Comparison of exposure-effect pathways to improve the assessment of human health risks of (hydroxylated) PCBs and brominated flame retardants

J. Legler[1], C.J.K. Buitenhuis[1], P.H. Cenijn[1], A. C. Gutleb[1], M van Velzen[1], T. Malmberg[2], Å. Bergman[2], A. Brouwer[1]

[1]Institute for Environmental Studies, Vrije Universiteit, Amsterdam, The Netherlands
[2]Department of Environmental Chemistry, Stockholm University, Sweden

Introduction

The European Union-financed project "Comparison of Exposure-Effect Pathways to Improve the Assessment of Human Health Risks of Complex Environmental Mixtures of Organohalogens (COMPARE)" started in January 2001 and will conclude in September 2004 (see also http://www.compare-project.info/). The main objective of the COMPARE project is to investigate comparative pathways of early life-stage exposure and long-term effects for several classes of organohalogens, including the polychlorinated biphenyls (PCBs) and the flame retardants polybrominated bisphenols and –diphenylethers, and their hydroxylated metabolites. Ultimately this research will provide a mechanism-based approach for the assessment of human health risks from exposure to organohalogens with a main focus on their phenolic metabolites.

The specific aims of the COMPARE project are:

1. To synthesize, identify and characterize different classes of organohalogens and their major hydroxylated metabolites in human blood plasma
2. To study and compare the maternal-to-fetal transfer kinetics (rodents, birds), the role of specific hormone binding proteins (e.g. transthyretin) as facilitated transport routes, and the comparative long-term adverse developmental (reproductive and behavioural) effects of early life-stage exposure to some representative congeners of different classes of organohalogens
3. To develop and use (total) exposure and/or effect markers for organohalogens in human blood samples, and to study possible exposure-health effect relationships in adult human individuals (osteoporosis) and the possible neurobehavioral impact of early (fetal) exposure to phenolic organohalogens in human infants
4. To perform an integrated (laboratory animal and human epidemiological exposure and effect) and comparative (several classes of organohalogens and their phenolic metabolites) risk assessment for exposure to complex mixtures of environmental chemicals

Highlights of the results of various aims of the multi-partner COMPARE project will be presented (see also Weiss *et al*[1]). The research carried out at the Institute for Environmental Studies is highlighted below. The goal of our research is to determine the effects of prenatal exposure to (hydroxylated) organohalogens using *in vitro* and *in vivo* (rodent) models. Model test compounds to determine long-term adverse developmental effects have been selected based on their presence in human plasma and the environment,

and/or their high volume use in Europe. Due to their prevalence in human plasma, the hydroxylated PCB metabolites 4-OH-CB107 and 4-OH-CB187, as well as 6-OH-BDE47 and 2,4,6- tribromophenol, were selected as test compounds. BDE 47 has been included as a test compound due to its relatively high levels in the environment and biota, whereas tetrabromobisphenol A (TBBPA) was selected as a model high volume production BFR. The potency of these compounds to disrupt endocrine systems was tested *in vitro*, using estrogen and dioxin-responsive reporter gene assays, as well as measurement of competitive binding to the thyroxine (T4) plasma binding protein transthyretin (TTR). The *in vivo* studies involved prenatal exposure of rats to test compounds during critical stages of gonadal development and were focussed on low dose effects. Several endpoints were investigated, including endocrine (thyroid and sex steroid hormones) effects, developmental landmarks, sexual and neurobehavioural development. Blood plasma and tissue levels of test compounds were analysed to determine transplacental transfer of (hydroxylated) organohalogens.

