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Cytotoxic effects and aromatase inhibition by xenobiotic endocrine disrupters alone and in combination ☆

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Abstract

Xenobiotics may cause long-term adverse effects in humans, especially at the embryonic level, raising questions about their levels of exposure, combined effects, and crucial endpoints. We are interested in the possible interactions between xenobiotic endocrine disrupters, cellular viability and androgen metabolism. Accordingly, we tested aroclor 1254 (A1254), atrazine (AZ), *o,p′*-DDT, vinclozolin (VZ), *p,p′*-DDE, bisphenol A (BPA), chlordecone (CD), nonylphenol (NP), tributylin oxide (TBTO), and diethylstilbestrol (DES) for cellular toxicity against human embryonic 293 cells, and activity against cellular aromatase, but also on placental microsomes and on the purified equine enzyme. Cellular viability was affected in 24 h by all the xenobiotics with a threshold at 50 μM (except for TBTO and DES, 10 μM threshold), and aromatase was inhibited at non-toxic doses. In combination synergism was observed reducing the threshold values of toxicity to 4–10 μM, and aromatase activity by 50% in some cases. In placental microsomes the most active xenobiotics rapidly inhibited microsomal aromatase in a manner independent of NADPH metabolism. Prolonged exposures to low doses in cells generally amplified by 50 times aromatase inhibition. These xenobiotics may act by inhibition of the active site or by allosteric effects on the enzyme. Bioaccumulation is a feature of some xenobiotics, especially chlordecone, DDT and DDE, and low level chronic exposures can also affect cell signaling mechanisms. This new information about the mechanism of action of these xenobiotics will assist in improved molecular design with a view to providing safer compounds for use in the (human) environment.

Keywords: Xenobiotics; Endocrine disrupters; Aromatase; Cytochrome P450 reductase; Steroidogenesis; Pesticides; PCB; Bisphenol A; Diethylstilbestrol; Nonylphenol; Human 293 embryonic cells; Equine testis; Combined effects

Introduction

The production, use, and release into the environment of xenobiotics continue to increase worldwide; some of these are widespread in many life-forms, are persistent and bioaccumulate through food chains raising questions about the validity of current threshold exposure levels, mechanisms of action and combined effects, synergism, addition, or antagonism of toxic effects. The xenobiotics used in this work have been selected on the basis of their widespread use and large number of applications, their penetrability into mammalian tissues, and their endocrine disrupting potential, leading to hormone-dependent diseases such as cancers, or reproductive disorders (Toppari et al., 1996). Such effects have been reported for polychlorobiphenyls (PCB) including aroclor, on rabbit embryotoxicity (Seiler et al., 1994), AZ on female rat sexual maturation (Ashby et al., 2002), BPA on recurrent miscarriages (Sugiura-Ogasawara et al., 2005), CD on male fertility and DES on adverse developmental effects after maternal exposure (Daston et al., 1997), DDT and its metabolite DDE on sexual precocity (Krstevska-Konstantinova et al., 2001), NP on human and mouse spermatozoa impairments (Fraser et al., 2006), TBTO on female and male rat development (Makita et al., 2003, 2004), and VZ on pregnancy and sexual maturation (Wolf et al., 2004). Although some of these xenobiotics, DDT, DDE, or PCB (A1254), have been banned in many countries, their use and production continue in several developing ones, and they are

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still found in human or animal tissues throughout the world (Colborn et al., 1993). Moreover, the lipophilic nature and poor degradability of these compounds promote their persistence and accumulation in food chains. Research evidence indicates combined effects particularly synergism demand studies on the effects of mixtures containing low doses xenobiotics.

In humans, the decline and well-known regional differences in semen quality (Jorgensen et al., 2001), the increasing incidence of testicular cancers (Jacobsen et al., 2000; Ohlson and Hardell, 2000), and increasing rates of developmental abnormalities in male and female reproductive tracts (Toppari and Skakkebaek, 1998; Sultan et al., 2001) during the last five decades, have been related to environmental contaminants. All these phenomena were called the testicular dysgenesis syndrome (Skakkebaek, 2002). For instance, the consumption of PCB-contaminated fish appears to shorten New York women's menstrual cycles (Mendola et al., 1997).

Xenobiotics can disrupt endocrine functions in several ways. These include interactions with transport proteins, hormone receptors, metabolic enzymes, and disruption of cell signaling processes. The most studied mechanisms of xenobiotic actions involve receptor-mediated processes (Massaad et al., 2002). For instance, DDT, DDE, NP, BPA (Blair et al., 2000), and many PCBs (Andersson et al., 1999) are reported to exert their effect via estrogen receptor binding. Other xenobiotic actions have been documented, including interferences to human steroid hormone binding globulin (Déchaud et al., 1999), increased serum estrogenic bioactivity (Paris et al., 2006), and disruption in gene expression of steroidogenic enzymes (Walsh et al., 2000; Walsh and Stocco, 2000). However, little is known concerning the direct effect of xenobiotics on steroidogenic enzymes (Vinggaard et al., 2000; Raun Andersen et al., 2002; Richard et al., 2005).

Cytochrome P450 aromatase, the product of the *CYP19* gene, is a member of the P450 superfamily. It is the only steroidogenic enzyme responsible for the irreversible bioconversion of androgens into estrogens (Simpson et al., 1994) and is the determinant in the control of the androgen/estrogen balance in the body. Aromatase levels are crucial in various tissues including gonads (Sipahutar et al., 2003), and implicated in numerous physiological functions (Simpson et al., 1997), and pathologies such as hormone-dependent cancers for which several classes of inhibitors have been designed (Séralini and Moslemi, 2001). Abnormalities in aromatase expression have been found in a wide range of structural and functional disorders in reproduction, development, cell and sexual differentiation, growth and maintenance of sexual behavior. Additionally, this enzymatic activity is present in different phyla including almost all vertebrates and several invertebrate species (Le Curieux-Belfond et al., 2001).

A few *in vitro* studies have highlighted the interaction of some xenobiotics with aromatase. Sanderson et al. (2000) demonstrated that the herbicide AZ stimulates aromatase activity, in contrast the fungicide Fenarimol and the herbicide Roundup inhibit aromatase activity in the human placenta and in JEG3 cell line, depending on the dose and time of exposure (Vinggaard et al., 2000; Richard et al., 2005).

This study was designed to evaluate the *in vitro* effects of selected known endocrine disrupters, on cellular toxicity, then at lower non-toxic doses on aromatase and cytochrome P450 reductase activities. This reductase is the second moiety of the aromatase enzyme complex; it serves as electron donor in the reaction, but it is not involved directly in androgen binding and metabolism. Several members of the P450 superfamily are known to be responsible for xenobiotic metabolism in many tissues. Two different mammalian aromatases that have been cloned, and well-characterized, the human and equine enzymes, are compared at a biochemical level (Moslemi et al., 1997; Séralini et al., 2003). The compounds were tested, alone or in combination, on both human placental microsomal and purified equine testicular aromatase preparations or in human embryonic 293 cells transfected with the human aromatase cDNA. These efforts will contribute to a better understanding of the mechanism(s) of action of these xenobiotics.

Materials and methods

Xenobiotics and chemicals. The xenobiotics tested in this study (Table 1, Fig. 1) were purchased from Fluka Chemika (St. Quentin Fallavier, France), except for A1254, a PCB which was a gift from Dr. Stephen Safe (Texas A&M University, USA). The xenobiotics as 0.5% solutions in DMSO were further diluted with Eagle's serum-free modified Minimum Essential Medium (EMEM, Abcys, Paris, France) and adjusted to pH 7.4. 4-Androsten-3,6,17-trione (AT) was

Table 1
Xenobiotics used in this work are known to be able to interfere with sexual steroid receptors in animals and are tested here in combination on human aromatase

Name (abbreviation)	Chemical name and formula	Common usage
Androstenetrione (AT)	4-Androsten-3,6,17-trione [$C_{19}H_{25}O_3$]	Aromatase inhibitor (positive control)
Aroclor 1254 (A1254)	A congener of polychlorinated biphenyls with 2 biphenyl rings and up to 54% chloride in the compound [$C_{12}H_5Cl_5$]	Electrical insulator, cosmetics
Atrazine (AZ)	2-Chloro-4-ethyl-amino-6-isopropylamino-1,3,5-triazine [$C_8H_{14}ClN_5$]	Herbicide
Bisphenol A (BPA)	2,2-*bis*-(4-Hydroxyphenyl)-propane [$C_{15}H_{16}O_2$]	Industry, pesticide adjuvant
Chlordecone (CD)	Decachloro-pentacyclo(5.2.1.0.0.0)decane-4-one [$C_{10}Cl_{10}O$]	Insecticide, fungicide
Diethylstilbestrol (DES)	[E]-3,4-*bis*[4-hydroxyphenyl]-3-hexene [$C_{18}H_{20}O_2$]	Drug, synthetic estrogen
p,p′-DDE (DDE)	1,1-Dichloro-2,2-*bis*(4-chlorophenyl) ethene [$C_{14}H_8Cl_4$]	DDT metabolite
o,p′-DDT (DDT)	1,1,1-Trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane [$C_{14}H_9Cl_5$]	Insecticide
Nonylphenol (NP)	Nonylphenol [$C_{15}H_{24}O$]	Surfactant, detergent, cosmetics
Tributyltin oxide (TBTO)	*bis*(Tributyltin) oxide [$C_{24}H_{54}OSn_2$]	Biocide, anti-fouling agent
Vinclozolin (VZ)	3-(3,5-Dichlorophenyl)-5-methyl-5-vinyloxazolidine-2,4-dione [$C_{12}H_9Cl_2NO_3$]	Fungicide

Their abbreviations, molecular formulas and common usages are presented.

