IN VIVO STEROIDOGENIC EFFECTS OF SEVERAL BROMINATED FLAME RETARDANTS IN WISTAR RATS

Cantón RF[1], Nijmeijer S[1], Daamen F[1], van der Ven L[2], van den Berg M[1].

[1]Institute for Risk Assessment Sciences (IRAS), PO Box 80.176, NL-3508 TD Utrecht, the Netherlands. [2]National Institute for Public Health and the Environment (RIVM), PO Box 1, 3720 BA Bilthoven, The Netherlands.

Abstract
The five major BFRs are hexabromocyclododecane (HBCD), tetrabromobisphenol-A (TBBPA) and three commercial mixtures of polybrominated diphenylethers (PBDEs) (penta-, octa-, deca-BDE), which are extensively used as flame retardants (FRs). Concentrations of PBDEs have been rapidly increasing during the last decennia in the environment and wildlife, including e.g. human breast milk. In addition, a number of endocrine (mainly *in vitro*) effects have been reported of these compounds. *In vivo* effects of some of these BFRs (penta- and deca-BDE mixtures), TBBPA and HBCD on the production of sex hormones (androgens and estrogens) were studied in Wistar rats after 28 d of oral exposure. Ovary and adrenal tissues were used for respectively CYP19 and CYP17 enzyme activity measurements. Effects on steroidogenic enzymes from these *in vivo* studies were conducted within the framework of the European project FIRE.

Introduction
Brominated flame retardants (BFRs) are chemicals used in many synthetic materials to prevent fire ignition processes. From an environmental and toxicological point of view BFRs have become an important group of organohalogen compounds, which include among others polybrominated diphenylethers (PBDEs; commercial penta-, octa- and deca-BDE mixtures), tetrabromobisphenol A (TBBPA) and hexabromocyclododecane (HBCD). Some BFR compounds were already found in the environment as long as 25 years ago, but nowadays these compounds can be found globally in many biota with a higher preference for lipid-rich organisms, e.g. fatty fish and marine mammals [1-3].
Concentrations of these BFRs in different environmental and biological samples (e.g. house dust or human milk) have been increasing during the last decades, although recent studies suggest that PBDE levels decrease slowly in some parts of the industrialized world [4]. This slow decline is probably related to the European ban and voluntary withdrawal from the North American market of the penta- and octa-BDE mixture, during the last years. *In vitro* studies have shown that certain BFRs, like PBDEs, can affect thyroid hormone homeostasis by acting as potent competitors of T_4 for binding to human thyroid hormone transport protein (TTR, transthyretin) [5]. In addition, some PBDEs or their metabolites have also shown in vitro interactions with the estrogen receptor α and steroidogenesis [6]. In this study effects on stereodogenic enzymes are reported from the *in vivo rat* studies that were conducted within the framework of the EU project FIRE (28d repeated oral dose studies with HBCD, TBBPA and two commercial mixtures pentaBDE and decaBDE).

Materials and Methods
Animals. Wistar rats were purchased from Harlan (Horst, NL), or bred at the RIVM facilities. All four BFRs were industrial formulations and donated by the Bromine Scientific and Environmental Forum (Brussels, Belgium). The commercial pentaBDE mixture was active carbon purified from brominated dioxin-like compounds prior to use in the animal experiments. The compounds were tested in 28d repeated oral dose toxicity studies (OECD407 protocol) as described earlier by Van der Ven et al, 2006 [7]. Briefly, exposure started after at least one week of acclimatization with 8 to 12 week old rats. Dose

ranges varied between 3-3000 mg per kg body weight for TBBPA, 0.3-200 mg/kg for HBCD, 0.27-200 mg/kg for the purified penta-BDE mixture and 1.87-60 mg/kg for deca-BDE. For precise assessment of dose-response relationships, the animals were distributed among eight dose groups (including control). This setup enables benchmark (BMDL) calculations [8] i.e. the 5% lower confidence bound of the critical effect dose (CED) at a critical effect size (CES), which was defined at 10 % for most parameters.

