

In vitro screening of the endocrine disrupting potency of brominated flame retardants and their metabolites

Timo Hamers¹, Jorke H Kamstra¹, Edwin Sonneveld², Albertinka J Murk³, Bart N Zegers⁴, Jan P Boon⁴, Abraham Brouwer²

¹Institute for Environmental Studies (IVM), Amsterdam NL

²BioDetection Systems (BDS), Amsterdam NL

³Wageningen University - Toxicology Group, Wageningen NL

⁴Royal Netherlands Institute for Sea Research (NIOZ), Den Burg NL

⁵Umeå University, Umeå S

Introduction

Substantial evidence is recently becoming available that brominated flame retardants (BFRs) are potential endocrine disruptorsⁱ. The toxicological profile of BFRs, however, is too incomplete and insufficient to perform human and ecological risk assessment. To fill these gaps, the EU funded research program FIREⁱⁱ was started in December 2002. This program aims at the identification and toxicological characterization of the most potent and environmentally relevant BFRs and their possible risk for human and wildlife health. As part of a hazard identification approach, twenty-seven BFRs have been selected within the framework of FIRE for pre-screening their endocrine-disrupting potencies. Selection of test compounds was based on a maximal variation in physico-chemical characteristics of BFRs within the test set, allowing the establishment of quantitative structure-activity relationships (QSARs)^{iii,iv}. In addition, environmental relevance (*e.g.* high production volumes and persistence) and availability for testing were used as selection criteria. BFRs were tested in seven different *in vitro* bioassays for their potency to interfere via estrogenic, thyroidal, androgenic, progestagenic, and Ah-receptor mediated pathways. Metabolisation rates of BFRs were determined using phenobarbital-induced rat liver microsomes. Finally, the endocrine disrupting potency of the metabolites was determined in the same *in vitro* bio-assays and compared to the potency of the parent compounds.

Material and Methods

Nineteen out of the twenty-seven test compounds were poly-brominated diphenyl ethers (PBDEs), *i.e.* triBDEs 19, 28, 38, 39, tetraBDEs 47, 49, 79, pentaBDEs 99, 100, 127, hexaBDEs 153, 155, 169, heptaBDEs 181, 183, 185, 190, nona-BDE 206, and deca-BDE 209. Three hexabromocyclododecane (HBCDD) isomers (α , β , and γ) and a HBCDD technical mixture (HBCDD-TM; about 90% γ isomer) were included in the test set, as well as the hydroxylated compounds tetrabromobisphenol-A (TBBPA), 2,4,6-tribromophenol (246TBP), and ortho-hydroxylated BDE 47 (6OH-BDE 47). The test set was completed by TBBPA-bis(2,3)dibromopropylether (TBBPA-DBPE). BDEs 28, 47, 49, 99, 100, 153, 155, 169, 181, 183, 190, 206, and 209 and 6OH-BDE47 and 246TBP were obtained from Stockholm University (G.

Marsh/Å. Bergman), BDEs 19, 38, 39, 79, 127, and 185 were bought from Accu Standard. TBBPA was obtained from Aldrich, HBCDD TM from Dead Sea Bromine Group, HBCDD isomers from Cambridge Isotope Laboratories, and TBBPA-DBPE from RIVO IJmuiden (P. Leonards).

Ah-receptor (DR), estrogen receptor (ER), androgen receptor (AR) and progesterone receptor (PR) agonistic and antagonistic activities of the test compounds were determined in CALUX® reporter gene bioassays (BioDetection Systems, NL) as described elsewhere^{v,vi,vii}. Two assays were used to test thyroid disrupting potency. Competition with thyroid hormone precursor thyroxine (T4) for T4 binding sites on carrier proteins such as transthyretin (TTR) was measured in the T4-TTR competition assay^{viii} and thyroid hormone triiodothyronine (T3) mimicking or inhibiting potency in the T-Screen^{ix}. Finally, the potency to inhibit estradiol sulphation was tested in the E2SULT inhibition assay^x.

Based on their *in vitro* endocrine disrupting potency, BFRs were classified for each bio-assay. Using hierarchical cluster analyses and principal component analysis (PCA), pattern recognition was performed to find common features among the tested BFRs depending on common toxicological profiles determined in the bio-assays.

Endocrine disrupting potency of the test set was not only determined for the parent compounds, but also for their metabolites. Metabolites were prepared by incubating BFRs in hepatic microsomal suspensions from phenobarbital-induced male Wistar rats with NADPH as electron donor at 37°C. To estimate optimum incubation periods, mixtures of three to six BFRs were incubated for 0, 15, 30, and 90 minutes, and the decrease of parent compound in each mixture was followed in time by GC-MS analysis. BFRs for which significantly decreased levels of parent compounds were found, were then incubated individually to prepare metabolite-containing microsomal extracts for testing in the *in vitro* assays described above.