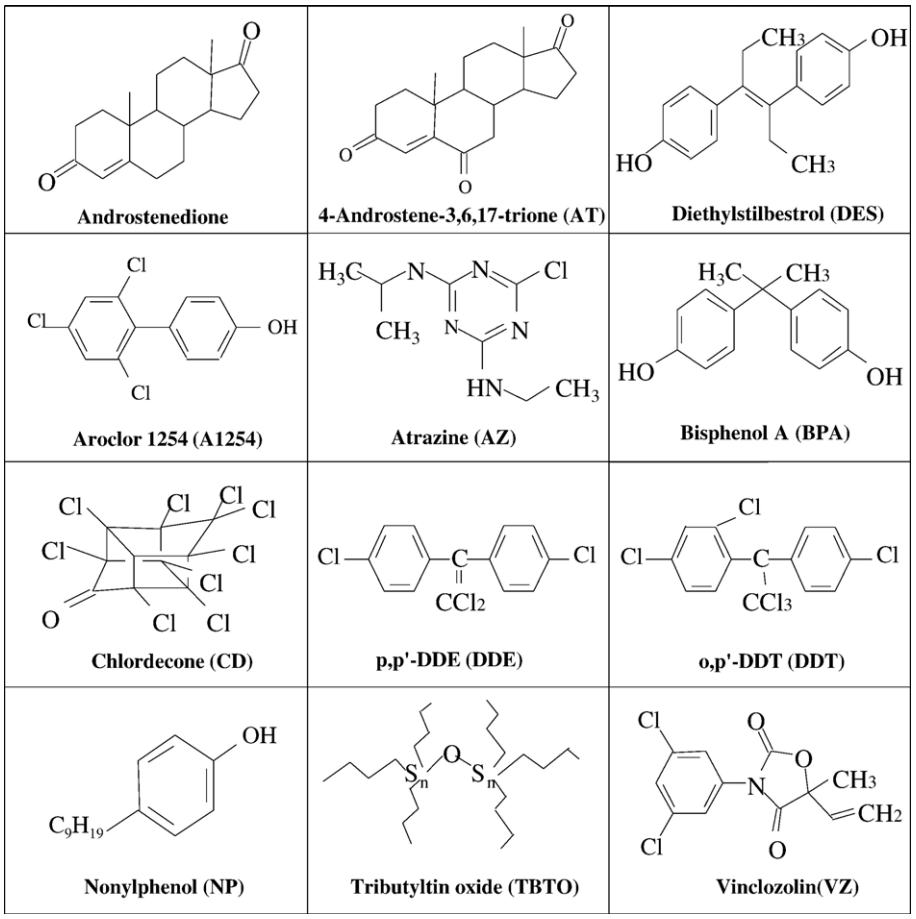

Fig. 1. Chemical structures of the xenobiotics studied, and androstenedione a natural substrate for aromatase.

purchased from Steraloids (Wilton, NH, USA). The [1β-^{3}H] androstenedione (specific activity, 25.3 Ci/mmol; 958.3 GBq/mmol) was purchased from Dupont-New England Nuclear (Les Ulis, France). The DMSO (dimethyl sulfoxide) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) were from Sigma-Aldrich. MTT was prepared as a 5 mg/ml stock solution in PBS, 0.22 μm filtered and diluted to 1 mg/ml in serum-free EMEM.

Cell lines and transfections. The human embryonic kidney 293 cell line (ECACC 85120602) was provided by CERDIC (Sophia-Antipolis, France). Cells were grown in phenol red-free EMEM containing 2 mM glutamine, 1% non-essential amino acids, 100 U/ml of antibiotics (mix of penicillin, streptomycin and fungizone) and 10% fetal calf serum (Biowhittaker: Gagny, France). Fifty thousand cells per well were grown at 37 °C (5% $C0_2$, 95% air) during 48 h to 80% confluence in 24-well plates, washed with serum-free EMEM and then were exposed to various concentrations of xenobiotics in serum-free EMEM for 24 h for viability tests. Transfections after confluence with human aromatase cDNA (pCMV-HA plasmid, Auvray et al., 1998) were adapted from Boussif et al. (1995) and performed 48 h before xenobiotics treatments. Immediately before transfection, cells were washed and supplemented with 500 μl of serum-free EMEM. After, 2 μg of pCMV-HA plasmid and the desired amount of polymer solution (6 nmol of phosphate and 5.4 μmol of the polyethylenimine transfectant (PEI at 50 kDa) were each diluted into 50 μl of 150 mM NaCl and mixed. After 3 h of incubation at 37 °C, cells were supplemented with 500 μl of serum-containing medium EMEM. The cell line was used for viability measurements and transfected with aromatase cDNA before aromatase assessments.

Cell treatments and viability measurements. Xenobiotics in DMSO were dissolved in serum-free EMEM to the final concentrations and incubated 24 h with cells. After wash, cells were incubated with 250 μl MTT (1 mg/ml) per well. This enzymatic test, based on the cleavage of MTT into a blue colored product (formazan) by the mitochondrial enzyme succinate-dehydrogenase (Mossmann, 1983; Denizot and Lang, 1986; Auvray et al., 1999), was used to evaluate human cell viability. The plates were incubated for 3 h at 37 °C and 250 μl of 0.04 *N*-hydrochloric acid-containing isopropanol solution was added to each well. The plates were then vigorously shaken in order to solubilize the blue formazan crystals formed. The optical density was measured using a KONTRON-UVIKON 860 spectrophotometer at 560 nm for test and 720 nm for reference.

Human placental microsomes. Full-term placentas from young healthy and non-smoking women were collected immediately after delivery and microsomes (50 μg/ml otherwise specified) were prepared as previously described (Richard et al., 2005) adapted from Dintinger et al. (1989). Protein concentration was assessed according to Bradford (1976), using bovine serum albumin as standard. They were incubated for 15 min with various concentrations of the xenobiotics and aromatase and reductase activities assayed. The possible metabolism of xenobiotics in the placenta was tested with the 4 compounds giving the strongest inhibition. The inhibitory activities were compared before and after pre-incubation, performed for 15 min at 37 °C with human placental microsomes (100 μg protein) in 500 μl of Tris–maleate buffer in the presence (a) or absence (b) of 60 μM NADPH-H^+. The aromatization (15 min, 37 °C) was then started by adding 100 pmol of [1β-^{3}H] androstenedione substrate to each compound and, at the same time, NADPH-H^+ was added to b.

