce the indicator dye, and therefore do not generate a fluorescent signal. Briefly, the assay was carried out according to the manufacturer's recommendations (Promega). Cells were treated with PCB or vehicle for durations between 0 and 24 hours. CellTiter-Blue® Reagent was added at a volume of 20 µL to each well one hour before fluorescence was recorded. The fluorescence of the samples was measured at 590 nm on a DTX 880 Multimode Plate Reader (Beckman-Coulter, Inc., Fullerton, CA). The presence of PCBs in the culture media did not interfere with the viability assay procedures.

Detection of cell death

To detect the presence of necrotic cell death, treated cell populations were trypsinized and resuspended in a 1:1 mixture of 1X PBS and 0.4% Trypan Blue solution (Sigma) and counted under an inverse phase contrast microscope with a Neubauer improved hemacytometer (Hausser Scientific, Horsham, PA). Cells with an intact membrane (viable or apoptotic cells) are not permeable to Trypan Blue and remained unstained, while cells with a damaged membrane (necrotic cells) stain blue. Necrotic cell death is expressed as the percentage stained cells per total cell number counted. Three replicates per experiment were analyzed, and the experiment was repeated four times.

DAPI Staining of Apoptotic Nuclei: Following fixation with 4% paraformaldehyde, GT1-7 cells grown on 22 mm glass cover slips were rinsed for 5 minutes with PBS, and stained with 1 µg/ml DAPI in water for 20 min at room temperature. We then removed excess DAPI by a rinse with water, and mounted cover slips onto slides with VectaShield (Vector Laboratories, Burlingame, CA, USA). We characterized nuclear morphology and quantified chromatin condensation using an Olympus BX61 System fluorescence microscope at a magnification of 400X. Normal cells have a smooth, round nucleus characterized by faint staining. Apoptotic cells were identified by the presence of brightly labeled pyknotic nuclei. The number of neurons with condensed nuclei vs. the number of neurons with intact nuclei was scored in five randomly chosen microscopic fields (each field typically contained 25 – 50 cells). We counted a total of at least 200 cells per slide, and then reported the number of apoptotic cells as a percentage. Three cultures were analyzed per experimental condition for each experiment, and experiments were repeated three times.

Determination of caspase activation

The Apo-ONE Homogeneous Caspase-3/7 Detection system (Promega Corporation, Madison, WI) was used to determine whether caspase-3/7 was activated following treatment with the toxicants, using the protocol provided by the manufacture. Following incubation at room temperature for three hours, caspase-3/7 activation were estimated from the fluorescence of each sample at the excitation wavelength at 485 nm and the emission wavelength at 535 nm using a fluorescence reader DTX 880 Multimode Detector (Beckman-Coulter, Inc., Fullerton, CA).

To visualize activated (cleaved) caspases -8 or -9, GT1-7 cells grown on top of 22 mm glass coverslips in 6-well tissue culture were fixed in 4% paraformaldehyde for 10 minutes at 4°C and then rinsed in PBS. Non-specific binding of endogenous proteins to primary antibody was blocked by incubating coverslips in 5% normal goat serum and 5% bovine serum albumin in

PBS for 1 hour at room temperature. Cells were incubated for 1 hour at room temperature with rabbit polyclonal antibodies against mouse active caspase-8 or caspase-9. Following a rinse in PBS and incubation in an anti-rabbit secondary antibody conjugated to fluorescein (Vector Labs, Burlingame, CA, USA), slides were mounted with VectaShield mounting media (Vector Labs, Burlingame, CA, USA) and visualized on an Olympus BX61 System fluorescence microscope. Observations of the presence or absence of activated caspase staining were made for each treatment and time point.

Statistical analysis of results

The effects of each PCB were analyzed by two-way ANOVA. Variables for most studies were dose and duration. For the ICI 182,780 experiment, variables were treatment (PCB alone or PCB plus ICI 182,780) and dose. Experiments were performed identically for each collection on the triplicate cultures. Results of triplicate cultures for each experiment were considered to be one independent variable; and thus for statistical purposes, final N's were 3 – 4 per group. When a significant effect was observed, post-hoc comparisons were performed using Fishers PLSD/Tukey-b analysis. In all cases, $P < 0.05$ was considered statistically significant.

Results

GnRH peptide levels

The GT1-7 cell media were collected 1, 4, 8 or 24 hours following treatment with PCBs 74, 118, 153, or their combination (referred to hereafter as PCB Mix). For each individual PCB and the PCB Mix, two-way ANOVA showed significant main effects of both dose ($p < 0.005$) and duration ($p < 0.005$), as well as an interaction of dose and duration ($p < 0.005$). In general, GnRH peptide levels were higher in the PCB treatment groups when compared to corresponding vehicle treatment at early timepoints (1 and 4 hours; Figure 1) and lower in PCB-treated cultures at later time points (8 and 24 hours; Figure 1).

The nuclear estrogen receptor antagonist ICI 182,780 (1000 nM) was co-administered with PCB74, PCB118, PCB153, or PCB Mix (1 μ M dose, 24 hour timepoint) to **ascertain whether endocrine-disrupting effects of PCBs are manifested upon GnRH release**. Significant differences between groups were detected by ANOVA ($p < 0.001$). Post-hoc analysis demonstrated that ICI alone reduced ($p < 0.05$) GnRH peptide levels in control cultures (Figure 2). PCB treatment was associated with suppressed GnRH peptide levels ($p < 0.01$), which was partially reversed by co-treatment with ICI 182,780 ($p < 0.001$ compared to PCB with no ICI 182,780; Figure 2).

