Understanding Liver Toxicity

induced by Polybrominated Diphenyl Ethers

in Human Hepatocytes

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ABSTRACT

Poly Brominated Diphenyl Ethers (PBDEs) are known flame retardants with highly persistent and lipophilic in nature. The continued usage of PBDE in various products amplifies the human burden of PBDEs. It is therefore, important to study the potential toxicological and/or biological effects of PBDE exposure in human. In this study we investigated the mode of action of PBDE induced toxicity in human liver by exposing human hepatocarcinoma cells in a time (24-72h) and dose (0-100μM) dependent manner. The highest test dose caused an inhibition in cell viability up to 50% after 72h, whereas lower doses (<50μM) showed slight increase in cell viability. Likewise, higher doses caused significant accumulation of intracellular ROS over time. Further, increase in caspase-3 enzyme levels and DNA fragmentation showed that, lower brominated PBDEs induce liver toxicity through accumulation of toxic metabolites and reactive oxygen species over time leading to caspase-mediated apoptotic cell death.

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List of Abbreviations

%	Percent
°C	temperature in degree celsius
AFC	7-amino-4-trifluoromethyl coumarin
AhR	Arylhydrocarbon Receptor
ANOVA	Analysis of variance
ATM	Ataxia-telangiectasia-mutated kinase,
BCA	Bicinchoninic acid assay
BDE	Brominated di phenyl ether
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CAD	Caspase-activated DNase
CAR	Constitutive Androstane Receptor
cm	Centimetre
CO_2	Carbon-di-oxide
$CuSO_4$	Copper Sulphate Salt
Cyp1A1	Cytochrome P 450 1A1 enzyme

CYP2B	Cytochrome P 450 2B class enzymes
CYP3A4	Cytochrome P 450 3A4 enzyme
DCFH-DA	Dichlorodihydrofluorescein diacetate
DEVD	Asp-Glu-Val-Asp
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribo nucleic acid
DUOX	Dual Oxidases
EDTA	Ethylenediaminetetraacetic acid
EROD	Ethoxyresorufin 0-deethylase
FBS	Fetal Bovine Serum
FOXA3	Hepatocyte Nuclear Factor 3 γ
GSTM1	Glutathione S-transferase Mu 1
GSTs	Glutathione S-transferases
H_2O_2	Hydrogen Peroxide
H4IIE	Rat Hepatoma cell line
HBSS	Hank's Buffered Salt Solution
HCl	Hydrogen Chloride

HepG2	Human Hepatocarcinoma cell line
HRE	Hormone responsive elements
IC_{50}	Half maximal inhibitory concentration
IPA	Isopropanol
LXR	Liver X Receptor
MAPK	Mitogen-activated protein kinases
MCF-7	Human Breast Carcinoma Cell Line
MDR1	Multidrug resistance protein 1
MEM	Minimum Essential Medium
mM	Milli molar
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5- diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear factor-kappaB
ng/g	Nanograms per gram weight
nm	Nano metres
OH	Hydroxyl group
OH-BDE	Hydroxylated BDE metabolites

p53	Protein 53 or tumor protein 53
PAH	Polyaromated hydrocarbons
PARP	Poly (ADP-ribose) polymerase
PB	Phenobarbital
PBDE	Poly Brominated Diphenyl Ethers
PBS	Phospahe Buffered Saline
PCB	Polychlorinated bi phenyl
PI3K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
PLCg1	Phospholipase C-g1
POP	Persistent organic pollutants
PROD	Pentoxy-resorufin-O-deethylase
PXR	Pregnane X Receptor
ROS	Reactive oxygen species
S.E.M	Standard Error of Mean
SK-N-SH	Human neuroblastoma cell line
SOD	Superoxide dismutase

SXR	Steroid X receptor
TCDD	Tetrachlorodibenzo-p-dioxin
TE	Tris-EDTA
UDPGT/UGT	Uridine-diphosphate glucuronosyltransferase
μM	Micromolar

I. Introduction

1. General Introduction

Flame retardants are man-made chemicals used in the manufacture of a wide-range of commercial and house-hold products globally. When there is a fire, these chemicals are able to compete with the transmission of combustion process and mitigate the flames by forming radical species. Among the available range of flame retardants, brominated flame retardants (BFRs) are most preferred due to their increased thermal stability even at lower volumes. Poly Brominated Diphenyl Ethers (PBDEs) are one of the BFRs used extensively due to their greater efficiency. PBDEs are not chemically bound to polymers in order to aid the quick release and reduce flames in the event of fire. Based on the bromine content there are a total of 209 congeners of PBDEs used in commerce; the PBDEs are manufactured as mixtures of: penta-, octa-, and deca-BDE mixture (IPCS 1994). The reported global production in 2001 was 8000, 4000 and 56,000 metric tons per year for penta-, octa-, and deca-BDE mixtures, respectively (Birnbaum and Staskal 2004).

