

Inhibition of aromatase activity by methyl sulfonyl PCB metabolites in primary culture of human mammary fibroblasts

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Introduction

Methyl sulfonyl PCB metabolites (MeSO₂-PCBs) are persistent contaminants and are ubiquitously present in humans and the environment. Lipophilicity of MeSO₂- PCB metabolites is similar to the parent compounds and they have been detected in human milk, adipose, liver and lung tissue¹. 4-MeSO₂-PCB-149 is the most abundant PCB metabolite in human adipose tissue and milk at a level of 1.5 ng/g lipids². Human blood concentration of 4-MeSO₂-PCB-149 is approximately 0.03 nM^{2,3}. 3-MeSO₂-PCB-101 is the predominant PCB metabolite in muscle and blubber in wildlife, such as otter, mink and grey seal². In the environment, they have been linked to chronic and reproductive toxicity in exposed mink⁴. Additionally, some MeSO₂-PCBs have been shown to be glucocorticoid receptor (GR) antagonists⁵.

Since approximately 60% of all breast tumors are estrogen responsive^{6,7}, exposure to compounds that are able to alter estrogen synthesis through interference with the aromatase enzyme, can lead to changes in estrogen levels and possibly to accelerated or inhibit breast tumor growth. Therefore, it is important to identify exogenous compounds that can alter aromatase activity in addition to those compounds which have direct interaction with the estrogen receptor (ER). Aromatase (CYP19) comprises the ubiquitous flavoprotein, NADPH-cytochrome P450 reductase, and a unique cytochrome P450 that is exclusively expressed in estrogen producing cells⁸. Previous studies have revealed that expression of the aromatase gene is regulated in a species- and tissue specific

manner^{7,9,10}. In healthy breast tissue, the predominantly active aromatase promoter region I.4 is regulated by glucocorticoids and class I cytokines⁸.

Therefore, it is important to investigate possible aromatase inhibiting properties of MeSO₂-PCBs (as anti glucocorticoids?) in relevant human tissues. We used primary human mammary fibroblasts because of their role in breast cancer development. We compared the results in primary fibroblasts with results that were obtained when using the human adrenocortiocarcinoma cell line H295R. Previous studies proved these cells to be a suitable tool for studying inhibitory effects of xenobiotics on aromatase activity¹¹. The aim of this study was to investigate effects on aromatase by MeSO₂-PCB exposure and elucidate a possible mechanism of action.

Methods

Isolation of fibroblasts from human mammary tissue Fibroblast isolation was performed according to Van de Ven and co-workers¹², with modifications. Healthy mammary tissue was collected after informed consent, from women undergoing reduction mammoplasty in the St. Antonius Hospital in Nieuwegein, The Netherlands. Tissue collection was approved by the Medical Ethical Committee (TME/Z-02.09) of the St. Antonius Hospital. Fibroblasts were isolated and cultured in RPMI 1640, supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 1 mg/ml insulin until use.

Cell culture and exposure All exposures were performed at 37°C, 5% CO₂ for 24h. Fibroblasts were cultured and subsequently exposed to test compounds in 6-well plates. The human adrenocortiocarcinoma cell line H295R¹³ was obtained from the American Type Culture Collection (ATCC # CRL-2128) and grown in 1:1 Dulbecco's modified Eagle medium/Ham's F-12 nutrient mix (DMEM/F12) containing 365 mg/ml l-glutamine and 15 mM HEPES. The culture medium was supplemented with 10 mg/l insulin, 6.7 µg/l sodium selenite and 5.5 mg/l transferrin (ITS-G), 1.25 mg/ml bovine serum albumin, 100 U/l penicillin/100 µg/l streptomycin and 2% steroid-free replacement serum Ultrosor SF. Experiments with H295R cells were performed in 24-well plates.

