# INHIBITION OF APOPTOSIS IN MCF-7 CELLS GROWING IN SERUM-SUPPLEMENTED MEDIUM TREATED WITH LOW-HALOGENATED PCB 3 AND ITS HYDROXYLATED METABOLITES.

Anna Ptak., Agnieszka Rak., Ewa L Gregoraszczuk

Department of Physiology and Toxicology of Reproduction, Institute of Zoology, Jagiellonian University, Krakow, Poland.

## Introduction

Lower chlorinated PCBs, especially those with one or two free *para*-positions, are rapidly metabolized to hydroxy-PCBs. This does not necessarily imply, however, that hydroxylated PCBs are rapidly excreted, as evidenced by the presence of hydroxylated PCB metabolites at unexpectedly high levels in the blood of exposed animals and humans (Bergman et al., 1994; Sandau et al., 2000). Recently, high levels of lower chlorinated biphenyls, including PCB3, were measured in indoor and outdoor air and a surprisingly high percentage of PCB exposure through inhalation was calculated (Harrad et al., 2006; Ishikawa et al., 2007).

The results of last our reported study showed that PCB3 and its 4-OH-PCB3 metabolite had no effect on cell proliferation until after 168 hours of exposure, whereas 3,4-diOHPCB3 decreased cell proliferation at the highest concentration tested. This came somewhat as a surprise, since 4-OH-PCB3 and 3,4-diOH-PCB3 have both been shown to be very potent activators of the ER in cultured cells; they are, in fact, twice as potent as 5 pM E2 (Machala et al., 2004). The cell cycle analysis showed an increase of cells in G2/M phase after exposure to 3,4-diOH-PCB3 for an extended period of time. Cells have DNA surveillance mechanisms that enable cells that have accumulated too much DNA damage to initiate a process termed apoptosis, a form of cellular suicide (Loechler, 2002).

The aim of the present data was to evalute the effect of PCB3 and its hydroxylated metabolites on MCF-7 cell apoptosis. MCF-7 breast cancer cells were cultured in two different hormonal milieu, either by growing them in media treated with activated charcoal to remove substantial amounts of estrogen, thus mimicking the hormonal milieu of breast cancer cells in postmenopausal women, or by growing in media supplemented with 5% FBS, thus mimicking the hormonal environment of breast cancer cells in normal cycling women.

## Material and Methods

MCF-7 cells (human estrogen-receptor positive metastatic breast cancer cell line) were incubated with PCB3 and its hydroxylated metabolites (4-OH-PCB3 or 3,4-diOH-PCB3) at a concentration of 60 ng/ml. Two methods were applied to detect DNA fragmentation. The first method used the Cellular DNA fragmentation ELISA kit (Roche Molecular Biochemicals), following the manufacturer's instructions. This assay is based on the quantitative detection of bromodeoxyuridine (BrdU)-labeled DNA fragments. In brief, after exposure to BrdU for 24 hours, cells were reseeded on to a microplate ($10^5$ cells/well) and treated for 24 hours. Supernatant was removed and cells were lysed with the kit buffer. Results are expressed as relative absorbance at 405 nm. The data were analyzed using using Stastistica 6.0, by 1-way analysis of variance (ANOVA) followed by the Tukey honestly significant difference (HSD) multiple range test.

The second method used was the TUNEL (*Terminal deoxynucleotide transferase dUTP Nick End Labeling*) assay. Cells were harvested by trypsinization, and then fixed in 1% paraformaldehyde for 30 minutes on ice. After washing the cells twice with PBS, the fixed MCF-7 cells ($1x10^6$ cells/ml) were incubated in 70% ethanol for at least 12 hours at –20°C and then the ApoBrdU-IHC™ DNA Fragmentation Assay colorimetric Kit (BioCAT, USA) was used according to the manufacturer's protocol. To detect the difference between apoptotic cells (black color) and non-apoptotic cells, fixed cells were incubated in DAB and methyl green. The cells were observed and analyzed using a Nikon Eclipse E200 microscope.

## Results and Discussion

We observed that PCB3 and 4-OH PCB3 inhibited staurosporine-induced apoptosis only in cells growing in medium supplemented with 5% FBS (Fig. 1b); they had no effect on apoptosis when cells were grown in estradiol-deprived medium (Fig. 1a). 3,4-di-OH-PCB3 inhibited apoptosis under both conditions, in estradiol-deprived and estradiol-containing medium.

