BDE47 BATCH CONTAINING TRACES OF BROMINATED DIBENZOFURANS ALTERS CELL CYCLE REGULATION *IN VITRO*

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Introduction.

Polybrominated diphenyl ethers (PBDE) have been widely used as flame retardants in a variety of consumer goods, e.g. plastics, textiles, computer and electronic devices. They were available mainly in technical mixtures containing different brominated congeners. Penta- and octa-brominated congener mixes have been banned in Europe while deca-mixtures are still in use. PBDEs had a high production volume of 76.000t alone in the year 2001 (Kierkegaard et al., 2004). These compounds exhibit highly lipophilic properties and have been demonstrated to accumulate in the environment. Especially the tetrabromodiphenyl ether congener BDE47 (2,2',4,4'-tetra BDE) is predominating in many studies. Petreas et al. (2003) detected BDE 47 in serum and adipose tissue from California women in the late 1990s; concentrations ranged from 5 to 510 ng/g lipid (median 16.5 ng/g) whereas comparative serum samples from women residing in the same area in the 1960s revealed no detectable BDE 47. Alarming concentrations of BDE 47 in human mothers milk could be detected ranging from 3 to 272 ng/g lipid with a median value of 18.4 (median PBDEs 34 ng/g) (Schecter et al., 2003). Interestingly, human tissue samples from Europe show a ten to hundred times lower PBDE burden.

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 Aryl hydrocarbon 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. Induction of cytochrome P450 monooxygenase 1A1 (CYP1A1) is a well characterized marker of AHR activation, e.g. for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Appropriate bioassays (EROD assay and CALUX bioassay), using biomarkers to get rapid information about the induction of CYP1A1 activity, have been applied for PBDEs by different working groups. However, so far induction of CYP1A1 activity could not be observed with BDE47 (Peters et al., 2004; Behnisch et al., 2003). Anyway, CYP1A1 activation solely does not explain the toxic effects observed for TCDD. Therefore we investigated whether cell cycle regulation is affected by BDE47 since TCDD is known to cause a block in cell cycle progression in 5L wt rat hepatoma cells. Deregulation of proliferation is a possible endpoint of cellular toxicity. We tested a sample of BDE47 that contained traces of 2,3,7,8-tetrabromofuran (TBDF) representing impurities of technical PBDE mixtures what has been reported previously (Hanari et al., 2006).

Materials and methods.

Chemicals: The BDE47 sample used in our experiments was synthesised in the Institute of Sanitary Engineering, Water Quality and Solid Waste Management, University of Stuttgart, Germany. Synthesis was performed by bromination of non-brominated diphenyl ethers under conditions that do

not favour any generation of dioxins. Purification has been performed comparable to the method described by Marsh et al.(1999). Analytic screening of the BDE47 by high resolution GC-MS gave no indications of contaminations by dioxins. However, traces of brominated furans could be detected within the congener batch. Especially a 2,3,7,8-tetrabromo dibenzofuran (TBDF) has to be mentioned according to its endangering high dioxin-like potency. The origin of this contaminant remains to be elucidated since secondary formation can be excluded by the substitution pattern. BDE47 is poorly soluble in water. Stock solutions of 10 or 100 mM were prepared with 100 % dimethylsulfoxid (DMSO). ß-naphtoflavon (bNF) was purchased from Merck, Darmstadt.

Cell line and culture conditions: The rat hepatocytic cell line 5L and the Ah receptor deficient subclone $BP8^{AhR-/-}$ (Weiss et al., 1996) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM, Life Science, Karlsruhe, Germany) supplemented with 10 % fetal bovine serum (FBS, Invitrogen, Karlsruhe, Germany). Cells were incubated at 37° in a humidified atmosphere with 8 % CO_2.

Flow cytometry: Cells were seeded in 6-well plates (10^6 per well) and 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. Then DNA was stained with DAPI (Partec, Münster, Germany). Analysis of cell cycle distribution 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).

BrdU proliferation assay: Determination of DNA synthesis rate was performed using a BrdU assay kit (Roche diagnostics, Mannheim, Germany). 5L and BP8 cells were seeded in 96well plated (10^5 per well) over night and then exposed to BDE47 for 24h. After the incubation cells were incubated with a BrdU antibody for two hours and incorporation of BrdU into newly synthesised DNA was measured as a marker of proliferation. Analysis was done in a microplate reader (Molecular Devices, Ismaning, Germany).

Results and discussion.

