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

Martin van den Berg[1], Marjoke Heneweer[1], Maarten de Geest[1], Paul de Jong[2], Ake Bergman[3], Thomas Sanderson[1]

[1]Institute for Risk Assessment Sciences and Utrecht (NL)
[2]St. Antonius Hospital and Nieuwegein (NL)
[3]Stockholm University, Stockholm (S)

Introduction

Methyl sulfonyl PCB metabolites ($MeSO_2$-PCBs) are persistent contaminants and are ubiquitously present in humans and the environment. Lipophilicity of $MeSO_2$- PCB metabolites is similar to the parent compounds and they have been detected in human milk, adipose, liver and lung tissue[1]. 4-$MeSO_2$-PCB-149 is the most abundant PCB metabolite in human adipose tissue and milk at a level of 1.5 ng/g lipids[2]. Human blood concentration of 4-$MeSO_2$-PCB-149 is approximately 0.03 nM[2,3]. 3- $MeSO_2$-PCB-101 is the predominant PCB metabolite in muscle and blubber in wildlife, such as otter, mink and grey seal[2]. In the environment, they have been linked to chronic and reproductive toxicity in exposed mink[4]. Additionaly, some $MeSO_2$-PCBs have been shown to be glucocorticoid receptor (GR) antagonists[5].

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 cells[8]. 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 cytokines[8].

Therefore, it is important to investigate possible aromatase inhibiting properties of $MeSO_2$-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 adrenocorticocarcinoma cell line H295R. Previous studies proved these cells to be a suitable tool for studying inhibitory effects of xenobiotics on aromatase activity[11]. The aim of this study was to investigate effects on aromatase by $MeSO_2$-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-workers[12], 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% CO_2 for 24h. Fibroblasts were cultured and subsequently exposed to test compounds in 6-well plates. The human adrenocorticocarcinoma cell line H295R[13] 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 Ultroser SF. Experiments with H295R cells were performed in 24-well plates.

^{3}H-water release assay for aromatase activity Catalytic activity of aromatase was determined by performing a ^{3}H-water release assay, after Lephart and Simpson[14,15] with modifications[15]. 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. $MgSO_4$-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 formazan[19] 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-$MeSO_2$-PCB-132, 4-$MeSO_2$-PCB-132, 4-$MeSO_2$-PCB-149, or 4-$MeSO_2$-PCB–91 each resulted in concentration-dependent decrease of aromatase activity (from 100% to approximately 20%) (Fig. 1).

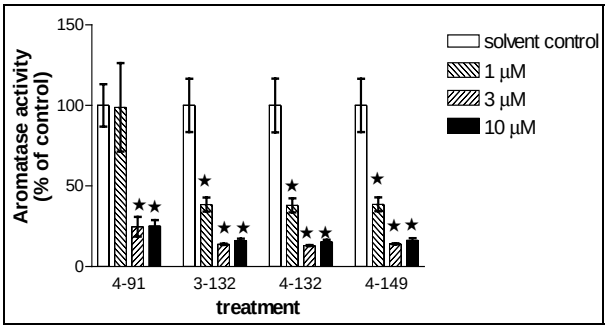

Fig. 1: Aromatase activity in primary human fibroblasts after 24h exposure to 0, 1, 3 or 10 µM of 3-$MeSO_2$-PCB -132, 4-$MeSO_2$-PCB -132, 4-$MeSO_2$-PCB -149, 4-$MeSO_2$-PCB -91. Error bars are standard errors. Bars represent average of triplicate measurements. (*) Significantly different from control ($p < 0.01$, except for 4-91: $p < 0.05$).

For 3-$MeSO_2$-PCB-132, 4-$MeSO_2$-PCB-132, and 4-$MeSO_2$-PCB-149, estimated IC_{50} values were < 1 µM. For 4-$MeSO_2$-PCB–91, the estimated IC_{50} 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 $MeSO_2$-PCBs just for the limited duration of the assay (6 hours), aromatase enzyme activity also decreased concentration dependently from 100% to 20% (Fig. 2).

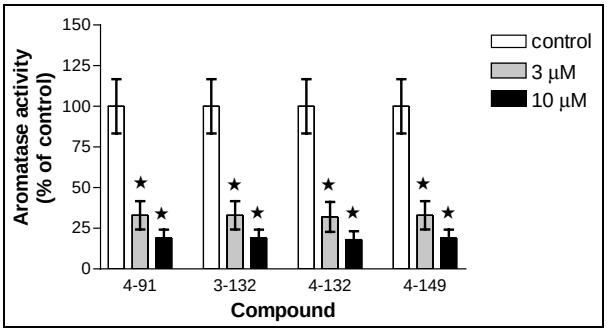

Fig. 2: Aromatase activity in primary human fibroblasts after 6h exposure to 0, 3 or 10 μM of 3-$MeSO_2$-PCB -132, 4-$MeSO_2$-PCB -132, 4-$MeSO_2$-PCB -149, 4-$MeSO_2$-PCB -91. Error bars are standard errors. Bars represent average of triplicate measurements. (*) Significantly different from control ($p < 0.05$).

