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Modulation of aromatase activity and mRNA by various selected pesticides in the human choriocarcinoma JEG-3 cell line

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Abstract

Aromatase enzyme plays a central role in steroidogenesis by converting androgens to estrogens and has been proposed as an important molecular target for many environmental endocrine disrupters chemicals. In this study, we have screened thirty selected pesticides with known, unknown or supposed effects on aromatase activity, for their ability to modulate aromatase activity in the human choriocarcinoma JEG-3 cell line after both short (2 h) and long exposure (24 h). All pesticides were tested at concentrations up to 10 μM that did not cause cytotoxicity after 24 h of exposure, as verified by the MTT viability assay. Four pesticides inhibited aromatase activity after 2 h of exposure: prochloraz (IC_{50} < 1 μM), fenbuconazole (IC_{50}= 1.1 μM), propiconazole (IC_{50}= 1.5 μM) and fenarimol (IC_{50}= 3.3 μM). Among them, prochloraz and fenbuconazole also exerted inhibitory effects after 24 h. Toxaphen (10 μM) and heptachlor (10 μM) inhibited aromatase activity after 24 h exposure only. Nine pesticides induced aromatase activity: aldrin, chlordane, cypermethrin, parathion-methyl, endosulfan, methoxychlor, oxadiazon, metolachlor and atrazine after 24 h of exposure, while tributyltin induced aromatase activity at 1 nM and 3 nM after both 2 h and 24 h of exposure respectively. To further investigate the mechanisms of aromatase induction we measured CYP19 mRNA expression and showed that methoxychlor, aldrin, chlordane and tributyltin induced the transcription of the *cyp19* gene. In addition, none of the aromatase inducers transactivated the retinoic acid receptor (RAR) in JEG-3 stably transfected with a RARE-luciferase plasmid while the RAR agonist TTNPB induced both aromatase and luciferase expression in these cells. Our results, which provide new data for fenbuconazole, as an inhibitor of human aromatase, and for eight pesticides as aromatase inducers, are discussed with regards to the regulation of aromatase expression in the JEG-3 cellular context.

Key words: pesticides; aromatase activity; aromatase mRNA; JEG-3; retinoic acid receptor.

1. Introduction

A number of environmental contaminants from agricultural, industrial and household origins can alter the normal functioning, synthesis and metabolism of endogenous hormones (Sonnenschein and Soto, 1998). These substances, termed Endocrine Disrupting Chemicals (EDCs), can act *via* multiple modes of action including either direct interaction with steroid hormone receptors, or by altering steroidogenesis. Cytochrome P450 aromatase (aromatase, CYP19) plays a crucial role in steroidogenesis by catalysing the rate-limiting step in the conversion of androgens into estrogens (Mendelson et al., 1985; Thompson and Siiteri, 1974). In human, aromatase is expressed in several tissues, and *cyp19* gene expression is differentially regulated by tissue-specific promoters (Simpson et al., 1993).

Several *in vitro* assays, developed from different tissues and species, have been described for the assessment of the effect of chemicals on aromatase. These include microsome assays using human placental microsomes (Vinggaard et al., 2000) or trout ovarian and brain microsomes (Hinfray et al., submitted; Monod et al., 1993), as well as cell-based assays using different cell lines such as the adrenocortical H295R (Sanderson et al., 2000), ovarian KGN (Nishi et al., 2001) or placental JEG-3 (Bahn *et al.*, 1981) cell lines.

Placenta is the main organ responsible for estrogen synthesis in pregnant women. The JEG-3 cell line are human choriocarcinoma cells derived from malignant placental tissues. As in placenta, JEG-3 cells endogenously express aromatase activity at detectable levels (Bahn *et al.*, 1981) and the *cyp19* gene expression is under the control of a distal promoter I.1 located adjacently upstream of exon I.1 in the gene (Means *et al.*, 1991). Various factors are involved in the up-regulation of *cyp19* gene expression in JEG-3 cells. These include protein kinase A activator (cAMP) (Harada *et al.*, 2003), retinoic acids (Sun *et al.*, 1998; Zhu *et al.*, 2002), phorbol ester protein kinase C activator 12-O-tetradecanoyl phorbol 13-acetate (TPA) and to a lesser extend IL-1β (Ritvos and Voutilainen, 1992). Since regulation of aromatase expression in these cells is the same as in placenta, JEG-3

cells have been proposed as a valuable tool for the assessment of a potential steroidogenesis disruption during embryonic and fœtal development (Drenth et al., 1998).

Several classes of pesticides have been identified as capable of altering this enzyme, either directly as competitive inhibitors of the catalytic activity, or indirectly through a modulation of its expression in cell-based assays. For instance, 2-chloro-s-triazines herbicides like atrazine, simazine and propazine *in vitro* (Sanderson et al., 2002; Sanderson et al., 2000), and p,p'-dichlorodephenyldichloroethane (p,p'DDE) *in vitro* and *in vivo* (You *et al.*, 2001) have been demonstrated to induce aromatase activity. Conversely, pesticides including imidazole-like fungicides such as prochloraz, triadimenol, propiconazole, triadimefon and fenarimol have been found to inhibit aromatase activity *in vitro* (Andersen et al., 2002; Vinggaard et al., 2000). Besides, many pesticides are currently used and increasingly detected in surface waters (IFEN, 2003; IFEN, 2004), and, for a number of them, there is a lack of information on their potential to interfer with aromatase.

In the present study, 30 pesticides were screened for their ability to modulate aromatase activity in the human choriocarcinoma JEG-3 cell line. The selected pesticides (Table 1) belong to different chemical classes and are of environmental concern, i.e. they are commonly found in surface waters as contaminants, and some are currently authorised for use. While the majority of them have not been tested yet for their effects on aromatase activity in this cellular model, some have been clearly described as aromatase inhibitors (prochloraz, fenarimol, propiconazole) or inducer (atrazine) in this or other cell systems or organisms, and were therefore used as reference pesticides. To our knowledge, there is no or limited information on aromatase alteration in a cellular system by the other pesticides tested in our study. By using the tritiated water assay after both short (2 h) and long (24 h) times of exposure, we identified either inhibition or induction, respectively, of aromatase enzymatic activity by the selected pesticides. Pesticides that were newly found to induce aromatase activity were further investigated for their action at the transcriptional level and through the retinoic acid receptor (RAR) signalling pathway.

2. Materials and methods

2.1. Chemicals

Radiolabelled [1β-^{3}H]androst-4-ene3,17-dione (1β-^{3}H-A) was from Perkin Elmer Life and Analytical Science (Courtaboeuf, France). The RAR pan-antagonist CD3106 was a generous gift from Serge Michel (Galderma Research, Sophia Antipolis, France) and the RAR pan-agonist P-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-napthalenyl-1-p ropenyl] benzoic acid (TTNPB) were provided by Tocris Cookson Ltd. (Bristol, United Kingdom). Dimethylsulfoxyde (DMSO), 4-hydroxyandrostenedione (4-OHA), forskolin and D-luciferine (luc), were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). All pesticides were from Sigma-Aldrich, except azimsulfuron (DuPont de Nemours, France), tributyltin, zinc pyrithione (Acros, Halluin, France) and sodium arsenite (Alpha Aesar, Bischheim, France). Stock solutions of chemicals were made up in DMSO and stored at –20°C. For cell exposure, all pesticides were diluted to the desired concentrations in the culture medium so that the final solvent concentration did not exceeded 0.1% v/v and would not affect cell viability.

