Multiparameter toxicity assessment of novel DOPO-derived organophosphorus flame retardants

Cordula Hirsch[1], Britta Striegl, Stephanie Mathes, Christian Adlhart, Michael Edelmann, Epifania Bono, Sabyasachi Gaan, Khalifah A. Salmeia, Lisa Hölting, Alice Krebs, Johanna Nyffeler, Regina Pape, Alexander Bürkle, Marcel Leist, Peter Wick, Stefan Schildknecht

C. Hirsch, P. Wick
Particles-Biology Interactions Laboratory, Swiss Federal Laboratories for Materials Science and Technology (Empa), CH-9014 St. Gallen, Switzerland
S. Gaan, K. A. Salmeia
Advanced Fibers Laboratory, Swiss Federal Laboratories for Materials Science and Technology (Empa) , CH-9014, St. Gallen, Switzerland

S. Mathes, B. Striegl, C. Adlhart, M.Edelmann
ZHAW; Life Sciences and Facility Management; Einsiedlerstr. 31;
8820 Wädenswil, Switzerland

S. Schildknecht, L. Hoelting, J. Nyffeler, A. Krebs, R. Pape, A. Bürkle, M. Leist
University of Konstanz, Universitaetsstr. 10;
78457 Konstanz, Germany

Running head: Flame retardant toxicity

Word count:
Reference numbers:
Number of greyscale illustrations:
Number of color illustrations:

Key words: flame retardants, OPFR, organophosphate, dermal absorption, neurotoxicity

[1]corresponding author

Acknowledgements:

We thank Xenia Mäder-Althaus and Laura Nel for excellent technical support.

Abstract

Halogen-free organophosphorus flame retardants are considered as replacements for the phased-out class of polybrominated diphenyl ethers (PBDEs). Toxicological information on new flame retardants however is still limited. Based on their excellent flame retardation potential, we selected three novel 9,10-dihydro-9-oxa-10-phosphaphenanthrene-10-oxide (DOPO)-derivatives and assessed their toxicological profile by a battery of *in vitro* test systems in order to provide toxicological information before their large-scale production and use. PBDE-99, applied as reference compound, exhibited distinct neuro-selective cytotoxicity at concentrations ≥ 10 µM. ETA-DOPO and EG-DOPO displayed adverse effects at concentrations > 10 µM in test systems reflecting the properties of human central and peripheral nervous system neurons, as well as in a set of non-neuronal cell types. DOPO and its derivative 6,6'-(ethane-1,2-diylbis(azanediyl))bis(6H-dibenzo[1,2]oxaphosphine 6-oxide (EDA-DOPO) were neither neurotoxic, nor generally cytotoxic, nor did they exhibit an influence on neural crest cell migration, inflammatory activation or the integrity of human skin equivalents at concentrations ≤ 400 µM. Furthermore no impact on algae growth and the viability of daphnids, could be detected up to 1000 mg/l (water soluble fraction).. Based on its superior flame retardation properties, its biophysical features suited for use in polyurethane foams, and its low cytotoxicity, EDA-DOPO emerges as a candidate for the replacement of currently applied flame retardants.

Introduction

Flame retardants are added to a wide range of materials in order to lower the risk of uncontrolled ignition and for the reduction of combustion rates. In recent years, the class of polybrominated diethyl ether retardants has been phased out, based on a growing understanding of their abundant prevalence in the environment and their persistence and bioaccumulation in the food chain (Covaci et al. 2011; Shaw et al. 2010; Hites et al. 2004). A developing number of observations indicated adverse influences of polybrominated flame retardants on endocrine functions mediated e.g. by thyroid-, androgen-, and estrogen-signaling pathways (Zhou et al. 2002; Li et al. 2013; Stroker et al. 2005), but also on kidney and liver integrity (Albina et al. 2010), the cholinergic system (Viberg et al. 2002), neuronal differentiation (Dingemans et al. 2007; Herbstman et al. 2010), and on the migration of neuronal precursors during development (Schreiber et al. 2010). Strict legal requirements on flammability properties are only one issue impeding the delicate choice for an alternative to brominated flame retardants. Beyond that, toxicological information plays an important role for human safety. Unfortunately such information is rather limited. As a promising approach for the substitution of polybrominated compounds, phosphorus flame retardants such as the inorganic red phosphorus or ammonium polyphosphate (Schartel et al. 2010), halogenated phosphorus flame retardants, and phosphonamidates and phosphoesters (Costa et al. 2007; van der Veen et al. 2012) are currently applied to meet regulatory flammability standards. Several of these replacements are additives and not covalently bound to their host material, thus allowing their diffusion out of the host material by volatilization, leaching, or by mechanical abrasion (Marklund et al. 2003). In case of the chlorinated phosphorus flame retardant tris(chloropropyl)phosphate (TCPP), that was intensively applied as alternative for

brominated additives, concentrations in the environment, but also in indoor ambient air and in household dust, even exceeded levels of polybrominated diphenyl ethers (PBDEs) (Staat et al. 2005; Tollbäck et al. 2006; van der Veen et al. 2012; Martinez-Carballo et al. 2007). Recent observations indicated a carcinogenic potential of TCPP (Ni et al. 2007; WHO 1998), hence justifying concerns on whether the alternatives currently used actually represent a lower risk than the compounds they are supposed to replace (Behl et al. 2015). These safety concerns on the industrial application of phosphorus flame retardants deserve even more attention when it is considered that structurally related phosphodiester compounds, such as organophosphorus pesticides, were reported to adversely influence the development of the nervous system and are indeed also used as chemical warfare agents (Gonzalez-Alzaga et al. 2014; Munoz-Quezada et al. 2013).

One of the potential substituents, that represents the basis for the here introduced novel structures, is the halogen-free organophosphorus flame retardant 9,10-dihydro-9-oxa-10-phosphaphenanthrene-10-oxide (DOPO) that displays excellent flame retardation properties, and exhibited an only low acute toxicity potential in the currently available studies (Waaijers et al. 2013a, 2013b). In order to improve its flame retardation potential, and to influence its biophysical parameters such as its melting point, which is requested by the industry for application in different plastic materials, novel phosphonamidate and phosphorester derivatives of DOPO have been described as alternatives (Gaan et al. 2009, 2014; Buczko et al. 2014; Neisius et al. 2014) but were so far not evaluated with respect to their toxicological potential. In the combustion process, these phosphonamidates primarily act in the vapor phase by the formation of phosphorus-containing low energy radical species ($PO^{\bullet}$). These radical species interact with $^{\bullet}OH$ or $H^{\bullet}$ radicals (equation 1) that are formed during combustion, and therefore lead to a reduction in the release of heat, hereby favoring the extinguishing of the

flame. In a subsequent step, the reduced phosphorus compound interacts with a second $H^{\bullet}$ radical (or $^{\bullet}OH$), allowing a recycling of the $PO^{\bullet}$ species (equation 2)

(1) $PO^{\bullet} + H^{\bullet} \longrightarrow HPO$

(2) $2\ HPO + 2\ H^{\bullet} + O_2 \longrightarrow 2\ PO^{\bullet} + 2\ H_2O$

The application of such novel DOPO-derived phosphorus flame retardants is currently precluded by limited information regarding their adverse effects on human development and health. In the present study, we assessed three novel *bis*-DOPO-derived flame retardants in direct comparison to their parental compound DOPO and to the polybrominated diphenyl ether PBDE-99 with respect to their cytotoxic potential, their skin sensitization and skin penetration properties, and their neurotoxic and neurodevelopmental properties by a battery of different *in vitro* test systems – pursuing the 3R concept. We relate the cytotoxicity profile to viability data in Daphnids and growth rates of algae. These toxicological investigations, together with a comparison of their flame retardation properties when applied as additives in polyurethane foams, indicated that 6,6'-(ethane-1,2-diylbis(azanediyl))bis(6H-dibenzo[1,2]oxaphosphine 6-oxide (EDA-DOPO) combines both superior flame retardation features with a significantly lower cytotoxic potential and might therefore emerge as a new candidate for the substitution of currently applied flame retardants.

Experimental procedures

Synthesis of DOPO-derivatives

9,10-dihydro-9-oxa-10-phosphaphenanthrene-10-oxide (DOPO) was purchased from Metadynea GmbH (Austria), all other chemicals were purchased from Sigma Aldrich (Switzerland) and were used without further purification. NMR spectra were collected at

ambient temperature using a Bruker Avance 400 NMR spectrometer. ^{1}H and $^{13}C\{H\}$ NMR spectra were referenced against the solvent (DMSO-d6). $^{31}P\{H\}$ NMR spectra were referenced against external 85% H_3PO_4.

Synthesis of EG-DOPO: A four neck round bottom flask (1 l) connected to additional funnel, condenser, mechanical stirrer, thermometer and bubbler, was charged with DOPO (100 g, 0.46 mol), Ethylene glycol (14.35 g, 0.23 mol), 1-methylimidazole (39.88 g, 0.48 mol) and CH_3CN (300 ml). The reaction vessel was immersed in an ice bath and CCl_4 (72.93 g, 0.47 mol) was added under N_2 at a rate such that the reaction temperature did not exceed 10 °C. After complete addition, the reaction temperature was allowed to warm up to ambient temperature, and the reaction was refluxed for 3 h. The reaction was then cooled down to 40 °C, and poured into DCM (300 ml). The organic was then washed with water (3 x 500 ml) and brine (1 x 500 ml) and dried over Na_2SO_4. The organic solution was filtered and the volatiles were removed under reduced pressure. The solid was then dissolved in minimum amount of Ethanol. Water (4 l) was then added slowly with vigorous stirring. The mixture was then stirred overnight. The aqueous solution was decanted and the washing step was repeated until the product becomes hard solid. The product was collected and extra dried under vacuum at 60 °C. Yield: 72.5 g, (64 %), m.p. 110.4 °C and 147.6 °C. Set of diastereomers: ^{1}H NMR (DMSO-d_6, 400.18 MHz) δ (ppm): 8.23-8.20 (m, 2H), 8.15 (dd, 2H, J = 8.0 Hz, J = 1.5 Hz), 7.84-7.80 (m, 2H), 7.75-7.68 (m, 2H), 7.54 (ds, 2H, J = 3.7 Hz, J = 0.8 Hz), 7.45-7.40 (m, 2H), 7.34-7.30 (m, 2H), 7.20 (dd, ^{1}H, J = 2.7 Hz, J = 1.2 Hz), 7.18 (dd, ^{1}H, J= 2.7 Hz, J= 1.2 Hz), 4.28-4.08 (m, 4H). ^{13}C NMR (DMSO-d_6, 100.62 MHz) δ (ppm): 149.05 (d, J = 7.7 Hz), 136.19 (d, J = 7.2 Hz), 134.02 (d, J = 2.5 Hz), 130.88, 129.73 (d, J = 9.3 Hz), 128.67 (d, J = 15.2 Hz), 125.82, 125.07, 124.62 (d, J = 11.8 Hz), 121.80 (d, J = 11.8 Hz), 121.36 (d, J = 179.3 Hz), 119.84 (d, J = 6.6 Hz), 65.29 (dt, J = 5.9 Hz, J = 1.9 Hz). ^{31}P NMR (DMSO-d_6, 161.99 MHz) δ (ppm) 9.91

