Cytotoxic effects of TBBPA and its interactions with signalling pathways in mammalian cells

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Introduction

Toxic effects of TBBPA published so far have been recently reviewed by Birnbaum and Staskal[1]. The LC_{50} indicating the acute toxicity *in vivo* due to a single oral dose in mice and rats were higher than 4 to 5 g/kg[2], however, systematically long-term *in vivo* studies are missing. Weak estrogenic effects have been described by Meerts *et al.*[3], demonstrating for TBBPA less pronounced activity than for other brominated bisphenols. The same group described competitive interactions *in vitro* with human transthyretin (TTR). In binding affinity assays they could demonstrate that TBBPA binds to TTR ten times more effectively than T_4. However, the available toxicological data are still extremely limited. For a comprehensive risk assessment valid data are insufficient.

Mitogen-activated protein kinases (MAPKs) play very important roles in cellular signalling pathways. Some of them can be inhibited by contact with small molecules interacting with specific binding sites. Interferences of chemicals with kinase or phosphatase activities can possibly trigger cell death or organismic disease states[4]. MAPKs were found in all eukaryotes and in mammals. Three well-tied cascade modules have been identified: The extracellular signal-regulated protein kinases (ERK 1 and 2), the c-*jun* N-terminal kinase or stress-activated protein kinases (JNKs/SAPKs), and p38. MAPKs are evolutionary well conserved enzymes that connect cell-surface receptors to different regulatory targets within the cell, however, they also respond to chemical and physical stresses thereby regulating almost all cellular processes like gene expression, proliferation, differentiation, adaptation, and apoptotic cell death[5].

The aim of this study was to evaluate possible cytotoxic effects, and to gain insights into the underlying molecular mechanisms respectively the corresponding cellular signalling processes. This approach would allow to identify sensitive end-points of cellular toxicological responses. For these molecular toxicological investigations established cell lines should be used, in order to have a suitable model for appropriate toxicological studies.

Methods and Materials

Chemicals: TBBPA and BPA were synthesised in the Institute of Siedlungswasserbau, University of Stuttgart, Germany. Both chemicals are poorly soluble in water. Stock solutions of 10 or 100 mM were prepared with 100% dimethylsulfoxid (DMSO).

Cell lines and culture conditions: Cal-62 human thyroid anaplastic carcinoma were purchased from the German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany (DSMZ No. ACC 448) The cells were cultured in Dulbecco's modified Eagle medium (DMEM, Life Science, Karlsruhe, Germany) supplemented with 10 % fetal calf serum (FCS). NRK (normal rat kidney) epithelial cells were obtained from the American Type Culture Collection (ATCC CRL-6509). These cells were cultured in a mixture of F12 Nutrient Mixture and DMEM (50:50) containing 1% Ultroser G (serum substitute, Life Technologies GmbH, Eggenstein, Germany). The final concentration of Ultroser was equivalent to 5 % FCS. A549 cells, human epithelial alveolar type II-like lung cells, were obtained from American Type Culture Collection (ATCC CCL-185). The cells were grown in DMEM, supplemented with 10 % FCS, 2 mM L-glutamine. Cells were incubated at 37° in a humidified atmosphere with 8% CO_2.

Determination of cell growth and cytotoxicity (MTT-Assay): Cells (5×10^4 per well) were grown in 6-well plates (Corning, NY, USA) for 4 to 7 days. 24 h after seeding, the cells were exposed to TBBPA or BPA at different concentrations. At certain time points (24 h, 48 h, 72 h, 96 h, 120 h) after addition of the chemicals the cells were trypsinized and counted with an haemocytometer (Casy 1, Schärfe System) by discriminating between necrotic and apoptotic cells. For a better comparison, proliferation is given in cell numbers related to the starting cell number. Viability was determined in 96-well plates (5×10^4 cells per well) using the MTT assay Kit (Roche, Basel, Switzerland) according to the instructions of the manufacturer. All experiments were carried out in triplicate. Dose-response relations were described by the sigmoidal function $y = y_0 + a * \exp(- \exp(x-x_0) / b)$ using Sigma Plot 8.0 (SPSS Science Software GmbH, Erkrath, Germany), where y is the viability of the cells compared to the solvent controls (y_0).

Flow cytometry: Cells were seeded in 6-well plates (10^6 per well) and grown overnight, and were than exposed to TBBPA for 24 or 48 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 analysed using ModFit software (Verity Software House Inc., Topsham, Maine, USA).