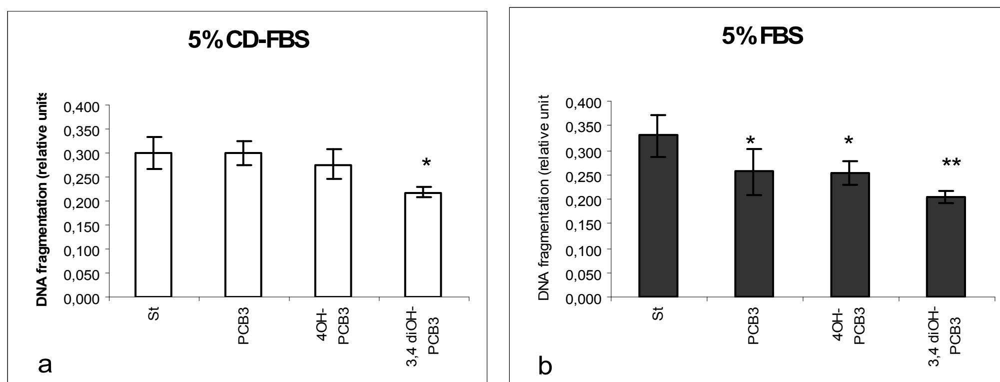


Fig. 1 Effect of PCB 3 and its hydroxylated metabolites (4-OH-PCB3 or 3,4-diOH-PCB3) combined with staurosporine (1μM) on apoptosis in cells growing in medium a) supplemented with 5% FBS-CD or b) 5% FBS. Control cells obtained staurosporine only. * ($p < 0.05$), ** ($p < 0,01$)

All the compounds tested, namely PCB3 (Fig.B), 4-OH PCB3 (Fig.C) and 3,4-di-OH-PCB3 (Fig.D), inhibit staurosporine-induced apoptosis in cells growing in medium supplemented with 5% FCS compared with control cells growing in the presence of staurosporin (Fig.A).

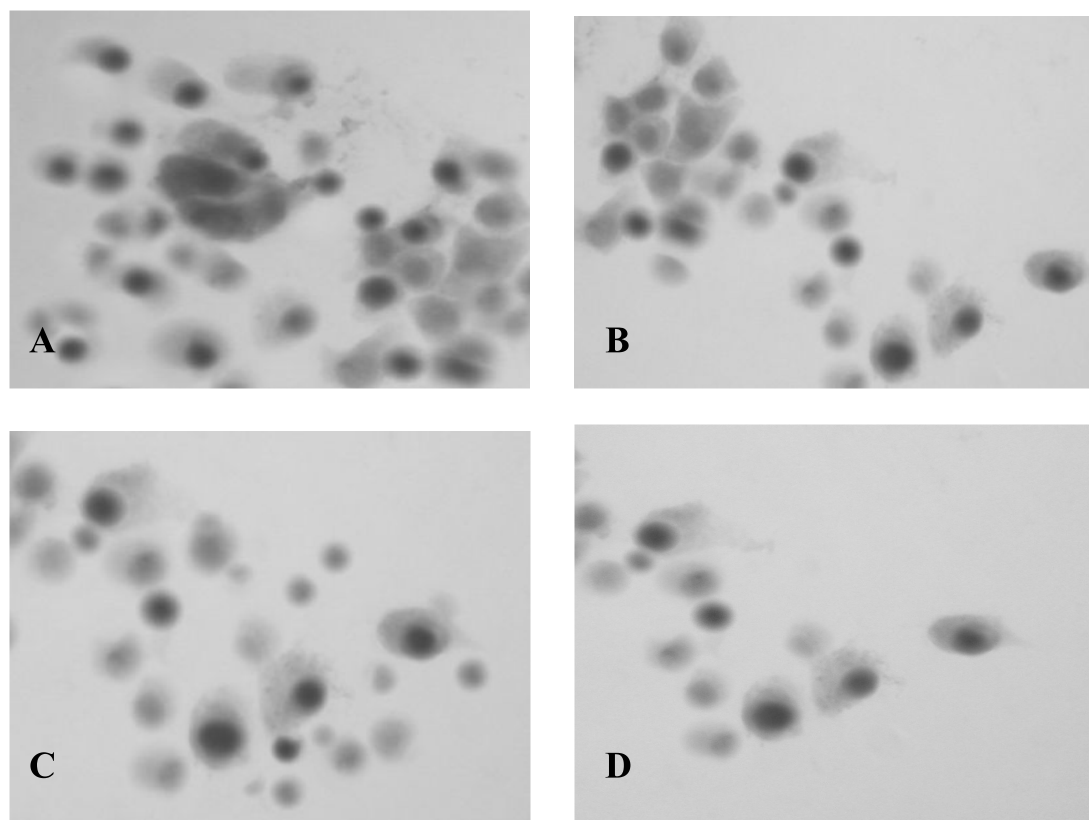


Fig. 2 MCF-7 breast cancer cells were cultured in medium supplemented with 5% FBS. Control cells were treated with staurosporine only (A). PCB 3 (B) and its hydroxylated metabolites (4-OH-PCB3(C) or 3,4-diOH-PCB3(D)) combined with staurosporine (1μM).

