SUNLIGHT IRRADIATION OF HIGHLY BROMINATED POLYPHENYL ETHER FLAME RETARDANTS AS SOLIDS AND IN SOLUTION GENERATES BY-PRODUCTS THAT INDUCE AHR-RELATED MRNA IN CHICKEN EMBRYONIC HEPATOCYTES

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Introduction

Phase-outs and recent (Stockholm Convention) regulation of penta- and octa-BDE flame retardants (FRs), which were produced in large volumes, has resulted in an increase in the demand for replacement brominated (B) FRs. There are now numerous BFRs currently in use including highly brominated chemicals such as tetradecabromo-1,4-diphenoxybenzene (TeDB-DiPhOBz, also known as 4'-PeBPO-BDE208 and SAYTEX 120) and 2,2',3,3',4,4',5,5',6,6'-decaBDE (BDE-209). Investigations on their environmental behaviors (i.e. photolytic stability, potential effects and toxicological impact on a given species) are urgently needed, especially for TeDB-DiPhOBz, which is broadly used in solid plastic and wire/cable. TeDB-DiPhOBz is also an alternative FR to BDE-209 that was added to the list of exemptions in polymeric applications[1].

The chemical structure of TeDB-DiPhOBz contains three fully brominated aromatic rings connected by two oxygen atoms. Similarly, BDE-209 consists of two fully brominated aromatic rings connected by one oxygen atom. Their similar chemical structures means that they exhibit some common properties, i.e. low volatility, high log K_{ow} and low bioavailability, and thus they are not predicted to be bioaccumulative in the environment[2]. Furthermore, when these two BFRs are dissolved in organic solvent they are photolytically unstable and rapidly degraded via stepwise, reductive debromination[3-6]. A recent and extremely important finding that we made was that degradation by natural sunlight of TeDB-DiPhOBz and BDE-209 in organic solution can generate by-products that affect *in vitro* expression of genes, especially aryl hydrocarbon receptor (AhR)-mediated *CYP1A4* mRNA expression with an induction of up to thousands of fold[7].

The present study reports: 1) gene expression profiles in CEH administered sunlight irradiated (SI) degradation by-product mixtures of SI-TeDB-DiPhOBz, SI-BDE-209 or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); 2) an evaluation of whether SI-TeDB-DiPhOBz and SI-BDE-209 mixtures induce AhR-related CYP1A4/5 mRNA in concentration-dependent manner; 3) an investigation of whether gene changes in (1) and (2) comparatively occur for TeDB-DiPhOBz and BDE-209 solids after sunlight irradiation; and 4) the identity of photodegraded by-products related to induced gene activity observed in (1), (2) and (3).

Materials and Methods

Chemicals To the best of our knowledge, pure standards for TeDB-DiPhOBz or its possible degradation by-products are not yet commercially available. Technical SAYTEX-120 (TeDB-DiPhOBz; Lot# 0GN01-$I0)

and BDE-209, in solid powder form, were kindly supplied by Wellington Laboratories (Guelph, ON, Canada). Purity of BDE-209 was reported to be greater than 98% by its supplier.

Sample Preparation Detailed information on sunlight irradiation of TeDB-DiPhOBz and BDE-209 in solvents was provided in a previous publication[7]. To investigate whether degradation occurs in a solvent-free system, TeDB-DiPhOBz or BDE-209 solid powder was exposed to natural sunlight. In brief, solid powder of two chemicals was transferred into quartz weighing bottles, respectively, and the exact masses were 0.01458 g (TeDB-DiPhOBz) and 0.00896 g (BDE-209). The quartz bottles were sealed with plastic paraffin film, and put in an open house roof-top location in Ottawa, Canada. The sunlight irradiation was naturally continuous over a three month period, from July 1 to October 8, 2014.

Chicken Embryonic Hepatocyte Assay, RNA Isolation and cDNA Synthesis Two independent chicken embryonic hepatocyte (CEH) cultures were prepared for the following chemical administrations: 1) sunlight irradiated BFR by-products generated in the solvent systems, and 2) sunlight irradiated by-products generated from the BFR solid forms. Methods for the CEH cell culture and DNA preparation have been described previously[7].

Aryl Hydrocarbon Receptor (AHR)-related ToxChip PCR Array The custom chicken RT^2 Profiler PCR Array was built by SABiosciences (Qiagen, Valencia, CA, USA) according to our specifications. Each 96-well array contained three identical sets of 27 target genes and 5 control genes, allowing 3 technical replicates to be screened per plate. The 27 target genes were identified as potential biomarkers of TCDD exposure in CEH exposed to 0.03 nM and 1.0 nM TCDD[7]. The 5 control genes included 2 internal control genes, a positive PCR control, a reverse transcription control, and a test well for genomic DNA contamination.

