AHR SIGNALLING PATHWAY IN RAT HEPATOMA CELLS INDUCED BY TRACES OF BROMINATED DIBENZOFURANS IN BDE47 SAMPLES?

Strack S[1], Wahl M[1], Weiss C[1], Jay K[2], Kuch B[3], Krug HF[1]

Forschungszentrum Karlsruhe, [1]Institute of Toxicology and Genetics, and [2]Institute of Technical Chemistry, Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany
[3]Universität Stuttgart, Institute for Sanitary Engineering, Water Quality and Solid Waste Management, Bandtäle 2, *70569* Stuttgart, Germany

Introduction

Brominated flame retardants (BFR) and various decomposition products can be detected world wide in the environment, but also regional monitoring programs prompt concern by observed concentrations in human and wild life. Risk assessment for this novel class of persistent organic pollutants is an important premise for preventive and sustainable policies, for which reliable toxicological data bases are essential.

BDE47 is an environmentally relevant tetrabromodiphenyl ether (2,2',4,4' tetra BDE). Structural similarities with halogenated dibenzodioxins and dibenzofurans refer to the possibility that similar toxic effects can be caused by BDE47, and indications for an involvement of the Ah receptor (AhR) would suggest a 'dioxin-like' behaviour. The aim of our study is to gain insights into mechanisms of underlying sub acute effects due to basic functions in mammalian cells, including signalling and regulatory gene expression processes. The pivotal question is, however, if BDE47 can interact in an agonistic way with the Ah receptor, and if it is responsible for the subsequent nuclear translocation of that transcription factor as well as for the expression of several corresponding target genes. Appropriate bioassays (EROD assay and CALUX bioassay), using biomarkers to get rapid information about the induction of Cytochrome P450 monooxygenase (CYP1A1) activity, have been applied by different working groups. However, so far induction of CYP1A1 activity could not be observed with BDE47.[1,2]

In our experiments we use the rat hepatoma cell line 5L wt and an AhR deficient sub clone BP8$^{AhR-/-}$ to analyze on a protein level possible CYP1A1 induction by BDE47 in presence and also absence of the Ah receptor. The substances we use for exposures of the cells were synthesised and purified at the University of Stuttgart (Kuch). GC-MS analyses of different batches did not show either dioxin or dibenzofuran impurities. The formation of brominated dibenzofurans was unlikely due to the conditions of synthesis but because of the same molecular mass as the diphenyl ethers, traces of dibenzofurans could not be excluded completely.

The results of our *in vitro* experiments, as described here, give several indications that our BDE47 showed weak but clear effects on AhR which are usually caused only by classical potent dioxin-like compounds. Compared to 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) or other well known AhR agonists, such as ß-naphthoflavon, the induction of CYP1A1 expressions and a G1 arrest in the cell cycle of 5L wt cells are of minor intensity. However, these effects are significant and reproducible, and can be explained, if the brominated BDE47 molecules act in an agonistic way, but with a lower affinity to the Ah receptor than TCDD or equivalent dibenzofurans.

Materials and methods

Chemicals: BDE47 was synthesized in the Institute of Sanitary Engineering, Water Quality and Solid Waste Management, Universität Stuttgart, Germany. Synthesis was performed by bromination of non-brominated diphenyl ether and by bromination of BDE15 (4,4´-dibromodiphenyl ether, Sigma Aldrich) under conditions that do not favor any generation of dioxins. The educts were purified by sulfuric acid treatment in n-heptane followed by column clean-up (Al_2O_3 b). The purity was controlled via HRGC/LRMS. The bromination was carried out in refluxing CCl_4. Bromine in CCl_4 was added subsequently and the proceeding of the reaction was controlled using HRGC/LRMS. After bromination the organic solution was washed with a solution of potassium hydroxide in water (1 N). Following a rotavaporation, solvent exchange to n-heptane and sulfuric acid treatment a column clean-up (Al_2O_3 b) using the eluents n-heptane, n-heptane/dichloromethane 98:2, n-heptane/dichlormethane 90:10, n-heptane/dichloromethane 1:1 has been performed. BDE-47 is eluted with the last fraction. Analytic screening of the BDE47 by HRGC/LRMS GC-MS gave no indications of contaminations by dioxins. BDE47 is poorly soluble in water. Stock solutions of 10 or 100 mM were prepared with 100 % dimethyl sulfoxide (DMSO). ß-naphthoflavone was purchased from Merck, Darmstadt.