To check whether aromatase inhibition by $MeSO_2$-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-$MeSO_2$-PCB-132, 4-$MeSO_2$-PCB-132, 4-$MeSO_2$-PCB-149, or 4-$MeSO_2$-PCB–91, did not alter levels of aromatase promoter I.4-specific RNA transcripts, which were elevated by simultanous exposure to 30 nM DEX (EC_{50} value) (Fig. 3).

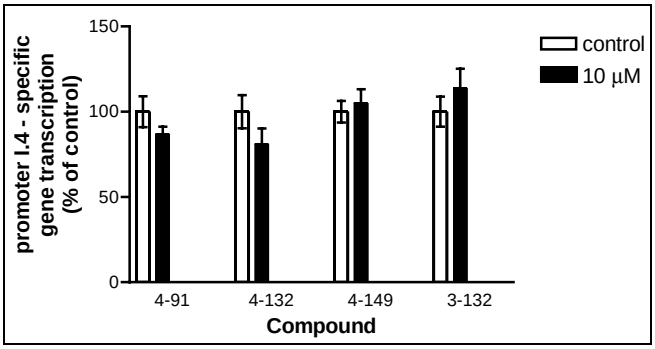

Fig 3: Aromatase promoter I.4-specific gene transcription in primary human fibroblasts after 24h exposure to EC_{50} of dexamethasone and 0 or 10 μM of 3-$MeSO_2$-PCB -132, 4-$MeSO_2$-PCB -132, 4-$MeSO_2$-PCB -149, or 4-$MeSO_2$-PCB -91. Error bars are standard errors. Bars represent average of triplicate measurements.

DEX-treated H295R cells were also exposed to different concentrations of 3-$MeSO_2$-CB -132, 4-$MeSO_2$-CB -132, 4-$MeSO_2$-CB –149 or 4-$MeSO_2$-CB – 91, which resulted in concentration-dependent inhibition of aromatase activity (Fig. 4). EC_{50} values were 0.9, 2, 1.8 and 0.6 μM, respectively. Cytotoxicity was not observed at concentrations up to 10 μM (data not shown).

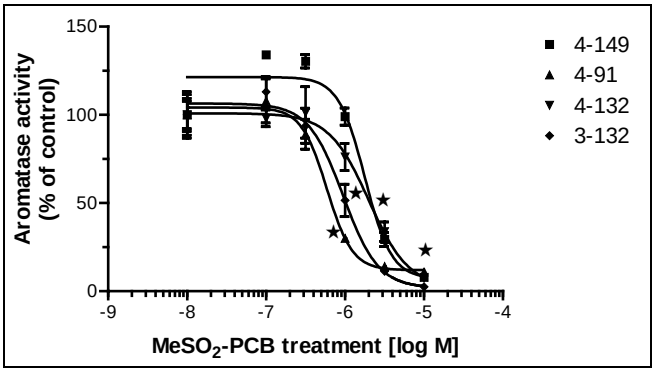

Fig 4: Aromatase activity in DEX-treated H295R cells after 24h exposure to different concentrations of 3-$MeSO_2$-PCB -132, 4-$MeSO_2$-PCB -132, 4-$MeSO_2$-PCB -149, 4-$MeSO_2$-PCB -91. Error bars are standard errors. Points represent averages of triplicate measurements. (*) Significantly different from control ($p < 0.01$).

Discussion

When primary human fibroblasts were exposed to $MeSO_2$-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 $MeSO_2$-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 $MeSO_2$- 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 $MeSO_2$-PCBs

have affinity for the GR and are able to interfere with the glucocorticoid signaling pathway[5]. Therefore, we expected the $MeSO_2$-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 EC_{50} 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 EC_{50} of DEX and different concentrations of $MeSO_2$-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 $MeSO_2$-PCBs in primary human mammary fibroblasts.

