ANALYSIS OF BROMINATED FLAME RETARDANTS AND BROMINATED DIBENZODIOXINS AND BIPHENYLS FOR Ah RECEPTOR ACTIVATION USING THE CALUX® BIOASSAY

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Brominated flame retardants (BFRs) such as polybrominated diphenylethers (PBDE) and polybrominated biphenyls (PBB) are known to bioaccumulate and consequently pose a potential threat to environmental and human health. Additionally, BFRs can be contaminated with brominated dioxins and polybrominated biphenyls (PBB), or on incineration these compounds may be produced. Levels of BFRs in environmental matrices and human tissues are well-documented[1, 2]. Although effects on thyroxine hormone levels, behavior and interference with the Ah-receptor and estrogen receptor have been reported for BFRs[3], the current knowledge of toxicological actions of BFRs is rather limited. The aim of this study was to investigate whether commonly used BFRs and associated compounds elicit dioxin-like toxicity in the cell based CALUX® reporter gene bioassay. In the CALUX® bioassay, compounds like dioxins that activate the Ah receptor cause firefly luciferase to be expressed. The amount of light produced by the luciferase reporter is directly proportional to the degree of Ah receptor activation. Several types of compounds were analyzed for dioxin-like activity: pure compounds used as flame retardants; pure compounds such as brominated dioxins and biphenyls, which are potential contaminants of flame retardants and commercial mixtures of BFRs.

Materials and Methods

Materials. BFR and PBB standards were purchased from AccuStandard, Inc. (New Haven, CT). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,7,8-tetrabromodibenzo-*p*-dioxin (TBDD), PBDE 47 and PBDE 99 were purchased from Wellington Laboratories (Guelph, Ontario). The purity of decabromodiphenyl ether was 85.5%, and the purity of all other compounds was equal to or greater than 98%. Firemaster BP-6, PHT4, and BP4A were technical mixtures.

CALUX® Assay. XDS has developed a cell line (mouse hepatoma H1L1) that was stably transfected with a vector that contains the gene for firefly luciferase under transactivational control of the aryl hydrocarbon receptor[4]. Serial dilutions of the compounds of interest were prepared in dimethyl sulfoxide. Prior to dosing the cells, the DMSO solutions were suspended in cell culture medium and the medium added to monolayers of the cells grown in 96 well culture plates. In addition to the samples, a standard curve of TCDD was assayed (a two-fold dilution series beginning at 0.5 ng TCDD/ml). The plates were incubated for 20 hours at 37^{o}C in a humidified CO_2 incubator to allow optimal luciferase gene expression. Following incubation, the medium

was removed and the cells were examined microscopically for viability. The induction of luciferase activity was quantified using the luciferase assay kit from Promega (Madison, WI).

Data analysis: The response for each concentration of each pure compound that reached a maximal response was analyzed at least three times. Data for the dose response series were fit to a sigmoid curve described by the Hill Equation using least squares best fit modeling. The values for the maximal response and concentrations associated with 20-80% of the maximal response (EC_{20-80}) were determined from the derived Hill Equation. The maximal response for each of the compounds was compared to the maximal response for TCDD using a two tailed student's t-test with $\alpha = 0.05$.

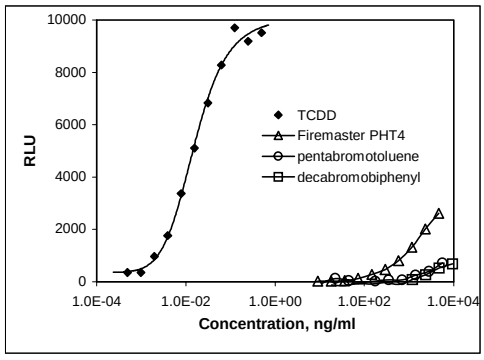
Results and Discussion

Figure 1A and B show that all of the BFRs were several orders of magnitude (10^5-10^6) less active than 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Based on these results the pure compounds would only make a significant contribution to the overall dioxin-like toxicity when present at the highest concentrations that have been measured in environmental samples.

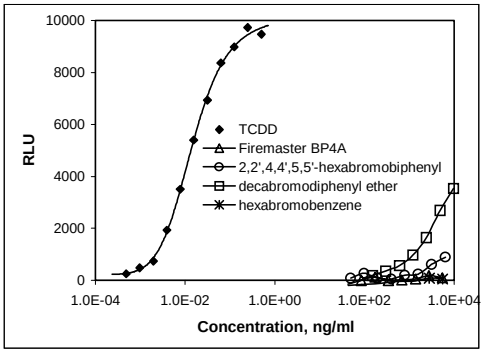
The most active BFRs in the CALUX® assay were Firemaster BP-6 (hexabromobiphenyl mixture – main component 2,2',4,4',5,5'-hexabromobiphenyl), Firemaster PHT4 (primary component tetrabromophthalic anhydride) and decabromodiphenyl ether (PBDE 209). The main component of Firemaster BP-6, 2,2',4,4',5,5'-hexabromobiphenyl, was much less active than the mixture. This suggests that the activity of Firemaster BP-6 could be primarily associated with other bromobiphenyls in the mixture, possibly including mono-ortho PBB. Likewise, it seems that the activity of decabromodiphenyl ether (PBDE 209 – purity 85.5%) may be associated with the contaminants, as pure brominated diphenyl ethers (PBDE 47 and PBDE 99 – purity >98%) were inactive (data not shown) and it is unlikely that diphenyl ethers could exist in a planar conformation due to the ether linkage. Compounds that bind to the Ah receptor, and are active in the CALUX® bioassay, tend to have a planar conformation.

