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Prioritization of hazards of novel flame retardants using the mechanistic toxicology information from ToxCast and Adverse Outcome Pathways

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Abstract

Background: Flame retardants (FRs) are used in most consumer products and textiles to comply with current flammability standards. After the restriction of polybrominated diphenyl ethers, a large number of chemically diverse replacement FRs are increasingly used, but the risk they represent is not yet properly evaluated and their toxicity pathways are still poorly understood.

Approach: We collected in vivo toxicological information on 62 (including 52 non-regulated) FRs and established five prioritization categories (Cat I to V) based on data availability and toxicological concern. We then considered available in vitro toxicological data from ToxCast, as a complement to in vivo information. By combining these information sources, we then explored relevant toxicity mechanisms for nine selected FRs (Cat I) using the AOP (Adverse Outcome Pathway) framework.

Results: For 20 FRs (Cat V), toxicological data on mammals were absent. Data available were scarce for another 22 FRs, of which 14 FRs (Cat II) may be of toxicological concern. We found substantial information for only ten replacement FRs, of which nine (Cat I) present some toxicological concern: tris-2-chloroethyl phosphate (TCEP), tris(1,3-dichloropropyl)phosphate (TDCIPP), triphenyl phosphate (TPhP), tricresyl phosphate (TMPP), tetrabromobisphenol A (TBBPA), tri-*n*-butyl phosphate (TNBP), tri(2-butoxyethyl) phosphate (TBOEP), tris(1-chloro-2-propyl) phosphate (TCIPP), 2-ethylhexyl diphenyl phosphate (EHDPP). ToxCast results confirmed in vivo based categorization for several FRs and identified potential molecular targets. For the nine Cat I FRs, we identified several molecular targets, health outcomes and some potential AOPs. However, the complete toxicity pathways leading from molecular targets to adverse health outcomes are still unknown, with the exception of TBBPA-induced neurotoxicity.

Conclusions: The approach presented in this study was particularly useful for the categorization of a large group of replacement FRs with relatively low data availability. We highlight priority compounds that critically need more toxicological studies or FRs for which regulatory measures could be envisaged. Our research also suggests that high toxicity indicated by ToxCast is particularly relevant for predicting higher hazard in vivo. Finally, we indicate several gaps and directions for future research, such as molecular targets that could be tested in vitro and health outcomes for cohort studies.

Keywords: Flame retardants, ToxCast, AOP, Prioritization, Hazard, Mechanisms of toxicity

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Introduction

Flame retardants (FRs) are compounds or mixtures added to polymers of consumer products, particularly textiles, furniture, electronics and electrical equipment, and building materials to delay the spread of fire and comply with flammability standards. FRs belong to a common group defined by its function but they are chemically quite diverse. Two major sub-groups of organic FRs are traditionally recognized, i.e., the brominated FRs (BFRs) and the organophosphate FRs (OPFRs). A third category is the chlorinated FRs, most of them being OPFRs or dechlorane-based compounds. The long used and most studied polybrominated diphenyl ethers (PBDEs) are under a strict ban after being included in Annex A of the Stockholm Convention on Persistent Organic Pollutants (POPs) (http://www.pops.int). However, flammability standards remain in place for many products, and consequently, other FRs such as OPFRs and novel BFRs are used as replacements. Many of these replacement FRs and their metabolites are consistently found at relatively high levels in both indoor and outdoor environments, and also in human matrices, as revealed e.g., by monitoring of indoor dust and ambient air, and human biomonitoring ([44], for reviews see [12, 19, 58, 69, 71]). People are thus exposed to these chemicals on a daily basis, possibly over a lifetime, and this may raise a serious concern for public health. It is, therefore, essential to help regulators to assess the risks FRs may pose to the population, by monitoring the levels to which people are exposed and evaluating their hazards for human health. In this paper, we specifically focus on the latter aspect, i.e., toxicity and hazard-based prioritization.

Toxicological studies have mostly relied on in vivo animal testing to evaluate the levels of hazard of chemicals, serving as a basis for regulatory decisions. However, in the last decade, the use of in vitro experiments coupled with mechanistic toxicology studies has increased, and may progressively overcome in vivo toxicology that raises both ethical, financial and efficiency issues [1]. To this end, the United States Environmental Protection Agency (US EPA) has launched the Toxicity Forecaster (ToxCast) and Toxicity Testing in the 21st century (Tox21) projects that use high-throughput screening methods and computational toxicology approaches to help rank and prioritize chemicals (https://www.epa.gov/chemical-research/toxicity-forecasting) [27]. Thanks to these projects, a large number of chemicals, including FRs, have been tested in vitro over a large spectrum of assays and all the results are openly accessible through the interactive Chemical Safety for Sustainability (iCSS) ToxCast dashboard (https://actor.epa.gov/dashboard/) [57]. In this paper, "ToxCast" refers to both ToxCast and Tox21 results.

To evaluate potential effects on human health based on in vitro testing, it is also necessary to have a wide knowledge of the specific mechanisms leading from initial effects of chemicals at the molecular level (i.e., those captured in ToxCast and Tox21) to actual adverse health effects. The Adverse Outcome Pathway (AOP) conceptual framework has been proposed to describe a chain of individual key events (KEs) linking a molecular initiating event (MIE) to an adverse outcome (AO) [2]. The AOP knowledge base (AOP-KB) represents the central repository for all AOPs developed with support from international authorities like the Organisation for Economic Co-operation and Development (OECD), European Union (EU) or US EPA (https://aopkb.oecd.org/index.html). To date, the major module of the AOP-KB is the AOP-wiki (https://aopwiki.org/), which provides a detailed text-based description of AOPs in a structured environment and is accessible online to encourage crowd-sourcing for AOP development.

The aim of the present paper is to support research and regulatory efforts by prioritizing novel FRs regarding their hazards, i.e., potential toxicity. The overall strategy was to combine information from the literature (in vivo effects of FRs), the ToxCast database and the AOP-wiki to define prioritization categories among a list of 62 identified FRs (focusing on the 52 non-regulated), and to define gaps in knowledge on their toxicity. Categorization was first established by considering (i) current regulatory status, and (ii) in vivo original research papers and reports (searching for available public literature, primarily via PubMed). We then considered available in vitro toxicological data as well, using the information from the ToxCast database, to complement in vivo categorization and get some insights on molecular targets. By combining these information sources, nine priority FRs were identified, and we have explored in detail relevant toxicity mechanisms using the AOP framework. This allowed us to propose some plausible mechanisms for the observed AOs and identify specific gaps for future research in terms of potential molecular targets and predicted AOs.

Results and discussion

In our research, we focused on a priority list of 62 synthetic organic FRs that was established by experts from the Human Biomonitoring for Europe (HBM4EU) program (scoping document is accessible in the following link: https://www.hbm4eu.eu/the-substances/flame-retardants/, see also Table 1). HBM4EU represents a joint effort of 28 countries, the European Environment Agency and the European Commission with the aim of providing evidence of the actual exposure of citizens to chemicals and the possible health effects to support policy making. Although this list does not cover all FRs present on the

Table 1 Categorization of flame retardants based on in vivo toxicological data (availability and toxicological concern)

