Chapter 4

Oestrogens, Xenoestrogens and Hormone-Dependent Cancers

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Additional information is available at the end of the chapter

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1. Introduction

The hormonal microenvironment surrounding endocrine-sensitive tissues may play an important role in the carcinogenesis of these tissues. Epidemiological evidence strongly suggests that steroid hormones, primarily oestrogens (E2), are implicated in ovarian and breast carcinogenesis.

The breast is an endocrine-sensitive organ. The development of the breast from puberty through the cycles of pregnancy, lactation and involution is regulated through hormonal controls. Epidemiological studies have demonstrated that 50% or more of breast cancers are environmental in origin. Epidemiological evidence linking breast cancer incidence to oestrogen exposure and the ability of oestrogen to drive the growth of breast tumours in vivo is well documented in clinical studies. In addition, the mechanism of oestrogen action on the growth of breast cancer cells in animal models and in vitro has been extensively described in experimental studies. The involvement of oestrogen in the progression of breast cancer is the basis for the successful use of endocrine therapy as a treatment for breast cancer. In addition to the physiological steroidal oestrogens, many compounds have been found to have oestrogenic activity such that the human breast can be exposed to environmental oestrogens from a variety of sources. The link between breast cancer and the use of the oral contraceptive pill has been extensively studied, and a study involving a million women has documented an increase in breast cancer following the use of hormone replacement therapy (HRT). Such findings demonstrate that the development of breast cancer can be influenced at all stages of life after puberty through the voluntary exposure to exogenous oestrogens.

Ovarian cancer is the fourth-ranking cause of cancer-related death in women from Western countries. The natural history of this cancer is characterised by the potential for particularly aggressive local invasion. Unfortunately, these tumours are often diagnosed at an advanced

stage (i.e., 70% of tumours are discovered at stage III). Although all cell types of the human ovary may undergo neoplastic transformation, the vast majority (80-90%) of tumours are derived from ovarian surface epithelium (OSE). One of the hypotheses regarding the causes of ovarian cancer argues that the repeated cycles of ovulation-induced trauma and repair of the OSE during ovulation, without pregnancy-induced rest periods, contribute to ovarian cancer development. This "incessant ovulation" hypothesis suggests that regenerative repair of OSE cells that occurs during ovulation results in the accumulation of mutations, which predisposes this cell layer to tumourogenesis. There is also growing experimental evidence that oestrogens may play an important role in ovarian carcinogenesis. Use of HRT for menopause-related symptoms could be associated with an increased risk of ovarian cancer incidence or mortality.

2. Endogenous oestrogens

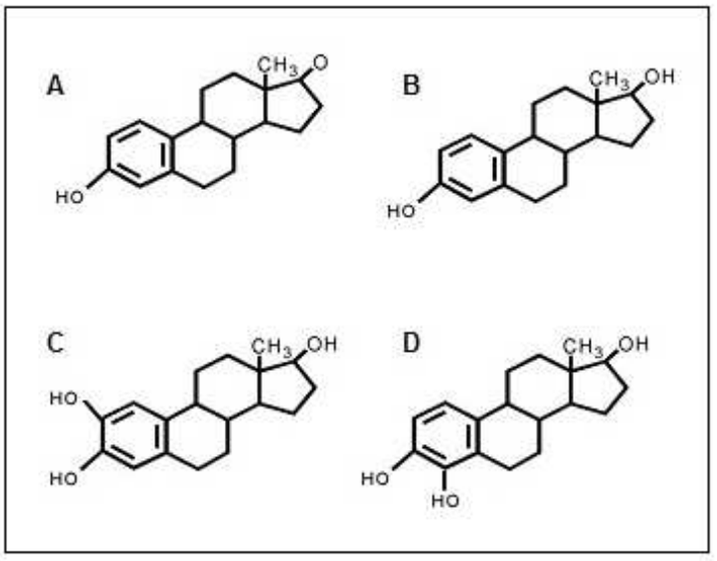

Figure 1. A) Oestrone (E1); B) Oestradiol (E2); C) 2-hydroxyoestradiol (2-OH-E2); D) 4-hydroxyoestradiol (4-OH-E2)

2.1. Carcinogenesis of oestrogens

Oestrogens are believed to play a critical role in the etiology of breast and ovarian cancer through two distinct pathways. First, the products of oestrogen metabolism damage DNA by forming adducts and oxidised bases, leading to mutations in oncogenes and tumour suppressor genes that normally control cell growth and proliferation [1]. Second, oestrogens may alter the expression of specific genes, which stimulate growth and proliferation of epithelial cells in the breast and ovary. Notably, oestradiol-17β has been classified as a carcinogen by the International Agency for Research on Cancer. Thus, natural oestrogens levels in men and women have the potential to act as carcinogens. For example, early menarche and late menopause are risk factors for breast cancer, due to longer oestrogen exposures. It is well established that chronic exposure to elevated oestrogen levels

contributes to carcinogenesis of multiple reproductive organs. Ovaries are not only the principal source of oestrogens in premenopausal women but are the key target tissue of oestrogen activity. Oestradiol and oestrone are mainly produced by follicular cells. Ovarian tissue oestrogen levels are at least 100-fold higher than the circulating levels and those in the follicular fluid of ovulatory follicles are even higher [2]. Additionally, active oestrogens are formed from circulating oestrone sulphate or oestradiol sulphate, as the result of de-conjugation by sulphatase [3]. The local release of biologically active oestrogens from conjugates and their further metabolism prolong the effect of oestrogen on peripheral tissues [4]. Oestrogen is essential to the function of the female reproductive system and a major regulator of growth and differentiation in normal ovaries. Furthermore, oestrogens are required for the proliferation and differentiation of healthy breast epithelium.

2.2. Classic ER mediated activity

The classic oestrogen receptors (ERs) are nuclear hormone receptors that act as transcription factors, regulating genes involved in homeostasis, development and metabolism. Two forms of ER have been identified, ERα and ERβ. While ERβ is predominantly expressed by granulosa cells, theca cells, surface epithelium, and CL, oocytes have also been reported to express the receptor. Both receptors are ligand-inducible nuclear hormone receptors. The classic mechanism of ER action involves binding of the ER to its ligand, resulting in receptor dimerisation, interaction with consensus oestrogen-response elements (EREs), and recruitment of transcriptional co-regulators, resulting in the formation of a complex that modulates the transcription of oestrogen target genes. The best-described nuclear receptor cofactors are the p160 family of co-activators, namely, SRC-1, SRC-2, and SRC-3; however, the cofactor complexes that mediate the ultimate outcome of ER signalling are complicated with more than 300 cofactors described in the literature [5]. Numerous genes with diverse functions in energy production, cell growth, cell cycle regulation, and cytoskeleton organisation, whose expression is induced or repressed by oestrogen, have been identified by microarray analysis. Oestrogen receptors have been shown to interact with other transcription factors, co-activator proteins, and tyrosine kinase growth-factor receptors and to cross–talk with other signal transduction pathways [6]. Through regulating gene expression, oestrogen functions as a potent stimulus for proliferation and inhibition of apoptosis, which may lead to the development of cancer.

2.3. Non-genomic ER mediated activity

ER signalling may occur in a ligand-dependent, non-genomic (extra-nuclear) pathway. This pathway involves the activation of other signal transduction pathways that lead to rapid responses, generally within minutes, to oestrogen exposure. The mechanism of non-genomic ER signalling is not clear, but is potentially mediated by a membrane-associated receptor. A G-protein coupled receptor known as GPR30 mediates rapid oestrogen signalling independent of ERs, which can lead to activation of the MAPK or phosphoinositide-3-kinase (PI3) kinase signalling cascades, fluctuations in intracellular calcium, or stimulation of cAMP production [7]. E2 mediation activates the MAPK and PI3 pathways that are the

major effectors of cell proliferation and cell survival. Deregulations of cell proliferation, differentiation, and apoptosis may allow cells that have harboured mutations in proto-oncogenes and tumour suppressor genes to survive and expand clonally.

2.4. Metabolism of oestrogens

There is growing evidence that E2 and its metabolites may be involved in breast cancer development. Endogenous E2 metabolites may play an important role by influencing the growth of oestrogen-sensitive target cells, both stimulating and inhibiting proliferation [8]. It is generally known that both the biosynthesis and metabolism of E2 occur in cancerous breast tissues. The cytochrome P450-dependent monooxygenases (CYP) are responsible for the biosynthesis and metabolism of endogenous compounds such as steroid hormones. 2-hydroxyestadiol (2-OH-E2) and 4-hydroxyestradiol (4-OH-E2) are two major hydroxylated metabolites of E2 formed by cytochrome P450 1A1 and 1B1, respectively. The catechols 2-OH-E2 and 4-OH-E2 can be oxidised to quinones, which are putative tumour initiators, and the 4-hydroxylated form of E2 appears to be one of the most genotoxic metabolites of E2 in the breast epithelium. The 4-OH-E2 to 2-OH-E2 concentration ratio has been reported to be 4:1 in a human breast cancer extract [9]. The catechol-*O*-methyltransferase (COMT), which methylates catechol estrogens, prevents their conversion to quinones. There are reports that 2-hydroxylated E2/E1 are better substrates for COMT than their 4-hydroxylated isomers [10,11]. Thus, the 2OH-E2/4OH-E2 ratio may be a critical parameter of the carcinogenicity of E2.