Reductase and aromatase preparations. The equine testes served as a source for the purification of aromatase and reductase because of the particularly high content of the enzymatic complex in mammals (Lemazurier et al., 2001). The equine aromatase offers a good and well-characterized comparative mammalian model (Séralini et al., 2003) in which the active site is comparable to the human

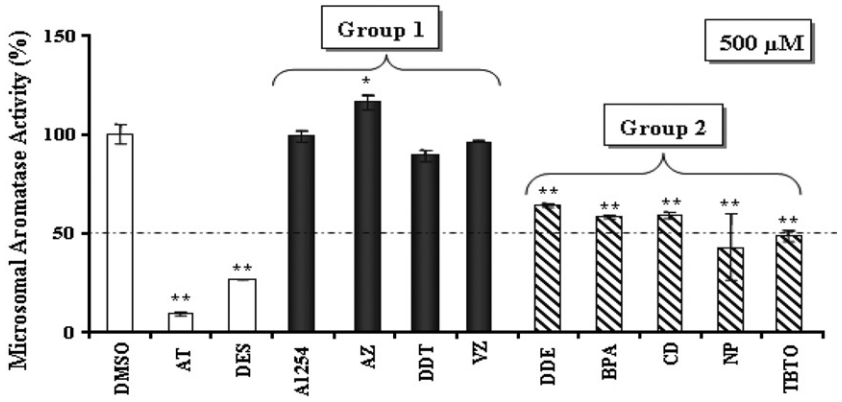

Fig. 2. Results of the initial screening of the xenobiotics on human microsomal placental aromatase. Human placental microsomes were incubated at 37 °C for 15 min at pH 7.4, with different xenobiotics at 500 µM. The results are expressed as % inhibition of control values (0.5% DMSO does not affect activity). AT, a well-known aromatase inhibitor and DES served as positive controls. All data are the mean±standard error (SEM). All the experiments were repeated 3 times in triplicate ($n=9$). Two categories separate compounds that present (group 2) or not (group 1) some inhibitions at this high dose of xenobiotics.

enzyme (Auvray et al., 1998). Reductase was obtained from microsomal preparations and separation of the aromatase on a hydrophobic-interaction column (ω-aminohexyl-Sepharose 4B). The aromatase was subsequently purified to homogeneity on successive chromatographic separation columns including concanavalin affinity as previously reported (Moslemi et al., 1997). Reductase and aromatase were further concentrated using Filtron Mini-Ultrasette 30K (Gelman Laboratory) to obtain an optimal activity in the reconstituted system. At least a 1:5 ratio of cytochrome P450 aromatase/reductase was needed (Moslemi et al., 1997). In this study purified aromatase (94 pmol) and reductase (602 pmol) were mixed in a total volume of 1 ml of Tris–maleate buffer (pH 7.4) and equilibrated at 37 °C for 15 min, with various concentrations of xenobiotics.

Enzymatic assays. The reductase assays were performed by measuring the absorbance corresponding to the reduced form of cytochrome *c* in the presence of NADPH-H^+ as previously described (Richard et al., 2005), adapted from Vibet et al. (1990). Reductase (66 µg protein) was pre-incubated with various concentrations of xenobiotics dissolved in DMSO at 37 °C for 15 min. After pre-incubation, 85 µl of 60 µM NADPH-H^+ was added and the reaction started by the addition of 100 µl of 500 µM cytochrome *c* and allowed to proceed at 37 °C for 2 min in a final volume of 2.5 ml of 0.2 M sodium phosphate buffer (pH 7.4). Results are expressed as variation in absorbance over the 2-min incubation. The general measurement of aromatase activity used the tritiated water release assay (Thompson and Siiteri, 1974) with slight modifications adapted from Dintinger et al. (1989). The assay was performed with microsomes (50 µg proteins) incubated with various concentrations of xenobiotics alone or in combination, as previously described (Richard et al., 2005). The assay was modified for transfected 293 cells exposed to various concentrations of xenobiotics alone or in combination. Cells were washed with serum-free EMEM and incubated for 45 min with 200 nM [1β-^{3}H] androstenedione at 37 °C (5% $C0_2$, 95% air). After adding 500 µl of charcoal: dextran T-70 suspension (7%:1.5%), the mixture was left at 4 °C for 5 min, and then centrifuged at 2700×*g*, 4 °C for 10 min. Supernatants (500 µl) and radioactivity were measured by scintillation counting.

Statistical analysis. All data are presented as the mean±standard error (SEM). The experiments were repeated 3 times in triplicate and in most cases, expressed as the percentage of controls. Statistically significant differences from controls were determined by a Student's *t*-test using significant levels of 0.01 (**).

Results

In order to test the combined effects at low doses (5–10 µM each), we first screened the selected xenobiotics (Fig. 1) by incubating 500 µM of each with a microsomal preparation of human placental aromatase. Two groups were identified. Group 1, A1254, AZ, DDT and VZ, did not significantly inhibit the enzyme while group 2, DDE, BPA, CD, NP and TBTO, caused ~50% inhibition of the aromatase. Androstenetrione (AT), 0.75 µM, a well-known aromatase inhibitor, and DES, 75 µM, a

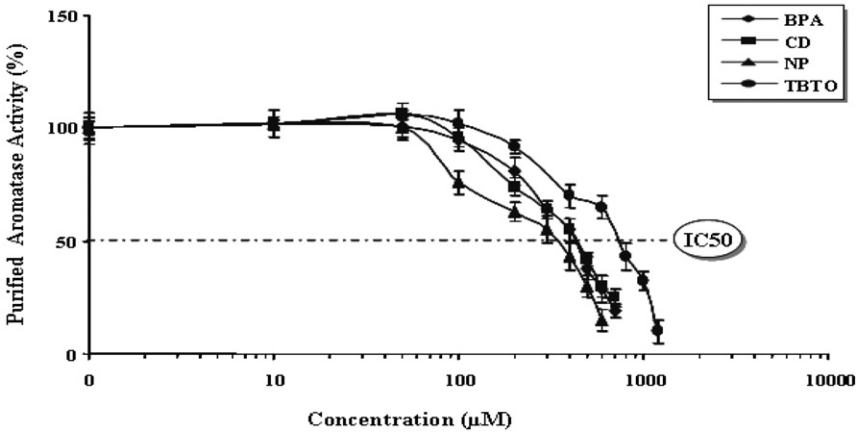

Fig. 3. Dose-dependent inhibition of purified reconstituted equine aromatase (94 pmol with 602 pmol reductase), serving as mammalian model, by BPA, CD, NP and TBTO.

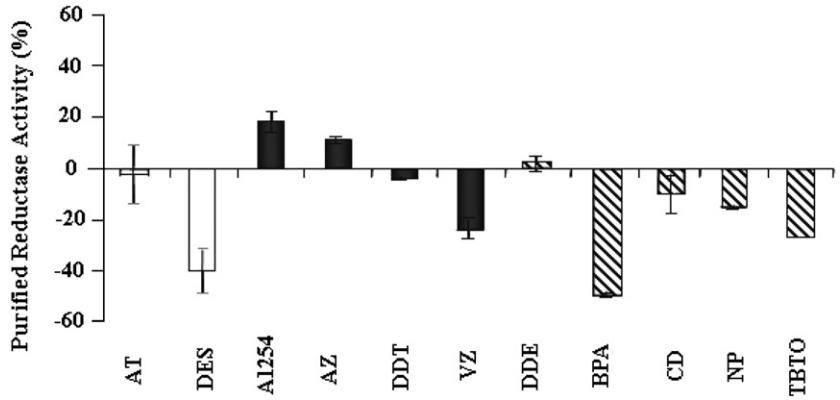

Fig. 4. Direct effects of xenobiotics on the purified reductase moiety of the aromatase complex. The activities were determined by subtracting the absorbance, between 0 and 2 min of incubation, and presented as percent of DMSO control alone. The maximal concentrations tested were AT (1 μM), DES (150 μM), CD (500 μM), TBTO (2000 μM), other xenobiotics (1250 μM).

synthetic non-steroidal estrogen, under the same conditions caused 90% and 75% inhibition, respectively.

We compared the IC50 values for the 4 most efficient xenobiotics of group 2 against the microsomal human and purified equine aromatase (Figs. 2 and 3). The inhibitory effect observed with BPA, CD, and NP was in the same range for both mammalian species and the curves were comparable (Fig. 3) indicating a direct effect on the enzyme, probably within the active site. TBTO had a stronger action on human aromatase. Reductase (the ubiquist electron donor moiety not responsible for steroid binding to the aromatase complex) inhibition is reported in Fig. 4. The reductase IC50 was reached for BPA at 1250 μM, whereas for aromatase it was obtained with 435 μM. The inhibitory effects of xenobiotics on both microsomal (Fig. 2)