Western blotting: For SDS-PAGE, cells were washed twice with ice-cold PBS and lysed in SDS-sample buffer. After centrifugation for ten minutes at 21.000 *g*, the protein content of the lysates was determined according to Bradford[6]. Gel electrophoresis and blotting was carried out according to standard protocols[7]. MAPK antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). Phospho-specific MAPK antibodies were purchased from New England Biolabs (Beverly,USA-p-ERK and p-P38) and Cell Signalling Technology (Beverly, USA - p-JNK).

Results and Discussion

Cytotoxicity of TBBPA and BPA: Cytotoxicity of the selected cell lines following exposure to TBBPA was determined after 24 hours using the MTT assay. To compare impacts of TBBPA with a non-brominated substance, bisphenol A (BPA) was included into the assay. In figure 1, typical dose-response curves for the NRK cells are given. All tested cell lines showed a significant reduction of viability in the applied concentration range of 25 and 200 µM (14 - 108 mg/l). From the sigmoid curves the LC_{50} concentrations were calculated. NRK prove to be most sensitive to TBBPA, with a calculated LC_{50} of 52 µM. A549 and Cal-62 were less sensitive with calculated LC_{50} values of 168 and 200 µM. The incubations with the non-brominated BPA did not show any toxic effect in the tested concentration range. In contrast to the brominated compound, the mitochondrial enzymatic activity is clearly enhanced up to a concentration of 50 µM. Between 75 and 200 µM, this increased activity decreased again, however, at high concentrations such as 200 µM it was equal to the activity of the solvent control. This observation clearly demonstrates that bisphenol A causes the observed *in vitro* cytotoxic effects, solely in its brominated form

Figure 1: Dose response curves for TBBPA and BPA in NRK cells (MTT- assay)

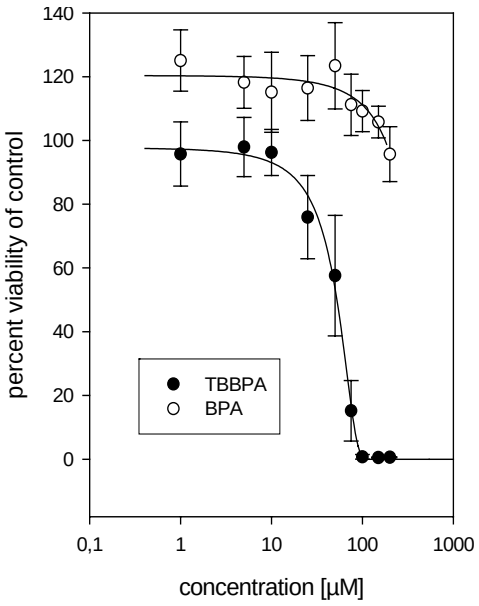
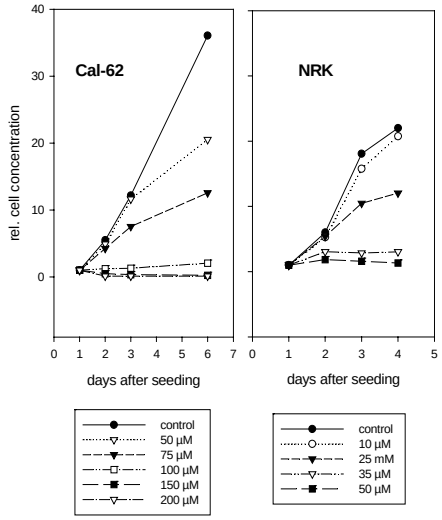


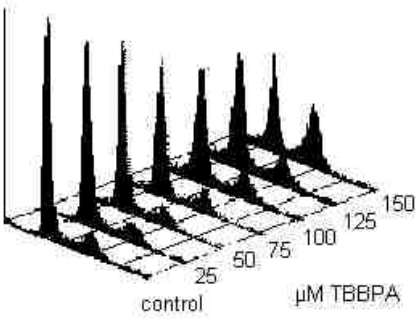
Figure 2: Growth of Cal-62 and NRK cells exposed to TBBPA



To analyse the mitogenic effect imposed by increasing concentrations of TBBPA, we examined the cell cycle phase distribution of the cells exposed for 24 hours. In the Cal-62 cells, a clear increased arrest of the cells in the G2/M-phase becomes evident at concentrations < than 50 µM (Fig. 3). The NRK cells obviously arrested in the G1/G0 phase. Similar to the NRK, the A549 also arrested in the G1/G0- phase, however, the lung cells were less sensitive. A continuous increase of cells arresting in the G1/G0- phase starts at concentrations greater than 50 µM (results not shown).