2.2. Cell viabiliy

The cellular viability was measured by using the methyl-thiazol-tetrazolium (MTT) assay (Mosmann, 1983). MTT was dissolved in culture medium to a concentration of 0.5 mg/ml and added to the cells after the culture medium had been removed. After 1 hr of incubation at 37°C, the medium was removed and the purple formazan product was dissolved in isopropanol. Plates were read at 570 nm against a 660 nm reference wavelength on a microplate reader (BioTek Instruments, France). Cell viability was expressed as a percentage of the corresponding control value.

2.3. JEG-3 choriocarcinoma cells cultures

JEG-3 human choriocarcinoma cells (ATCC # HTB-36) were kindly provided by Pr. Seralini (University of Caen, France). The cells were maintained in DMEM supplemented with 10 % of

foetal calf serum (FCS), 5 % Hepes buffer, 5% of non essential amino acids and 1 % antibiotic (penicillin-streptomycin) in a 5 % CO_2 humidified atmosphere at 37 °C. For chemical exposure, JEG-3 were seeded in 24-well culture plates (Dominique Dutscher S.A., Saint Quentin Fallavier, France) at a density of 50,000 cells/well in DMEM without phenol red, supplemented with 3 % of dextran-coated charcoal treated foetal calf serum (DCC-FCS) and 1 % antibiotic (penicillin-streptomycin). Cells were left to attach for 24 h before changing culture media and exposing the cells to the xenobiotics for 2 h or 24 h. Under these conditions, the cell monolayers were 80% confluent at the time of running the aromatase assay and *cyp19* mRNA measurement.

2.4. Aromatase assay

The catalytic aromatase activity was determined by using the tritium release assay as described previously (Drenth et al., 1998; Lephart and Simpson, 1991) with minor modifications. Briefly, 0,4 ml of DMEM-3% DCC-FCS, containing 80 nM of [1β-^{3}H] androst-4-ene3,17-dione (25,3 Ci/mmol) was added to the wells following pesticides treatment. After incubation for 2 h at 37°C, plates were placed on ice and 400 µl of medium was transferred to a borex-tube (VWR, Fontenay-sous-Bois, France). The medium was extracted with 2 ml of diethyl ether, vortexed for 30 sec and centrifuged for 10 minutes at 3000 g. This step was repeated once. Two hundred µl of the aqueous phase obtained were then mixed with 200 µl of a 5 % wt/v charcoal suspension, vortexed for 30 sec and centrifuged at 3000 g for 20 min. A 150 µl aliquot was removed and mixed with 750 µl of scintillation liquid OptiPhase 'Hi safe' 3 in 24-well flexiplates (Perkin Elmer Life and Analytical Science, Courtaboeuf, France) and counted for 2 min in a Liquid Scintillation Counter (Wallac Microbeta, Perkin Elmer, France). Under our experimental conditions, the apparent Km and Vmax in JEG-3 cells were found to be 82.6 nM and 66.04 nmol/min/mg respectively. The specificity of the aromatase assay was verified by using 4-OHA, an irreversible inhibitor of the catalytic activity of aromatase that blocks the formation of estrone (Brodie *et al.*, 1981). Results were expressed in % of basal aromatase activity in solvent treated cells (control).

Two times of exposure were used: firstly, a 2 h short time exposure to the test chemical concomitantly with β-^{3}H-A was used in order to assess direct competitive effects on the aromatase enzymatic complex (e.g. allosteric factors or post-translational events like phosphorylation) (Balthazart *et al.*, 2001; Heneweer *et al.*, 2004); secondly a 24 h exposure to the test chemical prior to changing the media and starting the aromatase assay for 2 h, to take into account the possible effects of chemicals on aromatase gene expression through interference at the transcription or (post)translation levels.

2.5. Cyp19 mRNA measurement

Cyp19 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were quantified directly from crude JEG-3 cell lysates exposed 24 h to inducers of aromatase activity with the QuantiGene Reagent system (Genospectra, Fremont, USA), according to the manufacturer's instructions. This assay is based upon the branched DNA technology, which relies on cooperative hybridisation between the target mRNA and a specific probe set. The probe set consists of three types of oligonucleotides: capture extenders (CEs), label extenders (LEs), and blocking probes (BLs), whose sequences were selected based on the sequences of the target and house keeping genes: human aromatase (Accession Number X13589) and human GAPDH (Accession Number AF 261 085). The cells were lysed in QuantiGene Lysis Working Reagent at 50°C for 30 minutes. 80 µl of lysate containing either 60,000 cells for cyp19 mRNA quantification or 1,000 cells for the house keeping gene quantification GAPDH, were transferred to a separate 96-wells capture plate. The appropriate probe set and the lysis reagent (LWR) were then added to the lysates. The capture plates were sealed and incubated at 53 °C for 18 h. After addition of amplifier, label probe and substrate reagents, the plates were transferred to the luminometer (Victor^{2}, Perkin Elmer, France), and the luminescence intensity was read. *Cyp19* mRNA expression was normalised by the GAPDH mRNA expression for which no variation was observed under pesticides treatment. Results were expressed as fold induction ± standard deviation of the solvent treated cell control.

2.6. *Stable transfection*

The stably transfected cell line JEG-3-RLN was obtained as already described (Balaguer et al., 2001). Briefly, we transfected JEG-3 cells with the retinoid responsive gene RARE3-tk-LUC^{+}-SVNeo plasmid (Balaguer et al., 2001). Selection of resistant clones by G418 was performed at 1 mg/ml. JEG3 clones that expressed retinoic acid (RA)-inducible luciferase were identified using photon-counting cameras (Argus 100 from Hamamatsu and Night Owl from Berthold). The most responsive clone was isolated and called JEG3-RLN.

2.7. *Luciferase assay*

For transactivation assays, the stably transfected JEG-3-RLN cells were seeded in DCC-FCS medium at a density of 5.10^4 cells per well of 96-well white opaque tissue culture plates, and left to incubate for 24 hours. After cell exposure, the medium was replaced by 50 µl of medium containing 3.10^4 M D-luciferin and the luminescence was read after 5 minutes in a MicroBeta Wallac microtiter plate luminometer. Results are expressed as percent of maximal luciferase induction by the RAR pan-agonist TTNPB (Loeliger et al., 1980).

2.8. *Data analysis*

In all assays, each compound was tested at various concentrations in at least three independent experiments, which always included positive (TTNPB or forskolin) or negative (solvent) controls. All experimental data were expressed as means ± standard deviation (SD) of triplicates of exposure. Data were subjected to statistical analysis by one way analysis of variance (ANOVA) followed by Dunnett's bilateral posthoc test. A value of $p < 0.05$ was considered significant. The SPSS™ software version 10.1 was used for the statistical analysis. For the aromatase assay, non-linear regression analysis of enzyme kinetic data was performed with Sigma Plot™ 8.0 in order to calculate apparent Km and Vmax values. EC_{50} and IC_{50} of RARtransactivation and aromatase inhibition were calculated with the Regtox 6.3 Microsoft Excel macro (Vindimian, 2006).