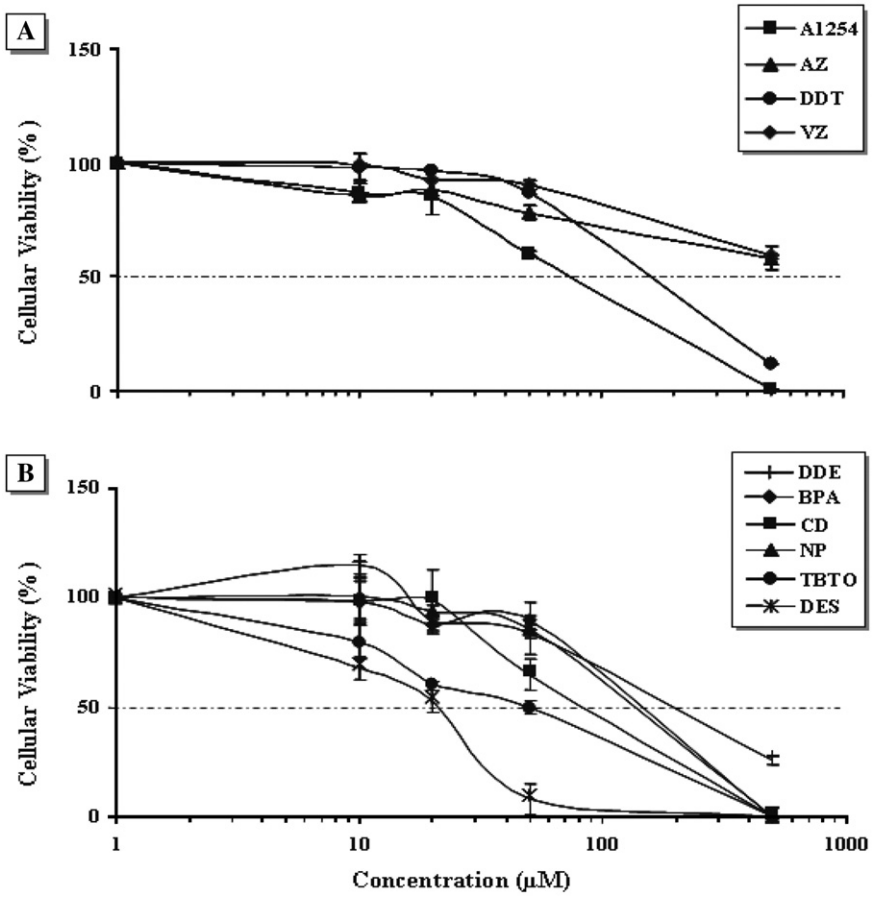

Fig. 5. Cytotoxic effects (percent of control) of xenobiotics from group 1 (A) or 2 (B) on 293 human embryonic cells in serum-free medium. They are evaluated by the mitochondrial succinate dehydrogenase activity after 24 h (n=9).

and purified aromatase (Fig. 3) were mainly exerted on P450 aromatase rather than on reductase. Slight increases of reductase activity occurred with A1254 and AZ, two of the weaker inhibitors of group 1 (Fig. 4).

Human embryonic cells were much more sensitive to these xenobiotics. Cytotoxic effects were significant at 50 μM and greatest for group 2 compounds (Fig. 5). Cell death involving mitochondrial dysfunction was demonstrated by measurement of the succinate dehydrogenase activity (Mossmann, 1983; Denizot and Lang, 1986; Auvray et al., 1999). TBTO and DES had LD50 ~20 μM and were not included in further studies. The remaining xenobiotics were tested in combinations, 4–10 μM concentrations of each compound that in total did not exceed the 20 μM concentration at which the compounds individually showed no activity (Fig. 6). Group 1 compounds with the addition of DDE caused a 40% decrease in cell proliferation but not when DDE was excluded or for binary combinations (Fig. 6A). Group 2 compounds when all combined had no significant effect on cellular proliferation but in binary combinations either slightly increased proliferation, DDE+BPA, DDE+NP or decreased proliferation, CD+BPA, CD+NP, NP+BPA (Fig. 6B).

Inhibition of aromatase was more pronounced than cellular toxicity at 20 μM concentrations. Individual compounds in group 1 showed ~20% inhibition which was increased for some binary combinations, A1254+AZ, AZ+VZ, DDT+VZ, and DDT+DDE, but reduced or unchanged with others, AZ+DDT, A1254+VZ, A1254+DDT (Fig. 7A). The inhibition was at least 25% greater in cells than that on microsomal preparations indicating a possible new pathway of action. An unexpected and apparently unpredictable increase in aromatase activity was found for some binary combinations, AZ+DDT, DDE+CD (Figs. 7A and B). In general group 2 compounds alone or in combination were more active than group 1 inhibiting the enzyme by up to 50% (Fig. 7B), TBTO was not considered for these aromatase inhibition because of its marked effect on cellular viability. The effect of DDE on the multiple combination of group 1 compounds (Fig. 7A) leads to a greater inhibition than the multiple combination of group 2 compounds (Fig. 7B).

The xenobiotics (BPA, CD, NP and TBTO) with the strongest inhibitory activity against aromatase in embryonic cells and microsomes were tested individually and in binary combinations against human microsomal and purified equine aromatase, in order to understand the mechanism of action (Fig. 8). The binary mixtures at their IC25 concentrations significantly amplified their inhibitory effect alone, up to 6- to 7-fold. A pronounced synergism was observed that reached 90% with CD+TBTO against the purified aromatase. The synergistic effect was greatest on the purified enzyme indicating a direct effect on it by these xenobiotics. In placental microsomes, the

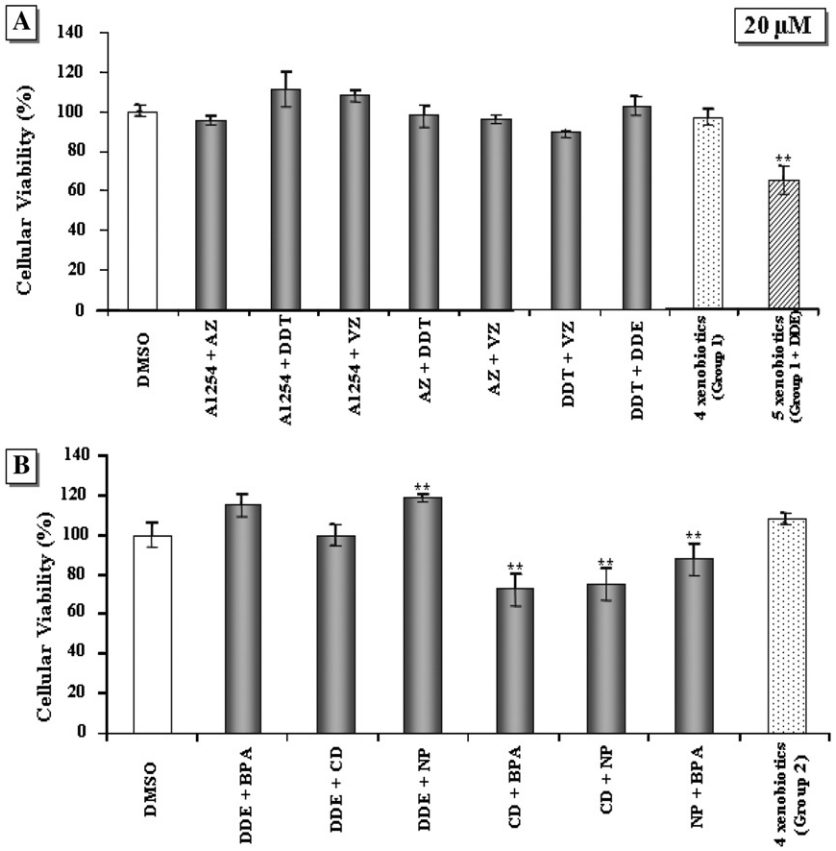

Fig. 6. Combined effects on cell viability of sublethal concentrations of xenobiotics alone. (A) Group 1; (B) group 2 tested in combinations of 2, 4 or 5 made up to a final concentration of 20 μM ($n=9$).

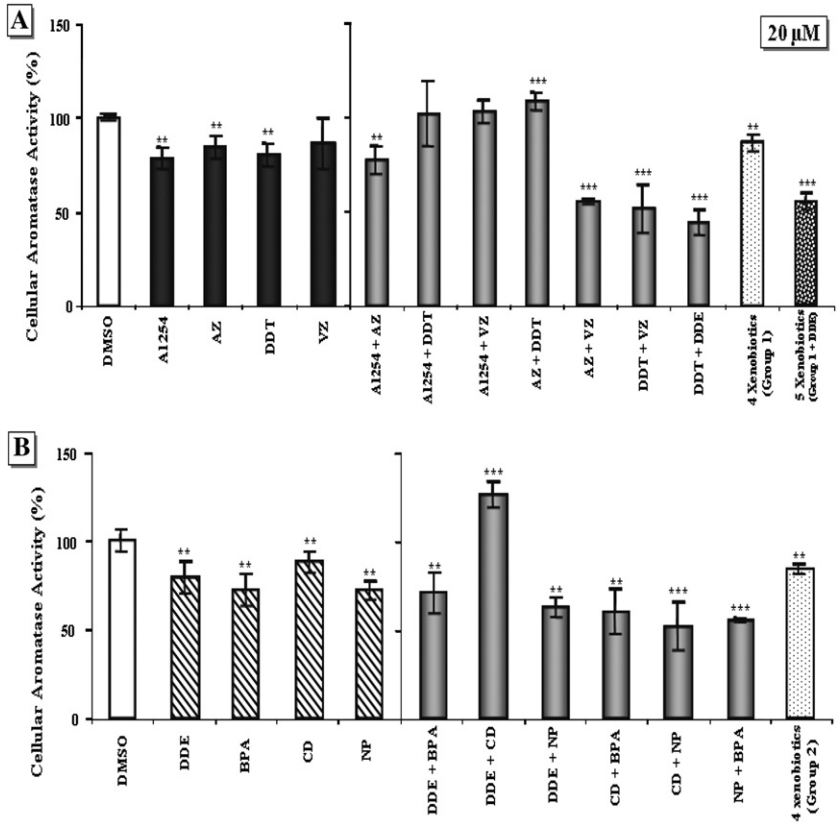

Fig. 7. Combined effects on cellular aromatase, from 293 embryonic cells, for sublethal concentrations of xenobiotics alone or in combination. (A) Group 1; (B) group 2 with the total concentration of 20 µM ($n=9$). $^{**}p<0.01$, the significant effects are observed in comparison to controls (**) and to xenobiotics alone (***).

individual substances acted independently of NADPH metabolism (Fig. 9).

