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NEUROTOXICITY OF POLYBROMINATED DIPHENYL ETHERS (PBDEs)

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“What is a scientist after all?

It is a curious person looking through a keyhole,

the keyhole of nature, trying to know what’s going on.”

- Jacques Costeau -

Summary

Polybrominated diphenylethers (PBDEs), a class of brominated compounds used as flame retardants, are widespread and persistent contaminants, which accumulate in the environment, in animals, in the food chain, and in humans. Several studies have highlighted that the toxicity of this substances impacts the nervous system during development, as perinatal exposure to PBDEs has been shown to affect behavior, in particular motor and cognitive activities. The present research project investigated the neurotoxicity of PBDEs through an *in vitro* approach. The potential interactions between different PBDEs congeners, and between PBDEs and PCBs, another class of persistent contaminants, to which humans are also exposed, was assessed utilizing the Loewe additive model and the Bliss independence criterion. Additionally, a potential mechanism of PBDEs neurotoxicity was investigated, by studying the involvement of glutamate, the main neurotransmitter of central nervous system.
The major findings presented in my thesis confirm the validity of *in vitro* models as alternatives to *in vivo* approaches to assess the toxicity of neurotoxicants. Results show that PBDEs cause neuronal toxicity by a mechanism involving in part the over-activation of ionotropic glutamate receptors, followed by oxidative stress leading to cell toxiucity and cell death. Moreover, co-exposure to two PBDEs congeners or a PBDe and a PCB has been shown to modify the toxicity of single compounds, suggesting that the study of interactions, supported by mathematical models, is an important issue that should be considered in risk assessment.

Key words: PBDEs; PCBs; glutamate; *in vitro* toxicology; interactions

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1. Introduction

1.1. Preamble

Toxicologists have long recognized the need to develop acceptable alternatives to conventional animal toxicity testing in risk assessment process, in order to address problems related to the increasing number of toxicants in the environment and in the working places, the large costs and time required for laboratory testing, and the concern of animal welfare activists (Costa, 1998).

In vitro testing methods present many practical advantages:

- data obtained from in vitro procedures are based on simplified approaches that require less time and cost to yield information;
- uniform and controlled chemical and physical environment;
- possibility to perform toxic exposure continuous or intermittent;
- exposure parameters strictly controlled;
- small amounts of chemical needed;
- systemic effects bypassed;
- human materials available.

On the other hand, in vitro approaches have some limitations:

- it is difficult to extrapolate in vitro toxicity data to animal or human in order to define the risk assessment;
- they do not take into account the distribution of the toxicant in the body, the route of administration, and the metabolism of the substance;
- target concentration is unknown;
- compensatory mechanisms cannot be determined;
- single tests cannot cover all targets and mechanisms.

The use of *in vitro* approaches is useful to study biochemical, functional and structural alterations of single cellular populations, advantaging analysis of complex systems, such as central nervous system (CNS).

Organotypic explants, tissue slice cultures, primary cell preparations, and established cell lines are, in a decreasing order of complexity, *in vitro* models to evaluate xenobiotic toxicity. With the exception of cell lines, all other approaches involve the use of cells or tissues directly derived from animals. Organotypic explants and tissues slices allow preserving the architecture and biochemical processes of that organ or portion; primary cultures and cell lines allow studying the effects of toxicants on separate cell types (e.g. in brain neurons, astrocytes, oligodendrocytes). In particular, cell lines provide a large amount of information, and reproducibility of results is better in cell lines than primary cultures. In contrast, cell line are tumor-derived cells; thus, effects in these cells may not always mimic those occurring in wild-type cells (Costa, 1998).

1.2. *In vitro* approaches and risk assessment

To characterize hazard dose-effect relationship, type of exposure, variability of sensibility, and description of uncertainty have to be considered. In general, approaches used to obtain dose-response relationship include NOAEL (No-observed-adverse-effect level) value, the highest dose at which no statistical significance from control is observed, or a LOAEL (Lowest-observed-adverse-effect level) value, which represents the lowest dose able to cause a significant effect. Uncertainty factors, which consider intraspecies and interspecies variability are often applied to these values for extrapolation to humans. These values are usually derived either from studies in humans, or, more often from animal experiments, but *in vitro* systems can also be useful in this regard. In fact, over the past decades, an increasing number of test systems for evaluating the possible toxicological hazard of chemical compounds have been developed and supported, for example by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) of National Toxicology Program in USA. Many *in vitro* testing not including animal models, but lower level of organization than organisms, have been used to risk assessment of toxicants. However, even if *in vitro* systems have been useful in studying molecular mechanisms of action of toxic substances and present many advantages (listed above), they are not widely accepted in hazard identification in human risk assessment (Goldoni et al., 2003). Usually, in *in vitro* studies, only IC_{50} value, or the concentration capable to cause the death of 50% of cells, has been considered, but not other parameters or reference doses. However, whereas the LD50 *in vivo* is the dose which causes 50% death of animals, thus is a parameter of systemic toxicity,

because involves the entire organism, in *in vitro* models represents a specific toxic effect on a specific cell system, for example neuronal cells, or lung cells, etc (Goldoni et al., 2003).

To compare and to extrapolate relevant doses to *in vivo* and humans from *in vitro* studies, the benchmark dose (BMD) seems to be more appropriate. The use of BMD was proposed in 1984 as an alternative to the NOAEL and LOAEL for setting regulatory levels such as reference doses (RfDs), acceptable daily intakes (ADIs) (Setzer and Kimmel, 2003). BMD is a more quantitative way to obtain threshold doses from dose-response curve; in fact, whereas NOAEL and LOAEL are discrete doses from a study, the BMD approach involves modeling the dose-response curve in the range of observable data, and then using that model to interpolate an estimate of the dose that corresponds to a specific level of response, e.g. 5 or 10% for quantal data, or some predefined change in response from control from controls for continuous data. A measure of uncertainty is also generally calculated, and the lower confidence the lower extreme of the confidence interval at 95% of the BMD is called the BMDL. Unlike the NOAELs and LOAELs, BMDs are not constrained to be one of the experimental doses, and may thus be a more consistent basis for dose-response assessment. NOAELs and LOAELs depend on sample size and on chosen doses, so they will be higher in studies with a smaller sample size, giving wrong information, or at least data related to that experiment. Moreover, and this is a very important issue about using BMD instead of the traditional approaches, the slope of the dose-response curve is not considered in NOAEL/LOAEL approaches, yielding more information, and thus reducing uncertainty in risk assessment (Goldoni et al., 2003; Setzer and Kimmel, 2003).

In conclusion, even if *in vitro* approaches have evident limitation related to the low complexity of the systems, they present other important advantages, in terms of use of animals, costs and time, and reproducibility. In particular, they may yield important data about the study of mechanisms of a toxic substance, and on doses relevant to risk assessment, opportunely modified by uncertainty factors.

2. Neurotoxicity

Neurotoxicity defines any adverse effect on the structure and function of the nervous system induced by exogenous or endogenous factors, including biological, chemical or physical agents (Philbert et al., 2000; Tilson et al., 1995).

The nervous system is perhaps the most complex biological system and consists of many different cell types organized on highly structured patterns. Neurons are responsible for the reception, integration, transmission and information storage. They can be classified based on their anatomical location (e.g. cerebellar, striatal), cellular structure (e.g. granule, pyramidal) or function (e.g. neuroendocrine) and primary neurotransmitter (e.g. dopaminergic, glutamatergic) (Kandel, 2000). Glial cells are the second major cell type in the nervous system. They provide support and nutrition, maintain homeostasis, form myelin, participate in signal transmission, and act as phagocytic cells. Astrocytes are characteristic star-shaped glial cells in the brain. They perform many functions, including the formation of the blood-brain-barrier (BBB), the provision of nutrients, and also play a central role in repair processes (Kandel, 2000). Both neurons and glials cells are generated from the neuroepithelial cells in the walls of the embryonic neural tube. Neural stem cells undergo symmetric or asymmetric cell division and can differentiate into neurons, astrocytes and oligodendrocytes (Johe, 1996; Reynolds, 1992). They are present not only in the embryo, but also in adult brain regions maintaining a neurogenic potential such as the subventricular zone (SVZ) of the lateral ventricle (Reynolds, 1992). Another important cell population present in the nervous system is the microglia, which is of mesodermal origin. These cells are usually in a resting state, but become active macrophage-like cells in response to injuries (Vilhardt, 2005).

Certain unique characteristics of the nervous systems make it particularly susceptible to various kinds of insults. Even thought the brain constitutes approximately 2% of the body mass, its metabolic rate and energy requirements accounts for at least 25% of oxygen consumption (Magistretti, 2000). This oxygen consumption, together with the high content of polyunsaturated fatty acids and iron that can promote lipid peroxidation, and the low levels of antioxidant enzymes such as catalase and glutathione peroxidase, make the nervous system more vulnerable to oxidative stress, as compared to other organs (Evans, 1993). In addition, the limited capability of neurons to regenerate also exacerbates the consequences of brain damage.

The development of the nervous system follows a regulated program that takes place through different developmental steps. This creates windows of susceptibility to adverse interference

not present in the mature brain. A proper function of the nervous system requires a precise number of cells in the right place with the correct characteristics, and this is achieved by proper cell proliferation, migration, and lastly differentiation when cells aquire specific properties (Rodier, 1994). Embryonic development also requires death of the excess cells. Programmed cell death plays a critical role in the development of the nervous system, and interference with this multifactorial fine regulated process can result in loss of cells that should not have been eliminated (Henderson, 1996; Ikonomidou et al., 2001; Johnson and Deckwerth, 1993). Any alteration in these specific steps may lead to different degrees and kinds of impairments in normal functions of central nervous system. However, the consequences of a developmental damage may not be evident until a critical age, when a deficit may be revealed or exacerbated by aging or exogenous influences.

2.1. Neurotoxicity in *in vitro* models

Because of the rather complex and heterogeneous structure of the nervous system, different neurotoxic insults can affect neurological function in different specific ways. The assessment of neurotoxic effects *in vivo* can be done at multiple levels of the nervous system organization by performing behavioral, neurophysiological, neurochemical and neuroanatomical analysis. However, these approaches provide limited information on the mechanistic events within the neurotoxic processes. *In vitro* models, instead, are powerful systems for the investigation of cellular function perturbation induced by neurotoxicants, and their value should be exploited further for neurotoxicity testing.

Most *in vitro* systems in neurotoxicological experiments make use of mammalian cells. Neuroblastoma and glioma cell lines, derived from spontaneous occurring tumors, are used frequently as models to investigates intracellular mechanisms in neurons and glia cells. By using homogeneous population of cells growing indefinitely *in vitro*, the design and execution of neurotoxicological investigations become facilitated. On the other hand, primary cultures allow the study of cells with differentiated character. Certainly, a combined use of the various *in vitro* models available is a powerful strategy to carry out mechanistic studies on the effects of neurotoxicants.

In fact, in neuronal cell cultures several end-points may be measured and standardized, such as cytotoxicity, cell death, membrane permeability, mitochondrial function, energy regulation,

synthesis of macromolecules and secretion of neurotransmitters (Costa, 1998; Nicotera et al., 1992).

A particular mention is necessary for oxidative stress. Nervous system has a very high oxygen turnover which together with a high quantity of polyunsaturated fatty acids and iron and antioxidant activity, and is particularly susceptible to oxidative stress. Antioxidant defense systems prevent formation/accumulation of oxygen metabolites. Oxidative stress occurs due to a disturbances in the balance between the antioxidant defense systems and the generation of ROS (reactive oxygen species), where excessive amounts of ROS leads to disruption of the cellular integrity (Betteridge, 2000; Sies and Cadenas, 1985). ROS are very reactive with all biological macromolecules, such as proteins, nucleotides, carbohydrates and lipids, above all with polyunsaturated fatty acids. Mitochondria are the main site generating ROS, such as the superoxide anion, hydroxyl radical, singlet oxygen and hydrogen peroxide (Morel and Barouki, 1999). In mitochondria, ROS are produced during respiration as a product of complex-I (NADH/ubiquinone oxireductase) and complex-III (ubiquinol/cyt c oxireductase) activity. Approximately 2% of the oxygen reacting in the respiratory chain causes the formation of superoxide radicals, which can be dismutated into hydrogen peroxide. The Fenton reaction, catalyzed by Cu^{2+} and $Fe^{2+,}$ can thus transform hydrogen peroxide into a more reactive ROS, the hydroxyl radical (Djordjevic, 2004). The mitochondria are also the main site of antioxidant defences. The defence system includes glutathione, glutathione peroxidase, glutathione reductase, superoxide dismutase, NADP dehydrogenase and vitamins E and C (McGowan et al., 1996; Sato et al., 1995). *In vitro* approaches are particularly useful to evaluate in punctual and precise way cellular oxidative damage. In fact, in vitro models allow to study, both through kinetic and time-fixed experiments, several parameters of oxidative stress, such as reactive oxygen species, lipidic peroxidation, oxidative damages to proteins and DNA (Reistad et al., 2005; Vettori et al., 2005).

2.2. A specific biomarker of neurotoxicity: glutamate

The amino acid L-glutamate is considered to be the major mediator of excitatory signals in the mammalian central nervous system and is involved in most aspects of normal brain function including cognition, learning and memory (Danbolt, 2001; Fonnum, 1984). Glutamate also plays important roles in the development of central nervous system, such as synapse induction and elimination, cell migration, differentiation and death. Endogenous

glutamate released by granule cells in culture stimulates neurite outgrowth (Pearce et al., 1987). Furthermore, glutamic acid is involved in signaling pathways also in peripheral organs and tissues as well as in endocrine cells (Moriyama et al., 2000).

Brain contains large amounts of glutamate (about 5-15 mmol per kg wet weight depending on the region), but only a small fraction is normally present in the extracellular fluid (its concentrations is in the range of 3-4 μM) (Danbolt, 2001). As a consequence, the concentration gradient of glutamate across the plasma membrane is several thousand-fold, and is in a dynamic equilibrium which is highly sensitive to changes in the energy supply. Glutamate is continuously released from cells, when they run out of energy, and then is removed from extracellular fluid, with a rapid turnover.

Glutamate taken up by the cells may be used for metabolic purposes (protein synthesis, energy metabolism) or used as a transmitter. In nerve terminals, reuse as a transmitter is straightforward. Glutamate is transported into synaptic vescicles by a vescicular glutamate transporter and then released by exocytosis. In astrocytes, glutamate taken up from the extracellular fluid may be converted to glutamine which is released to the extracellular space, taken up by neurons and recoverted to glutamate inside neurons. This trafficking of glutamate and glutamine between astrocytes and neurons has been proposed to be a major pathway by which glutamate is recycled (Danbolt, 2001).

2.3. The dual role of glutamate: neurotransmission and toxicity

Glutamate is both indispensable and highly toxic. Intracellular glutamate is generally considered non-toxic, even if not completely inert, and it may serve as an intracellular messenger in some cells and participates in regulating expression of glutamate transporters on cell surface. In contrast, glutamate is thereby toxic when is present at high concentration in the extracellular fluid. In fact, as glutamate is the main transmitter in central nervous system, and is involved in several fundamental processes of differentiation, outgrowth, synaptic transmission of nervous cells and cell elimination, it exerts its signaling role also by activating glutamate receptors. These receptors are located on the plasma membrane of cells expressing them. The glutamate concentration in extracellular space determines the expression and the activation of glutamate receptors, which are found on most cellular elements in nervous system (dendrites, nerve terminals, neuronal cell bodies as well as glial cells). Evidence accumulated over the past years indicates that the excessive activation of some glutamate

receptors is harmful for cells, leading to neuronal damage and death (Meldrum, 1993; Olney, 1990; Whetsell and Shapira, 1993). For example, the activation of ionotropic glutamate receptors leads to the influx of Na^+ and Ca^{2+} which have to be pumped out the cell again in a process requiring energy. Thus, neurons become more vulnerable to glutamate after energy deprivation. Moreover, Ca^{2+} acts as second messenger in the cell and the increasing intracellular concentration may cause a cascade of events which lead to cell death. Furthermore, glutamate may cause mitochondrial damage (Danbolt, 2001; Schinder et al., 1996) and enhance the rate of generation of reactive oxygen species (ROS) (Danbolt, 2001; Savolainen et al., 1995), both mechanisms by which nervous cells may get important damage, and then death For this reason, it is of critical importance that extracellular glutamate concentration is kept low.

2.4. Families of glutamate receptors proteins

Glutamate may activate two different families of receptors: ionotropic receptors (iGluR) and metabotropic receptors (mGluR).

The three classes of iGluR (NMDA, AMPA and kainate receptors) exist as macromolecular complexes that combine into numerous receptor assemblies (Bigge, 1999; Bleakman and Lodge, 1998; Dingledine et al., 1999; Michaelis, 1998). The complexity of the system is amplified by the different intracellular signal transduction cascades and the intracellular events that are involved. NMDA receptors mediate the slow component, whereas AMPA receptors contribute to the fast component of excitatory postsynaptic currents. NMDA receptors are activated by the presynaptic release of glutamate and instantaneous depolarization of the postsynaptic membrane via colocalized AMPA receptors (Seeburg et al., 1998). When the NMDA receptor detects simultaneously these two signals, it results in controlled Ca^{2+} influx through the ion channel that is essential for activity-dependent synaptic modulation. Ionotropic glutamate receptors are postsynaptic ligand-gated ion channel receptors (NMDA, AMPA and kainate receptors), and secure fast synaptic transmission (Acher et al., 2010).

All iGluRs are integral membrane proteins that assemble as heteromeric or homomeric receptors from subunits within their respective families, and this multimeric nature of iGluRs allows them to be tremendously diverse and adaptable, accounting for their presence in a broad range of processes. Post-transcriptional and post-translational modifications impart

additional diversity and adaptability (McFeeters and Oswald, 2004). All iGluR subunits contain three transmembrane domains (M1, M3 and M4) and a re-entrant membrane loop (M2) on the cytoplasmic side that lines the inner channel pore and defines the distinct ion selectivity of the ion channel. The extracellular amino-terminal domain of each subunit includes a necessary component of the glutamate recognition site (S1), making it a selective receptor modulation via different mechanisms. The M3-M4 loop includes a second required component of the glutamate recognition site (S2) and RNA splice variants that affect receptor desensitization. The intracellular carboxyl terminus is involved in signal transduction and receptor anchoring, and contains phosphorylation sites that modulate receptor activity (Bigge, 1999).

The binding of glutamate to iGluRs is a key step in the mechanism of rapid excitatory synaptic transmission among nerve cells within the mammalian central nervous system (CNS). iGluRs are important in the development and function of the CNS and are implicated in learning and memory formation. Furthermore, iGluRs seem to be associated with certain neurological and psychiatric diseases (e.g. stroke, epilepsy, ischemia-related brain damage, trauma, sustained-seizure damage, Huntington's disease, ALS, Parkinsonism-dementia-like syndrome, Alzheimer's disease) and are therefore considered as potential drug targets (Nishizawa, 2001).

iGluRs are tetrameric, ligand-gated ion channels and have been divided into three different classes on the basis of protein sequence identity and ligand selectivity: AMPA, kainate (K) and NMDA receptors. iGluRs couple the energy of agonist binding to the opening of a transmembrane ion pore, allowing influx of Na^+, K^+ or Ca^{2+} ions and thereby cause membrane depolarization and neuronal excitation to produce an electrical signal from the chemical stimulus. Two regions, S1 and S2, of the receptor protein have been shown to constitute the ligand-binding core of the receptors and it has been verified that this core is necessary and sufficient for achieving binding properties similar to that of the membrane-bound receptor. Recombinant, soluble constructs of the ligand-binding core of several iGluRs have been produced and the structures of several agonists and antagonists in complex with these constructs have been determined.

Glutamate also activates metabotropic glutamate receptors, which modulate its release, postsynaptic response, as well as the activity of other synapses (Acher et al., 2010; Danbolt, 2001).

This family of glutamate receptors consists of G-protein-coupled receptors. Eight subtypes have been identified and classified into three groups (I-III) based upon sequence homology,

transduction mechanism and pharmacological profile. Group I includes some receptors which are coupled to Gq and activate phospholipase C and thereby the inositol triphosphate and diacylglycerol production, while group II and group III receptors couple to Gi/G0 and inhibit adenyl cyclase (AC) (Acher et al., 2010; Danbolt, 2001). Group I receptors are mostly located postsynaptically, thus their activation increases excitability. On the other hand, group II/III receptors are generally presynaptic and their activation reduces glutamate release.