3. Results

3.1. Effects of model compounds on aromatase activity in JEG-3 cells

We first used model aromatase inhibitors and inducing compounds to check the validity of the aromatase response in JEG-3 cells in our assay conditions. The competitive inhibitor 4-OHA caused inhibition of aromatase activity in JEG-3 cells after both 2 and 24 h of exposure (Fig.1.a). The strong inhibition observed after 2 h at 10 nM suggested that short term exposure is suitable to detect competitive inhibition at the active site of the enzyme. The up-regulation of aromatase expression in this cellular assay was verified by using TTNPB, the RAR pan-agonist, and forskolin, the adenylate cyclase stimulator, as model activators of aromatase gene expression through RAR (Sun et al., 1998) or cAMP pathways (Harada et al., 2003), respectively. After a 24 h exposure, TTNPB and forskolin (Fig 1.b) caused a significant induction of both aromatase enzymatic activity (3.5-fold by forskolin and 2.5-fold by TTNPB) and *cyp19* mRNA (5-fold by forskolin and 2-fold by TTNPB) . No effect of these two compounds on aromatase expression could be detected after 2 h of exposure (data not shown). In addition, induction of aromatase by TTNPB was clearly mediated by the RAR since it was blocked by CD3106, the RAR pan-antagonist (Fig. 1b).

3.2. Effects of pesticides on aromatase activity in JEG-3 cells

Preliminary MTT tests showed that, at 10µM, none of the pesticides altered significantly the cellular viability after 24 h of exposure, excepted TBT, which was cytotoxic right from 0.1 µM (data not shown). Pesticides were then firstly tested in single dose (10 µM) for their effect on aromatase activity after 2 and 24 hours. Thereafter, when significant effects were observed, several concentrations were assayed so that dose-response curves could be established. The results obtained for aromatase inhibition (Fig. 2) and induction (Fig. 3) are detailed below.

Among the thirty pesticides tested, six inhibited aromatase activity in the JEG-3 cell line (Fig. 2). After 2 h of exposure, prochloraz, fenbuconazole, propiconazole and fenarimol at 10µM inhibited 95 %, 86 %, 72 % and 48 % of basal aromatase activity, respectively (Fig.2.a). Their

respective IC_{50} were < 1 µM, 1.1 µM, 3.3 µM and 10 µM. After 24 h of exposure, prochloraz and fenbuconazole inhibited 83 % and 28 % of basal aromatase activity, with IC_{50} of 2.4 µM and 15.8 µM, respectively (Fig. 2b). Fenarimol and propiconazole, which were inhibtors after 2 hours, showed no effect after long time exposure (Fig.2.b). Conversely, toxaphen and hepatchlor that did not modulated aromatase activity after 2 h, significantly reduced this enzyme activity by a factor two after 24 h of exposure (Fig.2.a and 2.b). Their respective IC_{50} were 11 µM and 16 µM. These differences in the ability of pesticides to inhibit aromatase may reveal divergent modes of action of the pesticides to alter aromatase expression, i.e. either by direct competitive action with [1β-^{3}H] androst-4-ene3,17-dione at the active site of the enzyme or by inhibiting gene transcription.

After 24 h of exposure, ten pesticides significantly induced aromatase activity right from 3 nM (TBT), 3 µM (aldrin, chlordane, cypermethrine, methoxychlor) or 10 µM (endosulfan, parathion-methyl, oxadiazon, metolachlor, atrazine) (Fig.3.a). The maximal aromatase induction ranged between 154 ± 19 % for metolachlor and 405 ± 81 % for aldrin. Among the pesticides tested, TBT was the only one found to induce this enzyme at both times of exposure. It significantly induced aromatase activity by a factor 2.5 at 10 nM and 3 nM after 2 h and 24 h of exposure respectively (Fig.3.b). The enzyme inhibition observed at 24 h at the highest concentration can be explained by strong cytotoxicity of this compound at 10 nM, as verified by the MTT assay.

3.3. Effects of aromatase inducers on cyp19 mRNA expression

To assess whether aromatase induction by pesticides could be linked to activation of *cyp19* gene transcription, we measured CYP19 mRNA expression after exposure of JEG-3 cells for 24 h to the active pesticides. GAPDH mRNA expression was used as a reference and was not affected by the treatments in these experiments (results not shown). Among the ten pesticides that induced aromatase activity in our experiments, only four were found to induce significantly *cyp19* mRNA expression (Fig.4): methoxychlor (10 µM), aldrin (10 µM), chlordane (10 µM) and tributyltin (1nM) with fold induction values over control ranging from 1.7 ± 0.1 to 2.2 ± 0.5. The effects of oxadiazon (1.1-fold), atrazine (1.2-fold), cypermethrine (1.4-fold), endosulfan (1.5-fold),

metaloachlor (1.4-fold) and parathion (1.3-fold) were found not statistically significant as compared to the solvent control.

3.4. RAR-mediated aromatase activity induction

In the JEG-3 choriocarcinoma cells, retinoids are some of the factors involved in the up-regulation of the *cyp19* gene expression (Sun et al., 1998; Zhu et al., 2002). Because pesticides have been recently described to act as agonists of RARs (Lemaire et al., 2005), we tested the hypothesis that this signalling pathway could be involved in aromatase induction in JEG-3 cells. For this purpose, JEG-3 cells stably transfected with the luciferase gene under the control of retinoic acid receptor response element (RARE) were established and used to assess luciferase induction by the selected pesticides. In these cells (JEG-3-RLN), TTNPB induced RARE-luciferase with an EC50 of 1.4 nM and reached maximal transactivation at 10 nM (Fig. 5). This induction was specifically RAR dependent since it was completely inhibited by the RAR antagonist CD3106 at 0.1 µM. The CD3106 alone had no significant inhibitory effect on the basal luciferase activity.

Among the ten pesticides that induced aromatase activity in this study, none induced luciferase activity in the JEG-3-RLN cells (results not shown). Only TBT at 1 nM slightly increased luciferase (by a factor 2) in a reproducible manner, however this effect was considered as non specific of the RAR since it was not reversed by CD3106. Overall, these results show that pesticides have no agonistic effect on RAR mediated transcription in JEG-3-RLN cells, and, as a consequence, suggest that induction of aromatase activity by pesticides is not mediated by RARs in JEG-3 cells.

4. Discussion

A number of xenobiotics including several pesticides can modulate, either positively or negatively, the catalytic activity or mRNA expression of human aromatase *in vitro* in cellular models (Sanderson et al, 2001, Sanderson et al, 2002, Nativelle-Serpentini et al, 2003). In the present study, we newly identified several pesticides of environmental concern that belong to various chemical classes, for their ability to alter the catalytic activity and the gene expression of

aromatase in the human choriocarcinoma JEG-3 cells. Moreover, first attempt to explore possible mechanisms responsible for aromatase induction by pesticides showed that RARs are not involved in this response.

In JEG-3 cells, previous studies have reported that aromatase is expressed at relatively high basal levels as compared to other cellular models like the human adrenocortical cell line (H295R) or the human breast cancer cell line MCF-7 (Sanderson et al, 2001), and is also inducible by several exogenous factors like cAMP analogues (Sanderson et al, 2001) and retinoids (Zhu et al, 2002), thus making them as a suitable model for both inhibition and induction aromatase studies. In our assay condition, aromatase expression was up-regulated by forskolin, an adenylate cyclase activator, and by TTNPB, a full agonist of RARs after overnight exposure, and was rapidly inhibited (after 2 hours) by the competitive inhibitor 4OHA (Fig. 1). These results led us to further use both times of exposure in the screening of pesticides in order to consider both direct inhibition through chemical interaction with the active site of the aromatase enzyme and an effect resulting from an alteration of regulation of the aromatase gene expression. The 30 pesticides tested exerted differential effects with either inhibition, induction, or lack of response of aromatase activity in JEG-3 cells.