Microsomal fraction. Microsomes were isolated from rat ovaries and adrenals and measured for CYP19 (ovary) and CYP17 (adrenal) activity. Tissues were weighed and homogenized in 10 volumes of TRIS-HCl buffer (TRIS-HCl 50mM; 1.15% KCl) using a potter Elvehjem device. After that, the tubes were centrifuged for 25 minutes at 15,000 rpm at 4°C. The supernatant was pipetted into a clean ultra-centrifuge tube and centrifuged for 1:15hr at 47,000 rpm at 4°C. Then, the supernatant was decanted and the pellet resuspended in a sucrose solution (0.25 M). After that, 3 µl of suspension in tubes was taken with 147 µl milliQ for protein measurement and the microsome suspension was frozen in aliquots (50/100 µl) at -70 °C and stored until further use.

CYP19 (aromatase) assay.The catalytic activity of aromatase was determined based on the tritiated water-release method of Lephart et al., [9]. The specificity of the aromatase assay based on the release of tritiated water was verified by measuring the production of estrone that is the aromatization product of androstenedione, using a 125I-labeled double-antibody radioimmunoassay kit (DSL-8700; ICN,Costa Mesa, CA), and by using 4-HA, an irreversible inhibitor of the catalytic activity of aromatase, to block the formation of tritiated water from 1ß-3H-androstenedione.

CYP17 enzymatic assay.After addition of 0.1uM Pregnenolone (precursor) the catalytic activity of CYP17 was determined in microsomes 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 of pregnenolone metabolism into mineral and glucocorticoids, the enzyme 3β-hydroxysteroid dehydrogenase was simultaneously blocked with Trilostane (1µM). SU 10863 (1µM) was used as a positive control for CYP17 inhibition[10].

Data analysis. To identify statistically significant differences among means, a one-way ANOVA was done using SPSS (12.0.1 for Windows) software. For graphs and plotting of dose-response curves, Prism 3.0 (GraphPad Software Inc., San Diego, CA, USA) was used.

