IN VITRO EFFECTS OF SELECTED BROMINATED FLAME RETARDANTS ON THE ADRENO CORTICAL ENZYME (CYP17)
A novel endocrine mechanism of action?

Rocio Fernandez Canton[1], Thomas Sanderson[1], Sandra Nijmeijer[1], Åke Berkman[2], Martin van den Berg[1]

[1]Institute for Risk Assessment Sciences, (IRAS), Utrecht University, Utrecht, The Netherlands
[2]Department of Environmental Chemistry and Analytical Chemistry, Stockholm University, Stockholm, Sweden

INTRODUCTION

Fire incidents have decreased over the last 20 years partly due to regulations requiring addition of flame retardants (FRs) to materials. These compounds can be divided into different chemical classes: inorganic, nitrogen, phosphorus and halogen containing flame retardants (usually brominated or chlorinated).
Not surprisingly, the use of brominated flame retardants (BFRs) in a variety of commercial and household products has increased over the years due to their low cost and high effectiveness.
Consequence of the high production of BFRs is that these compounds are now readily detectable in air, water, birds, fish, marine mammals, and in human adipose tissue and blood [1-4]
The five major BFRs are hexabromocyclododecane (HBCD), tetrabromobisphenol-A (TBBPA) and three commercial mixtures of polybrominated diphenyl ethers (PBDEs) (penta, octa, deca), which are extensively used as FRs at high production volume levels.
In addition, concentrations of PBDEs concentration have been rapidly increasing during the last 10 years in human breast milk from European and American women (Betts, 2002) and a number of endocrine (in vitro) effects have been reported [3, 5]. Consequently, the concern about BFRs and their metabolites with respect to their potential as endocrine disruptors (EDs) has been growing.
Studies in our laboratory are focused on potential interactions of a wide range of BFRs with sex hormone synthesis and metabolism. Previous results from our research group[6], showed inhibitory and inductive effects on aromatase (CYP19) (the key enzyme that converts androgens to estrogens) by certain BFRs, in particular the hydroxylated PBDEs and several bromophenols.
In the present study, the effects of ten of these BFRs on CYP17 activity were investigated. This enzyme also catalyzes an important step in the sex steroidogenesis and is responsible for the biosynthesis of dehydroepiandrosterone (DHEA). DHEA, produced in the adrenal gland, is the

most abundant sex steroid hormone in human blood and has been investigated for its relationship with anti-obesity, anti-tumor, anti-aging and anti-cancer effects [7, 8]. In these experiments we used the human adrenocortical carcinoma cell line (H295R cell line) to assess possible effects of these BFRs on CYP17 activity.

MATERIALS and METHODS

Cell culture conditions

H295R cells were obtained from the American Type Culture Collection (ATCC CRL-2128) and grown in culture under conditions published previously [9]. Wells (24-well plates) were seeded with 1 ml of cell suspension per well. The culture medium was changed 24h after seeding, during which time the cells attached to the plate and reached almost confluence, after which the cells were exposed to the test chemicals. These were added to the wells at various concentrations using 1 µl of stock solutions dissolved in DMSO.

Chemicals

In this study, cells were exposed to the following BFRs: tetrabromobisphenol-A (TBPPA), 2,4,6-tribromophenol (TBP), 6OH-2,2',4,4' tetraBDE47 (6-hydroxy- tetrabrominated diphenyl ether), HBCDD (hexabromocyclododecane-D) and the polybrominated diphenyl ethers (PBDEs) numbers 47, 49, 99, 100, 183 and 209. The compounds were synthesized at the Wallenberg Laboratory (Stockholm University, Sweden) and received at concentrations of 2.5mM. Concentrations tested ranged from from 0.01µM up to 10µM to study possible inhibition or induction of CYP17 in these cells

Enzymatic activity (CYP17) assay

After addition of 0.1uM Pregnenolone (precursor) the catalytic activity of CYP17 was determined in exposed H295R cells based on the production of its product dehydroepiandrosterone (DHEA), which was measured using a RIA kit (Radioimmunoassay #IM1138, Immunotech, Bechman Coulter Company). In order to measure CYP17 activity without interference by metabolism of pregnenolone, to mineralo- and glucocorticoids, the enzyme 3β-hydroxysteroid dehydrogenase was simultaneously blocked with trilostane (1 µM). SU 10863 (1µM) was used as a positive control for CYP17 inhibition.

MTT assay

Cell viability, as an indicator of cytotoxicity, was determined by measuring the capacity of H295R cells to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan [10]. MTT is reduced to the blue-colored formazan by the mitochondrial enzyme succinate dehydrogenase, which is considered a sensitive measure of mitochondrial function. In short, the cells in each well on the 24-well plate were incubated for 30 min, at 37˚C with 0.5 ml of MTT (1mg/ml) dissolved in culture medium without serum. Then, the MTT solution was removed, after which the cells were washed twice with PBS. The formazan formed in the cells was extracted by adding 1 ml of isopropanol and incubation for 10 min at room temperature. The isopropanol fraction was measured spectrophotometrically at 595 nm (FLUOstar Galaxy V4.30-0/ Stacker Control V1.02-0, BMG Labtechnologies).

Statistical analysis
All experiments were done in duplicate and every concentration was tested in quadruplicate. All results are presented as means with their standard deviations.

RESULTS and DISCUSSION

Very little information is available about possible interaction of xenobiotics with CYP17. This in spite of the fact that this enzyme plays a major role in steroidogenesis by synthesizing weak androgens in the adrenal gland.

