COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT (COT)

Follow-up to scoping paper on phosphate-based flame retardants and the potential for developmental toxicity

Background

1. Due to the stringent requirements of the Furniture and Furnishings (Fire) (Safety) Regulations introduced in 1988 in the UK, the use of flame retardants is greater in the UK than the rest of Europe. The legislation has set levels of fire resistance for domestic furniture, furnishings and upholstery products that are largely achieved by the use of chemical flame retardants.

2. Until recently, brominated flame retardants (BFRs) such as polybrominated diphenyl ethers (PBDEs) were the most common chemical flame retardant used for furnishing and textiles (Hendriks and Westerink, 2015). The bans and restrictions on the use of PBDEs have led to an increase in the use of alternative chemical flame retardants (Dodson *et al.*, 2012; Stapleton *et al.*, 2011). In 2010, the Department for Environment, Food and Rural Affairs (Defra) conducted a review of alternative flame retardant technologies and concluded that, while many of the alternatives have been well researched and considered fit for purpose, there are many other flame retardants that have not been adequately assessed for either their long term performance as a flame retardant or for their potential impact on humans following exposure (Defra, 2010).

3. Alternative chemical flame retardants include (organo)phosphate-based flame retardants (PFRs), or commercial mixtures of PFRs and non-PBDE BFRs (Dodson et al., 2012; Rock et al., 2018). These compounds share some structural similarity with organophosphate (OP) pesticides, which have been shown to interfere with neurodevelopment by cholinergic and non-cholinergic pathways (Pope, 1999).

4. In October 2018, the COT considered the potential of PFRs to cause developmental toxicity (TOX/2018/39) and requested further information on the structural characteristics of PFRs to assess their potential to interact with the active site of acetylcholinesterase (AChE). This paper aims to address these aspects by further discussing the cholinergic mode of action (MoA), as well as investigating potential non-cholinergic MoAs and associated toxicity.

Introduction to PFRs

5. PFRs are effective flame retardants for textiles as the triaryl phosphate esters impart durable flame resistance to hydroxyl-containing fibre-forming polymers such as cellulose (Defra, 2010; IPCS, 1997). PFRs may be grouped into non-halogenated

triaryl phosphates[1] (e.g. triphenylphosphate (TPHP) and tricresylphosphate (TCP)), and halogenated PFRs (e.g. tris (2-chloroisopropyl) phosphate (TCPP), tris(2-chloroethyl) phosphate (TCEP) and tris (1,3-dichloro-2-propyl) phosphate (TDCPP) (IPCS, 1997). The addition of the halogen to the PFR structure reduces the water solubility and vapour pressure of the flame retardant thereby increasing the retention of the flame retardant in the polymer (IPCS, 1997).

6. Phosphate esters are produced by chemical synthesis via condensation reactions. For example, TCP is made by heating either phosphorous pentachloride or phosphorous oxychloride with phenol and cresol, respectively (ATSDR, 2012). As cresol is often derived from petroleum refining, it is available in grades containing a mixture of ortho-, meta- and para- isomers. The Agency for Toxic Substances and Disease Registry (ATSDR) report that as a consequence, the resulting TCP is produced as a complex mixture of isomers of ortho-TCP, meta-TCP and para-TCP (ATSDR, 2012). Ortho-TCP is a potent neurotoxicant (Weiner and Jortner, 1999) and whilst its presence in commercial TCP mixtures is low (<0.1 %), it may contribute to the overall toxicity of TCP mixtures (ATSDR, 2012).

7. PFRs have a structural similarity with OP pesticides as they share the same generic OP chemical structure (Dishaw *et al.*, 2014) (Figure 1). The generic structure is comprised of a central phosphorous atom (P) with a phosphoric (=O) bond, a leaving group (X) and two other side groups (R1 and R2) (Elersek and Filipic, 2011).