Synthesis of EDA-DOPO: A four neck round bottom flask (1 l) connected to additional funnel, condenser, mechanical stirrer, thermometer and bubbler, was charged with DOPO (75 g, 0.35 mol), Ethylenediamine (8.34 g, 0.14 mol), Triethylamine (38.62 g, 0.38 mol) and $CHCl_3$ (400 ml). The reaction vessel was immersed in an ice bath, and CCl_4 (58.70 g, 0.38 mol) was added under N_2 at a rate such that the reaction temperature did not exceed 10 °C. After complete addition, the reaction temperature was allowed to warm up to ambient temperature and stirred overnight. The white product was then collected by filtration and was washed with $CHCl_3$, Ethanol and then water, respectively. The product was collected and extra dried under vacuum at 60 °C. Yield: 60.1 g, (88.7%), m.p. 255-257 °C. Set of diastereomers: ^{1}H NMR (400.18 MHz, DMSO-d_6) δ (ppm): 8.17- 8.11 (m, 4H), 7.80-7.76 (m, ^{1}H), 7.75-7.70 (m, 3 H), 7.56-7.49 (m, 2H), 7.43-7.38 (m,2H), 7.30-7.25 (m, 2H), 7.18-7.13 (m, 2H), 5.76-5.71 (m, 2H), 2.89-2.81 (m, 4H). ^{13}C NMR (100.62 MHz, DMSO-d_6) δ (ppm): 149.41 (d, J = 7.2 Hz), 135.97 (d, J = 6.8 Hz), 132.74, 130.37, 129.41 (d, J = 9.6 Hz), 128.34 (d, J = 14.3 Hz), 125.42 (d, J = 0.8 Hz), 125.24 (d, J = 161.7 Hz), 124.27, 124.08 (d, J = 10.7 Hz), 121.92 (d, J = 11.5 Hz), 120.05 (d, J = 5.8 Hz), 41.75 (d, J = 5.7 Hz). ^{31}P NMR (161.99 MHz, DMSO-d_6) δ (ppm): 15.29, 15.25.

Synthesis of ETA-DOPO: A four neck round bottom flask (1 l) connected to additional funnel, condenser, mechanical stirrer, thermometer and bubbler, was charged with DOPO (150 g, 0.63 mol) , Ethanolamine (21.2 g, 0.34 mol), Triethylamine (77.23 g, 0.76 mol) and $CHCl_3$ (400 ml). The reaction vessel was immersed in an ice bath, and CCl_4 (117.40 g, 0.76 mol) was added under N_2 at a rate such that the reaction temperature did not exceed 10 °C. After complete addition, the reaction temperature was allowed to warm up to ambient temperature and stirred overnight. The organic solvent was then concentrated under reduced pressure and the white precipitate was collected by filtration. The filtrate was then completely evaporated

under reduced pressure. The white product was then combined and washed with ethanol and water successively. The product was collected and extra dried under vacuum at 60 °C. Yield: 135.8 g, (80 %), m.p. 177-178 °C. Set of diastereomers: ^{1}H NMR (400.18 MHz, DMSO-d_6) δ (ppm): 8.25 (t, J = 7.2 Hz, J = 1H), 8.21-8.12 (m, 3H), 7.93-7.83 (m, 2H), 7.74-7.69 (m, 1H), 7.68-7.59 (m, 2H), 7.48-7.39 (m, 3H), 7.34 (dt, J = 0.8 Hz, J = 7.6 Hz, 1H), 7.31-7.26 (m, 2H), 7.15 (ddd, J = 1.0 Hz, J = 8.1 Hz, J = 14.0 Hz, 1H), 5.87-5.80 (m, 1H), 4.13-3.94 (m, 2H), 3.11-2.91 (m, 2H). ^{13}C NMR (100.62 MHz, DMSO-d_6) δ (ppm): 149.35-149.15 (8 resonances), 136.29-135.87 (8 resonances), 134.03 (d, J = 2.4 Hz), 132.75 (d, J = 2.2 Hz), 130.92 (d, J = 0.8 Hz), 130.40), 129.93 (dd, J = 2.0 Hz, J = 9.3 Hz), 129.43 (d, J = 9.6 Hz), 128.75 (dd, J = 1.1 Hz, J = 15.1 Hz), 128.27 (dd, J = 3.2 Hz, J = 14.3 Hz), 125.90-125.87 (3 resonances), 125.41, 125.29 (dd, J = 2 Hz, J = 163.0 Hz), 125.09, 124.66 (d, J =11.7 Hz), 124.30, 124.06 (dd, J = 1.3 Hz, J = 11.1 Hz), 122.02-121.84 (8 resonances), 121.57 (d, 178.8 Hz), 120.14-120.05 (4 resonances), 119.92-119.83 (4 resonances), 66.19-66.03 (m), 40.43 (d, J = 7.0 Hz). ^{31}P NMR (161.99 MHz, DMSO-d_6) δ (ppm): 14.59, 14.57, 9.83, 9.79 (Buczko et al. 2014; Salmeia et al. 2015)

UL94-HB (horizontal burning) fire tests

The UL-94 (ATSM D 4986 or ISO 9772) flammability standard test was applied to classify the flame retardation potential of the organophosphorus compounds embedded as additives in polyurethane (PU). The PU foams were cut to the dimension of 150 mm x 50 mm x 13 mm (length x width x thickness). Test specimens were marked at 25 mm, 60 mm, and 125 mm. The test specimens were placed on an iron grid in a horizontal position with a cotton indicator beneath. A 38 mm flame was then applied to the end of the specimen for 60 s and then removed. The time required for burning of a 100 mm long segment was recorded. The flame retardation potential of the different compounds tested was classified according to the UL-94

horizontal flammability criteria. Classification: HF 1: burning time after removal of external flame: ≤ 2 s in 80 % of the test specimens and ≤ 10 s in the remaining 20 % of test specimens. Afterglow time for each individual specimen: ≤ 30 s. Damaged length for each specimen < 60 mm. No ignition of cotton indicator by burning drops or particles. HF 2: same conditions as HF 1 with the exception that cotton is ignited by buring drops or particles. HBF: burning rate ≤ 40 mm/min over a distance of 100 mm or burning length ≤ 125 mm. UL 94 horizontal ratings classifications HF1 > HF2 > HBF > no ratings.

Toxicity assessment in human alveolar epithelial cells

The human alveolar epithelial cell line A549 (ATCC: CCL-185) was grown in Roswell Park Memorial Institute (RPMI-1640) medium (Sigma-Aldrich) supplemented with 10% FCS (Lonza), 0.2 mg/ml L-glutamine (Gibco), 50 µg/ml penicillin (Gibco), 50 µg/ml streptomycin (Gibco), and 100 µg/ml neomycin (Gibco) in humidified atmosphere at 37°C and 5% CO_2 (thereafter referred to as complete cell culture medium and standard growth conditions, respectively). Cells were subcultured at approximately 80-90% confluency using 0.5% Trypsin-EDTA (Sigma-Aldrich). For viability assessment, 1.5 x 10^4 cells were seeded in 200 µl complete cell culture medium per well of a 96-well plate and grown over night under standard growth conditions. Thereafter, cells were treated for 48 h with 200 µl standard growth medium per well containing increasing concentrations of the respective flame retardants or $CdSO_4$ as the positive control. The CellTiter96® AQueous One Solution (Promega) containing MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H) as the water soluble tetrazolium compound was used according to the manufacturer's protocol and detected at 490nm.

Toxicity assessment in human macrophages

The human monocytic cell line THP-1 (ATCC: TIB-202) was grown as a single cell suspension in complete cell culture medium at standard growth conditions. Subculturing was performed by replacement of medium at cell concentrations starting from 8 x 10^5 cells/ml. Cell concentrations were not allowed to exceed 10^6 cells/ml. To differentiate THP-1 monocytes into macrophages, cells were grown in the presence of 200 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 72 h. For viability assessment, 8 x 10^4 cells were seeded in 200 µl complete cell culture medium containing 200 nM PMA per well of a 96-well plate and differentiated for 72 h under standard growth conditions. Thereafter, medium was removed, cells were washed twice with pre-warmed PBS and increasing concentrations of flame retardants or $CdSO_4$ were added in 200 µl complete cell culture medium per well for 48 h. The CellTiter96® AQueous One Solution (Promega) containing MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H) as the water soluble tetrazolium compound was used according to the manufacturer's protocol and absorption was detected at 490 nm.

3D epidermal in vitro model

Human primary keratinocytes (PR3D-HPEK-50, CELLnTEC, Bern, Switzerland) were grown in flasks in proliferation medium (CNT-PR, CELLnTEC) until sub-confluency was reached. The cells were then seeded into transwell inserts containing polycarbonate membranes with a pore size of 0.4 µm at a density of $3.3*10^5$ cells/cm^2 and grown for 3 days in CNT-PR until confluency was reached. Differentiation was initiated by the addition of 3D barrier medium (CNT-PR-3D, CELLnTEC) for 18 h to allow development of cell-to-cell adhesion structures.