³H-water release assay for aromatase activity Catalytic activity of aromatase was determined by performing a ³H-water release assay, after Lephart and Simpson^{14,15} with modifications¹⁵. An assay based on the method of Lowry^{16,17} for protein determination was performed to correct for differences in cell numbers between the wells.

Promoter-specific mRNA levels RNA was isolated from fibroblasts using the RNA Instapure System (Eurogentec, Liège, Belgium) Specific primers, designed by Bouraïma^{11,18}, for aromatase promoter region I.4 were used. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using the Access RT-PCR System (Promega, Madison, WI, USA). Annealing temperature was 60°C, extension lasted 1 minute and 30 cycles were performed. MgSO₄-concentration was 1mM. Amplification product of 475 bp was detected using 2% agarose gel electrophoresis and ethidium bromide staining. Intensity of the stains was quantified using a FluorImager (Molecular Dynamics, USA).

Test for cell viability Mitochondrial function as an indicator for cell viability, was assessed by measuring the capacity of human primary fibroblasts to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan¹⁹ after 24h exposure to test compound.

Data analysis For graphs and plotting of dose-response curves, Prism 3.0 (GraphPad Software Inc., San Diego, CA, USA) was used. All error bars are presented as standard error of the mean (S.E.M.). To identify statistically significant differences among means, a one-way ANOVA followed by Dunnett's posteriori-test were performed.

Results

Exposure of primary human fibroblasts for 24h to 0, 1, 3, or 10 μM of 3-MeSO₂-PCB-132, 4-MeSO₂-PCB-132, 4-MeSO₂-PCB-149, or 4-MeSO₂-PCB-91 each resulted in concentration-dependent decrease of aromatase activity (from 100% to approximately 20%) (Fig. 1).

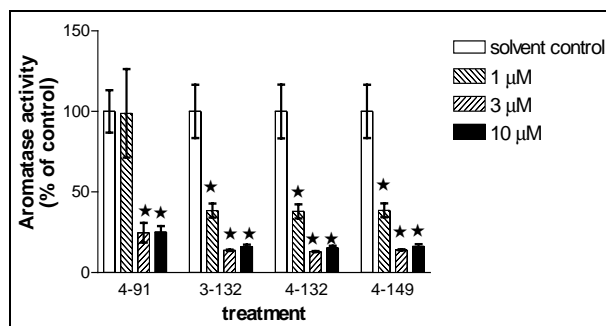


Fig. 1: Aromatase activity in primary human fibroblasts after 24h exposure to 0, 1, 3 or 10 μM of 3-MeSO₂-PCB-132, 4-MeSO₂-PCB-132, 4-MeSO₂-PCB-149, 4-MeSO₂-PCB-91. Error bars are standard errors. Bars represent average of triplicate measurements. (*) Significantly different from control ($p < 0.01$, except for 4-91: $p < 0.05$).

For 3-MeSO₂-PCB-132, 4-MeSO₂-PCB-132, and 4-MeSO₂-PCB-149, estimated IC₅₀ values were < 1 μM . For 4-MeSO₂-PCB-91, the estimated IC₅₀ value was between 1 and 3 μM . Cytotoxicity was not observed at concentrations up to 10 μM (data not shown). When primary fibroblasts were exposed to different concentrations of the MeSO₂-PCBs just for the limited duration of the assay (6 hours), aromatase enzyme activity also decreased concentration dependently from 100% to 20% (Fig. 2).

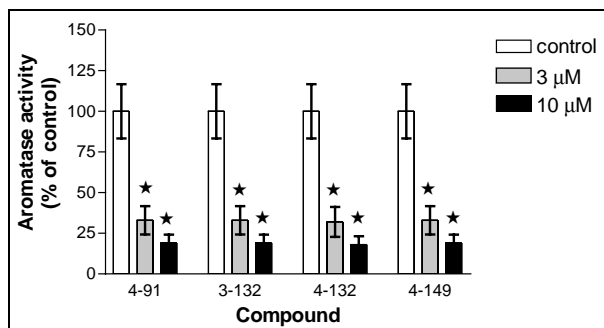


Fig. 2: Aromatase activity in primary human fibroblasts after 6h exposure to 0, 3 or 10 μM of 3-MeSO₂-PCB -132, 4-MeSO₂-PCB -132, 4-MeSO₂-PCB -149, 4-MeSO₂-PCB -91. Error bars are standard errors. Bars represent average of triplicate measurements. (*) Significantly different from control ($p < 0.05$).