GT1-7 cell viability

GT1-7 cell viability was measured by the CellTiter-Blue® Cell Viability Assay. We noted no significant differences in cellular viability from 0 to 24 h of exposure between untreated cultures and cultures treated with DMSO, the vehicle for PCBs (data not shown). For each PCB, two-way ANOVA showed significant main effects of both dose ($p < 0.005$) and duration ($p < 0.005$), as well as an interaction of dose and duration ($p < 0.005$). Effects varied for the individual congeners, but in general, PCB74 and PCB118 had inhibitory effects on relative cell viability at the intermediate doses and at all doses by the 24 hour timepoint (Figure 3A and 3B). PCB153 affected cell viability at varying doses depending upon timepoint (Figure 3C). The PCB Mix had the least effect on viability (Figure 3D).

Necrotic and apoptotic cell death

To differentiate between effects of PCBs on necrotic or apoptotic cell death, Trypan blue staining (Figure 4A) or DAPI labeling (Figure 4B) were used. Quantification of necrotic cell

death (Figure 5) showed that PCBs caused little necrosis until the 8 hour timepoint, at which the highest concentration of PCB118 or PCB153 caused a significant increase. After 24 h treatment, all of the PCBs caused a significant increase in necrotic cell death.

We also examined the effects of PCBs on apoptotic cell death (Figure 4B, Figure 6). Staurosporine, a protein kinase inhibitor that induces apoptosis in neuronal cells (Krohn et al., 1998), served as a positive control. Staurosporine consistently induced cell death in GT1-7 cells (data not shown). For the PCBs, by 4 hours of exposure, all of the PCBs induced an increase in the percentage of apoptotic nuclei, and PCB118 and the PCB Mix had effects at the 1 hour timepoint. Notably, the low and intermediate concentrations of PCBs were most likely to induce apoptosis for all of the PCBs, with the highest concentrations having the least effect (Figure 6).

Caspase activation was assessed at the 4 hour timepoint. For the caspase-3/7 assay, PCB74, PCB118, and the PCB Mix had similar effects, with the low and intermediate concentrations of PCBs causing about a 2-fold increase in caspase-3/7 activity (Figure 7). PCB153 did not significantly affect caspase-3/7 activity (Figure 7).

Immunofluorescence for cleaved (activated) caspases -8, and -9 were visualized by fluorescence microscopy. Treatment with PCBs resulted in detection of activated caspase-9 in all PCB treatments but not the vehicle (Figure 4C, 4D). No immunolabeling was observed for caspase-8 for any of the treatments (Figure 4E, 4F).

Discussion

The current study investigated effects of three PCBs, each representing a member of different PCB classes, on GnRH release from the GT1-7 cell line, and on neuroendocrine toxicity due to apoptotic and necrotic cell death. Our results show that PCB74 (coplanar), PCB118 (dioxin-like coplanar), and PCB153 (non-coplanar), significantly affected both GnRH peptide levels and cell survival in the hypothalamic GT1-7 cell line. In general, individual PCBs had an overall stimulatory effect on GnRH peptide levels at early time points and lower concentrations, and inhibitory effects on GnRH peptide at the later time points and higher concentrations. Treatment with PCBs also affected cell survival, resulting in a decrease in viability of up to 60% in GT1-7 cells treated with PCBs for 24 hours. Moreover, the three congeners induced both necrotic and apoptotic cell death, as indicated by cell morphology and DNA fragmentation.

Although there were some small differences, for the most part, lower concentrations of PCBs induced apoptosis, while higher concentrations caused necrotic cell death. Thus, PCBs caused both endocrine disrupting and neurotoxic effects in the GT1-7 cell line. Further, effects of a mixture of the three PCBs were not substantially different from those of the individual congeners. Therefore, at the dosages used in the current study, little additivity or synergism could be ascertained.

PCB effects on GnRH peptide concentrations

The three classes of PCB congeners altered GnRH peptide levels in GT1-7 cell media. Effects of PCB74, PCB118, and the PCB Mix were similar: in general, GnRH peptide concentrations were elevated at intermediate to high dosages at the 4 hour timepoint, and lower at the 8 and 24 hour timepoints compared to DMSO controls. PCB153 was slightly different in its effects, as it was the only PCB to be associated with a change at the 1 hour timepoint (increased GnRH peptide at the lowest and highest concentration). By 24 hours, GnRH peptide levels were lower in PCB153 treated cells at the three highest concentrations.