Prochloraz, propiconazole, and fenarimol have been described as aromatase inhibitors in various *in vitro* assays using cell-free or cellular systems derived from different tissues (see Table 1 for references). Accordingly, these pesticides were also potent aromatase inhibitors in our study, procholoraz being the most active one (Fig. 2). In addition, fenbuconazole, another imidazole fungicide, was newly identified as a potent inhibitor of aromatase activity with an IC50 of 1.1 µM. This study represents, to our knowledge, the first report on its effect on human aromatase. Its inhibitory action on fish aromatase *in vitro* was also observed in our laboratory using both brain and ovary microsomes from rainbow trout (Hinfray et al., submitted). Imidazole chemicals have been described as reversible inhibitors of the human aromatase activity (Ayub and Levell, 1988). The same mechanism of action has also been suggested for fenarimol, a chlorinated fungicide and well-

described aromatase inhibitor in different biological systems (Hinfray et al., submitted; Hirsch et al., 1987; Sanderson et al., 2002; Vinggaard et al., 2000). This correlates well with the stronger inhibitions observed after 2 h co-exposure with the radioactive substrate in comparison to the longer exposure protocol. The latter did not included co-incubation of the test chemical with the radiolabeled substrate during the aromatase assay, which explained that inhibitory effects were partly reversed for prochloraz and fenbuconazole, the most potent inhibitors in JEG-3, and completely reversed for fenarimol and propiconazole. Such result also illustrates that, in a chemical screening purpose using cellular assays, short term incubation is adapted to highlight competitive inhibition by chemicals at the active site of the enzyme.

The patterns of aromatase inhibition by toxaphen and heptachlor were quite different since their effect was only observed after 24 h exposure. This suggested involvement of down-regulation of aromatase expression by these compounds rather than an inhibition at the active site. Different effects of toxaphen on aromatase have been reported depending on the cellular model used. In JEG-3 cells, toxaphene was previously described without any effect on aromatase activity in JEG-3 at concentrations up to 3 µM after 18 h of exposure (Drenth *et al.*, 1998). Since we observed an inhibitory effect only at 10 µM, it is likely that the discrepancy with the previous cited study is due to different exposure conditions. This pesticide is an antagonist of the Estrogen-related receptor alpha-1 (ERRα-1), an orphan nuclear receptor (Yang and Chen, 1999). In breast (SK-BR-3) and liver (HepG2) cells, its binding to the ERRα-1 led to an inhibition of aromatase expression, which is regulated in part by an ERRα-1-modulating promoter in these cells (Yang and Chen, 1999). Although ERRα-1 is strongly expressed in JEG-3 cells (Mehta et al., 2002), it is not known whether the promoter I.1 that regulates aromatase expression in these cells is modulated by this receptor, which could explain the down-regulation observed in our study. Heptachlor was previously shown without effect on aromatase activity neither in human placental (Vinggaard et al., 2000) nor in rainbow trout (Hinfray et al., submitted) microsomal assays. In our study, it inhibited aromatase activity in JEG-3 cells only after 24 h of exposure at 10 µM, which corroborates the view that this

pesticide does not act as a competitive inhibitor of the enzyme but rather potentially regulates aromatase expression. Conversely to our finding, hepatchlor was reported to slightly increase aromatase activity in KGN cells (Morinaga et al., 2004). Although the mechanism responsible for its action is not known, these differences may be due to the tissue-specific regulation of aromatase activity (Simpson et al., 1993).

Some differential effects on aromatase induction were observed. Ten pesticides induced the enzymatic activity (Fig. 3) while induction of aromatase mRNA was observed only after exposure to metolachlor, aldrine, chlordane and TBT (Fig. 4).

Aromatase gene expression is regulated in a tissue-specific fashion by different promoters that are differently regulated by cellular transcription factors. As a consequence, depending on the studied tissue and/or the mechanism of action of a given chemical, different results can be found when comparing the effect of a same chemical in several cell lines or microsomal assays. For instance, TBT demonstrated different effects *in vitro* depending on the cellular model used (see Table 1). In both H295R (Sanderson *et al.*, 2002) and KGN cells (Ohno et al., 2004), TBT caused a concentration-dependant decrease of aromatase activity which was correlated with cytotoxicity at concentrations above 30 nM. Conversely, it caused a dose-dependant increase of both aromatase activity and mRNA in human choriocarcinoma cell lines (JAR, JEG-3 and BeWo cell lines) at concentrations ranging from 10 to 100 nM after 48 hours of exposure (Nakanishi *et al.*, 2002), as we report in our study although at lower concentrations and after shorter time of exposure (Fig. 3b and 4). The mechanism responsible for this induction was not mediated by cAMP (Nakanishi et al., 2002), but it was recently shown to involve the binding of TBT to the retinoid X receptor (RXR) and the further activation of the RXR homodimer but not the RXR-RAR heterodimer (Nakanishi et al., 2005). This supports our data that TBT has low influence on retinoic acid receptor (RAR)-mediated gene transcription in JEG-3 cells.

The triazine-containing herbicides like atrazine and vinclozolin induced aromatase activity in the adrenocortical H295R cells by increasing intracellular cAMP levels, possibly through inhibition of

phosphodiesterase activity (Sanderson et al., 2002) at 1 μM for atrazine and 30 μM for vinclozolin, but had no effect in the ovarian KGN cells (Morinaga et al., 2004; Ohno et al., 2004). In our assay conditions, atrazine, induced aromatase activity only at the highest tested concentration (10 μM), whereas vinclozolin (10 μM) had no effect on this enzyme. In H295R cells, steroidogenic CYPs are induced by synthetic cAMP analogues or through stimulation of adenylate cyclase by forskolin, which results in increased cAMP-mediated protein kinase A pathway leading to increased aromatase gene transcription. Besides, aromatase transcription is regulated by other specific factors in JEG-3 cells such as retinoic acid receptor, TPA and Il-β which might modulate the sensitivity of these cells to xenobiotics that are able to increase intracellular cAMP.

Methoxychlor, cypermethrine, aldrin, endosulfan and chlordane have been rarely studied regarding their *in vitro* effects on aromatase activity. Cypermethrin and chlordane were described to cause a slight inhibition of aromatase activity in KGN cells (Morinaga *et al.*, 2004) and in ERRα–1 transfected HepG2 cells (Yang and Chen, 1999), respectively. Methoxychlor, aldrine and endosulfan were unable to modulate this enzyme in KGN cells (Morinaga *et al.*, 2004). In JEG-3 cells, these five compounds significantly induced aromatase activity after a 24 h exposure. Again, the discrepancy between our results and those found in literature could be due to the different regulation of aromatase expression in JEG-3 cells in comparison with *in vitro* models derived from ovarian or hepatocyte carcinomas (Simpson et al., 1993). Finally, methyl-parathion, oxadiazon and metolachlor were here newly identified as inducers of aromatase activity in JEG-3 cells. Their mechanisms of intracellular action in human cells as EDCs are unknown although a very recent study has described oxadiazon and metolachlor as potent agonists of the PXR (Lemaire et al., 2006). Whether this receptor is present and can regulate aromatase expression in JEG-3 cells remains to be evaluated.

A fairly unexpected result was the finding that some of the pesticides that induced aromatase activity had no effect on mRNA production, but could suggest that regulation events occurred at the post-translational level. Such hypothesis is based on similar observation that have been previously

described in JEG-3 cells pre-exposed to steroidal (4OHA, atamestane) and non steroidal (fadrozole, vorozole, aminoglutethimide) aromatase inhibitors (Harada et al., 1999; Yue and Brodie, 1997). These compounds increased intracellular immunoreactive aromatase protein, but not aromatase mRNA, through inhibition of aromatase protein degradation provided by a stabilisation of the enzyme-substrate complex (Harada et al., 1999; Yue and Brodie, 1997). Further studies using inhibitor of protein synthesis, such as cycloheximide, or immunoreactive detection of aromatase protein in JEG-3 cells will be necessary to assess whether inhibition of aromatase protein turnover may explain aromatase induction by these pesticides.

