Risk of Endocrine Contaminants (RENCO) - Aims and a Summary of Initial Results

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Abstract

The full name of this EU project is "Assessment of Human Risk for Adverse Effects of Endocrine Active Environmental Organohalogen Contaminants" (ENV4-CT96-0170). It is aimed *to provide a scientific basis for assessment of risks for adverse health effects, with special emphasis on developmental effects in human infants, following background (environmental) exposure to endocrine active organohalogen substances (OHS).* The efforts will be concentrated on hydroxylated PCB and related phenolic organohalogens with a high fetal accumulation potential. The research program includes synthesis of "new" Organohalogen Substances (OHS), chemical characterization, X-ray crystallography of OHS and binding proteins, toxicological experimental studies and epidemiological studies in Sweden, Latvia and the Netherlands.

The results after one year of work are summarized: A large number of hydroxylated PCB congeners and polybrominated diphenyl ethers (PBDE) have been synthesized and characterized. Methods for reliable analysis of human plasma have been developed and analysis of PCB, DDT, DDE, hexachlorobenzene (HCB), hydroxy-PCB, pentachlorophenol (PCP) in human plasma samples of non-fish and fish consumers have been carried out. CALUX measurements have been performed on plasma samples. PCP concentrations are high but differ between Latvian and Swedish males. OH-PCB are present in similar concentrations as PCB in the two sampling groups. Competition studies of PBDE prior to and after in vitro metabolism for the T_4 binding to transtyretine (TTR) show that PBDE metabolites are strong competitors for the binding site on the protein. Initial studies on the crystal structure of PCP and tetrabromobisphenol A binding to TTR has been performed. A protocol has been agreed on by the RENCO working group for a prospective study in the Netherlands aiming at endocrine and neurodevelopmental effects of perinatal exposure to OHS in human infants.

ENDOCRINE DISRUPTERS

Introduction

The RENCO project is an interdisciplinary project for studies on potential hormonal effects of OHS. The project is in the initial phase concentrated on potential thyroidogenic effects but will also in a latter phase include potential sex hormone effects. The project may be described by the specific aims listed below:

- To develop a two-tiered method for *identification* of potential endocrine disruptors in environmental and human samples. This two-tiered method involves bioassays for analysis of endocrine activity and chemical analysis for structural identification of "unknown" compounds that affect endocrine activity in the bioassays (CALUX). The bioassays to be developed will be based on an aspect of the mechanism of action of endocrine active compounds. The chemical analysis will consist of gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS).
- To *synthesize* and chemically characterize "unknown" organohalogen compounds with structural resemblance to thyroid and sex hormones that are indicated by the bioassays as potential endocrine disruptor. Examples of organohalogens that show endocrine disrupting potential, or show structural resemblance to endocrine disruptors are: hydroxylated PCB, pentachlorophenol (PCP), tetrabromobisphenol A (TBBA) and hydroxylated polybrominated diphenyl ethers (hydroxy-PBDE). These synthesized compounds will be used as reference compounds for analytical purposes and as model compounds in experimental bioassays, X-ray crystallography and toxicological studies.
- To provide detailed information on *structural requirements* for endocrine disrupting effects; structure-activity relationships. This will be achieved by combining ligand-binding competition studies on some selected biomacromolecules (receptors, transport proteins) with X-ray crystallography studies for structural refinement of protein-ligand interaction. In addition, these protein-ligand interaction studies will be compared to functionality of the "putative" endocrine disrupting compounds on endocrine-mediated gene expression assay systems (CALUX).
- To study the *toxicological impact* of some model compounds on endocrine (thyroid, estrogenic) disruptive potential in conjunction with investigations on neurodevelopmental, reproduction and immunological functions in rat offspring born to mothers exposed to these model compounds during pregnancy. In addition, toxicokinetic studies on the model compounds will be included in these perinatal exposure studies. The data obtained should serve as a base to enable extrapolation from animal to man.
- To *measure in humans* the presence of endocrine active xenobiotics and their metabolites in human maternal and cord blood plasma in conjunction with analyses of thyroid and sex hormone levels. In addition, these blood measurements will be correlated with epidemiological and clinical data on birthweight, growth, physical landmarks, neurobehavioral, reproduction and immunological parameters. Also, identified endocrine active organohalogens and their metabolites will be quantified by chemical methods in plasma from adult males with high or with no consumption of contaminated fish, to establish a base of quantitative data from humans with varying exposure.

A summary of the results obtained within the frame of the RENCO project after one year of studies will be given.