Overall, these studies on GnRH peptide suggest that PCBs may initially stimulate but then inhibit GnRH synthesis and/or secretion. The mechanisms by which GnRH peptide levels are altered by PCBs across time are likely attributable to changes in: (i) GnRH decapeptide biosynthesis; (ii) the release of GnRH from GT1-7 cells, independently of changes in biosynthesis; (iii) GnRH peptide degradation; and/or (iv) number of viable GT1-7 cells. The GnRH measured in culture media may have been secreted from live GT1-7 cells, but it is also possible that cellular stores of the decapeptide were released upon membrane disintegration from dying cells. Here, the initial increase in GnRH does not coincide with a loss in cell viability, making it unlikely that the GnRH levels measured can be attributed to dying cells dumping their stores of the decapeptide. At the earlier time points, the elevated GnRH levels are more likely to be due to a stimulatory action of PCBs on release of peptide stores. Because PCBs have been shown to act through several neurotransmitter receptor systems, some of which can affect GnRH release and whose receptors are expressed on GT1 cells (e.g., serotonin, dopamine, norepinephrine – Khan & Thomas, 1997; Mariussen & Fonnum, 2001; Mariussen et al., 1999), the activation of these neurotransmitter receptors may have the transient action of causing a release of GnRH. However, the number of viable GT1-7 cells probably plays a role in the longer timepoints, as GT1-7 cell viability was decreased, and apoptosis and necrosis increased, over time (see below).

At the molecular level, the actions of PCBs on endocrine systems are thought to be mediated, at least in part, by nuclear estrogen receptors. Therefore, we tested PCBs together with ICI 182,780, a nuclear estrogen receptor antagonist that can act at both estrogen receptors α and β (Wijayarathne et al., 1999), and which has previously been shown to block some effects of estradiol in GT1-7 cells (Gore 2002) and other experimental models (Lee et al., 1999). In the current study, ICI 182,780 caused a partial reversal of the effects of the individual PCBs or their mixture on GnRH peptide secretion, suggesting **potential involvement of an estrogen receptor-dependent mechanism, but also indicating that other mechanisms must be involved**. We were surprised by the consistency of this result across the three classes of PCBs, as the three dimensional structure, degree of chlorination, and dose of a PCB congener contributes not only to its toxicity, but also to its interaction with steroid hormone receptors. Congeners that are lightly chlorinated tend to be more estrogenic, whereas the heavily chlorinated congeners may act as a weak estrogen receptor agonist or antagonist in a dose-dependent manner (Safe et al., 1994; Bonefeld-Jorgensen et al., 2001). It is possible that a wider range of concentrations and duration of treatment may be necessary to better differentiate the role of the estrogen receptor in mediating these processes, since current experiments were performed at the 24 hour timepoint at a single concentration (1 μ M) of PCBs.

In addition, it should be stressed that these PCBs may also act through other non-nuclear estrogen receptors, or through non-estrogenic pathways altogether. In primate GnRH neurons, ICI 182,780 does not block the estradiol-stimulated release of GnRH that is mediated through GPR30, the membrane G-protein coupled estrogen receptor (Noel et al., 2009). In fact, ICI 182,780 has been described as an agonist at the orphan receptor GPR30 (Thomas et al., 2005). However, because PCBs have a relatively low binding affinity for GPR30 *in vitro* (Thomas and Dong, 2006), PCB action on other non-estrogen membrane receptors, e.g. neurotransmitter receptors (Seegal et al., 1991; Khan & Thomas, 1997; Mariussen & Fonnum, 2001; Mariussen et al., 1999) may also account for some of the observed effects. Future experiments investigating the potential role of GPR30 or other membrane receptors will provide a more complete understanding of the mechanisms underlying the observed effects of PCBs on GnRH release.

PCB effects on apoptotic and necrotic cell death

Previous studies have shown that PCBs can be neurotoxic, and that the mechanism can involve either or both apoptotic and necrotic cell death. Using primary fetal cortical neuron cultures, Sanchez-Alonso and colleagues (2003) found that neuronal apoptosis reached 30 – 50% within 4 hours of incubation with 100 μ M PCB77 (a dioxin-like, coplanar PCB), which was also more cytotoxic than the non-coplanar congener PCB153. These effects may be tissue-specific. Howard et al. (2003) found that the non-coplanar PCB47 (1 μ M) induces apoptosis in primary cultured hippocampal neurons, but not cortical neurons. In the same study, the dioxin-like coplanar PCB77 (1 μ M) had no effect on apoptosis in either cell type (Howard et al., 2003), a discrepancy which may be attributable to the lower dose used. A recent study by Ndountse and Chan (2008) found that the non-coplanar congener PCB99 had greater potency for apoptotic cell death in human neuroblastoma cells compared to a dioxin-like coplanar PCB126 and or the PCB mixture, Aroclor 1254 (Ndountse and Chan, 2008). In the same study, necrotic cell death was also observed, and was attributed to the secondary necrosis that follows apoptotic cell death. Necrosis may also be the primary form of cell death caused by PCBs. For instance, a study by Johansson and colleagues (2006) found that both PCB126 and PCB153 induce necrotic cell death in AtT20 pituitary cells, although PCB126 was more potent in this regard. Merritt and Foran (2007) found that PCB153 caused necrotic cell death in two human glioblastoma cell lines at doses as low as 5000 nM. Similarly, Mariussen et al. (2002) found that Aroclors 1242 and 1254 and PCB153, but not PCB126, caused extensive cell death in cultured rat cerebellar granule cells after 24 h treatment duration with doses as low as 12.5 μ M. Together, this literature suggests neurotoxic effects of PCBs, particularly due to a combination of apoptotic and necrotic mechanism. However, prior to the current study, to our knowledge this question of whether and how PCBs may exert such effects in a neuroendocrine cell system has not been addressed.

