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Elucidating toxicological mechanisms of current flame retardants using a bacterial gene profiling assay

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ABSTRACT

Flame retardants are ubiquitously used chemicals that have been shown to contaminate environments. Toxicological data is largely limited, with little insight into their molecular modes of action that may give rise to their toxic phenotypes. Such insight would aid more effective risk assessments concerning these compounds, while also improving molecular design. We therefore used a bacterial stress-gene profiling assay to screen twelve currently-used flame retardants to obtain mechanistic insights of toxicity. Both brominated and organophosphate flame retardants were tested. All compounds showed statistically significant inductions of several stress genes when compared to control treatments. Triphenyl phosphate, tris(2-butoxyethyl) phosphate, tris(1,3-dichloro-2-propyl)phosphate, tris(butyl)phosphate, and tetrabromobisphenol A elicited (at least) two-fold inductions for any of the stress genes. When looking at absolute induction levels, the promoters induced are indicative of protein perturbation, DNA integrity and membrane integrity. However, normalising for the different induction potentials of the different stress genes and clustering using hierarchical and k-means algorithms indicated that in addition to protein and DNA damage, some compounds also resulted in growth arrest and oxidative damage. This research shows that this assay allows for the determination of toxicological modes-of-action while clustering and accounting for induction potentials of the different genes aids better risk assessment.

1. Introduction

Flame retardants (FRs) are compounds that are designed to prevent the initiation and spread of fire. Several FRs also serve roles as plasticisers (Meeker and Stapleton, 2010). Due to the need for safer consumer products, FRs are found in nearly all manufactured items. Given its ubiquitous use, these compounds are produced in large volumes, which are predicted to increase due to the ever-growing global population and urbanisation. Some of these FRs are shown to diffuse out of the materials, thereby contaminating surrounding environments (Covaci et al., 2006; De Wit et al., 2010) and biota (Covaci et al., 2011). Accordingly, many compounds have been detected in household dust (Abdallah and Harrad, 2009; Ali et al., 2011; Harrad et al., 2010; Dodson et al., 2012), while some have even been found as far as the Arctic, indicating long-range atmospheric transport and a high level of persistence associated with these compounds (De Wit et al., 2010). Exposure to these FRs is therefore not limited solely to humans, and these compounds are likely to pose a significant risk to the environment. Given their persistence and hydrophobicity, bioaccumulation of these chemicals is also possible; *e.g.* traces of FRs were detected in human mother's milk (Abdallah and Harrad, 2011; Lignell et al., 2009).

Several previously-used FRs have been shown to elicit adverse health effects. The compound 2,3-dibromopropanol, for example, was used in childrens' sleepwear to render it "safe" in the context of retarding the initiation and spread of fire. However, as it was shown to be highly mutagenic, it was banned from further use (Blum et al., 1978). As recently as 2013, hexabromocyclododecane (HBCD) was initially considered safe, but is now selected to be phased out of use by 2015 after years of use, due to its persistence (De Wit et al., 2010), bioaccumulation (Law et al., 2008), and toxicity (Ema et al., 2008). The industry is therefore constantly, but mainly *post factum*, adapting to new regulations, with new compounds and/or mixtures being regularly introduced. Initially, brominated flame retardants (BFRs) were the compounds of choice due to their low cost and high performance (Birnbaum and Staskal, 2004). However, their persistence, bioaccumulation, and toxicity urged the industries to explore alternatives. So-called novel BFRs, which are structurally similar to their toxic predecessors, and organophosphate flame retardants (PFRs), are currently used, with PFRs being preferred given their high concentrations being detected in house dust (Stapleton et al., 2009). Several toxicological endpoints are used to assess their threat to health, including measuring acute toxicity by monitoring lethal dose values, skin sensitisation, carcinogenic potential, immuno-, reproductive effects, and genotoxicity. However, in-depth knowledge on the molecular mechanisms (*i.e.* mode-of-action) by which compounds may elucidate their toxicological profile is

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still lacking for most of these currently-used FRs. Mode of action (MOA) studies are often limited to investigating endpoints related to hormone-dependent pathways, and even then, conclusions are often contradictory. Some mechanistic insights have been obtained for tetrabromobisphenol A (TBBPA), hexabromocyclododecane, and more recently tris(1,3-dichloro-2-propyl)phosphate (TDCPP). Studies have shown interference of these compounds with a wide variety of cellular processes, and in particular, production of reactive oxygen species in a number of different organisms (Xue et al., 2009; Hendriks et al., 2014; Choi et al., 2011; Ronisz et al., 2004).

We adopt here a bacterial gene profiling assay to gain further insight into the MOA of twelve FRs. All compounds except for 9,10-dihydro-9-oxa-10-phosphaphenanthrene (DOPO) have recently been detected in house dust (Dodson et al., 2012), indicating broad use of these FRs. DOPO was included since it is accepted as a suitable alternative to halogenated FRs given its lack of acute and chronic toxicity (Waaijers et al., 2013a; Salmeia and Gaan, 2015). This screening assay allows for the rapid and cost-effective assessment of compounds to induce several stresses when compared to other eukaryotic screening assays, while maintaining reproducibility comparable to OECD-approved whole-organism acute toxicity tests (Dardenne et al., 2007a). Stresses measured include oxidative stress, protein degradation, DNA damage, membrane damage, and growth arrest. Bacterial biosensors are frequently used to assess ecotoxicological impacts of compounds since they are particularly useful in compound screening and classification according to MOA (Nobels et al., 2010, 2011; Tajima, 2003; Dardenne et al., 2007b; Sommers and Mackay, 2005). Clustering analysis by hierarchical and k-means clustering algorithms were used to determine clusters of potential MOAs.