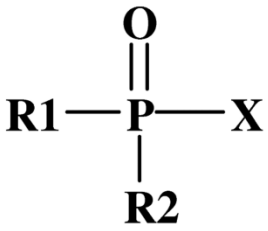
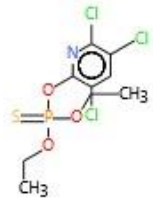
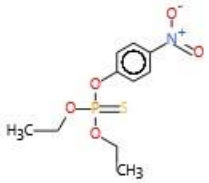
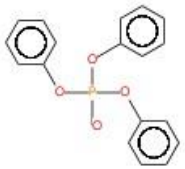
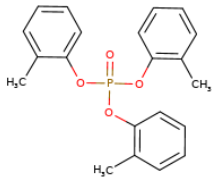
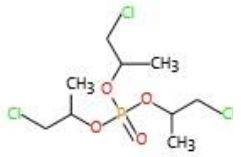
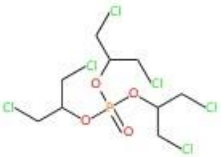
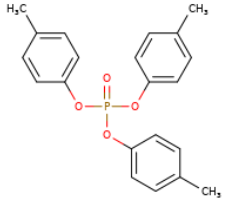
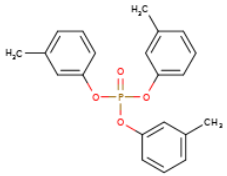


Figure 1. Generic structure of organophosphates

8. The structures of non-halogenated (TPHP and TCP-mixed isomers), halogenated PFRs (TCPP and TDCPP) and two OP pesticides (chlorpyrifos and parathion) are presented in Table 1 (ATSDR, 2012; ChemID, 2018; PubChem, 2018).

[1]also called triaryl phosphate esters

Table 1. Physicochemical properties of non-halogenated and halogenated PFRs and OP pesticides

Physical Property	Organophosphate Pesticides		Non-halogenated PFRs		Halogenated PFRs	
Chemical Name CAS RN	Chlorpyrifos (2921-88-2)	Parathion (56-38-2)	Triphenylphosphate (TPHP) (115-86-6)	Ortho-tricresylphosphate (o-TCP) (78-30-8) (<0.1% in PFR)	Tris (2-chloroisopropyl) phosphate (TCPP) (13674-84-5)	Tris (1,3-dichloro-2-propyl) phosphate (TDCPP) (13674-87-8)
Chemical Structure						
Chemical Name CAS RN/ formulae			para-tricresylphosphate (p-TCP) (78-32-0)	Meta-tricresylphosphate (m-TCP) (563-04-2)		
Chemical Structure						

Mode of action

9. OP compounds such as OP pesticides and PFRs have been associated with both cholinergic and non-cholinergic mechanisms of neurotoxicity (ATSDR, 2012). The cholinergic mechanism functions via inhibition of AChE and is generally well researched and described (Elersek and Filipic, 2011). The non-cholinergic mechanisms are less well understood.

Cholinergic MOA - Inhibition of AChE

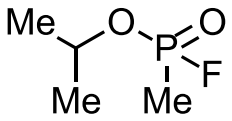
10. OP neurotoxicity occurs in both animals and humans via the phosphorylation and subsequent inhibition of AChE due to a nucleophilic reaction of the leaving group to a critical serine residue within the AChE active site. The reverse hydrolysis reaction to reactivate the AChE is slow resulting in AChE inhibition. This inhibition causes an accumulation of the neurotransmitter acetylcholine and an overstimulation of cholinergic receptors (Pope, 1999), ultimately leading to cholinergic intoxication syndrome (Dishaw, 2015).

11. The natural reaction of AChE to cleave acetyl choline is shown below where E is AChE, AX is acetylcholine and X is the choline moiety. The critical intermediate is the EAX complex where the serine of the AChE is acetylated and then reactivated by a hydrolysis reaction. With organophosphates this serine is phosphorylated and the hydrolysis reaction to reactivate the enzyme is much slower resulting in inhibition of the enzyme.

$$E + AX \rightarrow EAX \rightarrow EA + X \rightarrow E + A$$

12. The inhibition of AChE is dependent on three main factors, 1) the affinity of the OP for the acetylcholine binding site on AChE; 2) the propensity of the leaving group to be cleaved from the phosphor moiety by the AChE leaving the phosphor moiety free to phosphorylate a critical serine in the AChE active site; and 3) the rate at which the serine can be dephosphorylated. In the reaction with acetylcholine, the hydrolysis reaction to reactivate the enzyme takes place in microseconds. The hydrolysis reaction to release the phosphor group is much slower and can be in the order of hours to days.