3D growth and differentiation was induced by complete removal of medium from the upper transwell compartment and the replacement of CNT-PR-3D differentiation medium in the lower compartment. Cells were allowed to differentiate for 11 days with 3 medium changes per week in the lower compartment and direct cell-to-air contact on the top. Flame retardants were suspended at a concentration of 200 mM in PBS (phosphate buffered saline) containing 5 % DMSO (dimethyl sulfoxide) and 100 µl were applied on top of the epithelial barrier model according to the OECD guidelines for chemicals testing on Reconstructed Human Epidermis "*In vitro* skin irritation tests". 5 % SDS (sodium dodecyl sulfate) was used as cytotoxic chemical (positive control) and 5 % DMSO in PBS as negative control. The epidermal models were treated for 15 min at 37 °C and washed 3 times with PBS, before they were further cultivated for 48 h in fresh medium. Cell viability in the epidermal models was measured by resazurin reduction to resorufin (PrestoBlue® Assay). Cell viability values (fluorescence signals) were normalized to epidermal models thickness. Tissues were later washed with PBS and fixed in 10 % formalin at room temperature for 2.5 h. The membranes carrying the 3D epidermis were removed from the transwell plates and washed using PBS. The 3D epidermis was dehydrated with the consecutive addition of: 70 % EtOH for 1 h; 96 % EtOH for 1 h; 96 % EtOH for 1 h, 100 % EtOH for 1 h, 100 % EtOH for 1 h; extraction solvent 1 h; extraction solvent 1.5 h, paraffin 1.5 h, paraffin 1.5 h. Paraffin blocks were cut with a microtome (Zeiss, Hyrax M40) into 5 µm slices that were heated at 55°C for 1 h. Samples were re-hydrated in xylol and decreasing ethanol concentrations (2 min) and placed in water for 2 min, before they were stained with hematoxylin for 15 s. After this staining step, the slides were `blued` under rinsing tap water (5 min) and subsequently applied to 75 % EtOH and 96 % EtOH for 2 min in order to stain them with alcoholic eosin (4 min). Finally the slides were washed using 96 % ethanol and dehydrated with abs. EtOH and xylol. Samples were mounted with DPX mounting medium (Sigma-Aldrich) and analyzed by microscopy (Olympus IX 81, Programm Cell Sense).

Skin sensitization assay

Generation of the stable cell line KeratinoSens was previously described in detail (Emter et al. 2010). Briefly, HaCaT cells were stably transfected with a luciferase reporter gene under the control of a copy of the antioxidant response element (ARE) of the human aldo-keto-reductase family 1, member C2 (AKR1C2) gene. KeratinoSens cells were maintained in DMEM containing glutamax (Gibco/Invitrogen), 9 % FCS and 0.5 mg/ml G418. For experiments, the cells were seeded onto 96-well plates at a density of 10.000 cells per well in 125 µl medium without G418. After 24 h, medium was replaced by 200 µl DMEM containing 1% FCS and the compounds tested. After a 2 day incubation period at 37°C, 5% CO_2, the cells were washed once with PBS and a Resazurin reduction assay (PrestoBlue®) was carried out in order to compare the viability of the differently treated cells. The fluorescence intensity was measured at 530 nm_{ex} and 590 nm_{em}. After another washing step with PBS cell lysis was performed using 20 µl/well passive lysis buffer (Promega, Duebendorf, CH) for 20 min at RT. Luciferase activity was measured by applying 50 µl luciferase substrate automatically to each well and detecting thereby a luminescence signal.

Quantitative detection of flame retardants in porcine skin by confocal Raman spectroscopy

The vertical distribution of topically applied DOPO derivatives and reference compounds was investigated on segments of porcine ears from freshly slaughtered animals. The skin was separated from the cartilage, depilated, and placed into Franz type diffusion cells (xxx cm^2). The acceptor solution was PBS buffer containing XXX % of IGEPAL® CA-630 (Sigma Aldrich). Test compounds were dispersed at a concentration of 400 µM (2 % in case of caffeine) in PBS buffer containing XXX % of IGEPAL® CA-630 300 µl of this mixture including a blank solution were topically applied to the porcine skin and allowed to penetrate

for 60 min. Then, the skin was removed from the Franz cells, gently washed, and placed to the optical window of the inverse confocal Raman microscope (Model 3510 SCA, *River Diagnostics)*. The Raman microscope is equipped with a 60x oil-immersion objective. The 785 nm excitation laser was used to acquire spectra in the fingerprint region (400 – 1800 cm^{-1}, integration time 5 s) starting from the skin's surface down to a depth of 60 µm (step size of 2 µm). The acquisition was repeated at five positions at least. Averaged concentration profiles of flame retardants and reference compounds were extracted by fitting (unrestricted classical least squares, third order polynomial baseline) the Raman spectra with reference spectra of keratin (as representative of all proteins), water, ceramide 3 and cholesterol (as representatives of all lipids), NMF (fixed composition of natural moisturizing factor), lactate, urea, and the solid test compounds using Skin Tools software. Concentrations of test substances are given as molar fraction relative to keratin rather than correcting for the Raman signal attenuation by mathematical models (Franzen et al. 2013). Response factors of the test compounds had been determined against BSA solutions assuming similar Raman cross-sections for keratin and BSA as described in the literature (Caspers et al. 2001; Fleischli et al. 2013). Since EDA-DOPO, ETA-DOPO, and EG-DOPO were only partially soluble in chloroform, their spectra were calibrated against DOPO assuming the same Raman cross section for the biphenyl group. Due to the nature of the unrestricted fit, negative fitting coefficients can be obtained and the values must be compared to those of the blank. The fitting coefficients of the blank treated skin were also used to estimate the level of detection. The depth resolution of the confocal microscope was determined to be 8 µm at FWHM leaving an uncertainty of approx. 4 µm in differentiating between truly permeated compounds and compounds sitting at the surface of the skin.

Toxicity assessment of central nervous system neurons

LUHMES cells are conditionally immortalized human fetal ventral mesencephalic neuronal precursor cells that were obtained by clonal selection. Differentiated LUHMES cells show a clear dopaminergic phenotype which was described in detail previously (Schildknecht et al. 2009; Scholz et al. 2011). Cells were propagated in Advanced DMEM/F12 (Gibco/Invitrogen, Darmstadt, Germany), 1x N2 supplement (Invitrogen), 2 mM L-glutamine (Gibco), and 40 ng/ml recombinant bFGF (R+D Systems; Minneapolis, MN). The differentiation process was initiated by addition of differentiation medium consisting of advanced DMEM/F12, 1x N2 supplement, 2 mM L-glutamine, 1 mM dibutyryl-cAMP (Sigma), 1 µg/ml tetracycline (Sigma), and 2 ng/ml recombinant human GDNF (R+D Systems). After 2 days, cells were trypsinized and collected in Advanced DMEM/F12 medium. Cells were seeded onto 24-well plates at a density of 160.000 cells/cm^2. The differentiation process was continued for additional 4 days. Fully differentiated LUHMES were treated with the various flame retardants for 48 h from day 6-8 of differentiation. For visualization of morphology, cells were fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.2 % Triton X-100, washed with PBS, blocked with 1 % BSA (Calbiochem, San Diego, CA) in PBS for 1 h and stained with a polyclonal anti-β-III-tubulin antibody (Sigma) in 1% BSA/PBS at 4°C over night. After washing, the secondary antibody (anti-mouse-IgG, Alexa 488, Molecular Probes; 1:1000) in 1% BSA/PBS was added for 1 h, nuclei were stained by Hoechst dye H-33342 (1 µg/ml) for 20 min. For visualization, an Olympus IX 81 microscope (Hamburg, Germany) equipped with a F-view CCD camera was used. For quantitative evaluation of the neurite area, the β-III-tubulin-stained cells were analyzed using an automated microplate-reading microscope (Array-Scan II® HCS Reader, Cellomics, Pittsburgh, PA) equipped with a Hamamatsu ORCA-ER camera (resolution 1024 x 1024; run at 2 x 2 binning). Nuclei were identified as objects according to their intensity, size, area and

shape. A virtual area corresponding to the cell soma was defined around each nucleus. The total β-III-tubulin pixel area per field minus the soma areas in that field was defined as neurite mass.

Toxicity assessment of peripheral nervous system neurons

The human pluripotent stem cell (hPSC) line H9 (WA09 line) was obtained from WiCell (Madison, WI, USA). Import of cells and experiments were authorized under license # 170-79-1-4-27 (Robert Koch Institute, Berlin, Germany). Cells were cultured according to standard protocols (Thomson et al. 1998) and differentiated into dorsal root ganglia-like cells as described earlier (Chambers et al. 2012), with slight modifications. Briefly, neural differentiation was promoted by adding neural differentiation medium and the combination of 6 small molecule pathway inhibitors (noggin (35 ng/ml; R&D Systems Minneapolis, USA) dorsomorphin (600 nM; Tocris Bioscience, Bristol, UK), SB-431642 (10 µM; Tocris Bioscience, Bristol, UK), CHIR99021 (1.5 µM; Axon Medchem, Vienna, USA), SU5402 (1.5 µM; Tocris Bioscience, Bristol, UK) and DAPT (5 µM; Merck, Darmstadt, Germany). On day 8 of differentiation (DoD 8) cells were cryopreserved. For each single experiment, cells were thawed in neural differentiation medium supplemented with CHIR99021 (1.5 µM), SU5402 (1.5 µM) and DAPT (5 µM). For the peripheral neurotoxicity test (PeriTox-test), test chemicals were serially diluted in differentiation medium containing BDNF, GDNF and NGF (25 ng/ml; R&D Systems Minneapolis, USA) and added to the cells at day 3 after thawing. After 48 h, neurite area and viability were assessed as described earlier (Krug et al. 2013). Briefly, cells were loaded with 1 µM calcein-AM and 1 µg/ml H-33342 for 1 h at 37°C. For image acquisition, an Array-Scan VTI HCS (high contenting imaging microscope) (Cellomics, Waltham, MA USA) was used. In an automated procedure, all H-33342^{+}/calcein^{+} cells were analyzed as viable cells.

Neural crest cell (NCC) migration assay

Human embryogenic stem cells (H9 hESC line) (Wisconsin International Stem Cell Bank, Madison, WI, USA) were plated on a confluent layer of Mitomycin C-treated murine bone marrow-derived stromal MS5 cells in KSR medium (DMEM, 15% serum replacement, 1x GlutaMax, non-essential amino acids, β-mercapto ethanol). After 12 days, medium was changed to N2 medium (DMEM/F12 containing glucose, insulin, apo-transferrin, putrescine, selenite, progesterone, sonic hedgehog, fibroblast growth factor 8, brain derived neurotrophic factor, ascorbic acid). After 21 days, rosettes were picked and plated onto poly-L-ornithine/laminin/fibronectin coated plates in N2 medium. After 7 days, cells were FACS sorted for positive expression of p75 and HNK-1 and expanded for 28 days in N2 medium supplemented with EGF (20 ng/ml) and FGF_2 (20 ng/ml). For the following 28 days, cells were passaged 4-5 times for expansion, detached by accutase (PAA, Pasching, Austria) and cryopreserved in 90% FCS, 10% DMSO. For the analysis of neural crest cell migration, silicone stoppers (Platypus Technologies, Madison, WI, USA) were inserted in a 96 well plate coated with poly-L-ornithine/laminin/fibronectin. Freshly thawed NCCs were seeded at a density of 100.000 cells/cm^2 in N2 medium containing EGF and FGF_2 and allowed to attach for one day. Stoppers were removed and the cells were allowed to migrate into the center of the well. Compounds tested in this study were applied for 24 h. For single cell detection, cells were stained with H-33342 (1 μg/ml) and calcein-AM (0.5 μM). Viability was assessed by counting the number of H-33342 and calcein double-positive cells outside the migration zone, migration was detected by acquisition of the number of H-33342 and calcein-double positive cells with the migration area.