Name	Abbreviation	CAS number	Data availability	Numbers of available in vivo publications**	Toxicological concern	In vivo prioritization categories	Tested in ToxCast (Y-Yes, N-No)
Tris-2-chloroethyl phosphate	TCEP	115-96-8	Substantial	20/10	High	Cat I*	Y
Tris(1,3-dichloropropyl)phosphate	TDCIPP	13674-87-8	Substantial	52/17	High	Cat I*	Y
Triphenyl phosphate	TPHP	115-86-6	Substantial	39/24	High	Cat I*	Y
Tricresyl phosphate	TMPP	1330-78-5	Substantial	17/10	High	Cat I*	Y
Tetrabromobisphenol A	TBBPA	79-94-7	Substantial	69/36	Some	Cat I	Y
Tri(2-butoxyethyl) phosphate	TBOEP	78-51-3	Substantial	18/4	Some	Cat I	Y
Tris(1-chloro-2-propyl) phosphate	TCIPP	13674-84-5	Substantial	14/8	Some	Cat I	Y
2-ethylhexyl diphenyl phosphate	EHDPP	1241-94-7	Substantial	13/10	Some	Cat I	Y
Tri-*n*-butyl phosphate	TNBP	126-73-8	Substantial	17/11	Some	Cat I	Y
Dibromoneopentylglycol	DBNPG	3296-90-0	Scarce	2/2	High	Cat II*	Y
2,4,6-Tribromophenol	2,4,6-TBP	118-79-6	Scarce	5/3	High	Cat II*	Y
Isopropyl triphenyl phosphate	ip-TPP	68937-41-7	Scarce	9/4	High	Cat II*	Y
2,2-Bis(chloromethyl)trimethylenebis[bis(2-chloroethyl) phosphate]	V6	38051-10-4	Scarce	0/0	High	Cat II*	N
Decabromodiphenylethane	DBDPE	84852-53-9	Scarce	5/2	Some	Cat II	N
Tetrabromoethylcyclohexane	DBE-DBCH	3322-93-8	Scarce	9/1	Some	Cat II	Y
Hexabromobenzene	HBB	87-82-1	Scarce	6/3	Some	Cat II	Y
Pentabromotoluene	PBT	87-83-2	Scarce	2/1	Some	Cat II	N
Tris(2-ethylhexyl) phosphate	TEHP	78-42-2	Scarce	4/3	Some	Cat II	Y
Cresyl diphenyl phosphate	CDP	26444-49-5	Scarce	2/1	Some	Cat II	Y
Tris(2,3-dibromopropyl)isocyanurate	TDBP-TAZTO	52434-90-9	Scarce	5/3	Some	Cat II	N
Resorcinol bis(diphenylphosphate)	RBDPP	125997-21-9, 57583-54-7	Scarce	2/0	Some	Cat II	N
Dechlorane 602	DDC-DBF	31107-44-5	Scarce	1/1	Some	Cat II	N
Triethyl phosphate	TEP	78-40-0	Scarce	6/4	Some	Cat II	Y
Dechlorane Plus	DDC-CO	135821-03-9	Substantial	11/4	Low	Cat III	N
1,2-Bis(2,4,6-tribromophenoxy)ethane	BTBPE	37853-59-1	Scarce	4/1	Low	Cat IV	N
Tri-iso-butyl phosphate	TIBP	126-71-6	Scarce	1/1	Low	Cat IV	Y
2-(2-Hydroxyethoxy)ethyl 2-hydroxypropyl 3,4,5,6-tetrabromophthalate	HEEHP-TEBP	20566-35-2	Scarce	0/0	Low	Cat IV	N
2,4,6-Tris(2,4,6-tribromophenoxy)-1,3,5-triazine	TTBP-TAZ	25713-60-4	Scarce	0/0	Low	Cat IV	N
Tris(tribromoneopentyl)phosphate	TTBNPP	19186-97-1	Scarce	0/0	Low	Cat IV	N
Bisphenol A bis(diphenylphosphate)	BPA-BDPP	5945-33-5 , 181028-79-5	Scarce	1/0	Low	Cat IV	N
Bis(2-ethylhexyl)tetrabromophthalate	BEH-TEBP	26040-51-7	Scarce	9/3	Low/some	Cat IV/II	Y
2-Ethylhexyl-2,3,4,5-tetrabromobenzoate	EH-TBB	183658-27-7	Scarce	8/2	Low/some	Cat IV/II	N
N,*N*′-ethylenebis(tetrabromophthalimide)	EBTEBPI	32588-76-4	Insufficient	0/0	–	Cat V	N
Melamine polyphosphate	Melamine polyphosphate	20208-95-1, 15541-60-3	Insufficient	0/0	–	Cat V	N
Hexachlorocyclopentenyldibromocyclooctane	DBHCTD (HCDBCO)	51936-55-1	Insufficient	1/0	–	Cat V	N
Octabromotrimethyphenyl indane	OBTMPI	1084889-51-9, 1025956-65-3,893843-07-7, 155613-93-7	Insufficient	0/0	–	Cat V	N
Pentabromobenzyl acrylate	PBB-Acr	59947-55-1	Insufficient	0/0	–	Cat V	N
1,2,5,6-tetrabromocyclooctane	TBCO	3194-57-8	Insufficient	2/0	–	Cat V	N
2,3,5,6-tetrabromo-p-xylene	TBX	23488-38-2	Insufficient	0/0	–	Cat V	N
Pentabromoethylbenzene	PBEB	85-22-3	Insufficient	0/0	–	Cat V	Y
Tri-*n*-propyl-phosphate	TnPP	513-08-6	Insufficient	1/0	–	Cat V	N
Pentabromophenoxy-nonabromodiphenyl ether	4′-PeBPO-BDE208	58965-66-5	Insufficient	0/0	–	Cat V	N
Tribromoneopentyl alcohol	TBNPA	1522-92-5	Insufficient	0/0	–	Cat V	Y
Hexabromocyclodecane	HBCYD	25495-98-1	Insufficient	0/0	–	Cat V	N
Dibromostyrene	DBS	31780-26-4	Insufficient	0/0	–	Cat V	N
1,3-Bis(2,3-dibromopropyl)-5-(2-propen-1-yl)-1,3,5-triazine-2,4,5(1H,3H,5H)-trione	BDBP-TAZTO	75795-16-3	Insufficient	0/0	–	Cat V	N
1-(2,3-Dibromopropyl)-3,5-diallyl-1,3,5-triazine-2,4,6(1H,3H,5H)-trione	DBP-TAZTO	57829-89-7	Insufficient	0/0	–	Cat V	N
Pentabromophenol	PBP	608-71-9	Insufficient	0/0	–	Cat V	Y
2,4-Dibromophenol	DBP	615-58-7	Insufficient	0/0	–	Cat V	N

Table 1 (continued)

Name	Abbreviation	CAS number	Data availability	Numbers of available in vivo publications**	Toxicological concern	In vivo prioritization categories	Tested in ToxCast (Y-Yes, N-No)
Dechlorane 603	DDC-Ant	13560-92-4	Insufficient	0/0	–	Cat V	N
Dechlorane 604	HCTBPH/Dec 604	34571-16-9	Insufficient	0/0	–	Cat V	N
Diethylphosphinic acid	Diethylphosphinic acid	813-76-3	Insufficient	0/0	–	Cat V	N
2,4,4′-Tribromodiphenyl ether	BDE-28	41318-75-6				Regulated	N
2,2′4,4′-Tetrabromodiphenyl ether	BDE-47	5436-43-1				Regulated	Y
2,2′,4,4′,5-Pentabromodiphenyl ether	BDE-99	60348-60-9				Regulated	Y
2,2′,4,4′,6-Pentabromodiphenyl ether	BDE-100	189084-64-8				Regulated	N
2,2′,4,4′,5,5′-Hexabromodiphenyl ether	BDE-153	68631-49-2				Regulated	Y
2,2′,4,4′,5,6′-Hexabromodiphenyl ether	BDE-154	207122-15-4				Regulated	N
2,2′,3,4,4′,5′,6-Heptabromodiphenyl ether	BDE-183	207122-16-5				Regulated	N
2,2′,3,3′,4,4′,5,5′,6,6′-Decabromodiphenyl ether	BDE-209	1163-19-5				Regulated	Y
Hexabromocyclododecane	HBCDD	3194-55-6, 25637-99-4, 1093632-34-8				Regulated	Y
Tris(2,3-dibromopropyl) phosphate	TDBPP	126-72-7				Regulated	Y

Prioritization categories

Cat I: substantial toxicological data indicating toxicological concern

Cat II: scarce toxicological data indicating toxicological concern

Cat III: substantial toxicological data indicating lower toxicological concern

Cat IV: scarce toxicological data indicating lower toxicological concern

Cat V: insufficient toxicological data to evaluate the toxicological concern

* High toxicological concern

** All vertebrates/mammals

market, it is especially relevant for the identification of priority compounds for the European Union.

FRs regulatory status and categorization using in vivo toxicity data

Regulatory status

As the first criterion, the regulatory status of the compounds was explored and those compounds that are already subject to restrictions on new use were not further categorized (see Additional file 1: Table S1). We searched whether compounds were listed in following lists: Stockholm Convention on POPs, Annex XIV and Annex XVII of European chemical regulation REACH, the list of substances of very high concern (SVHC), categories assigned by the World Health Organization (WHO) International Agency for Research on Cancer (IARC), and eventual others (see below). PBDEs and hexabromocyclododecane are listed in Annex A of the Stockholm Convention on POPs, meaning that "parties must take measures to eliminate the production and use of the chemicals" (http://chm.pops.int/TheConvention/ThePOPs/AllPOPs/tabid/2509/Default.aspx). Tris(2,3-dibromopropoyl) phosphate (TDBPP) is included in the ECHA restriction list (Annex XVII to REACH) with the following condition: "shall not be used in textile articles, such as garments, undergarments and linen, intended to come into contact with the skin". We decided to categorize the ten above-mentioned substances as "regulated" and did not pursue their analysis further. Among the other FRs, tris-2-chloroethyl phosphate (TCEP) is included in Annex XIV of REACH and in the candidate list of SVHCs for authorization, tetrabromobisphenol A (TBBPA) and dibromoneopentylglycol (DBNPG) are categorized by the IARC as "Probably carcinogenic to humans" (Cat 2A) or "Possibly carcinogenic to humans" (Cat 2B), respectively. Tris(1,3-dichloropropyl)phosphate (TDCIPP) is included in the Proposition 65 list of chemicals known to cause cancer by the California EPA [29]. TDCIPP and TCEP have also been placed under some regulatory controls, mostly for children's toys and equipment, in several states of the US (http://www.saferstates.com/toxic-chemicals/toxic-flame-retardants). Although these can be considered as strong arguments for toxicological concern, they do not imply a strict ban or strong restriction of the substance at large scales. We, therefore, decided to include TCEP, TBBPA, DBNGP and TDCIPP in the prioritization work, as well as all other remaining FRs. For these 52 "non-regulated" FRs from the initial list, we then established prioritization categories based on toxicological data availability and toxicological concern.