13. The nerve agents sarin and soman have a high affinity for AChE due to the presence of fluorine in the leaving group (Figure 2), which allows for rapid cleavage of this bond and subsequently the phosphor group to phosphorylate the critical active site serine of AChE (Elersek and Filipic, 2011; Moshiri *et al.*, 2012). In contrast, the leaving groups of less toxic OP compounds, such as pesticides, usually contain alkyl or aryl functional groups with alkoxy functional groups as side groups. These have a lower affinity for the AChE active site and the bond between the phosphor group and the leaving group is stronger and so less readily cleaved, resulting in a much lower rate of inhibition of the AChE (Elersek and Filipic, 2011). As PFRs generally have larger alkyl chains in the leaving/side groups they may also exhibit reduced affinity for AChE and therefore limited neurotoxicity of PFRs via inhibition of AChE may be anticipated. Dishaw (2015) suggested that PFRs do not have a strong binding affinity for AChE and exhibit low acute toxicity compared with OP pesticides.



Sarin (Isopropylmethylphosponofluoridate)

Figure 2. Structure of sarin

14. Thus, chemical structure is a strong predictor of AChE reactivity of OP chemicals. Steric hindrance[2] in the binding pocket increases with the increasing length or branching of alkyl substituents (Dishaw, 2015) and highly hydrophobic groups do not allow cleavage of the leaving group from the phosphor moiety, thereby reducing the reactivity of the OP compound (Ballentyne and Marrs, 1992).

15. An early study tested the inhibitory activity of various halogenated and non-halogenated PFRs on AChE, isolated from organs of the electric ray *Torpedo ocellata* (at concentrations that are considered to be more realistic in terms of human exposure). Authors reported that phosphate esters are not potent AChE inhibitors, when compared to the OP pesticide diisopropyl phosphorofluoridate used as the control (Eldefrawi et al., 1977). Other authors noted that human exposure to PFRs is usually chronic and low level, rather than the acute exposure to high doses that are associated with OP pesticide cholinergic toxicity (Abou-Donia et al., 2016).

Non-cholinergic MoA

16. Exposure to OPs, such as ortho-TCP, is also associated with Organophosphate-Induced Delayed Neurotoxicity (OPIDN) (also referred to as Organophosphate-Induced Delayed Polyneuropathy), a neurodegenerative disorder characterised by a latent period of several weeks between exposure and the manifestation of neurological effects (e.g. ataxia or paralysis) (Weiner and Jortner, 1999).

17. The target for OPIDN is proposed to be neuropathy target esterase (NTE), rather than inhibition of AChE, as there is a good correlation between inhibition of the enzyme by OP compounds and their ability to cause OPIDN (Jokanovic *et al.*, 2011). NTE belongs to the same protein family of serine esterases as AChE (Elersek and Filipic, 2011). The role of NTE is not completely understood, however it is noted that sufficient NTE must be irreversibly inhibited before OPIDN develops (Ehrich *et al.*, 1997). Therefore the delay in initiation of neurological effects is thought to be due to this progressive inhibition of NTE by reaction with OP compounds (Jokanovic *et al.*, 2011).

18. A number of structural features appear to be essential for the neurotoxicity observed in OPIDN including the presence of an ortho-methyl group in an aromatic series (as seen in ortho-TCP, see Figure 3). Importantly, with the triaryl phosphates, metabolic activation to a neurotoxic metabolite must occur. This metabolism occurs

[2] Steric hindrance is the slowing or stopping of a chemical reaction due to a molecules structure. Larger molecules or those with higher numbers and/or size of alkyl groups on the carbon with the leaving group leads to slower reaction rates.

more readily when there is a substituent at the ortho position on the phenyl ring, as seen with ortho-TCP. This forms a cyclic phosphate similar to saligenin, a highly neurotoxic metabolite that inhibits NTE. For example, ortho-TCP is metabolised to phenyl saligenin. Esters with no ortho-substituents, such as TPHP, are not neurotoxic as metabolism does not occur. In addition, neurotoxicity is decreased by further substitution on the phenyl ring with additional methyl groups in the meta or para positions by providing alternative hydroxylation pathways without the formation of a cyclic ester due to steric hindrance (i.e. meta-TCP). The size of the substituent on the ortho position also affects the neurotoxicity potency. Larger and more branched substituents i.e. a butyl group, interfere with metabolic activation to neurotoxic metabolites, due to steric hindrance (ATSDR, 2012; Weiner and Jortner, 1999).