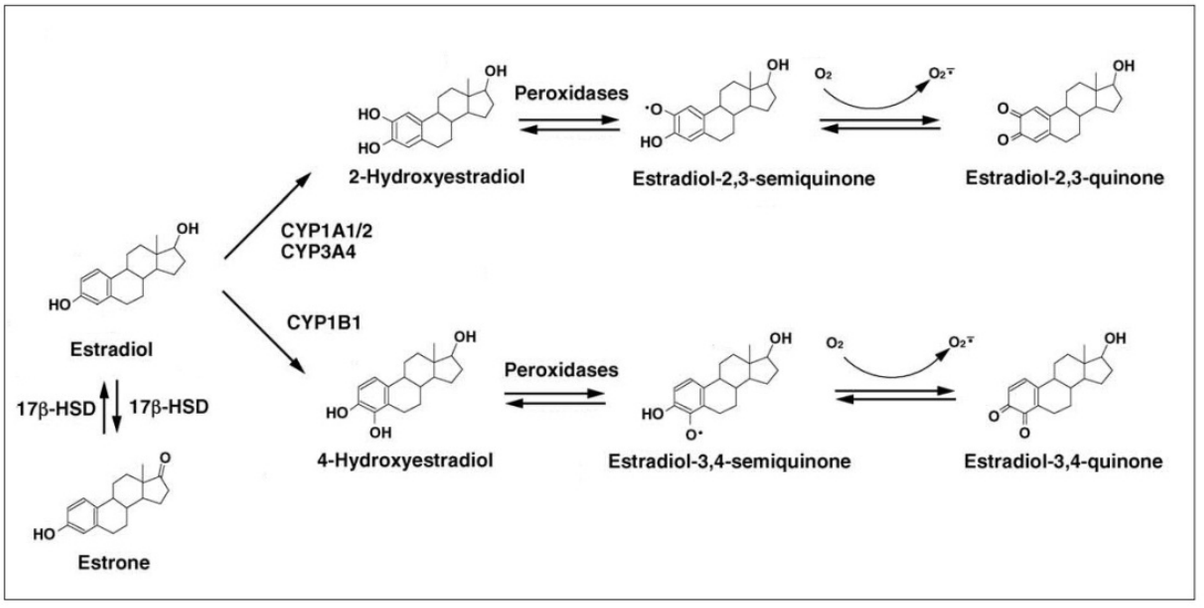

Figure 2. Major metabolic pathway in cancer initiation by oestrogens

We propose that local activation of the cytochrome P450 enzymes CYP1A1 and CYP1B1 by E2 may generate active metabolites that affect apoptosis and thereby promote mammary carcinogenesis. To test this hypothesis, we measured the ability of E2 to induce CYP1A1 and CYP1B1 and assessed the influence of the parent compounds and their hydroxylated metabolites on apoptosis.

The previously published results demonstrated that E2 increased CYP1B1 protein expression after 48 h of cell culture but had no effect on CYP1A1 protein levels [12], Figure 3. CYP1B1 has been suggested to play key roles in initiating breast cancers in humans, as this enzyme is active in catalysing oestradiol to a 4-hydroxylated metabolite. Cytochrome P450 enzymes, the products of the *CYP* genes, are components of the oestrone hydroxylase enzyme system. Elevated 4-hydroxyoestrogen production has been associated with breast tumours [13].

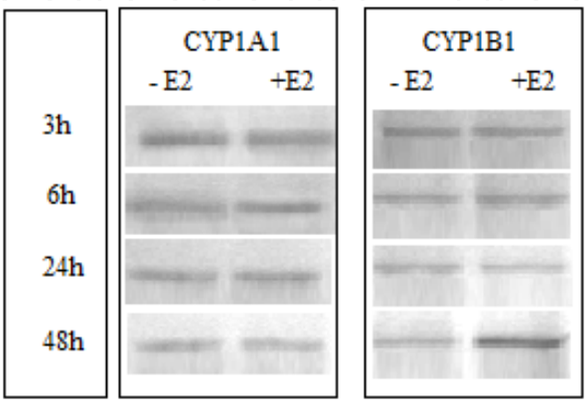

Figure 3. The effect of oestradiol (1nM) on expression of the CYP 1B1 protein in MCF-7 cells [12]

2.5. Action of E2 and its hydroxylated metabolites (2-OH-E2 and 4-OH-E2) on MCF-7 breast cancer cell proliferation

In our previously published data [12], we demonstrated that E2 can be locally metabolised to their hydroxylated derivatives via cytochrome P450 enzymes in breast cancer MCF-7 cells. Additionally, E2 hydroxylated metabolites had a time-dependent affect on MCF-7 cell proliferation [14]. While E2 and 4-OH-E2 elicited a significant increase in cell proliferation over the entire time of exposure, 2-OH-E2 resulted in an increase of cell proliferation only after a long incubation period, Figure 4.

Our observations were consistent with the results of [15], who demonstrated that 4-OH-E2 is more oestrogenic than 2-OH-E2. In [16] it was indicated that certain oestradiol metabolites, i.e., 4-OH-E2 and 16-OH-E2, are able to mimic the effects of 17β-oestradiol on proliferation and markers of tumour metastasis. The effect was more pronounced for 16-OH-E2. In contrast, the metabolite 2-OH-E2 did not show any significant effect on these parameters. The effect of the various oestrogen metabolites appears to be dependent on their ability to bind to the oestrogen-receptor. The most potent oestrogen regarding an influence on proliferation, apoptosis and metastasis is 17β-oestradiol. As this oestrogen appears to be metabolised intracellularly, the direction of oestradiol metabolism may influence breast cancer risk in certain predisposed women. Enhanced metabolism towards 2-OH-E2 may even be protective, as this metabolite is rapidly converted into 2-methoxyestradiol, which has been shown to be a potent anti-proliferative and anti-angiogenic agent in various tumour cells [17].

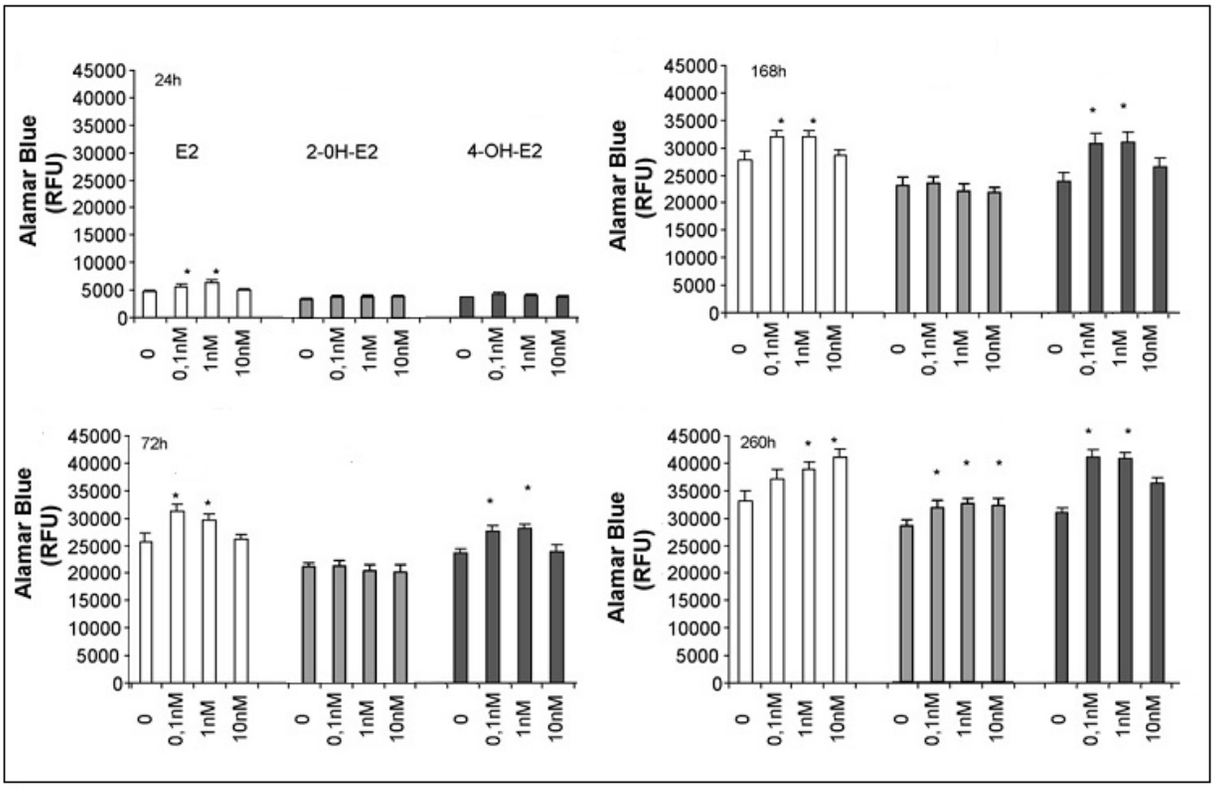

Figure 4. Action of E2 and its hydroxylated metabolites 2-OH-E2 and 4-OH-E2 on MCF-7 cell proliferation after 24, 72, 168, and 260 h of exposure to 0.1, 1.0 or 10nM concentrations of these compounds. (*) $p < 0.05$ [14]

2.6. Molecular mechanisms of E2 and its hydroxylated metabolites (2-OH and 4-OH)

The human sex hormone-binding globulin (SHBG), a glycoprotein that specifically binds plasma androgens and oestradiol, participates in the mechanism of action of oestradiol in breast cancer cells. The SHBG protein has been detected, using reverse transcriptase-polymerase chain reaction (RT-PCR), in ZR-75-1, MCF-7 and MDA-MB-231 cells, as well as in 11 breast tissue samples [18]. Although the data concerning the cell lines were convincing, no evidence for mRNA translation has been presented. Due to its unique property to regulate bioavailable oestradiol, several epidemiological studies have implicated SHBG as having a role in breast cancer, and it has been suggested that plasma SHBG levels are inversely associated with breast cancer risk in post-menopausal women. According to [19], for SHBG to be biologically relevant, the interaction of SHBG with its membrane-binding site (SHBG-R) requires the occurrence of a precise sequence of events. First, SHBG must bind to a membrane (through SHBG-R), and a ligand must then interact with the SHBG bound to the membrane. It is only at this point that the biological effect is elicited. If the ligand binds to SHBG before the protein binds to the membrane, it is blocked from interacting with membranes, Figure 5. In breast cancer MCF-7 cells, SHBG binds to the membrane, and through cyclic adenosine monophosphate (cAMP) induction and protein kinase A (PKA) activation, it inhibits oestradiol-induced cell proliferation [20].