Cell line and culture conditions: The rat hepatocytic cell line 5L wt and the Ah receptor deficient sub clone $BP8^{AhR-/-}$ were employed.[3] Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Life Science, Karlsruhe, Germany) supplemented with 10 % heat-inactivated fetal-calf serum (FCS) and 2 mM L-glutamine. Cells were incubated at 37° C in a humidified atmosphere with 8 % CO_2.

Flow cytometry: Cells were seeded in 6-well plates (10^6 per well), grown overnight and exposed to BDE47 for 24 hours. For cell cycle analysis, attached and floating cells were harvested, combined and fixed with an ethanolic solution containing DAPI as DNA stain. The cells were stained for 24 to 40 h at –20°C and then flow cytometry was carried out in a LSR-flow cytometer (Becton Dickinson, San Jose, CA, USA). Fluorescence emission of 30.000 cells was determined and the cell cycle phase distribution was analyzed using ModFit software (Verity Software House Inc., Topsham, Maine, USA).

Western blotting: Cells were exposed in 6-well plates (Corning, NY, USA) for 24 h (10^6 cells per well). SDS-PAGE has been performed as described previously.[4] AhR antibodies were obtained from Biomol (Hamburg, Germany) and CYP1A1 antibodies from Santa Cruz Biotechnology (Santa Cruz, USA).

Fluorescence microscopy: Cells were seeded and grown up in chamber slides (Nunc) until the monolayer was semi confluent. After exposure for 3 hours, cells were fixed with 4 % paraformaldehyde and treated with primary antibodies against AhR (Biomol Hamburg, Germany) and with secondary antibodies labeled with CY3 (Jackson-Immuno-Research). Nuclear DNA was stained with Hoechst dye (Bisbenzimide H 33258, Sigma). Fluorescence imaging was performed with an Axiovert 100 (Zeiss, Jena, Germany) using an oil immersion, 100x objective.

Results and discussion

Purity of BDE47 samples: The purity of all substances used in these experiments was checked by gas chromatography-mass spectrometry. The results of the GC-MS analysis of our BDE47 are shown in figure 1. Amongst contamination by inert aliphatic squalen several pentabromo-diphenyl ethers could be identified. No evidence of any impurity by dibenzo-p-dioxins could be detected. However, the resolution of the applied GC-MS was not high enough to exclude completely minimal traces of brominated dibenzofurans hidden in the signals of the diphenyl ethers.