To this end, in the present study, we quantified cellular viability in response to individual PCBs or a mix, and determined whether this was due to apoptosis or necrosis. First, we found that the individual PCBs diminished cell viability, with PCB74 and PCB118 having such actions by the first hour of treatment. PCB153 was associated with a diminution of viability beginning at the fourth hour of treatment. The PCB Mix was, surprisingly, the least likely to cause diminished GT1-7 cell viability. Second, we ascertained effects of PCBs on necrosis. This mechanism for cell death was not observed until 8 hours (PCB118, PCB153, both at the highest concentrations) to 24 hours (all PCBs, particularly at the higher concentrations). Third, effects of PCBs on apoptosis were evaluated, with PCB74 and PCB118 having the greater potencies for induction of apoptotic cell death, especially at low to intermediate concentrations. This latter result with PCB74 and PCB118 is consistent with the decreased viability detected in cells treated with these two congeners. Differences in results among the PCBs suggest that the relative neurotoxic potency of individual PCB congeners for induction of neuronal cell death is dependent upon PCB structure.

As a whole, our observations also show that lower doses of PCBs induce apoptosis, and higher doses induce necrosis (c.f. Figures 5 and 6). This result is consistent with other studies establishing a dose-dependent relationship between apoptosis and necrosis in neuronal cells exposed to toxicants (Bonfoco et al., 1995). However, the level of apoptotic cell death induced by PCBs in the present study is not as extensive as that observed in previous studies, a discrepancy likely due to differences in cell lines and methodology. In the dose range used in the current study, the loss of cell viability after 4 hours of treatment can be attributed to the induction of apoptosis. In contrast to previous studies, here we found that the three classes of PCBs had similar potencies for induction of apoptosis at the dose range studied. At later timepoints, the loss of cell viability is due primarily to the induction of necrotic cell death. It is possible that some of the necrotic cells observed were due to the secondary necrosis that

follows apoptosis. **We also observed some apoptotic and necrotic cells after 24 h in the vehicle treatments alone. Serum starvation alone induces apoptosis over time, including in the GT1-7 cell line (Srinivasan et al., 1996).** However, differences between vehicle and PCB treatments indicate that the PCBs themselves are inducing these responses.

Apoptosis proceeds via the activation of caspases, a family of cysteine aspartate specific proteases (reviewed in Nuñez et al., 1998). **Stimulation of membrane death receptors activates caspase-8 and initiates the extrinsic apoptotic pathway, while release of mitochondrial signaling factors and subsequent cleavage of caspase-9 activates the intrinsic apoptotic pathway.** Regardless of the pathway initially activated, caspase-3 is widely regarded as the effector caspase, and is activated by both death receptor- and mitochondrial-mediated apoptosis. Once caspase-3 is activated by cleavage, the cell reaches a “point of no return” in the development of apoptosis. Thus, caspase-3 activation (assayed in concert with caspase-7) provides a useful marker of early apoptosis.

The present study demonstrates that coplanar and dioxin-like coplanar PCB congeners (PCB74, PCB118) and their mixture activate caspase-3, consistent with induction of apoptotic cell death. Moreover, we observed the activation of caspase-9 by all of the PCB treatments, which is associated with the mitochondrial-mediated intrinsic pathway of apoptosis. Our results are consistent with other studies that have demonstrated activation of caspase-9 following exposure to a non-coplanar (Ghosh et al., 2007) and dioxin-like coplanar (Hsu et al. 2007) PCB. By contrast, caspase-8 was not activated by any PCB treatment, suggesting that this mechanism is not in play in the GT1-7 cell neurotoxicity.

Environmental endocrine disruption by PCBs

Developmental apoptosis, a process that is regulated by activation of estrogen receptors (reviewed in McCarthy, 2008), plays a critical role in sexual differentiation of the hypothalamus and is necessary for normal maturational processes involved in the attainment and maintenance of reproductive function. PCBs may disrupt developmental apoptosis through actions on estrogen receptors as well as by other mechanisms of action in the nervous system. GT1-7 cells were originally isolated from developing GnRH neurons that have both mature neuronal properties but are not post-mitotic due to transformation with the SV-40 large T antigen (Mellon et al., 1990). Other exogenous compounds have effects on the developing brain *in vivo* at a range of micromolar concentrations similar to the one used in this study. For instance, a recent study found that the phytoestrogen genistein affects developmental apoptosis in the brain of zebrafish embryos at micromolar levels (Sassi-Messai et al., 2009). It is reasonable to postulate that PCB-induced apoptosis or necrosis may be related to the neurotoxic effect of these EDCs on the reproductive axis, and that different molecular mechanisms may operate in the induction of cell death, depending on the structure of the PCB congeners, the neuronal population studied, **and the developmental stage.** Therefore, our results have considerable relevance to exposures of wildlife, humans and laboratory animals to PCBs, particularly when exposure occurs during key developmental life stages such as the period of sexual differentiation (Dickerson & Gore, 2007).