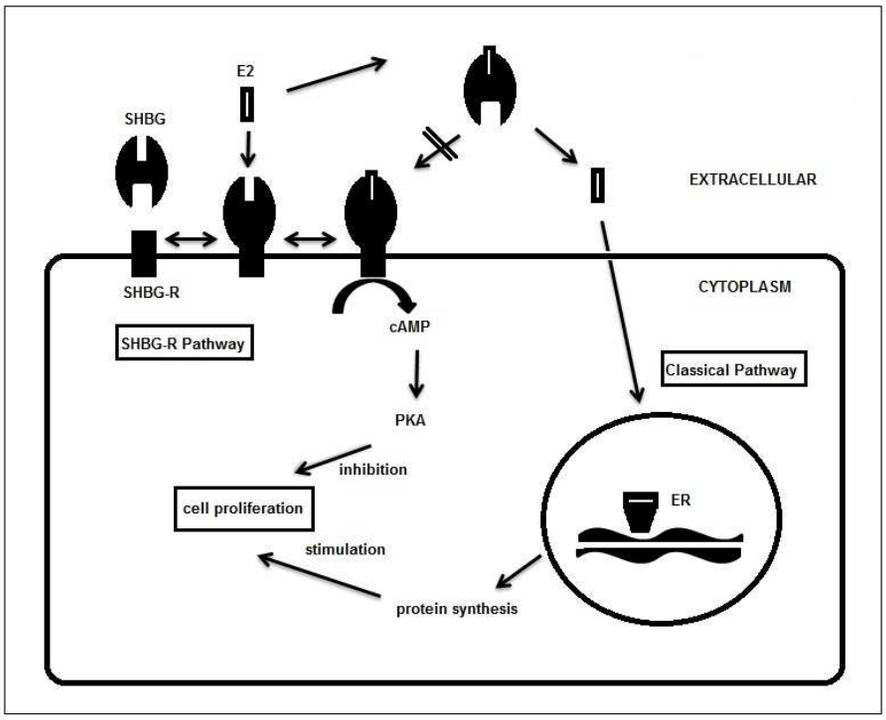

Figure 5. Model for SHBG-mediated signalling pathway

Despite extensive years of research, the action of hydroxylated metabolites of E2 (2-OH-E2 and 4-OH-E2) on SHBG intra/extracellular secretion as well as gene and protein expression in extrahepatic tissue has not yet been investigated. Our data demonstrated that hydroxylated metabolites of E2, with a potency of E2 > 4-OH-E2 > 2-OH-E2, increased intracellular and had no effect on extracellular SHBG levels [21], Figure 6.

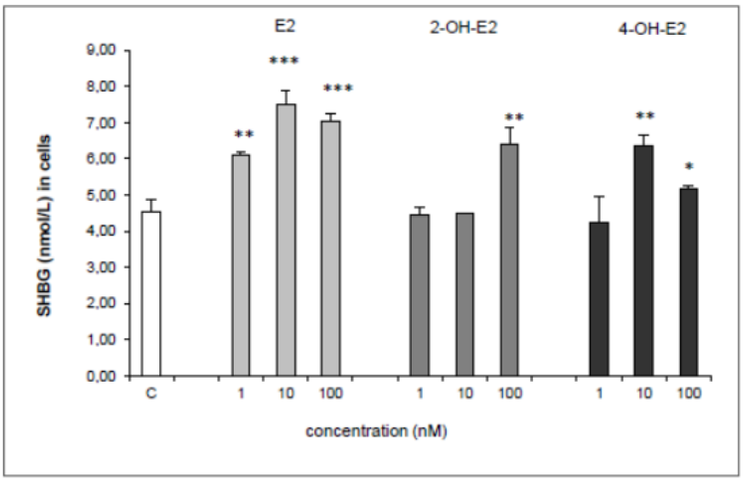

Figure 6. The effect of E2, 2-OH-E2 and 4-OH-E2 on intracellular SHBG levels in MCF-7 cells. (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$ [21]

Additionally, we demonstrated that E2, 2-OH-E2 and 4-OH-E2 increased SHBG mRNA and protein expression [21], Figure 7.

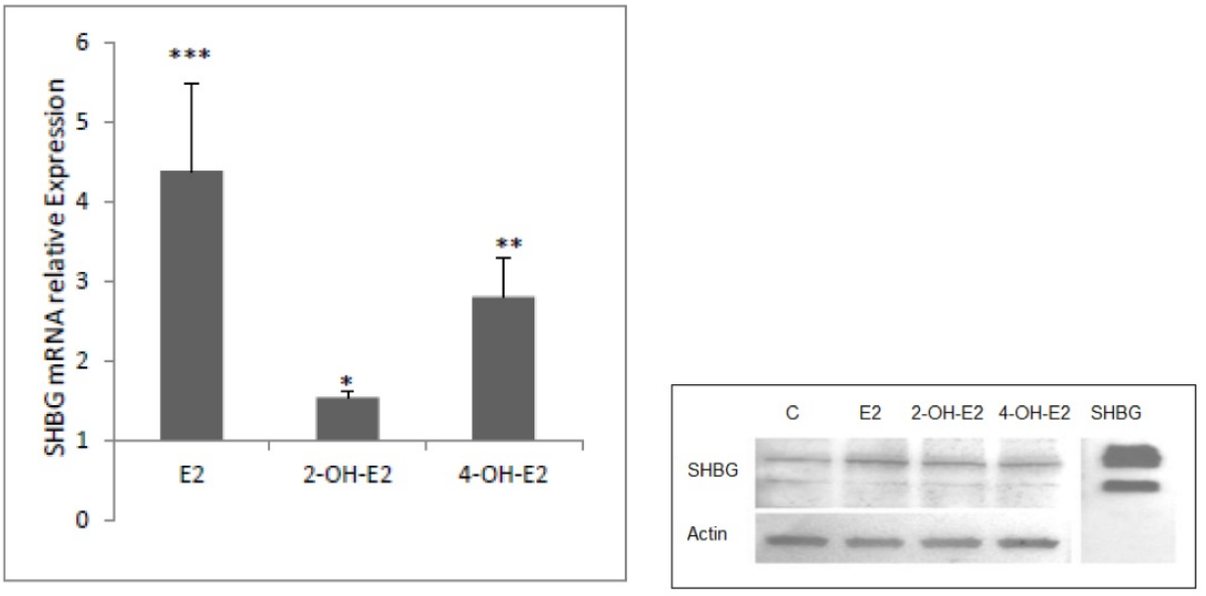

Figure 7. The effects of E2, 2-OH-E2 and 4-OH-E2 on SHBG mRNA and protein expression in MCF-7 cells. SHBG mRNA determined by real time PCR. (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$ [21]

In addition to classical transcriptional actions, oestrogens also trigger rapid intracellular signalling events typically associated with membrane receptors. Oestradiol, a biologically potent oestrogen, can induce rapid activation of many signalling molecules, including cAMP/PKA and MAPK pathways [22]. An increasing number of studies indicate that the cellular action of E2 can be initiated at the plasma membrane, through membrane versions of oestrogen receptors (mERs) or via other membrane-resident E2-binding proteins [23]. The binding of E2 to the membrane receptor causes an increase of intracellular cAMP levels [23]. As a second messenger, cAMP is involved in a variety of intracellular signalling pathways that lead to diverse physiological effects, including the control of cell proliferation. The majority of cAMP signalling in eukaryotic cells is through PKA. It has been reported that activation of the cAMP/PKA pathway induces growth inhibition in a variety of human cancer cells, including the MCF-7 cell line [23]. Mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinases (ERK1 and ERK2), are also rapidly stimulated by oestrogens in various cell types, including breast cancer cells. Additionally, it has been reported that the cAMP/PKA and the MAPK pathways are connected in MCF-7 cells [23,24]. Notably, ERK can be activated or inhibited by cAMP and E2, although activation of the cAMP/PKA suppresses the activity of ERK1/2 [25]. However, controversies also exist regarding the extent to which E2 may influence ERK1/2 activation [26]. We have shown that E2 and its hydroxylated metabolites do not activate cAMP/PKA and ERK1/2 in breast cancer cells and confirmed previously published data, which demonstrated a lack of ERK1/2 activation in a breast cancer cell line [27], Figure 8, 9. The observed reversible action of PD98059 on cell proliferation can be explained via the actions of hydroxylated estrogens, which similarly to E2, stimulate secretion of a number of growth factors that affect MAPK activity [28].