Ten different brominated flame retardants (BFRs) were studied in the H295R adrenocarcinoma cell line to find potential interactions with CYP17. In order to study this interaction, a new method was developed that allowed experiments in small in vitro systems. BFRs were added to the cell culture at concentrations ranging from 0.01μM to 10 μM and possible inhibitory or inductive effects on CYP17 activity were measured after 24h of exposure.

HBCDD, BDE99 and BDE100 showed significant inhibition of CYP17 activity but these effects were seen only at the highest concentration (10 μM) with a 50% decrease of the control activity. At these concentrations no signs of cytotoxicity were observed in the cells.
A strong inhibition of CYP17 was found when the cells were treated with the major hydroxy metabolite of BDE47 (6OH-BDE47), causing a concentration dependent decrease of almost 90% at 10 μM. Previous studies also showed that this metabolite decreased aromatase activity by 95% at 7.5 μM, while analysis of cytotoxicity (MTT) showed a 40% decrease in cell viability at 7.5 μM. This indicates that inhibition of both CYP19 and CYP17 may, at least partly, be caused by cytotoxicity of this compound [6].

A maximum of two-fold induction of CYP17 activity was found if H295R cells were exposed for 24 h to the phenolic compounds TBP and TBBPA. TBBPA was a potent inducer of CYP17 activity, causing in 50% induction at the lowest concentration tested (0.01μM). In the case of TBP, a higher concentration (10 μM) of this compound was needed to induce CYP17 activity to a similar level . As with the observed effects on aromatase, a phenolic structure with bromine atoms adjacent to the hydroxy group was identified as necessary for possible interactions with CYP17. Our recent results show a possible new mechanistic pathway through which some endocrine disruptors, such as certain BFRs, may interfere with the endocrine system. However, further studies are necessary to establish if present concentrations in humans and wildlife are high enough to exert interactions with CYP17 or CYP19.

FIRE nr	Compound (10 uM)	Induction CYP 17 (% of control)	Inhibition CYP 17 (% of control)
1	BDE-47	n.e.	n.e.
2	BDE-49	n.e.	n.e.
3	BDE-99	n.e.	< 50%
4	BDE-100	n.e.	< 50%
5	BDE-183	n.e.	n.e.
6	BDE-209	n.e.	n.e.
7	Tribromophenol	> 200%	n.e.
	TBBPA	> 200%	n.e.
9	HBCDD	n.e.	< 50%
10	6OH-BDE-47	n.e.	< 10%

Table 1. The effects of BFRs on in vitro CYP17 enzymatic activity H295R cells. Relative induction values were calculated by comparing CYP17 values to the control (DMSO 100% activity) at 10 µM.
n.e. = no effect

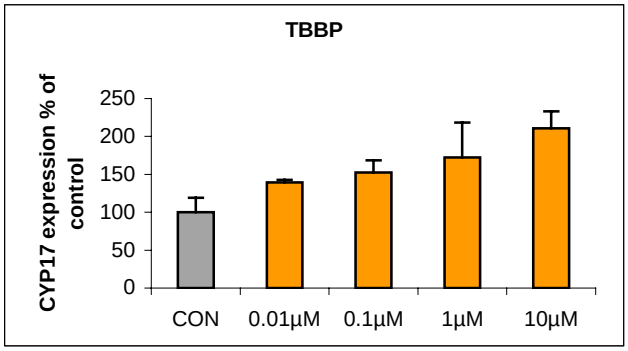

Figure 1. Induction of CYP17 after 24h. exposure to different concentrations of TBBP - A.

ACKNOWLEDGMENTS
This work was supported financially by European Union project (FIRE) number QLRT-2001-00596

REFERENCES

1. Hites, R.A., *Polybrominated diphenyl ethers in the environment and in people: a meta-analysis of concentrations.* Environ Sci Technol, 2004. 38(4): p. 945-56.
2. de Wit, C.A., *An overview of brominated flame retardants in the environment.* Chemosphere, 2002. 46(5): p. 583-624.
3. Darnerud, P.O., et al., *Polybrominated diphenyl ethers: occurrence, dietary exposure, and toxicology.* Environ Health Perspect, 2001. 109 Suppl 1: p. 49-68.
4. Meironyte Guvenius, D., A. Bergman, and K. Noren, *Polybrominated diphenyl ethers in Swedish human liver and adipose tissue.* Arch Environ Contam Toxicol, 2001. 40(4): p. 564-70.
5. Cordle, F., *The use of epidemiology and clinical toxicology to assess environmental health problems.* Regul Toxicol Pharmacol, 1984. 4(3): p. 236-48.
6. Rocío F.Cantón et al., *Effects of brominated flame retardants on activity of the steroidogenic enzyme aromatase (CYP19) in H295R human adrenocortical carcinoma cells in culture*. Proceedings of 23rd International Symposium on Halogenated Environmental Organic Pollutants and POPs, Vol 61, p.104-6 (Boston 2003)
7. Labrie, F., et al., Endocrine and intracrine sources of androgens in women: inh*ibition of breast cancer and other roles of androgens and their precursor dehydroepiandrosterone.* Endocr Rev, 2003. 24(2): p. 152-82.
8. Ciolino, H., et al., *Dehydroepiandrosterone inhibits the expression of carcinogen-activating enzymes in vivo.* Int J Cancer, 2003. 105(3): p. 321-5.
9. Sanderson, J.T., et al., *2-Chloro-s-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity?* Toxicol Sci, 2000. 54(1): p. 121-7.
10. Denizot, F. and R. Lang, *Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability.* J Immunol Methods, 1986. 89(2): p. 271-7.