Statistics

Values are expressed as the mean ± SD. If not otherwise indicated, experiments were performed at least three times with three technical replicates in each experiment. Data were analyzed by one-way ANOVA or Student´s t-test as appropriate, differences were determined by Bonferroni´s post hoc test (Prism or Origin software). If not otherwise indicated, means were considered as statistically significant at $p < 0.05$.

Results

Synthesis and flame retardation properties of DOPO-derivatives

During combustion of the host material, the organophosphorus flame retardant DOPO primarily acts in the gas phase and forms low energy $PO^{\bullet}$ radicals that can interact with $H^{\bullet}$ or $^{\bullet}OH$ radicals and thus lower the amount of released energy (Neisius et al. 2014). In order to modulate the biophysical properties of DOPO for an optimization of its use in polyurethane foams and to further improve its flame retardation potential, three novel *bis*-DOPO derivatives comprising an alkyl spacer linked via N-and O-atoms to the central phosphate moieties (Fig. 1a) were synthesized as recently described (Qiang et al. 2011; Gaan et al. 2013). The flame retardation potential was evaluated by subjecting the new compounds, embedded into polyurethane, to the UL-94 horizontal burning fire test (Fig. 1b). When added to soft polyurethane foams, the three novel bridged DOPO-derivatives achieved higher flame retardation ratings compared with the two chlorophosphates TCCP and TCEP that served as widely applied reference compounds (Fig. 1b). The flame retardation potential of the DOPO-derivatives was significantly higher compared with their parental compound DOPO. (UL 94 classification: HF 1 > HF 2 > HBF) (Fig. 1b). The fire burning test clearly indicated that the

novel *bis*-DOPO derivatives display superior flame retardation properties compared with the compounds they are supposed to replace. These features prone them as potential new candidates for use in several plastic materials of everyday common household goods, posing however also the question on their adverse influence on human health.

Acute toxicity assessment of DOPO-derivatives

Based on their superior flame retardation potential combined with their beneficial properties for application in polyurethane foams, the three DOPO-derivatives were tested in a series of different *in vitro* models in order to obtain initial information on their potential harmful properties. To allow a comparison with published toxicological data of other flame retardants, DOPO but also PBDE-99, as representative for the class of polybrominated diethyl ethers, were tested in parallel in all assays applied in this work. Inhalation is considered as one of the dominating routes of exposure for flame retardants. Therefore, we first applied the human lung epithelial cell line A549 as well as human macrophages, differentiated from the monocyte cell line THP-1, and exposed them to the respective flame retardants for a period of 2 days. While DOPO, EDA-DOPO, and PBDE-99 exhibited no signs of cell damage at concentrations up to 400 μM, acute toxicity in both cell models was detected with ETA-DOPO and EG-DOPO at concentrations > 20 μM (Fig. 2). These findings confirm observations in the literature, describing the absence of cell damage by PBDE-99 in A549 cells in the same concentration range (Kim et al. 2014). Another report assessed DOPO toxicity in PC12 and B35 neuroblastoma cell models and also observed no damage in a comparable concentration range as applied herein, further confirming the low cytotoxic potential of DOPO (Hendriks et al. 2014).

For cell types primarily involved in the inflammatory response, such as macrophages or astrocytes, previous reports indicated an inflammatory activation in response to their exposure to flame retardants (Koike et al. 2014). We hence tested the release of the proinflammatory cytokines TNF-α and IL-8 in differentiated THP-1 macrophages and could not observe any significant release (Suppl. Fig. 1a + b). It was recently reported that PBDE-47 and PBDE-209 would induce oxidative stress and thereby contribute to DNA damage (Pellacani et al. 2012). In A549 cells, no significant increase in reactive oxygen species could be observed (Suppl. Fig. 1c), nor were we able to detect DNA strand breaks in A549 or LUHMES cells exposed to the flame retardants tested in this work (Suppl. Fig. 2 + 3). As alternative readout for the assessment of a potential inflammatory activation evoked by the flame retardants, the translocation of cytosolic subunits of the NF-κB complex into the nucleus was investigated in astrocytes and, similar to the situation in macrophages, no influence could be detected (Suppl. Fig. 4).

For further comparison with published DOPO toxicity data, the standardized acute daphnia immobilization (OECD guideline 202) test was employed. An EC_{50} of roughly 1.5 mM was recently reported for DOPO (Waaijers et al. 2013). In our testing of EDA-DOPO, ETA-DOPO, or EG-DOPO, even the highest concentrations applied (water soluble fraction of a 1000 mg/l ≈ 2mM solution) resulted only in minimal immobilization of 5-10 % of the daphnids (Suppl. Fig. 5). In an alternative OECD-approved assay (OECD guideline 201), freshwater microalgae were exposed to the flame retardants. At the highest concentrations (water soluble fraction of a 1000 mg/l ≈ 2mM solution), EG-DOPO displayed a declined algae growth while EDA-DOPO and ETA-DOPO exhibited no effect (Suppl. Fig. 6).

In conclusion, our observations underline a low acute toxic potential of DOPO and its derivatives in non-neuronal cells and in lower freshwater organisms. The application of DOPO and PBDE-99 allowed some overlap with previously published data and confirmed

them. These observations allow a comparison of our data on the novel DOPO-derivatives with other reports and enable an integration of the herein presented toxicological data into a network of data in the literature.

Interaction of DOPO-derivatives with epidermal barriers.

Dermal uptake of flame retardants present in ambient air, household dust, or by direct contact with plastic-containing materials, emerged as a major uptake route besides ingestion and inhalation. To assess their influence on the integrity of the epidermis, a 3D human *in vitro* epidermal model was topically exposed to high concentrations (10 mM, 50 mM, 200 mM) of the respective flame retardant for 48 h. Even at the highest concentrations applied onto the Stratum corneum, all flame retardants tested displayed no detectable influence on the architecture of the epidermis (Fig. 3a). As positive control, SDS (5 %) was added to the model and resulted in a complete disintegration of the Stratum corneum and substantial cell death in the Stratum spinosum. Viability of the epidermis was detected by the PrestoBlue® assay and indicated no significant changes when exposed to the flame retardants (not shown). The observed absence of significant epidermal damage could either be the result of a negligible penetration of the compounds into the epidermis, or it could be the consequence of a higher resistance of epidermal cells compared with other cell types. To address the aspect of penetration into a complex skin structure, the different flame retardants were topically applied on segments of porcine ears from freshly slaughtered animals. Uptake was detected in a non-invasive manner by application of confocal Raman spectroscopy (Fig. 3b). Caffeine was used as positive control, indicating a significant penetration of ETA-DOPO and a moderate uptake of PBDE-99, while all other compounds displayed no detectable penetration into the skin within the investigated time period of 1 h.

In order to analyze the irradiative potential of the various flame retardants when in direct contact with epidermal cells, a keratinocyte-based cell model optimized to detect electrophilic skin sensitizing compounds was utilized in the next step. With this model, covalent modifications of active site cysteine residues in Keap1 by electrophiles lead to its dissociation from Nrf2 that subsequently accumulates in the nucleus to activate the expression of genes with an antioxidant response element (ARE) in their promoter (Emter et al. 2010). Treatment of the reporter construct-carrying keratinocytes with the various flame retardants revealed an increase in ARE-dependent luciferase expression by ETA-DOPO and EG-DOPO that was however inversely correlated with a decline in cell viability. Under the experimental conditions employed in Fig. 3c, the rise in luciferase signal hence can not be attributed to a specific innate response of the cells, but must rather be interpreted as the consequence of cell death-associated processes. Comparable effects were also observed with higher concentrations (>10 μM) of the positive control benzylidine acetone. PBDE-99 however lead to a concentration-dependent moderate increase in ARE-dependent luciferase expression in the absence of significant cell death, thus indicating a skin sensitization potential of PBDE's. DOPO and EDA-DOPO exhibited neither an induction of luciferase activity nor an adverse effect on cell viability (Fig. 3c) in agreement with the observations of Fig. 2 made with human lung cells and macrophages. Taken together, these findings/results confirmed the cytotoxic potential of ETA-DOPO and EG-DOPO when added to 2D cell models.

Based on the assays applied herein, a direct adverse influence of the flame retardants on epidermal integrity appears to play no significant role. There are also no reports indicating a direct degenerative influence of flame retardants present in household dust on human skin. More importantly, however, is the fact that some of the compounds have the potential to penetrate the Stratum corneum and hence can enter the human body. Our observations on the penetration of DOPO-derivatives was limited to only 1 h. Whereas uptake of ETA-DOPO and

PBDE-99 was apparent even after this short incubation period, it can not be excluded that under conditions of chronic interaction, also DOPO, EDA-DOPO, and EG-DOPO can penetrate the skin at quantitative amounts and hence enter the human body.