2. Materials and methods

2.1. Chemicals

Two classes of currently-used FRs were tested; PFRs and BFRs containing two novel brominated FRs (NBFRs). All compounds except TBPH and DOPO were obtained from Sigma-Aldrich (USA) (Table 1). TBPH and DOPO were obtained from Santa-Cruz Biotechnologies (USA), and Tokyo Chemical Industry (JAP), respectively. Stock solutions of 1 M in 100% DMSO (Thermo-Fisher Scientific, USA) were prepared for TPP, TBEP, TCEP, TnBP, TEP, DOPO, and TBBPA. Due to limited solubility at 1 M, stock solutions of 0.1 M were made up for TCPP, TDCPP, TBPH, TBC, and HBCD. The highest concentrations used per compound in the bacterial stress-gene assay are indicated in Table 1.

2.2. Bacterial strains

All *Escherichia coli* strains contain a specific stress gene promoter::*lacZ* fusion, as documented in Table 2. Each strain, except for *PQ37*, was based on the *E. coli* K12 derivative strain, SF1. SF1 strains contain the mutation *lac4169* which results in the deletion of the entire *lac* operon and *rpsL*, rendering the strains resistant to streptomycin (Eisentraeger et al., 2007). The *PQ37* strain is derived from *E. coli* GC4436 and contains the *lacZ* gene under the control of the *SfiA* promoter (Quillardet and Hofnung, 1985). All strains were stored at −80 °C in Viabank™ vials (Medical Wire and Equipment, UK) upon further use in the bacterial stress-gene profiling assay.

2.3. Bacterial stress-gene profiling assay

The bacterial stress-gene profiling assay has been performed as previously described (Dardenne et al., 2007a; Nobels et al., 2011). The assay was performed as three technical repeats in 96-well plates (Sterilin Ltd, UK). Each row of the 96-well plate contained a different strain. The resulting plates were incubated for 90 min at 37 °C while shaking (200 rpm). Optical density (600 nm) was measured to check for uniformity (pre-dose time point). Next, the compound to be tested was added to the wells at different concentrations in 5% DMSO. Again, optical density (600 nm) was measured to check for compound precipitation in the form of aggregates and precipitates that may result in an increased optical density (start exposure time point). Successive 1:2 dilutions were performed ultimately resulting in the lowest concentration being 1/128th of the highest concentration. The plates were allowed to incubate at 37 °C for 90 min while shaking (200 rpm), followed by a final measurement of the optical density (600 nm) (post-exposure time point). Cells were lysed for 15 min at 20 °C with 0.5 mg/ml polymyxin B sulphate (Sigma-Aldrich, USA) in lysis buffer (4% Triton X-100 (Sigma-Aldrich, USA)). Lysate was then transferred to 96-well plates containing ONPG buffer (40 mM $NaH_2PO_4.H_2O$, 70 mM $(NaHPO_4).2H_2O$, 10 mM KCl, 2 mM $MgSO_4.7H_2O$, 0.4% ortho-Nitrophenyl-β-galactoside) and optical density at 420 nm was immediately measured (start exposure time point). After the incubation step for 15 min at 20 °C, optical density was monitored again (post-exposure time point). The reduction ONPG (colourless) to ONP (yellow) by β-galactosidase is a direct measure of the activity of the promoters in response to treatment with FR compounds. Detailed specifications and performance of the assay were previously published (Dardenne et al., 2007a). Additionally, quality control of assay performance was evaluated throughout the course of experiments by reference compounds (Tables S1 and S2, Supplementary information). Stress gene inductions were comparable to previously achieved results with the same reference compounds (Dardenne et al., 2008).

2.4. Determination of optimal test concentrations

The maximum test concentrations used for each compound as depicted in Table 1 were determined by means of a prokaryotic proliferation assay. This was conducted on the strain containing the *ClpB* promoter since this strain consistently showed increased cytotoxicity to FR compounds. Briefly, the protocol involved inoculating and incubating

Table 1
All compounds with highest concentration tested and separated into their major classes of flame retardants.

Class	Chemical name	Abbreviation	CAS number	Highest concentration tested (μM)	$LogK_{ow}$	Supplier
PFR	Tris (1-chloro-2-propyl) phosphate	TCPP	13674-84-5	100	2.59	Sigma-Aldrich
	Triphenyl phosphate	TPP	115-86-6	75	4.59	Sigma-Aldrich
	Tris(2-butoxyethyl) phosphate	TBEP	78-51-3	300	3.75	Sigma-Aldrich
	Tris (1,3-dichloro-2-propyl) phosphate	TDCPP	13674-87-8	100	3.65	Sigma-Aldrich
	Tri(2-chloroethyl)phosphate	TCEP	115-96-8	5000	1.44	Sigma-Aldrich
	Tris(butyl) phosphate	TnBP	126-73-8	300	4.00	Sigma-Aldrich
	Triethyl phosphate	TEP	78-40-0	5000	0.80	Sigma-Aldrich
	9,10-dihydro-9-oxa-10-phosphaphenanthrene	DOPO	35948-25-5	5000	1.87	Tokyo Chemical Industry Co.
BFR	Hexabromocyclododecane	HBCD	3194-55-6	12.5	5.62	Sigma-Aldrich
	Tetrabromobisphenol A	TBBPA	79-94-7	25	5.90	Sigma-Aldrich
	Bis(2-ethylhexyl) tetrabromophthalate	TBPH	26040-51-7	25	11.95	Santa-Cruz Biotechnology
	Tris(2,3,-dibromopropyl) isocyanurate	TBC	52434-90-9	12.5	7.37	Sigma-Aldrich