Discussion

In this study we report a model for screening hormonal interferences between xenobiotics and androgen metabolization in mammalian steroidogenesis, at the active site of the aromatase complex. Except for a few reports (Pelissero et al., 1996; Vinggaard et al., 2000; Heneweer et al., 2004), little is known about direct xenobiotic effects on aromatase activity and comparative studies of the purified enzyme, and the enzyme in subcellular fractions (microsomes) and human embryonic cells. This enzyme is responsible for estrogen production and the crucial androgen/estrogen balance necessary for normal embryonic and fetal development, even in the male (Carreau et al., 2006), in many species. The lipophilic compounds used here may be more easily dissolved and metabolized in membranes of the endoplasmic reticulum (Cribb et al., 2005), where the aromatase complex is located (Simpson et al., 1994); but their chemical structures do not allow a prediction of their activity even though the aromatase active site has been well characterized by site-directed mutagenesis (Auvray et al., 1998).

Human embryonic 293 cells have been proven to be very suitable for assessing the hormonal activities of xenobiotics after transfection (Kuiper et al., 1998). Their cellular viability was affected by all xenobiotics tested here at 50 µM (except for TBTO and DES, 10 µM). All the xenobiotics tested disrupt cellular aromatase at low doses (20 µM) and more in binary or multiple combinations. The more potent ones exert a direct action at the microsomal level and on the purified enzyme within a few minutes in two mammalian species, equine and human. Letcher et al. (1999) have previously reported the cytotoxic effects, on placental JEG3 cells, of some organochlorines at very low concentrations (1 nM) while concentrations of 1–10 µM were needed to produce a maximal decrease in aromatase activity. This differential sensitivity towards xenobiotics depends on time and cell culture conditions. For instance, in our group, the aromatase inhibitions by lindane and BPA were greater after 18 h on 293 cells than after direct interaction with microsomes or purified enzyme during 15 min, this was confirmed in several cases. Bioavailability and/or prolonged exposure to lower doses may amplify endocrine disruptions. Interestingly, the threshold of inhibition is 50 times lower in embryonic cells suggesting possible bioaccumulation (Streit, 1992) or a specific metabolic effect (Cribb et al., 2005), and/or some action on cell signaling pathways (Frigo et al., 2005). Such long-term effects may amplify endocrine disruptions which are not predictable by short-term experiments. Moreover, they do not always appear on a linear portion of a dose-response curve (Gelderblom et al., 2001; Nativelle-Serpentini et al., 2003; Benachour et al., 2007). Some compounds that do not show inhibitions on microsomes

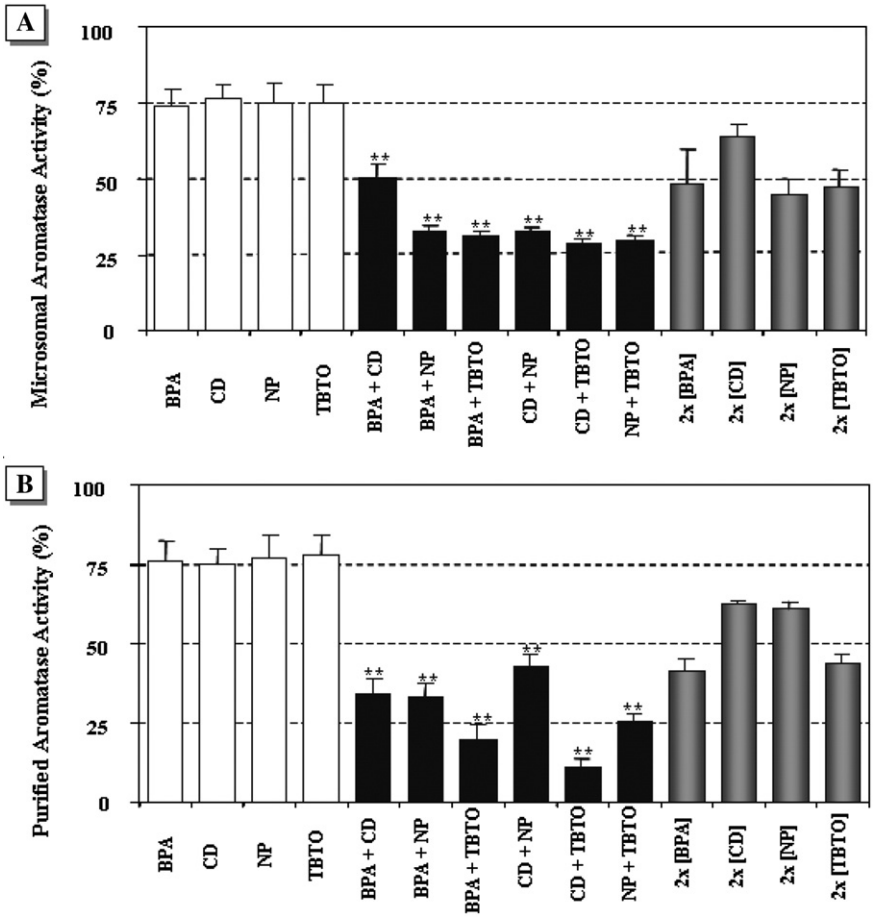

Fig. 8. Effects of xenobiotics at IC25 on microsomal (A) or purified (B) aromatase. Alone (white bars); binary combinations (black bars) and 2× IC25 (grey bars). $^{**}p<0.01$.

became active at 50-fold lower doses in cells within 24 h, at 20 μM alone or at 4–10 μM in binary combinations. These concentrations are far below the LD50 described *in vivo* that range from 146 μM for TBTO to 34 mM for VZ in rats, and around the "Acceptable Daily Intake (ADI)" for DDT and VZ. Cytotoxicity does not appear to involve aromatase since cells can survive without this enzyme. Moreover, aromatase is inhibited at 2- to 3-fold lower doses than mitochondrial succinate dehydrogenase which is involved in cell death and measured by the MTT assay. The most cytotoxic "steroid-like" compounds, such as BPA, CD, NP and TBTO, are the best aromatase inhibitors at non-toxic doses but their interaction with the ubiquist electron donor reductase may participate in general cell damage (Wang et al., 2007). Despite the variations in their chemical structures their inhibitory effects were more pronounced on the aromatase moiety than the ubiquitous P450 reductase component of the complex.

The majority of the current studies are generally interested in the effects of only one chemical at a time, whereas organisms are daily exposed to mixtures of various products in the environment, and it has been underlined that the priority of toxicity studies should focus on mixtures (Feron et al., 2002; Tichy et al., 2002; Lydy et al., 2004; Monosson, 2005). The synergism found in binary combinations of these xenobiotics confirm this view and emphasize the need for more extended studies in this field. Substances that caused no visible cytotoxic effects under our conditions at 20 μM become cytotoxic and/or aromatase disrupters in combination from 4 to 10 μM, probably by multiple pathways of actions, including modulation of aromatase gene expression (Richard et al., 2005). This should be taken into account in the calculation of thresholds of "Acceptable Daily Intake (ADI)". The combinations generally amplify all effects and most often aromatase inhibition, even on the purified enzyme. They are rarely compensatory but in more complex mixtures of 4–5 compounds this is the case in microsomal preparations at 150–400 μM, but whole cell preparations are usually affected at 40-fold lower doses.