Cytotoxicity of flame retardants in central nervous system neurons

Previous studies on the toxicity of halogenated flame retardants indeed identified a wide spectrum of different cell types and organs affected, but they explicitly highlighted the adverse effects on neuronal integrity and on neurodevelopmental processes (Hendriks et al. 2014). For investigations on the influence of the DOPO derivatives on central nervous system neurons, we applied human LUHMES cells that can be differentiated into mature post-mitotic neurons within 6 days (Schildknecht et al. 2009; Scholz et al. 2011). Fully differentiated LUHMES (day 6) were treated with varying concentrations of the different flame retardants (Fig. 4a) for a period of 2 days (day 6-8) (Fig. 4b). For visualization of cell morphology, cells were fixed and stained with an anti-β-III-tubulin antibody. In our previous studies with the LUHMES cells, we observed that neurite architecture serves as a far more sensitive marker for neuronal integrity than classical cell viability assays (Krug et al. 2013; Schildknecht et al. 2013). For the quantitative assessment of total neurite mass, an automated microscope system with an analysis algorithm allowing the detection of total neurite mass was applied (Fig. 4c). In addition to that, cell viability was detected by following the reduction of resazurin and by the lactate dehydrogenase (LDH) release assay as well as by the detection of intracellular levels of ATP and reduced glutathione (GSH) (Supp. Fig. 7). In summary, all assays indicated a degeneration of neurites evoked by ETA-DOPO, EG-DOPO, and PBDE-99 at concentrations > 10 μM, while DOPO and EDA-DOPO exhibited no influence at concentrations up to 100 μM. These results were obtained with fully differentiated neurons. To investigate the influence of the flame retardants on neurite outgrowth when exposed

during the differentiation process, LUHMES were treated from day 0 to day 2 of their differentiation (Suppl. Fig. 8 and 9). Qualitatively, the same observations as with fully differentiated cells were made with the exception that EDA-DOPO also revealed a moderate, but significant influence on total neurite area. This effect was however not reflected by cell viability markers, respectively by the intracellular levels of ATP and GSH (Suppl. Fig. 8 and 9). In order to introduce an alternative assay to investigate more subtle changes in neurite integrity, we investigated the migration of mitochondria in neurites of differentiated LUHMES (Suppl. Fig. 10). Treatment of the cells with the parkinsonian toxin MPP^+, applied herein as a positive control, resulted in a significant decline in the velocity of mitochondrial migration in neurites, while all flame retardants under conditions where neurite integrity was not affected (25 µM, 12 h), exhibited no significant influence on mitochondrial migration (Suppl. Fig. 10). At high flame retardant concentrations, an influence on mitochondrial velocity was indeed observed with ETA-DOPO, EG-DOPO, and PBDE-99, however this effect was directly correlated with neurite degeneration and hence can not be considered as an independent toxicity parameter.

In conclusion, analysis of neurite mass and integrity in both differentiating and fully differentiated LUHMES and its comparison with markers of general cell viability indicated that the flame retardants investigated evoke no neuro-specific degeneration. Instead, the observed impact on neurites could rather be considered as consequence of general cell degenerative processes.

Toxicity of flame retardants in neurons of the peripheral nervous system

Neurons of the central nervous system (CNS) are protected by the blood brain barrier in an organism. It can hence be speculated that neurons of the peripheral nervous system *per se*

represent a more vulnerable target either by their more direct exposure to environmental toxicants *in vivo* or by intrinsic features distinguishing them from CNS neurons. To analyze potential differences between central and peripheral neurons with respect to their response towards the flame retardants tested, we generated peripheral neurons from human stem cell-derived neural precursors and treated these cells for a period of 48 h with the respective flame retardants during neuronal differentiation (Fig. 5a). Similar to the observations made with the CNS neurons, EG-DOPO and PBDE-99 significantly affected neurite integrity (Fig. 5b) and cell viability (Fig. 5c) while DOPO, EDA-DOPO and ETA-DOPO exhibited a moderate inhibitory influence on neurite outgrowth and general cell viability at concentrations > 50 µM. These observations indicate that no significant difference between central and peripheral neurons *in vitro* exists with respect to their degeneration in response to the flame retardants investigated. Although we did not explore/examine/analyze the underlying mechanisms leading to cell death, the parallel decline in neurite mass and in cell viability observed both in central- and in peripheral neurons, together with comparable responses of non-neuronal cells (Fig. 2 + 3), again suggest that DOPO and its derivatives evoke a cell type independent mechanism of cell damage.

Influence of flame retardants on neural crest cell migration

Adverse effects of a compound on the nervous system are not necessarily correlated with its direct toxicity or its influence on neuronal differentiation. Instead, only subtle changes in neural precursor migration during early development can have profound influence on the subsequent development of the nervous system. We therefore tested the flame retardants in a stem cell-derived human neural crest cell migration assay (Fig. 6). Cells were allowed to migrate into a cell-free area, migration as well as cell viability on the individual cell level were assessed as readouts. A quantitative analysis was conducted by an algorithm that in

addition detects cell numbers in order to exclude a contribution of cell proliferation to the migration events analyzed (Fig. 6a). While DOPO and EDA-DOPO displayed neither an inhibitory influence on cell migration nor on cell viability, ETA-DOPO and EG-DOPO both showed a concentration-dependent decline in cell migration and a decline in cell viability (Fig. 6b). PBDE-99 indicated a moderate, but significant inhibition of cell migration in the absence of impaired cell viability. Similar effects were observed with ETA-DOPO and EG-DOPO in a relatively narrow concentration range of ca. 30-60 µM. In this range, migration was far more sensitive in its response towards these two flame retardants compared with the adverse influence on cell viability. In all neuronal assays, ETA-DOPO and EG-DOPO showed cytotoxic properties at significantly lower concentrations of flame retardants compared with DOPO and EDA-DOPO.

Comparison and prioritization of organophosphorus flame retardants

In the present work, different *in vitro* test systems for the assessment of potential adverse effects of the three novel DOPO-derivatives were implemented. To allow a better comparison between the individual compound's safety profile, the IC_{50} values determined by these assays were plotted in a single graph (Fig. 7). Due to limitations in solubilization of the compounds at maximal residual DMSO levels of 0.1 %, maximal concentrations in two-dimensional cell culture experiments were limited to 400 µM. Within this concentration range, some assays displayed no reduction e.g. in viability and hence allowed no calculation of IC_{50} values. EDA-DOPO, in all assays utilized, exhibited no harmful influence while the average IC_{50} values of ETA-DOPO and EG-DOPO were in the range of 20-100 µM in the models applied. The three novel DOPO-derivatives tested in the present work all exhibited a superior flame retardation potential compared with their parental compound DOPO. The present initial analysis employing a variety of different *in vitro* assays concordantly adverted a cell-damaging

potential of ETA-DOPO and EG-DOPO in a concentration range comparable to the toxicity potential of PBDE-99. EDA-DOPO displayed superior flame retardation features compared with its parental compound DOPO and at the same time evoked no harmful influence in all cell types and assays employed in this work.

Discussion

Acute toxicity of DOPO derivatives in cotext with current literature data

In the present work, novel halogen-free *bis*-DOPO compounds linked via alkyl spacers by N- and O-bonds were for the first time analyzed for their potential harmful influence by a set of cellular *in vitro* assays as well as their influence on Daphnid viability and algal growth. The three novel compounds were designed in order to possess an improved flame retardation potential, but also for a wider range of applications, based on their higher melting points and increased stability, that is a prerequisite for their use in the production process of plastic materials (Buczko et al. 2014; Gaan et al. 2013, 2015; Neisius et al. 2014; Salmeia et al. 2015; Qiang et al. 2011). In contrast to the current practice to investigate potential harmful properties of flame retardants after their introduction into the market, we applied here a series of different human *in vitro* screening assays to allow a fundamental toxicological classification before their production and application in industrial scales. Compared to the vast literature on the class of polybrominated diethyl ethers, covering aspects such as indoor and outdoor contamination levels, human uptake routes, accumulation in different organs and tissues, and their toxic effects in a variety of *in vitro* and *in vivo* test systems (Watkins et al. 2011; Stapleton et al. 2014; Lyche et al. 2015; Liagkouridis et al. 2015), only limited data on

DOPO toxicity and no information on the harmful influence of *bis*-DOPO derivatives are so far available.

In order to allow a direct comparison of the toxicological data presented in this study with published data on polybrominated diphenyls, the parental compound DOPO, as well as PBDE-99 as a representative example for the class of PBDE's, were incorporated in all experiments of this work. At concentrations up to 400 µM, DOPO displayed no influence on all parameters investigated herein. These findings verify data from a previous study by Hendriks *et al.*, in which rat adrenomedullary pheochromocytoma PC12 cells and rat neuroblastoma B35 cells, that were treated for 24 h with maximal concentrations of 100 µM DOPO, exhibited no signs of alterations in cell viability, generation of reactive oxygen species, or in intracellular Ca^{2+} levels (Hendriks et al. 2014). In a standardized Daphnia immobilization assay (OECD Guidelines for the Testing of Chemicals, Test No. 202), treatment of young daphnids with the water soluble fraction of a 1000 mg/l suspension (ca. 2 mM), DOPO, added for 48 h was reported to show no significant influence on their viability (Waaijers et al. 2013). Our toxicological assessment of the three *bis*-DOPO compounds with the same standardized Daphnid model revealed no significant influence up to the highest concentrations applied (water soluble fraction of a 1000 mg/l suspension) (Suppl. Fig. 5). Based on this information, we applied DOPO as negative control reference compound in our experimental setups to allow a direct comparsion with currently available literature data and to enable an integration of the information generated in the present work into the existing network of toxicological data on the different classes of flame retardants. As positive control, we have chosen PBDE-99, as a representative example of the class of polybrominated diphenyls. Among the vast number of PBDE variants produced, five congeners (PBDE-47, PBDE-99, PBDE-100, PBDE-153, and PBDE-154) predominate in human tissues and account for more than 90 % of the PBDE's detected in the human body (Stasinska et al.

2014). Literature data on PBDE-99, on the one hand, reported an absence of adverse effects in non-neuronal cells up to 100 μM that was further confirmed by our observations made with the A549 or THP-1 cells or with keratinocytes (Fig. 2 and 3). On the other hand, epidemiological investigations focusing on the correlation between PBDE exposure in early development with deficits in psychomotor and IQ performance in later life, indicated a profound influence on the development and function of the neuronal system (Shy et al. 2011; Gump et al. 2014; Herbstman et al. 2010; Chevrier et al. 2010; Chao et al. 2011). Our observation on the selective influence of PBDE-99 on central and peripheral neuronal integrity was fundamentally supported by other *in vitro* observations in the literature indicating a pronounced selective toxicity in neuronal cells (Schreiber et al. 2010). These observations clearly indicate that the cell type selective toxicity of PBDE's is adequately represented by the *in vitro* models employed in the present study. Combined with the observations made with DOPO that served as negative control in our work, the information on the toxicological response to PBDE-99 allows a direct comparison with the relatively large number of *in vitro* observations in the literature. Data on PBDEs in the literature allow some correlation between their toxicity in cell culture *in vitro* systems, their levels in different human organs and tissues and their adverse effects on psychomotor functions in humans. Under the assumption of comparable volatilization rates and bioaccumulation properties of PBDEs and *bis*-DOPO compounds, the data presented herein allow first speculations on the potential interference of *bis*-DOPO compounds with (neuro)-developmental processes *in vivo*. The ultimate approach to address these questions indeed presumes the inclusion of *in vivo* test models in the compilation of an adverse profile of these compounds.