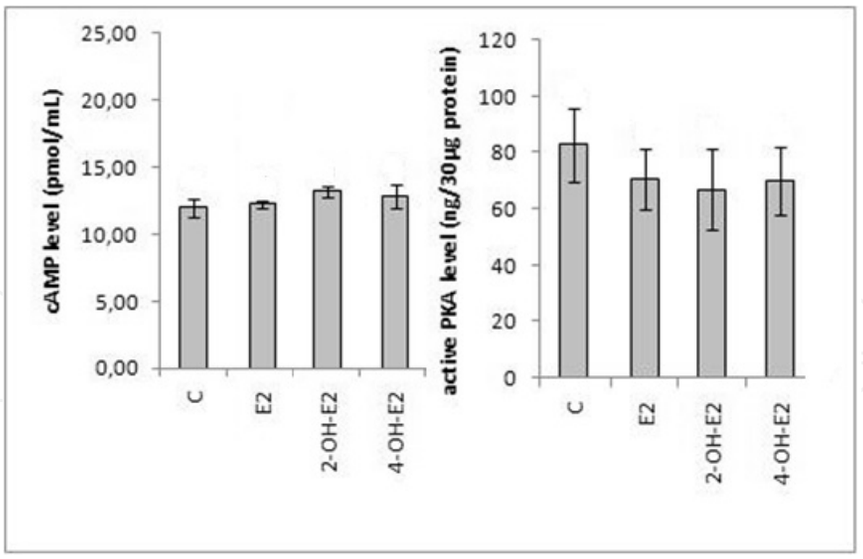

Figure 8. The effects of E2, 2-OH-E2 and 4-OH-E2 on cAMP and PKA levels in MCF-7 cells. [27]

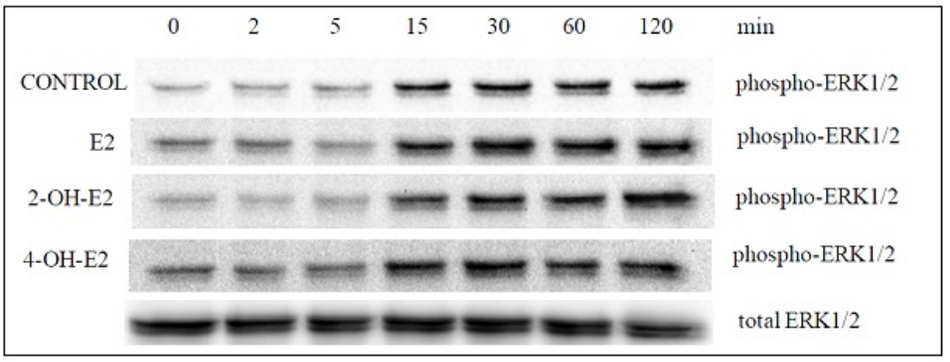

Figure 9. Time-dependent effects of E2, 2-OH-E2 and 4-OH-E2 on phospho- and total ERK1/2 protein expression in MCF-7 cells. [27]

3. Xenoestrogens

Xenoestrogens form a diverse group of man-made chemicals that have been released into the environment from agricultural spraying (herbicides, pesticides), by-products of industrial processes and waste disposal of polychlorinated biphenyls (PCBs) and dioxins or as discharges from treatment systems (alkyl phenols). They are also found in household products in daily use, such as plastics (bisphenol A, phthalates) and flame retardants (polybrominated organics) and cosmetic products (e.g., parabens, cyclosiloxanes). They may be present in diets, and because they are lipophilic, they can pass up the food chain dissolved in animal fat and accumulate in humans at the top of the food chain.

3.1.1. Polychlorinated biphenyls and breast cancer

Polychlorinated biphenyls (PCBs) are industrial chemicals that have been used as hydraulic fluids, dielectric fluids for transformers and cooling fluids for capacitors in the formulation of lubricating and cutting oils and as plasticisers in prints, copy paper, adhesives, sealants

and plastics. PCBs are complex chemical mixtures that comprise theoretically 209 congeners. Although the production and use of these compounds were banned in the late 1970s, a significant portion of the PCBs purchased by industry are still in use, mostly within capacitors and transformers. These compounds remain in our environment and are routinely found in human serum, breast milk and adipose tissue, including breast tissue. PCB exposure has been classically thought to alter normal endocrine signalling by mimicking endogenous hormone action by binding to hormone receptors, blocking receptors, or through interference with steroid metabolism. The relationship between exposure to PCBs and breast cancer has been addressed in numerous epidemiological studies since the early 1990s [29].

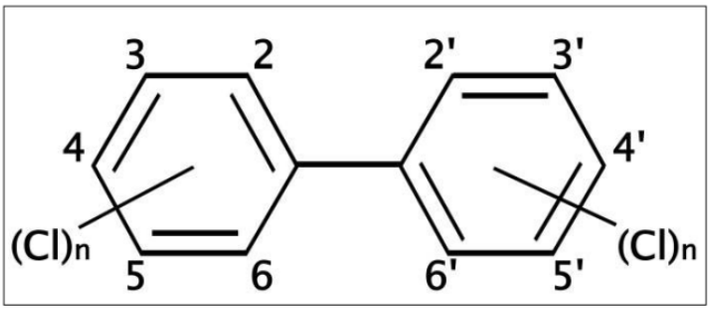

Figure 10. Polychlorinated biphenyls (PCBs)

3.1.2. Metabolism of polychlorinated biphenyls

PCBs are not only efficacious inducers but also substrates for cytochrome P450 enzymes. This enzyme metabolises PCBs, as well as other environmental compounds that enter the body, into by-products that are mutagenic and carcinogenic. Notably, lower chlorinated PCBs (such as PCB3) are rapidly metabolised and therefore called "episodic congeners" [30]. One member of the CYP1 family, CYP1A1, has been shown to play an important role in the metabolism of PCBs [31,32].

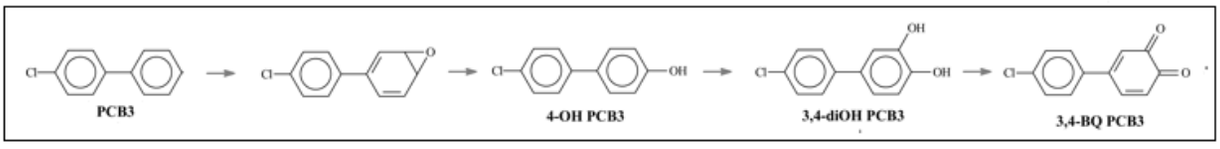

Figure 11. The metabolic activation of the 4-chlorobiphenyl (PCB3)

We demonstrated previously in MCF-7 breast cancer cells that PCB3 modestly increases CYP1A1 but not CYP1B1, protein levels and activity, Figure 12. This alteration may change metabolic activation pathways of PCB3 itself or local oestrogen metabolism and excretion [32]. The metabolic activation of PCBs and/or E2 by CYPs leads to the formation of arene oxide intermediates and reactive quinones, which can bind covalently to macromolecules such as DNA, RNA and proteins [33].

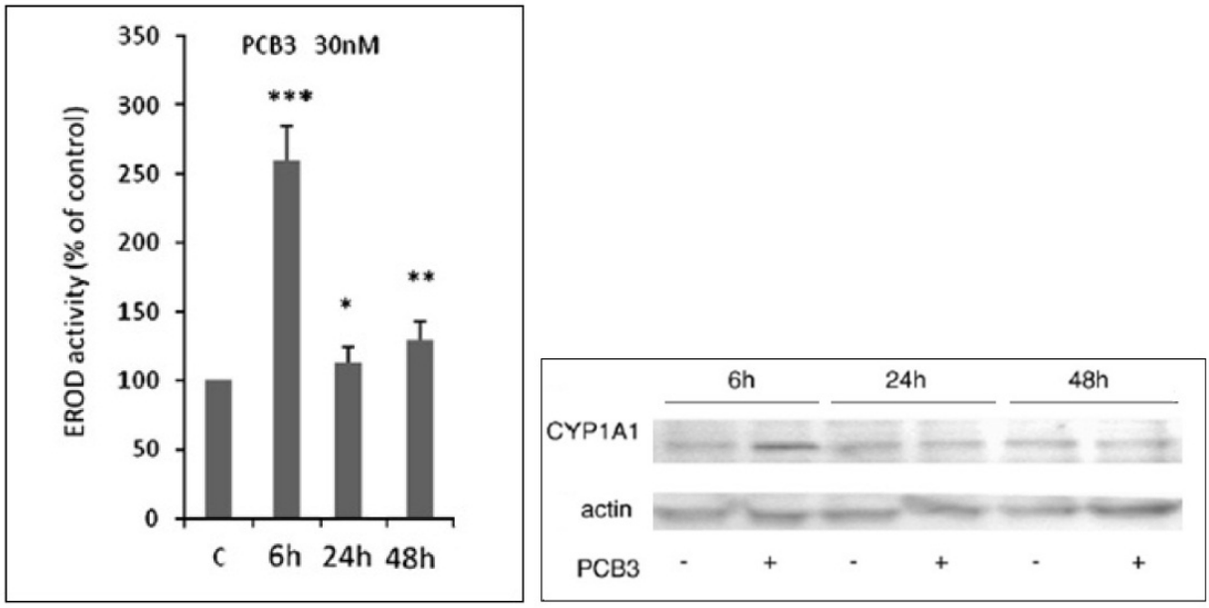

Figure 12. The effects of PCB3 on CYP1A1 activity (EROD assay) and protein levels in MCF-7 cells (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$ [32]

3.1.3. Action of PCB3 (4-chlorobifenyl) and its metabolites (4-diOH-PCB3 and 3,4-diOH-PCB3) on MCF-7 human breast cancer cell proliferation and apoptosis

The balance between proliferation and apoptosis determines tissue homeostasis under physiological conditions. Apoptosis is recognised as a major barrier that must be circumvented by tumour cells to survive and proliferate under stressful conditions. Apoptosis can be initiated via two different types of signals: intracellular stress signals (intrinsic pathway) and extracellular ligands (extrinsic pathway). Previous studies have provided conflicting data regarding the proliferation and apoptosis effects of PCBs on breast cancer cells. As opposed to the action of oestrogen on MCF-7 proliferation, we showed that PCB3 and its metabolite 4-OH-PCB3 had no effect on cell proliferation at any time during exposure, while at the highest concentration, 3,4-diOH-PCB3 decreased cell proliferation [14]. Additionally, we showed that PCB3 and both of its hydroxylated metabolites had no effect on caspase-8 (extrinsic pathway) and caspase-9 (intrinsic pathway) activity when cells were grown in medium deprived of oestrogen, but they reduced caspase-9 activity when cells were grown in medium supplemented with serum containing oestradiol, Figure 13. Interestingly, a decrease in DNA fragmentation was observed upon treatment with 3,4-diOH-PCB3 in both culture conditions, suggesting that 3,4-diOH-PCB3 inhibits a caspase-independent pathway of cell death [34].