In vivo toxicological data collection

To search for toxicological data, we used PubMed and/or Web of Science as resources for finding original research papers (in vivo studies with vertebrates or other evidences, e.g., from human cohort studies). The search query was as follows "Full name, or abbreviation(s) of the compound AND toxicity". When too few results (less than 10) were retrieved, the search with only "full name or abbreviation(s) of the compound" was additionally performed. Cohort and epidemiology studies using metabolites as markers of exposure were also considered as additional evidence for the toxicity of the parent compound. For example, bis(1,3-dichloro-2-propyl) phosphate (BDCIPP) in urine is listed in the reference for TDCIPP, and studies using diphenyl phosphate (DPhP) as a marker of exposure were included as references for two major parent compounds, i.e., triphenyl phosphate (TPhP) and 2-ethylhexyl diphenyl phosphate (EHDPP) [13]. Around 200 research papers that included information on FR toxicity in vivo were found following these literature searches, with a heterogeneity in the number of retrieved papers for each compound (see Table 1 and Additional file 1: Table S1). The detailed list of references for each FR can be found in Additional file 2: Table S4.

In addition, we considered reports from several regulatory agencies such as the US-EPA, European Food Safety Authority (EFSA), WHO, National Research Council (NRC), EU, UK Environment Agency, Norwegian Environment Agency (NEA), US National Toxicology Program (NTP) and the US Agency for Toxic Substances and Disease Registry (ATSDR).

Categorizations for data availability and for toxicological concern

To evaluate and categorize the amount of data available, we considered both reports and original papers that described any toxicological effects of chemical exposure in a vertebrate system, with higher weight given to mammalian studies (see Additional file 1: Table S1). We have used three categories in terms of information availability for individual FRs, i.e., insufficient, scarce or substantial. Insufficient means we could not find any in vivo toxicity data in mammals; scarce means there was at least one paper addressing toxicity in mammals or at least one agency report describing mammalian studies; substantial means we found at least ten papers with in vivo toxicity results, including at least four publications reporting mammalian toxicity (i.e., papers or documented reports). For more than a third of the non-regulated FRs from the priority list (i.e., 20 out of 52), we did not find any accessible data on in vivo toxicity in mammals (see Table 1). For the majority of the remaining FRs, the information remains scarce (22 FRs), and substantial documentation of in vivo toxicity has been found for only ten FRs.

We then evaluated hazard level of FRs (those with scarce or substantial in vivo toxicity data) using three categories—low, some or high toxicological concern, considering any sub-lethal toxicological effect. In general, categorization depended on the effective doses at which effects were observed, consistency among studies (when enough studies are available) and categorization by regulatory agencies (when available). Criteria for each categories were as follow: high—information from the majority of research papers (mostly rodent studies and human cohorts, when available) and reports from regulatory agencies converge to conclude on higher hazard; some—variable evidence in the studies: some conclude moderate or high hazard while others conclude on low hazard; low—information from the majority of studies indicated lower hazard (higher weight was given to evidence from mammalian studies). Detailed arguments are provided in the "Comments on hazard levels" section of the Additional file 1: Table S1 and quantitative criteria for High/Moderate/Low hazard, derived from US-EPA criteria, are provided in Additional file 1: Table S2. The attribution to high/some/low toxicological concern categories depends on the amount of data availability, and was, therefore, done on a case-by-case basis, involving also our expert opinion. For example, TDCIPP was considered to have high toxicological concern based on converging conclusions from reports from agencies, such as US-EPA, many rodents and fish studies and human cohorts, while this category was attributed to DBNPG based mostly on one NTP report. In the case of Dechlorane Plus (DDC-CO), some studies in fish indicated potential for toxicity but several well-documented rodent studies concluded low hazard and we, therefore, decided to categorize it with a low human health toxicological concern. As for tri(2-butoxyethyl) phosphate (TBOEP), studies in rodents indicated moderate hazard while several fish studies indicated high hazard from which we concluded that it presents some toxicological concern. References to all the studies considered are presented in Additional file 2: Table S4.

Prioritization categories and recommended actions

Considering both the availability of data on in vivo toxicity and corresponding hazard evaluation, we established five priority categories: Cat V—data available are insufficient and hazard cannot be evaluated; Cat IV and III—lower toxicological concern based on scarce (Cat IV) or substantial (Cat III) information; and Cat I and II—some or high toxicological concern (high indicated with an asterisk) based on scarce (Cat II) or substantial (Cat I) information. The results of the categorization are

provided in Table 1 with full names and abbreviations of all FRs. Details on the data supporting the categorization are provided in Additional file 1: Table S1. It is important to note that this is entirely hazard-based, and other information (e.g., exposures) should also be taken into account for final decisions regarding priority and regulation.

Our categorization may provide indication on future actions that could be taken for each FRs, depending on their category. Thus, for compounds in Cat V, IV and II there is a clear need for more toxicological studies because only few or no studies were identified in our search. This is particularly urgent for Cat V (20 compounds), where no data on in vivo toxicity in mammals could be identified. Cat IV compounds (6 FRs) appear to be of lower priority especially in comparison to Cat II compounds (14 FRs). Cat I compounds (9 FRs) seem to be of priority and may be considered for regulatory measures. However, additional information on their toxicity in humans, especially from cohort or epidemiology studies, and on their mechanisms of toxicity are needed. Compounds highlighted with asterisk (*), i.e., Cat II* (2,4,6-tribromophenol (2,4,6-TBP), isopropyl triphenyl phosphate (ip-TPP), 2,2-bis(chloromethyl)trimethylenebis[bis(2-chloroethyl) phosphate] (V6) and DBNPG) and Cat I* (TCEP, TDCIPP, TPhP and Tricresyl phosphate (TMPP)), are of the highest priority considering their toxicological concern. Finally, Cat III (one FR only, DDC-CO) may be a better candidate replacement of the regulated FRs. For two compounds, namely Bis(2-ethylhexyl)tetrabromophthalate (BEH-TEBP) and 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB), the categorization regarding toxicological concern remained uncertain because the evidence was mostly based on studies of a chemical mixture (such as FireMaster 550), which contains a number of other FRs assessed in this study. These indicate rather moderate chronic toxicity but it is difficult to conclude on the exact hazard of each individual compound.

To further refine the prioritization, address uncertainties, identify potential molecular targets and get more insights into FR toxicity (especially for Cat V FRs, where no in vivo toxicity data were available), we have also explored available in vitro data. In this paper, we will only describe the search for in vitro data using the ToxCast dashboard. Detailed exploration of research publications where in vitro studies were reported is beyond the scope of the present study.

Using ToxCast data for "in vitro prioritization" and identification of molecular targets

Data collection using the iCSS ToxCast dashboard

The iCSS ToxCast dashboard provides an easy and open access to chemical screening data from ToxCast and Tox21 programs, for a total of 9076 chemicals using 1192 assays (status as of March 2018) (https://actor.epa.gov/dashboard/). We searched for each FR by CAS number or chemical name, and retrieved the (i) number of assays in which the FR had been tested, and (ii) the number of assays in which the FR was assigned as "active" according to the ToxCast classification. A chemical is considered "active" if any of the three regression parameters automatically derived for the obtained assay (i.e., constant, hill and gain–loss) sufficiently fits the dose–response curve [57]. Out of the total 62 assessed FRs, 28 were tested in ToxCast and/or Tox21 assays. Data from at least 113 assays and up to 882 assays per chemical were available (see Table 2). All chemicals were active in at least one assay (with the only exception of decabromodiphenyl ether (BDE-209), CAS 1163-19-5). However, the percentage of assays in which the FR was "active" substantially varied ranging from 0.23% for DBNPG to 22.3% for TBBPA.

We also looked in detail on the active hits to "verify" the AC50 (concentration of a chemical where 50% of the maximum response is achieved), which has been automatically derived within the high-throughput screening mode and stored in the databases. First, we selected the records where AC50 of individual FRs were relatively low (lower AC50 values indicate higher toxicity). To reduce the risk of selecting non specific hits that may occur when AC50 is close to the concentrations at which general cytotoxicity is observed [28], we used only AC50 values that were below the cytotoxicity limits provided in the ToxCast dashboard (see Table 2). Almost half of the FRs (13 of 28) can be considered as non cytotoxic in the concentration range tested (cytotoxicity limit of 1000 μM). For these FRs, we decided to select AC50 below 10 μM (this threshold is commonly used in in vitro screenings). For the other half of the FRs (14 of 28), cytotoxicity limits were rather low, ranging from 1.11 μM (for hexabromocyclododecane (HBCDD)) to 12.2 μM (for TBOEP), and we set an arbitrary threshold of 1 μM. For one FR (BDE-209), cytotoxicity limit was not available. Second, we confirmed the selected AC50 by examining the quality of individual dose–response curves (see details in Additional file 3: Figure S1).