The human adrenocorticocarcinoma 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 cells[11], although to a lesser extent than in primary fibroblasts. When DEX-treated H295R cells were exposed to different concentrations of $MeSO_2$-PCBs, a concentration-dependent decrease of aromatase activity was observed. IC_{50} 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 $MeSO_2$-PCB-exposure of approximately 10 μM is needed. In human blood, 0.03 nM 4-$MeSO_2$-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 $MeSO_2$-PCB exposure on aromatase activity is not expected. Looking at $MeSO_2$-metabolites of different PCB congeners, quite large differences in affinity for the GR are observed (Table 1). Johansson showed a weak GR-binding at $MeSO_2$-PCB concentrations of approximately 10 μM[5]. Our studies indicated cytotoxicity at concentrations larger than 10 μM. The IC_{50} values for aromatase inhibition in both fibroblasts and H295R cells are lower than the IC_{25} 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 of antagonism resulting in

physiological changes. The lack of decrease of mRNA levels after exposure to $MeSO_2$-PCBs, suggesting a GR-independent mechanism leads to our overall conclusion that the decrease in aromatase activity caused by $MeSO_2$-PCBs is mainly due to catalytic inhibition.

References

1 Noren, K.; Lunden, A.; Pettersson, E.; Bergman, A. Environ Health Perspect 1996, 104, 766-772.

2 Weistrand, C.; Noren, K. Environ Health Perspect 1997, 105, 644-649.

3 Letcher, R. J.; Lemmen, J. G.; van der Burg, B.; Brouwer, A.; Bergman, A.; Giesy, J. P.; van den Berg, M. Toxicol Sci 2002, 69, 362-372.

4 Lund, B.-O.; Orberg, J.; Bergman, A.; Larsson, C.; Bergman, A.; Backlin, B.-M.; Hakansson, H.; Madej, A.; Brouwer, A.; Brunstrom, B. Environ Toxicol Chem 1999, 18, 292-298.

5 Johansson, M.; Nilsson, S.; Lund, B. O. Environ Health Perspect 1998, 106, 769-772.

6 Quinn, A. L.; Burak, W. E., Jr.; Brueggemeier, R. W. J Steroid Biochem Mol Biol 1999, 70, 249-256.

7 Zhou, D.; Zhou, C.; Chen, S. J. Steroid Biochem. Molec. Biol. 1997, 61, 273-280.

8 Kamat, A.; Hinshelwood, M. M.; Murry, B. A.; Mendelson, C. R. Trends Endocrinol Metab 2002, 13, 122-128.

9 Bulun, S. E.; Simpson, E. R. Breast Cancer Res Treat 1994, 30, 19-29.

10 Reed, M. J.; Purohit, A. Clin Endocrinol (Oxf) 2001, 54, 563-571.

11 Heneweer, M.; van den Berg, M.; Sanderson, J. T. Toxicol Lett 2004, 146, 183-194.

12 van de Ven, J.; Donker, G. H.; Sprong, M.; Blankenstein, M. A.; Thijssen, J. H. J Steroid Biochem Mol Biol 2002, 81, 237-247.

13 Gazdar, A. F.; Oie, H. K.; Shackleton, C. H.; Chen, T. R.; Triche, T. J.; Myers, C. E.; Chrousos, G. P.; Brennan, M. F.; Stein, C. A.; La Rocca, R. V. Cancer Res 1990, 50, 5488-5496.

14 Lephart, E. D.; Simpson, E. R. Methods Enzymol 1991, 206, 477-483.

15 Sanderson, J. T.; Seinen, W.; Giesy, J. P.; van den Berg, M. Toxicol Sci 2000, 54, 121-127.

16 Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. J. Biol. Chem 1951, 193, 265-275.

17 Rutten, A. A.; Falke, H. E.; Catsburg, J. F.; Topp, R.; Blaauboer, B. J.; van Holsteijn, I.; Doorn, L.; van Leeuwen, F. X. Arch Toxicol 1987, 61, 27-33.

18 Bouraima, H.; Lireux, B.; Mittre, H.; Benhaim, A.; Herrou, M.; Mahoudeau, J.; Guillon-Metz, F.; Kottler, M. L.; Reznik, Y. Eur J Endocrinol 2003, 148, 457-461.

19 Denizot, F.; Lang, R. J Immunol Methods 1986, 89, 271-277.

20 Letcher, R. J.; Van Holsteijn, I.; Drenth, H.-J.; Norstrom, R. J.; Bergman, A.; Safe, S.; Pieters, R.; Van den Berg, M. Toxicol Appl Pharmacol 1999, 160, 10-20.

21 Sanderson, J. T.; Boerma, J.; Lansbergen, G. W.; van den Berg, M. Toxicol Appl Pharmacol 2002, 182, 44-54.

22 Cohen, M. H.; Johnson, J. R.; Li, N.; Chen, G.; Pazdur, R. Clin Cancer Res 2002, 8, 665-669.