3.1.4. Molecular mechanisms of PCB3 and its hydroxylated metabolites (4-diOH-PCB3 and 3,4-diOH-PCB3)

Similarly to endogenous oestrogens, xenoestrogens can also contribute to the activity of the SHBG pathway and the MAP kinase pathways. Several studies have demonstrated that

human SHBG binds phytoestrogens, fatty acids and 4-OH-2′,3′,4′,5′-PCB and 4-OH-2,2′,3′,4′,5′-PCB [35]. Our previously published data indicated that 3,4-diOH-PCB3 does not interact with SHBG through the membrane-binding site or directly. However, an increase in intracellular SHBG levels was noted under the influence of 3,4-diOH-PCB3 in MCF-7 cells [34], Figure 14.

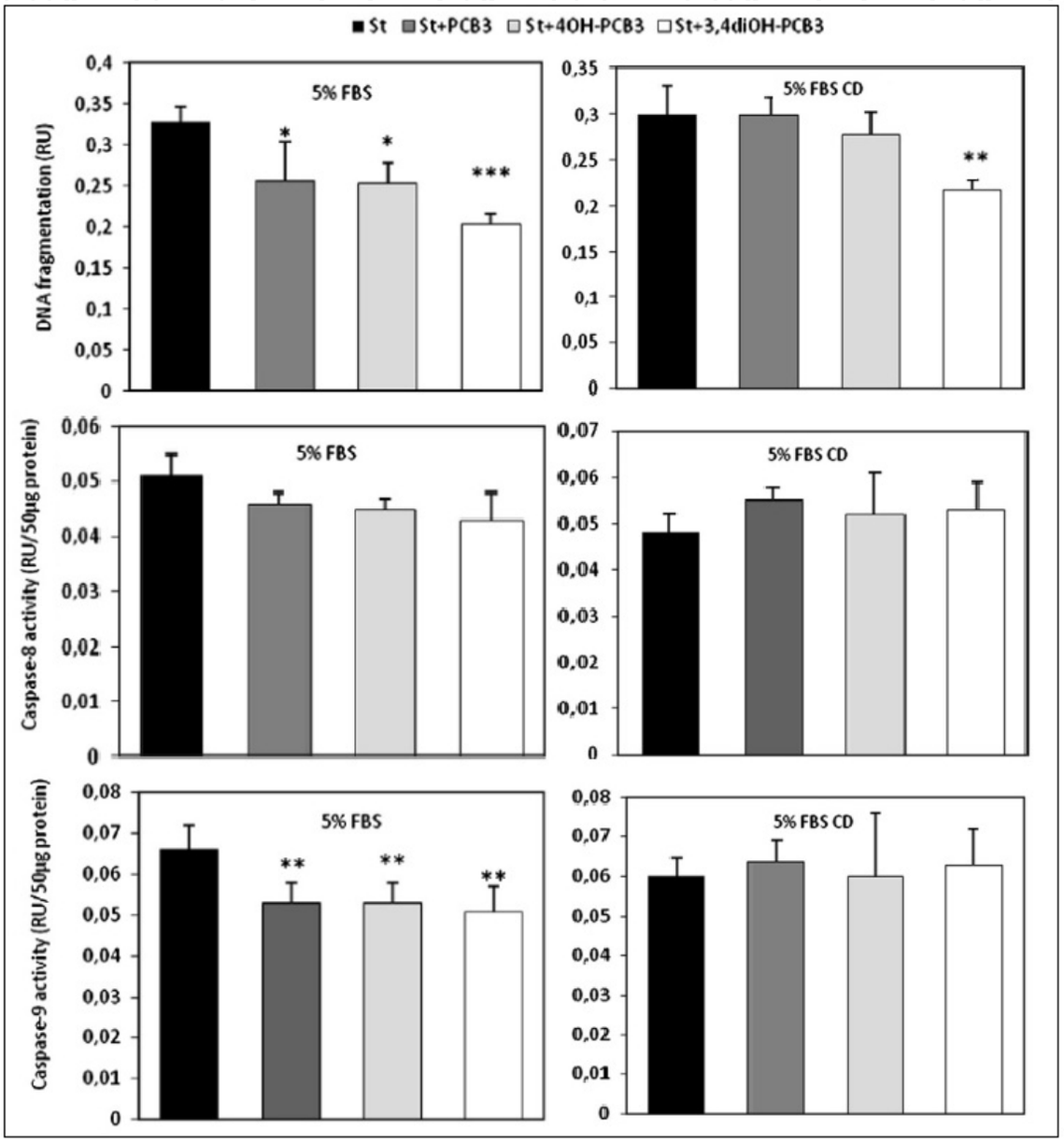

Figure 13. Effects of PCB3 (300 nM) and its metabolites on DNA fragmentation, as determined by ELISA; caspase-8 activity and, caspase-9 activity after 24 h of growth in medium supplemented with 5% FBS (left panel) or deprived of estrogen and by treatment with activated charcoal-dextran (5% FBS CD) (right panel). Staurosporine (St; 0.1 μM) was added during the last 3 h to induce apoptosis. (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$ [32]

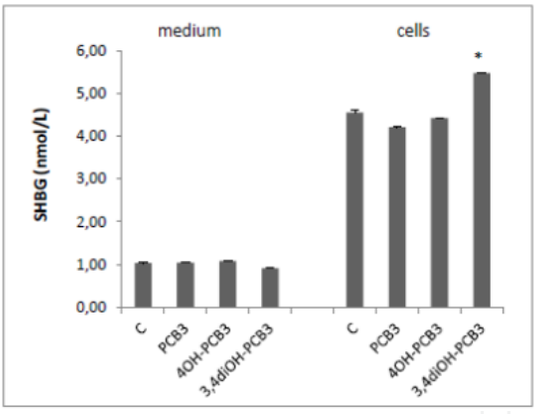

Figure 14. The effects of PCB3, 4-OH-PCB3 and 3,4-di-OH-PCB3 on extracellular (medium) and intracellular (cells) SHBG levels' in MCF-7 cells. (*) p<0.05 [34]

Furthermore, 3,4-diOH-PCB3 is ineffective in the activation of cAMP. Moreover, using a PKA inhibitor, we demonstrated that neither PCB3 nor its metabolites act through PKA [34], Figure 15.

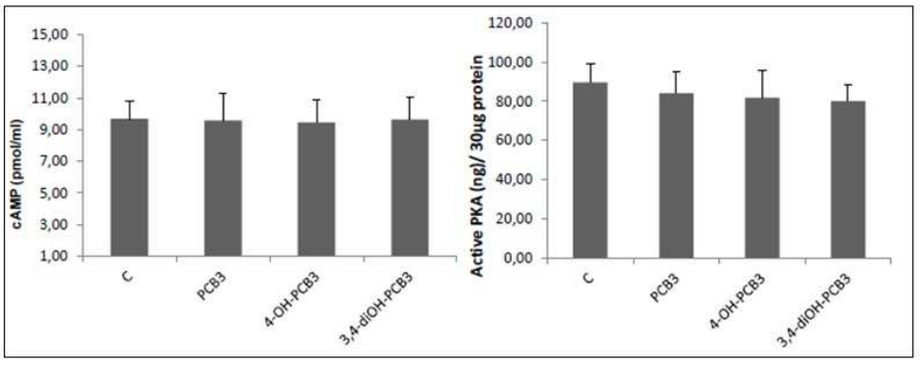

Figure 15. The effects of PCB3, 4-OH-PCB3 and 3,4-di-OH-PCB3 on cAMP and PKA levels in MCF-7 cells. ($p < 0.05$) [34]

Thus, the anti-proliferative action of 3,4-diOH-PCB3 is not due to inhibition of the ERK1/2 pathway via the SHBG/AMP/PKA pathway, but rather a direct inhibitory action on the ERK1/2 system [34], Figure 16.

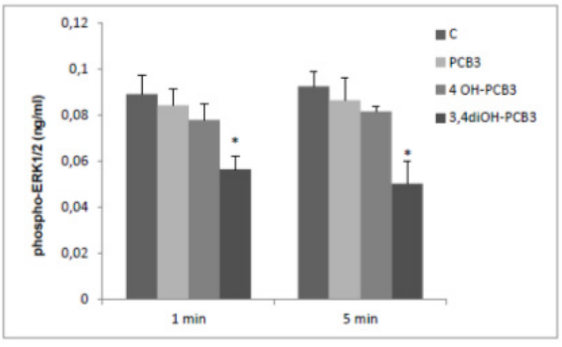

Figure 16. The effect of PCB3, 4-OH-PCB3 and 3,4-di-OH-PCB3 on phospho- ERK1/2 levels in MCF-7 cells. (*) p<0.05 [34]

Others have demonstrated that xenoestrogens can lead to the oscillating activation of ERK. Compounds from different classes of endocrine disruptors such as phytoestrogen (coumestrol), organochlorinated pesticides and their metabolites (endosulfan, dieldrin, DDE) and detergents (p-nonylphenol) can produce the same time-dependent activation pattern for ERKs [36]. These xenoestrogens produced rapid (3–30 min after application), concentration-dependent ERK1/2 phosphorylation. In addition, [37] demonstrated that the mitogenic effect of PCB153 was ERK1/2-mediated. Moreover, they showed that inhibition of ERK1/2 with PD98059 completely blocks the mitogenic effect of PCBs.