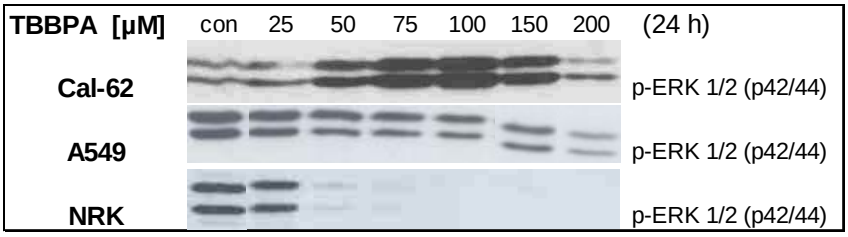
Effects of TBBPA on proliferation and cell cycle phase distribution: Growth curves of untreated and treated cells were generated using a haemocytometer to monitor the result of cell proliferation as well as cell death in culture. Under the chosen culture conditions untreated cells grew exponentially within the first 3 to 4 days after seeding. Treatment with TBBPA caused a considerable decrease in growth rate at 25µM in NRK, whereas the growth rates of A549 and Cal-62 decreased at concentrations > 75µM. An almost complete inhibition of growth could be observed when the TBBPA concentrations exceeded 150 µM in Cal-62 and A549, or 50 µM in NRK respectively. The growth curves of the Cal-62 and NRK cells are given in figure 2.

Figure 3: A G2/M cell cycle arrest in Cal-62 cells after incubation with TBBPA for 24 h



Modulation of MAP kinase pathways: The activation patterns of the mitogen-activated protein (MAP) kinase pathways (ERK, JNK, p38) seems to represent a promising approach to provide

Figure 4: Different ERK-activation pattern in TBBPA exposed cell lines



more detailed insights into chemical stress-induced cellular responses, the liable mechanisms, and their underlying signalling processes. We therefore modulations of the Raf-MEK-ERK pathway. This pathway is linked mainly with proliferation and survival of the cells, although induction of apoptosis has also been described[8]. The activation or deactivation of ERK 1/2 at the end of this MAPK cascade was detected with phospho-specific antibodies and the results are presented in figure 4. In NRK and A549 cells, ERK 1/2 shows a high basal activation that is reduced by increasing concentrations of TBBPA. This result is consistent with the observation in the proliferation assays. In Cal-62 cells, ERK is activated by increasing concentrations of TBBPA with a maximum at 100 µM. Time dependent analysis of the activation of ERK1/2 in these cells shows a sustained phosphorylation inducing also toxic effects (results not shown). A possible reason for that effect can be an inhibition of a specific phosphatase that is responsible for the essential dephosphorylation of the involved MAPKs.

Conclusions

To conclude, our studies demonstrate the impact of TBBPA on basic cellular regulations at concentrations in the low micro molar range. Fortunately there is still an apparent gap between the effective concentrations and those observed in the environment. The varying effectiveness of TBBPA in different mammalian cells exclude a non-specific mode of action and suggest the potential of various adverse effects in different organs.

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References

1 Birnbaum L.S., Staskal D.F. (2004) Environ Health Perspect.;112(1):9-17.
2 WHO (1995) Environmental Health Criteria 172, Geneva: World Health Organisation
3 Meerts I.A., Letcher R.J., Hoving S., Marsh G., Bergman A., Lemmen J.G., van der Burg B., Brouwer A. (2001) Environ Health Perspect. Apr;109(4):399-407.
4 English J., Pearson G., Wilsbacher J., Swantek J., Karandikar M., Xu S., Cobb M.H. (1999) Experimental Cell Research 253, 255-270
5 Pouysségur J., Volmat V., Lenormand P. (2002) Biochem. Pharmacology 64, 755-763
6 Bradford M.M.. (1976) Anal Biochem. May 7;72:248-54.
7 Laemmli U.K.(1970) Nature, 227, 680-685
8 Chang L., Karin M. (2001) Nature. 2001 Mar 1;410(6824):37-40.