Given that PCB74, PCB118, and PCB153 are congeners frequently found in environmental (Lake et al., 1995), wildlife (Kunisue et al., 2003), and human lipid and serum samples (Freels et al., 2007; Park et al., 2007; Larsen et al., 1994; Lanting et al., 1998), results from this study are relevant for ecological exposures. As the human body burden of the three PCB congeners used in this study varies considerably by individual and tissue, and it is difficult to extrapolate from *in vitro* to *in vivo*, the doses in our study were chosen to approximate human levels. (Freels et al., 2007; Park et al., 2007; Larsen et al., 1994; Lanting et al., 1998). **Because PCBs differentially accumulate into microenvironments in living tissues, with levels reaching approximately 50 ppb in neonatal human brains (Lanting et al., 1998), developing neurons**

may be exposed to micromolar concentrations of these toxicants. This study establishes the role of PCBs in the induction of apoptosis in GnRH GT1-7 cells as a model for developing hypothalamic neurons. Future experiments in animal models will apply the present observations in GT1-7 cells on the effects of PCB exposure on the developing GnRH neurosecretory system.

Acknowledgments

The authors would like to thank Dr. Edward Mills for use of cell culture facilities. This work was supported by NIH ES012272 (ACG), NIH ES07784 (ACG), NIH T32 ES07247 (SMD), NSF 04-615 (SMD), and NSF 0446112 (MJW).

BIBLIOGRAPHY

- Altmann L, Mundy WR, Ward TR, Fastabend A, Lilienthal H. Developmental exposure of rats to a reconstituted PCB mixture or Aroclor 1254: effects on long-term potentiation and [³H]MK-801 binding in occipital cortex and hippocampus. *Toxicol Sci* 2001;61:321–330. [PubMed: 11353141]
- Bonefeld-Jørgensen EC, Andersen HR, Rasmussen TH, Vinggaard AM. Effect of highly bioaccumulated polychlorinated biphenyl congeners on estrogen and androgen receptor activity. *Toxicology* 2001;158:141–153. [PubMed: 11275356]
- Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci U S A* 1995;92:7162–7166. [PubMed: 7638161]
- Coccini T, Randine G, Castoldi AF, Grandjean P, Ostendorp G, Heinzow B, Manzo L. Effects of developmental co-exposure to methylmercury and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153) on cholinergic muscarinic receptors in rat brain. *Neurotoxicology* 2006;27:468–477. [PubMed: 16455139]
- Costa LG, Fattori V, Giordano G, Vitalone A. An in vitro approach to assess the toxicity of certain food contaminants: methylmercury and polychlorinated biphenyls. *Toxicology* 2007;237:65–76. [PubMed: 17553607]
- Dickerson SM, Gore AC. Estrogenic environmental endocrine-disrupting chemical effects on reproductive neuroendocrine function and dysfunction across the life cycle. *Rev Endocr Metab Disord* 2007;8:143–159. [PubMed: 17674209]
- Freels S, Chary LK, Turyk M, Piorkowski J, Mallin K, Dimos J, Anderson H, McCann K, Burse V, Persky V. Congener profiles of occupational PCB exposure versus PCB exposure from fish consumption. *Chemosphere* 2007;69:435–443. [PubMed: 17583774]
- Gafni J, Wong PW, Pessah IN. Non-coplanar 2,2',3,5',6-pentachlorobiphenyl (PCB 95) amplifies ionotropic glutamate receptor signaling in embryonic cerebellar granule neurons by a mechanism involving ryanodine receptors. *Toxicol Sci* 2004;77:72–82. [PubMed: 14600284]
- Ghosh S, De S, Dutta SK. Altered protein expressions in chronic PCB-153-induced human liver (HepG2) cells. *Int J Toxicol* 2007;26:203–212. [PubMed: 17564901]
- Gore AC. Organochlorine pesticides directly regulate gonadotropin-releasing hormone gene expression and biosynthesis in the GT1-7 hypothalamic cell line. *Mol Cell Endocrinol* 2002;192:157–170. [PubMed: 12088877]
- Gore AC. Developmental programming and endocrine disruptor effects on reproductive neuroendocrine systems. *Front Neuroendocrinol* 2008;29:358–374. [PubMed: 18394690]
- Gore AC, Wu TJ, Oung T, Lee JB, Woller MJ. A novel mechanism for endocrine-disrupting effects of polychlorinated biphenyls: direct effects on gonadotropin-releasing hormone neurones. *J Neuroendocrinol* 2002;14:814–823. [PubMed: 12372006]
- Howard AS, Fitzpatrick R, Pessah I, Kostyniak P, Lein PJ. Polychlorinated biphenyls induce caspase-dependent cell death in cultured embryonic rat hippocampal but not cortical neurons via activation of the ryanodine receptor. *Toxicol Appl Pharmacol* 2003;190:72–86. [PubMed: 12831785]
- Hsu PC, Pan MH, Li LA, Chen CJ, Tsai SS, Guo YL. Exposure in utero to 2,2',3,3',4,6'-hexachlorobiphenyl (PCB 132) impairs sperm function and alters testicular apoptosis-related gene expression in rat offspring. *Toxicol Appl Pharmacol* 2007;221:68–75. [PubMed: 17445852]