3. Polybrominated diphenylethers (PBDEs): a class of brominated flame retardants

3.1. Use and occurrence

Flame retardants (FR) comprise a diverse group of chemicals which are widely used in many applications, including industrial and consumer products such as the manufacture of electronic equipment, textiles, plastic polymers and in the car industry. The annual consumption is currently over 1.5 million tonnes (Segev et al., 2009). The use of FR is primarily to prevent fire-related damage and to protect materials against ignition, and in the past few decades it has contributed in reducing the incidence of fires (Costa et al., 2008).

There are more than 175 different types of FR, commonly divided into four major groups: inorganic FRs, organophophorus FRs, nitrogen-containing FRs and halogenated organic FRs. Although FRs chemically differ one from another, share a general mechanisms of action, with some differences depending on the subtype (Segev et al., 2009).

Inorganic FRs are added as fillers into the polymers and are considered immobile; organophosphorous FRs are widely used both in polymers and textile cellulose fibers. Nitrogen-containing FRs inhibit the formation of flammable gases and are primarily used in polymers containing nitrogen, such as polyurethane and polyamide; the most important nitrogen-based FRs are melamine and its derivates. Halogenated organic FRs are usually made by chlorine or bromine. Brominated flame retardants (BFR) are more numerous than chlorinated FRs, due to their efficiency and because at high temperatures the decomposition products are less volatile than those derived from chlorinated compounds (Murphy, 2001).

Among brominated compounds, some were removed from the market following their contamination of animal feed in the 1970 (Dunckel, 1975). Others, such as tetrabromobisphenyl A (TBBPA), hexabromocyclododecane, and polybrominated diphenyl ethers (PBDEs) are still widely used (Alaee et al., 2003). PBDEs are extensively used in a variety of consumer products, are chemically similar to the long banned polychlorinated biphenyls (PCBs) and can exist in 209 possible congeners. Defined by their degree of bromination, PBDEs have been marketed mainly as mixtures of penta-, octa- and decabrominated BDE (Fonnum and Mariussen, 2009). PentaBDE and octaBDE have been banned in several states in USA and in Europe, whereas decaBDE are still commercialized and are the most widely used PBDE globally (Costa and Giordano, 2007). The decaBDE constitute more than 90% of the total PBDEs usage, and those mixtures contain BDE-209.

The octaBDE mixture includes several hexa to nona brominated isomers, while pentaBDE mixtures are composed of about 30% tetraBDE (BDE-47), 55% pentaBDE (BDE-99, BDE-100) and about 5% hexaBDE (BDE-153, BDE-154) (Fonnum and Mariussen, 2009). PBDEs are not fixed in the polymer product through covalent binding, but are simply added to the polymers, and thus are easily released into environment. In the last twenty years, PBDEs have become ubiquitous persistent organic pollutants; they bioaccumulate in the environment, biomagnify up the food chain (Hale et al., 2003; Law et al., 2006).

3.2. PBDEs: environmental contamination

As recently reviewed by Costa et al. (2008), the ubiquitous presence of PBDEs in the environment, in animals and humans has been confirmed by several studies. In particular, the highest concentrations of PBDEs have been detected in outdoor air, sediments, sludge, soil; in indoor air and house dust; in several food commodities; and in birds, fish and terrestrial animals. PBDEs have also been found in human tissues, such as serum, blood, adipose tissue and breast milk (Fonnum and Mariussen, 2009). The most abundant PBDEs congeners in environment, animals and human biological samples include five tetra-, penta- and hexa-BDE congeners (BDE-47, -99, -100, -153, -154) and also the widely used decaBDE (BDE- 209) (Darnerud et al., 2001), which can be broken down to the lower brominated congeners commonly found in humans (Soderstrom et al., 2004).

3.3. PBDEs in humans: exposure and sources

Human exposure to PBDE has been documented in numerous biomonitoring studies. Main sources of exposure to PBDES in adults is thought to be house dust ingestion and in general indoor environment, and consumption of contaminated food (Lorber, 2008), but also occupational exposure has been documented (Schecter et al., 2009; Segev et al., 2009). If presence of PBDEs in human serum and adipose tissue is confirmed all around the world, a large difference has been observed among PBDEs levels in samples from North America (200 ng/g lipid) and Europe and Asia (about 5 ng/g lipid) (Glynn et al., 2010; Hites et al., 2004; Lorber, 2008; Schecter et al., 2005; Trudel et al., 2010; Vizcaino et al., 2010; Vorkamp et al., 2010).

Among foods, the highest concentrations of PBDEs were described in fish, meats, and dairy products, depending on their origin. Meat is considered the major source from diet in U.S., whereas in other regions, it is estimated to be fish (Costa et al., 2008).

The fact that PBDEs have been detected also in cord blood samples means that foetus is also exposed throughout the prenatal period, because these compounds are able to cross the placenta (Frederiksen et al., 2009), and similar concentrations have been found in maternal and fetal blood (Costa et al., 2008). Significant levels of PBDEs have been measured in human breast milk, particularly in North America. For example, mean levels of all congeners of PBDEs in human milk in 2002-05 were measured, and concentrations of 3.7 ng/g of lipid, 1.57 ng/g of lipid and 73.9 ng/g of lipid were found in Europe, Japan and USA, respectively. More recent measurements were carried out, describing results in agreement with those previously reported (Petreas et al., 2011; Roosens et al., 2010).

To support the idea that the major source of exposure to PBDEs is represented by house dust, recent findings have been published (Costa et al., 2008; Johnson et al., 2010; Vorkamp et al., 2011; Wang et al., 2010). For example, it has been observed a strong relationship between dust and serum concentrations of several predominant PBDE congeners, providing evidence that dust is a primary route of PBDEs exposure (Johnson et al., 2010).

For toddlers in particular, dust has been estimated to account for 80% of PBDE exposure (Wilford et al., 2005). This represents a big concern, because infant and toddlers spend more time at home, have increased hand-to-mouth activity; in addition, their dietary preferences and exposure from breast milk, result in a overall greater ingestion of PBDEs than adults.

Occupational exposure to PBDEs may also occur in different settings, including PBDE manufacturing plants; facilities where PBDE-containing products, such as polyurethane foam or electronics, are manufactured or recycled; in offices where employees spend a large amount of time working with computers or other electronic devices; during installation or removal of carpet; and finally recently emerged the case of workers in car industry (Schecter et al., 2009; Segev et al., 2009).

3.4. General toxicology of PBDEs

PBDEs have low acute toxicity, with oral LD50s of > 5 g/kg. Upon chronic exposure, target organs are the liver, the kidney and the thyroid gland. Among the family of PBDEs, different congeners seem to share a similar toxicological profile, with decaBDE being less potent than

other lower brominated congeners. For example, in subchronic toxicity studies in rat, no-observed-effect-levels are usually in the g/kg/day range for decaBDE, but less than 10 mg/kg/day for pentaBDE (Costa et al., 2008).

Toxicokinetic studies in adult animals have indicated that absorption, metabolism and excretion of PBDEs are congener-, species- and gender-dependent (Costa et al., 2008; de Wit, 2002; Hakk and Letcher, 2003). Exposure to mixture of PBDEs displayed a similar congener pattern of distribution in the brain, adipose tissue, kidney and lung for most congeners (Huwe 2008). Polybrominated isomers may be metabolized to lower brominated congeners, while lower brominated congeners are metabolized to mono- and di-hydroxylated metabolites (e.g. 6-OHBDE-47), which may have a different toxic potency. With regard to excretion, it has been observed that young animals have a reduced ability to excrete PBDEs, which contributes to a higher body burden. Generally, PBDEs are not considered to be genotoxic (Costa et al., 2008), even if rodents exposed to BDE-209 show an increased incidence of hepatocellular carcinomas and thyroid adenomas. PBDEs can be fetotoxic, but usually at maternally toxic doses, and there is no evidence of teratogenicity. Even if PBDEs are chemically similar to PCB, they do not activate the Ah receptor-AhR nuclear translocator protein-XRE complex, although they can bind to the Ah receptor. However, it has been reported that PBDEs are capable to induce mixed-type monoxygenase in vivo, and also they may to induce phase II metabolizing enzymes, such as uridine diphosphoglucuronosyl transferase (UDPGT). It has been observed that PBDEs can inhibit CYP activity, such as aromatase (Costa et al., 2008).

It has been shown that PBDEs may interact as antagonists or agonists of androgen, progesterone, and estrogen receptors (Costa and Giordano, 2007). Reproductive toxic effects of PBDEs have also been reported (Kuriyama et al., 2005).

3.5. Developmental neurotoxicity

As infant and toddler seem to be exposed to high levels of PBDEs, because of ingestion of house dust and ingestion of breast milk, the potential adverse effects of PBDEs on the development central nervous system is a great concern (Costa et al., 2008). Moreover, several findings provided by animal studies indicated that perinatal exposure to PBDEs may cause alterations in the domains of motor activity and cognitive functions. In particular, a series of studies have shown that exposure of neonatal mice and rats to various PBDEs congeners (BDE -47, -99, -153, -183, -203, -206, -209) as a single oral dose, in most cases on PND 10,

causes hyperactivity, and impairs learning and memory normal functions (Costa et al., 2008). Although evidences are available on levels of PBDEs in serum, breast milk, adipose tissue, there is only little information on possible developmental adverse effects in humans from PBDE exposure. Only a few epidemiological studies have been published on neurotoxicity of PBDEs in humans. Roze et al. (2009) conducted a prospective cohort study of 62 mother-infant, and they reported several associations between maternal blood PBDEs levels, measured during the 35th week of pregnancy and their 5-6 year-old children motor, cognitive and behavioural performances. Correcting for socio-economic status and gender, maternal PBDEs levels were associated to diminished manipulative abilities (BDE-154), decreased sustained attention (BDE-47, -99, -100) and verbal memory (BDE-153), and with better selective attention (BDE-47) and behavior (BDE-47, -99, -100). These effects were described for the following levels (expressed as median) of BDE-47 (0.9 ng/g lipid weight), BDE-99 (0.2 ng/g lipid weight), BDE-100 (0.2 ng/g lipid weight), BDE-153 (1.6 ng/g lipid weight), BDE-154 (0.5 ng/g lipid weight). A recently study suggested in a similar way that PBDEs can impair infant neurodevelopment (Herbstman et al., 2010). This longitudinal cohort study included 329 mothers who gave birth in New York (US), prenatal exposure to BPDEs was assessed through measurements in cord blood samples and median concentrations (ng/g lipid weight) of PBDEs were the following: BDE-47 (11.2), BDE-85 (0.7), BDE-99 (3.2), BDE-100 (1.4), BDE-153 (0.7), BDE-154 (0.6), BDE-183 (0.6). Infant neurodevelopment was assessed at 12, 24 and 36 months and negative association were described with mental and psychomotor functionalities.

Information about the potential mechanisms of PBDEs neurotoxicity are still limited, but Costa and Giordano (2007) proposed two general possible ways of action: PBDEs may impair brain development interfering indirectly on thyroid hormones homeostasis, and may also exert a direct effect on central nervous system. Thyroid hormones have an important role in brain development (LaFranchi et al., 2005), and PBDEs may interfere with the production, or with the transport system of these hormones. The key role of thyroid during the development of nervous system has been demonstrated, and abnormalities brain functions have been described when thyroid was impaired. In particular, PBDEs have been reported to decrease levels of total and free T4 in adult animals, in adolescent animals, and following developmental exposure (Costa et al., 2008). Given that thyroid hormones are known to play a relevant role in brain development, and that hypothyroidism has been associated with a large number of neuroanatomical and behavioral effects (Haddow et al., 1999), this latter effect has been particularly investigated. PBDEs exposure effects on thyroid functions may be

relate to an enhanced metabolism and excretion of T4,or to an interaction of PBDEs with the thyroid hormone transport system. Another possible hypothesis is that PBDEs may interfere with thyroid hormone transport. Evidences reported Meerts et al. (Meerts et al., 2000) that several PBDEs could interact with transthyretin (TTR), one of the thyroid hormone binding proteins in plasma, thereby displacing T4. Moreover PBDEs metabolites, in particular 6-OH-BDE-47, were shown to be most potent in displacing T4 from TTR (Hamers et al., 2006; Meerts et al., 2000).

PBDEs may also directly cause neurotoxic effects in neuronal and glial cells. Few studies have described biochemical/molecular changes occurring in the central nervous system of animals following in vivo developmental exposure to PBDEs (Costa and Giordano, 2007). PBDEs may interfere with signal transduction pathways, and intracellular signals, such as the translocation of protein kinase C (PKC), stimulation of arachidonic acid release, inhibition of calcium uptake in cerebellar granule neurons (Kodavanti and Derr-Yellin, 2002; Kodavanti et al., 2005). Impairment of calcium homeostasis have also been seen in microsomes and mitochondria isolated from several brain regions of adult male rats (Reviewed by Costa et al. 2008). BDE-99 has been shown to cause apoptotic cell death in human astrocytoma cells (Madia et al., 2004), and a similar effect has also been observed with DE-71 in cerebellar granule cells (Reistad et al., 2006), and with BDE-47 in hippocampal neurons and human neuroblastoma cells (Giordano et al., 2008; He et al., 2008a; He et al., 2008b). Oxidative stress may be induced by PBDEs in neurotoxicity mechanism. In fact, DE-71 and BDE-47 were shown to cause oxidative stress in human neutrophil granulocytes (Reistad and Mariussen, 2005), an effect shared by other brominated fire retardants (Reistad et al., 2005; Reistad et al., 2007), Furthermore, BDE-47 was reported to induce oxidative stress in also in SH-SY5Y human neuroblastoma cells, in rat hippocampal neurons, and in fetal liver hematopoietic cells (Reviewed by Costa et al. 2008).

3.6. Polychlorinated Biphenyls (PCBs)

Polychlorinated biphenyls are a class of chlorinated aromatic hydrocarbons that were introduced to the market in the 1930's, with applications in numerous industrial products, such as diluents, dielectric fluids for transformers and capacitors, hydraulic fluids, additives in different types paint and cement, and in insulating material for windows. As PBDEs, PCBs are widespread and persistent environmental contaminants, and although their production and

use have been strictly banned in developed countries for the last three decades, PCBs are still found in the environment, in animals, foods and biological samples (Fonnum and Mariussen, 2009; Ndountse and Chan, 2009).

The PCBs family includes 209 possible congeners. They are generally divided in three main classes, depending on the chlorine substitution pattern. The coplanar PCBs, also called dioxin-like PCBs, have chlorine substitution in the *para*- and *meta*- position; the mono-*ortho*-substitute PCB congeners, which may achieve coplanarity; and the isomers with two or more *ortho*-substitution, which are always non-coplanar (Fonnum and Mariussen, 2009). PCBs are commercially marketed as mixtures with different degrees of chlorination, with ensuing different properties. In the commercial mixtures and in the environment the non-coplanar, *ortho*-substituted PCBs are the most dominant. In particular, PCB-153 is the non-planar, non-dioxin like PCB, isomer that appear most abundant in the environment, in human serum and in mammalian tissues (Vettori et al., 2006).

Because their lipophilic nature and resistance towards biotic and abiotic decomposition, PCBs tend to accumulate in lipidic tissues, including brain, and to further accumulate in higher trophic levels through the food chain (Fonnum and Mariussen, 2009; Mariussen et al., 2002; Vettori et al., 2006) The highest levels of PCBs in the brain have been found in polar sea gulls from the islands of Svalbard and it was in the range of 0.9-29.5 mg/kg brain (wet wt.), corresponding to about 3-90 µmol/kg (Gabrielsen et al., 1995).

The PCBs exhibit a wide range of biological and toxicological properties. Epidemiological studies after accidental and occupational exposure have shown that PCBs may affect the central nervous system (Mariussen et al., 2002). In vivo studies on rodents, avians and monkeys exposed to PCBs confirmed neurobehavioral effects observed in humans: both pre- and postnatally hyperactivity, effects on learning or memory and alteration in motor activity were described (Tilson et al., 1990).

Humans are exposed to PCBs through diet, especially fish, and these compounds are present in blood and breast milk, suggesting that infants may be exposed during pregnancy and lactation (Llansola et al., 2009).

The mechanisms of PCBs toxicity are not well known. It has been observed that PCBs interfere with dopaminergic system (Vettori et al., 2006) and disturb the thyroid-and steroid hormone metabolism (Mariussen et al., 2002). Moreover, several studies reported also that these compounds impact calcium homeostasis in neuronal cells (Kodavanti et al., 1993; Mundy and Guise, 1999). This may induce oxidative stress, and thus causing cell death. ROS formation has been described also PC12 cell line (Vettori et al., 2006), human granulocytes

(Voie et al., 2000) and in rat brain synaptosomes (Voie and Fonnum, 2000). Llansola et al. (2009) observed that cerebellar neurons exposed to PCB-126 and PCB-153 impair the glutamate-nitric oxide-cGMP pathway. Ndountse and Chan (2009), exposed human SHS5-SY neuroblastoma cells to the PCBs mixture Aroclor1254 and to two PCB congeners (coplanar, non-ortho PCB-126 and non-coplanar PCB-99), and found that all the compounds were able to affect the expression of NMDA receptors, and that calcium intracellular levels was impaired as well. Recently, perturbation of Ca^{2+} induced by non-coplanar PCBs were related to the ryanodine receptors activation (Pessah et al., 2010).

4. Interactions among toxicants

Interactions among substances *in vivo* and *in vitro* are studied for the assessment of combined favorable/adverse effects of treatments in the pharmaceutical field (Jonker et al., 2005). In recent years, however, there has been a growing interest in studying the interactions among environmental toxicants and food contaminants (Groten et al., 2001), mainly because humans are habitually exposed to mixture of chemicals, and from a toxicological point of view it is important and interesting to understand if substances interact to modify their potency and effects.

Several toxicants, at relatively low concentrations, may cause varying degrees of neurological effects particularly on the developing nervous system. Therefore, the study of interactions among neurotoxicants in *in vitro* models, where the molecular mechanisms of toxicity are usually studied, represents today an emerging field in the experimental neurotoxicology (Cory-Slechta, 2005). In the literature, it may be confusing to define the terms "additivity", "synergism" and "antagonism", because of the methods adopted to describe them. In fact, without the use of any mathematical model to describe interactions, "synergism" is erroneously defined as an increased effect as compared to the effect of single toxicants composing the mixture. However, in some cases this observed effect could be additive or even antagonistic, depending on the curves that describe the response/effect of a toxicant on a cellular line, when its concentration increases. Additivity means that two or more toxicants act without any interaction among them. In other words, the overall effect is not different from what we expect from concentration-effect/response relationship of the single compounds (Groten et al., 2001). We can speak about of antagonism and synergism when the overall effect of mixture is respectively lower and higher than expected (Groten et al., 2001). From a molecular point of view, it is possible simplify, imaging the cell as a system having a number of target sites for the toxicant. Every toxicant has a specific affinity for similar or different binding sites. When compounds bind their targets, induce such quantifiable direct/non-direct effect. Interaction means that the affinity is positively/negatively modified by the presence of other toxicants. This may occur as a direct modification of binding constant, a modification of the cause/effect chemical pathway, or an amplification of the observed effect induced by the binding. To define the specific molecular pathway is complex and hard, especially when is totally unknown. In most cases, when macroscopic effects are assessed in *in vitro* systems (viability, cell count, % of apoptotic or necrotic cells, oxidative stress, etc), the molecular action of the toxicant cannot be entirely defined; however, the significance of interaction

remains valid, as it quantifies the "deviation" from additivity, independently of its nature (Goldoni and Johansson, 2007). Some relatively simple mathematical models to study interactions have been defined, though not all scientists agree on their biological plausibility (Greco et al., 1995). However, they are extremely useful to describe the interactions among toxicants in particular in *in vitro* models, that ensure a good reproducibility of experiments, leading that the system may be described by mathematical functions.

Two are the models mostly used in several forms or equations to study interactions among toxicants, the Bliss Independence criterion and the Loewe Additivity model.