Though PBDEs have strong advantage in resisting fires, the ability of these compounds to be released out of the polymers has raised concerns globally. Because of their high volume production, these anthropogenic chemicals have become ubiquitous, contaminating soil, air, and wildlife. Eventually increased levels of these environmental contaminants have been found in human blood, adipose tissue, and breast milk (EFSA 2011). Because of concerns of environmental, ecological and human contamination by PBDE, attempts have been made to cease usage and production of penta- and octa-BDE

mixtures. However, continuing usage of the different PBDE congeners in various products and their disposal magnifies the environmental burden of PBDEs; also this environmental contamination is expected to last for decades due to their high persistence. Additionally, deca-BDE mixture which mainly contains BDE-209 has been reported to undergo microbial or UV/sun-light influenced degradation into lower-brominated congeners (Gerecke et al 2005), thereby increasing the environmental burden. It is therefore, important to study the potential toxicological and/or biological effects caused by PBDE exposure in human as well as in wild-life.

2. PBDE: Structure and Main Congeners

PBDEs are brominated aromatic compounds, and their basic structure consists of two phenyl rings linked by an ether (-o-) bond. Each phenyl ring can hold up to five bromine atoms, and there are 209 possible congeners based on the position and number of the bromine substitutes (Fig.1).

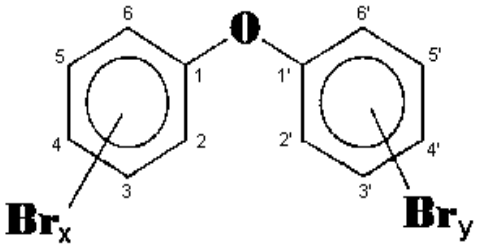


Figure 1. Basic structure of PBDEs showing various possible bromine substitutions (INCHEM) (http://www.inchem.org/documents/ehc/ehc/ehc162.htm)

PBDEs which are used as mixtures in commerce are available as PentaBDE, OctaBDE and DecaBDE mixtures. Each mixture has been named according to their bromine content, PentaBDE consists of PBDE core with 4-6 bromine atoms, whereas OctaBDE

consists PBDE core with 6-8, and DecaBDE mainly consists fully substituted PBDEs (10 bromines). The chemical stability of PBDEs differs with the number of substituted bromine atoms and structure, however in general, congeners with very few (up to three) or very high (nine or ten) number of bromine atoms are highly vulnerable to abiotic transformation and are least stable (Eriksson et al., 2004; Christiansson et al., 2009).

The production of PBDEs was about 80,000 tons annually, until their use in commerce was banned or voluntarily ceased (eg. USA in year 2003) globally (EFSA 2011). PBDEs are mainly used in polymers, textiles, construction and electrical equipment, electronics, upholstery and furniture. Because of their intensive usage PBDEs have been detected in environment (air, soil and water) as well as biota including human (Harrad et al., 2010; Sjodin et al., 2006; Vrkoslavova et al., 2010; Law et al., 2006b; Muir et al., 2006). This is also because these substances are highly lipophilic and persistent. Among various congeners detected in humans, the most common were the PentaBDE mixtures (such as Great Lakes DE-71^{TM} or Bromkal 70-5DE^{TM}); these were found at higher levels (Hites 2004). They were also the most prevalent compared to the highest produced BDE-209 (deWit 2002).

Main congeners of commercial PentaBDE mixture (DE-71) are 2,2',4,4'-TetraBDE (BDE47) and 2,2',4,4',5-pentaBDE (BDE99) (Fig. 2), and they make up to 80% of this total mixture (Sjödin *et al*. 1998; Chen *et al*. 2006a). This mixture was predominantly used as an additive in polyurethane foam, upholstery and furniture, phenolic and epoxy resins, packaging materials and textiles, which are believed to be in use still (WHO 1994, NICNAS 2001, EU 2001).