Categorization based on in vitro toxicity using ToxCast results

Two criteria from the ToxCast in vitro data, i.e., (1) the percentage of "positive hits" (assays in which the FR is active) and (2) the presence or absence of "verified hits" (confirmed AC50 below 10 μM or below cytotoxicity limit), were used to classify FRs into three categories. "Low in vitro toxicity" compounds (8 compounds) were active in less than 10% of the tested assays and there were no verified hits. For example, TCEP and

Table 2 Prioritization of flame retardants based on in vitro results from the ToxCast assays

Abbreviation	In vivo prioritization	ToxCast prioritization	Final prioritization	Cytotoxic limit (µM; as found in ToxCast)	Number of assays tested	% positive hits	Number of assays with AC50 < 1 µM	Number of assays with AC50 < 10 µM	Number of "verified hits"
TCEP	Cat I*	L	Cat I*	1000	874	0.34	0	0	0
TDCIPP	Cat I*	H	Cat I*	6.71	883	14.84	2		2
TPHP	Cat I*	H	Cat I*	5.2	882	12.81	4		2
TMPP	Cat I*	H	Cat I*	3.91	562	10.14	4		2
TBBPA	Cat I	H	Cat I	8.22	882	22.34	2		1
TBOEP	Cat I	M	Cat I	12.2	566	5.83	7		1
TCIPP	Cat I	M	Cat I	1000	546	2.38	6	4	3
EHDPP	Cat I	H	Cat I	2.08	566	19.96	4		4
TNBP	Cat I	M	Cat I	1000	883	2.04	0	6	4
DBNPG	Cat II*	L	Cat II*	1000	874	0.23	1	0	0
2,4,6-TBP	Cat II*	M	Cat II*	6.27	566	13.78	1		0
ip-TPP	Cat II*	M	Cat II*	1.9	509	21.02	6		0
DBE-DBCH	Cat II	M	Cat II	1000	113	2.65	0	1	1
HBB	Cat II	M	Cat II	1000	113	0.88	0	1	1
TEHP	Cat II	M	Cat II	3.41	882	6.35	6		3
CDP	Cat II	H	Cat II	3.24	509	18.27	6		2
TEP	Cat II	L	Cat II	1000	390	0.51	0	2	0
TIBP	Cat IV	M	Cat IV	1000	513	2.73	4	3	2
BEH-TEBP	Cat IV/II?	M	Cat IV/II?	1000	546	2.01	1	2	1
PBEB	Cat V	M	Cat V	1000	416	3.85	3	6	5
TBNPA	Cat V	L	Cat V	1000	113	1.77	0	0	0
PBP	Cat V	H	Cat II	5.53	113	22.12	2		2
BDE-47	Regulated	L	Regulated	7.78	113	7.08	0		0
BDE-99	Regulated	L	Regulated	1000	113	2.65	0	0	0
BDE-153	Regulated	L	Regulated	1000	113	1.77	0	2	0
BDE-209	Regulated	L	Regulated	NA	242	0.00	0	0	0
HBCDD	Regulated	M	Regulated	1.11	602	17.77	4		3
TDBPP	Regulated	H	Regulated	2.41	566	18.02	18		5

ToxCast prioritization

L (low): < 10% of positive hits and no "verified hit"

M (moderate): > 10% of positive hits and no "verified hit" OR < 10% of positive hits and "verified hit(s)"

H (high): > 10% of positive hits and "verified hit(s)"

Positive hit: assay for which the compound is indicated as "active" in ToxCast

"Verified hit": AC50 $\leq$ 10 µM or AC50 $\leq$ 1 µM (if cytotoxicity limit is close to, or bellow, 10 µM), and verified after examining the dose–response curve

* High toxicological concern

tetrabromodiphenyl ether (BDE-47) were categorized as low. TCEP was active in only 0.34% of 874 assays and AC50 for the active assays were all above 10 µM, and BDE-47 was active in 7.1% of 113 assays and there was no AC50 below 1 µM. On the other hand, "high in vitro toxicity" compounds (8 compounds) were active in more than 10% of the tested assays with at least one verified hit. For example, TDCIPP was active in 15% of 883 assays and two AC50 below 1 µM were verified after DR curve examination. Finally, "moderate in vitro toxicity" compounds (12 compounds) were either active in less than 10% of tested assays with at least one verified hit, or active in more than 10% of tested assays but with no verified hit. For example, Tris(2-ethylhexyl) phosphate (TEHP) was active in 6.4% of 882 assays and three AC50 below 1 µM were confirmed after DR curve examination.

Comparing categorizations based on in vitro (ToxCast) vs. in vivo data and refining prioritization categories

We then compared the categorization based on in vitro ToxCast data (Table 2) with the in vivo prioritization of 18 Cat I-IV FRs tested in ToxCast (discussed in the previous section; Table 1). All compounds with High in vitro toxicity based on ToxCast results also presented some or high toxicological concern based on in vivo data (Cat I and II). Those were 6 out of 18 compounds—TDCIPP, TPhP, TMPP, TBBPA, EHDPP, and Cresyl diphenyl phosphate (CDP). Compounds with Moderate in vitro toxicity based on ToxCast were categorized for having either low (Tri-iso-butyl phosphate, TIBP), some (TBOEP, Tris(1-chloro-2-propyl) phosphate (TCIPP), tri-*n*-butyl

phosphate (TNBP), tetrabromoethylcyclohexane (DBE-DBCH), hexabromobenzene (HBB) and TEHP) or high in vivo toxicological concern (2,4,6 TBP and ip-TPP). Finally, compounds with low in vitro toxicity were considered to have some (triethyl phosphate (TEP)) or high (TCEP, DBNPG) toxicological concern in vivo. TCEP is probably the best example, with one of the lowest percentage of positive hits in ToxCast (0.34% of 874 assays) but high toxicological concern in vivo (it is on the list of SVHC and in the Annex XIV of REACH). These inconsistencies between in vitro results from ToxCast and in vivo effects could be due to the lack of assays corresponding to the toxicological endpoints of these compounds. Another explanation would be that these FRs have a poor bioavailability in in vitro assays (e.g., binding to plastic, rapid degradation), which would be consistent with their high cytotoxicity limits in ToxCast (1000 μM for TCEP, DBNPG and TEP). It is also important to note that metabolic activation, which is not addressed in ToxCast in vitro testing, may play an important role in the in vivo effects of these FRs. Interestingly, we did not record any cases where in vivo was "low" but in vitro evidence indicated "high" toxicity.

Taken together, by comparing in vivo to in vitro ToxCast categorizations for 18 chemicals, our research suggests that High in vitro toxicity based on ToxCast results may provide a good indication that the compound is toxic also in vivo. On the other hand, Low or Moderate in vitro toxicity from ToxCast cannot be directly used to predict the level of hazard in vivo. This observation is not based on a large scale systematic analysis and could be due to our rather conservative approach when attributing the toxicological concern based on in vivo data, or due to the criteria we used for establishing ToxCast-based prioritization categories. However, this is most likely due to missing hits (false negatives) and insufficient range of tests that do not represent all possible molecular targets. Indeed, another case study reached the same conclusions when comparing in vivo toxicity of *ortho* phthalates to ToxCast based prioritization using different ranking methods (Toxicological Priority Index and statistical methods) [51].

Finally, we used ToxCast results as an indication for the compounds for which availability of in vivo toxicological data were insufficient [i.e., Cat V compounds—pentabromoethylbenzene (PBEB), tribromoneopentyl alcohol (TBNPA) and pentabromophenol (PBP)] or for which the toxicological concern remained uncertain (two in Cat IV/II but only BEH-TEBP was tested in ToxCast/Tox 21). For PBP, ToxCast results suggest that this compound has High in vitro toxicity based on ToxCast results (more than 20% of positive hits and 2 "verified" hits), and this seems to be a good indication of in vivo toxicological concern, as discussed above. We, therefore, decided to move PBP into Cat II. This is also in agreement with an in vitro study showing that PBP is a very potent competitor for thyroxine (T4) binding to transthyretin (TTR) [43]. For the other three compounds with insufficient in vivo toxicological data (TBNPA and PBEB), or uncertain in vivo categorization (BEH-TEBP), Low or Moderate in vitro toxicity based on ToxCast results cannot be used alone to predict the level of hazard in vivo, as also discussed above. We therefore kept these compounds in Cat V or Cat II/IV.

In conclusion, results from the ToxCast assays provided indications of potential toxicity (hazard) that was useful for prioritization, in complement to in vivo information. For several FRs, it further confirmed the categories attributed based on in vivo data. In one case (PBP), we used ToxCast results as additional evidence to complement insufficient in vivo data and to change the categorization. When ToxCast and in vivo prioritizations were contradictory, the in vivo prioritization had preference and was not modified.

Identification of potential molecular targets for individual FRs using ToxCast results

Since ToxCast primarily serves to identify potential molecular targets of chemicals and provide insights into the mechanisms of toxicity, we have explored this information source for FRs considering the verified hits (i.e., assays for which AC50 was below or equal to 10 μM, or bellow cytotoxicity limit, and that were manually verified; see Additional file 3: Figure S1). For five of the tested FRs, there was no assay with an AC50 below 10 μM or below cytotoxicity limit (see Table 2). For the other 23 FRs, more than half of the hits (108 assays in total) were not confirmed after examining the dose–response curve (64 out 108). This may reflect some imperfections in the design of ToxCast assays and their analysis in identifying false positive. This issue is known and ToxCast analysis includes a last step that flags potential false positive and false negative findings. However, this flagging step, which is fully automated, remains error-prone, and manual verification of individual hits is therefore highly recommended [57].