4.1. Approaching the study of interactions of mixtures

The first step to study interactions among toxicants is to plan in the right way the experiments, in order to obtain good-quality dose-response curves, from which parameters of the functions will be derived. The term "Dose-response relationship" define the relationship between the amount of a toxicant administered and the subsequent effect. The Dose-response curve (DR-curve) is the mathematical function which fits in the best way that change as a function of the increase of dose of the toxicant. In *in vitro* models, it is common referring to the terms Concentration-response relationship and Concentration-response curve. The effect measured in the *in vitro* system is usually a change in cell viability, an increase of cellular death (both necrosis and apoptosis), or a decrease in cell number.

The Dose-effect curve (DE-curve) is the mathematical function of best fit which shows the trend of a specific effect as a function of the concentration/dose of the toxicant.

The Hill function is the most used function to fit the DR/DE curves. Biological plausibility and adaptability to several biochemical processes and simplicity are important characteristics of Hill function, allowing to use it in combination studies (Goldoni and Johansson, 2007; Goldoni et al., 2003; Goutelle et al., 2008; Hill, 1910).

The formula E=E(x) of Hill function can be generalized as follows:

$$E = E_{max} * \frac{x^n}{(IC_{50})^n + x^n} \quad , \qquad (1a)$$

where *x* is the concentration of the toxic substance, and *n* is a parameter related to the slope of the curve (introduced by Hill); E_{max} is the concentration of *x* that causes the highest effect (Goldoni et al., 2003). The model can be extended to the case when we have a decrease of a

biological parameter (e.g. a decrease in cell viability) despite of an increasing effect. It refers to the case in which we do not consider the effect (mortality), but the proportion of living cells (viability), if the maximum viability is 1 and the minimum is 0, V=1 and E=1-mortality. In this situation the dose-response equation will be the following:

$$\frac{x^n}{(IC_{50})^n + x^n}, \qquad (1b)$$

where V is the viability.

From a biological point of view, *n* represents the "cooperativity index" and is related to the idea that the affinity of a ligand to a binding site might be affected by the concentration of already bounded ligand molecules. Due to conformational changes in macromolecules, or for steric effects, the binding sites may be reciprocally influenced by the binding of molecules which may modify the binding of additional ligands (Goldoni and Johansson, 2007). A *n* value >1 indicates that when a molecule binds its specific target site, it increases the binding affinity of the next molecule. When $n<1$, the situation is the opposite.

4.2. The Bliss Independence Criterion

The model is based on the assumption that two or more toxic agents act independently from one another (Bliss, 1939; Greco et al., 1995). Thus, the target sites of the toxicants are not overlapping, suggesting that the mechanism of action of the compounds in the mixture are completely independent. In some cases, this assumption is supported by data, in particular when drugs with different target sites are used in *in vitro* models before further studies of poly-therapy (La Monica et al., 2009).

Additivity, which is represented by the non interaction curve, is described the following Bliss formula for two toxicants:

$$E(x,y)=E(x)+E(y)-E(x)*E(y), \text{ or } V(x,y)=V(x)*V(y) \qquad (2)$$

where x and y are the two toxicants, E is the effect (such as mortality), and V the viability in the case when not the effect but living cells are considered.

As these equations define the non-interaction curve, every E significantly higher than expected in the combination experiments, signifies synergism. In contrast, when E is significantly lower than that expected additivity surface means antagonism.

Even if the non interaction among toxicants is demonstrated, the Bliss model presents important limitations (Goldoni and Johansson, 2007): (1) In case of steep dose response/effect curves, synergism could be overestimated. This is particularly true when toxicants are considered independent without any scientific evidence: the mutual dependence, not evaluated by the model, particularly when the effect/response is under 50% of the total, is considered as a synergistic effect. (2) The biological plausibility of the model is poor: except some specific cases, e.g. when some macroscopic parameters are measured (cell viability or death, oxidative stress, DNA damage and so on), toxicants tend to have at least in part common target sites. The model gives therefore an excessive simplification of the non-interaction curve.

4.3. The Loewe Additivity Model

The assumption of this model is the opposite to the Bliss independence criterion, and in particular that two or more toxic agents act on the same biological sites (or different indistinguishable binding sites), by the same mechanism of action, and they differ only in potency (Berenbaum, 1985; Greco et al., 1995). Even if the same mechanism of action is assumed, this does not mean that the DR/DE curves of single toxicants are parallel (Goldoni and Johansson, 2007).

The hypothesis of the model is the following:

$$\exists\, X_1, X_2 : E(x_1, x_2) = E(X_1) = E(X_2), \qquad (3)$$

Where X_1 and X_2 are the concentrations of toxicants in single exposure experiments, and x_1 and x_2 those in combined exposure experiments. This means that it is fundamental defining the equation of the DR/DE curve of single compounds, to entirely apply this model.

The non interaction curve (additivity) is described in a n-dimension space by the following formula, when we observe a response/effect (E) increasing with the concentration of the n considered toxicants:

$$\frac{x_1}{X_1} + \frac{x_2}{X_2} = 1 \qquad (4)$$

There will be synergism when $\frac{x_1}{X_1} + \frac{x_2}{X_2} < 1$ and antagonism when $\frac{x_1}{X_1} + \frac{x_2}{X_2} > 1$.

This model has also some limitations (Goldoni and Johansson, 2007): (1) It should be used only with simple systems, where the main parameters are measurable. The most simple one is

the effect of combined inhibitors on enzyme activity. Its use in toxicology is recommended with *in vitro* models, because the parameters of the experiments are relatively controllable, whereas its use in complex biological systems is only an approximation. (2) If dose-response/effect curves are very steep, a partial independence of target sites is estimated by the model as antagonism, which can be therefore overestimated.

4.4. Which one is the best model?

Although the two general methods have been compared with appositely studied software (Dressler et al., 1999), no agreement on which of the two models is more appropriate exists. However, the Loewe additivity model is generally preferred, because of a higher biological plausibility (Greco et al., 1995). The Bliss independence criterion is preferred when it is demonstrated that the two compounds have independent mechanism of action, and presumably different target sites . A current application of Loewe additivity approach is based on the isobolographic method, which does not consider the entirely DR/DE curve but only the IC_{50}. When both models have been applied, often the general results and conclusions coincide.

Furthermore, as discussed in Goldoni and Johansson (2007) the use of several *in vitro* tests, of asynchronous exposure (Goldoni et al., 2008), and the summary of overall data, could add important elements for the choice of a model over another (Goldoni and Johansson, 2007).

5. Aims of the study

On the basis of the available literature about PBDEs, I have chosen to approach the study of PBDEs neurotoxicity through an *in vitro* model. By using a human neuroblastoma cell line, the first part of the study is concentrated on the interaction between different PBDE congeners, in order to understand if co-exposure to a PBDE mixture may modify the toxic potency of the single compounds. This field may add important information in better characterizing the risk assessment after exposure to PBDEs. Furthermore, the *in vitro* model used is particularly suitable for the application of the Loewe additivity model and the Bliss independence criterion, through which I analyzed interaction data.

Secondly, with the same cellular model, it is of particular toxicological relevance the study of interactions between different classes of contaminants which tend to accumulate in environment, animal and food, and which present chemical similarities, such as PBDEs and PCBs.

Finally, the last part of the project has addressed the potential mechanism of neurotoxicity of the most abundant congener among PBDEs, BDE-47. In particular, during the last period of the PhD program, that I spent at the Dept. Environmental and Occupational Health Sciences, University of Washington (Seattle, WA), I assessed the role of glutamate of the toxicity of BDE-47 on granule cerebellar neurons from 7-day-old mice, a suitable model to study glutamatergic system and neuro-developmental toxicity.

In summary, the aims of the project were the following:

- To study the interaction between BDE-47 and BDE-99 in a neuronal in vitro model, applying Loewe additivity model and Bliss independence criterion, and to relate cell viability after combined exposure to oxidative stress.
- Since humans and wildlife are rarely exposed to a single contaminant, but rather to mixtures, to investigate the potential interactions between selected congeners of two widespread and persistent classes of contaminants, PBDEs and PCBs, in promoting cytotoxicity.
- To investigate the potential role of glutamate in BDE-47 toxicity, on a suitable neurodevelopmental model, including granule cerebellar neurons from 7-days-old mice.

6. Materials and Methods

6.1. Materials

BDE-47 was purchased from ChemService (West Chester, PA, USA) and BDE-99 from Chiron (Trondheim, Norway), while 5-(and-6)-carboxy-2'-7'-dichloro-fluorescein diacetate (Carboxy-H_2-DCFDA) was from Molecular Probes (Milan, Italy). (+)-5-methyl-10,11-dihydro-5H-dibenzo[a.d] cyclohepten-5,10-iminemaleate (MK-801), PCB-126 and PCB-153, anhydrous dimethylsulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), N-buthanol, and thiobarbituric acid were purchased from Sigma-Aldrich (Milan, Italy). Ethanol was bought from Carlo Erba (Milan, Italy), anhydrous isopropanol and 1,1,3,3-tetraethoxypropane (malondialdehyde) were obtained from Fluka Chemie (Buchs, Switzerland), and the protein bicinchoninic acid assay was from Pierce Chemical (Rockford, IL). 2,3-Dihydroxy-6-nitro-sulfamoylbenzo [f] quinoxaline (NBQX), (RS)-1-Aminoindan-1,5-dicarboxylic acid (AIDA), (2S)-2-Amino-2-[(1S,2S)-2-carboxycy
cloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495), (RS)-α-Methylserine-O-phosphate (MSOP) were from Tocris Cookson (Ellisville, MO). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA).

For cultures, Dulbecco's Modified Eagle's Medium (DMEM), Foetal Bovine Serum (FBS), Penicillin (5000 units/ml) and Streptomycin (5000 μg/ml), L-Glutamine (100X solution) and Trypsin (0.05%, EDTA 0.02% in PBS) were purchased from Celbio (Milan, Italy). Flasks and 96-well plates were obtained from Costar, Corning Inc. (Corning, NY).

6.2. Cultures of human neuroblastoma cells

The human neuroblastoma SK-N-MC cell line was purchased from The American Type Culture Collection (ATCC, Rockville, MD). Cultures of SK-N-MC were prepared as described by Vettori et al. (2006). Briefly, cells were seeded and grown at the density of 40.000 cells/cm^2 in DMEM medium supplemented with 10% foetal bovine serum, 25 units/ml of penicillin + 25 μg/ml of streptomycin and glutamine 1X. Cells were maintained at 37 °C in a 5% CO_2 humidified incubator and sub-cultured twice a week. For the MTT and the

thiobarbituric acid reactive substances (TBARS) assays, and for measurement of reactive oxygen species (ROS), exponentially growing cells were collected and were suspended at $1,5x10^5$ cells/ml concentration in fresh medium. Thirty six hours after seeding, cells were exposed to different concentrations of toxicant or vehicle.

6.3. Cultures of cerebellar granule neurons

Cultures of cerebellar granule neurons (CGNs) were prepared from 7-day-old C57Bl/6J mice killed by decapitation after CO2 narcosis, as described by Giordano et al. (2006). Briefly, after removal of the meninges from the whole brain, cerebella were rapidly dissected, tissues were cut into small cubes. Cerebella matrix was digested at 37 °C for 30 min in 1.5 mg/ml papain solution (1.5 mg/ml, DNase, plus Mg^{2+} in HBSS buffer). At the end of digestion, HBSS buffer was added and tissue was sedimented at 900 rpm x g for 5 min at 4 °C, then the supernatant was carefully removed and cerebellar tissue was resuspended in HBSS buffer. The next step was the mechanic dissociation by trituration of the matrix, using a long-stem Pasteur pipette. After dissociation the cell suspension was centrifuged in a refrigerated centrifuge at 900g for 5 min. The cell pellet was resuspended in complete growth medium consisting of Neurobasal A medium containing 1.5 mM GlutaMAX, 250 µg/ml Fungizone, Gentamicin, KCl 26 mM, and B27, a medium supplement with a newly improved formulation that substitutes serum. Cells were seeded at the concentration of 0.6×10^6 cells/ml in plates previously coated with Poly-D-lysine 200 µg/ml. After 1 hour cells were washed and fresh complete medium war replaced. Cells were maintained at 37 °C in a 5% CO_2 humidified incubator. At day 4 after CGNs preparation, neurons were treated with cytosine arabinofuranoside (AraC) 3 µM in complete Neurobasal A medium, containing B27 minus AO (without antioxidants) to prevent glial proliferation and obtain neuron-enriched cultures. Four days after AraC treatment, 50% of medium was replaced with fresh complete medium, and from day 10 after preparation, neurons were differentiated and ready to experiments.

6.4. Cell treatments

Human neuroblastoma cell line: PBDEs were dissolved in DMSO to obtain stock solutions of 25 mM, which were diluted appropriately at the time of use in free-serum medium. Final concentration of DMSO did not exceed 0.1%, and did not alter cell viability. In co-exposure

experiments, combinations of different concentrations of PBDEs or PCBs were added simultaneously to the medium freshly prepared without serum. Cells were incubated with chemicals for 24 h for cytotoxicity assay, and for the assessment of TBARS levels, while for measurement of ROS formation the exposure time was 3 h.

Cultures of cerebellar granule neurons: BDE-47 was dissolved in DMSO to obtain stock solution of 25 mM, which was diluted appropriately at the time of use in medium-B27 minus AO and without GlutaMAX, in the case of cytotoxicity (exposure 24 h) and TBARS (exposure 12 h) tests, as well as all the glutamate receptors antagonists tested. For measurement of glutamate release, ROS production and cytoplasmic free Ca^{2+} assays, BDE-47 and all the glutamate receptors antagonists tested stock solutions were dissolved in Locke's buffer. Before starting exposure to BDE-47 5 µM, cells were washed with the proper buffer, then neurons were incubated for 30 min at 37 °C with ionotropic or metabotropic gluatamate receptors antagonists or BAPTA-AM. Supernatants were removed and BDE-47 and antagonists/BAPTA-AM were simultaneously added.

6.5. Cytotoxicity assay

The MTT assay was carried out to evaluate cell viability (Mossman, 1983). The method used in this study is described by Vettori et al. (2006). After exposures, cell survival was quantified by a colorimetric method using the metabolic dye MTT. Culture medium was removed and replaced with 500 µl/well of buffer solution containing 2 mg/ml MTT. After incubation for 2 h at 37 °C, the MTT solution was removed, and the formazan reaction product was dissolved in 250 µl of DMSO. Absorbance was read at 570 nm, and the results expressed compared to unexposed controls. Untreated controls and blanks were incubated in the same plates and under the same conditions.

6.6. Trypan blue exclusion test

To evaluate cell membrane damage characteristic of necrosis and late apoptosis, cells were harvested and an aliquot of the cell suspension was mixed with an equal volume of 0.4 % Trypan blue in phosphate-buffered saline (PBS). Cells were scored at the phase contrast microscope using a Neubauer improved counting chamber. Samples were cultured in triplicate.

6.7. Measurement of L-glutamate release

Exposure conditions in L-glutamate release studies were identical to those used in the citotoxicity assay, excepted for time of exposure. In this case, time-course experiments were performed, and several time-points were considered. Buffers from treated cells were collected, and cells were scraped to calculate protein content. After measurement of pH, supernatants were collected, and determination of L-glutamate was carried out using the Amplex Red Glutamic Acid/Glutamate Oxidase Assay kit (Molecular Probes, Invitrogen). This kit is designed for continuously detecting glutamic acid or for monitoring glutamate oxidase activity in a fluorescence microplate reader. In the assay, l-glutamic acid is oxidized by glutamate oxidase to produce α-ketoglutarate, NH3 and H2O2. Hydrogen peroxide reacts with Amplex Red reagent (supplied by the kit) in a 1:1 stoichiometry in the reaction catalyzed by horseradish peroxidase (HRP) to generate resorufin. According to manifacturer's guidelines, reagents were prepared and by using excitation in the range of 530-560 nm and emission at about 590 nm, measure of fluorescence was carried out.

6.8. Measurement of Reactive Oxygen Species (ROS) formation

ROS production was measured by fluorescence, using 5-(and-6)-carboxy-2'-7'-dihydrodichlorofluorescein diacetate (Carboxy-H_2-DCFDA) as described by Giordano et al. (2006). Upon entering cells Carboxy-H_2-DCFDA is de-esterificated and then oxidizated by ROS to its fluorescent form. In a typical experiment, SK-N-MC cells were washed with HEPES 20 mM (in HBSS) and then pre-incubated for 30 min (37 °C) with Carboxy-H_2-DCFDA (20 μM), which was added from a stock solution in DMSO and diluted in HEPES. The quantity of DMSO never exceeded 0.1%, and was also added to the blank. Cells were washed with HBSS to remove extracellular Carboxy-H_2-DCFDA. After treatments (at 37 °C), the incubation medium was removed, and a solution of Tris-HCl-TritonX and a cell dissociation solution (Sigma) was added for 10 minutes. Cell lysates were scraped from the dishes and the extracts were centrifuged. The supernatant was collected, and the fluorescence was immediately read with a fluorescence spectrophotometer (Cary Eclipse, Varian, Palo Alto, CA, USA) looking at the fluorescence peak between 510 and 550 nm (excitation=480 nm). Fluorescence values were normalized for protein content, determined by the BCA protein assay.

6.9. Measurement of lipid peroxidation

Cellular ThioBarbituric Acid Reactive Substances (TBARS) were measured according to the method of Vettori et al. (2006). In a typical experiment, after three cycles of freezing and thawing (-80 °C and 37 °C), controls and treated cells were centrifuged at 3000 g for 5 min. 200 µl of supernatant were diluted with 200 µl of 0.2 M orthophosphoric acid. After vortexing, 25 µl of a 0.11 M thiobarbituric acid (TBA) solution prepared in 0.1 M NaOH were added and the vortexed solution was incubated at 95 °C for 45 min. TBARS were extracted adding 500 µl of n-butanol and 50 µl of a saturated solution of NaCl. After a vigorous mixing, the reaction mixture was centrifuged at 3000 g for 10 min and the upper solution was collected. TBARS concentrations were measured in fluorescence (excitation=515 nm, emission peak between 520 and 570 nm). Malondialdehyde was used as a standard for the calibration curve. TBARS concentrations were normalized for the number of viable cells as assessed by the Trypan Blue exclusion test. Samples were cultured in triplicate and experiments were performed three times.

6.10. Intracellular calcium measurement

Neurons plated in 35-mm glass-bottomed dishes were loaded with the Ca^{2+}-sensitive fluorescent dye Fluo-4/AM (5 µM) and placed on the stage of an inverted microscope. The dye in the cytoplasmic portion of the cells was excited, and fluorescence images were captured at 3 seconds intervals by a charge-coupled device camera (Princeton Scientific Instruments, Trenton NJ). Fifty cells in each treatment group were analyzed using MetaMorph software (Molecular Devices). Fluorescence measurements were normalized as $\Delta F/F$ ($F- F_0/F$; F was the intensity value obtained during the experiment, and F_0 was the baseline intensity value).

6.11. Statistical analysis and assessment of threshold doses on dose-response curves

Data were expressed as the mean ± SD of at least three independent experiments. Differences in effects at different PBDE concentrations (single exposure) were assessed by means of one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's *post-hoc* tests. SPSS

15.0 software (SPSS inc., Chicago, IL) was used and a p value of 0.05 was always considered as significant. The lowest concentration which produced a significant effect on viability was defined as Lowest Observed Adverse Effect Level (LOAEL), while the highest concentration which did not produce any significant effect on viability was defined as No Observed Adverse Effect Level (NOAEL). Benchmark dose (BMD10), the concentration at which 10% effect on viability was observed, and its lowest 95% CI extreme (BMDL10), were calculated by means of Benchmark Dose Software (BMDS) 1.4.1 (US EPA, Washington, DC).
Concentration-response curves for each PBDE were extrapolated applying to the experimental data the Hill function (Hill, 1910):

$$Viability\,(V) = V_0\left(1 - \frac{x^n}{(IC_{50})^n + x^n}\right) \qquad (5)$$

Where V_0 is the maximum of viability (e.g. 1 or 100%), n is the cooperation index and is related to the slope of the curve and x is the concentration of the compound.

6.12. Mathematical models to study the interactions

The Loewe additivity model and the Bliss independence criterion were applied using the equations reported in Goldoni and Johansson (2007) and the significance of the interactions was calculated with the method proposed by Vettori et al. (2006) and Goldoni and Johansson (2007), considering the minimum and the maximum curves on the basis of the errors of the Hill function parameters for single compounds.