In our studies we used a BDE47 sample that was contaminated with TBDF. Technical PBDE mixtures have been shown to contain TBDF and other polybrominated furans (Sanders et al., 2005) as impurities. We found that our BDE47 batch causes deregulation of cell cycle progression in 5L wt rat hepatocytes but not in the $BP8^{AHR-/-}$ subclone as detected by measurement of the DNA content and analysis of DNA synthesis rate.

Probably TBDF alone acted in a dioxin-like manner in all our studies. We could detect induction of the marker enzyme CYP1A1 both on mRNA and protein level (data not shown). Enhanced CYP1A1 levels have been associated with toxicity and cancer but do not explain the underlying mechanism. We focussed on cell cycle regulatory effects because TCDD is well known to block cell cycle progression in 5L hepatocytes. In our experiments the TBDF-contaminated BDE47 sample caused an increasing arrest in the G1-phase of cell cycle dose-dependently (fig. 1) after 24h as observed for TCDD with 1nM and another positive control, beta-naphtoflavon (bNF) with 10µM. Saturation was reached already at 1µM of BDE47. Underlining these results, S-phase of the cell cycle was decreased in 5L beyond BDE47 concentrations of 1µM after 24h whereas in $BP8^{AHR-/-}$ cells similar effects became

apparent only at highest doses (fig. 2). The comparison of 5L hepatocytes and BP8 subclones suggests strongly the involvement of the AHR.

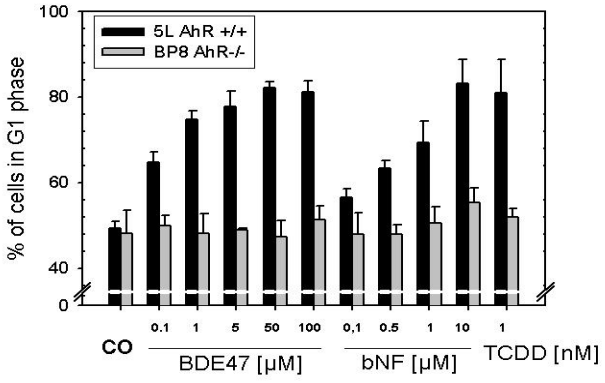

Fig. 1. Cell cycle alteration by a BDE47 sample, beta-naphtoflavon (bNF) and TCDD in 5L wt rat hepatoma cells and the AhR deficient sub clone BP8$^{AhR-/-}$. Increase in cells residing in the G1-phase of the cell cycle is displayed after 24h incubation.

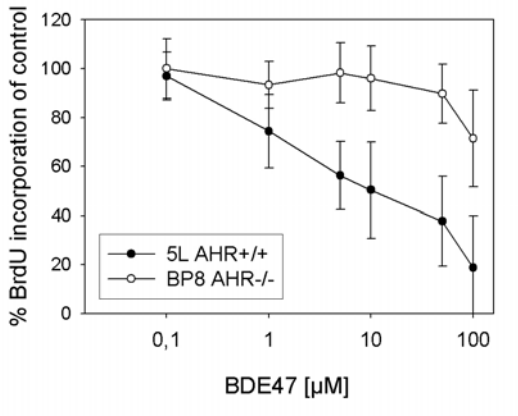

Fig. 2. Reduction of proliferation by a BDE47 sample assayed by BrdU incorporation into newly synthesised DNA in 5L wt rat hepatoma cells and the AhR deficient sub clone BP8$^{AhR-/-}$ after 24h.

Conclusions.

We focussed in our studies on effects of PBDE on AHR beyond induction of CYP1A1. Our results show that BDE47 containing TBDF was able to induce a block in cell cycle progression of 5L wt rat hepatoma cells which could not be detected in AHR deficient BP8 subclones. TBDF action in dioxin-like manner is not surprising and they are already taken into consideration for TEF-evaluation (Van den Berg et al., 2005). Whether these furans accompany PBDEs in technical mixtures and in the environment needs to be investigated since toxicological data are rare. In this study the contaminated BDE47 is representative for technical mixtures with impurities by brominated furans what has been reported previously (Hanari et al., 2005). In the future we plan to investigate the dioxin-like potency of TBDF on target genes other than CYP1A1. Highly purified PBDE congeners without any contamination have been reported to act as AHR antagonists (Peters et al., 2006). The consequences of PBDE-antagonism on AHR endogenous function in cellular metabolism and of non-coplanar effects remain to be elucidated.

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