Several authors have previously reported a synergistic interaction between two weak estrogenic compounds *in vitro* and *in vivo*. Arnold et al. (1996) reported that a 1:1 mixture of dieldrin, with endosulfan or toxaphene exerted a 160- to 1600-fold higher potency for activating the transcriptional activity of the human estrogen receptor gene, transfected in yeast cells, than either compound alone. They also reported that chlordane, which had no activity alone in their model, but when added to this binary combination significantly enhanced the potency of the three chemicals tested. Furthermore, exposure to PCB (aroclor 1254) did not affect the body growth but with TBT led

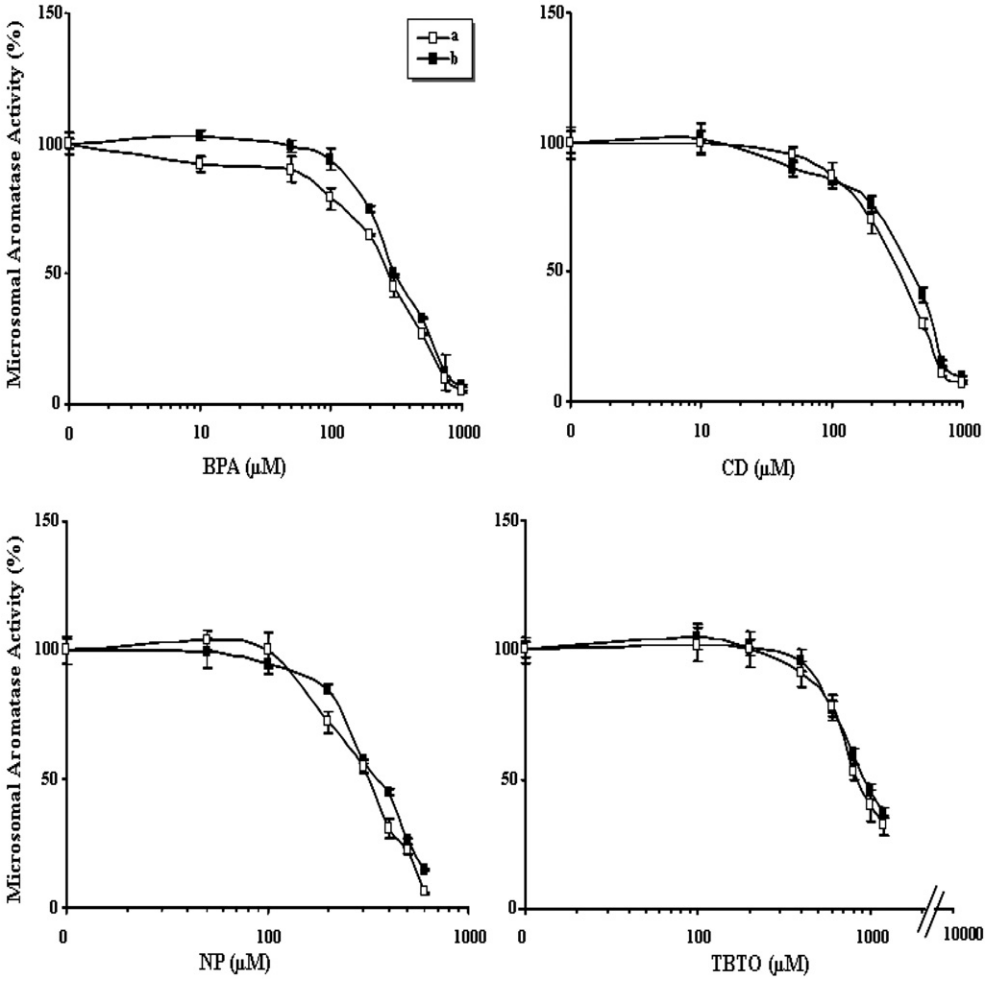

Fig. 9. Effects of metabolism of xenobiotics on microsomal aromatase. Human placental microsomes (100 μg protein) were pre-incubated at 37 °C for 15 min with BPA, CD, NP, and TBTO in the presence (a) or absence (b) of 60 μM NADPH-H^+ in a final volume of 0.5 ml Tris–maleate buffer. Aromatization (at 37 °C for 15 min) was started by adding 100 pmol of [1β-^{3}H] androstenedione plus 60 μM NADPH-H^+ to b.

to a decreased body growth. Exposure to PCB-TBT mixture resulted in an approximately additive decrease of body growth and enzymatic activity of glutathione-*S*-transferase (GST) in young carp (Schmidt et al., 2005). Kemadjou Njiwa et al. (2004) showed that aroclor 1254-DDT mixture synergistically altered spermatic release.

In our study, the combination of two xenobiotics significantly amplified their effects on a short-term basis and directly on the enzyme. Two complementary structures may more easily clutter up the active site or alter the enzyme. Thus a synergistic effect can be expected both at the receptor and the enzymatic levels, and this could explain some *in vivo* toxic effects since organisms are exposed to combinations of multiple agents. Our results complement those reported by other authors. For example, the herbicide AZ that inhibits gonadal aromatase activity in developing alligators *in vivo* at a concentration of 14 ppm (Crain et al., 1997), and in human adrenocortical carcinoma H295R cells in culture (Sanderson et al., 2000), was found to inhibit aromatase activity in this study. These actions appear to be time-dependent and also vary according to species or cells. The model presented here by utilizing the purified enzyme alongside cellular and microsomal aromatase systems makes it possible to discriminate between different mechanisms of action that may cause toxic effects in the human and other organisms. We have shown that xenobiotic toxicity does not necessarily require metabolic transformation in the placenta. Placentally derived microsomes are known to contain several xenobiotic-metabolizing enzymes, XMEs, that are functional throughout pregnancy (Hakkola et al., 1998). In some cases, these XMEs may activate xenobiotics and, in the other cases, the presence of some xenobiotics may suppress or enhance the metabolic action of XMEs. Additionally, it should be noted that, apart from its steroid metabolizing action, human placental aromatase may also contribute to xenobiotic metabolism.

It is noteworthy that induction of gene expression by estrogenic action does not exclude interference with the enzyme active site. Thus, from these results and others, it is apparent that xenobiotic disruption of estrogen biosynthesis can result either from inhibition or induction of aromatase gene expression, via estrogen receptor or non-receptor mechanisms, and from alterations of aromatase activity directly and indirectly. Moreover, hormone-related abnormalities are not always due to the

presence or absence of a single endogenous hormone but also to their imbalance in the body. The effects of xenobiotics are linked to the real tissue concentrations of these pollutants, their bioavailability, bioaccumulation and stability, time of exposure and are variable with time. Our results show unambiguously that xenohormones may interfere at a crucial point of the steroidogenic pathway, namely at the aromatase level.

Furthermore, in these experiments, some inhibitory levels of xenobiotics in cells are close to the ADI. For example, the ADI of DDT is 1.2 mg/kg, and 0.15 mg/kg for Bisphenol A, for which our active dose is 0.9 mg/kg. For Vinclozolin the ADI is 0.6 mg/kg, and we used 1.4 mg/kg. Thus our 4–20 μM doses can be considered as low. Some of these compounds are persistent, they have a long half life (several days to some years) and thus can be bioaccumulated in the food chain since they are hydrophobic (like Nonylphenol, Coldham et al., 1998). Chu et al. (2003) noticed that PCB have high bioaccumulation potential and may affect a number of biological/physiological processes, including disruption of the endocrine system function, lipid metabolism and reproduction. PCB, DDT and metabolites contaminate human blood in Japan (Minh et al., 2006), women mammary fat tissue in Argentina (Munoz-de-Toro et al., 2006) and milk in British mothers around 0.5–2.3 μg/g (Kalantzi et al., 2004). In fact, these levels are approximately in the same range of the doses used here (1.3 μg/g).

In conclusion, synergism between two or more xenobiotics significantly amplified their toxic effects in short-term studies involving the aromatase enzyme in cells and on the isolated enzyme. The complementary structures of these compounds may block the active site and/or alter the enzyme allosterically. Synergistic effects can be expected both at the receptor and the enzymatic levels and should be taken into account when explaining some *in vivo* disturbances, since organisms are commonly exposed to combinations of multiple agents. This knowledge could make it possible to improve the prevention of some pathologies and also allow the study of structural changes that reduce toxic effects leading to safer products being brought to market.

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References

Andersson, P.L., Blom, A., Johannisson, A., Pesonen, M., Tysklind, M., Berg, A.H., Olsson, P.E., Norrgren, L., 1999. Assessment of PCBs and hydroxylated PCBs as potential xenoestrogens: *in vitro* studies based on MCF-7 cell proliferation and induction of vitellogenin in primary culture of rainbow trout hepatocytes. Arch. Environ. Contam. Toxicol. 37, 145–150.

Arnold, S.F., Klotz, D.M., Collins, B.M., Vonier, P.M., Guilette Jr., L.J., McLachlan, J.A., 1996. Synergistic activation of estrogen receptor with combinations of environmental chemicals. Science 272, 1489–1492.