Results and Discussion

Methoxylated PCB congeners, known to be retained in human plasma[1], have been prepared via methods previously described[2] and/or via new improved routes for synthesis. Since several of the hydroxy-CBs accumulated in plasma have the hydroxy group in a phenyl ring with a 4-hydroxy-2,3,5-tri- or 4-hydroxy-2,3,5,6-tetrachlorophenyl structure it is possible to prepare these OH-CBs as shown in Figure 1. Further, as many as 31 PBDE congeners have been prepared for the Renco project. The structures of all PBDE congeners synthesized so far and the methods used for their preparation are shown by Sjödin et al[3].

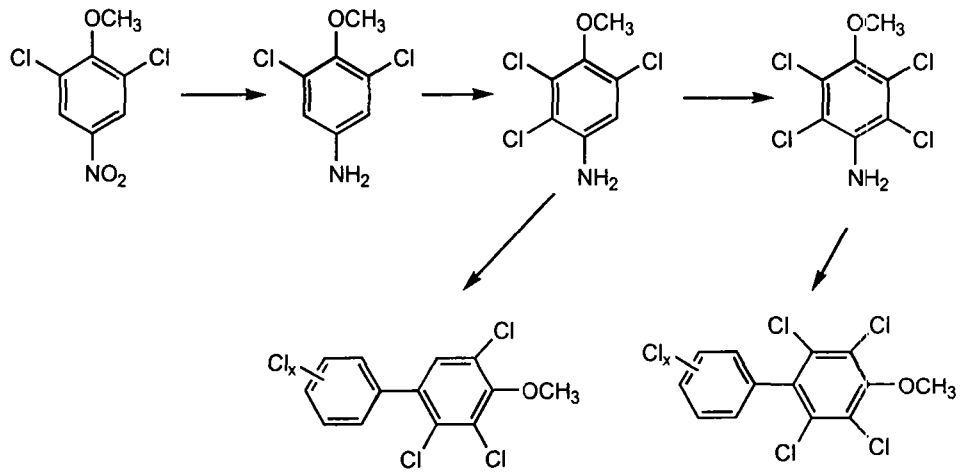


Figure 1. Pathway for the synthesis of certain *para*-substituted polychlorobiphenylols.

Chemical analysis of a pooled human plasma sample from a blood-donor central in Stockholm has shown the presence of more than 100 phenolic (hydroxylated) OHS [4]. Pentachlorophenol is the dominating compound in the plasma but an additional number of polychlorinated, polybrominated and mixed chlorinated and brominated phenols are also present. As many as 30 of the hydroxylated OHS present in the plasma are PCB metabolites. Tetrabromobisphenol A (TBBPA) have been indicated in the human plasma. Further details on this study is given at this symposium by Klasson Wehler[4]. The occurrence of all these phenolic type of compounds may be of endocrinological importance and toxicological studies are requested for determination of their thyroidogenic and sex hormone related activities.
Also, a number of polybrominated diphenyl ethers (PBDE), substances used as flame retardants, have been determined in human blood plasma. The concentrations are in the low ppb (ng/g lipid weight) range and at least two orders of magnitude lower than the concentration of PCB.

A previous report[1] on the presence and pattern of hydroxy-PCB in humans is confirmed in plasma samples from ca. 40 Swedish non-fish eaters and in fish eaters. The concentrations of the total hydroxy-PCB are only slightly lower than the concentration of PCB in the blood. The ratio of 4-hydroxy-2,3,3',4',5-tetraCB (4-OH-CB107), a major OH-PCB in the plasma, and

2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) is determined to be between 0.08 and 0.35 in their plasma. No relation between this ratio and fish consumption was observed in the Swedish material.

Blood samples were taken and analysed as part of an epidemiological study of non-fish eaters and fish eaters from Sweden and Latvia. The plasma concentrations of CB-153 was well correlated ($r^2 = 0.992$) to the total PCB concentrations, based on 18 PCB congeners. Higher PCB, hydroxy-PCB, DDE and hexachlorobenzene concentrations were detected in the Swedish males eating fish and the concentrations were correlated with fish intake and age. PCB and OH-PCB concentrations (ng/g fresh weight) versus age ar shown for fish eaters and non-fish eaters in Figure 2. The corresponding data for the Latvian cohort is not yet available. Non-fish consumers in Sweden had higher concentrations of PCP than the fishermen. Higher concentrations of PCP were determined in Swedish than in Latvian males. Interestingly no obvious age-related increase in the PCB or DDE concentrations were observed for the non-fish eaters. Correlation of OHS concentrations with endocrine related parameters of the blood are under way for both the Swedish and Latvian cohorts.