Comparison of in vitro and in vivo flame retardant burdens

Toxicological results obtained with *in vitro* models raise the question on actual *in vivo* concentrations in humans that are largely influenced by the biophysical properties of a given compound. The most comprehensive literature on indoor and outdoor contamination levels and on the compound's organ- and tissue accumulation rates is currently available for PBDEs. A growing number of studies analyzing PBDE levels in different cohorts of various countries were published in recent years (Chao et al. 2007; Roze et al. 2009; Eskenazi et al. 2013). Exposure of infants was routinely observed as significantly higher compared with adults, largely as a result of the higher rate of oral, nasal, and dermal uptake, as well as of different toxicokinetic parameters compared with adults (Toms et al. 2009; Rose et al. 2010). PBDE-99 was identified in office dust at concentrations of ca. 900 ng/g dust (Watkins et al. 2013). While uptake of house dust by adults was in the range of 20-50 mg/day, those of smaller children were found to reach values of 100-200 mg/day (Wilford et al.; U.S. EPA 2008). For children, these numbers imply a daily uptake of PBDE-99 in the range of 75 ng/day. Based on the lipophilicity and the ability to passage the placental barrier, breastfed infants are exposed to a ca. 300 fold higher oral dose of PBDE congeners per day compared with adults (Toms et al. 2009; Rose et al. 2010; Costa et al. 2014). Given these numbers, average blood levels of PBDEs in adults in the United States were in the range of 10-300 ng/g lipid and infant levels were reported almost an order of magnitude higher (Costa et al. 2007; Toms et al. 2009; Rose et al. 2010). Assuming a uniform distribution of the compounds in blood, these numbers equal steady state PBDE concentrations in the range of 10-100 nM in blood (Costa et al. 2014). Considering their lipophilicity, accumulation of PBDEs in lipid-rich tissues such as the brain, kidneys, liver or fat thus can easily lead to local concentrations in the low micromolar range and hence are in the range of the concentrations tested in this work.

Epidermal uptake of organophosphorus flame retardants

Recent pharmacokinetic calculations indicated that dermal uptake represents the second most important contributor to PBDE body burden, only exceeded by uptake via ingestion (Abdallah et al. 2015). DOPO and its *bis*-DOPO derivatives are additives and hence are not covalently bound to their host material, implying an inherent tendency for volatilization. Considering their primary use in plastic materials, processed in everyday consumer products such as household appliances or furniture, dermal contact with DOPO derivatives will play a central role following the introduction into the market. We therefore investigated the influence of the different DOPO-derivatives on the integrity of a three-dimensional *in vitro* epidermal model, their penetration potential, and their cytotoxic potential when in contact with cultured keratinocytes. Apical application of flame retardants at concentrations of up to 200 mM (in 5% DMSO in PBS) resulted in no apparent disintegration of the epidermal architecture. The Stratum corneum represents the outermost layer of the epidermis and consists of nucleus- and organelle-free corneocytes that are constantly replaced by desquamation. Direct contact of the flame retardants with the Stratum corneum apparently had no influence on its integrity, but evoked the question on the influence of the flame retardants when in contact with organelle-containing cells of the Stratum granulosum or Stratum spinosum. In order to address this question, we first analyzed how deep apically applied flame retardants can penetrate an intact epidermis. Analysis by Raman microscopy indicated a detectable penetration of ETA-DOPO and to a lesser extent of PBDE-99 into porcine skin with a maximal penetration depth of less than 10 µm. The Stratum corneum has an average depth of 20-40 µm. These findings hence indicate that penetration of externally applied flame retardants can be efficiently deferred by the Stratum corneum, however it has to be considered that the incubation interval employed in Fig. 3b was limited to only 1 h in contrast to the chronic exposure with flame retardants under everyday conditions. The assessment of the toxicity of EDA-DOPO, ETA-DOPO, and EG-DOPO in a two-dimensional keratinocyte cell culture model revealed IC_{50} values (Fig. 3c) for the respective flame retardants that were comparable to our observations in macrophages or

lung epithelial cells (Fig. 2). These high concentrations are rather unlikely to occur by exposure to ambient air and household dust. Furthermore, in a living organism, penetrated compounds are continuously distributed by the vascular system. By taking these considerations into account, it can be concluded that a direct harmful influence of the flame retardants tested in this study on epithelial barriers plays only a neglectable role. However, the observed import of extracorporal flame retardants via the skin apparently represents a potential route for their uptake which is an essential parameter in the evaluation of their safety profile.

Neurotoxic potential of DOPO-derivatives

Studies on the harmful potential of polybrominated flame retardants highlighted their explicit adverse influence on the neuronal system (Costa et al. 2007, 2014). Exposure to PBDE's in the early development of test animals indicated signs of slower motor skill development, an influence on synaptic plasticity, and changes in hippocampal long term potentiation in rats and mice (Viberg et al. 2006; Dingemans et al. 2007; Ta et al. 2011). Recent human epidemiological studies confirmed a correlation between early development exposure to various PBDEs and a reduction in psychomotor development, motorfunction, and IQ in childhood age (Chevrier et al. 2010; Herbstman et al. 2010; Chao et al. 2011; Gump et al. 2014). Plasma levels of PBDE's detected in these studies were usually not associated with acute neurodegenerative events. Brain development in its early stages however is characterized by timely and spatially tightly regulated phases of precursor proliferation, migration, and differentiation. It hence can be assumed that only moderate interferences by environmental toxicants in these stages might lead to miswiring of neuronal circuits, or to changes in the ratio between different neuronal types. This would prefer levels of particular

neurotransmitters at the expense of others, as shown for an inhibition of the cholinergic system which is involved in learning and memory (Viberg et al. 2003).

Based on these observations in the literature, we applied a series of *in vitro* test systems to study the potential harmful influence of the organophosphate flame retardants on the integrity of differentiated and differentiating neurons of the central and peripheral nervous system and on the influence on neural crest cell migration. In our previous studies on *in vitro* neurodegeneration, we elaborated the quantitative assessment of total neurite mass and the migration of mitochondria in neurites as readouts that displayed a far more pronounced sensitivity compared to standard cytotoxicity assays such as resazurin or MTT reduction or the LDH release assay. Interestingly, both neurite integrity and mitochondrial migration in a concentration- and time-dependent manner closely reflected the decline in central and peripheral neuron viability detected by resazurin reduction, LDH release, or uptake of calcein-AM (Fig. 4 + 5) (Suppl. Fig. 10). Furthermore, the concentration-dependent toxicity evoked by ETA-DOPO and EG-DOPO in the neuronal models occurred in a modestly lower concentration range compared with the non-neuronal models applied in Fig. 2 and 3. In both models of central and peripheral nervous system neurons, comparable profiles of cell damage and toxicity were observed in fully differentiated cells and when the cells were exposed during differentiation (Fig. 4) (Suppl. Fig. 7 + 8 + 9). In contrast to that, and in agreement with previous reports in the literature, PBDE-99 exhibited a distinct neurotoxic potential with a detrimental influence on central and peripheral neurons at concentrations > 10 µM while in non-neuronal models such as macrophages, lung epithelial cells, or keratinocytes, no significant toxicity up to 400 µM was observed. These findings clearly illustrate that DOPO and its derivatives possess a rather cell type independent toxicity profile. Previous reports on the damage evoked by PBDEs in the nervous system not only highlighted their influence on neuronal differentiation, but also the influence on neural precursor cell migration (Zimmer et

al. 2014). An inappropriate migration of precursor cells in early brain development would entail significant disturbance in neuronal function. DOPO and EDA-DOPO exhibited neither an influence on neural crest cell migration nor on their viability at concentrations up to 100 µM. Both ETA-DOPO and EG-DOPO affected cell viability in the same concentration range as observed with the assays applied in Figs. 2,3,4, and 5. Interestingly, human neural crest cells displayed no decline of viability up to 100 µM PBDE-99 (Fig. 6) in contrast to the compound's influence on differentiating or fully differentiated central- and peripheral neurons (Fig. 4 + 5). Migration of these neural crest cells however was inhibited by PBDE-99. These observations were fully corroborated by a previous report utilizing murine neural crest cells (Zimmer et al. 2014), in which an inhibition of migration in the absence of cell death in a concentration range of up to 100 µM was observed. It was not the scope of the present work to investigate the selective neurotoxicity of PBDE-99, however the data provided herein and in the literature indicate an interaction with neuro-selective targets that are expressed after the migration of immature neurons to their ultimate position in the brain, but before an outgrowth of neurites is inhibited.

The present work represents the first toxicological assessment of the DOPO-derivatives EDA-DOPO, ETA-DOPO, and EG-DOPO with an explicit focus on cellular *in vitro* models. The comparison with PBDE-99 as a representative example for the class of polybrominated diphenyl ethers convincingly demonstrated that ETA-DOPO and EG-DOPO possess cell-type independent cytotoxic properties at concentrations > 10 µM while DOPO and EDA-DOPO display no comparable effect at concentrations higher than 100 µM as can be deduced from the graphical summary of all IC_{50} values calculated from the experiments of this work (Fig. 7). The herein provided information now allows a targeted design of *in vivo* experiments addressing questions on the compound's uptake, distribution, and accumulation in different organs and tissues, as well as their influence on developmental processes. A combination of

the present *in vitro* data with *in vivo* observations in test animals would provide a solid platform for a qualified initial assessment of the adverse potential of the DOPO-derivatives EDA-DOPO, ETA-DOPO, and EG-DOPO when exposed to man.

Figure Legends

Fig. 1 (**a**) Structures of 9,10-dihydro-9-oxa-10-phosphaphenanthrene-10-oxide (DOPO) and the novel bridged DOPO derivatives EDA-DOPO, ETA-DOPO, and EG-DOPO. The derivatives are characterized by variations in the heteroatom attached to the phosphorus center of the respective DOPO monomer, comprising two phosphate- (EG-DOPO), two phosphoramidate- (EDA-DOPO), or a combination of phosphate- and phosphoramidate-moieties. (**b)** UL-94 horizontal burning fire test. The respective flame retardants were added to polyurethane foam at the concentrations as indicated. The concentration in the host material was based on the weight of polyol used in the manufacturing process of polyurethane foams. The density of the polyurethane foam was approximately 50 kg/m^3. Based on the inherent reactivity of DOPO, its final concentration in the foam was limited to 5 %. UL-94 horizontal rating classification: HF 1 > HF 2 > HBF > no rating. TCPP (Tris(2-chlorisopropyl)phosphate) and TCEP (Tris-(2-chlorethyl) phosphate) were included in the fire tests as representative examples of widely applied chlorophosphates.