Ashby, J., Tinwell, H., Stevens, J., Pastoor, T., Breckenridge, C.B., 2002. The effects of atrazine on the sexual maturation of female rats. Regul. Toxicol. Pharmacol. 35, 468–473.

Auvray, P., Moslemi, S., Sourdaine, P., Galopin, S., Séralini, G.-E., Enguehard, C., Dallemagne, P., Bureau, R., Sonnet, P., Rault, S., 1998. Evidence for new non-steroidal human aromatase inhibitors and comparison with equine aromatase inhibition for an understanding of the mammalian active site. Eur. J. Med. Chem. 33, 451–462.

Auvray, P., Sourdaine, P., Moslemi, S., Séralini, G.-E., Sonnet, P., Enguehard, C., Guillon, J., Dallemagne, P., Bureau, R., Rault, S., 1999. MR 20492 and MR 20494: two indolizinon derivatives that strongly inhibit human aromatase. J. Steroid Biochem. Mol. Biol. 70, 59–71.

Benachour, N., Sipahutar, H., Moslemi, S., Gasnier, C., Travert, C., Séralini, G.-E., 2007. Time and dose-dependent effects of roundup on human embryonic and placental cells and aromatase inhibition. Arch. Environ. Contam. Toxicol. 53, 126–133.

Blair, R.M., Fang, H., Branham, W.S., Hass, B.S., Dial, S.L., Moland, C.L., Tong, W., Shi, L., Perkins, R., Sheehan, D.M., 2000. The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. Toxicol. Sci. 54, 138–153.

Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., Behr, J.P., 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. Proc. Natl. Acad. Sci. U.S.A. 92, 7297–7301.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Carreau, S., Delalande, C., Silandre, D., Bourguiba, S., Lambard, S., 2006. Aromatase and estrogen receptors in male reproduction. Mol. Cell. Endocrinol. 246, 65–68.

Chu, F.L., Soudant, P., Hale, R.C., 2003. Relationship between PCB accumulation and reproductive output in conditioned oysters *Crassostrea virginica* fed a contaminated algal diet. Aquat. Toxicol. 65, 293–307.

Colborn, T., Vom Saal, F.S., Soto, A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ. Health Perspect. 101, 378–384.

Coldham, N.G., Sivapathasundaram, S., Dave, M., Ashfield, L.A., Pottinger, T.G., Goodall, C., Sauer, M.J., 1998. Biotransformation, tissue distribution, and persistence of 4-nonylphenol residues in juvenile rainbow trout (*Oncorhynchus mykiss*). Drug Metab. Dispos. 26, 347–354.

Crain, D.A., Guillette, L.J., Rooney, A.A., Pickford, D.B., 1997. Alterations in steroidogenesis in alligators (*Alligator mississippiensis*) exposed naturally and experimentally to environmental contaminants. Environ. Health Perspect. 105, 528–533.

Cribb, A.E., Peyrou, M., Muruganandan, S., Schneider, L., 2005. The endoplasmic reticulum in xenobiotic toxicity. Drug Metab. Rev. 37, 405–442.

Daston, G.P., Gooch, J.W., Breslin, W.J., Shuey, D.L., Nikiforov, A.I., Fico, T.A., Gorsuch, J.W., 1997. Environmental estrogens and reproductive health: a discussion of the human and environmental data. Reprod. Toxicol. 11, 465–481.

Déchaud, H., Ravard, C., Claustrat, F., De la Perriere, A.B., Pugeat, M., 1999. Xenoestrogen interaction with human sex hormone-binding globulin (hSHBG). Steroids 64, 328–334.

Denizot, F., Lang, R., 1986. Rapid colorimetric assay for cell growth and survival modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods 89, 271–277.

Dintinger, T., Gaillard, J.L., Moslemi, S., Zwain, I., Silberzahn, P., 1989. Androgen and 19-norandrogen aromatization by equine and human placental microsomes. J. Steroid Biochem. 33, 949–954.

Feron, V.J., Cassee, F.R., Groten, J.P., van Vliet, P.W., van Zorge, J.A., 2002. International issues on human health effects of exposure to chemical mixtures. Environ. Health Perspect. 110, 893–899.

Fraser, L.R., Beyret, E., Milligan, S.R., Adeova-Osiguwa, S.A., 2006. Effects of estrogenic xenobiotics on human and mouse spermatozoa. Hum. Reprod. 21, 1184–1193.

Frigo, D.E., Vigh, K.A., Struckhoff, A.P., Elliott, S., Beckman, B.S., Burow, M.E., McLachlan, J.A., 2005. Xenobiotic-induced TNF-alpha expression and apoptosis through the p38 MAPK signaling pathway. Toxicol. Lett. 155, 227–238.

Gelderblom, W.C.A., Abel, S., Smuts, C.M., Marnewick, J., Marasas, W.F.O., Lemmer, E.R., Ramljak, D., 2001. Fumonisin-induced hepatocarcinogenesis: mechanisms related to cancer initiation and promotion. Environ. Health Perspect. 109, 291–300.

Hakkola, J., Pelkonen, O., Pasanen, M., Raunio, H., 1998. Xenobiotic-metabolizing cytochrome P450 enzymes in the human feto-placental unit: role in intrauterine toxicity. Crit. Rev. Toxicol. 28, 35–72.

Heneweer, M., van den Berg, M., Sanderson, J.T., 2004. A comparison of human H295R and rat R2C cell lines as *in vitro* screening tools for effects on aromatase. Toxicol. Lett. 146, 183–194.

Jacobsen, R., Bostofte, E., Engholm, G., Hansen, J., Olsen, J.H., Skakkebaek, N.E., Moller, H., 2000. Risk of testicular cancer in men with abnormal semen characteristics: cohort study. BMJ 321, 789–792.

Jorgensen, N., Andersen, A.G., Eustache, F., Irvine, D.S., Suominen, J., Petersen, J.H., Andersen, A.N., Auger, J., Cawood, E.H., Horte, A., Jensen, T.K., Jouannet, P., Keiding, N., Vierula, M., Toppari, J., Skakkebaek, N.E., 2001. Regional differences in semen quality in Europe. Hum. Reprod. 16, 1012–1019.

Kalantzi, O.I., Martin, F.L., Thomas, G.O., Alcock, R.E., Tang, H.R., Drury, S.C., Carmichael, P.L., Nicholson, J.K., Jones, K.C., 2004. Different levels of polybrominated diphenyl ethers (PBDEs) and chlorinated compounds in breast milk from two U.K. Regions. Environ. Health Perspect. 112, 1085–1091.

Kemadjou Njiwa, J.R., Muller, P., Klein, R., 2004. Binary mixture of DDT and Arochlor1254: effects on sperm release by *Danio rerio*. Ecotoxicol. Environ. Saf. 58, 211–219.

Krstevska-Konstantinova, M., Charlier, C., Craen, M., Du Caju, M., Heinrichs, C., De Beaufort, C., Plomteux, G., Bourquiqnon, J.P., 2001. Sexual precocity after immigration from developing countries to Belgium: evidence of previous exposure to organochlorine pesticides. Hum. Reprod. 16, 1020–1026.

Kuiper, G.G., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., Van der Saag, P.T., Van der Burg, B., Gustafsson, J.A., 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinology 139, 4252–4263.

Le Curieux-Belfond, O., Moslemi, S., Mathieu, M., Séralini, G.-E., 2001. Androgen metabolism in oyster *Crassostrea gigas*: evidence for 17β-HSD activities and characterization of an aromatase-like activity inhibited by pharmacological compounds and a marine pollutant. J. Steroid Biochem. Mol. Biol. 78, 359–366.

Lemazurier, E., Sourdaine, P., Nativelle, C., Plainfosse, B., Séralini, G.-E., 2001. Aromatase gene expression in the stallion. Mol. Cell. Endocrinol. 178, 133–139.

Letcher, R.J., Van Holstein, I., Drenth, H.-J., Norstrom, R.J., Bergman, A., Safe, S., Pieters, R., van den Berg, M., 1999. Cytotoxicity and aromatase (CYP19) activity modulation by organochlorines in human placental JEG-3 and JAR choriocarcinoma cells. Toxicol. Appl. Pharmacol. 160, 10–20.

Lydy, M., Belden, J., Wheelock, C., Hammock, B., Denton, D., 2004. Challenges in regulating pesticide mixtures. Ecol. Soc. 9, 1–15.

Makita, Y., Tanaka, A., Omura, M., Ogata, R., 2003. Effects of simultaneous administration of tributyltin (TBT) and *p,p′*-DDE on female offspring of Wistar rats. J. Toxicol. Environ. Health 66, 2337–2347.