Real-time Reverse Transcription Polymerase Chain Reaction (real-time RT-PCR) Multiplex real-time RT-PCR assays were employed to measure CYP1A4, CYP1A5 and β-actin (normalizer gene) mRNA abundance in CEH exposed to DMSO, SI-TeDB-DiPhOBz and SI-BDE-209 by use of dual-labeled fluorescent hydrolysis probes.

Instrumental Analysis Identification and quantification of furans was carried out using an Agilent 1200 liquid chromatographic (LC) system, coupled with an Agilent 6250A quadrupole-time-of-flight mass spectrometer (Q-TOF)-MS (Agilent Technologies, Mississauga, ON, Canada). For each run, 2 uM purine (*m/z* 119.0363) and 50 nM HP-0921 (*m/z* 805.9854) were introduced into the Q-TOF with toluene as reference masses.

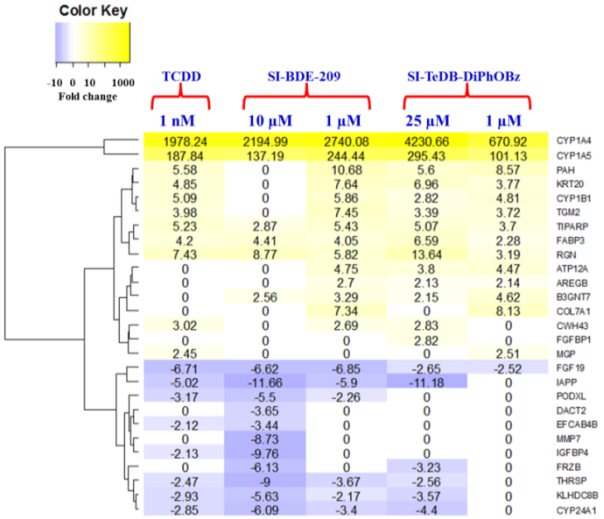

Figure 1. Transcriptional profiles of 27 AhR-responsive genes on the Avian ToxChip PCR array following exposure to TCDD, SI-TeDB-DiPhOBz and SI-BDE-209.

Results and Discussion:

The 27 dioxin-responsive genes, which were selected based on results of microarray analysis (data not shown), were determined in CEH following exposure to 1 and 25 μM SI-TeDB-DiPhOBz and 1 and 10 μM SI-BDE-209, and showed greatly similar profiles compared to TCDD (Figure 1). Hierarchical clustering of the 27 genes was conducted based on mRNA expression levels, and two genes, CYP1A4 and CYP1A5, were separated into an independent group from all other genes due to their extremely large fold-changes, on average 1-3 orders of magnitude higher than those of other genes. In terms of both their specific fold changes, the other 25 genes were further classified into two very clear sub-clusters that show consensus up-regulation or down-regulation directions (see Figure 1) following exposure to any of these three examined chemicals.

Both SI-TeDB-DiPhOBz and SI-BDE-209 induced CYP1A4/5 mRNA in a concentration-dependent manner, but mRNA expression of both seemed not to reach plateaus at the currently examined concentrations. Maximal levels of CYP1A4 mRNA expression induced by SI-TeDB-DiPhOBz and SI-BDE-209 were 5620-fold and 2480-fold, respectively, which were comparable with fold change values obtained from the PCR array technology. Maximal levels of CYP1A5 mRNA expression were 295-fold and 333-fold following exposure to SI-TeDB-DiPhOBz and SI-BDE-209, respectively. Since concentration-dependent plateaus for both were not achieved, the relative potencies (ReP) and the derived effective concentrations (ECs) could not be calculated in this study. To address whether the observed results can occur in a more real environmental situation, irradiation of the solid forms of TeDB-DiPhOBz and BDE-209 was also conducted. With DMSO as a control, CYP1A4 mRNA in CEH was significantly altered by SI-TeDB-DiPhOBz or SI-BDE-209 at 1.5 μM or greater. The greatest fold changes were 5.3±2.1 and 66±6.9 for SI-TeDB-DiPhOBz and SI-BDE-209, respectively. These results demonstrated that photolytic degradation of TeDB-DiPhOBz and BDE-209 also occurs in a solvent-free system, and can generate by-products that induce the alteration of CYP1A4 mRNA in CEH.