7. Results and Discussions

7.1. Low concentrations of the brominated flame retardants BDE-47 and BDE-99 induce synergistic oxidative stress mediated neurotoxicity in human neuroblastoma cells

7.1.1. Results

Through MTT assay we tested the effects of combined exposure to BDE-47 and BDE-99 on viability of SK-N-MC human cells, and the Hill function was used to fit the experimental points. IC_{50} values of BDE-47 and BDE-99 were calculated, and they were 10.8 ± 0.7 μM and 33.9 ± 1.6 μM, respectively, as shown by Figure 1(a-b). The IC_{50} of BDE-47 was lower than that of BDE-99, but in both cases, the cooperativity index *n* was >1. Also values of the other threshold doses, such as NOAEL, LOAEL, BMD10 and BMDL10 for BDE-47 were about half of those found for BDE-99 (Fig. 1a-b).

Cell viability was measured by using several combinations of different concentrations of BDE-47 and BDE-99, and then compared with that expected by the non-interaction surface, calculated starting from the equations of concentration-response curves of the single compounds. As described by Table 1, the combinations of BDE-47 and BDE-99 at which synergistic/antagonistic interactions were significant with both Loewe and Bliss models are reported. The 3-D graphs in Figure 2(a-b) showed the entire non-interaction surfaces with both models and all experimental data points are presented. The combined concentrations of BDE-47 of 1-2.5 μM and BDE-99 of 5-30 μM gave rise to synergism, whereas the combination of concentrations of BDE-47 of 10-15 μM and BDE-99 of 5-50 μM described prevalently antagonistic effects.

In order to confirm mathematical results from interactions study, a biological and concrete effect was assessed for those concentrations at which the greatest synergistic/antagonistic interactions were observed, and presence of oxidative stress was evaluated. In particular, we chose the TBARS assay as a marker of lipid peroxidation, and DCF-DA test, as a marker of ROS formation. The effects of PBDEs on ROS levels (measured after a 3 h incubation) are

shown in Fig. 3. Exposure to 1 μM BDE-47 alone or 5 μM BDE-99 alone did not induce any significant increase in intra-cellular ROS levels as compared to controls. However, a combined exposure caused a highly significant ($p<0.01$) increase in ROS levels. Higher concentrations of BDE-47 (15 μM) and of BDE-99 (20 μM) when present alone, significantly increased ROS levels. However upon combined exposure, levels of ROS were lower than after each individual compound (Fig. 3).

In figure 4 results of the lipid peroxidation experiments after single and combined exposures are shown. Treatment of 1 μM of BDE-47 alone did not cause any significant effect on lipid peroxidation, while the exposure to 5 μM BDE-99 alone showed a modest but significant increase as compared to controls TBARS levels ($p<0.05$). On the other hand, the combined effect was significantly higher than the single exposures ($p<0.01$). When cells were treated with 15 μM BDE-47 and 20 μM BDE-99 in single and combined experiments, TBARS levels were significantly different from controls in all cases ($p<0.01$), but combined exposure did not induce any significant increase as compared with the two single exposures.

7.1.2. Discussion

For the first time, the possible interactions between two common PBDE congeners, using an *in vitro* approach has been addressed. The two congener involved in the study, BDE-47 and BDE-99, are among the most abundant congeners in the environment, and they have been usually found at the highest concentrations in human tissues and biological samples (Petreas et al., 2003; Schecter et al., 2005).

The *in vitro* approach chosen for the study is represented by a human neuroblastoma cell line, as a good model to study neurotoxicity. SK-N-MC cells were treated with the selected compounds, alone or in combination, and the Loewe and Bliss models were used to assess interactions. The main finding of this study was that the *in vitro* combined exposure to BDE-47 and BDE-99 induced synergistic and antagonistic effects depending on the PBDE concentration.

As first step to approach interaction study, the two concentration-response curves for cell viability after 24 h exposure to BDE-47 or BDE-99 were carried out, and IC_{50}, NOAEL, LOAEL, BMD10 and BMDL10 values were calculated. Importance was given to benchmark dose, because its concept represents an improvement of the traditional practice in risk assessment of chemicals (Sand et al., 2004). In fact, BMD and BMDL, differently from

NOAEL and LOAEL, are directly calculated from dose-response fitting curve and therefore reduce the uncertainty in risk assessment (Goldoni et al., 2003). In the present study, the BMD10 value was calculated as the dose producing a 10% change in cell viability as compared to controls, which is usually the first significant effect point in *in vitro* models and corresponds therefore to an IC_{10}. Moreover, Lower BMD (BMDL) was considered, because is an estimate of its lowest extreme in the 95% CI and should be considered as the lowest threshold dose (Goldoni et al., 2003). Calculate BMD10 and BMDL values allows to compare *in vitro* and *in vivo* data (Goldoni et al., 2003), giving significance to results., Differences among IC_{50} found for BDE-47 and BDE-99 (about a factor of three) were maintained in the threshold doses, in particular for BMDL (about a factor of two), due to similar cooperativity index >1. Therefore, BDE-47 had a general higher neurotoxic potency than BDE-99 at all concentrations.

The peer-reviewed literature is generally in agreement with the present findings. The fact that BDE-47 and BDE-99 could induce cytotoxicity and other cellular effects, such as apoptosis, LDH release, translocation of PKC, changes in intracellular calcium and AA release in a range of concentration of 1-50 μM, and 1-100 μM (Llansola et al., 2007; Madia et al., 2004), respectively, was already shown by several studies in human or rat neuronal or astroglial cells (Costa and Giordano, 2007; Dingemans et al., 2007; He et al., 2009; Kodavanti and Derr-Yellin, 2002),

On the other hand, limited data are available on levels of PBDE found in brain tissue after *in vivo* exposure. Neurotoxic effects in rats at BDE-99 brain concentrations of about 0.4 μM are reported by Cheng et al. (2009), while Reistad et al. (2006) have measured concentrations of 0.5 μM for BDE-99 and 0.4 μM for BDE-47 in the rat brain 74 hours after i.p. injection of 13.2 mg/kg DE-71 (a penta-BDE mixture), without observing neurotoxic effects. Staskal et al. (2006) found that concentrations of BDE-47 and BDE-99 in rat brain tissue 5 days following a single i.v. administration (1 mg/Kg) were about 0.02 μM and 0.04 μM, respectively. Thus, neurotoxic effects *in vivo* have been found at brain concentrations of BDE-47 and BDE-99 about one order of magnitude lower than BMDL observed in the present *in vitro* study. Differences in exposure modalities and uncertainties factors in comparing *in vitro* and *in vivo* data should be considered in understanding them. On the other hand, the metabolization of the PBDEs *in vivo* with the formation of highly toxic metabolites and the neurotoxic indirect effect that can be induced by PBDEs as endocrine disruptors *in vivo* cannot be observed with simple neuronal *in vitro* models (Costa et al., 2008). In humans, it is only possible to make a

theoretical estimation about brain levels of PBDEs, and it would be in the nM range, based on levels found in blood (0.21-580 ng/g lipid) (Costa and Giordano, 2007; Giordano et al., 2008). In the present study, interactions were studied combining several concentrations of BDE-47 (range 1-15 μM) and BDE-99 (range 5-50 μM), and comparing them with non-interaction surfaces calculated with the Loewe and Bliss mathematical models, and the major finding is that the type of interaction varied depending on the range of concentrations considered. In particular, at concentrations of BDE-47 below its threshold doses (1-2.5 μM) and in a wide range of BDE-99 concentrations below its IC_{50} (5-30 μM), prevalently synergistic effects were observed, independently of the model used. In contrast, at concentrations of BDE-47 near its IC_{50} (10-15 μM) and in a wide range of BDE-99 concentrations, below and over its IC_{50} (5-50 μM), prevalently antagonistic effects were observed. In the peer reviewed literature studies of other compounds tested in combined exposure both *in vivo* and *in vitro*, for which the nature of the interactions varied along the concentration range using the isobolographic method are available (Faessel et al., 1999; Gessner, 1995). On the other hand, the mathematical methods used in the present study, which consider all the non-interaction surface and not only the trend of IC_{50}, is particularly useful in assessing different types of interactions along all the range of concentrations of the tested toxicants (Goldoni and Johansson, 2007).

We thought to confirm results obtained by applying Loewe and Bliss models on viability data, evaluating combined exposure effects on oxidative stress, as it is thought to be one important mechanism of direct neurotoxicity of PBDEs (Costa and Giordano, 2007; Giordano et al., 2008). Thus, markers of oxidative stress, assessed both at early (3 hours) (ROS) and late (24 hours) (TBARS) times of exposure were considered at the combinations of concentrations, at which synergistic/antagonistic effects were more pronounced. The results found upon exposure to BDE-47 or BDE-99 alone were consistent with those previously reported (Giordano et al., 2008; He et al., 2008a; He et al., 2009; He et al., 2008b). Interestingly, the result of the oxidative stress measurements (ROS and TBARS) confirmed the interactions found in the cell viability experiments (Fig. 3,4). The finding of synergistic or antagonistic interactions when measuring both cell viability and markers of oxidative stress, further supports the idea that the latter plays an important role in PBDE neurotoxicity.

A reciprocal influence of these two PBDEs congeners on their respective hypothetical binding sites, with an increased affinity or a different kinetics of binding is the hypothesis which may explain synergism. In particular, low concentrations of BDE-47 seemed to modulate the effect

of BDE-99, but not the opposite, because the range of concentrations at which synergism was observed was wide for BDE-99 and very narrow for BDE-47. The other possibility is that the binding of PBDEs to their target sites could also enhance the cellular permeability, exacerbating their toxic effects. Kinetic studies on PBDEs intracellular concentrations at different times are necessary to substantiate this hypothesis.

In contrast, the antagonistic interaction may be explained by other mechanisms. As suggested by Goldoni et al. (2008), the antagonism may be caused by a saturation of intracellular target sites by the toxicant present at higher concentrations (BDE-99). Finally, the reduction in overall toxicity may thus be related to inability of the most toxic compounds (BDE-47) to bind to the same hypothetical targets sites. However, the binding of toxicant to a putative target inside the cell may activate some cellular mechanism of defense which modulate and reduce the toxicity of other compounds. Additionally, it is not possible to exclude the hypothesis that the two PBDEs, because of their hydrophobic properties, may interact with each other.

7.1.3. Figures and Tables

Figure 1

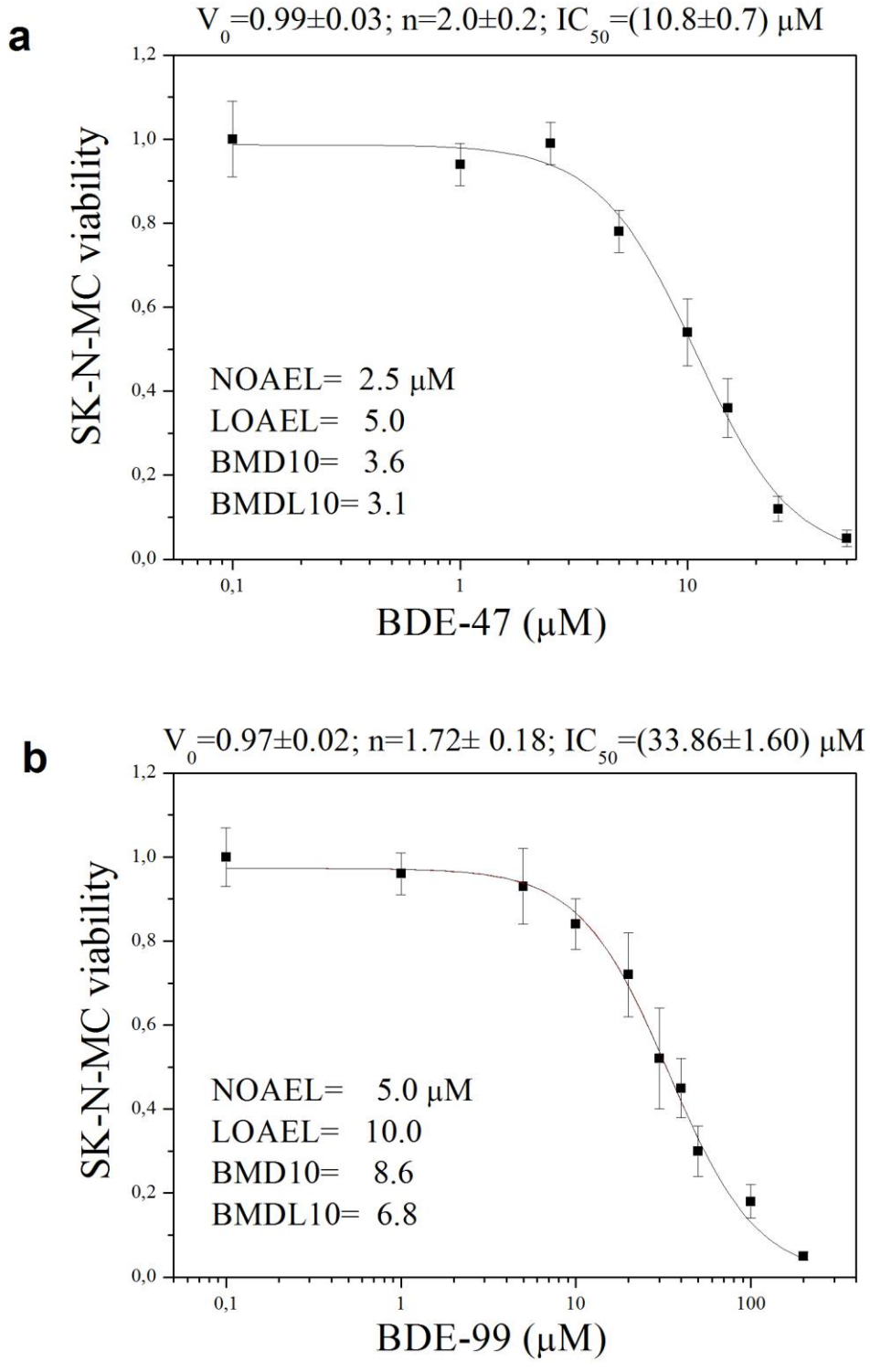
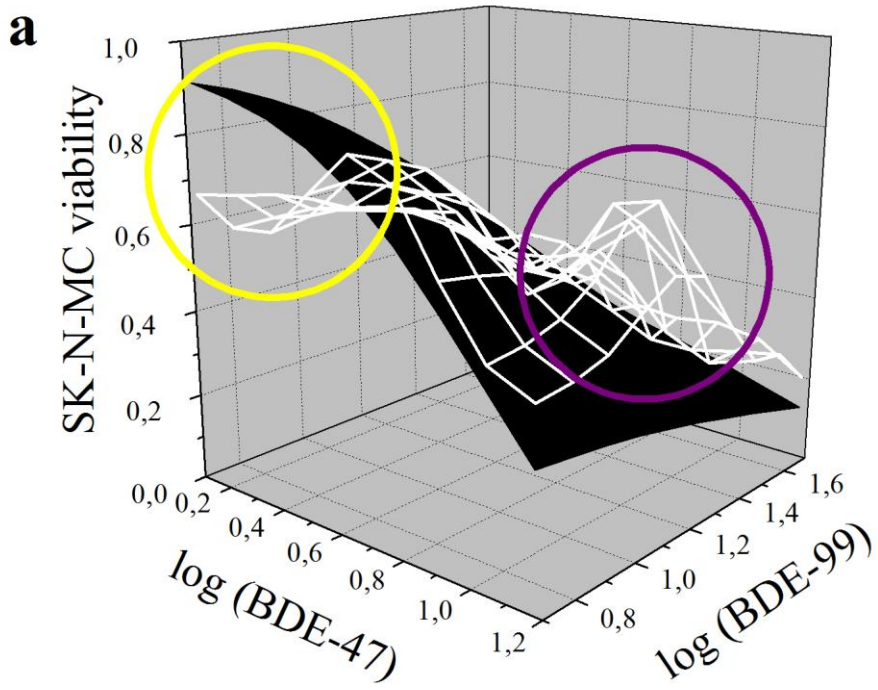


Figure 2


a
SK-N-MC viability
1,0
0,8
0,6
0,4
0,2
0,0
0,2
0,4
0,6
0,8
1,0
1,2
log (BDE-47)
0,8
1,0
1,2
1,4
1,6
log (BDE-99)

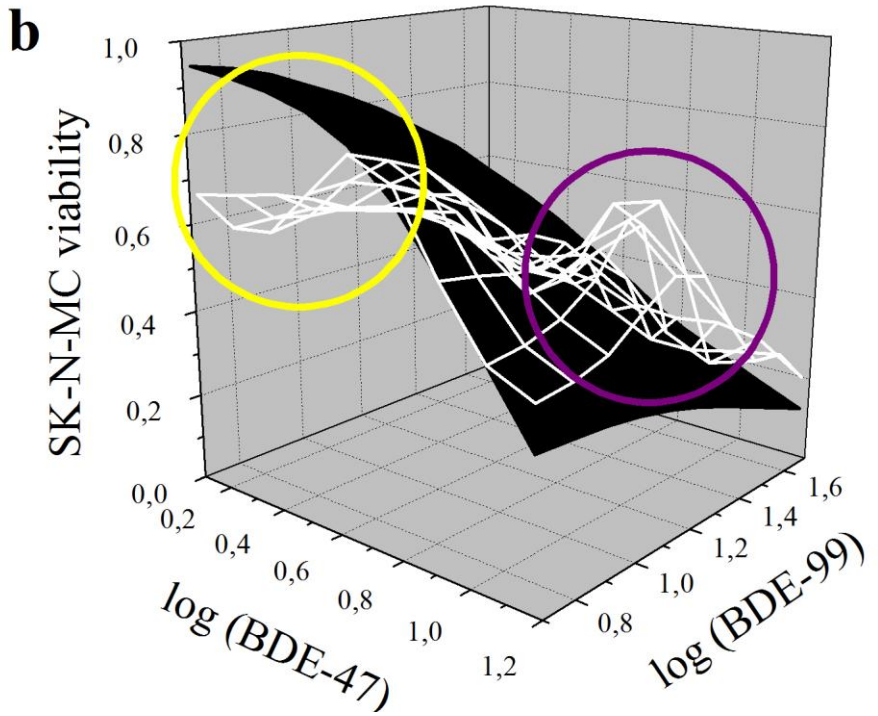

b
SK-N-MC viability
1,0
0,8
0,6
0,4
0,2
0,0
0,2
0,4
0,6
0,8
1,0
1,2
log (BDE-47)
0,8
1,0
1,2
1,4
1,6
log (BDE-99)