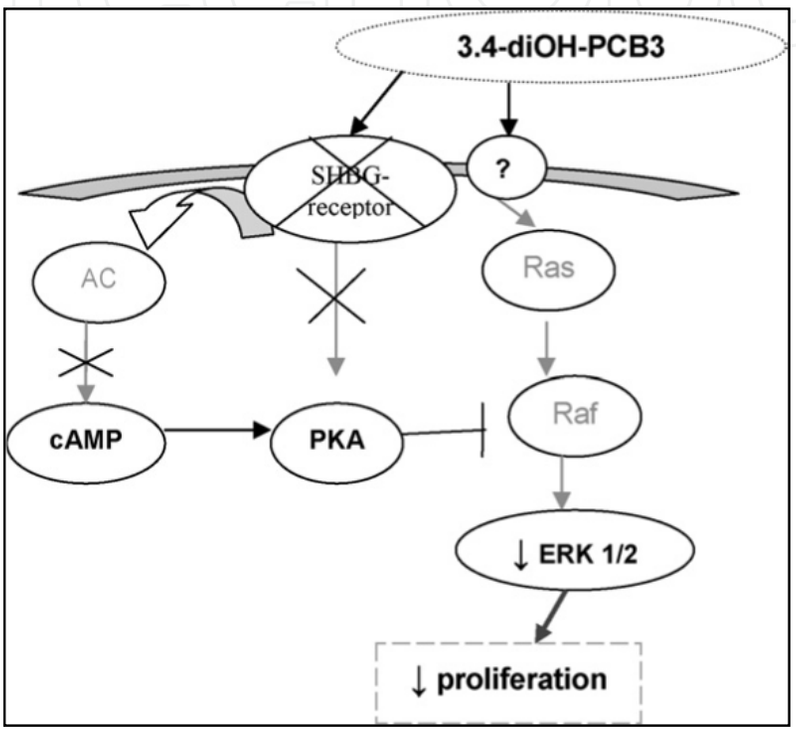

Figure 17. Proposed mechanism of antiproliferative action of 3,4-di-OH-PCB3 in MCF-7 cells [34]. The down arrow indicates a ERK1/2 decrease and proliferations decrease. AC, adenylyl cyclase; Ras-Raf, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase.

3.2. Bisphenol A and ovarian cancer

Bisphenol A (BPA) is a small monomer (228 Da) commonly used as plasticiser in the manufacture of polycarbonate plastics and epoxy resins. It is present in a multitude of products, including the interior coatings of food cans, milk containers, and baby formula bottles, as well as in dental composites and sealants. BPA was found in the serum, milk, saliva, and urine of humans at nanomolar concentrations. Many studies in the United States, Europe, and Japan have documented BPA levels ranging from 0.2 to 18.9 ng/mL (0.5–100 nM) in adult and foetal human serum [38]. Epidemiological studies have highlighted the correlation between the increase of BPA levels in the environment and the incidence of cancer in humans. BPA can act as an endocrine disruptor and a mitogenic substance inducing cell proliferation. As a mitogen, BPA induces susceptibility to neoplastic transformation [39].

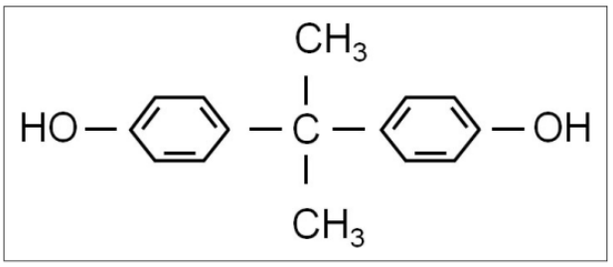

Figure 18. Bisphenol-A (BPA)

3.2.1. BPA action on cell proliferation

BPA can increase cell proliferation in a dose-dependent manner in OVCAR- 3 human ovarian cancer cells [40], MCF-7 breast cancer cells [41] and HeLa cells [42], Figure 19.

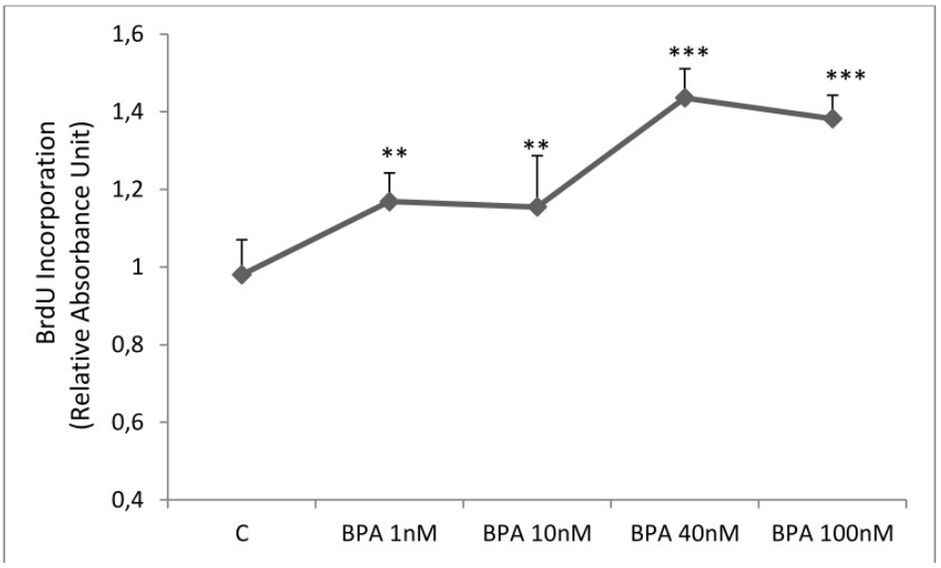

Figure 19. The effect of BPA on the proliferation of OVCAR-3 cells. (**) $p<0.01$, (***) $p<0.001$ [40]

Alterations in the mechanisms controlling cell cycle progression play a relevant role in the pathogenesis of different types of human neoplasia. As the cell cycle is regulated by the coordinate action of cyclin-dependent kinases (cdk), specific cyclin proteins and cdk inhibitors, we focused on cell cycle associated gene analysis. The cell cycle has four sequential phases: the S phase, when DNA replication occurs, and the M phase, when the cell divides into two daughter cells, as well as two gap phases referred to as G1 and G2. The G1-S transition in normal cells requires phosphorylation of the retinoblastoma protein (Rb) by cyclin D/Cdk4 or cyclin D/Cdk6 in mid G1 and by cyclin E/Cdk2 complexes later in G1. As cells progress into the S phase, cyclin A is expressed and becomes the primary cyclin associated with Cdk2. Progression from G2 into mitosis requires the activity of the Cdk and Cdc2 (also known as Cdk1) complexed with cyclin B, which has been shown to phosphorylate proteins regulated during mitosis. Basal and mitogen-induced cell growth is regulated by cell cycle inhibitors, which can bind to and inhibit the Cdks (such as p15, p16, p21, p27, p57). The results of our study [40] demonstrated that BPA promotes G1 to S-phase

progression by stimulating the expression of cyclin D1, CDK4, E2F1, E2F3 and PCNA while inhibiting the expression of inhibitors (p21WAF1/CIP1, Weel-1 and GADD45 alpha) and progression from G2 into mitosis by stimulating the expression of cyclin A in ovarian cancer cells OVCAR-3, Figure 20. The results of our study [40] additionally demonstrated that the suppression of p21/WAF mRNA, a marker of poor overall survival in ovarian cancer patients, was under the influence of BPA.

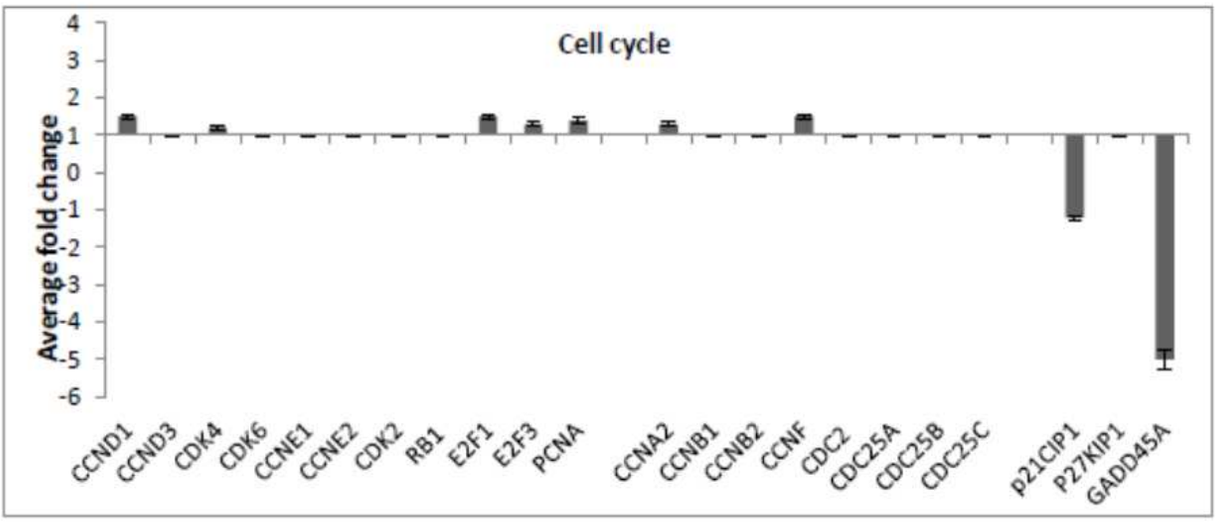

Figure 20. Selected cell cycle gene expression changes in OVCAR-3 cells exposed to BPA identified by real-time PCR [40]

3.2.2. BPA action on apoptosis

Studies on the effects of BPA on apoptosis have generated conflicting results, largely due to the micromolar concentrations of BPA utilised by most studies. BPA, in doses of 10, 1 and 0.1 mM, was a potent inhibitor of apoptosis in MCF-7 cells [43]. In contrast, BPA increased the expression of Bax (pro-apoptotic) and concomitantly decreased the expression of Bcl2 (pro-survival) at both protein and mRNA levels in granulosa cells [44]. In addition, BPA at a concentration of 100 μM decreased cell viability and increased necrosis in human endometrial endothelial cells (HEECs) [45].