Finally, we further investigated the mechanisms of aromatase induction by pesticides by evaluating their ability to elicit a RAR-mediated transcriptional activation. Indeed, the CYP19 promoter in JEG-3 cells contains RARE and aromatase activity is regulated by RARs, the main isoform present in these cells being the RARα (Sun et al., 1998). The finding that none of the pesticides that induced aromatase activity transactivated the RARα in JEG-3 might be related to the recent finding that five organo-chlorinated pesticides, including aldrin, chlordane, and endosulfan, could transactivate the RARβ and the RARγ but not the RARα in HeLa cells stably transfected with specific RAR-responsive reporter gene (Lemaire et al., 2005). Taken together, our results demonstrate that some pesticides may induce aromatase activity through transcriptional activation of the CYP19 gene, but this transactivation is unlikely to be mediated by RARα in JEG-3 cells. As discussed above, other signalling pathways, such as RXR or cAMP/protein kinase A, could be involved in the transcriptional activation of aromatase gene by these pesticides.

When considering the physiologic consequences of the modulating effects on aromatase by pesticides, it is important to relate the present findings to the levels of human exposure. We have shown that several pesticides were able to modulate human aromatase activity, the most potent ones being prochloraz, fenbuconazole and propiconazole as aromatase inhibitors and TBT, aldrin and chlordane as aromatase inducers. Except for TBT that was active at nM concentrations, most compounds were active in the 1-10 μM range (corresponding to 0.1 to 4 ppm). For pesticides,

reported internal doses in human fluids are usually in the low to middle ppb range (CDC 2005), which are lower than the active concentrations that we found in this study. Thus, it would appear that normal human exposure to the chemicals investigated in this study would be unlikely to cause reproductive dysfunction through aromatase disruption. However, regarding the potential risk following exposure to such compounds, no definite conclusion can be drawn from a single *in vitro* assay. First, because some pesticides have been shown to be active on different mechanisms involved endocrine disruption such as interaction with androgen or estrogen receptors, as well as with hormone metabolism. For instance, chlordane, an inducer of aromatase in this study, is also an agonist of the estrogen receptor and an antagonist of the androgen receptor (Lemaire et al., 2006; Kojima et al., 2004). Thus, the same chemical may be able to interact in vivo with different endocrine targets and generate alteration in regulatory pathways with associated physiological consequences. Second, because humans are continuously exposed to multiple chemical agents that can potentially act as endocrine disrupters and that are capable of accumulating in the organism. Hence, because of possible interactive action between different chemicals and on different endocrine targets, assessment of *in vivo* endocrine disrupting effects of the pesticides is needed in order to conclude on their potential risk for human health.

5. Conclusion

In this study fenbuconazole was identified as a new inhibitor of human aromatase activity. In addition, we found out new inducers of aromatase activity, namely aldrin, chlordane, cypermethrine, methyl-parathion, endosulfan, methoxychlor, oxadiazon and metolachlor. The observed induction was linked to a transcriptional activation by four out of ten pesticides, while for the six others, post-transcriptional regulation might be involved. However, due to the complexity of the aromatase regulation at different levels of gene expression, the diversity of the chemicals tested and the specificity of the cellular model, further work is still to be done in order to elucidate the mechanisms of aromatase activation by toxicants in these cells. Nonetheless, this study provides

support for the use of JEG-3 cells for the assessment of both inhibition and induction of aromatase by pesticides. Moreover, when compared with the different data reported in literature, our results emphasise the view that tissue-specific up-regulation of aromatase activity must be taken into account for the choice of the cell-based model to be used in the *in vitro* assessment of potential aromatase disruption in human. Finally, because several of these pesticides are currently used in many agricultural applications and are increasingly found in surface waters, the present study, together with other recent publications on the endocrine effects of pesticides (Andersen et al., 2002; Kojima et al., 2004; Lemaire et al., 2005; Lemaire et al., 2006), strengthens the view that these chemicals may contribute, at least in part, to the occurrence of reproductive adverse effects in both human and aquatic organisms.

Acnowledgements

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Table 1. Summary of the studied pesticides according to the chemical classes, their use and published data on their ability to modulate aromatase activity by using *in vitro* assays.

Chemicals	Chemical class[a]	CAS N°	Use[b]	Reported effect on aromatase activity in microsome assays[c]	Reported effect on aromatase activity in cell based assays[c]	Effect on aromatase activity in our study[c]	Effect on aromatase mRNA in our study[c]
Atrazine	AZO	1912-24-9	I	n.e.[d]	↑[h], n.e.[f]	↑	n.e.
Tributyltin (TBT)	OM	1461-22-9	AF	n.e.[d]	n.e.[f], ↑[g], ↓[i]	↑	↑
Methoxychlor	OC	72-43-5	I / A	n.e.[d]	n.e.[f]	↑	↑
Endosulfan	OC	115-29-7	I / A	n.e.[d], ↓[e]	↓[f]	↑	n.e.
Aldrin	OC	309-00-2	I	n.e.[d]	↓[f]	↑	↑
Cypermethrine	PYRE	67375-30-8	I / A	n.e.[d]	↓[f]	↑	n.e.
Chlordane	OC	57-74-9	I / A	n.e.[d]	-	↑	↑
Parathion methyl	DIC	298-00-0	I /A	n.e.[d]	-	↑	n.e.
Oxadiazon	OXA	19666-30-9	H	n.e.[d]	-	↑	n.e.
Metolachlor	OC	51218-45-2	I	n.e.[d]	-	↑	n.e.
Fenarimol	PYR	60168-88-9	F	↓[d,e]	↓[h,f,g]	↓	n.d.
Prochloraz	AZO	67747-09-5	F	↓[d,e]	↓[h]	↓	n.d.
Propiconazole	AZO	262-104-4	F	↓[d,e]	↓[h,f]	↓	n.d.
Heptachlor	OC	76-44-8	I	n.e.[d,e]	↑[f]	↓	n.d.
Fenbuconazole	AZO	114369-43-6	F	↓[d]	-	↓	n.d.
Toxaphene	OC	8001-35-2	I / A	-	-	↓	n.d.
Benomyl	CAR	17804-35-2	A / F / N	n.e.[d]	↑[f]	n.e.	n.d.
Vinclozolin	DIC	50471-44-8	F	n.e.[d,e]	↑[h], n.e.[f]	n.e.	n.d.
*o,p'*DDT	OC	50-29-3	I / A	n.e.[d]	n.e.[f]	n.e.	n.d.
Mecoprop	OC	16484-77-8	H	n.e.[d]	-	n.e.	n.d.
Aminotriazole	AZO	61-82-5	H	n.e.[d]	-	n.e.	n.d.
Diuron	PHE	330-54	H	n.e.[d,e]	-	n.e.	n.d.
Isoproturon	PHE	34123-59-6	H	n.e.[d]	-	n.e.	n.d.
Fipronil	PRZ	120068-37-3	I	n.e.[d]	-	n.e.	n.d.
Bupirimate	PYR	41483-43-6	F	n.e.[d]	-	n.e.	n.d.
Permethrine	PYRE	52645-53-1	I / A	n.e.[d]	-	n.e.	n.d.
Azimsulfuron	SULF	120162-55-2	H	n.e.[d]	-	n.e.	n.d.
Pretilachlor	OC	51218-49-6	H	-	-	n.e.	n.d.
Zinc Pyrithione	OM	1121-31-9	F/AF	-	-	n.e.	n.d.
Sodium Arsenite	-	7784-46-5	F	-	-	n.e.	n.d.