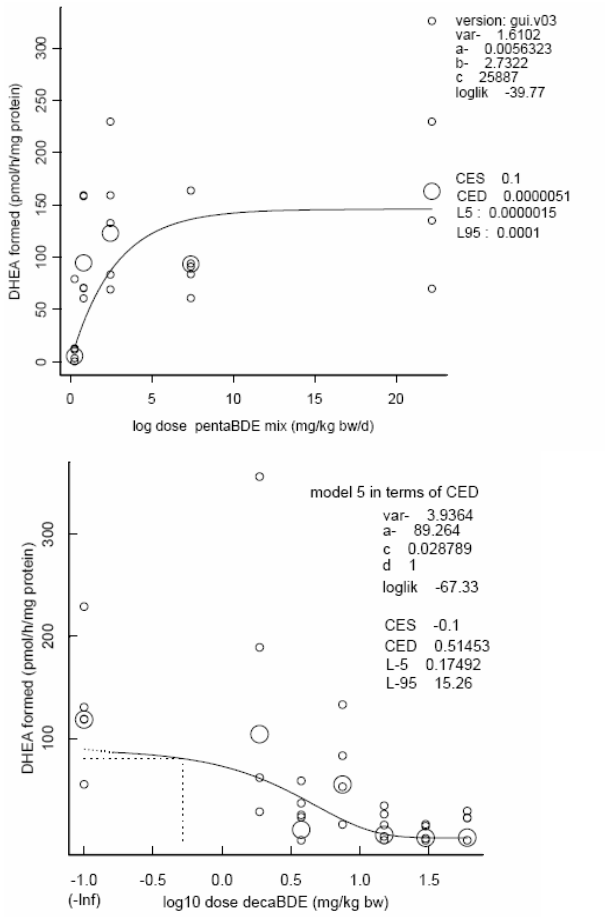
Results and Discussion

Uptake from the gastrointestinal tract was effective for all compounds, since hepatic concentrations of TBBPA, HBCD, penta and deca-BDE increased with dose levels in both males and females. Remarkably, in the case of HBCD, the compound was more persistent in females. In the ovaries of TBBPA exposed rats trend towards induction of CYP19 could be measured in all dose groups, but this increase was not statistically significant due to large individual variations. No changes in adrenal CYP17 activity were observed in the TBBPA exposed rats. The average aromatase activity in the ovaries of rats from the HBCD exposed groups (above 10 mg/kg bw) was on average higher compared to the control group, but again a statistical dose dependent increase could not be established due to high individual differences. Furthermore, the adrenal production of DHEA was increased in both females and males exposed to HBCD compared to the controls, although not statistically significant. However, this increase was not found at the highest dose level. These results might indicate that effects of HBCD on adrenal CYP17 activity could be compensated by a hormonal feed back mechanism at the higher dose level. A significant dose dependent increase of DHEA production by CYP17 could be measured in the female adrenals from the 28-days penta-BDE study (Figure. 1A). A concurring average increase in ovarian aromatase activity was also observed, although again not statistical significant. Even at the highest dose levels of decaBDE no effects on CYP19 activity were seen the in ovaries, but CYP17

activities were decreased statistically significant in the female adrenals (Figure. 1B). In view of the often high individual variation for the above steroidogenic effects based on administered dose, the CYP17 effects by pentaBDE (and decaBDE) were also analyzed based on internal hepatic concentrations. These analyses indeed confirmed an inducting effect of pentaBDE and inhibiting effect of decaBDE on adrenal CYP17 activity as the more sensitive effects of these compounds in rat. From our results we can also conclude that in general female rats seem to be more sensitive than males to BFRs with respect to effects on steroidogenesis. So far, most of the information regarding interaction of BFRs and sex steroidogenesis has been obtained with *in vitro* experiments, but these results are actually the first to report interaction in the *in vivo* situation. As the interactions between of BFRs and especially CYP17 were observed at lower dose levels our results might have implications for risk assessment of these compounds.

Figure. 1 The effects of pentaBDE (A) mixture and decaBDE (B) on adrenal CYP17 in the rat after 28 days oral exposure.

Figure. 1A



Acknowledgements

This work described in this paper was fully supported by FIRE European project with a contract number QLRT-2001-00596.

References:

1. de Wit, C.A., *An overview of brominated flame retardants in the environment.* Chemosphere, 2002. **46**(5): p. 583-624.
2. Law, R.J., et al., *Levels and trends of polybrominated diphenylethers and other brominated flame retardants in wildlife.* Environ Int, 2003. **29**(6): p. 757-70.
3. 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.
4. Sellstrom, U., et al., *Temporal trend studies on tetra- and pentabrominated diphenyl ethers and hexabromocyclododecane in guillemot egg from the Baltic Sea.* Environ Sci Technol, 2003. **37**(24): p. 5496-501.
5. Meerts, I.A., et al., *Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin in vitro.* Toxicol Sci, 2000. **56**(1): p. 95-104.
6. Olsen, C.M., et al., *Brominated phenols: characterization of estrogen-like activity in the human breast cancer cell-line MCF-7.* Toxicol Lett, 2002. **129**(1-2): p. 55-63.
7. van der Ven, L.T., et al., *A 28-day oral dose toxicity study enhanced to detect endocrine effects of hexabromocyclododecane in Wistar rats.* Toxicol Sci, 2006. **94**(2): p. 281-92.
8. Slob, W., *Dose-response modeling of continuous endpoints.* Toxicol Sci, 2002. **66**(2): p. 298-312.
9. Lephart, E.D. and E.R. Simpson, *Assay of aromatase activity.* Methods Enzymol, 1991. **206**: p. 477-83.
10. Canton, R.F., et al., *In vitro effects of brominated flame retardants and metabolites on CYP17 catalytic activity: a novel mechanism of action?* Toxicol Appl Pharmacol, 2006. **216**(2): p. 274-81.