A hallmark of apoptosis is the fragmentation of nuclear DNA. The intrinsic apoptosis pathway is activated when intracellular stress signals lead to the release of cytochrome c and other pro-apoptotic proteins within the mitochondria, which is regulated by members of the Bcl-2 family of proteins. The consequent release of cytochrome C leads to the formation of the apoptosome (cytochrome C, Apaf-1 and procaspase-9) in which procaspase-9 is auto-activated, thereby activating the executioner caspases. The extrinsic pathway is activated when extracellular ligands (such as Fas ligand, TNFα, TRAIL) bound to the death receptor domains of these receptors, recruit adaptor proteins (such as FADD and TRADD) and initiator caspase-8 and -10 (DISC). Both apoptotic pathways lead to the activation of the executioner caspases-3, -6 and -7, which are the main proteases that

degrade the cell. A hallmark of apoptosis is the fragmentation of nuclear DNA. Endonuclease G is essential for DNA fragmentation during caspase-independent apoptosis. In response to apoptotic stimuli, it is also released from the mitochondria into the cytosol, where it translocates to the nucleus and generates oligonucleosomal DNA fragmentation.

In our previously published study, we demonstrated that inhibition of the caspase-3 activity had no effect on DNA fragmentation in cells exposed to BPA [40]. We demonstrated that BPA acts by suppressing the expression of pro-apoptotic genes, such as FAS, FADD, RAIDD, caspase-8, -10, -3, -6, -7, CAD, Bax, Bak, Bok and Apaf-1, and inducing the expression of pro-survival genes, such as Bcl-x and Mcl-1. Moreover, BPA activates the caspase-independent apoptotic pathway via the induction of endonuclease G gene expression, Figure 21. The absence of an effect of BPA on DNA fragmentation is hypothesised to result from the simultaneous activation of the caspase-independent pathway and an inhibition of caspase-dependent DNA fragmentation. The observed induction of p53 (a nuclear transcription factor that accumulates in response to cellular stress, including DNA damage and oncogene activation) and suppression of caspase-3 and -7 is thought to activate the DNA repair process. Therefore, despite the observed induction of endo G gene expression, an action of BPA on DNA fragmentation was not observed [40].

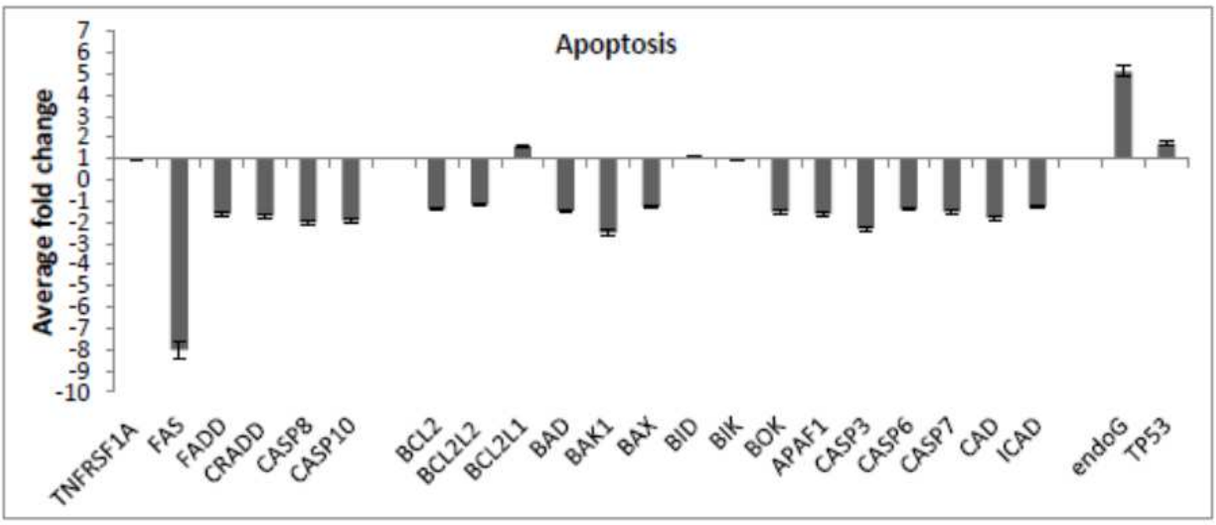

Figure 21. Selected apoptosis gene expression changes in OVCAR-3 cells exposed to BPA identified by real-time PCR [40]

3.2.3. Mechanism of action of BPA in OVCAR-3 cell cycle regulation

BPA can elicit rapid responses in cells via the involvement of a non-genomic signalling pathway though the activation of second messenger systems. Our data demonstrated that BPA stimulated proliferation of ovarian epithelial cancer cells via the induction of a rapid activation of ERK1/2, Stat3, and Akt signalling systems [46], Figure 22, 23. Previous studies demonstrate that BPA triggers a rapid biological response through the phosphorylation of Stat3, ERK1/2 and Akt in 3T3-L1 cells [47] and ERα/β-positive and -negative breast cancer cells [48].

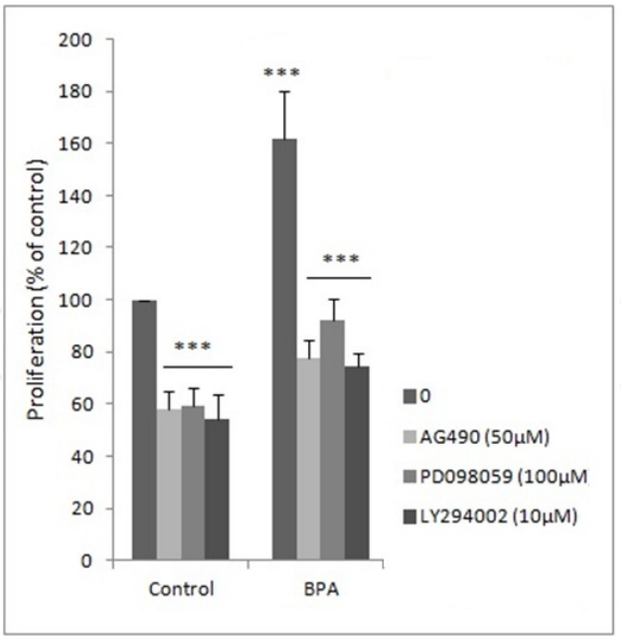

Figure 22. The effect of BPA on cell proliferation. OVCAR-3 cells were pretreated with AG490 (50 μM), PD098059 (100 μM) or LY294002 (10 μM) for 1 hr, then the cells were treated BPA (8 ng/ml) and an alamarBlue assay was performed. (***) $p<0.001$ [46]

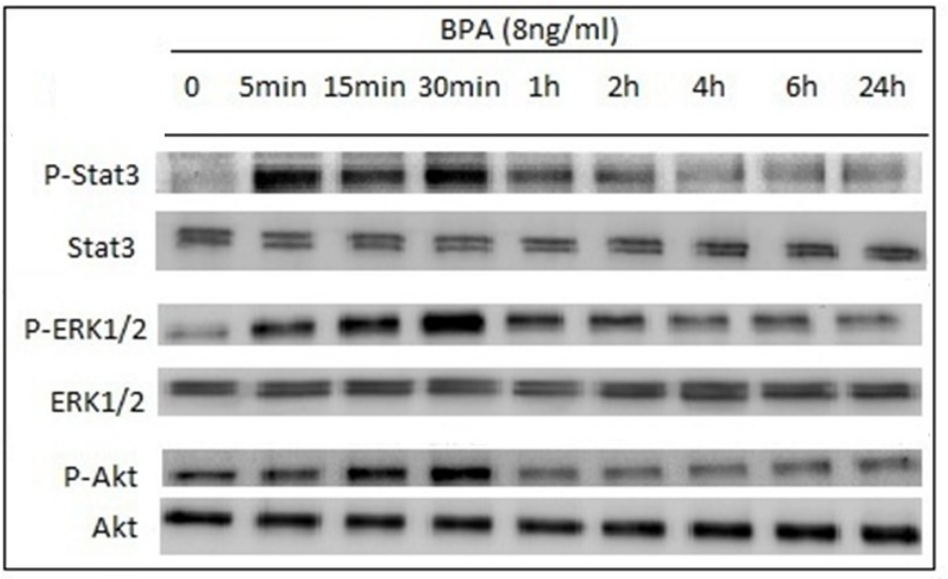

Figure 23. The effects of BPA on the activation of Stat-3, ERK1/2 and Akt in OVCAR-3 cells. Western blot analysis was performed following BPA treatment in a time-dependent manner (5, 15, and 30 min and 1, 2, 4, 6 and 24 hrs). Total Stat-3, ERK1/2 and Akt were used to normalise the level of phosphorylated Stat-3, ERK1/2 and Akt, respectively [46]

3.3. Combinatory effects of endo- and exogenous oestrogens.

Recently, an increasing amount of data has demonstrated that xenoestrogens can interfere with endogenous oestrogens. In our previously published data, we investigated the contribution of specific representative PCBs and PBDEs on the oestrogenic action of

endogenous oestradiol on the proliferation and apoptosis of the breast cancer cell line MCF-7. The structure of polybrominated diphenyl ethers (PBDEs) is similar to that of PCBs. PBDEs are a series of 209 possible congeners that differ in the number and position of bromine atoms. These chemicals are widely used as flame retardants in plastic foams, textiles, electronic devices, and building materials. As a result, they are widely found in the environment and can be found in human blood and milk. Some of the PBDE congeners most commonly found in human samples are BDE-47, BDE-99, and BDE-153 [49].

Our data demonstrated that all PCB congeners (118, 138, 153 and 180) and all concentrations tested increased MCF-7 cell proliferation while decreased cell apoptosis to a greater extent than oestradiol. However, in co-treatment experiments with oestradiol, cell proliferation decreased in comparison to the cell proliferation observed for cells exposed to PCB congeners alone, and no additional effect on cell apoptosis was observed [50], Figure 24.