Fig. 2 Determination of acute cytotoxicity. Human alveolar epithelial cells (A549) and human monocytes (THP-1), differentiated into macrophages, were treated with the various flame retardants for 48 h. Viability was detected by following the reduction of the tetrazolium compound MTS. All data are mean ± SD (n=3) *$p < 0.05$.

Fig. 3 Interaction of DOPO-derivatives with epidermal barriers. (**a**) Influence of flame retardants on epidermal integrity. The air-exposed Stratum corneum of a 3D epidermal *in vitro* model generated from primary human keratinocytes was topically exposed to the various flame retardants (200 mM) for 48 h, SDS served as positive control. The tissues were then fixed, sliced and stained with hematoxiline and eosine in order to allow a discrimination of the different epidermal layers. The loose layer beneath the Stratum basale represents the filter membrane. (**b**) Penetration of flame retardants into skin. For the assessment of quantitative penetration profiles, porcine skin was topically exposed to the various flame retardants (400 µM) for 1 h. Confocal Raman microscopy was then applied to obtain a depth profile for each compound. The graph illustrates the amount of the respective flame retardants and the positive control caffeine relative to keratin content as a function of depth. (**c**) Keratinocyte sensitization. To determine the skin sensitization potential of the flame retardants tested, human HaCaT keratinocytes were stably transfected with a construct coding for the luciferase gene under control of the antioxidant response element (ARE) that is activated upon exposure of the cells with electrophilic compounds. Luciferase activity was detected after a 2 day incubation period with the various flame retardants in the concentrations as indicated. Benzylidine acetone served as positive control. In parallel, viability of the cells was assessed by the PrestoBlue® reduction assay. All data are mean ± SD (n=3) $*p < 0.05$.

Fig. 4 Toxicity in neurons of the central nervous system. (**a**) Mature human dopaminergic neurons (LUHMES) were treated with the different flame retardants for 48h from day 6 to day 8 of LUHMES differentiation as indicated in scheme (**b)**. Cell morphology was visualized by fixation and staining of the cells with an anti-β-III-tubulin antibody (yellow), nuclear DNA was stained with Hoechst H-33342 (blue). (**c)** For quantitative assessment of total neurite mass, cells were analysed by an automated microscope system with an imaging algorithm

allowing a dissimilation between cell bodies and cell extensions. The graph represents the total neurite mass detected following the respective treatments. All data are mean ± SD (n=3) *$p < 0.05$.

Fig. 5 Toxicity in neurons of the peripheral nervous system. (**a**) Differentiation scheme for the generation of human dorsal root ganglia-like cells. The pluripotent stem cell line hESC-H9 was differentiated in a 2-step scheme into peripheral neurons. (**b**) Flame retardants were applied at DoD 3 (Days of Differentiation) post thawing for a period of 48 h, neurite outgrowth was investigated by staining of the cells with calcein-AM (cell morphology) and Hoechst 33342 (nuclei). (**c**) Quantitative assessment of cell numbers, as well as of neurite mass was conducted with an automated microscope system. Image analysis was performed with optimized algorithms allowing the detection of cell numbers by counting of nuclei. Neurite growth was quantitatively assessed by detection of the entire calcein signal subtracted by a defined area surrounding the nucleus defined as cell body. Data are mean ± SD (n=3) *$p< 0.05$.

Fig. 6 Influence of flame retardants on neural crest cell migration. (**a**) Neural crest cells were differentiated from the human embryonic stem cell line hESC-H9. The two images of control and EG-DOPO treated cells illustrate the general principle of the assay. The area inside the imaginary dotted ring is covered in the beginning by silicone inserts while cells are seeded into the surrounding area. Following removal of the inserts, cells are allowed to migrate into the cell-free area. For the delineation and identification of single cells, nuclei were stained with Hoechst-33342 (blue), cell bodies were stained with calcein (green). For better visualization, areas of blue and green overlay were depicted in red. (**b**) Detection of migration and cell viability. Following removal of the stoppers, cells were allowed to migrate in the

presence of the flame retardants for 24 h. The number of cells within the imaginary ring was detected and compared to the cell number in the entire well to assess a contribution by cell proliferation events. Viability was detected by comparison of the number of calcein-positive cells with the total cell number in the well. Data are mean ± SD (n=3) *$p < 0.05$.

Fig. 7 Synopsis of IC_{50} values. The plot summarizes the IC_{50} values obtained from the assays applied in this work. In the *in vitro* assays, maximal flame retardant concentrations were limited to 400 µM given a maximal residual DMSO concentration of 0.1 %. Due to this limitation, for some compounds no IC_{50} values were assessed. Calculated IC_{50} values are illustrated on the lower segment of the plot. The data points at the top of the plot indicate that a clear toxicity was not observed at test concentrations of 100-400 µM. n.d.: not determined.

References

Abdallah MA, Pawar G, Harrad S (2015) Evaluation of in vitro vs. in vivo methods for assessment of dermal absorption of organic flame retardants: a review. Environ Int. 74:13-22

Albina ML, Alonso V, Linares V, Bellés M, Sirvent JJ, Domingo JL, Sánchez DJ (2010). Effects of exposure to BDE-99 on oxidative status of liver and kidney in adult rats. Toxicology. 271(1-2):51-6

Behl M, Hsieh JH, Shafer TJ, Mundy WR, Rice JR, Boyd WA, Freedman JH4, Hunter ES, Jarema K, Padilla S, Tice RR (2015) Use of alternative assays to identify and prioritize organophosphorus flame retardants for potential developmental and neurotoxicity. Neurotoxicol Teratol. 2015 Sep 16. doi: 10.1016/j.ntt.2015.09.003.

Buczko A, Stelzig T, Bommer L, rentsch D, Heneczkowski M, Gaan S (2014) Bridged DOPO derivatives as flame retardants for PA6. Poly Degrad Stab 107:158-165

Caspers P, Lucassen G, Carter E, Bruining H, Puppels G (2001) In vivo confocal Raman microspectroscopy of the skin: Noninvasive determination of molecular concentration profiles, J Invest Dermatol 116 (3):434-442

Chambers SM, Qi Y, Mica Y, Lee G, Zhang XJ, Niu L, Bilsland J, Cao L, Stevens E, Whiting P, Shi SH, Studer L (2012) Combined small-molecule inhibition accelerates developmental timing and converts human pluripotent stem cells into nociceptors. Nat Biotechnol. 30(7):715-20

Chao HR, Tsou TC, Huang HL, Chang-Chien GP (2011) Levels of breast milk PBDEs from southern Taiwan and their potential impact on neurodevelopment. Pediatr Res. 70(6):596-600

Chao HR, Wang SL, Lee WJ, Wang YF, Päpke O (2007) Levels of polybrominated diphenyl ethers (PBDEs) in breast milk from central Taiwan and their relation to infant birth outcome and maternal menstruation effects. Environ Int. 33(2):239-45

Chevrier J, Harley KG, Bradman A, Gharbi M, Sjödin A, Eskenazi B (2010) Polybrominated diphenyl ether (PBDE) flame retardants and thyroid hormone during pregnancy. Environ Health Perspect. 118(10):1444-9

Costa LG, de Laat R, Tagliaferri S, Pellacani C (2014) A mechanistic view of polybrominated diphenyl ether (PBDE) developmental neurotoxicity. Toxicol Lett. 230(2):282-94

Costa LG, Giordano G (2007) Developmental neurotoxicity of polybrominated diphenyl ether (PBDE) flame retardants. Neurotoxicology. 28(6):1047-67

Covaci A, Harrad S, Abdallah MA, Ali N, Law RJ, Herzke D, de Wit CA (2011). Novel brominated flame retardants: a review of their analysis, environmental fate and behaviour. Environ Int. 37(2):532-56

Dingemans MM, Ramakers GM, Gardoni F, van Kleef RG, Bergman A, Di Luca M, van den Berg M, Westerink RH, Vijverberg HP (2007) Neonatal exposure to brominated flame retardant BDE-47 reduces long-term potentiation and postsynaptic protein levels in mouse hippocampus. Environ Health Perspect. 115(6):865-70

Emter R, Ellis G, Natsch A (2010) Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers in vitro. Toxicol Appl Pharmacol. 245(3):281-90

Eskenazi B, Chevrier J, Rauch SA, Kogut K, Harley KG, Johnson C, Trujillo C, Sjödin A, Bradman A (2013) In utero and childhood polybrominated diphenyl ether (PBDE) exposures and neurodevelopment in the CHAMACOS study. Environ Health Perspect. 121(2):257-62

Fleischli F, Mathes S, Adlhart C (2013) Label free non-invasive imaging of topically applied actives in reconstructed human epidermis by confocal Raman spectroscopy. Vib Spectrosc 68:29-33

Franzen L, Selzer D, Fluhr J, Schaefer U, Windbergs M (2013) Towards drug quantification in human skin with confocal Raman microscopy. Eur J Pharm Biopharm 84 (2):437-444

Gaan S, Liang S, Mispreuve H, Perler H, Naescher R, Neisius M (2015) Flame retardant flexible polyurethane foams from novel DOPO-phosphonamidate additives. Polym Degrad Stab 113:180-188

Gaan S, Neisius M, Mercoli P, Liang S, Mispreuve H, Naescher R (2013) Phosphonamidates, production methods and flame retardant applications. WO2013020696 A2

Gaan S, Rupper P, Salimova V, Heuberger M, Rabe S, Vogel F (2009) Thermal decomposition and burning behavior of cellulose treated with ethyl ester phosphoramidates. Effect of alkyl substituent on nitrogen atom. Polym Degrad Stab 94:1125-1134

González-Alzaga B, Lacasaña M, Aguilar-Garduño C, Rodríguez-Barranco M, Ballester F, Rebagliato M, Hernández AF (2014) A systematic review of neurodevelopmental effects of prenatal and postnatal organophosphate pesticide exposure. Toxicol Lett. 230(2):104-21

Gump BB, Yun S, Kannan K (2014) Polybrominated diphenyl ether (PBDE) exposure in children: possible associations with cardiovascular and psychological functions. Environ Res. 132:244-50

Hendriks HS, Meijer M, Muilwijk M, van den Berg M, Westerink RH (2014) A comparison of the in vitro cyto- and neurotoxicity of brominated and halogen-free flame retardants: prioritization in search for safe(r) alternatives. Arch Toxicol. 88(4):857-69

Herbstman JB, Sjödin A, Kurzon M, Lederman SA, Jones RS, Rauh V, Needham LL, Tang D, Niedzwiecki M, Wang RY, Perera F (2010). Prenatal exposure to PBDEs and neurodevelopment. Environ Health Perspect. 118(5):712-9