Figure 1. Dose response curves for BFRs.

A.



B.



Trace contaminants such as polybrominated dibenzo-*p*-dioxins, dibenzofurans and dioxin-like PBB can occur in BFRs. As discussed above, the low level of response in the CALUX® assay that is seen for BFRs could be caused by trace contamination of the standards by polybrominated

dibenzo-*p*-dioxins, dibenzofurans or dioxin-like PBB. Contamination of TBDD in BFR at only parts per million levels would be sufficient to produce the response seen for the most active BFRs. An additional concern associate with the use of BFR is that improper incineration of these compounds can result in the production of polybrominated dibenzo-*p*-dioxins, dibenzofurans and dioxin-like PBB[5].

In the CALUX® assay the brominated analogue of TCDD and dioxin-like PBB showed equivalent, or in the case of the PBB compounds, greater activity than their chlorinated analogues (see Figure 2 and Table 1). Therefore the health risks associated with the use of BFRs could be substantially caused by the presence of contaminating brominated dioxin-like compounds or the formation of these compounds when BFRs are burned during waste incineration.

Figure 2. Dose response curves for selected polybrominated and polychlorinated dibenzodioxins and biphenyls.

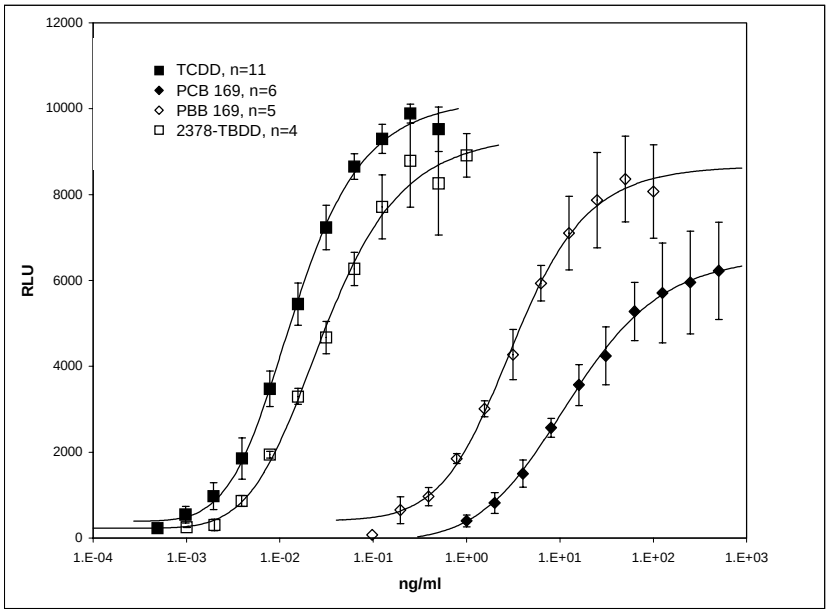


Table 1. Results from the comparison of the dose response curves for the active dibenzodioxins, dibenzofurans and selected coplanar polychlorinated biphenyls to 2,3,7,8-tetrachlorodibenzo-p-dioxin. All values are based on and reported as ng/ml.

Compound	WHO-TEF[6]	CALUX REP, based on EC_{50}	REP Range, (EC_{20} to EC_{80})	B_{max} = TCDD B_{max} 2 tailed, $\alpha = 0.05$	Efficacy, % of TCDD B_{max}
TCDD	1	1.00 +/- 0.01			
2378-TBDD		0.49 +/- 0.07	0.38 to 0.60	yes	96%
PCB 77	0.0005	0.0014 +/- 0.0004	0.012 to 0.017	no	53%
PBB 77		0.015 +/- 0.003	0.007 to 0.030	no	83%
PCB 169	0.01	0.0011 +/- 0.0003	0.0007 to 0.0017	no	69%
PBB 169		0.0047 +/- 0.0007	0.0047 to 0.0053	no	82%

Conclusions

In regards to activation of the Ah receptor, the health risks associated with BFRs could be substantially caused by the presence of contaminating dioxin-like compounds or the formation of brominated dioxin-like compounds during the incineration of BFR containing materials. Brominated coplanar PBB and 2,3,7,8-TBDD activated the CALUX® assay at equivalent or higher levels than the corresponding chlorinated compound. We have previously reported that the results for environmental samples using the CALUX® assay correlate well with GC/MS data, but the CALUX® results tend to be higher[7]. Some of this difference could be due to the contribution of brominated or mixed halogenated compounds to the total TEQ of the sample and experiments are being planned to test this hypothesis. If brominated and mixed halogenated compounds are found to make a significant contribution to total TEQ values, the CALUX® assay could provide a relatively easy method for estimating the total dioxin-like activity in a sample, including the contributions from all brominated and mixed halogenated compounds. Application of this assay could contribute to the collection of data on occurrence and contribution of both brominated and chlorinated dioxins, dibenzofurans, biphenyls and BFRs to the TEQ value in environmental, biological or human samples. In contrast, chemical analysis of brominated and mixed halogenated dioxins, furans and biphenyls can be very difficult and expensive due to the large number of possible combinations.

Acknowledgements

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