(a) AZO : azole ; CAR : carbamate ; DIC : Dicarboximide ; OC: organochlorine ; OM : Organometal ; OP : Organophosphorus ; OXA : oxadiazole ; PHE : phenylurea ; PRZ : pyrazole ; PYR : pyrimidine ; PYRE : Pyrethroid ; SULF: Sulfonylurea ; *(b)* A : acaricide, F: fungicide, H: herbicide, I: insecticide, AF: antifouling ; *(c)* n.e. : no effect, ↑ : aromatase induction, ↓ : aromatase inhibition, - : no data could be found in the literature, n.d. : not determined ; *(d)* in rainbow trout brain and ovary microsomes (Hinfray et al. submitted) ; *(e)* in human placental microsomes (Andersen et al., 2002; Vinggaard et al., 2000) ; *(f)* in KGN cells (Morinaga et al., 2004; Ohno et al., 2004) ; *(g)* in JEG-3 cells (Nakanishi et al., 2002; Vinggaard et al., 2000); *(h)* in H295R cells (Sanderson et al., 2002) ; *(i)* in KGN cells (Saitoh et al., 2001).

Figure legends

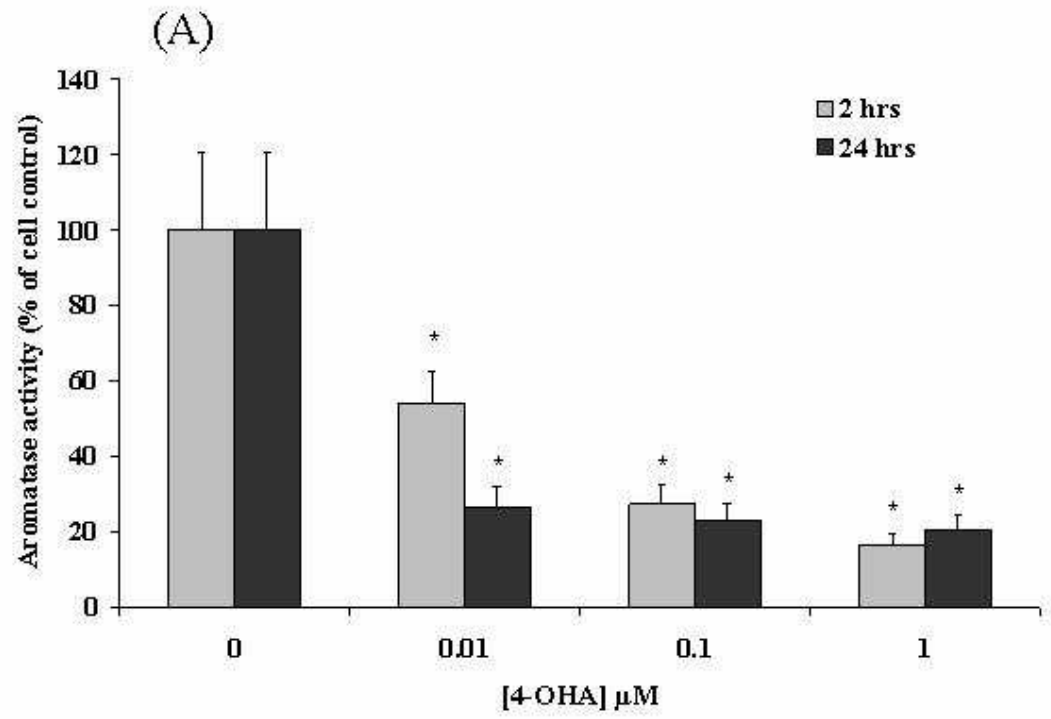
Fig.1. Effects of model compounds on aromatase expression in JEG-3 cells. (A) Time-and concentration-dependant inhibition of aromatase activity by 4-OHA. (B) Induction of enzymatic activity and mRNA expression by forskolin, TTNPB, and CD3106 after a 24-hr exposure. Values are means of triplicates ± SD. * : statistically different values from solvent control ($p < 0.05$).

Fig.2. Pesticides as inhibitors of aromatase activity in JEG-3 cells (A) after 2 h of exposure with radiolabelled androstenedione, and, (B) after 24 h of exposure prior to the aromatase assay. Values are means of triplicates ± SD. * : statistically different values from solvent control ($p < 0.05$).

Fig.3. Pesticides as inducing agents of aromatase activity (A) after 24 h of exposure or (B) after both 2 and 24 h of exposure to TBT. Values are means of triplicates ± SD. * : statistically different values from solvent control ($p < 0.05$).

Fig.4. Effects of pesticides found to induce aromatase activity on the expression of the *cyp19* mRNA in JEG-3 cells after 24 h of exposure. All chemicals were tested at 10 µM except TTNPB (1µM) and tributyltin (3 nM). Values are means of triplicates ± SD and are representative of three independent experiments. * indicates a statistically significant induction as compared to control cells ($p < 0.05$).

Fig.5. Concentration-response curve of RAR-dependant luciferase activity in JEG-3 cells stably transfected with the RAREx3-TK-Luciferase gene (JEG-3-RLN). JEG-3-RLN were exposed for 16 hours to several concentrations of the RAR agonist TTNPB or of the RAR antagonist CD3106 either alone or in combination with TTNPB 10 nM. Each value represents the mean of triplicate of exposure ± SD.


(A)
Aromatase activity (% of cell control)
140
120
100
80
60
40
20
0
2 hrs
24 hrs
*
0
0.01
0.1
1
[4-OHA] µM

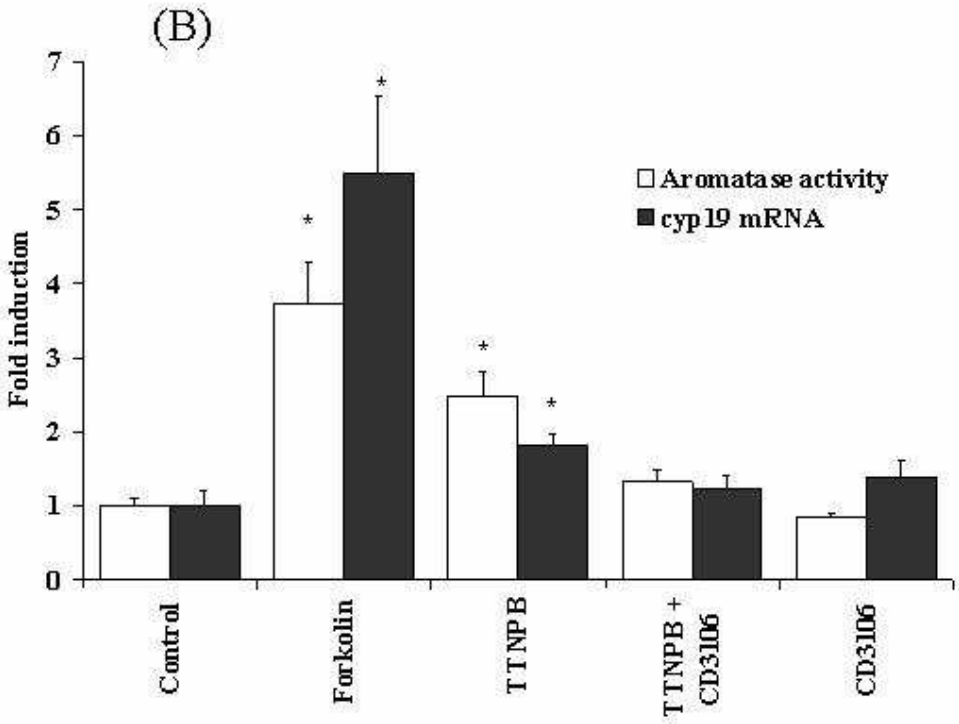

(B)
Fold induction
7
6
5
4
3
2
1
0
Aromatase activity
cyp19 mRNA
*
Control
Forkolin
TTNPB
TTNPB + CD3106
CD3106