A detailed look at the molecular targets of the verified hits revealed that the majority of the hits (24 out of 44) related to drug metabolism: one assay corresponded to CIS-activation of NR1I3 (also named CAR for constitutive androstane receptor) response element, one assay corresponded to CYP2C19 enzymatic activity and 22 hits corresponded to activation of Pregnane X Receptor (PXR, also named SXR or NR1I2) (see Table 3). In agreement with the ToxCast/Tox21 results, potent activation of PXR by several OPFRs and their metabolites

Table 3 Potential molecular targets ("verified hits") identified in ToxCast assays

Abbreviation	Number of "verified hits"	Molecular target	AC50 (μmol/L)	Name of assay
TDCIPP	2 (2)	PXR	0.388	ATG_PXRE_CIS_up
		PXR	0.358	ATG_PXR_TRANS_up
TPHP	2 (4)	PXR	0.704	ATG_PXR_TRANS_up
		CYP2C19	0.804	NVS_ADME_hCYP2C19
TMPP	2 (4)	PXR	0.530	ATG_PXRE_CIS_up
		PXR	0.857	ATG_PXR_TRANS_up
TBBPA	1 (2)	PPAR	0.93	ATG_PPRE_CIS_up
TBOEP	1 (7)	PXR	0.988	ATG_PXRE_CIS_up
TCIPP	3 (10)	PXR	1.03	ATG_PXR_TRANS_up
		PXR	4.68	NVS_NR_hPXR
		PXR	3.84	ATG_PXRE_CIS_up
EHDPP	4 (4)	PXR	0.530	ATG_PXRE_CIS_up
		PXR	0.279	ATG_PXR_TRANS_up
		VDR	0.829	ATG_VDRE_CIS_up
		ER1	1.07	ATG_ERa_TRANS_up
TNBP	4 (6)	PXR	3.80	ATG_PXRE_CIS_up
		PXR	5.75	ATG_PXR_TRANS_up
		TSPO	1.29	NVS_MP_hPBR
		TSPO	1.38	NVS_MP_rPBR
DBE-DBCH	1 (1)	AR	1.55	TOX21_AR_LUC_MDAKB2_Agonist
HBB	1 (1)	AR	7.94	TOX21_AR_LUC_MDAKB2_Agonist
TEHP	3 (6)	PXR	0.364	ATG_PXRE_CIS_up
		PXR	0.243	ATG_PXR_TRANS_up
		VDR	0.617	ATG_VDRE_CIS_up
CDP	2 (6)	PXR	0.735	ATG_PXR_TRANS_up
		MMP(mit)[a]	1.05	TOX21_MMP_ratio_down
TIBP	2 (7)	PXR	1.78	ATG_PXRE_CIS_up
		PXR	1.58	ATG_PXR_TRANS_up
BEH-TEBP	1 (3)	GR	4.96	NVS_NR_hGR
PBEB	5 (9)	PXR	7.94	ATG_PXRE_CIS_up
		PXR	6.92	ATG_PXR_TRANS_up
		RXR	5.50	ATG_RXRb_TRANS_up
		uPAR	4.07	BSK_3C_uPAR_up
		Tissue factor	4.37	BSK_3C_TissueFactor_down
PBP	2 (2)	PPARγ	0.374	TOX21_PPARg_BLA_antagonist_ratio
		p53	0.932	TOX21_p53_BLA_p3_ratio
HBCDD	3 (4)	MMP(met)1[a]	0.892	BSK_BE3C_MMP1_up
		PAI	0.954	BSK_hDFCGF_PAI1_down
		Proliferation	0.970	BSK_hDFCGF_Proliferation_down
TDBPP	5 (18)	PXR	0.188	ATG_PXR_TRANS_up
		PXR	0.410	NVS_NR_hPXR
		NR1I3	0.553	ATG_PBREM_CIS_up
		TSPO	0.152	NVS_MP_hPBR
		TSPO	0.113	NVS_MP_rPBR

Color code indicates to which target the assay is related: bright blue—PXR; dark blue—other related to drug metabolism; orange—VDR; red—PPAR; green—TSPO; purple—AR; yellow—various unique targets

"Verified hit": AC50 ≤ 10 μM or AC50 ≤ 1 μM (if cytotoxicity limit is close to, or bellow, 10 μM), and verified after examining the dose–response curve

PXR pregnane X receptor, *CYP2C19* cytochrome P450 family 2 subfamily C polypeptide 19, *PPAR* peroxisome proliferator-activated receptor, *VDR* vitamin D receptor, *ER1* estrogen receptor 1, *TSPO* translocator protein, *AR* androgen receptor, *MMP(mit)* mitochondrial membrane potential, *GR* glucocorticoid receptor, *RXRβ* retinoid X receptor beta, *uPAR* urokinase receptor, *MMP(met)1* matrix metallopeptidase 1, *PAI* plasminogen activator inhibitor, *NR1I3* nuclear receptor subfamily 1 group I member 3

[a] MMP abbreviation is used for two different names in ToxCast: mitochondrial membrane potential (we added "(mit)") and matrix metallopeptidase (we added "(met)")

has also been reported in research papers by Kojima and colleagues [33, 34]. This is somehow expected due to the well-established role of PXR in drug metabolism, and PXR is a common target for many chemicals, as evidenced by the high frequencies of positive hits for all chemicals tested in ToxCast in PXR related assays (29% for the assay "ATG_PXR_TRANS_up" and almost 50% for the assay "ATG_PXRE_CIS_up") (for review, see [73]). However, the fact that several FRs (TDCIPP, TPhP, TMPP, TBOEP, TCIPP, EHDPP, TNBP, TEHP, CDP, TIBP, PBEB and TDBPP) activated PXR with low AC50s ranging from 0.188 to 1.03, raises the issue of possible mixtures effects that may have relevance with regard to human health outcomes. Indeed, recent research has identified non-xenobiotic roles for PXR such as increase of metastasis and drug resistance that may contribute to poor prognosis in cancer (for review, see [49]). PXR may also play an important role in the development of metabolic syndromes [21, 62] and is associated with hepatic steatosis or neurodevelopmental toxicity via two existing AOPs (AOPs 60 and 8, https://aopwiki.org/oecd_page).

Among other verified hits, we found Peroxisome proliferator-activated receptor (PPAR for TBBPA and PBP) that plays important roles in several diseases including diabetes and obesity (for review, see [25]). In addition, TBBPA was active in another two PPARγ-related ToxCast assays that are not shown in Table 3 because AC50 were slightly above 1 μM. This is in agreement with other reports of PPARγ activation by TBBPA at concentrations below 1 μM in cell culture and zebrafish [54, 55]. Androgen receptor (AR) activity appears to be affected by DBE-DBCH (also known as TBECH) and HBB. Androgenic activity of DBE-DBCH has already been reported in several in vitro system and in zebrafish, and gonadal masculinization (an expected effect of androgenic activity) has also been observed in frogs [3, 30, 31, 39, 53]. Four hits indicate effects of TNBP and TDBPP on function and kinetics of Translocator protein (TSPO, also named peripheral benzodiazepine receptor—PBR). TSPO is an outer mitochondrial membrane protein that was initially thought to regulate steroidogenesis but recent studies seriously question this function and its exact role remains to be established (for review, see [59]). Another two verified hits related to the activation of the Vitamin D Receptor (VDR) response element were found for TEHP and EHDPP. Vitamin D may play an important protective role against cancer (for review, see [18]). Finally, some other positive assay responses were detected for only one FR, such as Estrogen Receptor 1 (ER1) for EHDPP, p53 for PBP, mitochondrial membrane potential (MMP(mit)) for CDP, glucocorticoid receptor (GR) for BEH-TEBP, retinoid X receptor beta (RXRβ) for PBEB and others (see Table 3). Many of these targets are known to play a role in various pathologies. For example, the *p53* gene is mutated in more than half of human cancers [37]. RXRβ is a binding partner regulating transcriptional function of several nuclear receptors (e.g., retinoic acid receptor, thyroid hormones and VDR) and it, therefore, regulates several processes in development or diabetes [64, 74]. Hits related to changes in proliferation (for HBCDD) or mitochondrial membrane potential (for CDP) may be associated with general cytotoxicity. AC50 for these assays are indeed very close to the cytotoxicity limits reported in ToxCast for both chemicals (see Tables 2, 3).

Taken together, ToxCast results were useful for identifying potential molecular targets. There were several different targets related to various processes, probably reflecting the structural variety of FRs. However, a few targets (namely PXR, PPARγ, AR, TSPO and VDR) were shared by several (up to 12) FRs, raising the issue of potential additive mixture effects. Identifying targets also gives insights into mechanisms of toxicity, although additional information is needed to understand the full pathway leading from target to health outcome.

Identification of plausible mechanisms of toxicity for Cat I FRs, using AOP-wiki

We used the AOP-wiki to organize and rationalize all the information collected previously (research papers, reports and in vitro ToxCast results). Our aims were to identify (1) plausible mechanisms of toxicity, primarily for the observed in vivo AOs, and (2) gaps in knowledge, where experimental data are needed to clarify molecular targets, adverse outcomes, and toxicity pathways. In this work, we only focused on Cat I compounds for which there was sufficient information available and that seemed to present a toxicological concern. We would like to note that the information on AOPs presented in the paper reflects the state of the art as of June 2018, when the information from AOP-Wiki was collected, and may differ from the current status, because of the dynamic nature of the AOP-Wiki. Links to snapshots of the AOPs reflecting their status at the time when we collected information are provided in Table 4 and in Additional file 1: Table S3. Current status of all AOPs can be found in AOP-wiki (https://aopwiki.org/aops).