Hites RA (2004). Polybrominated diphenyl ethers in the environment and in people: a meta-analysis of concentrations. Environ Sci Technol. 38(4):945-56

Kim JS, Klösener J, Flor S, Peters TM, Ludewig G, Thorne PS, Robertson LW, Luthe G (2014) Toxicity assessment of air-delivered particle-bound polybrominated diphenyl ethers. Toxicology. 317:31-9

Koike E, Yanagisawa R, Takigami H, Takano H (2014) Penta- and octa-bromodiphenyl ethers promote proinflammatory protein expression in human bronchial epithelial cells in vitro. Toxicol In Vitro. 28(2):327-33

Krug A, Balmer N, Matt F, Schonenberger F, Merhof D, Leist M (2013) Evaluation of a human neurite growth assay as specific screen for developmental neurotoxicants, Arch Toxicol 87 (12):2215-2231

Li X, Gao Y, Guo LH, Jiang G (2013). Structure-dependent activities of hydroxylated polybrominated diphenyl ethers on human estrogen receptor. Toxicology. 309:15-22

Liagkouridis I, Cousins AP, Cousins IT (2015) Physical-chemical properties and evaluative fate modelling of 'emerging' and 'novel' brominated and organophosphorus flame retardants in the indoor and outdoor environment. Sci Total Environ. 524-525:416-26

Lyche JL, Rosseland C, Berge G, Polder A (2015) Human health risk associated with brominated flame-retardants (BFRs). Environ Int. 74:170-80

Marklund A, Andersson B, Haglund P (2003) Screening of organophosphorus compounds and their distribution in various indoor environments. Chemosphere. 53(9):1137-46

Martínez-Carballo E, González-Barreiro C, Sitka A, Scharf S, Gans O (2007) Determination of selected organophosphate esters in the aquatic environment of Austria. Sci Total Environ. 388(1-3):290-9

Muñoz-Quezada MT, Lucero BA, Barr DB, Steenland K, Levy K, Ryan PB, Iglesias V, Alvarado S, Concha C, Rojas E, Vega C (2013) Neurodevelopmental effects in children associated with exposure to organophosphate pesticides: a systematic review. Neurotoxicology. 39:158-68

Neisius M, Lutz M, Rentsch D, Hemberger P, Gaan S (2014) Synthesis of DOPO-Based Phosphonamidates and their Thermal Properties. Ind Eng Chem Res 53:2889-2896

Ni Y, Kumagai K, Yanagisawa Y (2007) Measuring emissions of organophosphate flame retardants using a passive flux sampler. Atmos Environ 41:3235-3240

Pellacani C, Buschini A, Galati S, Mussi F, Franzoni S, Costa LG (2012) Evaluation of DNA damage induced by 2 polybrominated diphenyl ether flame retardants (BDE-47 and BDE-209) in SK-N-MC cells. Int J Toxicol. 31(4):372-9

Qiang Y, Mack GA, Junzuo W (2011) Process for the preparation of DOPO-derived compounds and composition thereof. Patent WO 2011123389 A1

Rose M, Bennett DH, Bergman A, Fängström B, Pessah IN, Hertz-Picciotto I (2010) PBDEs in 2-5 year-old children from California and associations with diet and indoor environment. Environ Sci Technol. 44(7):2648-53

Rosanna DP, Salvatore C (2012) Reactive oxygen species, inflammation, and lung diseases. Curr Pharm Des. 18(26):3889-900

Roze E, Meijer L, Bakker A, Van Braeckel KN, Sauer PJ, Bos AF (2009) Prenatal exposure to organohalogens, including brominated flame retardants, influences motor, cognitive, and behavioral performance at school age. Environ Health Perspect. 117(12):1953-8

Salmeia K, Gaan S (2015) An overview of some recent advances in DOPO-derivatives: Chemistry and flame retardant applications. Polymer Degradation and Stability 113:119-134

Schildknecht S, Karreman C, Pöltl D, Efrémova L, Kullmann C, Gutbier S, Krug A, Scholz D, Gerding HR, Leist M (2013) Generation of genetically-modified human differentiated cells for toxicological tests and the study of neurodegenerative diseases. ALTEX 30(4):427-44

Schildknecht S, Pöltl D, Nagel DM, Matt F, Scholz D, Lotharius J, Schmieg N, Salvo-Vargas A, Leist M (2009) Requirement of a dopaminergic neuronal phenotype for toxicity of low concentrations of 1-methyl-4-phenylpyridinium to human cells. Toxicol Appl Pharmacol. 241(1):23-35

Scholz D, Pöltl D, Genewsky A, Weng M, Waldmann T, Schildknecht S, Leist M (2011) Rapid, complete and large-scale generation of post-mitotic neurons from the human LUHMES cell line. J Neurochem. 119(5):957-71

Schreiber T, Gassmann K, Götz C, Hübenthal U, Moors M, Krause G, Merk HF, Nguyen NH, Scanlan TS, Abel J, Rose CR, Fritsche E (2010). Polybrominated diphenyl ethers induce

developmental neurotoxicity in a human in vitro model: evidence for endocrine disruption. Environ Health Perspect. 118(4):572-8

Shaw SD, Blum A, Weber R, Kannan K, Rich D, Lucas D, Koshland CP, Dobraca D, Hanson S, Birnbaum LS (2010). Halogenated flame retardants: do the fire safety benefits justify the risks? Rev Environ Health. 25(4):261-305

Shy CG, Huang HL, Chang-Chien GP, Chao HR, Tsou TC (2011) Neurodevelopment of infants with prenatal exposure to polybrominated diphenyl ethers. Bull Environ Contam Toxicol. 87(6):643-8

Staaf T, Ostman C (2005) Organophosphate triesters in indoor environments. J Environ Monit. 7(9):883-7

Stapleton HM, Misenheimer J, Hoffman K, Webster TF (2014) Flame retardant associations between children's handwipes and house dust. Chemosphere. 116:54-60

Stasinska A, Heyworth J, Reid A, Callan A, Odland JØ, Trong Duong P, Van Ho Q, Hinwood A (2014) Polybrominated diphenyl ether (PBDE) concentrations in plasma of pregnant women from Western Australia. Sci Total Environ. 493:554-61

Stoker TE, Cooper RL, Lambright CS, Wilson VS, Furr J, Gray LE (2005). In vivo and in vitro anti-androgenic effects of DE-71, a commercial polybrominated diphenyl ether (PBDE) mixture. Toxicol Appl Pharmacol. 2005 Aug 22;207(1):78-88.

Ta TA, Koenig CM, Golub MS, Pessah IN, Qi L, Aronov PA, Berman RF (2011) Bioaccumulation and behavioral effects of 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) in perinatally exposed mice. Neurotoxicol Teratol. 33(3):393-404

Thomson J, Itskovitz-Eldor J, Shapiro S, Waknitz M, Swiergiel J, Marshall Vand J, Jones M (1998) Embryonic stem cell lines derived from human blastocysts, Science 282 (5391):1145-1147

Tollbäck J, Tamburro D, Crescenzi C, Carlsson H (2006) Air sampling with Empore solid phase extraction membranes and online single-channel desorption/liquid chromatography/mass spectrometry analysis: determination of volatile and semi-volatile organophosphate esters. J Chromatogr A. 1129(1):1-8

Toms LM, Sjödin A, Harden F, Hobson P, Jones R, Edenfield E, Mueller JF (2009) Serum polybrominated diphenyl ether (PBDE) levels are higher in children (2-5 years of age) than in infants and adults. Environ Health Perspect. 117(9):1461-5

U.S. EPA, 2008. Chiled-Specific Exposure Factors handbook (Final report). U.S. EPA, Ed.). U.S. Environmental Protection Agency, Washington, DC.

van der Veen I, de Boer J (2012) Phosphorus flame retardants: properties, production, environmental occurrence, toxicity and analysis. Chemosphere. 88(10):1119-53

Viberg H, Fredriksson A, Eriksson P (2002). Neonatal exposure to the brominated flame retardant 2,2',4,4',5-pentabromodiphenyl ether causes altered susceptibility in the cholinergic transmitter system in the adult mouse. Toxicol Sci. 67(1):104-7

Viberg H, Fredriksson A, Jakobsson E, Orn U, Eriksson P (2003) Neurobehavioral derangements in adult mice receiving decabrominated diphenyl ether (PBDE 209) during a defined period of neonatal brain development. Toxicol Sci. 76(1):112-20

Viberg H, Johansson N, Fredriksson A, Eriksson J, Marsh G, Eriksson P (2006) Neonatal exposure to higher brominated diphenyl ethers, hepta-, octa-, or nonabromodiphenyl ether, impairs spontaneous behavior and learning and memory functions of adult mice. Toxicol Sci. 92(1):211-8

Waaijers SL, Bleyenberg TE, Dits A, Schoorl M, Schütt J, Kools SA, de Voogt P, Admiraal W, Parsons JR, Kraak MH (2013b) Daphnid life cycle responses to new generation flame retardants. Environ Sci Technol. 47(23):13798-803

Waaijers SL, Hartmann J, Soeter AM, Helmus R, Kools SA, de Voogt P, Admiraal W, Parsons JR, Kraak MH (2013a) Toxicity of new generation flame retardants to Daphnia magna. Sci Total Environ. 463-464:1042-8

Watkins DJ, McClean MD, Fraser AJ, Weinberg J, Stapleton HM, Sjödin A, Webster TF (2011) Exposure to PBDEs in the office environment: evaluating the relationships between dust, handwipes, and serum. Environ Health Perspect. 119(9):1247-52

Watkins DJ, McClean MD, Fraser AJ, Weinberg J, Stapleton HM, Webster TF (2013) Associations between PBDEs in office air, dust, and surface wipes. Environ Int. 59:124-32

WHO, 1998. Environmental Health Criteria 209. International Program on Chemical Safety. World Health Organization, Geneva.

Wilford BH, Shoeib M, Harner T, Zhu J, Jones KC (2005) Polybrominated diphenyl ethers in indoor dust in Ottawa, Canada: implications for sources and exposure. Environ Sci Technol 39(18):7027-35

Zhou T, Taylor MM, DeVito MJ, Crofton KM (2002). Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption. Toxicol Sci. 66(1):105-16

Zimmer B, Pallocca G, Dreser N, Foerster S, Waldmann T, Westerhout J, Julien S, Krause KH, van Thriel C, Hengstler JG, Sachinidis A, Bosgra S, Leist M (2014) Profiling of drugs and environmental chemicals for functional impairment of neural crest migration in a novel stem cell-based test battery. Arch Toxicol. 88(5):1109-26