Figure 3

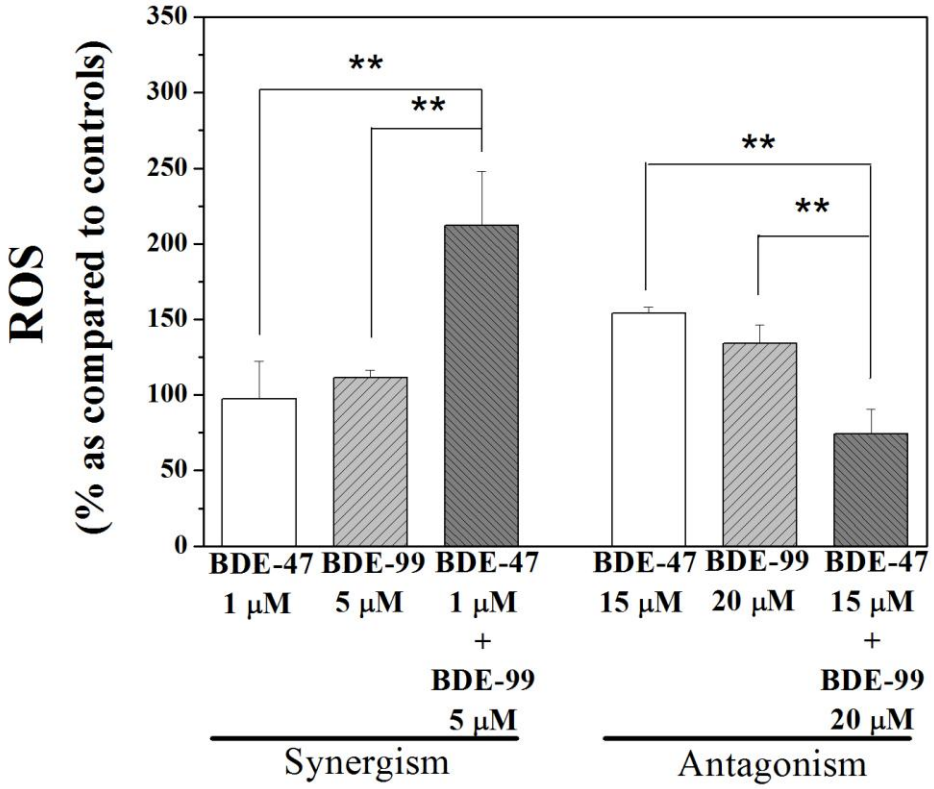
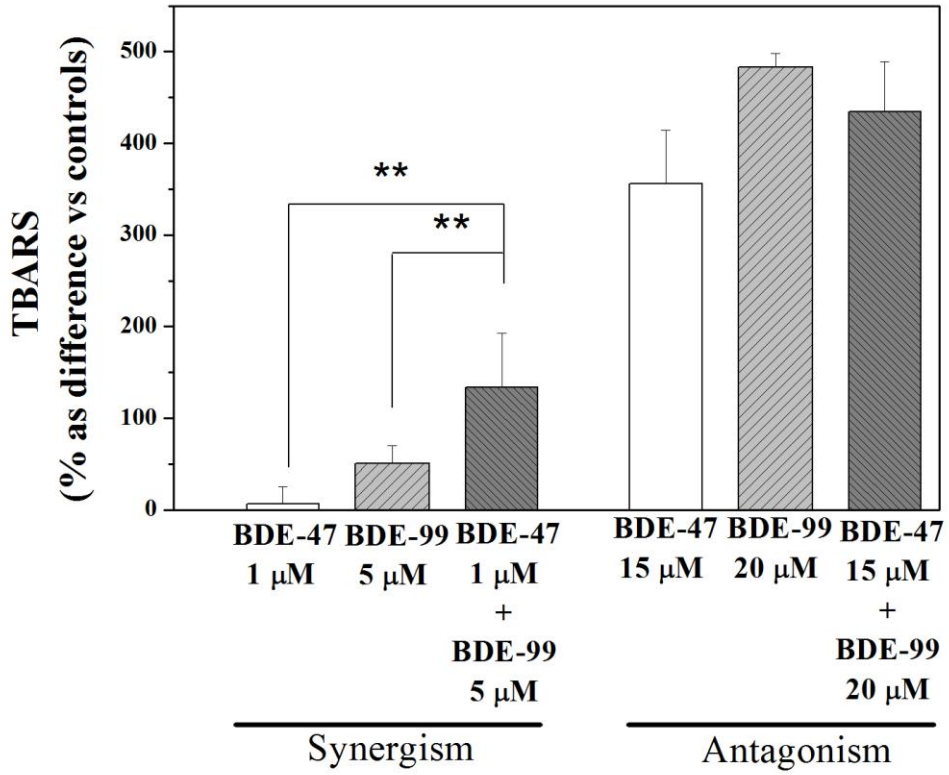

ROS
(% as compared to controls)
350
300
250
200
150
100
50
0
**
**
**
**
BDE-47 1 μM
BDE-99 5 μM
BDE-47 1 μM + BDE-99 5 μM
BDE-47 15 μM
BDE-99 20 μM
BDE-47 15 μM + BDE-99 20 μM
Synergism
Antagonism

Figure 4


TBARS
(% as difference vs controls)
500
400
300
200
100
0
**
**
BDE-47
1 μM
BDE-99
5 μM
BDE-47
1 μM
+
BDE-99
5 μM
BDE-47
15 μM
BDE-99
20 μM
BDE-47
15 μM
+
BDE-99
20 μM
Synergism
Antagonism

Legend to figures

Fig. 1 Concentration–response curves reporting variation in SK-N-MC cell viability after 24 h exposures to BDE-47 (a) or BDE-99 (b). Cell viability values are normalized to controls. Viability was measured by the MTT reduction assay, as described in Methods. The figures show the mean (± SD) of two separate experiments, each carried out in 4 replicates, the parameters of the fitting functions, and the NOAEL, LOAEL, BMD10 and BMDL10 values.

Fig. 2 Comparison between experimental data points on cell viability and the non-interaction surface calculated by the Loewe additivity model (a) and the Bliss independence criterion (b). The black 3D-surfaces represent the non-interaction curves calculated by model equations. The white curves represent the experimental viability for different combination of concentrations of BDE-47 and BDE-99; the yellow circles contain the concentrations at which synergistic interactions were observed; the purple circles contain the concentrations at which antagonistic interactions were observed.

Fig. 3 Reactive Oxygen Species (ROS) levels upon single and combined exposures to BDE-47 and BDE-99. ROS were measured 3 h after PBDEs exposure, as described in Materials and Methods. Results represent the mean (± SD) of three separate experiments, each carried out in duplicate (**p < 0.01, significantly different from single exposure). % increase vs control $= 100 * (\frac{ROS_{treated}}{ROS_{control}} - 1)$.

Fig. 4 Lipid peroxidation (TBARS levels) upon single and combined exposures to BDE-47 and BDE-99. TBARS levels were measured 24 h after PBDEs exposure, as described in Materials and Methods. Results represent the mean (± SD) of three separate experiments, each carried out in duplicate (**p < 0.01, significantly different from single exposure). % increase vs control $= 100 * (\frac{TBARS_{treated}}{TBARS_{control}} - 1)$.

Table 1: Comparison between experimental and theoretical non-interaction values expected by the Loewe and Bliss models in combined exposure experiments.

BDE-47 (μM)	BDE-99 (μM)	$V_{th,Loewe}$	$Range_{th,Loewe}$	$V_{th,Bliss}$	$Range_{th,Bliss}$	$V_{Experimental}$	$SD_{Experimental}$	Interaction
1	**5**	0.93**	0.88-0.98	0.96**	0.91-1.00	0.76	0.07	Synergistic
1	**10**	0.84**	0.79-0.90	0.89**	0.82-0.94	0.65	0.05	Synergistic
1	**20**	0.67**	0.61-0.72	0.71**	0.65-0.77	0.51	0.05	Synergistic
1	**30**	0.51*	0.47-0.55	0.55**	0.51-0.59	0.47	0.03	Synergistic
2.5	**5**	0.86**	0.80-0.92	0.92**	0.85-0.97	0.63	0.07	Synergistic
2.5	**10**	0.76**	0.71-0.83	0.85**	0.77-0.91	0.63	0.02	Synergistic
2.5	**20**	0.59**	0.54-0.64	0.68**	0.61-0.75	0.52	0.04	Synergistic
10	**10**	0.40**	0.35-0.45	0.48*	0.42-0.54	0.55	0.08	Antagonistic
10	**20**	0.31**	0.26-0.36	0.39**	0.33-0.44	0.67	0.02	Antagonistic
15	**5**	0.30**	0.25-0.35	0.33**	0.27-0.38	0.43	0.06	Antagonistic
15	**10**	0.26**	0.21-0.32	0.31**	0.25-0.36	0.50	0.11	Antagonistic
15	**20**	0.21**	0.17-0.26	0.24**	0.19-0.29	0.50	0.04	Antagonistic
15	**30**	0.17**	0.13-0.22	0.19**	0.15-0.23	0.30	0.06	Antagonistic
15	**40**	0.14**	0.11-0.19	0.15**	0.11-0.19	0.28	0.05	Antagonistic
15	**50**	0.12**	0.09-0.16	0.12**	0.09-0.15	0.20	0.04	Antagonistic

Viability of SK-N-MC in co-exposure experiments to different concentrations of BDE-47 and BDE-99 was assessed by the MTT assay, as described in Materials and Methods. The table shows the theoretical viability values expected by the Loewe additivity model ($V_{th,Loewe}$), the Bliss independence criterion ($V_{th,Bliss}$) and the ranges of expected V_{th} values. Experimental data presented are the mean (± SD) of two separate determinations (4 replicates for each experiment). When experimental data deviated significantly from the theoretical ones (**$p< 0.01$, *$p<0.05$) synergism or antagonism could be defined.

7.2. Synergistic toxicity between PBDEs and PCBs in human neuroblastoma cells

7.2.1. Results

The effects of BDE-47, BDE-99, PCB126 and PCB-153 on cell viability were evaluated by the MTT assay, and experimental points were fitted with the Hill function. As shown in Fig. 6a, the IC_{50} of BDE-47 (12.06 ± 1.01 μM) was about half of that of BDE-99 (30.77 ± 1.95 μM), while for the PCBs the IC_{50} of PCB-126 (9.88 ± 2.92 μM) was lower than that of PCB-153 (15.38 ± 0.76 μM) (Fig. 6b). Values of NOAEL, LOAEL, BMD10 and BMDL10 for each compound were calculated, and are shown in Table 2.

To assess the potential interactions between each PBDE with PCB-126 or PCB-153, cell viability was measured through several combinations of different concentrations of chemicals. These concentration ranges were selected using the equation concentration-response curves of single compounds. Data were compared with those expected by the non-interaction surface, calculated starting from the equation of concentration–response curves of the single compounds.

Tables 3-6 show the comparison between experimental and theoretical non-interaction values expected by the Loewe model in combined exposure experiments for all four mixtures. Data show the theoretical viability values expected by the Loewe additivity model ($V_{th,\ Loewe}$) and the ranges of expected V_{th} values. Experimental data presented are the mean (± SD) of two separate determinations (4 replicates for each experiment). Synergism or antagonism is indicated when experimental data are significantly different from the theoretical ones.

The 3-D graphs in Fig. 6 show the non-interaction surfaces created with the Loewe model and all experimental data points for the mixtures, i.e. BDE-47 with PCB-126 (a) or BDE-47 with PCB-153 (c). Figure 6a, c show the non-interaction surfaces and all experimental data points for mixtures of BDE-47 and PCB-126 (a) or of BDE-47 and PCB-153 (c). Synergistic effects were prevalently observed for BDE-47 and PCB-153 at all concentrations (Fig. 6c; Table 5), while additive effects were prevalently identified for BDE-47 and PCB-126, with synergistic effects at the highest concentration of BDE-47 (10 μM) (Fig. 6a; Table 3).

As shown in Fig. 6b and in Table 4, the nature of interactions between 5-10 μM of BDE-99 and all concentrations of PCB-126 was quite complex, with synergistic and antagonistic effects or simple additivity depending on the combination used. With higher concentrations of BDE-99 (20–30 μM), prevalently synergic effects were identified with PCB-126 and

synergistic/additive effects with PCB-153. On the other hand, concentrations of BDE-99 from 5 to 10 μM combined with PCB-153 showed prevalently synergistic effects (Fig. 6d; Table 6).

7.2.2. Discussion

The present study shows that PCBs and PBDEs in combined exposure give rise to interactions of different nature in an in vitro model with human SK-N-MC cells. PBDEs and PCBs are both widespread contaminants, and they have caused environmental concerns worldwide because of their ubiquitous bio-accumulative nature and their adverse effects on human health (Shao et al., 2008). PBDEs have been used for decades as flame retardants in different products. Main sources of PBDE exposure are the indoor environment and the diet, but the outdoor environment and occupational exposure have also been documented. PCB have accumulated in the environment and biota, and at the moment represent a significant public health concern (Pessah et al., 2010). Among PBDEs we selected for our experiments BDE-47 and BDE-99, the most common isomers found in wildlife and human tissues. In the case of PCBs, PCB-153, that has been identified as a major contributor to total PCB burden in humans, and PCB-126, as the dioxin-like representative congener, were selected. Concentration-response curves and values of $IC_{50,}$ NOAEL, LOAEL, BMD10 and BMDL10 of each compound were calculated to assess their specific toxicity in human neuroblastoma cells. BDE-47 was more potent than BDE-99, while PCB-126 was more toxic than PCB-153, as shown by differences in IC_{50} values and of other quantitative parameters presented in Table 2. These findings are generally in agreement with the literature. IC_{50} and threshold doses values of BDE-47 and BDE-99 are overlapping to the ones calculated by Tagliaferri et al. (Tagliaferri et al., 2010) in the same cell line. Also, the IC_{50} values found for PCB-153 and PCB-126 are supported by data from literature, as shown by Costa and Giordano (Costa and Giordano, 2007) in SH-SY5Y neuroblastoma cells. Vettori et al. (2006) in PC12 cell line found an IC_{50} value of about 200 μM for PCB-153, and Lin et al. (2006) tested PCB-153 and PCB-126 in two different breast cancer cell lines finding that the concentrations of both substances capable to inhibit of 50% the viability were higher than 100 μM. The potential interactions between PBDE and PCB congeners were investigated by combining several concentrations of BDE-47 (range 1-10 μM) and BDE-99 (range 5-30 μM) with PCB-126 (range 1-10 μM) or PCB-153 (range 1-15 μM), and comparing them with the Loewe non-interaction surface. The selected concentrations are from equations of concentration–response

curves of single compounds after 24 h exposure and they are lower or equal to their IC_{50} values. The results show that the nature of interactions is related to the PCBs structure, and that, depending on the concentration, the type of interaction varies. Both PBDEs, at relatively low concentrations, showed synergistic interactions with PCB-153, higher concentrations of BDE-47 combined with all concentrations of PCB-153 showed mainly synergistic interactions, while additivity was observed at high concentrations of BDE-99. Antagonism was sporadic, indicating that generally PCB-153 can amplify the toxic effects of PBDEs. The interaction between PBDEs and PCB-126 is more complex, indicating a different mechanism of combined action. Low doses of BDE-47 had prevalently additive effects with PCB-126, while BDE-99 at low doses show variable effects (antagonistic, additive, or synergistic, depending by the combination). In contrast, mainly the highest concentrations of PBDEs (around IC_{50} values of both PBDEs) had prevalently synergistic effects with all the concentrations tested of PCB-126, suggesting a possible role of PCB structure on the nature of the interaction with PBDEs. Further studies are necessary to better identify this mechanism. Our findings are supported by those studies where combinations of different compounds have been shown to lead to interactions that varied along the concentration range (Faessel et al., 1999; Gessner, 1995). Moreover, several studies have shown that toxicity depends on PCB chemical structure (Baars et al., 2004; Kodavanti and Tilson, 1997; Sanders et al., 2005; Schantz et al., 1997; Shain et al., 1991; Tofighi et al., 2010). Finally, the application of the Bliss independence criterion gave consistent results (data not shown), indicating that the use of different models was in this study not crucial in defining synergism.

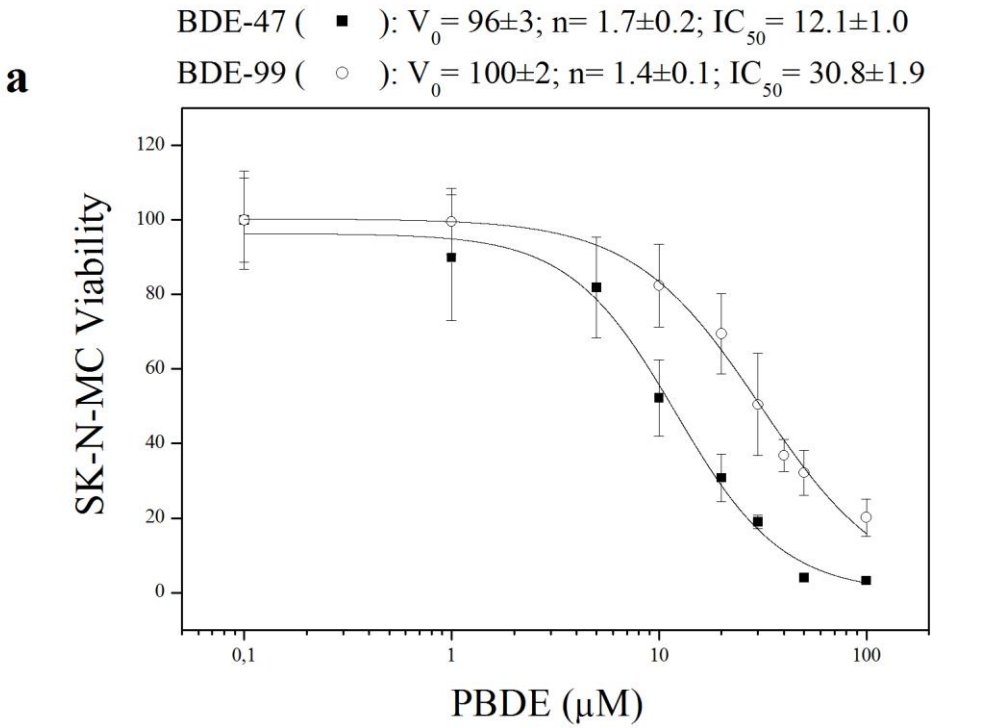
Furthermore, our results are in agreement with earlier findings provided by other authors (Eriksson et al., 2006; Gao et al., 2009; He et al., 2009; He et al., 2010), who investigated the effect of mixtures of PCBs and PBDEs trough an *in vivo* and *in vitro* approach, respectively. In particular, mice exposed on postnatal day 10 to a combined low dose of PCB-52 (a non-dioxin-like PCB congener, 1.4 μmol/kg body weight) and BDE-99 (1.4 μmol/kg), displayed developmental neurotoxic effects significantly more pronounced than PCB-152 alone (14 μmol/kg bw) (Eriksson et al., 2006). Gao et al. (2009) showed that a combined exposure of SH-SY5Y cells to BDE-47 (5 or 10 μM) and PCB-153 (5 μM) may exacerbate the effects of oxidative stress induced by BDE-47. Indeed, the authors found that the Reactive Oxygen Species (ROS) formation and DNA damage were dramatically increased if compared to corresponding cells treated with BDE-47 and PCB-153 alone. These findings were confirmed by He et al. (2010) , who demonstrated that the effect of the combination of BDE-47 and PCB-153 on DNA damage, DNA-protein cross-links and chromosome abnormalities in the

same cell line was higher than that produced by each compound, when present alone. In another study, He et al. (2009) explored the mechanism of BDE-47 and its interaction with PCB-153 evaluating the combined effect on cytotoxicity, intracellular Ca^{2+} level, apoptosis, caspases and death associated protein kinase (DAPK) expression on SH-SY5Y cells. They found that BDE-47 can interact with PCB-153 in enhancing cytotoxicity, intracellular calcium level and the expression of caspase-3, showing mostly synergistic interaction. In MCF-7 cells, Llabjani et al. (2010) investigated the effect of binary mixtures of PBDEs (BDE-47, BDE-153, BDE-183 or BDE-209) with or without the coplanar PCB-126 or the non-planar PCB-153 on biochemical alterations, assessed by IR spectroscopy with multivariate analysis. They found significant different spectra for PCB-126 and PCB-153, reflecting their different mechanisms. Interestingly, the treatment with PBDEs showed spectra similar to that of PCB-153. In particular, the most evident alterations induced by BDE-47 and PCB-126 were similar to the pattern observed with PCB-153 and PCB-126. In conclusion, the combined exposure to PBDEs and PCB-153 gave rise to an enhanced alterations in cell status, while mixtures of PBDEs and PCB-126 did not show the same synergism.

These results are in agreement with our results, as also in our study the interaction between PBDEs and PCB-126 appeared highly dependent by the combination used, as already discussed. The synergism observed between low concentrations of BDE-47 or BDE-99 and a wider range of concentrations of PCB-153 is very interesting from a toxicological point of view, because these compounds are widespread in the environment and in biota, at relatively low concentrations, and humans may be co-exposed to them. Their toxicological synergism suggest that the compounds are able to reciprocally modify the affinity of their target sites, enhancing the total effect on cell viability. BDE-47/BDE-99 and PCB-153 could be able to increase the intracellular availability of the compounds, with consequent exacerbated effects. Another possibility is that the simultaneous exposure has a synergistic effect on cellular defense mechanism impairment. Further studies are in progress to measure the intracellular content of PCBs and PBDEs in single and combined exposures, and to better characterize other cellular end-points. Whereas the nature interaction of both PBDEs and PCB-153 seems to be well defined, PBDEs and PCB-126 interact in a more complex manner. This suggests that from a toxicological point of view it is important to take into account that different congeners among PCBs may have a different potency in the presence of others toxicants.