Procedure to search for plausible toxicity pathways among existing AOPs

Over 200 AOPs are currently listed in the AOP-wiki to date (as of June 2018), of which only nine have been endorsed or approved, ten are under review and the large majority are under development, with variable AOP confidence. To identify plausible mechanisms of toxicity, we searched for those AOPs for which we found enough evidence (in ToxCast, research papers or

Table 4 Plausible AOPs for category I flame retardants

AOP number	AOP name (as of June 2018)	Status of AOP (as of June 2018)		Snapshot[b]	
		SAAOP status	OECD status	URL	Date (MM.DD.YYY)
8	Upregulation of thyroid hormone catabolism via activation of hepatic nuclear receptors, and subsequent adverse neurodevelopmental outcomes in mammals short	Included in OECD work plan	Under development	https://tinyurl.com/y9c4kh4v	01.04.2019
18	PPARα activation in utero leading to impaired fertility in males	Included in OECD work plan	EAGMST under review	https://tinyurl.com/yb8qwyro	01.04.2019
29[a]	Estrogen receptor agonism leading to reproductive dysfunction	Under development	–	https://tinyurl.com/y8hd6hnd	01.04.2019
51	PPARα activation leading to impaired fertility in adult male rodents	Included in OECD work plan	Under development	https://tinyurl.com/yahpasof	01.04.2019
64	Glucocorticoid receptor (GR) mediated adult Leydig cell dysfunction leading to decreased male fertility	Under development	_	https://tinyurl.com/ybjes6b8	01.04.2019
71	Modulation of adult Leydig cell function subsequent to glucocorticoid activation	Under development	_	https://tinyurl.com/ya3wbsve	01.04.2019
124	HMG-CoA reductase inhibition leading to decreased fertility	Under development	_	https://tinyurl.com/ycudbwgf	01.04.2019
144	Lysosomal damage leading to liver inflammation	Included in OECD work plan	Under development	https://tinyurl.com/yb7yu9fo	20.12.2016
152	Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity	Included in OECD work plan	Under development	https://tinyurl.com/yd6wzrbm	01.04.2019
163	PPARgamma activation leading to sarcomas in rats, mice, and hamsters	Under development	_	https://tinyurl.com/y9k2wmln	01.04.2019
200	Estrogen receptor activation leading to breast cancer	Under development	_	https://tinyurl.com/yat8frjl	01.04.2019
220	Chronic Cyp2E1 activation leading to liver cancer	Included in OECD work plan	EAGMST under review	https://tinyurl.com/y8qvefdm	17.11.2017

AOP Status (extracted from the Users' handbook [48]): SAAOP status (Society for the Advancement of AOPs): "Under development"—content still under development, not ready for formal review; "Included in OECD work plan"—an AOP development project has been reviewed, accepted into the OECD workplan, and the project number assigned. OECD status—reflects the progress after the AOP has been included in the OECD AOP Development Workplan. (OECD—Organisation for Economic Co-operation and Development; EAGMST—Advisory Group on Molecular Screening and Toxicogenomics)

[a] Applicability domain for AOP 29 is fishes and amphibians

[b] Snapshot representing the state of the AOP at the time when the data was collected for the Snapshot

reports) that the chemical affects several key events of the AOP, at relatively low levels of exposure (i.e., indication of higher toxic potential). We established a systematic and unbiased procedure to link the information from experimental research (as identified in original research papers, reports and ToxCast) with the AOPs existing in the AOP-wiki. A schematic representation of the procedure, with one illustrative example for TDCIPP is shown in Fig. 1. In brief, the complex information from literature, reports and ToxCast was re-structured into individual biological effects that could then be associated with KE(s)/MIEs/AOs from AOP-Wiki. For each of those

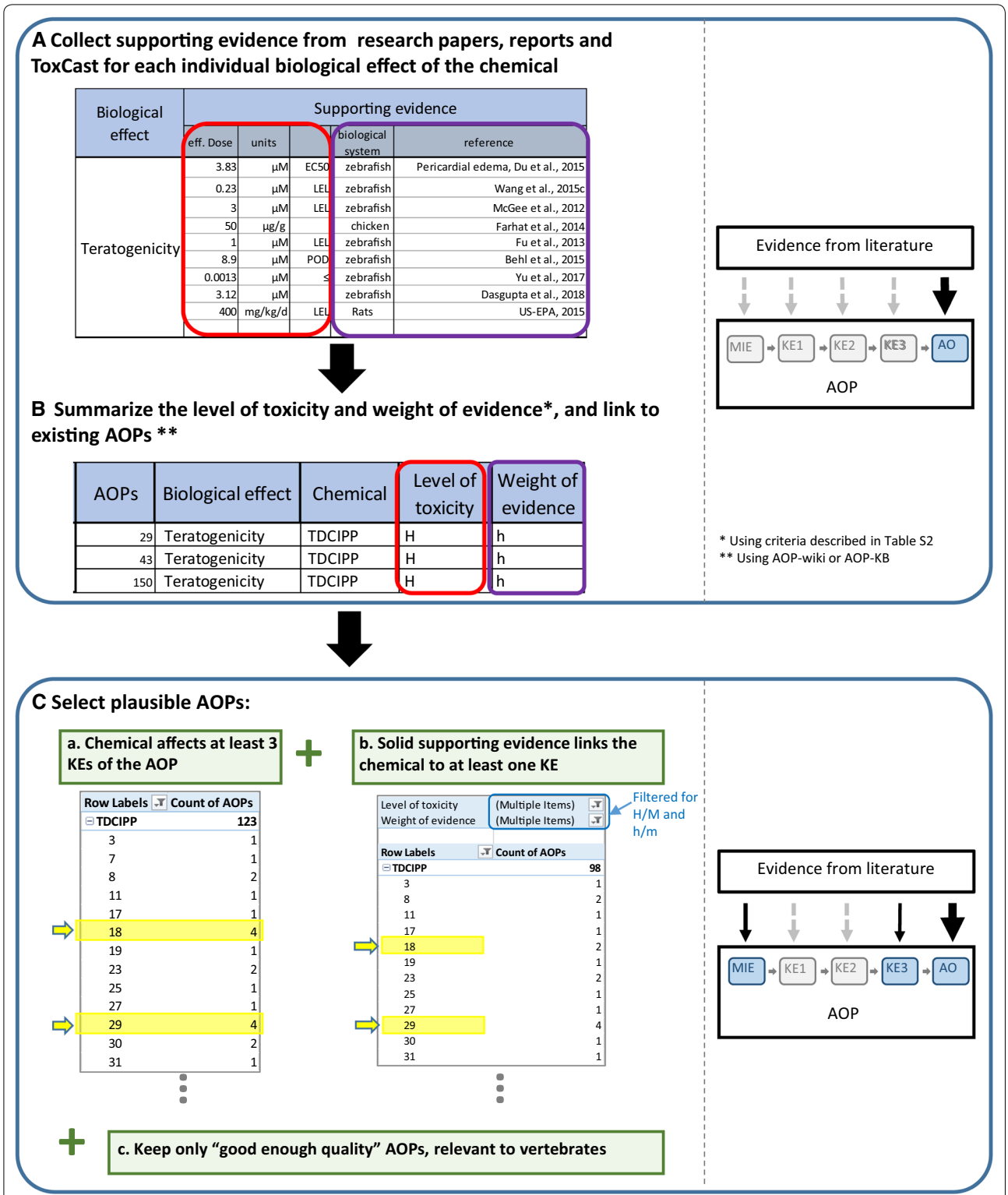

Fig. 1 Schematic presentation of the procedure for linking toxicological data on Category I FRs to existing AOPs and select plausible AOPs, an example for TDCIPP. The 3 main steps of the procedure are detailed and illustrated with extracts from Excel tables (**a**, **b**) and pivot tables (**c**) generated from the table shown in **b**. Full data corresponding to table shown in **a** are presented in Additional file 4: Tables S5. Schemes on the right illustrate the goal of steps A and B (linking evidence from literature to individual MIE/KE/AO and corresponding AOPs), and then **c** (selecting plausible AOPs). Plain arrows indicate that some supporting evidence links the chemical to the MIE/KE/AO and the thickness of the arrow refers to the weight of evidence and level of toxicity

described biological effects, we listed the references and the effect doses (one example with teratogenicity as a biological effect of TDCIPP is shown in Fig. 1a, and complete tables with all biological effects for all Cat I FRs can be found in Additional file 4: Tables S5). This documents the evidence and its weight in establishing links between the FR and the biological effect, and we summarized this by attributing categories "high", "moderate" or "low" to levels of toxicity (uppercase letters) and available weight of evidence (lowercase letters). The criteria for evaluating the levels of toxicity and weight of evidence are explained in details in Additional file 1: Table S2. Finally, we listed the AOPs from AOP-wiki that included corresponding biological effect, thereby linking the FR to existing AOPs. We collected all this information for each Cat I FR in an Excel table (structured as shown in Fig. 1b). This exercise linked Cat I FRs to 109 AOPs in total, of which the large majority are shared by several FRs (data not shown). TDCIPP, TPhP and TBBPA were linked to the highest number of AOPs (83, 80 and 79, respectively).