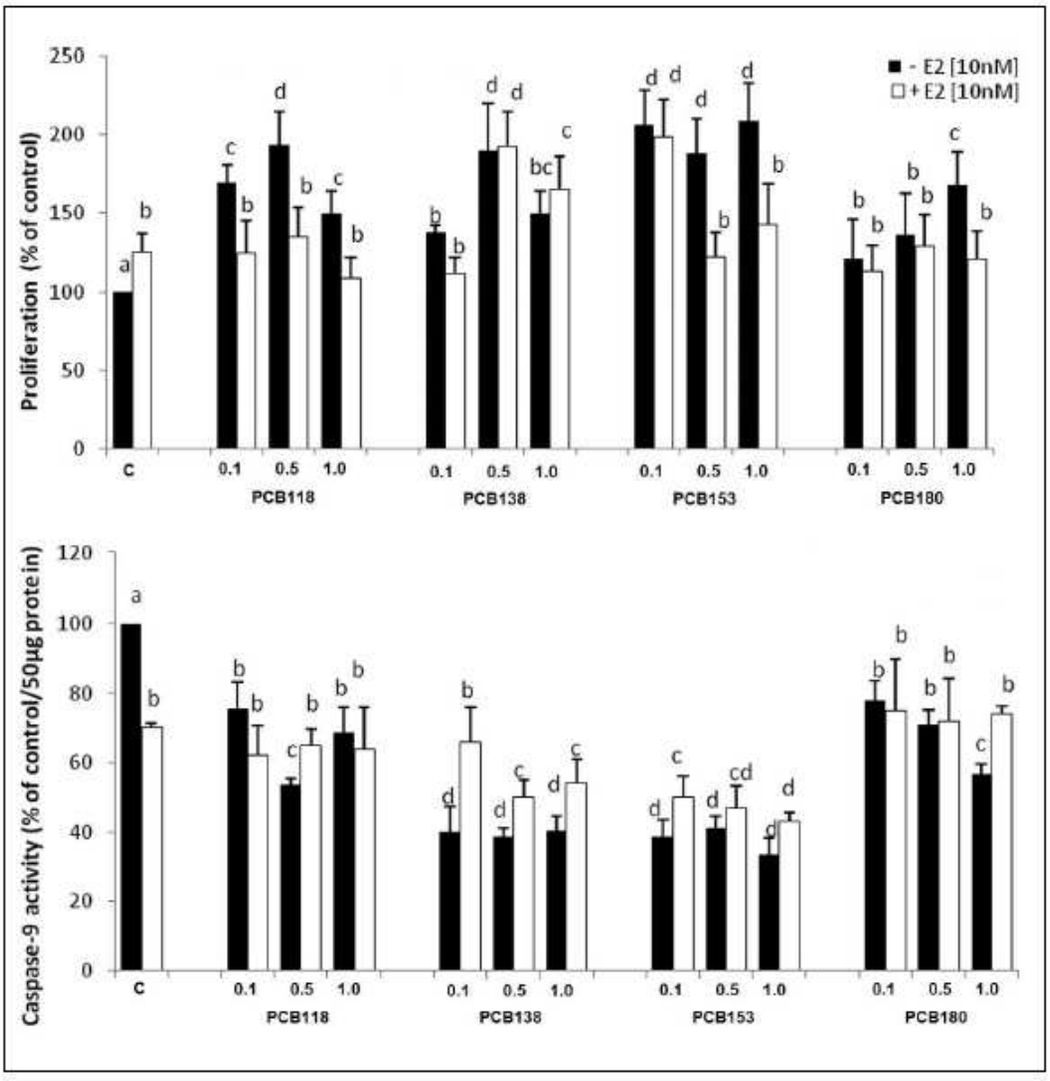

Figure 24. The effects of PCB (0.1, 0.5 and 1 mM) and oestradiol, alone (black bar) and combination treatment (open bar) on proliferation after 72 hours of culture and on caspase-9 activity after 24 hours of culture in MCF-7 cells. (a<b<c<d) $p < 0.05$ [50]

Despite the similarity in structure, PCBs and PBDEs had different effects on proliferation and apoptosis in MCF-7 breast cancer cells. None of the PBDE congeners tested (47, 99, 100, 209) had an effect on basal cell proliferation; however, all PBDE congeners tested significantly decreased basal cell apoptosis. In the presence of oestradiol, additive anti-apoptotic activity and an ability to induce cell proliferation were observed [51], Figure 25.

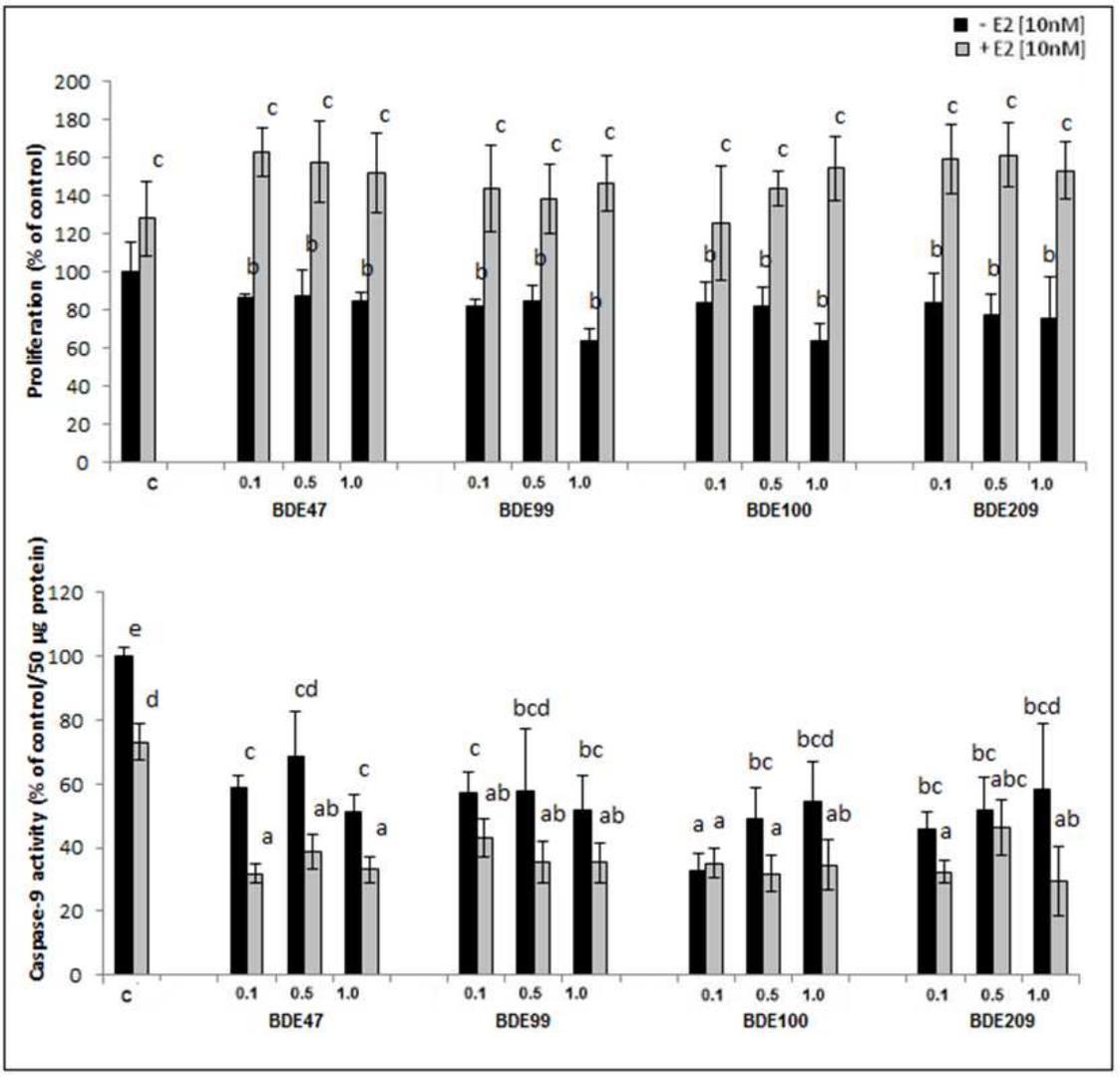

Figure 25. The effects of PBDE (0.1, 0.5 and 1 mM) and oestradiol, alone (black bar) and combination treatment (gray bar) on proliferation after 72 hours of culture and on caspase-9 activity after 24 hours of culture in MCF-7 cells. (a<b<c<d) $p < 0.05$ [51]

4. Conclusion

Despite a reduction in the doses of natural oestrogens used in HRT and contraceptives, as well as the production and use of environmental oestrogens, these compounds remain in our environment and are routinely found in human serum, breast milk and adipose samples, including breast tissue. Importantly, differences in activity as potential carcinogens are being studied. The potencies of these compounds in nuclear transcription reporter

assays, compared with the endogenous oestrogens, range from very weak (e.g., PCBs) to somewhat weak (e.g., bisphenol A) to strong (DDE). Moreover, both endogenous and exogenous oestrogens form metabolites during metabolism that act more strongly than their corresponding parental compounds. The results of a published study suggest that the genomic and nongenomic actions of the parent endogenous and exogenous oestrogens lead to the activation of secondary messenger systems responsible for proliferation and apoptosis. The results of our study indicate that it is necessary to consider local metabolism in peripheral tissues, such as ovary and breast. Therefore, an accurate assessment of the carcinogenicity of oestrogens and xenoestrogens requires an understanding of not only the potential mode of action of the parent compounds but also their hydroxylated metabolites. These include the promotion of tumorigenic progression, genotoxicity, and the developmental reprogramming that increases susceptibility to other carcinogenic events.

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