Methods

In vitro assays

The *in vitro* CALUX® assays for dioxin-like compounds (DR) and for estrogen-like compounds (ER) were obtained from BioDetection Systems, Amsterdam, The Netherlands. The *in vitro* ER-CALUX® assay measures luciferase expression in stably transfected human T47D breast cancer cells expressing endogenous estrogen receptors[2]. The dioxin-responsive DR-CALUX® assay uses rat hepatoma cells expressing endogenous Ah receptors[3]. In both assays, cells are seeded in 96 well plates, incubated with test compounds for 24 hours and luciferase protein is assayed by lysing the cells, adding luciferin substrate, and measuring light photon production. The T4-TTR competitive binding assay, which measures the ability of the test compound to interfere with the natural hormone T4 binding to the plasma transport protein TTR[4]. Briefly, test compounds are incubated for 24 hours in a test tube containing ^{125}I labeled T4, non-radioactive T4 and purified human TTR. Test compounds bound to TTR (thereby displacing T4 from the protein) are separated from unbound compounds over a gel column. Radioactivity in the column eluate is measured and indicates the TTR binding capacity of the test compound.

In vivo assays

Pregnant Wistar rats were orally dosed with test compounds during gestation day 10-16 as described previously[5,6]. The test compounds were: Aroclor 1254 (positive control, 25 mg/kg/day); 4-OH-CB107 (0.1, 0.5 and 5 mg/kg/bw/day); 4-OH-CB187 (0.5 and 5 mg/kg/bw/ day); TBBPA (25 mg/kg/day); 2,4,6-tribromophenol (25 mg/kg/day); BDE 47 (20 mg/kg/day); and 6-OH-BDE 47 (5 mg/kg/day). Starting on postnatal day (PND) 1, offspring were scored for developmental landmarks including onset of hair grow, pinna detachment, bilateral eye opening, vaginal opening and balanopreputial separation. Thyroid hormone- and estrogen levels were measured a various time points. Offspring were subjected to several different neurobehavioural tests, like open field, catalepsy test, sweet preference and brain auditory evoked potentials. Female offspring were monitored daily for estrous cycle length during 3 different periods in the experiment. The first period was directly after vaginal opening (PND 30-68), the second between PND 150-170 and the last

between PND 210-230. Plasma concentrations of test compounds were measured in pooled plasma samples of F1 pups at PND4, and of individual F0 mothers following weaning at PND24, according to methods described elsewhere[7].

Results and Discussion

In vitro endocrine disrupting effects of test compounds

The potency of the COMPARE test compounds to bind to and transactivate estrogen and dioxin receptors was investigated using the ER- and DR-CALUX® assays. In the ER-CALUX® assay, BDE 47 was the only compound which showed estrogenic activity, albeit by concentrations exceeding 1000 nM. The BDE 47 metabolite 6-OH-BDE 47 showed clear dose-response related antagonism on the ER-mediated response (see also Hamers *et al*[8]). Of the compounds tested for dioxin-like activity, only 6-OH-BDE induced luciferase activity in the DR-CALUX® assay, with a potency of about 2000 times less than TCDD. The PCB metabolite 4-OH-PCB107 showed antagonistic effects on both the ER and DR-CALUX® responses.

In the TTR-binding competition assay, all compounds tested, with the exception of BDE 47, showed displacement of radioactive labeled thyroxin (T4) and binding to the thyroid hormone transport protein TTR. The hydroxylated PCBs were the most potent inhibitors of T4 binding to TTR (up to 10x more potent than T4). The flame retardant TBBPA and metabolite 2,4,6 tribromophenol, were also more potent relative (upt to three times) to T4 in binding. The hydroxylated BDE 6-OH-BDE47 was about 20 times less potent than T4. These results suggest that binding of these compounds to the plasma transport protein TTR may be an important mechanism of disruption of thyroid hormone homeostasis.

Effects of prenatal exposure to hydroxylated PCBs and BFRs

Measurement of test compounds in prenatally exposed rats showed clear placental transfer of hydroxylated PCBs and BFRs from mother to fetus (Figure 1). F0 females still contained elevated levels of test compounds in plasma following weaning.

The effects of prenatal exposure to the PCB metabolites are described elsewhere[6], whereas analyses of tissues obtained following BFR exposure are still ongoing. Briefly, no effects were observed on developmental landmarks following exposure to both (metabolites of) PCBs and BFRs, indicating low, non-teratogenic exposure doses. Exposure to hydroxylated PCBs resulted in a marked reduction in plasma total thyroxin in offspring at PND 4. Behavioural studies with the PCB metabolite-exposed offspring have indicated some neurodevelopmental effects, such as impaired habituation in the open field test.