7.2.3. Figures and Tables

Figure 5



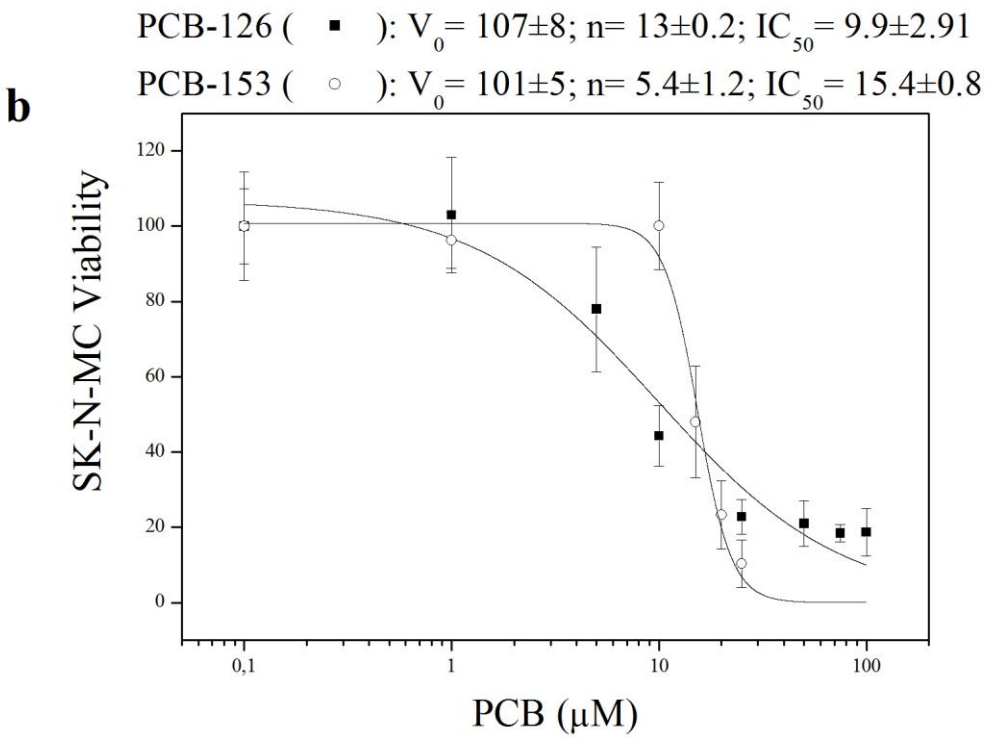
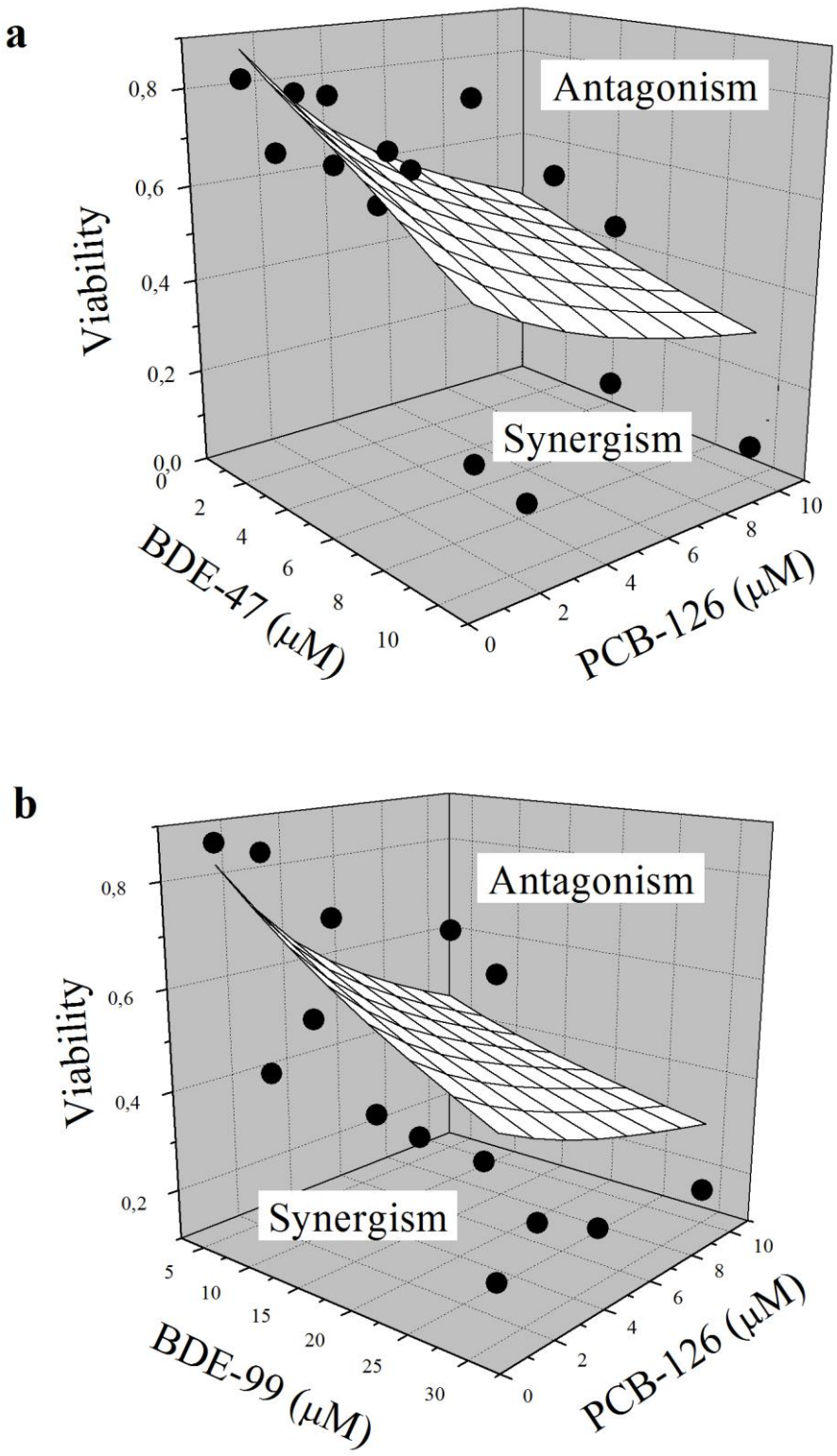
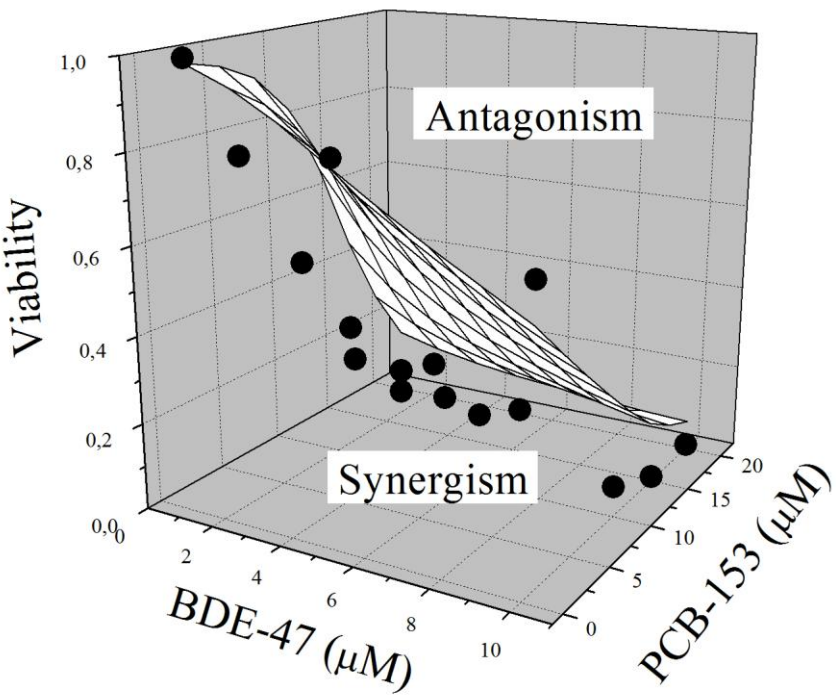


Figure 6


a
Antagonism
Synergism
Viability
0,8
0,6
0,4
0,2
0,0
0
2
4
6
8
10
BDE-47 (μM)
0
2
4
6
8
10
PCB-126 (μM)
b
Antagonism
Synergism
Viability
0,8
0,6
0,4
0,2
5
10
15
20
25
30
BDE-99 (μM)
0
2
4
6
8
10
PCB-126 (μM)

c


Antagonism
Synergism
Viability
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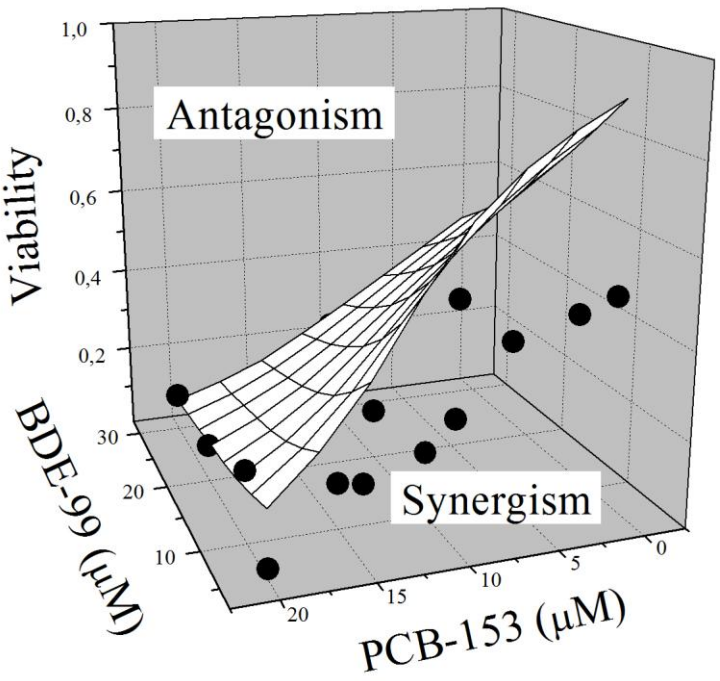
0,8
0,6
0,4
0,2
0,0
0
2
4
6
8
10
BDE-47 (μM)
0
5
10
15
20
PCB-153 (μM)

d


Antagonism
Synergism
Viability
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0,8
0,6
0,4
0,2
30
20
10
BDE-99 (μM)
20
15
10
5
0
PCB-153 (μM)

Legend to Figures

Fig. 5 Concentration–response curves reporting variation in SK-N-MC cell viability after 24 h exposure to BDE-47 and BDE-99 (a), PCB-126 and PCB-153 (b). Cell viability values are normalized to controls. The parameters of the fitting are also reported. Data represent the mean (± SD) of two separate experiments, each carried out in 8 replicates.

Fig. 6 The comparison between experimental data points on cell viability and the non-interaction surface calculated by the Loewe additivity model. The white 3-D-surfaces represent the non-interaction curves calculated by model equation. Black points represent the experimental viability values obtained for different combinations of concentrations of BDE-47 and PCB-126 (a), BDE-99 and PCB-126 (b), BDE-47 and PCB-153 (c), and BDE-99 and PCB-153 (d). The area over non–interaction surfaces implies antagonism, while the area under them implies synergism. Finally, the rotation of the surface in figure 6a-d depends on the better visualization of experimental data points.

Table 2. Values of BMD, BMDL10 NOAEL AND LOAEL for BDE-47, BDE-99, PCB-126, PCB-153 (μM)

	BMD	**BMDL10**	**NOAEL**	**LOAEL**
BDE-47	3.50	2.87	1	5
BDE-99	9.18	7.53	5	10
PCB-126	1.59	1.11	1	5
PCB-153	10.94	10.17	10	15

Table 3. Interaction between BDE-47 and PCB-126

BDE-47 (μM)	**PCB-126 (μM)**	**$V_{th,\ Loewe}$**	**Range $_{th,\ Loewe}$**		**$V_{experimental}$**	**$SD_{experimental}$**	**Interaction**
1	1	0.88	1.01	0.72	0.82	0.07	Additive
1	2.5	0.76	0.92	0.61	0.78	0.09	Additive
1	5	0.63	0.78	0.50	0.51	0.04	Additive
1	10	0.48	0.59	0.34	0.43	0.04	Additive
2.5	1	0.82	0.96	0.68	0.69	0.04	Additive
2.5	2.5	0.71	0.86	0.57	0.79	0.08	Additive
2.5	5	0.59	0.73	0.47	0.61	0.05	Additive
2.5	10	0.45	0.55	0.32	0.54	0.05	Additive
5	1	0.73	0.87	0.61	0.70	0.08	Additive
5	2.5	0,63	0.76	0.51	0.71	0.06	Additive
5	5	**0.53****	0.64	0.43	0.79	0.13	Antagonistic
5	10	0.40	0.51	0.28	0.46	0.05	Additive
10	1	**0.53****	0.62	0.45	0.24	0.05	Synergic
10	2.5	**0.47****	0.56	0.37	0.13	0.06	Synergic
10	5	**0.40***	0.49	0.30	0.31	0.07	Synergic
10	10	**0.32***	0.42	0.21	0.07	0.02	Synergic

Comparison between experimental and theoretical non-interaction values calculated by the Loewe models in co-exposure experiments with BDE-47/PCB-126 mixture. $V_{th,\ Loewe}$ are values expected by the Loewe additivity model, while the ranges of expected values is indicated as V_{th} values. Data presented are the mean (± SD) of two separate determinations (4 replicates for each experiment). (**$p < 0.01$, *$p < 0.05$).

Table 4. Interaction between BDE-99 and PCB-126

BDE-99 (µM)	PCB-126 (µM)	$V_{th,\ Loewe}$	$Range_{th,\ Loewe}$		$V_{experimental}$	$SD_{experimental}$	Interaction
5	1	0.83	0.96	0.69	0.87	0.14	Additive
5	2.5	**0.72***	0.87	0.58	0.84	0.06	Antagonistic
5	5	**0.60***	0.74	0.48	0.69	0.03	Synergic
5	10	**0.46****	0.56	0.33	0.61	0.04	Antagonistic
10	1	**0.74****	0.87	0.62	0.46	0.03	Synergic
10	2.5	**0.65****	0.78	0.53	0.54	0.04	Synergic
10	5	0.55	0.68	0.45	0.52	0.04	Additive
10	10	**0.42***	0.52	0.30	0.53	0.06	Antagonistic
20	1	**0.59****	0.69	0.50	0.44	0.04	Synergic
20	2.5	**0.53****	0.63	0.45	0.37	0.03	Synergic
20	5	**0.46****	0.54	0.35	0.27	0.03	Synergic
20	10	**0.36***	0.46	0.25	0.27	0.03	Synergic
30	1	**0.47****	0.50	0.44	0.21	0.03	Synergic
30	2.5	**0.43****	0.47	0.38	0.28	0.02	Synergic
30	5	**0.38****	0.43	0.31	0.21	0.02	Synergic
30	10	**0.31****	0.37	0.23	0.17	0.02	Synergic

Comparison between experimental and theoretical non-interaction values calculated by the Loewe models in co-exposure experiments with BDE-99/PCB-126 mixture. $V_{th,\ Loewe}$ are values expected by the Loewe additivity model, while the ranges of expected values is indicated as V_{th} values. Data are the mean (± SD) of two separate determinations (4 replicates for each experiment). (**$p < 0.01$, *$p < 0.05$).

Table 5. Interaction between BDE-47 and PCB-153

BDE-47 (µM)	PCB-153 (µM)	$V_{th, Loewe}$	$Range_{th, Loewe}$		$V_{experimental}$	$SD_{experimental}$	Interaction
1	1	0.98	1.05	0.92	0.99	0.13	Additive
1	10	**0.79****	0.90	0.68	0.46	0.09	Synergic
1	15	**0.43****	0.52	0.34	0.15	0.05	Synergic
1	20	**0.16****	0.27	0.09	0.05	0.03	Synergic
2.5	1	**0.93***	1.05	0.84	0.80	0.11	Synergic
2.5	10	**0.63****	0.73	0.55	0.32	0.07	Synergic
2.5	15	**0.33****	0.43	0.24	0.09	0.05	Synergic
2.5	20	**0.13****	0.23	0.07	0.00	0.05	Synergic
5	1	0.80	0.87	0.71	0.82	0.07	Additive
5	10	**0.45****	0.52	0.37	0.27	0.07	Synergic
5	15	**0.24****	0.32	0.16	0.07	0.04	Synergic
5	20	**0.10****	0.19	0.05	0.01	0.03	Synergic
10	1	**0.55***	0.62	0.49	0.64	0.07	Antagonistic
10	10	**0.27****	0.35	0.20	0.07	0.05	Synergic
10	15	**0.14****	0.23	0.09	0.01	0.03	Synergic
10	20	**0.07****	0.14	0.04	0.01	0.01	Synergic

Comparison between experimental and theoretical non-interaction values calculated by the Loewe models in co-exposure experiments with BDE-47/PCB-153 mixture. $V_{th, Loewe}$ are values expected by the Loewe additivity model, while the ranges of expected values is indicated as Vth values. Data are the mean (± SD) of two separate determinations (4 replicates for each experiment). (**$p < 0.01$, *$p < 0.05$).

Table 6. Interaction between BDE-99 and PCB-153

BDE-99 (µM)	PCB-153 (µM)	$V_{th, Loewe}$	$Range_{th, Loewe}$		$V_{experimental}$	$SD_{experimental}$	Interaction
5	1	**0.92****	0.99	0.85	0.51	0.03	Synergic
5	10	**0.66****	0.76	0.58	0.29	0.03	Synergic
5	15	**0.37****	0.46	0.28	0.18	0.04	Synergic
5	20	**0.16****	0.25	0.08	0.03	0.03	Synergic
10	1	**0.81****	0.89	0.74	0.42	0.03	Synergic
10	10	**0.52****	0.59	0.45	0.15	0.04	Synergic
10	15	**0.29****	0.37	0.21	0.11	0.03	Synergic
10	20	0.13	0.21	0.07	0.17	0.02	Additive
20	1	**0.62****	0.69	0.56	0.25	0.05	Synergic
20	10	**0.35****	0.42	0.28	0.12	0.06	Synergic
20	15	**0.20***	0.28	0.14	0.14	0.03	Synergic
20	20	0.10	0.17	0.05	0.09	0.02	Additive
30	1	**0.48****	0.53	0.44	0.26	0.04	Synergic
30	10	0.26	0.32	0.20	0.23	0.03	Additive
30	15	0.15	0.22	0.10	0.10	0.02	Additive
30	20	0.08	0.14	0.04	0.09	0.02	Additive

Comparison between experimental and theoretical non-interaction values calculated by the Loewe models in co-exposure experiments with BDE-99/PCB-153 mixture. $V_{th, Loewe}$ are values expected by the Loewe additivity model, while the ranges of expected values is indicated as V_{th} values. Data are the mean (± SD) of two separate determinations (4 replicates for each experiment). (**$p < 0.01$, *$p < 0.05$).

7.3. Role of glutamate in tetrabrominated diphenyl ether (BDE-47) neurotoxicity

7.3.1. Results

The potential involvement of glutamate receptors in BDE-47 toxicity was tested by co-treating CGNs with a NMDA receptor antagonist (MK-801, 5-10-25-50 μM), an AMPA/K receptor antagonist (NBQX, 5-10-25-50 μM), a Group I metabotropic receptor antagonist (AIDA, 100-250-500 μM), a Group II metabotropic receptor antagonist (LY341495, 10-50-100 nM), or a Group III metabotropic receptor antagonist (MSOP, 100-250-500 μM), together with BDE-47 (5 μM). Viability decrease was the measured end-point. As shown in Fig. 7, both NMDA and AMPA/K receptor antagonists showed a protective effect against mortality induced by BDE-47. Figure 8 shows the effect of metabotropic receptors (Group I-II-II) antagonists on viability of CGNs exposed to BDE-47; no protection was observed, suggesting that glutamate ionotropic, but not metabotropic receptors may be involved in BDE-47 toxicity.

The second step was to evaluate whether BDE-47 may cause a potential increase in glutamate extracellular concentration sufficient to activate its receptors. Time-course experiments were performed and glutamate levels in supernatants of cells exposed to BDE-47 was evaluated (data not shown). As shown in Fig. 9, the levels of glutamate in the extracellular space increased after BDE-47 exposure, with a significant difference as compared to controls after 30 minutes of exposure. NMDA and AMPA/K receptor antagonists (both at 10 μM) did not affect the action of BDE-47. These results indicate that BDE-47 5 μM increases glutamate levels outside the cells, which may in turn activate ionotropic receptors. Indeed, glutamate levels did not change when receptors were blocked by antagonists, suggesting that the increase of glutamate concentration was not dependent on the activation of receptors, but may be an upstream event.