To check whether aromatase inhibition by MeSO₂-PCBs was (partly) caused by down regulation of gene expression, fibroblasts were exposed to test compounds and promoter I.4-specific mRNA levels were assessed. Exposure to solvent control, 10 μM of 3-MeSO₂-PCB-132, 4-MeSO₂-PCB-132, 4-MeSO₂-PCB-149, or 4-MeSO₂-PCB-91, did not alter levels of aromatase promoter I.4-specific RNA transcripts, which were elevated by simultaneous exposure to 30 nM DEX (EC₅₀ value) (Fig. 3).

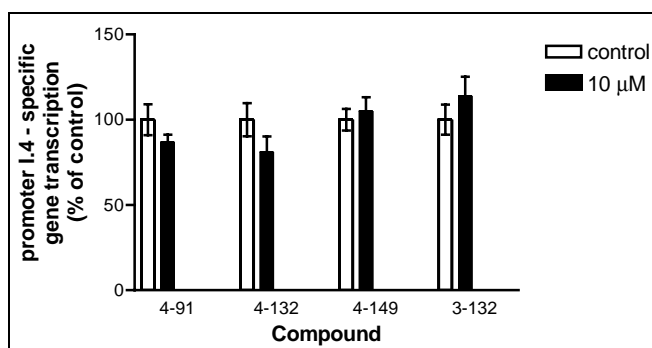


Fig 3: Aromatase promoter I.4-specific gene transcription in primary human fibroblasts after 24h exposure to EC₅₀ of dexamethasone and 0 or 10 μM of 3-MeSO₂-PCB -132, 4-MeSO₂-PCB -132, 4-MeSO₂-PCB -149, or 4-MeSO₂-PCB -91. Error bars are standard errors. Bars represent average of triplicate measurements.

DEX-treated H295R cells were also exposed to different concentrations of 3-MeSO₂-CB -132, 4-MeSO₂-CB -132, 4-MeSO₂-CB -149 or 4-MeSO₂-CB -91, which resulted in concentration-dependent inhibition of aromatase activity (Fig. 4). EC₅₀ values were 0.9, 2, 1.8 and 0.6 μ M, respectively. Cytotoxicity was not observed at concentrations up to 10 μ M (data not shown).

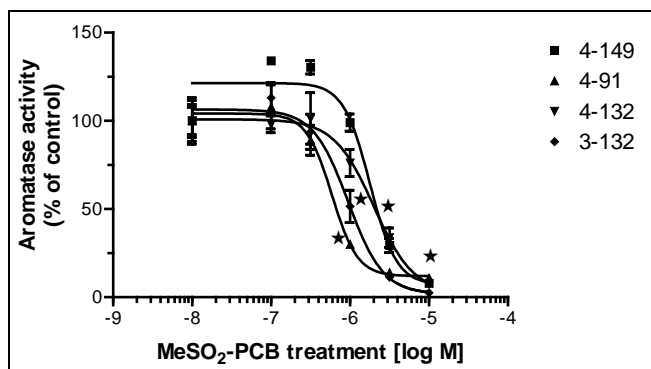


Fig 4: Aromatase activity in DEX-treated H295R cells after 24h exposure to different concentrations of 3-MeSO₂-PCB -132, 4-MeSO₂-PCB -132, 4-MeSO₂-PCB -149, 4-MeSO₂-PCB -91. Error bars are standard errors. Points represent averages of triplicate measurements. (*) Significantly different from control ($p < 0.01$).