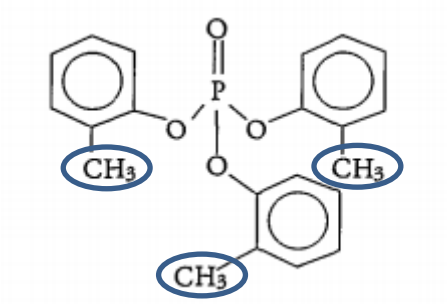


Figure 3. Illustration of the ortho-methyl groups as seen in ortho-TCP

19. Weiner and Jortner (1999) reviewed a series of acute and chronic OPIDN studies in hens with a number of triaryl phosphate compounds, including ortho-TCP, TCP mixed isomers (including ortho-TCP) as seen in commercial TCP3 and isopropyl phenyl phosphates. It was found that whilst isopropyl phenyl phosphates showed some neurotoxicity in acute studies, these were generally conducted at high doses (> 10,000 mg/kg bw). The authors reported that mixed isopropyl phenyl phosphates were much less potent for inducing OPIDN than TCP and ortho-TCP, the latter being the most potent. Authors reported that changes to the chemical synthesis of commercial TCP resulted in an isomeric mixture that had reduced neurotoxic potential when compared to ortho-TCP alone. It was also noted that the chemical structure of other PFRs, such as TPHP, does not contain the required functional groups to elicit OPIDN (Weiner and Jortner, 1999).

20. PFRs have also been shown to exert effects on other neurochemical mechanisms. For example, TCEP has antagonistic effects on the neurotransmitter gamma-aminobutyric acid (GABA) in mice (Umezu *et al.*, 1998). Gant *et al.* (1987) have shown that PFRs such as TPHP and ortho-TCP can bind to the GABA receptor and reduce GABA-induced chloride influx with IC_{50}s of 18.2 and 14.2 µM, respectively. Other PFRs tested (Antiblaze (mixture of cyclic phosphates) and β-

[3]Commercial TCP is a complex mixture containing the meta- and para isomers and mixed tricresyl and dicresyl phosphate esters. The ortho TCP isomer occurs only as a contaminant in commercial mixtures and usually at very low concentrations (<0.1 %) National Research Council (2000).

chloroethyl phosphate) had no effect on GABA-induced chloride influx. This property is unrelated to AChE inhibition and is not shared by the OP neurotoxic agents with the exception of soman that had an IC_{50} of 24 µM. TPHP and ortho-TCP also inhibited t-[35S]butylbicyclophosphorothionate ([35S]TBPS) binding to both the GABA receptor (IC_{50} 12.9 and 17.6 µM respectively) and voltage dependent chloride channel (10.3 and 1.5 µM respectively) and again were more potent than other OPs such as nerve agents. Gant et al. (1987) noted that the less potent anti-AChE inhibitor ortho-TCP is the most potent inhibitor of GABA receptor function. Overall, authors concluded that none of the OPs tests were as potent as TBPS in inhibiting chloride influx and therefore GABA receptors or voltage dependent chloride channels are primary targets for OP-induced seizures. However, they concluded that whilst GABA receptors are not the primary targets for PFRs, the inhibitory effects on these receptors may also contribute to their toxicity.

21. Dishaw et al. (2011) compared the neurotoxicity of four PFRs (TCEP, TCPP, TDCPP and tris (1,3- dibromopropyl) phosphate (TDBPP)) to chlorpyrifos using PC12 cells[4], an *in vitro* model for neurodevelopmental toxicity. TCEP and TCPP promoted cell differentiation into the cholinergic phenotype, whereas TDCPP (all doses tested) and TDBPP (high dose only) promoted differentiation into both cholinergic and noncholinergic dopaminerigic phenotypes. Authors suggested that the results demonstrate that different PFRs show divergent effects on neurodifferentiation, suggestive of different mechanisms of toxicity (Dishaw *et al.*, 2011).

22. Furthermore, TDCPP caused concentration-dependent neurotoxicity at levels equivalent to or lower than chlorpyrifos, with authors reporting effects with TDCPP at 10, 20 and 50 µM and with chlorpyrifos at 50 µM (Dishaw *et al.*, 2011). The authors concluded that the data indicates that PFRs have the potential to be developmental neurotoxins via disruption of the critical stages of brain development, such as causing deficiencies in the number of neurones and altered neurodifferentiation. This may lead to irrevocable changes in brain function. Overall, authors considered that the potency of PFRs for neurodevelopment toxicity was similar or greater than that of an OP pesticide (chlorpyrifos) (Dishaw *et al.*, 2011). The Committee noted previously, however, that high concentrations (10-50 µM) of PFRs were used in this study that are not considered to be relevant to human exposure and that there is only a single study.

