APPLICATIONS OF IN VITRO SCREENING TOOLS TO SUPPORT RISK ASSESSMENT OF BROMINATED FLAME RETARDANTS

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Introduction

About 25% of the annual flame retardant production consists of halogenated organics of which brominated flame-retardants (BFRs) are the most important group. This is due to the fact that bromine is the most efficient halogen in capturing free radicals, whereas brominated organohalogens decompose (i.e. deliver free radicals) at temperatures that are commonly found during combustion[1]. Although BFRs are life saving due to their flame-retarding characteristics, some may also pose a health risk to man and the environment, given their suspected endocrine disrupting (ED) potencies[2,3,4].

The EU-funded FIRE program[5] aims at the identification and toxicological characterization of the most potent and environmentally relevant BFRs and their possible risk for human and wildlife health. This paper presents an overview of the nine different applications for which a battery of in vitro bioassays was used within the hazard identification of BFRs of the FIRE program, namely

I. To screen the ED potency of BFRs;
II. To distinguish different toxicity profiles for BFRs, each with its own representative indicator BFR;
III. To determine the impact of metabolization on the persistence and ED potency of BFRs;
IV. To develop QSAR models for the ED potency of BFRs;
V. To provide mechanistic support for results from in vivo studies with BFRs;
VI. To identify BFR-metabolites with highest ED potency;
VII. To determine the impact of UV-illumination on the ED potency of BDE-209;
VIII. To confirm the concentration additivity concept for the ED potency of BFRs;
IX. To estimate the contribution of BFRs to the total ED potency in biota.

Material and Methods

For toxicity screening (application I) and profiling (II), as well as for metabolization experiments (III) and QSAR development (IV), a test set of 27 BFRs was selected consisting of the high production BFRs tetrabromobisphenol-A (TBBPA), polybrominated diphenylethers (PBDEs; 19 different congeners), and hexabromocyclododecane (HBCD; technical mixture and the alpha, beta, and gamma diastereoisomers). The test set also included 2,4,6-tribromophenol (246-TBP), ortho-hydroxylated BDE-47 (6OH-BDE-47), and tetrabromobisphenol-A-bis(2,3)dibromopropylether (TBBPA-DBPE).

In vitro metabolization required for applications III, IV, and VI was performed by incubating BFRs in hepatic microsomal suspensions from phenobarbital-induced male Wistar rats with NADPH as electron donor at 37°C[6]. 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[7]. BFRs for which significantly decreased levels of parent compounds were found, were then incubated individually to prepare metabolite-containing microsomal extracts for ED potency screening. BDE metabolites were identified by GC-MS according to Marsh et al[8].

To determine the impact of UV illumination on the ED potency of decabrominated BDE-209 (application VII), BDE-209 was dissolved in tetrahydrofuran and photochemically transformed by UV-light (290 nm; 90, 120, or 200 min) at Stockholm University (Dr. Bergman).

To confirm the concept of concentration additivity (application VIII), mixtures were prepared of environmentally relevant BFRs in the same ratio as they were analyzed in extracts from seal blubber, tern eggs, and fish (sandeel and sprat/herring). For each species composition three types of mixtures were prepared: (i) only PBDEs (congeners 28, 47, 99, 100, and 183), (ii) PBDEs and HBCD, and (iii) PBDEs, HBCD, and TBBPA. Measured ED potencies were compared to calculated ED potencies using

the concentration addition concept and the relative ED potencies of BFRs determined in the screening (application I).

To estimate the contribution of BFRs the total ED potency in biota (application IX), total ED potency of biota extracts was tested in in vitro bioassays, whereas the BFR contribution was calculated using the relative ED potencies determined in the in vitro screening of individual BFRs (application I).

A total number of seven different bioassays were available. 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[9,10,11]. 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[12] and thyroid hormone triiodothyronine (T3) mimicking or inhibiting potency in the T-Screen[13]. Finally, the potency to inhibit estradiol sulphotransferase was tested in the E2SULT inhibition assay[14]. For some applications, the whole battery of bioassays was used, whereas for other applications ED measurements were restricted to subsets of these seven bioassays. All ED measurements were performed as described elsewhere[15].