Results and discussion

Out of the total set of 27 BFRs tested for 12 different endpoints, highest responses were found in the AR CALUX® bioassay, the T4-TTR competition assay and the E2-SULT inhibition assay. Whereas none of the BFRs tested acted as AR-agonists, BDE 19 and BDE 100 were extremely potent AR-antagonists with IC₅₀-values <0.1 µM. Together with BDEs 47 and 49, these BFRs had an anti-androgenic potency higher than the anti-androgenic drug flutamide (IC₅₀ = 1.3 µM) that was used as a reference compound. Common structural features among these most potent AR-antagonists are the di-ortho (2,6) or ortho-para (2,4) bromine substitution on the one phenyl ring and the presence of an ortho bromine atom (2') on the other phenyl ring.

In the T4-TTR-assay, TBBPA and 246TPB were very potent T4 competitors with a TTR-binding affinity comparable to T4. TBBPA and 246TBP were also the most potent estradiol sulfotransferase inhibitors (IC₅₀ <0.1 µM), being one order of magnitude lower than the positive control pentachlorophenol. Although the other hydroxylated test compound 6OH-BDE47 was less potent than 246TBP and TBBPA in both the TTR and E2SULT assay (IC₅₀ = 1-10 µM), it was still the most potent of the rest of the test set, indicating that the presence of a hydroxyl-group clearly increases the potency of these compounds to interfere with TTR-mediated transport of thyroid hormone T4.

In the other *in vitro* bioassays, none of the tested BFRs had potencies with similar EC_{50} -values $<0.1 \mu\text{M}$. Still, these assays yielded plenty of information to discriminate between BFRs with high and low endocrine disrupting potency. With respect to PBDEs for instance, ER agonism was found for some low-brominated BDEs (\leq hexa-BDEs) whereas ER-antagonism was found only for high-brominated (\geq hepta-BDEs). Similar to the AR CALUX® bioassay, highest PR-antagonistic response was found for BDE 19 in the PR CALUX® bioassay, but the anti-PR potency of BDE 19 ($IC_{50}=0.8 \mu\text{M}$) was about 4000 times lower than for the breast cancer drug RU486, which was used as a reference compound. The highest DR-agonistic potency was found for BDEs 38, 79, 153 and 190 ($EC_{50} = 0.1\text{-}1.0 \mu\text{M}$). Especially the relatively high DR-agonistic potency of di-ortho-substituted BDE153 (2,2',4,4',5,5') is a remarkable result, given the lack of DR-activating potency reported for the identically substituted polychlorinated biphenyl (PCB 153)^{xi}. BDE 153 probably fits better in the DR-pocket than PCB 153 due to the presence of an ether-bridge between the two phenyl-rings, which is absent in PCB153.

Using pattern recognition methods (*i.e.* cluster analysis and PCA), the hydroxylated BFRs TBBPA and 246TBP with high TTR-binding and E2SULT inhibiting potency could clearly be distinguished from the 2,2'-substituted BDEs 19, 49, and 100 with high AR- and PR-antagonistic potency. Moreover, TBBPA-DBPE and BDEs 169 and 209 were clearly recognized as a group of compounds with almost no potency in any of the assays performed. For the remaining BFRs, ER-agonistic and ER-antagonistic responses seem to be the most discriminating factor, separating hepta-brominated BDEs, HBCDD TM, and gamma-HBCDD with low anti-estrogenic potency from lower brominated BDEs and alpha and beta isomers of HBCDD with no or very low estrogenic potency.

For twenty BFRs out of a total of twenty-seven tested in the *in vitro* metabolisation experiments, less than 80% of the original parent compound could be recovered after 90 minutes of incubation, indicating that more than 20% of the compound had been metabolized. Highest metabolisation rates were found for BDEs 19, 38 and 49 for which less than 10% of the parent compound could be recovered after 15 minutes of incubation. No or hardly any metabolisation was found for BDEs 127, 153, 169, 181, 183, 190, 206, and 209. In general, BDEs were somewhat easier oxidized by microsomal cytochrome P450 (CYP) than the corresponding PCBs, given the time-dependent decrease found for BDEs 47, 99, 100, and especially 155, that were supposed to be resistant to CYP metabolisation based on similar experiences with their PCB equivalents^{xii}.