23. Dishaw *et al.* (2011) also reported a structure-activity relationship between OP compounds and neurodifferentiation as PFRs that had similar halogenation substitution patterns also showed similar effects on neurodifferentiation. For example, authors reported that TDCPP and TDBPP, which differ only by the type of halogen substituent, both promoted differentiation into the cholinergic and dopaminergic neuronal phenotypes. TCEP and TCPP, however, which differ from TDCPP and TDBPP most notably by the number halogen substituents, showed

[4] The PC12 cell line was derived from a rat adrenomedullary tumor and represents a neuronal model cell due to its property of acquiring the characteristics of sympathetic neurons when exposed to nerve growth factor (NGF)

distinct effects on neurodifferentiation, promoting emergence of the cholinergic phenotype only. Therefore, it may be that the PFRs have different MoAs for neurotoxicity, depending on its chemical structure. Again, it should be noted that high concentrations (10-50 µM) of PFRs were used.

24. In a later paper studying effects in early life stage zebrafish, PFRs were demonstrated to elicit overt and neurodevelopmental toxicity at concentrations similar to, or below that of chlorpyrifos (PFRs 3.3-10 µM; chlorpyrifos 10 µM (Dishaw *et al.*, 2014).

Toxicity of PFRs

Human data

Neurotoxicity – AchE inhibition

25. There are many reports of neurotoxic effects in humans attributed to exposure to food items contaminated with ortho-TCP (ATSDR, 2012). It is noted that ortho-TCP occurs as a contaminant (<0.1 %) in commercial TCP mixtures used as PFRs. Studies with commercial mixtures of TCP suggest it is less neurotoxic than ortho-TCP alone, although ortho-TCP does contribute to the neurotoxicity of TCP mixtures (Weiner and Jortner, 1999).

Neurotoxicity – OPIDN

26. A 5-year old girl exposed, via inhalation, to TCEP in household timber (600 mg/kg wood) was reported to develop characteristics of OPIDN such as weakness in the arms and abdominal muscles. Nine months later she was admitted to hospital with dystelectatic pneumonia and spinal muscle atrophy. The delay in the onset of symptoms was characteristic of OPIDN (Abou-Donia *et al.*, 2016).

Neurotoxicity - OPICN

27. Abou-Donia *et al.* (2016) reported that PFRs and OP pesticides may also cause Organophosphate-Induced Chronic Neurotoxicity (OPICN). OPICN is a disorder involving neuronal degeneration and subsequent structural, functional, physiological, neurological and neurobehavioral abnormalities and is largely characterised by chronic neurobehavioural alterations[5]. This action can occur following a single acute exposure to OPs or following low sub-acute exposures at sub-lethal dose levels. It results in long-term cognitive deficits and sensorimotor dysfunctions, in the absence of acute cholinergic toxicity. Abou-Donia *et al.* (2016) does not report an example of PFRs causing OPICN in humans, however an

[5] OPICN may also be referred to as Chronic Organophosphate Induced Neuropsychiatric Disorder (COPIND). In the 1999 COT report and the 2014 COT statement on organophosphates, data on COPIND was reviewed. In 2014 the COT concluded that whilst there is an excess of multiple neuropsychiatric symptoms in people who have been exposed to organophosphates at levels insufficient to cause overt acute poisoning, it is difficult to determine whether the symptoms are a consequence of chemical toxicity or via psychological mechanisms that do not involve organophosphates.

example of tris(2-butoxyethyl) phosphate (TBEP) causing OPICN in Sprague Dawley rats is reported.

28. Limited examples of PFRs affecting neurobehaviour in children and infants have been reported in TOX/2018/39. Although few human data are available, the limited data does indicate a correlation between PFR exposure and reduced cognitive performance and poor social behaviours in children and infants (Castorina *et al.*, 2017; Lipscomb *et al.*, 2017).

Animal data

Neurotoxicity

29. A number of studies have reported PFRs causing neurotoxicity in adult animals.

30. TCEP administered as a single dose of 275 mg/kg bw via oral gavage caused seizures in female rats within 60 to 90 minutes of dosing. Following a 7 day treatment-free period, a necropsy was conducted and severe damage to the CA1 and CA3 regions of the hippocampus was observed. A single gavage dose of TnBP (1000 mg/kg bw) to rats induced a significant reduction in motor activity, 11 hours after dosing (ATSDR, 2012).