The inhibition of apoptosis is thought to play a central role in tumor promotion. . According to the prevailing hypothesis, the inhibition of apoptosis, which is intrinsically enhanced in preneoplastic cell clones, may play a central role in tumor promotion (Bursch et al., 1984). In fact, the inhibition of apoptosis in preneoplastic enzyme-altered foci has been demonstrated for phenobarbital, TCDD, and other liver tumor promoters (Bursch et al., 1984; Stinchcombe et al., 1995; Plant et al., 1998).

Several studies have investigated the correlation between PCB exposure and the incidence of breast cancer. Studies concerned with the sum of all PCB congeners present in breast adipose tissue or plasma, generally find no elevation in breast cancer risk (Gammon et al., 2002; Hoyer et al., 1998; Laden et al., 2001;Zheng et al., 2000). Other studies have found a correlation between high PCB levels and breast cancer in connection with specific CYP isoforms (Laden et al., 2002; Li et al., 2005; Moysich et al., 1999; Zhang et al., 2004), indicating that metabolism of either an exogenous compound like the PCBs themselves, or of endogenous compounds like estradiol, may play a role. Even so, the association between PCBs and breast cancer is questionable.

Many of these compounds are members of a large and diverse group of tumor-promoting compounds that support the clonal expansion of preneoplastic cell clones in rat liver thus enhancing the risk of malignant transformation (Schulte-Hermann et al., 1993). The molecular mechanisms underlying tumor promotion are poorly understood. A number of hypotheses exist, including the notion that the inhibition of apoptosis, which is intrinsically enhanced in preneoplastic hepatocyte clones, may play an important role (Bursch et al., 1984).

A number of 'non-dioxin-like' PCBs are either inactive or almost inactive as AhR agonists, but induce a battery of drug-metabolizing enzymes, including rat CYP2B1/2B2, known as phenobarbital-inducible genes (Connor et al., 1995; Giesy and Kannan, 1998). Therefore, these PCBs are sometimes categorized as 'phenobarbital-like' inducers. A variety of PCBs induce both CYP2B1/2B2 and 1A isozymes in rat liver, and therefore, have been categorized as 'mixed-type' inducers (Connor et al., 1995). Among the 'non-dioxin-like' PCBs there are also a number of congeners that act as tumor promoters in rat liver (Buchmann et al., 1991; Hemming et al., 1993; Connor et al., 1995). They congeners share in common with the tumor promoter phenobarbital the property of induction of a battery of certain drug-metabolizing enzymes, including CYP2B1/2B2 (Connor et al., 1995; Giesy and Kannan, 1998). The signaling pathway that leads to the 'phenobarbital-type' induction of CYP2B genes involves the constitutive androstane receptor (CAR), which acts as a transactivator of a distal enhancer in responsive genes called the phenobarbital-responsive enhancer module (Kawamoto et al., 1999). An analysis of CYP1A1 and CYP1B1 protein levels indicates that PCB3 is a relatively potent inducer of this enzyme (data not shown).

In summary, our results show that the action of PCB3 and its hydroxylated metabolite 4-OH-PCB3 on MCF-7 cells depends on the hormonal milieu. We have shown that apoptosis is inhibited in cells cultured in serum-supplemented medium but not in hormone-deprived medium. Moreover, we have shown that 3,4-di-OH, a PCB3 metabolite that is directly converted to 3,4-quinones, is the most potent antiapoptotic agent in MCF-7 cells. Inhibition of apoptosis was noted under both conditions. These results are consistent with the hypothesis that mammary tumor development can be initiated primarily by metabolism of xenoestrogens to quinones, which may react with DNA to induce oncogenic mutations. Further experiments are required to identify the mechanism of 3,4-di-OH action that is critical for tumor promotion and its relation to the inhibition of apoptosis.