For toxicity profiling, hierarchical cluster analyses (HCA) and principal component analysis (PCA) were used as pattern recognition methods to classify the BFRs into clusters with similar toxicity profiles[15]. For QSAR modelling, partial least regression methods were applied using Simca-P software (v10.5, Umetrics Inc. Umeå, Sweden)[7].

Results and Discussion

The **in vitro screening** (application I) demonstrated that in vitro ED potencies that had not or only marginally been described for BFRs so far, were shared by a wider set of BFRs than was ever tested before[15]. AR-antagonistic and PR-antagonistic potencies were found for 18 out of the 27 BFRs in the test set. Also potentiation of T3-mediated effects and E2SULT inhibition are relatively unknown effects of BFRs that were found for 9 and 8 BFRs, respectively. For some environmentally relevant BFRs, the ED potency was higher than for positive controls: BDE-100 was a better AR-antagonist than the clinical drug flutamide, 246-TBP and TBBPA were better TTR-binders than the natural ligand T4, and TBBPA was a better E2SULT-inhibitor than the well-known inhibitor pentachlorophenol.

Based on the similarity and dissimilarity in bioassay responses from the in vitro screening, BFRs were classified into five different **ED toxicity profiles** (application II)[15], i.e.

1. hydroxylated BFRs (TBBPA, 246-TBP) with high TTR-binding and E2SULT inhibiting potencies;
2. tri- to hexabrominated diphenylethers (BDE-19, 49, 100, 155) with a di-ortho [2,2'] substitution and no adjacent bromine substitutions with antiandrogenic, antiprogestagenic, and T3-potentiating potencies and to a lesser extent estrogenic activity;
3. HBCD (technical mixtures and individual diastereoisomers) with similar T3-potentiating activity as cluster 2, lower antiandrogenic and antiprogestagenic potencies than cluster 2, and low DR-agonistic potency;
4. tri- to hepta-brominated diphenylethers with lowest antiandrogenic and antiprogestagenic potencies but higher DR-agonistic potency than cluster 3;
5. relatively inactive "rest-BFRs", including tri- to deca-brominated diphenylethers.

Metabolization experiments with CYP2B induced rat microsomes (application III) yielded half-life estimates within the test period of 90 min for 15 out of the 26 tested BFRs[7]. Highest metabolization rates were found for low-brominated diphenylethers (<5 min for BDEs 19, 38, and 49). No or hardly any metabolization was found for BDEs 127, 153, 169, 181, 183, 190, 206, and 209. Metabolization of PBDEs seemed easier than of the corresponding PCBs: a time-dependent decrease was found for BDEs 47, 99, 100, and especially 155 (Fig. 1), of which the PCB equivalents were supposed to be resistant to CYP metabolization[16]. In addition, large differences were found between metabolization rates of different HBCD diastereoisomers. In contrast to the beta and the gamma isomers, the alpha isomer of HBCD hardly showed any decrease in the metabolization experiments with hepatic microsomes from CYP2B induced rats (Fig. 1) and fresh harbour seals found dead in the Dutch Wadden Sea[17]. This diastereoisomer-specific biotransformation of HBCD may be one explanation for the exclusive accumulation of HBCD alpha in marine mammals despite the domination of the gamma isomer (>90%) in HBCD technical mixtures.

The most significant **QSAR models** (application IV) were calculated for AR-antagonistic, PR-antagonistic, ER-agonistic, TTR-binding and E2SULT inhibiting potencies determined in the in vitro screening (application I), and for BFR-specific metabolization rates (application III)[7]. QSAR models

predicted AR-antagonistic, PR-antagonistic, and ER-agonistic potencies of PBDEs to be dependent on Br-substitutions in ortho-positions. Out of all PBDEs present in the commercial pentamix Bromkal 70-5DE, highest potencies (IC_{50}/EC_{50}-values of 0.1, 1.9, and 5 µM, respectively) were predicted for BDE-17. Highest CYP2B-mediated metabolization rates were predicted for low-brominated ortho-substituted PBDEs with free meta- and para-positions.