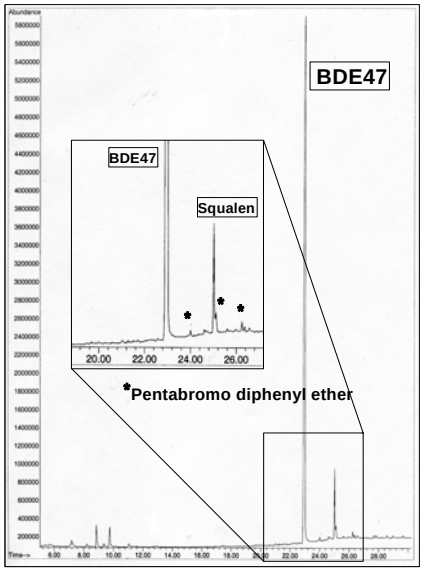


Fig 1. GC- chromatogram of the applied BDE47 substance

Cell cycle effects of BDE47: Flow cytometric measurements of 5L wt and BP8$^{AhR-/-}$ after DAPI staining show that the cell cycle is altered in a dose-dependent manner by BDE47 and by ß-naphthoflavone and TCDD as positive controls. After exposure with BDE47 for 24 h in 5L cells an increasing arrest in the G1 phase at concentrations >50 µM can be observed. When 5L wt were exposed to ß-naphthoflavone, a well known agonist of the AhR, G1 arrest is more pronounced. Importantly, in BP8$^{AhR-/-}$ cell cycle lacking AhR expression, neither of the compounds affects cell cycle progression. Thus, BDE47 and ß-naphthoflavone delay cell cycle progression via AhR similarly to the action of TCDD described previously.[3]

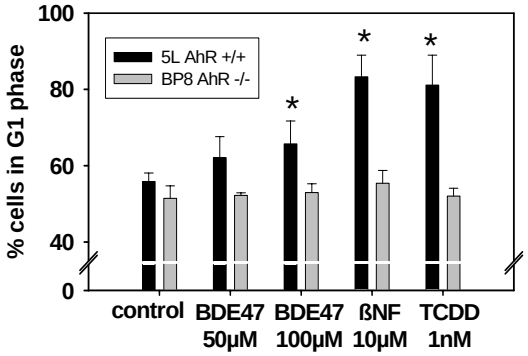
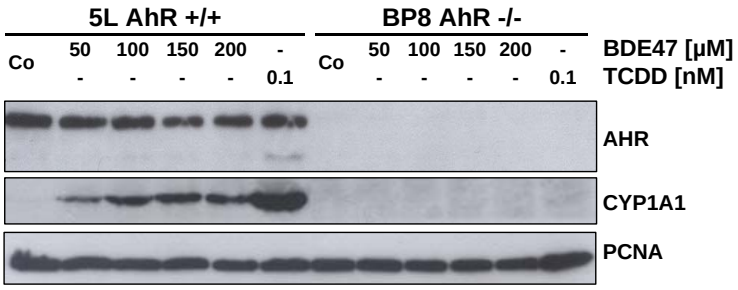
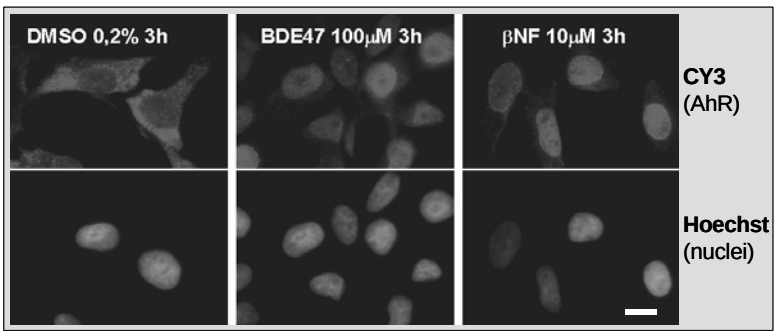


*Fig. 2. Cell cycle alteration by BDE47 and ß-naphthoflavone in 5L wt rat hepatoma cells and the sub clone BP8$^{AhR-/-}$, *p<0.05*

BDE47 induces CYP1A1 expression: The Ah receptor can be detected by western blotting only in 5L wt rat hepatocytes, but – as described previously - not in AhR deficient BP8$^{AhR-/-}$ cells.[3] Exposure to BDE47 for 24 hours leads to a dose-dependent induction of a CYP1A1 expression in 5L wt cells beginning at concentrations of 50 µM. A clear enhanced induction can be observed when 5L wt are exposed to the chlorinated TCDD, but in BP8$^{AhR-/-}$ no induction can be detected, suggesting that our BDE47 functions as a weak ligand for the AhR.

Fig. 3. Induction of CYP1A1 expression in 5L wt cells after treatment with BDE47 and TCDD for 24 hours. No induction of CYP1A1 expression in the AhR receptor deficient sub clone BP8$^{AhR-/-}$ can be observed.