- Hwang SG, Lee HC, Lee DW, Kim YS, Joo WH, Cho YK, Moon JY. Induction of apoptotic cell death by a p53-independent pathway in neuronal SK-N-MC cells after treatment with 2,2',5,5'-tetrachlorobiphenyl. *Toxicology* 2001;165:179–188. [PubMed: 11522376]
- Inglefield JR, Mundy WR, Shafer TJ. Inositol 1,4,5-triphosphate receptor-sensitive Ca(2+) release, store-operated Ca(2+) entry, and cAMP responsive element binding protein phosphorylation in developing cortical cells following exposure to polychlorinated biphenyls. *J Pharmacol Exp Ther* 2001;297:762–773. [PubMed: 11303068]
- Jin Z, El-Deiry WS. Overview of cell death signaling pathways. *Cancer Biol Ther* 2005;4:139–163. [PubMed: 15725726]
- Johansson C, Tofighi R, Tamm C, Goldoni M, Mutti A, Ceccatelli S. Cell death mechanisms in ArT20 pituitary cells exposed to polychlorinated biphenyls (PCB 126 and PCB 153) and methylmercury. *Toxicol Lett* 2006;167:183–190. [PubMed: 17049763]
- Juárez de Ku LM, Sharma-Stokkermans M, Meserve LA. Thyroxine normalizes polychlorinated biphenyl (PCB) dose-related depression of choline acetyltransferase (ChAT) activity in hippocampus and basal forebrain of 15-day-old rats. *Toxicology* 1994;94:19–30. [PubMed: 7801322]
- Kang KS, Park JE, Ryu DY, Lee YS. Effects and neuro-toxic mechanisms of 2, 2', 4, 4', 5, 5'-hexachlorobiphenyl and endosulfan in neuronal stem cells. *J Vet Med Sci* 2001;63:1183–1190. [PubMed: 11767051]
- Khan IA, Thomas P. Aroclor 1254 inhibits tryptophan hydroxylase activity in rat brain. *Arch Toxicol* 2004;78:316–320. [PubMed: 14997283]
- Khan IA, Thomas P. Aroclor 1254-induced alterations in hypothalamic monoamine metabolism in the Atlantic croaker (*Micropogonias undulatus*): correlation with pituitary gonadotropin release. *Neurotoxicology* 1997;18:553–560. [PubMed: 9291503]
- Khan I, Thomas P. Disruption of neuroendocrine control of luteinizing hormone secretion by aroclor 1254 involves inhibition of hypothalamic tryptophan hydroxylase activity. *Biol Reprod* 2001;64:955–964. [PubMed: 11207213]
- Kodavanti PR. Neurotoxicity of persistent organic pollutants: Possible mode(s) of action and further considerations. *Dose Response* 2006;3:273–305. [PubMed: 18648619]
- Koh JY, Wie MB, Gwag BJ, Sensi SL, Canzoniero LM, Demaro J, Csernansky C, Choi DW. Staurosporine-induced neuronal apoptosis. *Exp Neurol* 1995;135:153–159. [PubMed: 7589326]
- Krohn AJ, Preis E, Prehn JH. Staurosporine-induced apoptosis of cultured rat hippocampal neurons involves caspase-1-like proteases as upstream initiators and increased production of superoxide as a main downstream effector. *J Neurosci* 1998;18:8186–8197. [PubMed: 9763465]
- Kunisue T, Watanabe M, Subramanian A, Titenko AM, Tanabe S. Congener-specific patterns and toxic assessment of polychlorinated biphenyls in resident and migratory birds from Southern India and Lake Baikal in Russia. *Arch Environ Contam Toxicol* 2003;45:547–561. [PubMed: 14708671]
- Lake JL, McKinney R, Lake CA, Osterman FA, Heltshe J. Comparisons of patterns of polychlorinated biphenyl congeners in water, sediment, and indigenous organisms from New Bedford Harbor, Massachusetts. *Arch Contam Toxicol* 1995;29:207–220.
- Lanting CI, Huisman M, Muskiet FA, van der Paauw CG, Essed CE, Boersma ER. Polychlorinated biphenyls in adipose tissue, liver, and brain from nine stillborns of varying gestational ages. *Pediatr Res* 1998;44:222–225. [PubMed: 9702918]
- Larsen BR, Turrio-Baldassarri L, Nilsson T, Iacovella N, Domenico A, Montagna M, Facchetti S. Toxic PCB congeners and organochlorine pesticides in Italian human milk. *Ecotoxicol Environ Saf* 1994;28:1–13. [PubMed: 7523063]
- Lee A, Jackson J, Gooch J, Hilsenbeck S, Coronado-Heinsohn E, Osborne KC, Yee D. Enhancement of insulin-like growth factor signaling in human breast cancer: estrogen regulation of insulin receptor substrate-1 expression in vitro and in vivo. *Mol Endocrinol* 1999;10:787–796. [PubMed: 10319328]
- Lee JY, Kim JW, Cho SD, Kim YH, Choi KJ, Joo WH, Cho YK, Moon JY. Protective effect of ginseng extract against apoptotic cell death induced by 2,2',5,5'-tetrachlorobiphenyl in neuronal SK-N-MC cells. *Life Sci* 2004;75:1621–1634. [PubMed: 15261766]
- Mariussen E, Fonnum F. The effect of polychlorinated biphenyls on the high affinity uptake of the neurotransmitters, dopamine, serotonin, glutamate and GABA, into rat brain synaptosomes. *Toxicology* 2001;159:11–21. [PubMed: 11250051]