Makita, Y., Omura, M., Ogata, R., 2004. Effects of perinatal simultaneous exposure to tributyltin (TBT) and *p,p′*-DDE [1,1-dichloro-2,2-*bis*(*p*-chlorophenyl) ethylene) on male offspring of Wistar rats. J. Toxicol. Environ. Health 67, 385–395.

Massaad, C., Entezami, F., Massade, L., Benahmed, M., Olivennes, F., Barouki, R., Hamamah, S., 2002. How can chemical compounds alter human fertility? Eur. J. Obstet. Gynecol. Reprod. Biol. 100, 127–137.

Mendola, P., Buck, G.M., Sever, L.E., Zielesny, M., Vena, J.E., 1997. Consumption of PCB-contaminated freshwater fish and shortened menstrual cycle length. Am. J. Epidemiol. 146, 955–960.

Minh, T.B., Watanabe, M., Kajiwara, N., Iwata, H., Takahashi, S., Subramanian, A., Tanabe, S., Watanabe, S., Yamada, T., Hata, J., 2006. Human blood monitoring program in Japan: contamination and bioaccumulation of persistent organochlorines in Japanese residents. Arch. Environ. Contam. Toxicol. 51, 296–313.

Monosson, E., 2005. Chemical mixtures: considering the evolution of toxicology and chemical assessment. Environ. Health Perspect. 113, 383–390.

Moslemi, S., Vibet, A., Papadopoulus, V., Camoin, L., Gaillard, J.-L., 1997. Purification and characterization of equine testicular cytochrome P-450 aromatase: comparison with the human enzyme. Comp. Biochem. Physiol. 118, 217–227.

Mossmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Munoz-de-Toro, M., Beldomenico, H.R., Garcia, S.R., Stoker, C., De Jesus, J.J., Beldomenico, P.M., Ramos, J.G., Luque, E.H., 2006. Organochlorine levels in adipose tissue of women from a littoral region of Argentina. Environ. Res. 102, 107–112.

Nativelle-Serpentini, C., Richard, S., Séralini, G.-E., Sourdaine, P., 2003. Aromatase activity modulation by lindane and bisphenol-A in human placental JEG-3 and transfected kidney E293 cells. Toxicol. In Vitro 17, 413–422.

Ohlson, C.G., Hardell, L., 2000. Testicular cancer and occupational exposures with a focus on xenoestrogens in polyvinyl chloride plastics. Chemosphere 40, 1277–1282.

Paris, F., Jeandel, C., Servant, N., Sultan, C., 2006. Increased serum estrogenic bioactivity in three male newborns with ambiguous genitalia: a potential consequence of prenatal exposure to environmental endocrine disruptors. Environ. Res. 100, 39–43.

Pelissero, C., Lenczowski, M.J., Chinzi, D., Davail-Cuisset, B., Sumpter, J.P., Fostier, A., 1996. Effects of flavonoids on aromatase activity, an *in vitro* study. J. Steroid Biochem. Mol. Biol. 57, 215–223.

Raun Andersen, H., Vinggaard, A.M., Hoj Rasmussen, T., Gjermandsen, I.M., Cecilie Bonefeld-Jorgensen, E., 2002. Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity *in vitro*. Toxicol. Appl. Pharmacol. 179, 1–12.

Richard, S., Moslemi, S., Sipahutar, H., Benachour, N., Séralini, G.-E., 2005. Differential effects of glyphosate and roundup on human placental cells and aromatase. Environ. Health Perspect. 113, 716–720.

Sanderson, J.T., Seinen, W., Giesy, J.P., van den Berg, M., 2000. 2-Chloro-*s*-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity? Toxicol. Sci. 54, 121–127.

Schmidt, K., Staaks, G.B., Pflugmacher, S., Steinberg, C.E., 2005. Impact of PCB mixture (Aroclor 1254) and TBT and a mixture of both on swimming behavior, body growth and enzymatic biotransformation activities (GST) of young carp (*Cyprinus carpio*). Aquat. Toxicol. 71, 49–59.

Seiler, P., Fischer, B., Lindenau, A., Beier, H.M., 1994. Effects of persistent chlorinated hydrocarbons on fertility and embryonic development in the rabbit. Hum. Reprod. 9, 1920–1926.

Séralini, G.-E., Moslemi, S., 2001. Aromatase inhibitors: past, present and future. Mol. Cell. Endocrinol. 178, 117–131.

Séralini, G.-E., Tomiline, A., Auvray, P., Nativelle-Serpentini, C., Sourdaine, P., Moslemi, S., 2003. Molecular characterization and expression of equine testicular cytochrome P450 aromatase. Biochim. Biophys. Acta 1625, 229–238.

Simpson, E.R., Mahendroo, M.S., Means, G.D., Kilgore, M.W., Hinshelwood, M.M., Graham-Lorence, S., 1994. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr. Rev. 15, 342–355.

Simpson, E.R., Zhao, Y., Agarwal, V.R., Michael, M.D., Bulu, S.E., Hinshelwood, M.M., Graham-Lorence, S., Sun, T., Ficher, C.R., Qin, K., Mendelson, C.R., 1997. Aromatase expression in health and disease. Rec. Prog. Horm. Res. 52, 185–213.

Sipahutar, H., Sourdaine, P., Moslemi, S., Plainfossé, B., Séralini, G.-E., 2003. Immunolocalization of aromatase in stallion Leydig cells and seminiferous tubules. J. Histochem. Cytochem. 51, 311–318.

Skakkebaek, N.E., 2002. Endocrine disruptors and testicular dysgenesis syndrome. Horm. Res. 57 (S2), 43.

Streit, B., 1992. Bioaccumulation processes in ecosystems. Experientia 48, 955–970.

Sugiura-Ogasawara, M., Ozaki, Y., Sonta, S., Makino, T., Suzumori, K., 2005. Exposure to bisphenol A is associated with recurrent miscarriage. Hum. Reprod. 20, 2325–2329.

Sultan, C., Balaguer, P., Terouanne, B., Georget, V., Paris, F., Jeandel, C., Lumbroses, S., Nicolas, J., 2001. Environmental xenoestrogens, antiandrogens and disorders of male sexual differentiation. Mol. Cell. Endocrinol. 178, 99–105.

Thompson, E.A., Siiteri, P.K., 1974. Utilization of oxygen and reduced nicotinamide adenine dinucleotide phosphate by human placental microsomes during aromatization of androstenedione. J. Biol. Chem. 249, 5364–5372.

Tichy, M., Borek-Dohalsky, V., Rucki, M., Reitmajer, J., Feltl, L., 2002. Risk assessment of mixtures: possibility of prediction of interaction between chemicals. Int. Arch. Occup. Environ. Health 75, S133–S136 (Suppl).

Toppari, J., Skakkebaek, N.E., 1998. Sexual differentiation and environmental endocrine disruptors. Bailliere's Clin. Endocrinol. Metab. 12, 143–156.

Toppari, J., Larsen, J.C., Christiansen, P., Giwercman, A., Grandjean, P., Guillette, L.J.J., Jegou, B., Jensen, T.K., Jouannet, P., Keiding, N., Leffers, H., McLachlan, J.A., Meyer, O., Muller, J., Rajpert-De Meyts, E., Scheike, T., Sharpe, R., Sumpter, J., Skakkebaek, N.E., 1996. Male reproductive health and environmental xenoestrogens. Environ. Health Perspect. 104, 741–803.

Vibet, A., Dintinger, T., Maboundou, J.C., Gaillard, J.L., Divoux, D., Silberzahn, P., 1990. Estrogen synthetase in the horse: comparison of equine placental and rat liver NADPH–cytochrome *c* (P-450) reductase activities. FEBS Lett. 261, 31–34.

Vinggaard, A.M., Hnida, C., Breinholt, V., Arsen, J.C., 2000. Screening of selected pesticides for inhibition of CYP19 aromatase activity *in vitro*. Toxicol. in Vitro 14, 227–234.

Walsh, L.P., Stocco, D.M., 2000. Effects of lindane on steroidogenesis and steroidogenic acute regulatory protein expression. Biol. Reprod. 63, 1024–1033.

Walsh, L.P., McCormick, C., Martin, C., Stocco, D.M., 2000. Roundup inhibits steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression. Environ. Health Perspect. 108, 769–776.

Wang, S.L., Han, J.F., He, X.Y., Wang, X.R., Hong, J.Y., 2007. Genetic variation of human cytochrome P450 reductase as a potential biomarker for mitomycin C-induced cytotoxicity. Drug Metab. Dispos. 35, 176–179.

Wolf, C.J., LeBlanc, G.A., Gray, L.E., 2004. Interactive effects of vinclozolin and testosterone propionate on pregnancy and sexual differentiation of the male and female SD rat. Toxicol. Sci. 78, 135–143.