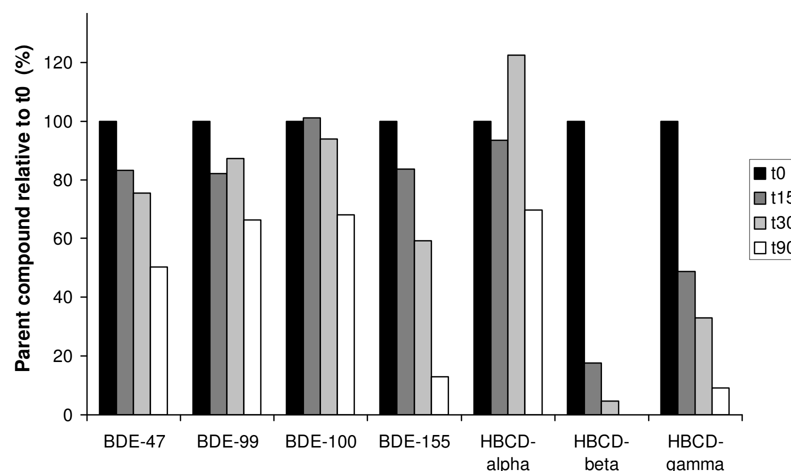

Fig. 1: Time-dependent decrease of BFRs after 15, 30, and 90 min of incubation with CYP2B induced rat microsomes.

In vitro results were used **to support interpretation of in vivo results** from rat experiments (application V) with TBBPA, PBDE-pentamix, and HBCD[18], which were selected as representative BFRs for clusters 1, 2, and 3 (application II). The in vitro results, which demonstrated TTR-binding for TBBPA and T3-potentiation for TBBPA, HBCD, and some PBDEs[15,19], were used to explain the decreased total T4 levels in plasma of TBBPA and HBCD exposed rats. These T4 decreases were accompanied with direct effects on the TH-axis or indirect TH-associated effects, such as thyroid gland activation, increased pituitary weight, and developmental effects in neurophysiological functions (HBCD and TBBPA), increased plasma-cholesterol levels (HBCD and PBDE-pentamix), and increased plasma protein levels (TBBPA, HBCD and PBDEs)[18]. Also, decreased weights of male reproductive organs in PBDE (pentamix) exposed rats may be attributed to BDE-100, for which antiandrogenic potency was determined in the AR-CALUX bioassay[15], or to pentamix constituents BDE-17, 66, 85, 138, and 154 for which the QSAR models predicted additional AR-antagonistic potency[7].

For most PBDEs, an increase in TTR-binding and E2SULT-inhibiting potencies was found after CYP2B-mediated metabolization. **Identification of hydroxylated metabolites with ED potency** (application VI) was only performed for the environmentally very relevant [2,2',4,4']-tetrabrominated diphenyl ether (BDE-47). Out of all six identified mono-hydroxylated metabolites, 3OH-BDE-47 and the bromine-shifted 4'OH-[2,2',4,5']-tetrabrominated diphenyl ether (4'OH-BDE-49) were formed in largest quantities. Altogether, both metabolites accounted for 85% and 72% of the observed TTR-binding and E2SULT-inhibition, respectively, by metabolized BDE-47 after 90 min of incubation.

Deca-brominated BDE-209 was classified as a relative inert BFR in cluster 5. DR-agonistic, TTR-binding, and E2SULT-inhibiting potencies of BDE-209, however, were significantly increased by UV-illumination. Probably, the DR-agonistic response can be attributed to the formation of polybrominated dibenzofurans. TTR-binding and E2SULT-inhibiting potencies suggest the presence of hydroxylated transformation products of which the origin remains unclear so far.

The **confirmation of the concept of concentration addition** (application VIII) was hampered by low ED potencies of BFRs most abundant in biota extracts and by low concentrations of the more potent environmentally relevant BFRs. Satisfying results were obtained, however, for PR-antagonistic, DR-antagonistic, ER-agonistic and AR-antagonistic responses. For TTR-binding and E2SULT-inhibition, concentration addition could not be confirmed due to additional problems with TBBPA losses by evaporation during preparation of the mixtures and with background activity of the blanks in the E2SULT assay.