- Mariussen E, Mørch Andersen J, Fonnum F. The effect of polychlorinated biphenyls on the uptake of dopamine and other neurotransmitters into rat brain synaptic vesicles. *Toxicol Appl Pharmacol* 1999;161:274–282. [PubMed: 10620485]
- Mariussen E, Myhre O, Reistad T, Fonnum F. The polychlorinated biphenyl mixture aroclor 1254 induces death of rat cerebellar granule cells: the involvement of the N-methyl-D-aspartate receptor and reactive oxygen species. *Toxicol Appl Pharmacol* 2002;179:137–144. [PubMed: 11906243]
- McCarthy MM. Estradiol and the developing brain. *Physiol Rev* 2008;88:91–124. [PubMed: 18195084]
- Mellon PL, Windle JJ, Goldsmith PC, Padula CA, Roberts JL, Weiner RI. Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. *Neuron* 1990;5:1–10. [PubMed: 2196069]
- Merritt RL, Foran CM. Influence of persistent contaminants and steroid hormones on glioblastoma cell growth. *J Toxicol Environ Health A* 2007;70:19–27. [PubMed: 17162496]
- Ndoutse LT, Chan HM. Role of N-methyl-D-aspartate receptors in polychlorinated biphenyl mediated neurotoxicity. *Toxicol Lett* 2009;184:50–55. [PubMed: 19022367]
- Noel SD, Keen KL, Baumann DI, Filardo EJ, Terasawa E. Involvement of G protein-coupled receptor 30 (GPR30) in rapid action of estrogen in primate LHRH neurons. *Mol Endocrinol* 2009;23:349–359. [PubMed: 19131510]
- Núñez G, Benedict MA, Hu Y, Inohara N. Caspases: the proteases of the apoptotic pathway. *Oncogene* 1998;17:3237–3245. [PubMed: 9916986]
- Park H, Lee SJ, Kang JH, Chang YS. Congener-specific approach to human PCB concentrations by serum analysis. *Chemosphere* 2007;68:1699–1706. [PubMed: 17509640]
- Ruiz P, Faroon O, Moudgal CJ, Hansen H, De Rosa CT, Mumtaz M. Prediction of the health effects of polychlorinated biphenyls (PCBs) and their metabolites using quantitative structure-activity relationship (QSAR). *Toxicol Lett* 2008;181:53–65. [PubMed: 18662755]
- Safe SH. Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. *Crit Rev Toxicol* 1994;24:87–149. [PubMed: 8037844]
- Salama J, Chakraborty TR, Ng L, Gore AC. Effects of polychlorinated biphenyls on estrogen receptor-beta expression in the anteroventral periventricular nucleus. *Environ Health Perspect* 2003;111:1278–1282. [PubMed: 12896846]
- Sánchez-Alonso JA, López-Aparicio P, Recio MN, Pérez-Albarsanz MA. Polychlorinated biphenyl mixtures (Aroclors) induce apoptosis via Bcl-2, Bax and caspase-3 proteins in neuronal cell cultures. *Toxicol Lett* 2004;153:311–326. [PubMed: 15454307]
- Sanchez-Alonso JA, Lopez-Aparicio P, Recio MN, Perez-Albarsanz MA. Apoptosis-mediated neurotoxic potential of a planar (PCB 77) and a nonplanar (PCB 153) polychlorinated biphenyl congeners in neuronal cell cultures. *Toxicol Lett* 2003;144:337–349. [PubMed: 12927351]
- Sassi-Messai S, Gibert Y, Bernard L, Nishio S, Ferri Lagneau KF, Molina J, Andersson-Lendahl M, Benoit G, Balaguer P, Laudet V. The phytoestrogen genistein affects zebrafish development through two different pathways. *PLoS ONE* 2009;4:e4935. [PubMed: 19319186]
- Seegal RF, Bush B, Brosch KO. Sub-chronic exposure of the adult rat to Aroclor 1254 yields regionally-specific changes in central dopaminergic function. *Neurotoxicology* 1991;12:55–65. [PubMed: 2014069]
- Stellman SD, Djordjevic MV, Muscat JE, Gong L, Bernstein D, Citron ML, White A, Kemeny M, Busch E, Nafziger AN. Relative abundance of organochlorine pesticides and polychlorinated biphenyls in adipose tissue and serum of women in Long Island, New York. *Cancer Epidemiol Biomarkers Prev* 1998;7:489–496. [PubMed: 9641493]
- Thomas P, Dong J. Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption. *J Steroid Biochem Mol Biol* 2006;102:175–179. [PubMed: 17088055]
- Thomas P, Pang Y, Filardo EJ, Dong J. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology* 2005;146:624–632. [PubMed: 15539556]
- Wijayarathne AL, Nagel SC, Paige LA, Christensen DJ, Norris JD, Fowlkes DM, McDonnell DP. Comparative analyses of mechanistic differences among antiestrogens. *Endocrinology* 1999;140:5828–5840. [PubMed: 10579349]

Figure 1.