To select the most relevant AOPs that would represent plausible mechanisms of toxicity for the FR, we applied three inclusion criteria (Fig. 1c). First, we kept only those AOPs for which the FR was documented to interact at the level of three or more KEs of the AOP (i.e., given AOP appears at least three times in the table, for a given FR). Second, we selected AOPs for which the experimental evidence indicated that FR was highly or moderately toxic (with high or moderate weight of evidence) for at least one of the KE. Third, we included only AOPs applicable to vertebrates and AOPs of minimum acceptable quality (i.e., where MIE and AO were documented or KE-relationships were described). A total of 12 plausible AOPs were found for all Cat I FRs, several of them being shared by several FRs. AOP number, full name, status and link to snapshots representing the status of the AOP at the time when we collected the data can be found in Table 4, and the comprehensive list of plausible AOPs for each Cat I FR, with their corresponding MIE and AO, is provided in Additional file 1: Table S3. We note here that the wiki nature of the AOP-wiki, which is important to encourage crowdsourcing, has some draw-backs that are important to keep in mind when interpreting the results of the AOP search. First, the quality of AOPs that are not yet approved or endorsed after official OECD evaluation panel is quite variable and some of them may not be fully reliable. Another limitation relates to the incomplete representativeness of records in AOP-wiki, where some biological processes are thoroughly covered and included in many different AOPs (e.g., thyroid hormone levels) but others (e.g., activation of progesterone receptor) are not included at all yet, and remain to be further incorporated.

Plausible AOPs for the observed AOs of Cat I FRs

Figure 2 illustrates the main results of this search, highlighting the plausible AOPs for the main health adverse outcomes reported in animal and/or human studies for several Cat I FRs. These AOs relate to reproductive toxicity, neurotoxicity and hepatotoxicity.

Considering liver-related AOs (hepatotoxicity and liver cancer), evidence for TDCIPP, TCEP, TMPP, TBBPA, TPhP and TNBP relied exclusively on animal studies [9, 10, 15, 67, 68, 72]. Our search identified AOP 144 (all AOPs listed in Table 4) as a plausible mechanism for TMPP-, TBBPA-, TPhP-, TNBP- and TDCIPP-induced hepatotoxicity, and AOP 220 as a plausible mechanism for TCEP-, TBBPA-, TNBP- and TDCIPP-induced liver cancer. Considering reproductive toxicity, effects of TCEP, TMPP, TDCIPP and TPhP exposure on male tract formation and semen quality have been reported in rodent studies and a few human studies found correlations between TDCIPP and TPhP exposure and semen quality [7, 9, 41, 42, 68, 72]. We found five plausible AOPs that lead to either of the two AOs related to male reproductive toxicity. Finally, considering neurotoxicity, several fish studies report effects of most Cat I FRs on locomotor activity and a couple of human studies report statistical associations between TPhP or TCEP exposure and neurobehavior/cognition in children [8, 24, 38]. Several animal studies analyzing the neurotoxic effects of TBBPA reached contradictory conclusions, ranging from LOAELs of 0.1 mg/kg/day in mice or 0.0064 µM in zebrafish to NOAELs of 1000 mg/kg/day in rats [16, 26, 32, 46, 47, 50, 77]. We also identified plausible AOPs for TPhP-, TBBPA-, TDCIPP- and TCEP-induced effects on neurobehavior/cognition. Our search, therefore, provides mechanistic supports for the major outcomes of several Cat I FRs, reinforcing the conclusion that these are plausible adverse effects of novel FRs, such as TBBPA- and TCEP-induced neurotoxicity, TDCIPP-induced liver cancer or TDCIPP- and TPhP-induced effects on male fertility. Whether these health outcomes will be observed in humans also depends on the levels of FRs to which people are exposed.

In addition to the three main adverse health outcomes shown in Fig. 2, teratogenicity also appears as a sensitive endpoint for novel FRs. Evidence in literature points to the teratogenic effects of most Cat I FRs in fishes (for example: [4, 14, 40, 47, 63]), and a few studies suggest that TCEP and TCIPP may be highly teratogenic in rodents [68]. We found one plausible AOP (AOP 29) for this endpoint, which is applicable to oviparous animals only. Nonetheless, this outcome may deserve further research attention.

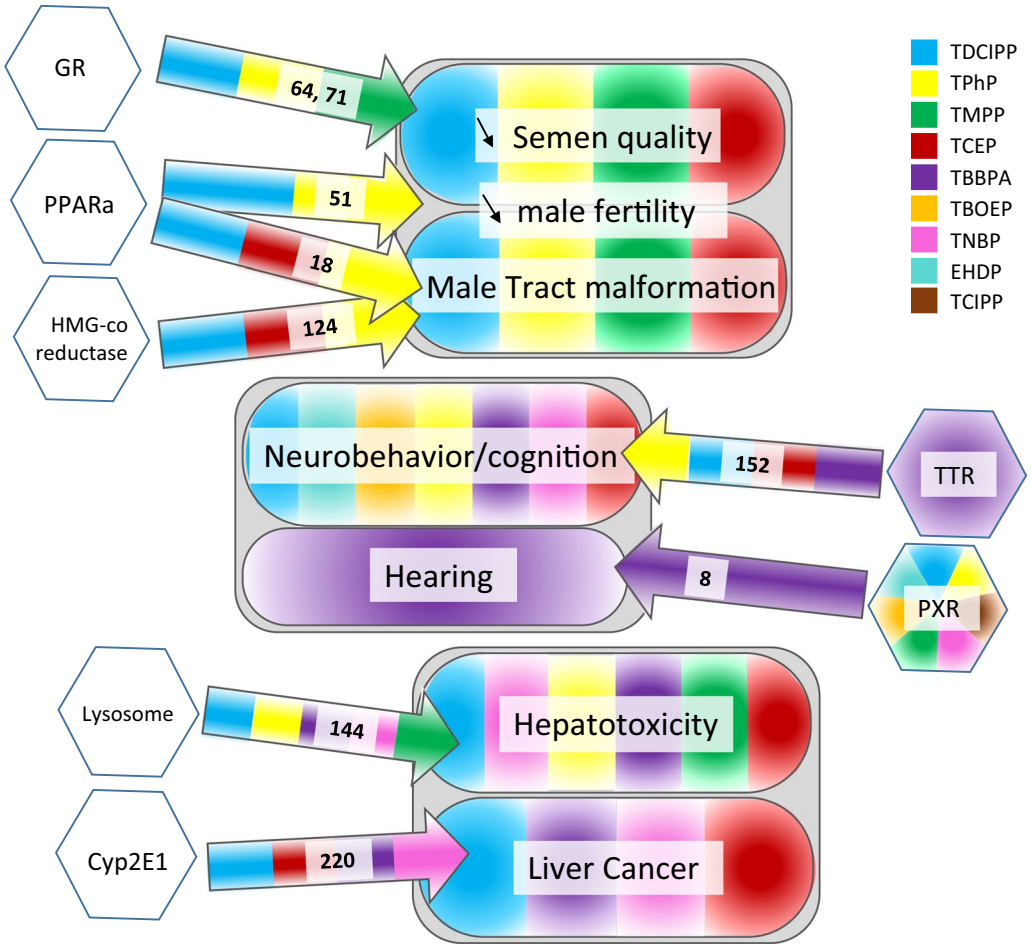

Fig. 2 Illustration of major results from literature/ToxCast/AOP search for Category I FRs. Major AOs are indicated (in ovals) with corresponding plausible AOPs (arrows with their number from the AOP-wiki) and MIE (in hexagons). A color code indicates for which Cat I FR the AOP is a plausible mechanism, or the AO or MIE has been reported. No colors at MIE indicate that the effects of FRs have not been reported so far or AC50 from ToxCast was not "verified" as explained in the text. Several other AOs, AOPs and MIEs not illustrated in this figure have been investigated and are listed in Additional file 1: Table S3. (Disclaimer: because of dynamic nature of AOP-Wiki, the information on AOPs presented in the paper reflects state of the art as of June 2018, when the information from AOP-Wiki was collected)

Identification of gaps in knowledge on mechanisms of toxicity of Cat I FRs

This exercise also revealed the critical lack of identified molecular targets that would lead to the AOs documented in in vivo toxicological and/or human studies: none of the MIEs of plausible AOPs has been convincingly shown to be a target for Cat I FRs based on the current evidence. The only exception is TBBPA, for which a molecular target that may lead to neurobehavioral outcomes (TTR, according to AOP 152) has been identified. This identified gap in knowledge can be tackled in different ways. One obvious way is to predict molecular interactions theoretically using the structure–activity (toxicity) relationships and then experimentally test potential targets corresponding to MIEs of well-established AOPs (mostly in vitro studies with MIE and FRs, and, perhaps also in vivo studies at later stages). For example, binding to TTR of novel FRs that have reported deleterious effects on Neurobehavior/cognition and affect T4 levels (AOP 152), might be of concern. Interestingly, Hill and colleagues have reported that several Cat I FRs enhance T4-TTR binding, strongly supporting the hypothesis that such FRs may bind to TTR [22]. It could also be interesting to test interactions of FRs with Cyp2E1, MIE of AOP 220 that leads to liver cancer through oxidative stress. This AOP appeared as a plausible mechanism of toxicity for several FRs but data on their interaction with Cyp2E1 are missing.