Fig. 1

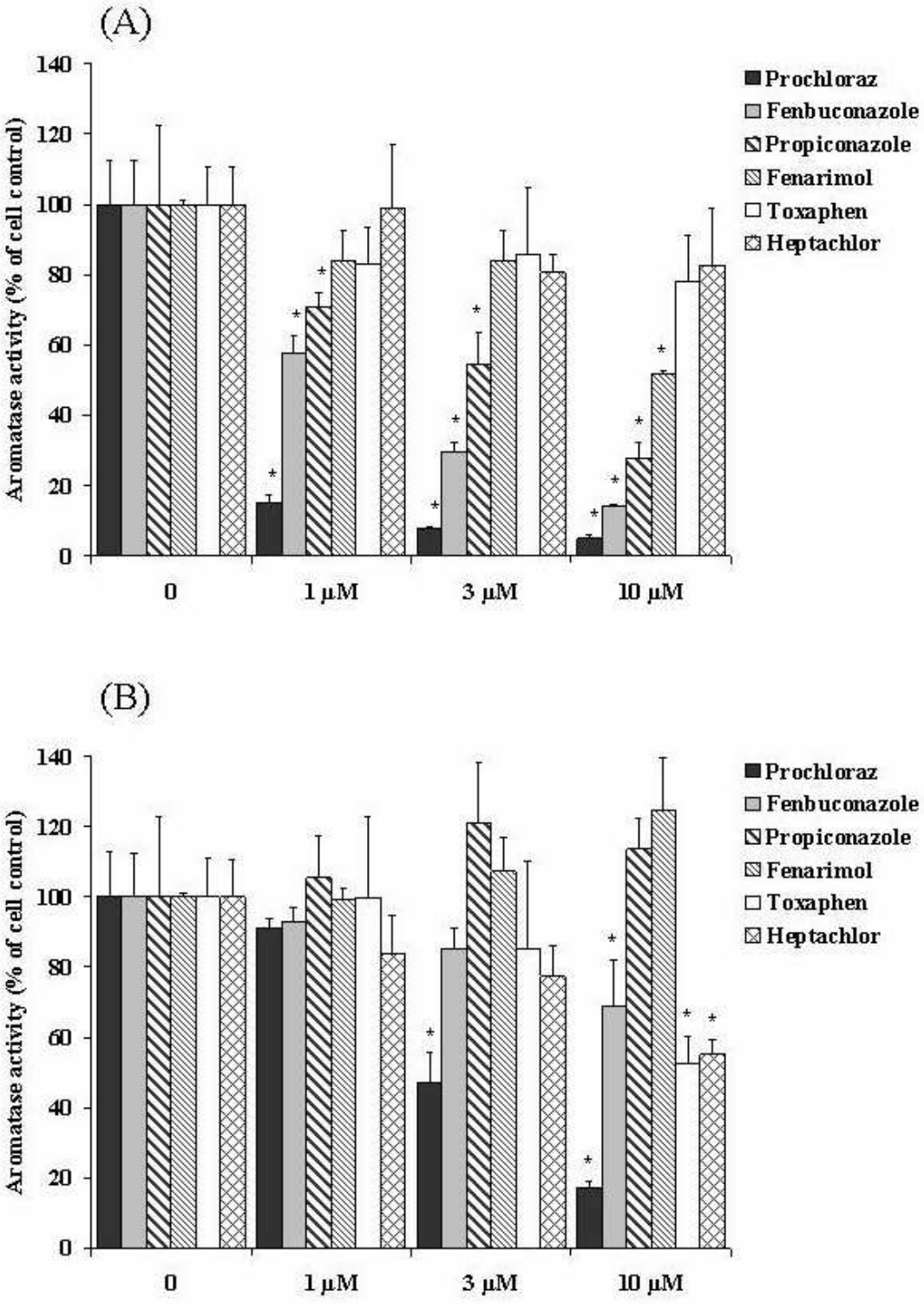

(A)
Aromatase activity (% of cell control)
140
120
100
80
60
40
20
0
0
1 µM
3 µM
10 µM
Prochloraz
Fenbuconazole
Propiconazole
Fenarimol
Toxaphen
Heptachlor
(B)
Aromatase activity (% of cell control)
140
120
100
80
60
40
20
0
0
1 µM
3 µM
10 µM
Prochloraz
Fenbuconazole
Propiconazole
Fenarimol
Toxaphen
Heptachlor