References


1. Bergman, A., Klasson-Wehler, E., Kuroki, H., 1994. Environ Health Perspect 102(5), 464-9.
2. Sandau CD, Ayotte P, Dewailly E, Duffe J, Norstrom RJ.Environ Health Perspect. 2000 Jul;108(7):611-6.
3. Harrad S, Hazrati S, Ibarra C.Environ Sci Technol. 2006 Aug 1;40(15):4633-8.
4. Ishikawa, Y., Noma, Y., Yamamoto, T., Mori, Y., Sakai, S., 2007. Chemosphere 67(7), 1383-93.
5. Machala M, Bláha L, Lehmler HJ, Plísková M, Májková Z, Kapplová P, Sovadinová I, Vondrácek J, Malmberg T, Robertson LW. Chem Res Toxicol. 2004 Mar;17(3):340-7.
6. Loechler, E.L., 2002. Environmental Mutagens and Carcinogens. In: Nature Encyclopedia of Life Sciences. Nature Publishing Group, New York, NY,http://www.els.net.
7. Bursch, W., Lauer, B., Timmermann-Trosiener, I., Barthel, G.,Schuppler, J., Schulte Hermann, R., 1984. Carcinogenesis 5,453–455.
8. Stinchcombe S, Buchmann A, Bock KW, Schwarz M. Carcinogenesis. 1995 Jun;16(6):1271-5.
9. Plant, N.J., Horley, N.J., Dickins, M., Hasmall, E., Elcombe, C.R.,Bell, D.R., 1998..Carcinogenesis 19, 1521–1527.
10. Gammon, M.D., Wolff, M.S., Neugut, A.I., Eng, S.M., Teitelbaum, S.L., Britton,J.A., Terry, M.B., Levin, B., Stellman, S.D., Kabat, G.C., et al., 2002.Cancer Epidemiol. Biomarkers Prev. 11 (8),686–697.

11. Hoyer, A.P., Grandjean, P., Jorgensen, T., Brock, J.W., Hartvig, H.B., 1998. Lancet 352 (9143),1816–1820.
12. Laden, F., Hankinson, S.E., Wolff, M.S., Colditz, G.A., Willett, W.C., Speizer,F.E., Hunter, D.J., 2001. Int. J. Cancer.91 (4), 568–574.
13. Zheng, T., Holford, T.R., Tessari, J., Mayne, S.T., Owens, P.H.,Ward, B., Carter,D., Boyle, P., Dubrow, R., Archibeque-Engle, S., et al., 2000. Am. J. Epidemiol. 152 (1), 50–58.
14. Laden, F., Ishibe, N., Hankinson, S.E., Wolff, M.S., Gertig, D.M., Hunter, D.J.,Kelsey, K.T., 2002. Cancer Epidemiol. Biomarkers Prev. 11 (12), 1560–1565.
15. Li, Y., Millikan, R.C., Bell, D.A., Cui, L., Tse, C.K., Newman, B., Conway, K.,2005. Breast Cancer Res. 7 (1), R12–R18.
16. Moysich, K.B., Shields, P.G., Freudenheim, J.L., Schisterman, E.F., Vena, J.E.,Kostyniak, P., Greizerstein, H., Marshall, J.R., Graham, S., Ambrosone, C.B., 1999. Cancer Epidemiol.Biomarkers Prev. 8 (1), 41–44.
17. Zhang, Y.,Wise, J.P., Holford, T.R., Xie, H., Boyle, P., Zahm, S.H., Rusiecki, J.,Zou, K., Zhang, B., Zhu, Y., et al., 2004.. Am. J. Epidemiol. 160 (12), 1177–1183.
18. Schulte-Hermann R, Bursch W, Kraupp-Grasl B, Oberhammer F, Wagner A, Jirtle R. Environ Health Perspect. 1993 Dec;101 Suppl 5:87-90.
19. Connor, K., Safe, S., Jefcoate, C.R., Larsen, M., 1995. Biochem. Pharmacol. 50, 1913–1920.Connor et al., 1995;
20. Giesy, J.P., Kannan, K., 1998. Crit. Rev. Toxicol. 28, 511–569
21. Buchmann, A., Ziegler, B., Wolf, A., Robertson, L.W., Durham, S.K., Schwarz, M., 1991. Toxicol. Appl. Pharmacol. 111, 454–468
22. Hemming, H., Flodström, M., Wängärd, L., Bergman, A.,
23. Kronevi, T., Nordgren, I., Ahlborg, U.G., 1993. Eur. J. Pharmacol. Environ. Toxicol. 248, 163–174.
24. Kawamoto, T., Sueyoshi, T., Zelko, I., Moore, R., Washburn,K., Negishi, M., 1999. Mol. Cell. Biol. 19, 6318–6322.

Acknowledgements.
Supported by State Committee for Scientific Research number 1929/PO1/06/31 from 2006- 2009