Nuclear translocation of AhR: A specific indication of an activation of the Ah receptor is the association of the transcription factor with its partner protein Arnt and the translocation from the cytosol into the nucleus. In the control sample (DMSO

Fig 4. Translocation of cytosolic AhR into the cell nucleus within a 3 hours period of exposure with BDE47 (100µM) and with ß-naphthoflavone (10 µM) observed with fluorescent microscopy. AhR was immunostained with CY3 and nuclear DNA was stained with Hoechst dye, objective 100x, oil-immersion (scale 10 µM).

0.2 %) the cytosolic distribution of the AhR (immunostained with CY3) after 3 hours can be seen. An exposure with BDE47 (100 µM) as well as with ß-naphthoflavone (10 µM) for 3 hours makes evident an obvious translocation into the nuclei, which have been stained with the DNA intercalating Hoechst dye.

Conclusions

In this study when 5L wt rat hepatoma cells were exposed to our BDE47 sample we found dioxin-like effects including nuclear translocation of the activated Ah receptor, G1 arrest of the cell cycle and significant CYP1A1 induction. On the contrary no alteration of the cell cycle and no induced expression of the CYP1A1 monooxygenase at all could be detected in the Ah receptor deficient sub clone BP8$^{AhR-/-}$. These results suggest an involvement of the Ah receptor in the cellular response to an acute BDE47 exposure in rat hepatocytes. Polybrominated dibenzo-p-dioxin impurities (PBDDs) of the applied substance could be excluded and the presence of significant amounts of dibenzofurans (PBDFs) in our sample seems unlikely. However, we cannot rule out that the observed effects may be caused by minimal amounts of PBDFs in our sample, particularly as PBDFs seem to have toxic properties that are similar to those of their chlorinated homologs, as recently reviewed by Birnbaum et al.[5]

On receptor level we observed a direct interaction with the AhR leading to a subsequent nuclear translocation. On protein and mRNA level (data not shown) we could detect a CYP1A1 upregulation dependent on the AhR, but we did not use up to now 7-Ethoxyresorufin-O-deethylation (EROD) as a marker to test an enzymatic activity of CYP1A1. Determination of CYP1A1 (EROD) activity in our experiments would be interesting, because other authors could not detect any CYP1A1 (EROD) activity subsequently to an exposure to different PBDEs in mammalian cells.[1,2] The complex pattern of potential interactions of PBDEs on the AhR pathway, recently has been revealed by Peters et al.[6] Testing agonistic as well as antagonistic effects of different PBDEs their results suggest that PBDEs can bind to AhR, but do not activate the AhR-Arnt-XRE complex.

To conclude, we found interactions of our BDE47 with AhR in hepatocytes, although we cannot exclude definitively that minimal traces of highly potent brominated dibenzofurans in our BDE47 sample were responsible for the observed effects. Studies of specific binding of BDE47 as a ligand to AhR would provide more certainty.

Acknowledgements

We acknowledge the financial support of this study by BWPLUS (BWR 24013).

References

1. Peters AK, van Londen K, Bergman Å, Bohonowych J, Denison MS, van den Berg M, Sanderson JT. *Toxicol Sci* 2004;82:488
2. Behnisch PA, Hosoe K, Sakai S. *Inviron Intern* 2003;29: 861
3. Weiss C, Kolluri SK, Kiefer F, Göttlicher M. *Experim Cell Res* 1996;226:154
4. Strack S, Sander M, Detzel T, Kuch B, Krug HF. *Organohalogen Comp* 2004;66:3843
5. Birnbaum LS, Staskal DF, Diliberto JJ. *Environ Int* 2003;29(6):855
6. Peters AK, Nijmeijer S, Gradin K, Backlund M, Bergman Å, Poellinger L, Denison MS, van den Berg M, *Toxicol Sci* 2006; Apr 6 (Epub ahead of print)