Analysis of the estrous cycle of female offspring showed a prolongation the estrous cycle length in all the 4-OH-CB107 and the 0.5 mg/kg/day 4-OH-CB187 groups, only in the last period of estrous cycle monitoring. This suggests an aging effect of prenatal exposure to the hydroxylated PCB metabolite. We are currently determining if this phenomenon is also present in the BFR-exposed groups, and investigating possible mechanisms underlying this aging effect.

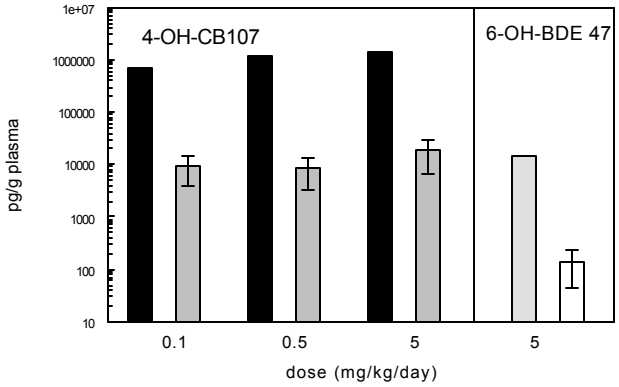

Figure 1. Plasma concentrations of 4-OH-CB107 and 6-OH-BDE 47 in rats following prenatal exposure (gestation day 10-16). Compounds were measured in pooled plasma samples of F1 pups (left bars) and in individual F0 mothers (n=3-4) (right bars).

Conclusions

The COMPARE project has resulted in a wealth of information about exposure, kinetics and effects of (metabolites of) PCBs and BFRs found in human plasma. This information is currently being integrated for risk assessment purposes. One important conclusion already evident is that for both the PCBs and BFRs analysed, transport to the fetal compartment both in humans and in animal models is apparent. Research in our laboratory with a rodent model has indicated that prenatal exposure to the PCB metabolites may result in profound effects later in life.

Acknowledgement

This study was financed by the European Union (Contract QLK4-CT2000-00261).

References

1. Weiss, J., Meijer, L., Sauer, P., Linderholm, L., Athanasiadis, I. and Bergman, Å. Third International Brominated Flame Retardants Conference, Toronto, Canada, 2004.
2. Legler, J., Van den Brink, C.E., Brouwer, A., Murk, A.J., Van der Saag, P.T., Vethaak, A.D., and Van der Burg, B. *Toxicol. Sciences*, 1999, 48:55-66.
3. Murk, A.J., Legler, J., Denison, M.S., Giesy, J.P., Van de Guchte, C. and Brouwer, A. *Fund. Appl. Toxicol.* 1996, 33:149-160.
4. Meerts, I.A.T.M., J.J. van Zanden, E.A.C. Luijks, I. van Leeuwen-Bol, G. Marsh, E. Jakobsson, Å. Bergman, and Brouwer, A. (1999). *Toxicol. Sciences.* 1999, 56(1): 95-104.
5. Meerts, I.A., Assink, Y., Cenijn, P.H., Van Den Berg, J.H., Weijers, B.M., Bergman, Å., Koeman, J.H., and Brouwer, A. *Toxicol Sci.* 2002, 68(2):361-7.
6. Buitenhuis, C.J.K., Cenijn, P., Bergman, Å., Gutleb, A.C., Legler, J. and Brouwer, A. Society of Toxicology 43rd Annual Meeting, Baltimore, U.S.A., 2004.
7. Hovander, L., Athanasiadou, M., Asplund, L., Jensen, S. and Klasson Wehler, E. *J. Anal. Toxicol.* 2000, *24*, 696-703.
8. Hamers, T., Kamstra, J.H., Sonneveld, E., Murk, A.J., Zegers, B.N., Boon, J.P., Andersson, P.L., and Brouwer, A. Third International Brominated Flame Retardants Conference, Toronto, Canada, 2004.