Several evidences about toxic mechanisms of PBDEs are available, and their ability to induce oxidative stress is confirmed by several studies (Costa et al., 2008). In order to investigate whether ionotropic receptors were involved in the induction of oxidative stress by BDE-47, we blocked glutamate receptors, and ROS production and TBARS levels were measured. Interestingly, both oxidative stress biomarkers resulted significantly inhibited by the co-exposure to both NMDA and AMPA/K receptors antagonists (10 μM) and BDE-47. After 1 hour of exposure to BDE-47, levels of reactive oxygen species (ROS) in CGNs were about 3-

fold as compared to controls. When cells were co-treated with MK-801 (10 μM) and NBQX (10 μM), ROS levels were in both cases not significantly higher that controls, indicating that the activation of receptors was involved in inducing oxidative stress (Fig. 10). Lipid peroxidation, measured by means of TBARS, was also evaluated, and similar results were obtained after 12 hours of exposure with BDE-47 without and with ionotropic glutamate receptor antagonists (Fig. 10).

In order to assess whether calcium was involved in BDE-47 toxicity and whether activation of ionotropic glutamate receptors, especially NMDA receptors, has a role in potential intracellular calcium changes, experiments of co-treatment with BAPTA-AM (5 μM), and BDE-47 5 μM were performed. We found that BAPTA-AM was able to protect CGNs against BDE-47 toxicity (Fig. 11a), but did not affect BDE-47 induced increase in extracellular glutamate (Fig. 11b). However, BAPTA-AM inhibited oxidative stress induction by BDE-47 (Fig. 11c). Together, these data suggest that also calcium is involved in BDE-47 toxicity, though the temporal and functional relationship with glutamate ionotropic receptors is still unknown.

We also measured intracellular calcium levels after BDE-47 exposure over time. As shown in Fig.12, BDE-47 was able to induce an increase in calcium levels within a few minutes, followed by a slow decrease. The increase of calcium levels appeared to be modulated by both glutamate ionotropic receptors. In particular, calcium levels observed after exposure to BDE-47 were 2 folds higher than that observed with NMDA receptor blocked, and more than 2 folds higher than what observed when the AMPA/K receptor was inactive. However, data about intracellular Ca^{2+} level changes are still too preliminary and should be confirmed in further experiments. Moreover, mechanisms by which Ca^{2+} increases should be further investigated. Calcium may enter the cell either through ionotropic receptors (especially NMDA receptors) or through calcium sensitive channels on plasma membrane, or Ca^{2+} may be released from stores on ER or mitochondria.

7.3.2. Discussion

This final study investigated the potential role of glutamate in BDE-47 toxicity in primary CGNs from 6/8-days-old mice. Glutamate is considered to be the major mediator of excitatory signals in the mammalian central nervous system and is involved in most normal brain functions, and as it is indispensable, it may also be highly toxic. Thus, it follows that it should

be kept at the right concentration in the right place at the right time. High levels of glutamate may overactivate its receptors, causing for example, excitotoxic neuronal death. In the present study, we exposed CGNs to specific antagonists of the main subtypes of both glutamate ionotropic and metabotropic receptors and to BDE-47 (5 µM). While antagonists of ionotropic glutamate receptors (NMDA and AMPA/K) protected neurons against BDE-47 toxicity, antagonists of metabotropic glutamate receptors did not.

These results suggested the involvement of glutamate ionotropic receptors in BDE-47 toxicity. Previously, Reistad et al. (2006) showed that in CGNs cultures, cell death induced by DE-71, a pentaBDE mixture, was significantly decreased (45%) by MK-801 (3 µM). Moreover, it has been demonstrated that other brominated flame retardants, such as tetrabromobisphenol A (Reistad et al., 2007), or other neurotoxicants, such as polychlorinated biphenyls (PCBs) (Mariussen et al., 2002; Ndountse and Chan, 2009) or methylmercury (Ndountse and Chan, 2008) or domoic acid (DA) (Giordano et al., 2007) may exert their toxic effects on neuronal cells by activating NMDA or AMPA/K receptors. In particular, Reistad et al. (2007) have demonstrated that MK-801 reduced viability of CGNs by 86%. A NMDA receptor antagonist (3 µM) partly inhibited Aroclor 1254-induced (A1254) cell death, and showed a significant protective effect on viability of neurons exposed to PCB-153 (Mariussen et al., 2002). In the same study, the authors tested also the AMPA/K receptor antagonist (NBQX at 10 µM), that described a neuroprotective effect against A1254-induced cell death, but it was less potent than MK-801 effect. The involvement of NMDA receptor antagonist was described also by Ndountse et Chan (2009). They investigated the effect of PCB-126, PCB-99 or A1254 on viability of SH-SY5Y neuroblastoma cells. Results show that the two PCB congeners and A1254 increased NMDA receptors expression on neurons, and that treatment with MK-801 (10 µM) caused a significant protection against PCB-mediated neurotoxicity (LDH release). Also the toxicity of domoic acid, which is an agonist of AMPA/K receptor, on CGNs was antagonized by NBQX and by MK-801.

Since NMDA and AMPA/K receptors appear to have a role in BDE-47 neurotoxicity and are activated by glutamate, we investigated whether BDE-47 could cause an increase in extracellular concentration of glutamate, sufficient to activate receptors. Results (Fig. 9) showed that BDE-47 was able to induce an increase of the extracellular concentration of glutamate, with a peak after 30 minutes, but NMDA and AMPA/K receptors antagonists did not interfere in this effect. This finding clearly suggests that the increase in glutamate in the extracellular space precedes the activation of ionotropic receptors. It is therefore possible that BDE-47 enhances extracellular glutamate levels by still unknown mechanisms, leading to

activation of NMDA and AMPA/K receptors which initiate a cellular cascade of events, including disruption of calcium homeostasis and induction of oxidative stress, ultimately resulting in cell death. Indeed both ionotropic receptor antagonists blocked BDE-47 induced oxidative stress, measured by ROS production and lipid peroxidation levels (Fig. 10).

Measurement of the effect of BDE-47 exposure on intracellular Ca^{2+} levels, shown in Fig. 12, revealed that BDE-47 impacts the normal homeostasis of calcium, though the potential underlying mechanisms have not been investigated. Enhancing in calcium concentration inside the cells may be related to glutamate receptors activation. In fact, Ca^{2+} may rise by the direct influx through NMDA receptors, through calcium permeable AMPA receptors or indirectly via depolarization induced opening of voltage-sensitive calcium channels (VSCCs) (Hilton et al., 2006). Recently, Reistad et al. (2007) demonstrated that the brominated flame retardant TBBPA was capable to induce calcium influx, elevation in extracellular glutamate, ROS formation, and cell death in CGNs. They found that MK-801 was able to block cell death, which is consistent with our findings, but had not significant effect on ROS formation. The authors suggested that the disturbance of calcium homeostasis and the activation of glutamate receptors may contribute to oxidative stress induction. The potential relationship between cytotoxicity, NMDA receptor activation, impairment of calcium homeostasis and ROS production was also observed by Mariussen et al. (2002), when mechanisms of toxicity of a PCB mixture in rat cerebellar granule neurons were investigated. The major conclusion was that cell death and ROS production were mainly mediated by the activation of NMDA receptor.

Although we demonstrated the perturbation of calcium homeostasis by BDE-47, as described by the increase of intracellular calcium (Fig. 12), and by the effect of treatment with the calcium chelator BAPTA-AM (Fig 11a, 11c), the sequence of events linking glutamate receptors activation, changes in calcium homeostasis, and induction of oxidative stress, is still elusive.

Taken together these results suggest that glutamate has an important role in BDE-47 toxicity. Further investigations are needed to elucidate the interaction of BDE-47 with glutamate and glutamate receptors.

7.3.3. Figures and Tables

Figure 7

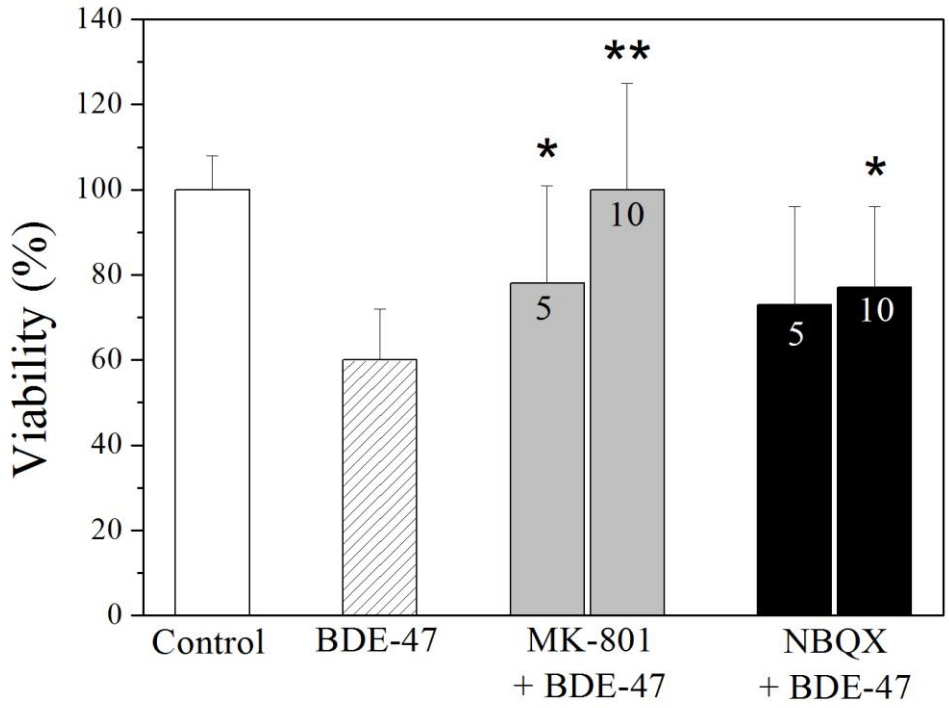
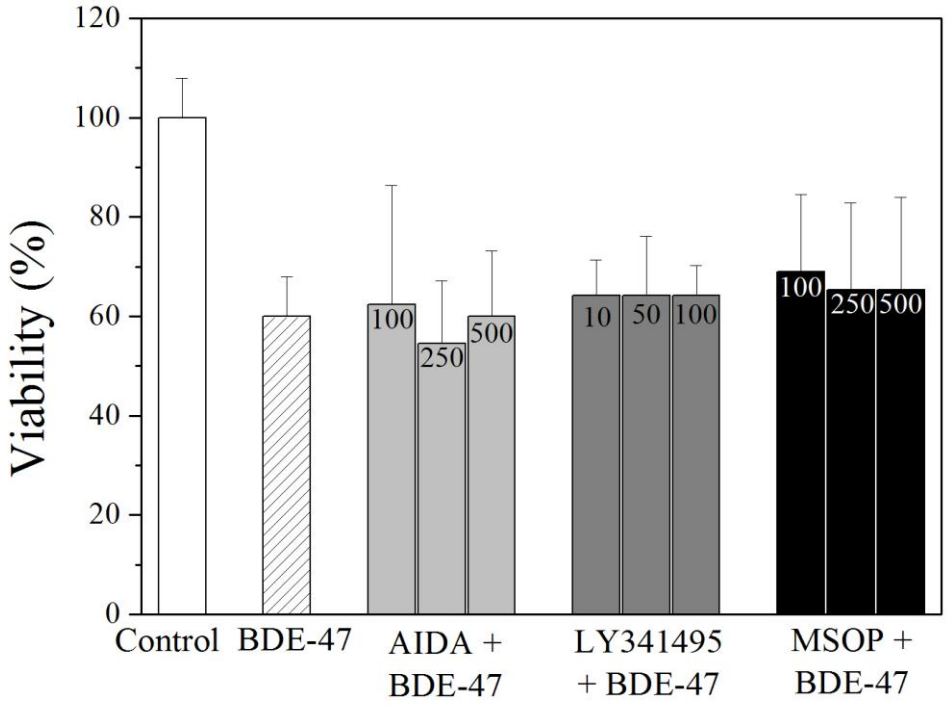


Figure 8


Viability (%)
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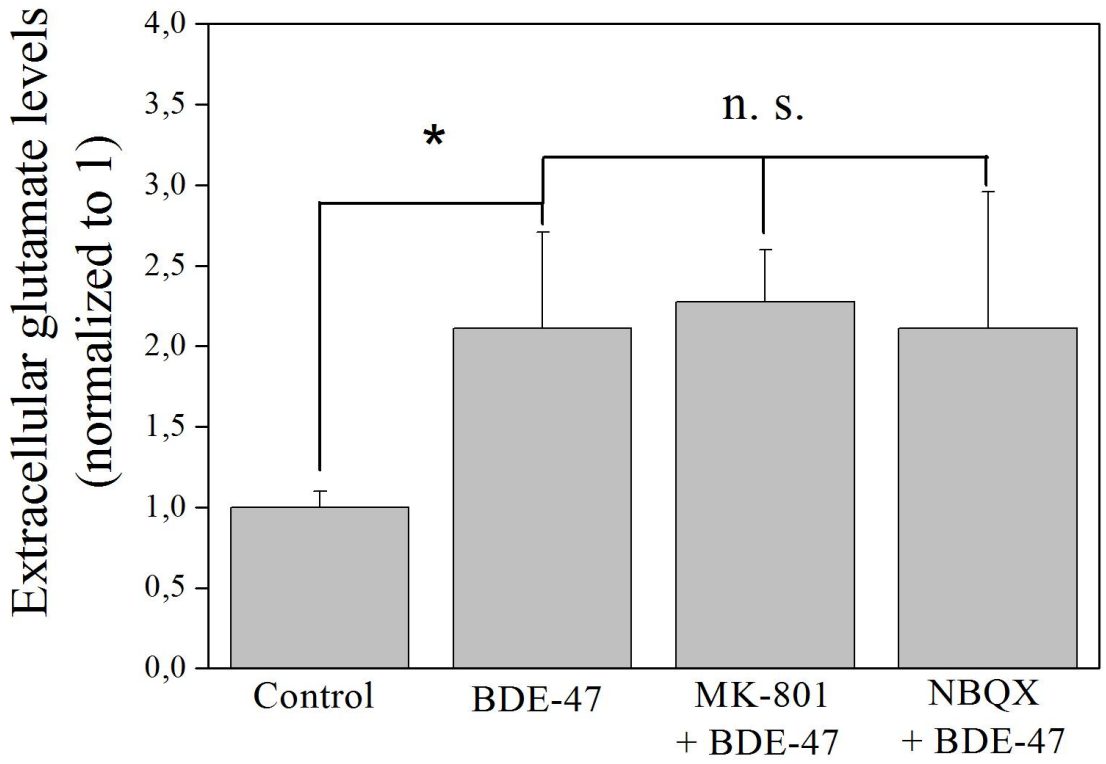
100
80
60
40
20
0
100
250
500
10
50
100
100
250
500
Control
BDE-47
AIDA + BDE-47
LY341495 + BDE-47
MSOP + BDE-47

Figure 9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3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
*
n. s.
Control
BDE-47
MK-801
+ BDE-47
NBQX
+ BDE-47

Figure 10

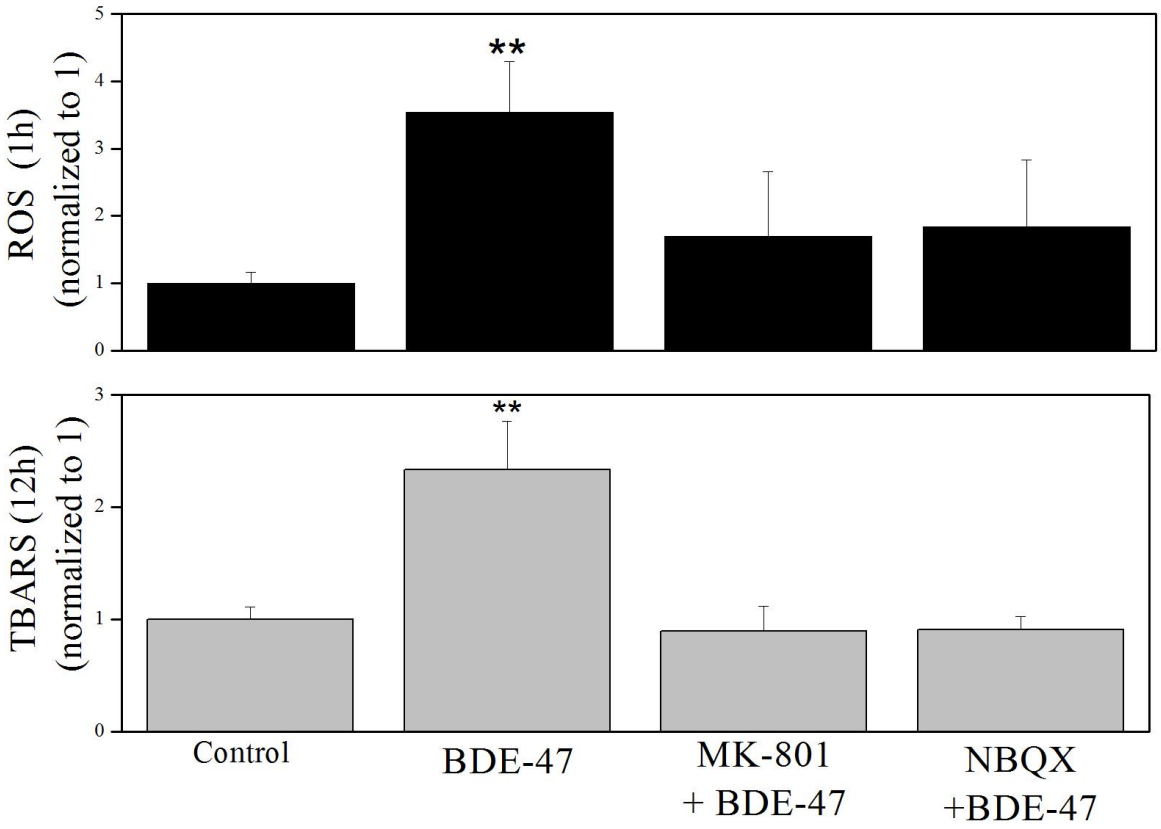
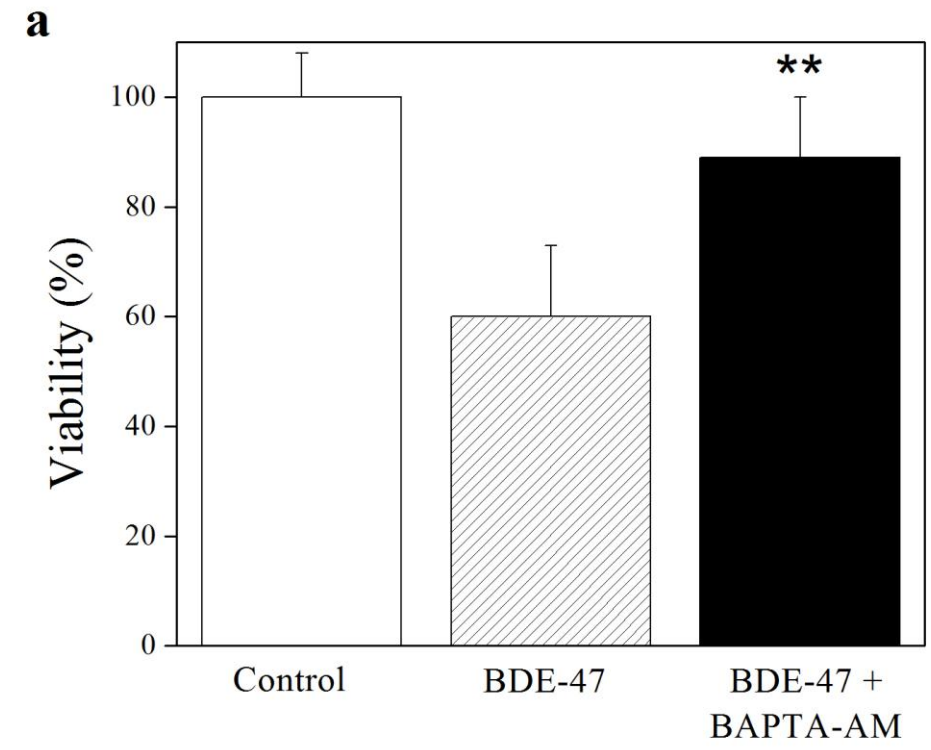
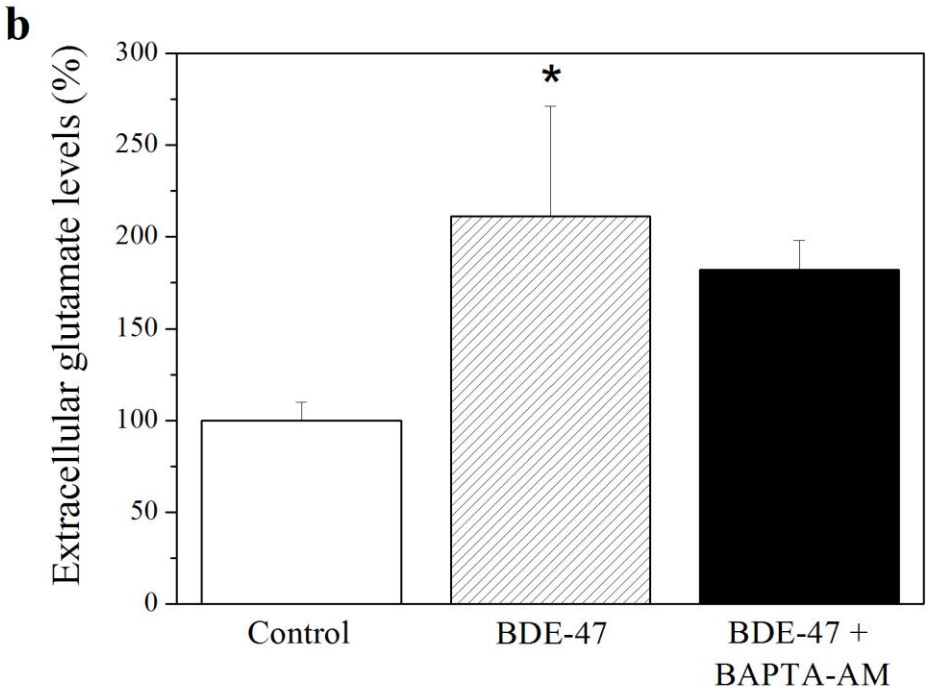