So far, the endocrine disrupting potency of metabolites has only been tested in T4-TTR competition assay and in AR, PR, and ER CALUX® bioassays. Compared to the parent compounds, a higher TTR-binding capacity was found for all metabolized BDEs, with highest T4-TTR competing potency for BDE 49 metabolites. Metabolites of BDEs 19, 28, 38, 39 and 155, of which the parent compounds had no TTR-binding capacity at all, showed largest increases in toxic potencies, with EC_{50} values equivalent to 0.1-1.0 μM of parent compound. After metabolisation, T4-TTR competing potency had decreased for TBBPA and 246TBP, and no more competing activity was found for TBBPA-DBPE and the alpha and beta isomers of HBCDD. After metabolisation, an increase in anti-androgenic potency was found for TBBPA-DBPE and in anti-estrogenic potency for BDE 28, whereas the parent compounds did not show any activity for these endpoints. For all other compounds tested in AR, PR, and ER CALUX® bioassays, similar antagonistic results were found for the metabolite fraction compared to the parent compounds. With respect to ER-agonistic

responses, no activity was found for any of the metabolite fractions except for a small response from BDE 185 metabolites.

Conclusions

Our results clearly demonstrate that brominated flame retardants as well as their oxidized metabolites can interfere with endocrine pathways. We can distinguish separate groups of BFRs based on their toxicological profile. Highest anti-AR potency was found for di-ortho (2,2') substituted BDEs, and highest T4-TTR competing and E2-SULT inhibiting potency for hydroxylated BFRs. For these *in vitro* endocrine pathways, potencies were higher than for clinical drugs, natural ligands, or positive controls. Endocrine disrupting potencies of the BFRs changed drastically after metabolisation of the parent compounds. This was most obvious in the T4-TTR competition assay. After completion, the *in vitro* dataset will be used within the FIRE program to establish QSARs and to select a number of compounds for *in vivo* testing. This will provide a valuable endocrine disruption based data-set of the environmentally most relevant BFRs for human and ecological risk assessment.

Acknowledgements

The work described in this paper was performed within the framework of the EU-funded project "Risk assessment of brominated flame retardants as suspected endocrine disruptors for human and wildlife health" (Acronym: FIRE) under contract number QLRT-2001-00596.

References

-
- i Legler J., Brouwer A. 2003. Are Brominated flame retardants endocrine disruptors? *Environ. Internat.* 29: 879-885.
- ii Risk assessment of brominated flame retardants as suspected endocrine disrupters for human and wildlife health (Acronym: FIRE). EU-funded project QLRT-2001-00596. see: www.rivm.nl/fire
- iii Andersson P.L., Örn U., Öberg K., Tysklind M. 2004. Chemical characterization and strategic selection of BFRs for PBT assessment. *Abstract BFR2004* Toronto.
- iv Harju M., Andersson P.L., Hamers T., Sonneveld E., Tysklind M. 2004. Multivariate QSAR modeling of the endocrine effects of polybrominated diphenyl ethers. *Abstract BFR2004* Toronto.
- v Murk A.J., Leonards P.E.G., Van Hattum B., Luit R., Van der Weiden M.E.J., Smit M., 1998. Application of biomarkers for exposure and effect of polyhalogenated aromatic hydrocarbons in naturally exposed European otters (*Lutra lutra*). *Environ. Toxicol. Pharm.* 6: 91-102.
- vi Legler J., Van den Brink C.E., Brouwer A., Murk A.J., Van der Saag P.T., Vethaak, A.D. Van der Burg B. 1999. Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. *Toxicol. Sci.* 48: 55-66.
- vii Sonneveld E. et al. (in prep.)
- viii Lans M.C., Klasson-Wehler E., Willemsen M., Meussen E., Safe S., Brouwer A. 1993. Structure-dependent, competitive interaction of hydroxyl-polychloro-biphenyls, -dibenzo-p-dioxines and -dibenzofurans with human transthyretin. *Chem.-Biol. Interactions* 88: 7-21.
- ix Gutleb A.C., Meerts I.A.T.M., Bergsma J.H., Schriks M., Murk A.J. 2004. T-Screen as a tool to identify thyroid hormone receptor active compounds. *Environ. Toxicol. Pharm.* (submitted)
- x Kester M.H.A., Bulduk S., Tibboel D., Meinel W., Glatt H., Falany C.N., Coughtrie M.W.H., Bergman A., Safe S.H., Kuiper G.G.J.M., Schuur A.G., Brouwer A., Visser T.J. 2000. Potent inhibition of estrogen sulfotransferase by hydroxylated PCB metabolites: A novel pathway explaining the estrogenic activity of PCBs. *Endocrinology* 141: 1897-1900.
- xi Van der Plas S.A., Gibbs C.M., Brouwer A. 1998. Antagonistic effects of mono- and di-ortho substituted polychlorinated biphenyls in the ethoxy-O-deethylase activity and the luciferase reporter gene assay in vitro. *Organohalogen Compounds* 37: 183-186
- xii Boon J.P., Van der Meer J., Allchin C.R., Law R.J., Klungsøyr J, Leonards P.E.G., Spliid H., Storr-Hansen E., McKenzie C., Wells D.E. 1997. Concentration-dependent changes of PCB patterns in fish-eating mammals: Structural evidence for induction of cytochrome P450. *Arch. Environ. Contam. Toxicol.* 33: 298-311