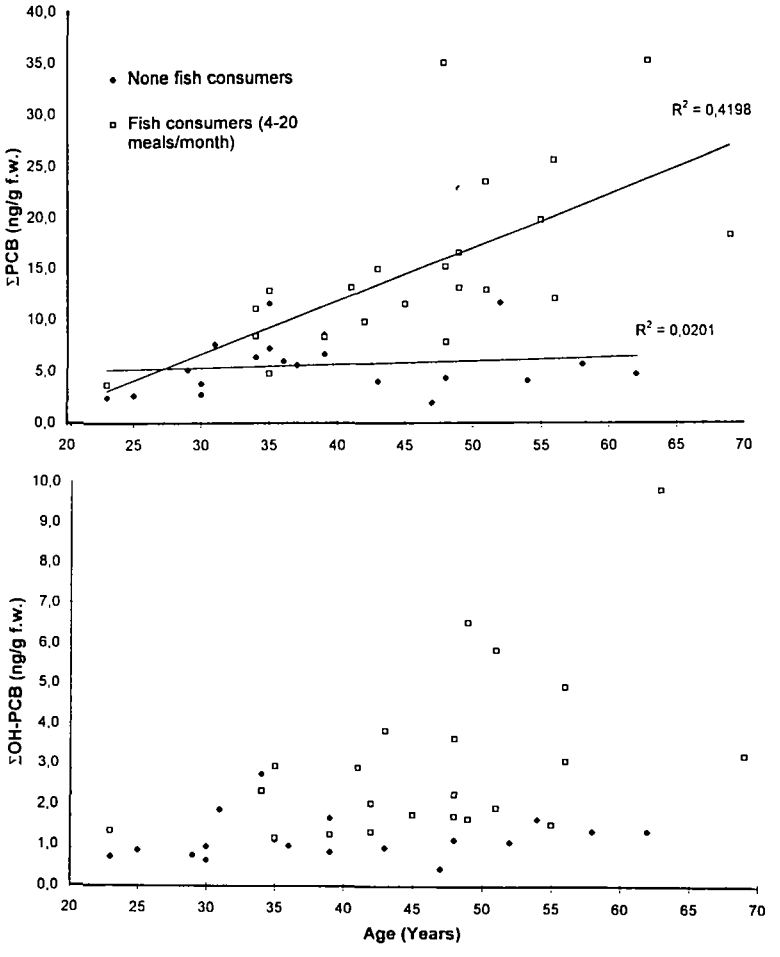
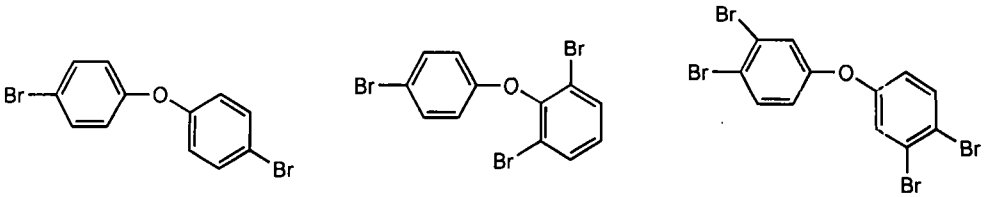


Figure 2. Total PCB concentrations in non-fish eater and fish consumers versus age (upper diagram) and total OH-PCB concentrations versus age (lower diagram).



BDE-15 BDE-32 BDE-77

Figure 3. Chemical structures of the three PBDE congeners tested for competition with T_4 for TTR.

Metabolism of seventeen PBDE congeners and *in vitro* competition binding assays on TTR have been initiated since potential hydroxylated metabolites of PBDE have strong structural resemblance with thyroxine (T_4) and triiodothyronine (T_3) [5]. Hepatic microsomes were prepared from Wistar rats after pretreatment with phenobarbital (PB, 0.1% w/v in the drinking water for 7 days); The T_4 assays were performed as described previously [6]. So far three PBDE have been studied; BDE-15, BDE-32 and BDE-77 (structures shown in Figure 3). The PBDE congeners (6 μM) were incubated for 10 and 30 min, respectively, with the PB induced rat liver microsomes. The OHS present in the microsomal preparation was extracted and used in the competition with T_4 for TTR. Since no reference compounds are available it was only possible to determine the competition by dilution technique. As shown in Figure 4, metabolites competing with T_4 is produced under the experimental conditions described for BDE-15 and BDE-77 but not for BDE-32. No competition was observed for any of the parent PBDE congeners. Similar results have previously been observed for 2,2',4,4'-tetraBDE (BDE-47) after microsomal transformation (unpublished). Also, a strong competing potency (5 times better than T_4) have been determined for TBBPA. It can be concluded that certain PBDE congeners are transformed to metabolites that compete with thyroxine for a transport protein, TTR, suggesting a potential endocrine disturbing effect of these PBDE metabolites if present in wildlife and humans. It is notable to see that also TBBPA is competing for the TTR binding site even though the complex between TBBPA and TTR may be difficult to form. This statement is based on difference electron electron density maps calculated for the complex.