ROS (1h)
(normalized to 1)
TBARS (12h)
(normalized to 1)
**
**
0
1
2
3
4
5
Control
BDE-47
MK-801
+ BDE-47
NBQX
+BDE-47

Figure 11


a
Viability (%)
100
80
60
40
20
0
**
Control
BDE-47
BDE-47 +
BAPTA-AM


b
Extracellular glutamate levels (%)
300
250
200
150
100
50
0
*
Control
BDE-47
BDE-47 +
BAPTA-AM

c

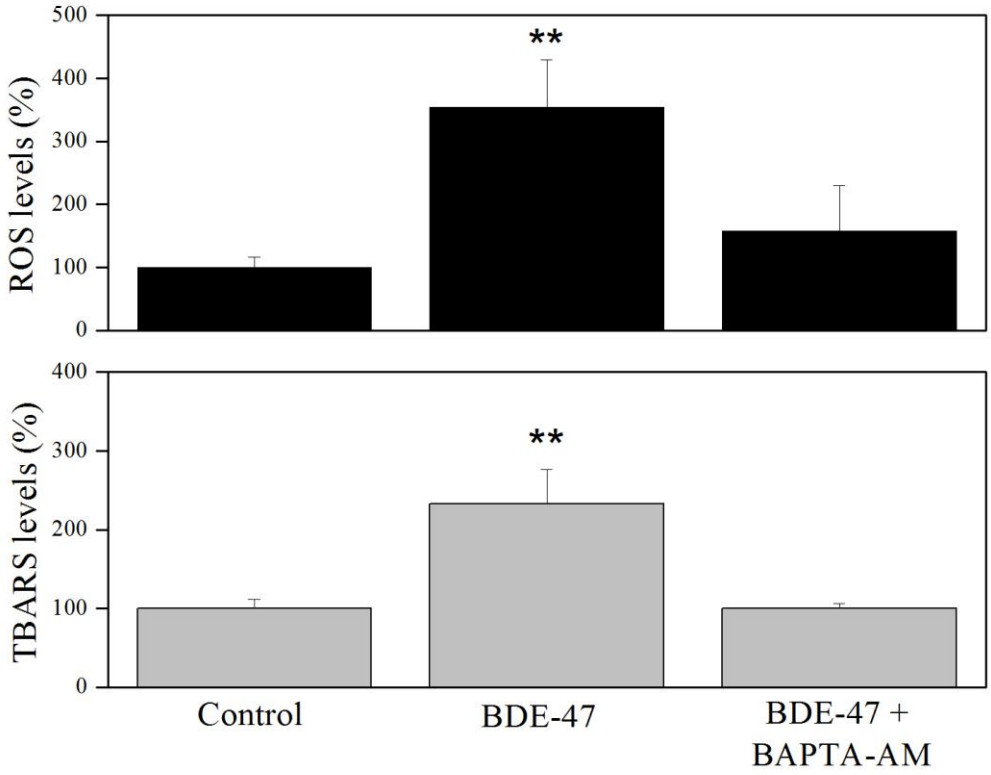
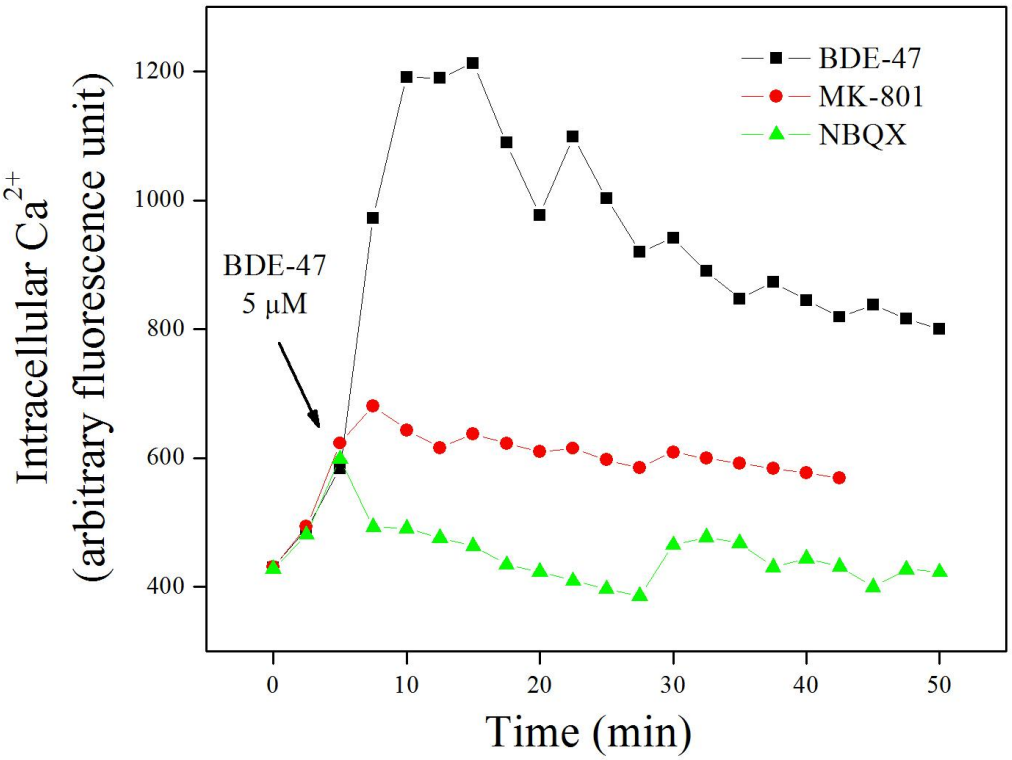

ROS levels (%)
500
400
300
200
100
0
**
TBARS levels (%)
400
300
200
100
0
**
Control
BDE-47
BDE-47 +
BAPTA-AM

Figure 12


Intracellular Ca^{2+}
(arbitrary fluorescence unit)
1200
1000
800
600
400
0
10
20
30
40
50
Time (min)
BDE-47
5 μM
BDE-47
MK-801
NBQX

Legend to Figures

Fig. 7 Variation in CGNs cell viability after 24 h exposure to BDE-47 5 µM and 5-10 µM of NMDA receptor antagonist (MK-801), and BDE-47 5 µM and 5-10 µM of AMPA/K receptor antagonist (NBQX). Cell viability values are normalized to controls. Control=Untreated cells; BDE-47=Cells exposed to BDE-47 5 µM alone; BDE-47+MK-801= Cells exposed to BDE-47 5 µM and 5-10 µM of MK-801; BDE-47+NBQX= Cells exposed to BDE-47 5 µM and 5-10 µM of NBQX. *=$p<0.05$ as compared to BDE-47; **=$p<0.01$ as compared to BDE-47. Data are reported as mean (±SD) of at least three separate experiments.

Fig. 8 Variation in CGNs cell viability after 24 h exposure to BDE-47 5 µM and several concentrations (100-250-500 µM) of Group I metabotropic receptor antagonist (AIDA), BDE-47 5 µM and several concentrations (10-50-100 nM) of Group II metabotropic receptor antagonist (LY341495), and BDE-47 5 µM and several concentrations (100-250-500 µM) of Group III metabotropic receptor antagonist (MSOP). Control=Untreated cells; BDE-47=Cells exposed to BDE-47 5 µM alone; BDE-47+AIDA= Cells exposed to BDE-47 5 µM and 100-250-500 µM of AIDA; BDE-47+ LY341495= Cells exposed to BDE-47 5 µM and 10-50-100 nM of LY341495; BDE-47+MSOP= Cells exposed to BDE-47 5 µM and 100-250-500 µM of MSOP.
Data are reported as mean (± SD) of at least three separate experiments.

Fig. 9 The figure shows the significant glutamate release induced by BDE-47 5 µM compared to control after 30 minutes of exposure. Moreover, co-treatment with glutamate ionotropic receptors (MK-801 and NBQX, both 10 µM) does not impact extracellular glutamate levels, at the same time-point. Glutamate levels values were normalized to controls, and data are presented as the mean (± SD) of three separate experiments.

Fig. 10 Reactive Oxygen Species (ROS) levels and lipid peroxidation (TBARS) upon exposure to BDE-47 (5 µM) and BDE-47 and MK-801 or NBQX (both at 10 µM) . ROS were measured 1 h after treatment, as described in Materials and Methods, while TBARS after 12 h. Results represent the mean (± SD) of three separate experiments (**$p < 0.01$, significantly different from controls).

Fig. 11 Cell viability (a), glutamate release (b) and oxidative stress (c) (ROS and TBARS) changes by co-treatment of CGNs with BDE 5 µM and BAPTA-AM 5 µM, compared to controls. Control=Untreated cells; BDE-47=Cells exposed to BDE-47 5 µM alone; BDE-47+BAPTA-AM= Cells exposed to BDE-47 5 µM and 5 µM of BAPTA-AM. Results are the mean (± SD) of three experiments in the case of cell viability and oxidative stress, the mean (± SD) of two separate assays for glutamate release. (a) ** =$p < 0.01$ as compared to BDE-47; (b) * =$p<0.05$ as compared to controls; (c) ** =$p<0.01$ as compared to controls.

Fig. 12 Very preliminary data about intracellular calcium levels changes after BDE-47 µM solely, and in presence of ionotropic receptors antagonists, MK-801 and NBQX (10 µM), and in presence of calcium chelator, BAPTA-AM (5 µM) and EGTA, over time (minutes). No statistic analysis has been performed, further experiments should be done.

8. Final conclusions

In conclusion, the studies presented in my thesis confirm the validity of *in vitro* models as an alternative to an *in vivo* approaches to assess the toxicity of neurotoxic substances, such as polybrominated diphenyl ethers.

Initially, the human neuroblastoma cell line (SK-N-MC) was used to assess the potential interaction between BDE-47 and BDE-99, two of the most abundant PBDE congeners in the environment and in biota. The study shows that the interactions between BDE-47 and BDE-99 varied along the concentrations of both PBDEs, and could be either synergistic or antagonistic. The Loewe additivity model and the Bliss independence criterion were used to analyze viability data, obtained by combined exposure experiments. Not only cell viability was considered as possible end-point, but also oxidative stress. The fact that a simultaneous co-exposure to BDE-47 and BDE-99 could induce synergistic neurotoxic effects, in particular at low concentrations of BDE-47, is of particular interest from a toxicological point of view. Indeed, humans are exposed to mixtures of PBDEs, most notably low levels of tetra- and penta-BDEs, such as BDE-47 and BDE-99.

As a second step, potential interactions between two different classes of environmental contaminants, PBDEs and PCBs, was assessed, by using the same cellular model. Also in this case the *in vitro* approach has proven to be suitable, and interesting results were obtained. In particular, the combined exposure to low concentrations of BDE-47 or BDE-99 and a wider range of concentrations of a non-dioxin like PCB congener, PCB-153, gave rise to an enhanced alterations in cell status. Mixtures of two PBDEs and PCB-126, a dioxin-like PCB, did not show the same synergism, if not at high concentrations of PBDEs. The synergism observed between low concentrations of BDE-47 or BDE-99 and a wider range of concentrations of PCB-153 is very interesting from a toxicological point of view, because these compounds are widespread in the environment and in biota, at relatively low concentrations, and humans may be co-exposed to them. Further studies are in progress to measure the intracellular content of PCBs and PBDEs in single and combined exposures, and to better characterize other cellular end-points. Whereas the nature of interaction of PBDEs and PCB-153 seems to be well defined, PBDEs and PCB-126 interact in a more complex manner. This suggests that from a toxicological point of view it is important to take into account that different congeners among PCBs may have a different potency in the presence of others toxicants.

Finally, the third part of the thesis investigated a potential mechanism underlying the cytotoxicity and neuronal death induced by BDE-47. Cerebellar granule neurons from 7-days old mice were isolated and cultured. Preliminary results show that glutamate has an important role in BDE-47 toxicity. A pharmacological approach by using antagonists of glutamate receptors demonstrated that ionotropic receptors (NMDA and AMPA/K), rather than the metabotropic ones, are involved in the mechanism of BDE-47 neurotoxicity. In particular, it is probable that BDE-47, by means of still not known molecular mechanisms, is able to cause an increase in extracellular glutamate levels, which in turns activates ionotropic receptors and induces oxidative stress and the disruption of calcium homeostasis, events that finally determine neuronal death. It remains unclear the exact relationship between increasing extracellular glutamate and increasing intracellular calcium, but both events have a key role in neurotoxic mechanism of BDE-47. Further studies are necessary to confirm these findings, and also to understand how BDE-47 can cause the increase of glutamate in the extracellular fluid. It should be clarified whether BDE-47 causes the release of glutamate or blocks its uptake, interfering with glutamate transporter or its vescicular release.

In summary, the studies carried out during my PhD research project highlight that PBDEs are environmental and food contaminants able to cause neuronal toxicity, by a mechanism which involves oxidative stress. Glutamate and the activation of its ionotropic receptors have a role in inducing toxicity, as well as the disruption of calcium homeostasis. Humans may be co-exposed to different congeners of PBDEs, and different food contaminants (such as PCBs), and these compounds can interact modifying the potency of single compounds. This suggest that the study of interactions, supported by mathematical models, is an important issue that should be considered in risk assessment.

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10. Appendix

Results from my research project on PBDE neurotoxicity is being published (see below). In addition to my main field of study, during the PhD program I was also involved in other activities. Below, you can find the list of publications on international journals, the participation to scientific meetings, and other activities carried out during the program.

List of scientific publications:

- C. Pellacani*, **S. Tagliaferri***, A. Caglieri, M. Goldoni, G. Giordano, A. Mutti, L. G. Costa. * These authors contributed equally to this work. "Synergistic toxicity between PBDEs and PCBs in human neuroblastoma cells". Submitted.

- Contribution in drafting the report "Scientific information on mycotoxins and natural plant toxicants", EFSA (CFP/EFSA/CONTAM/2008/01).

- Verzelloni E., Pellacani C., Tagliazucchi D., **Tagliaferri S.**, Calani L., Costa L.G., Brighenti F., Borges G., Crozier A., Conte A. and Del Rio D. "Antiglycative and neuroprotective activity of colon-derived polyphenol catabolites". Mol Nutr Food Res. 2011 Jan; in press.

- Goldoni M. and **Tagliaferri S.**, "Dose-response or dose-effect curves in *in vitro* experiments and their use to study combined effect of neurotoxicants: a practical approach.". In "Neurotoxicology in Vitro: Methods and Protocols", (Costa L.G., Giordano G., Guizzetti M.). In Press, 2011 Springer/Humana.

- **Tagliaferri S.**, Caglieri A., Goldoni M., Pinelli S., Alinovi R., Poli D., Pellacani C., Giordano G., Mutti A. and Costa LG. "Low concentrations of the brominated flame retardants BDE-47 and BDE-99 induce synergistic oxidative stress-mediated neurotoxicity in human neuroblastoma cells", Toxicol in Vitro 2010 Feb;24(1):116-122.

- La Monica S., Galetti M., Alfieri RR., Cavazzoni A., Ardizzoni A., Tiseo M., Capelletti M., Goldoni M., **Tagliaferri S.**, Mutti A., Fumarola C., Bonelli M., Generali D., Petronini PG. "Everolimus restores gefitinib sensitivity in

resistant non-small cell lung cancer cells lines", Biochem Pharmacol. 2009 Sept;78(5):460-468.

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- Caglieri A, Goldoni M, De Palma G, Mozzoni P, Gemma S, Vichi S, Testai E, Panico F, Corradi M, **Tagliaferri S**, Costa LG. "Exposure to low levels of hexavalent cromium: target doses and comparative effects on two human pulmonary cell lines", Acta Biomed. 2008;79 Suppl 1:104-15.

Participation (with oral presentation or poster) to congresses:

- **Tagliaferri S.**, Giordano G., Goldoni M., Costa L.G., Mutti A. "Ritardanti di fiamma: un rischio emergente per la tossicologia ambientale". Convegno Nazionale delle Scuole di Medicina del Lavoro, Taormina, ottobre 2010, 23.

- Pellacani C., **Tagliaferri S.**, Costa L. G, Caglieri A, Brighenti F, Crozier A, Del Rio D. Colon-derived phenolic catabolites protect against neuronal oxidative stress. In: 4th International Conference on Polyphenols and Health, abstract book. Harrogate International Centre, Harrogate, UK, December 7th-11th 2009.

- **S. Tagliaferri**, A. Caglieri, M. Goldoni, S. Pinelli, R. Alinovi, C. Pellacani, A. Mutti, LG. Costa. "Valutazione dell'azione combinata di BDE-99 e BDE-47 in cellule neuronali umane", XV Congresso Nazionale Società Italiana di Tossicologia, Verona, gennaio 2009, 223.

- A. Caglieri, M. Goldoni, P. Mozzoni, G. De Palma, M. Galetti, RR. Alfieri, **S. Tagliaferri**, LG. Costa, PG. Petronini, A. Mutti. "Espressione dell'eme-ossigenasi-1 in due linee cellular polmonari umane con diverso profile polimorfico per GSMT1 esposte a fumo di sigaretta", XV Congresso Nazionale Società Italiana di Tossicologia, Verona, gennaio 2009, 103.

I collaborated with Drs. Caglieri and Goldoni in the development of non invasive approaches for early diagnosis of lung diseases, and in particular, I was involved in the measure and evaluation of oxidative stress biomarkers (H_2O_2) in exhaled breath condensate.

I gave a lecture on Anticancer drugs in a Pharmacology course for Medical students, University of Parma, in 2009.

I also participated to a seminar of PhD students of Prevention Sciences program, held at the Dept. Internal Medicine, Nephrology and Prevention Sciences of University of Parma, on June 2009. The title of my presentation (in Italian) was "Utilizzo di modelli *in vitro* per lo studio delle interazioni".

At the moment, I'm involved in compiling the database "Screening of literature on bisphenol A" (NP/EFSA/CEF/2010/01).

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