Fig. 2

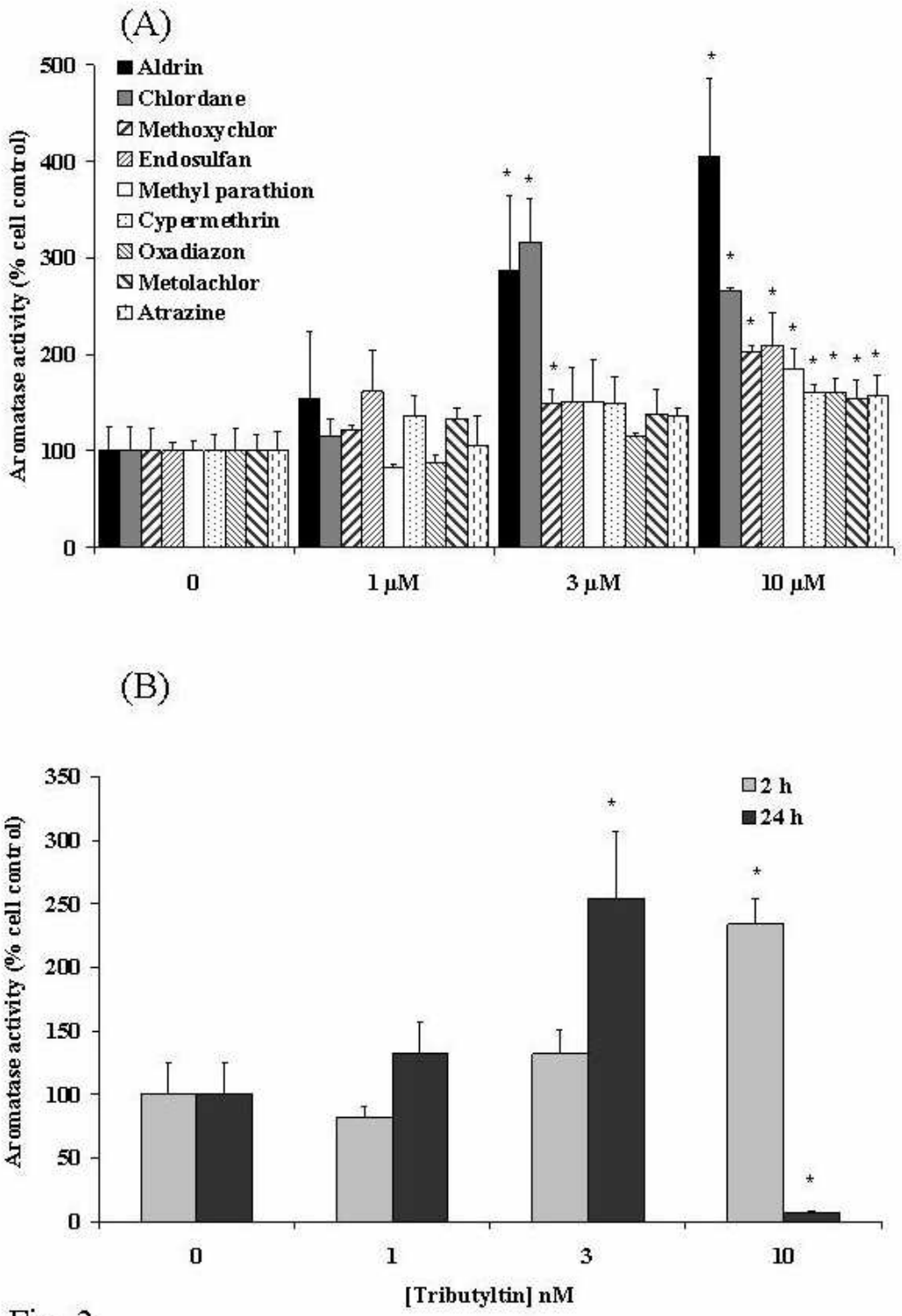

(A)
Aromatase activity (% cell control)
500
400
300
200
100
0
Aldrin
Chlordane
Methoxychlor
Endosulfan
Methyl parathion
Cypermethrin
Oxadiazon
Metolachlor
Atrazine
0
1 µM
3 µM
10 µM
(B)
Aromatase activity (% cell control)
350
300
250
200
150
100
50
0
2 h
24 h
0
1
3
10
[Tributyltin] nM

Fig. 3

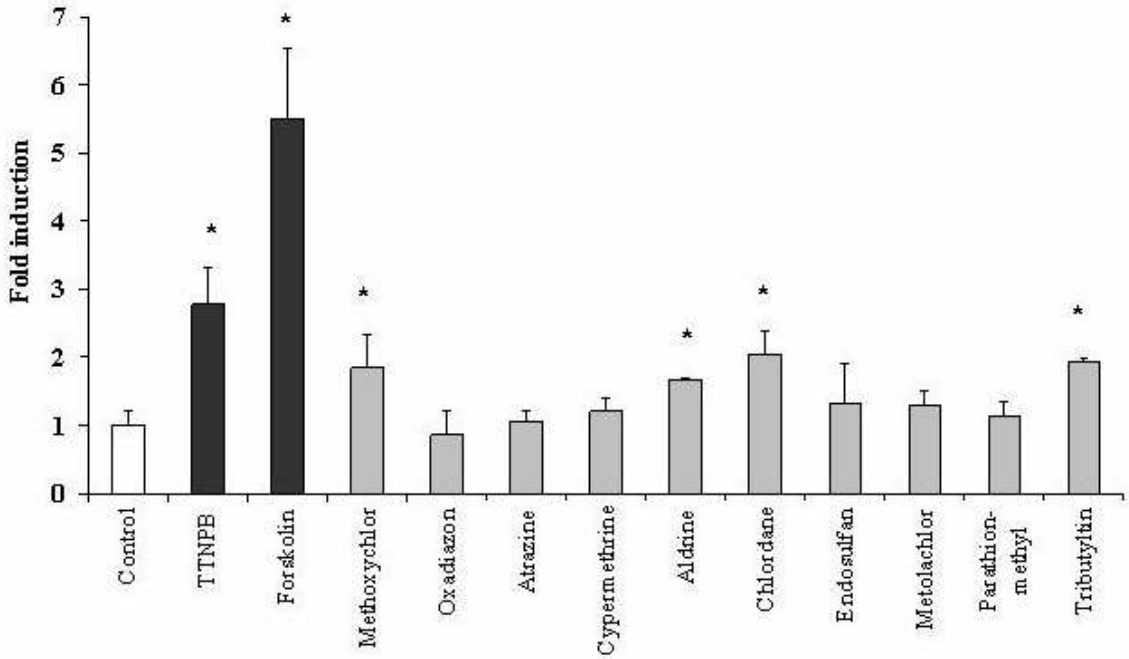

Fold induction
0
1
2
3
4
5
6
7
*
*
*
*
*
*
Control
TTNPB
Forskolin
Methoxychlor
Oxadiazon
Atrazine
Cypermethrine
Aldrine
Chlordane
Endosulfan
Metolachlor
Parathion-methyl
Tributyltin

Fig. 4

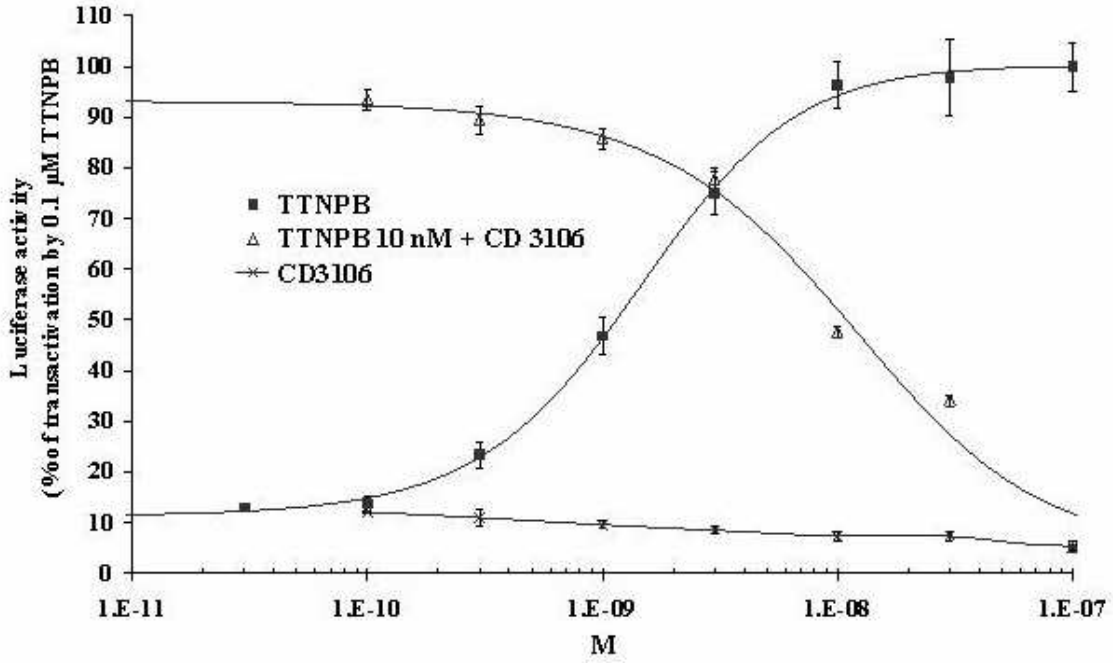

Luciferase activity
(% of transactivation by 0.1 µM TTNPB
110
100
90
80
70
60
50
40
30
20
10
0
TTNPB
TTNPB 10 nM + CD 3106
CD3106
1.E-11
1.E-10
1.E-09
1.E-08
1.E-07
M

Fig. 5