Another possible way to address the identified gaps is to search for mechanisms of toxicity, in the form of novel

AOPs, that would link known information on interaction with molecular targets to documented adverse outcomes affected by FRs. For example, the AOP 18 describes activation of PPARα leading to malformation of the male reproductive tract and reduced male fertility by decreasing levels of TSPO and STAR proteins. This AOP appears to be a plausible toxicity pathway for TDCIPP-, TCEP- or TPhP-induced reproductive toxicity, but available data suggest that the MIE, PPARα activation, is unlikely to be affected by these FRs ([23, 33], and results from ToxCast assays). TSPO (i.e., second KE in the AOP 18) may constitute an alternative MIE since several ToxCast assays suggested that it could be a target of TDCIPP and TPhP (AC50s slightly above 2 µM). In the case of TCEP, we could not find any molecular target in published papers or in ToxCast, whereas this was one of the novel FRs that presented the highest toxicological concern based on available in vivo data. A systematic and blind search for direct targets could be envisaged, using for example an approach similar to the one used for TPhP that led to the identification of carboxylesterases as specific targets [45].

Possible AOs of Cat I FRs predicted from AOP search

In addition to providing mechanistic supports for health outcomes associated with FR exposures, and identifying gaps for future research, the AOP search also highlighted some potential adverse health effects that may be of concern but have been overlooked or not yet studied. For example, we did not find any study addressing the incidence of breast cancer in relation to novel FRs, although there was experimental evidence on interactions of FRs with several KEs of AOP 200 that links mitochondrial dysfunction and oxidative stress to breast cancer as an AO. We also identified mechanistic evidence pointing towards an effect of TPhP and TBBPA on metabolic disorders but only very few studies addressed these adverse effects in animal models or in humans. For example, several in vitro studies reported adipogenesis and/or lipid accumulation and two studies showed increase in body weight associated with TPhP exposure in humans and rodents, and with TBBPA exposure in fish [6, 35, 52, 55, 56, 65, 66, 70]. In addition, activation of PPARγ (the MIE of AOP 72 that leads to obesity) by TPhP and TBBPA has been reported in many in vitro studies, two ToxCast assays and in zebrafish, with effective doses below 1 µM for TBBPA only (for example: [17, 54, 55]). Another pathway for TPhP may also involve carboxylesterases that are identified targets of TPhP and can cause hypertriglyceridemia in mice [45] but it does not correspond to any existing AOP and establishing this AOP would require more research evidence.

Conclusion

We collected toxicological information on novel FRs that are increasingly used as replacement for the regulated PBDEs. This allowed us to establish prioritization categories (based on data availability and toxicological concern) that indicate the actions that should be taken for those different FRs (see Fig. 3 and Table 1). Categorization remains uncertain for two FRs, BEH-TEBP and EH-TBB, because the evidence suggesting moderate hazard is based mostly on analysis of chemical mixtures, such as Firemaster 550 and BZ 54 [68]. Cat I FRs are of toxicological concern based on substantial information and they should be considered for regulation. We particularly highlight TDCIPP, TPhP, TCEP and TMPP, for which data suggest a high toxicological concern and to which people seem to be highly exposed [44]. Amongst the FRs with scarce data available, some appear to have a higher toxicological concern, such as DBNPG, 2,4,6-TBP, ip-TPP and V6. Decabromodiphenylethane (DBDPE) and 2,4,6-TBP would deserve particular focus in future

a

Prioritization classes based on data availability and toxicological concern	Actions needed
Cat I : High priority based on substantial information – 9 FRs	Cohort/epidemiology studies and further mechanistic characterization (see B)
Cat II :High priority based on limited information – 15 FRs	More in vivo studies
Cat III : Low priority based on substantial information – 1 FR	Suggested as replacement
Cat IV : Low priority based on limited information – 6 FRs	No immediate action
Cat V :insufficient information – 19 FRs	In vitro and in vivo studies

b

Gaps : indications for future research on Cat I FRs

Targets	AOs	Mechanism of toxicity
• in vitro (and in vivo?) test of potential targets (MIEs from plausible AOPs): TTR, Cyp2E1, ... • Identify potential alternative targets: e.g., leading to decrease TSPO or STAR (AOP 18)	More epidemiology and (animal) studies addressing: • Neurobehavior/cognition • Semen quality • Malformation of the male tract. (e.g. Kryptorchidism) • Liver Cancer • Teratogenicity • Breast cancer	• Examine intermediate KEs to validate some AOPs: e.g., levels of T4 (AOP 152), .. • Knowledge based elaboration of new AOPs. For example: ➢ TTR → Hearing deficiency. ➢ E2 SULT → Breast/uterine cancers. ➢ ER → decreased male fertility. ➢ PXR → hepatotoxicity. ➢ PR → decreased fertility. ➢ Ces → metabolic disorders

Fig. 3 Recommendation for future actions based on data availability, toxicological concern and future research on molecular targets, adverse health effects and mechanisms of toxicity of Category I FRs

research due to presumed higher exposure [5, 36, 60]. We would like to clearly indicate that these categorizations are entirely hazard-based. Establishing prioritization based on the actual risks to the population would require additional comprehensive documentation on exposures. Other prioritizations of FRs have already been performed, including a recent prioritization by a Swedish group [20]. In this paper, the prioritization criteria related mostly to environmental exposure (usage, environmental detection, classification in previous 6 prioritization lists and time trends in exposure in Sweden) but authors did not take into account hazard (toxicity) levels [20]. Our categorization addresses this substantial but missing information on toxicity.

Our search also highlighted gaps that prevent critical evaluation and risk assessment of novel FRs. First, the toxicity data available seemed insufficient or scarce for the large majority of novel FRs, calling for more studies. Even for the most studied Cat I FRs that had the highest toxicological concern, there was a critical lack of epidemiology or human cohort studies. Our search identified main health effects supported by plausible mechanisms of toxicity that would deserve particular attention—male fertility, neurobehavior, hepatotoxicity or metabolic disorders. Second, the complete toxicity pathways leading from molecular targets to adverse health outcomes were still unknown for prioritized novel FRs, with the exception of TBBPA, for which a link to neurotoxicity might be established. Our search gave several concrete indications of future research for elucidating those pathways including for example modulation of TTR leading to neurotoxicity or activation of Cyp2E1 linked to liver cancer. Our search relied primarily on information from the AOP-wiki in which some processes are poorly (if not) represented, and other mechanistic information could, therefore, have been missed. We would also like to mention that, with a few exceptions (for example [75, 76]), the design of a large majority of experimental studies does not correspond to the expected scenario of exposure to FRs, i.e., low doses and chronic exposures.

For 18 out of 62 chemicals, prioritization based on in vivo experimental data could be compared with prioritization based on in vitro results from the ToxCast program. Although there was some concordance between ToxCast prioritization and in vivo toxicity (especially for positive, i.e., toxic compounds), ToxCast in vitro results were not able to reliably predict low in vivo toxicity. This is in agreement with some other recent reports [51, 61].

It also appeared from this search that several potential molecular targets, AOPs, health outcomes and some KEs are shared by several Cat I FRs, raising the issue of potential mixture effects that should deserve more attention in future research. Interestingly, a recent study suggests that mixture effects may happen when several chemical, with different molecular targets, affect KEs shared by several AOPs and converging toward similar endpoints [11]. Correspondingly, our results, along with AOP network analysis, may identify combinations of FRs that affect various AOPs converging to specific KEs (e.g., thyroid hormone levels), and for which we may therefore expect mixture effects.

In summary, the approach presented in this study that integrates complex information from open literature, reports, ToxCast and AOP wiki was found to be particularly useful for categorization of a large group of FRs, identifying relevant health hazards and mechanisms of toxicity for replacement chemicals with relatively low data availability. Results also indicate future actions where, for example, derivation of updated reference doses (RfD) would be essential for the systematic regulatory risk assessment of this priority chemical group.

Additional files

Additional file 1: Table S1. Detailed information on data availability and toxicological concern of FRs. **Table S2.** Criteria applied for level of hazard and weight of evidence. **Table S3.** The comprehensive list of plausible AOPs for each Category I flame retardants with their corresponding MIE and AO.

Additional file 2: Table S4. Detailed references on in vivo toxicity (hazard) data for studied FRs.

Additional file 3: Figure S1. Criteria for "invalidating" a hit from ToxCast after examination of the dose-response curve, examples.

Additional file 4: Table S5. Detailed information and references on toxicity (hazard) data for Category I FRs.

Abbreviations

AOP: Adverse Outcome Pathway; AO: adverse outcome; BDCIPP: bis(1,3-dichloro-2propyl) phosphate; BFR: brominated flame retardant; DPhP: diphenyl phosphate; EU: European Union; FR: flame retardant; HBM4EU: Human Biomonitoring for Europe, EC H2020 Project; iCSS: interactive chemical safety for sustainability; KE: key event; MIE: molecular initiating event; NTP: National Toxicology Programme; OECD: Organisation for Economic Co-operation and Development; OPFR: organophosphate flame retardant; PBDE: polybrominated diphenyl ether; POP: persistent organic pollutant; PPAR: peroxisome proliferator-activated receptor; PXR: pregnane X receptor; SAAOP: Society for the Advancement of AOPsg; SVHC: substances of very high concern; T4: thyroxine; Tox21: toxicity testing in the 21st century; ToxCast: Toxicity Forecaster; TTR: transthyretin; US-EPA: United States Environmental Protection Agency; WHO: World Health Organization.

Authors' contributions

LoB: collected data (open literature, reports, ToxCast dashboard and AOP-wiki), rationalized and made categorizations, and drafted the manuscript; LuB: supervised the work and contributed to paper concept and writing, LM: contributed by the exposure and risk assessment issues. All authors reviewed the manuscript before submission. All authors read and approved the final manuscript.

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The authors declare that they have no competing interests.

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