Finally, the **contribution of BFRs to the total ED potency in biota extracts** (application IX) was estimated in a pilot study with 6 extracts from 4 animal species. Extracts from tern eggs, seal blubber, sandeel and sprat/herring showed TTR-binding and E2SULT inhibiting potency, but AR-antagonistic

and PR-antagonistic potencies were only found for the latter three. Although the concentration addition concept (application VIII) was not confirmed completely, the concept was still adopted here to calculate the contribution of the analyzed BFR-levels to the total ED potency, because (i) the concept is widely accepted for mixtures of compounds with a similar mode of action and (ii) previous results indicated that the concept may lead to an overestimation (but not an underestimation) of the ED potency of BFRs (worst case). Using the relative ED potencies of BFRs determined in the in vitro screening (application I)[15], the contribution of BFRs to the total ED potency was estimated in most cases to be <1%. E2SULT-inhibiting potencies (up to 7% in tern egg extracts) are an exception to this rule, but these results should be handled with care given the fact that concentration addition could not be checked at all for this mode of action.

In conclusion, in vitro bioassays proved to be useful tools to screen the possible ED potency of BFRs, to classify BFRs in clusters with similar toxicity profiles, and to help interpreting in vivo results. Pilot results indicated, however, that the contribution of non-metabolized BFRs to the total ED potency of biota extracts is low. The in vitro bioassays further demonstrated that different BFRs have different metabolization rates. Metabolization as well as photochemical transformation by UV light, however, may be important prerequisites for BFRs to exert their ED mode of action.

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References

1 Alaee M, Arias P, Sjödin A, Bergman Å. *Environ Int* 2003,29:683.
2 Darnerud PO, Eriksen GS, Johanesson T, Larsen PB, Viluksela M. *Environ Health Perspect* 2001,109(suppl 1):49.
3 Legler J, Brouwer A. *Environ Int* 2003,29,879.
4 Vos JG, Becher G, Van den Berg M, De Boer J, Leonards PEG. *Pure Appl Chem* 2003,75:2039.
5 RIVM. *Risk assessment if 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
6 Meerts IATM, Van Zanden JJ, Luijks EAC, Van Leeuwen-Bol I, Marsh G, Jacobsson E, Bergman Å, Brouwer A. *Toxicol Sci* 2000,56:95.
7 Harju M, Hamers T, Kamstra JH, Sonneveld E, Boon JP, Tysklind M, Andersson PL. *Environ Toxicol Chem* (submitted)
8 Marsh G, Athanasiadou M, Athanasiadis I, Sandholm A. *Chemosphere* 2006,63:690.
9 Murk AJ, Leonards PEG, Van Hattum B, Luit R, Van der Weiden MEJ, Smit M. *Environ Toxicol Pharm* 1996,6:91.
10 Legler J, Van den Brink CE, Brouwer A, Murk AJ, Van der Saag PT, Vethaak AD, Van der Burg B. *Toxicol Sci* 1999,48:55.
11 Sonneveld E, Jansen HJ, Riteco JAC, Brouwer A, Van der Burg B. *Toxicol Sci* 83:136.
12 Lans MC, Klasson-Wehler E, Willemsen M, Meussen E, Safe S, Brouwer A. *Chem-Biol Interactions* 1993,88:7.
13 Gutleb AC, Meerts IATM, Bergsma JH, Schriks M, Murk AJ. *Environ Toxicol Pharm* 2005,19,231
14 Kester MHA, Bulduk S, Tibboel D, Meinl W, Glatt H, Falany CN, Coughtrie MWH, Bergman A, Safe SH, Kuiper GGJM, Schuur AG, Brouwer A, Visser TJ. *Endocrinology* 2000,141:1897.
15 Hamers T, Kamstra JH, Sonneveld E, Murk AJ, Kester MHA, Andersson PL, Legler J, Brouwer A. *Toxicol Sci* 2006 doi:10.1093/toxsci/kfj187 (advance access published on April 6, 2006).
16 Boon JP, Van der Meer J, Allchin CR, Law RJ, Klungsøyr J, Leonards PEG, Spliid H, Storr-Hansen E, McKenzie C, Wells DE. *Arch Environ Contam Toxicol* 1997,33:298.
17 Zegers BN, Mets A, Van Bommel R, Minkenberg C, Hamers T, Kamstra JH, Pierce G, Boon JP. *Environ Sci Technol* 2005,39:2095.
18 Van der Ven LTM, Fernández-Cánton R, Germer S, Lilienthal H, Schrenk D, Van den Berg M, Piersma AH, Vos JG. *Organohalogen Comp* 2006, this issue.
19 Schriks M, Vrabie CM, Gutleb AC, Faassen EJ, Rietjens IMCM, Murk AJ. *Toxicol in Vitro* 2006,20:490.