Studies in progress

The RENCO project is in progress also with other studies. Some of these are summarized here. Thus, hydroxy-PCBs and -PBDEs will be studied for functionality effects using in vitro assays developed within the RENCO project.
PCP, hydroxy-PCB, hydroxy-PBDE, and a highly used flame retardant, TBBPA are under further investigations by X-ray diffraction methods for their binding to TTR.
Synthesis of hydroxy-PCB and hydroxy-PBDE will be prioritized for in depth endocrine related studies at developmental toxicological endpoints.
A protocol has been agreed on by the RENCO working group for a prospective study in the Netherlands aiming at endocrine and neurodevelopmental effects of perinatal exposure to OHS in human infants. Recruitment of pregnant mothers has started. Results will be expected within the next two years.

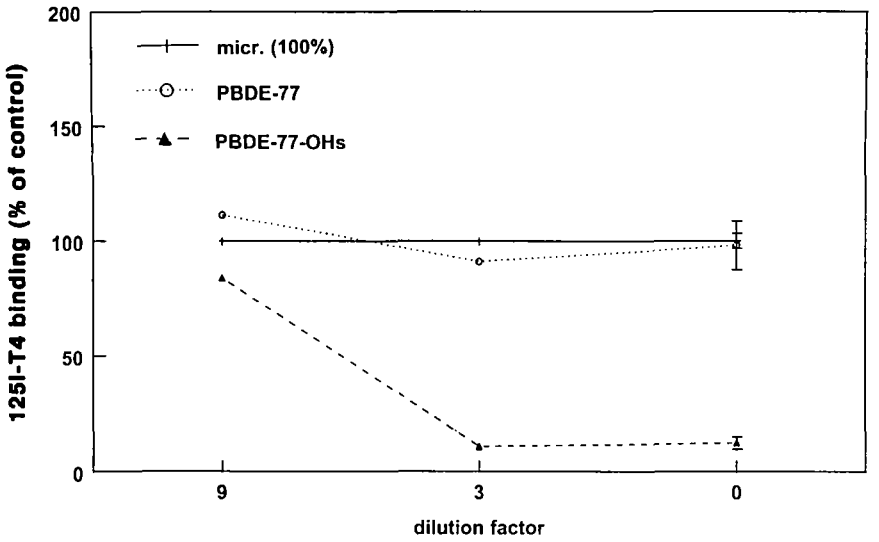


Figure 4 TTR-T4 competition binding of 3,3',4,4'-tetraBDE (BDE-77) prior to (dotted line) and of metabolites of BDE-77 after rat liver microsomal transformation of BDE-77.

Acknowledgements

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Literature Cited

(1) Bergman, Å., Klasson Wehler, E. and Kuroki, H. *Environ. Health Perspec.*, **1994**, 102, 464-469.
(2) Bergman, Å., Klasson Wehler, E., Nilsson, A., Kuroki, H. *Chemosphere*, **1995**, 30, 1921-1938.
(3) Sjödin, A., Bergman, Å, Jakobsson, E., Kierkegaard, A. And Sellström, U., *Organohalogen Compounds* **1997**. 17th International Meeting, Dioxin '97, Indianapolis, USA.
(4) Klasson Wehler, E., Hovander, L. And Bergman, Å. *Organohalogen Compounds* **1997**. 17th International Meeting, Dioxin '97, Indianapolis, USA.
(5) Brouwer, A., Morse, D., Lans, M., Schurr, G., Murk, A., Klasson-Wehler, E and Bergman, Å J. *Toxicol. and Industr. Health,* **1996**, submitted.
(6) Morse, D.C.; van Bladeren, P. J.; Klasson Wehler, E. and Brouwer, A. *Xenobiotica,* **1995**, 25(3), 245-260.