The effects of (A) PCB74, (B) PCB118, (C) PCB153, or (D) PCB Mix on GT1-7 GnRH peptide levels. The cells, seeded on 96-well plates, were treated for 1, 4, 8, and 24 hrs with 0.1% DMSO alone (vehicle) or with 0.1, 1, 10, or 100 μ M PCB. By 4 hr post-treatment, a stimulation of peptide was observed in PCB-treated cells in a dose-dependent manner that varied by compound. At late time points (8 hr, 24 hr) PCBs suppressed GnRH peptide. Results are expressed as average GnRH peptide levels $\pm$ S.E.M for each experimental average of pooled triplicate cultures ($N = 4$) per treatment. * $p < 0.05$, ** $p < 0.005$ vs. vehicle (DMSO).

ER Modulates PCB-Suppression of GnRH Peptide

Figure 2. Estrogen receptor antagonism modulates PCB-suppression of GnRH peptide release
Cells were treated for 24 hrs with 0.1% DMSO alone (vehicle), 1000 nM ICI alone, 1 μM PCBs, or 1 μM PCBs coadministered with ICI. Coadministration of PCB with ICI partially reversed PCB-induced suppression of GnRH peptide. Results are expressed as average GnRH peptide levels $\pm$ S.E.M for each experimental average of pooled triplicate cultures (N = 4) per treatment. *p < 0.05, **p < 0.005 vs. vehicle (DMSO).

Figure 3.

The effects of (A) PCB74, (B) PCB118, (C) PCB153, and (D) PCB Mix on GT1-7 cell viability. Fluorescence measured at 560_{Ex}/590_{Em} is proportional to the number of metabolically active cells. Samples at each timepoint were normalized to vehicle control fluorescence at 1 hr timepoint, and are expressed as percentage control. Individual congeners had inhibitory effects upon cell metabolism/viability vs. vehicle at 1 hr, whereas their combination only differed from control at the lowest dose at 4 hr. Each value, expressed as percentage control, is the mean $\pm$ S.E.M of four experiments run in triplicate. After 24 hrs treatment, all PCBs decreased viability. *p < 0.05, **p < 0.005 vs. vehicle (DMSO) at 1 hr baseline.

Figure 4.

Fluorescence assays of markers of cell death are shown. (A) Trypan Blue staining of GT1-7 cells is shown for a representative PCB118 treated culture. Normal cells appear unstained and necrotic cells (arrow) appear blue. (B) DAPI staining of GT1-7 nuclei is shown in a representative PCB74 treated culture. The arrow shows a condensed apoptotic nucleus, and the arrowhead shows a mitotic (non-apoptotic) nucleus. Scale bar = 10 μ m (panels A and B). (C and D) Cleaved caspase-9 immunofluorescence is shown for the 4 hour timepoint for a representative control (C) or PCB Mix (D) culture. Results for individual PCBs and the PCB Mix were similar, and all PCBs activated cleaved caspase-9 immunofluorescence. (E and F) Cleaved caspase-8 immunofluorescence is shown for the 4 hour timepoint for a representative control (C) or PCB Mix (D) culture. Results for individual PCBs were indistinguishable from

the PCB Mix (not shown). Caspase-8 immunofluorescence was not detectable in any of the PCB- or vehicle-treated cultures. Scale bar = 10 μ m (panels C – F).

Figure 5.

The effects of (A) PCB74, (B) PCB118, (C) PCB153, and (D) PCB Mix treatment upon GT1-7 trypan blue staining (necrotic cell death). Cells were incubated in absence (DMSO) or presence of PCB concentrations (0.1, 1, 10, or 100 μM) for different durations (1, 4, 8, 24 hr). Results are expressed as a percentage of blue stained cells characteristic of necrotic cells $\pm$ S.E.M for 4 triplicates of each experimental condition. We observed a slight but significant increase in necrotic cell death after 8 h treatment with the highest dose PCB118 or PCB153. After 24 h treatment, the highest dose PCB74 and two highest doses of PCB118 and PCB153 caused an extensive increase in necrotic cell death.

Figure 6.

The effects of (A) PCB74, (B) PCB118, (C) PCB153, and (D) PCB Mix on GT1-7 apoptotic cell death. Cells were incubated in absence (DMSO) or presence of PCB concentrations (0.1, 1, 10, or 100 μ M) for different durations (1, 4, 8, 24 hr). Results are expressed as a percentage pyknotic DAPI stained cells characteristic of apoptotic cells $\pm$ S.E.M for 4 triplicates of each experimental condition. We observed a significant increase in apoptotic cell death as early as 1 hr treatment with lower doses (0.1 or 1 μ M) PCB74, PCB118, or PCB Mix. After 24 h treatment, all PCBs caused a significant increase in apoptotic cell death.

Figure 7.

The effects of (A) PCB74, (B) PCB118, (C) PCB153, or (D) PCB Mix on caspase-3/7 activation. Cells were incubated in absence (DMSO) or presence of PCB concentrations (0.1, 1, 10, or 100 μM) for four hours. Results are expressed as fold increase of caspase-3/7 activation compared to control cells $\pm$ S.E.M for 4 triplicates of each experimental condition. Treatment with PCB74, PCB118 or the PCB Mix resulted in activation of caspase-3/7, while PCB153 did not significantly affect caspase-3/7 activity. * $p < 0.05$, ** $p < 0.005$ vs. vehicle (DMSO) at 4 hr.