Discussion

When primary human fibroblasts were exposed to MeSO₂-PCBs, a statistically significant decrease of basal aromatase activity was observed. A decrease in enzyme activity can be caused by direct catalytic inhibition of the enzyme and/or down regulation of gene expression. To test for catalytic inhibition, primary human fibroblasts were exposed to MeSO₂-PCBs for the duration of the aromatase assay only and a decrease of aromatase activity was observed. Because exposure of primary human fibroblasts was only for 6h, and no effects on the RNA level were observed after 24h exposure, little to no effect on RNA synthesis is likely to have taken place. This indicates a direct effect of the MeSO₂-PCBs on the aromatase enzyme. Letcher et al.²⁰ showed a decrease of aromatase activity in JEG-3 and JAR cells after exposure to several organochlorines. However, this was due to cytotoxicity. This was not the case in primary human fibroblasts. Some MeSO₂-PCBs

have affinity for the GR and are able to interfere with the glucocorticoid signaling pathway⁵. Therefore, we expected the MeSO₂-PCBs to inhibit aromatase activity in primary human fibroblasts due to their antiglucocorticoid properties and subsequent ability to downregulate promoter I.4 regulated aromatase activity. As a positive control we used the synthetic GR-antagonist RU486. The concentration-dependent decrease of aromatase promoter I.4-specific mRNA levels after exposure to the EC₅₀ of DEX and different concentrations of RU486 (data not shown), indicates that decrease of aromatase activity through downregulation of CYP19 mRNA is possible in these cells. However, exposure of fibroblasts to the EC₅₀ of DEX and different concentrations of MeSO₂-PCBs, did not result in any significant changes in aromatase promoter I.4-specific mRNA levels, indicating that interaction with the GR does not play a role in aromatase inhibition by MeSO₂-PCBs in primary human mammary fibroblasts.

The human adrenocortiocarcinoma cell line H295R has been shown to be a suitable tool for studying inhibitory effects on aromatase activity^{11,21}. Therefore, we compared the results obtained in primary human mammary fibroblasts with those obtained in H295R cells. 100 nM DEX also increased aromatase activity in H295R cells¹¹, although to a lesser extent than in primary fibroblasts. When DEX-treated H295R cells were exposed to different concentrations of MeSO₂-PCBs, a concentration-dependent decrease of aromatase activity was observed. IC₅₀ values were comparable in H295R cells and primary fibroblasts. The pharmacological agent Letrozole, decreases aromatase activity with 80 %²². To obtain a similar effect on aromatase activity, a MeSO₂-PCB-exposure of approximately 10 µM is needed. In human blood, 0.03 nM 4-MeSO₂-PCB-149 was detected^{2,3}. This means that there is a difference of 5 orders of magnitude between human body burden and pharmacologically active concentrations. Therefore, a biologically significant effect of MeSO₂-PCB exposure on aromatase activity is not expected. Looking at MeSO₂-metabolites of different PCB congeners, quite large differences in affinity for the GR are observed (Table 1). Johansson showed a weak GR-binding at MeSO₂-PCB concentrations of approximately 10 µM⁵. Our studies indicated cytotoxicity at concentrations larger than 10 µM. The IC₅₀ values for aromatase inhibition in both fibroblasts and H295R cells are lower than the IC₂₅ for GR-binding. This confirms our observations that aromatase activity can be inhibited without GR-binding and subsequent decrease of aromatase mRNA synthesis. Furthermore, an increase in affinity for the GR was not reflected in an increase in aromatase inhibition, suggesting that receptor binding does not always imply biologically significant agonism or antagonism resulting in

physiological changes. The lack of decrease of mRNA levels after exposure to MeSO₂-PCBs, suggesting a GR-independent mechanism leads to our overall conclusion that the decrease in aromatase activity caused by MeSO₂-PCBs is mainly due to catalytic inhibition.

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