31. Rats and mice (male and female) were administered 22-350 mg/kg bw/day and 44-700 mg/kg bw/day TCEP, respectively for 16 days via oral gavage. Serum cholinesterase activity was reduced in female, but not male, rats compared to controls. Mice (sex not stated) showed ataxia and convulsions during the first three days of treatment at 350 or 700 mg/kg bw/day (NTP, 1991).

32. In a 16-week oral (gavage) study, rats and mice were administered 22-350 mg/kg bw/day and 44-700 mg/kg bw/day TCEP, respectively. Female rats administered 175 (8/10 rats) and 350 mg/kg bw/day (10/10 rats) showed periods of hyperactivity after dosing, and periodic convulsions were seen in the high dose group, reduced serum cholinesterase activity and necrosis of the hippocampus and thalamus was reported, whereas necrosis was only seen in 2/10 males at 350 mg/kg bw/day (NTP, 1991).

33. In a 2-year oral (gavage) study, TCEP was administered to rats at 0, 44 or 88 mg/kg bw/day and mice at 0, 175 or 350 mg/kg bw/day. Degenerative lesions consisting of gliosis, mineralisation, haemorrhage, and/or haemosiderin accumulation occurred in the cerebrum and brain stem of more than 50 % of female rats receiving 44 or 88 mg/kg TCEP; similar lesions were seen in only a few dosed males. In mice, no neurological effects were reported in either sex (NTP, 1991).

34. For comparison, Hutter *et al.* (2013) reported maximum levels of TCEP in dust was 35 mg/kg. Assuming a 10 kg child ingests 200 mg dust per day, this equates to an intake of 7 µg/day (or 0.7 µg/kg bw/day). Moreover, (Stapleton *et al.*, 2009) calculated that the average cumulative exposure to flame retardants from dust

ingestion is 16 µg/day for children (1.6 µg/kg bw/day for a 10 kg child). Authors reported that PBDEs and TPHP and TDCPP account for the majority of exposure.

Summary

35. PFRs exhibit a structural similarity with OP pesticides as they share the same generic OP chemical structural. It is hypothesised that the presence of alkyl or aryl functional groups as leaving groups, as seen in PFRs, results in the leaving group having a lower affinity for the AChE active site, thereby causing less inhibition and subsequent neurotoxicity. Some studies have demonstrated AChE inhibition by PFRs but only at high concentrations.

36. It has also been hypothesised that PFRs may elicit similar toxicity as OP pesticides based on non-cholinergic mechanisms. Some PFRs and OP pesticides cause OPIDN although high concentrations were required for PFRs to elicit OPIDN via the inhibition of NTE. GABA receptors may also be inhibited by OP pesticides and some PFRs. However the toxicological significance of this, particularly at likely human exposure levels, remains unclear.

Questions for the Committee

Members are asked to provide general comments on the paper and in particular:

i. Whether any conclusions can be drawn from the information presented on the potential for developmental toxicity of PFRs?

NCET at WRc/IEH-C under contract supporting the PHE Secretariat
March 2018

Abbreviations

AChE	Acetylcholinesterase
ATSDR	Agency for Toxic Substances and Disease Registry
BFRs	Brominated flame retardants
[^{35}S]TBPS	t-[^{35}S]Butylbicyclophosphorothionate
CHAMACOS	Center for the Health Assessment of Mothers and Children of Salinas
COPIND	Chronic Organophosphate Induced Neuropsychiatric Disorder
COT	Committee on Toxicity of chemicals in food, consumer products and the environment
Defra	Department for Environment, Food and Rural Affairs
GABA	Gamma-aminobutyric acid
MoA	Mode of Action
NTE	Neuropathy target esterase
OP	Organophosphate
OPICN	Organophosphate-Induced Chronic Neurotoxicity
OPIDN	Organophosphate-Induced Delayed Neurotoxicity
PBDEs	Polybrominated Diphenyl Ethers
PFRs	Phosphate-Based Flame Retardants
TBEP	Tris(2-butoxyethyl) phosphate
TCEP	Tris(2-chloroethyl) phosphate
TCP	Tricresylphosphate
TCPP	Tris (2-chloroisopropyl) phosphate
TDBPP	Tris (1,3- dibromopropyl) phosphate
TDCPP	Tris (1,3-dichloro-2-propyl) phosphate
TPHP	Triphenylphosphate
TnBP	Tri-n-butyl phosphate

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