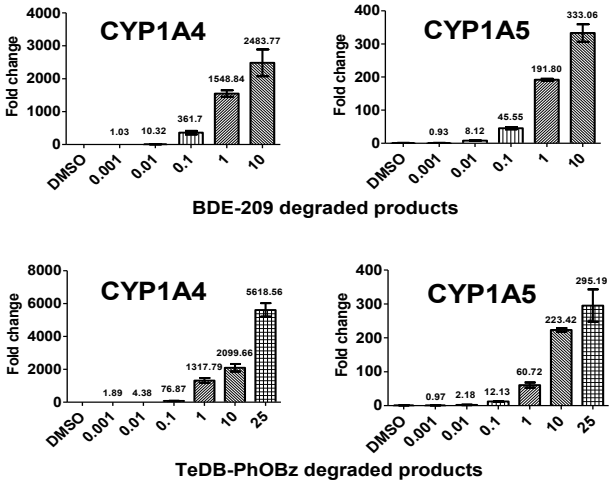

Figure 2. Concentration-dependent effects of SI-BDE-209 and SI-TeDB-DiPhOBz on CYP1A4 and CYP1A5 mRNA expression in chicken embryo hepatocyte cultures after exposure 36 h.

Given the lesser degree of BFR photodegradation and by-product formation for the BFRs as solids, identification of transformation products in SI-BFR (in addition to debrominated by-products[7]) was only possible for the solvent systems. Transformed products of BFR in solution were chemically identified following three criteria: 1) molecular masses were calculated based on their phenoxide ion $[M\text{-}Br+O]^-$, which was normally regarded as a precursor ion of PBDEs in an APPI(-) source using a toluene dopant; 2) the measured *m/z* value should be within 10 ppm of its calculated value, and any observed Br anion isotopic peak number and response ratio should match with the predicted structure; 3) a retention time gap should be observed between identified chemicals and products of debromination since the $[M\text{-}HBr]^-$ or $[M\text{-}Br_2]^-$ anions might produce furan structures in the APPI(-) source.

Following the above criteria, numerous mono- and di-furan structures were identified in addition to the debrominated products. We identified a Br_4-di-dibenzofuran structure, which exhibited exactly the same mass characteristics as predicted (i.e. molecular mass, Br isotope peak number and response ratio, retention gap with any products of debromination) (Figure 3). Based on the specific structural properties of dioxin-/furan-like compounds, these coplanar, brominated di-dibenzofurans might be responsible for AhR-mediated potencies. These chemicals also share structures that are similar to a photo-oxidation product of tryptophan, 6-formylindole[3,2-b]carbazole (FICZ), which is an endogenous ligand that binds with high affinity, and initiates AhR signaling at small concentrations (nM), and is metabolized to inactive metabolites[9,10]. For SI-BDE-209, Br_2 to B_6-dibenzofuran (BDF) homologs were identified, which is consistent with a study where di- to octa-BDFs were detected in decaBDE-containing plastics that had been exposed to sunlight[11]. PBDFs are active ligands for AhR-mediated signaling, which explains the TCDD-like gene expression in CEH exposed to SI-BDE-209, although the relative potency of the PBDF by-products is currently unclear.

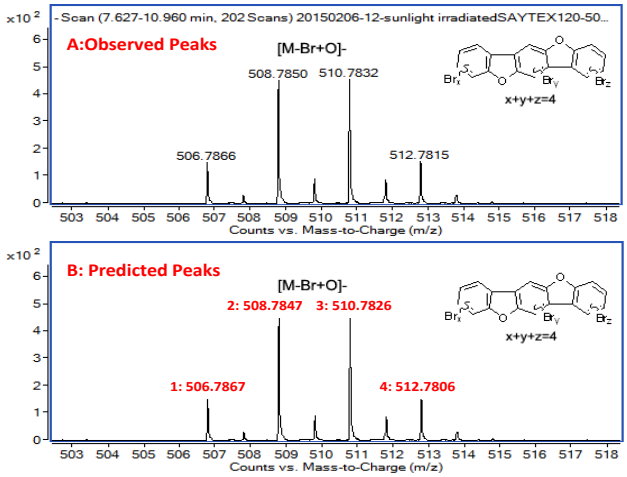

Figure 3. Observed (A) and predicted (B) peak number and *m/z* values of Br_4-di-dibenzofuran structure assumed to be formed from TeDB-DiPhOBz in solution during solar irradiation.

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