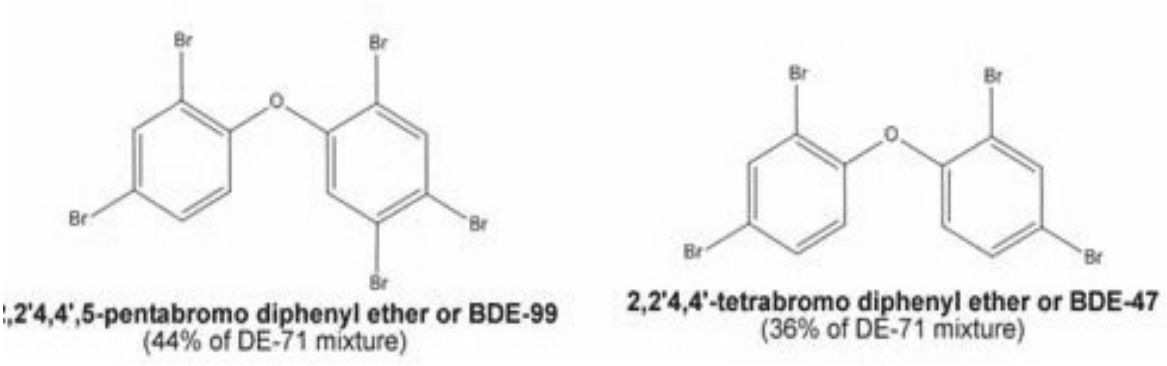


Figure 2. Typical structure of BDE-99 and BDE-47

According to the reports, the usage of PBDE in 2001, in US alone was 95% of the global production of PentaBDE. This could be one of the main reasons for significantly higher concentrations in US populations (ΣPBDEs = 35 ng/g lipid) compared to Europeans (ΣPBDEs = 2 ng/g lipid) (Hites 2004). A number of toxicological reviews have predominantly indicated that the lower brominated (four to six) PBDEs have greater potential to induce systemic or organ specific toxicity; At lower levels, it is observed, that these substances have greater bioavailability and bioaccumulation potential (EU, 2001, 2002, 2003; Darnerud *et al*., 2001; Hardy, 2002; McDonald, 2002; Health Canada, 2004).

3. Environmental exposures and tissue levels

PBDEs are released into environment through various routes and processes, such as during the manufacturing process, use in commerce, use of articles containing PBDEs, or at the time of recycling/incineration. During any of these phases PBDEs can be released into air, water and soil/landfills. OF all the environmental compartments, air has been identified as the major source of PBDE exposure. For example, in Europe up to 43 tonnes of penta-BDE was released into environment through volatilization from polyurethane foam containing articles alone (ECB, 2002). Most importantly indoor air (house dust) has been related to 80-90% human exposures in several studies and

especially toddlers are at the greater risk (Fischer et al., 2006, Thomsen et al., 2002). Another significant source of exposure arises from consumption of food of animal origin. PBDEs accumulate greatly in food chain because of their persistent and lipophilic nature,.

Among the dietary sources of exposure, fish is the major contributor followed by meat and dairy products. In Europe, consumption of seafood has been related to detection of PBDEs in human biological samples in several studies. Higher serum PBDE levels have been detected among daily consumers of fish and people consuming fatty acid supplements (e.g. omega-3) (Thomsen *et al*., 2008; Sjödin *et al*., 2000). In North America, PBDE levels are one to two orders of magnitude higher than in Europeans, and the contributing factors are house dust followed by consumption of meat and dairy products (Wu *et al*., 2007, Johnson *et al*., 2010). Recently a strong positive correlation between consumption of poultry or red-meat and serum PBDE levels has been reported in the US population (Fraser *et al*., 2009). Also children (particularly toddlers) are highly exposed to PBDEs, mainly BDE-47 and -99 via feeding (breast feeding). Several studies reporting highest levels detected in breast milk supported this observation. Exposure levels in children (<3 years) have been calculated to be 3-6 times higher than adults (EFSA 2011). Overall, PBDEs are detected in many human specimens such as blood, liver, adipose tissue, breast milk and placental tissue (Meironyté *et al*. 1999; Sjödin *et al*., 2008). BDE-47, -99, -100, -153, -154 (components of DE-71 mixture) were predominantly detected in human tissue and they account for 90% of total human body burdens due to PBDEs.

Among all PBDE congeners, the levels of lower brominated congeners (BDE-47, -99, -100) were most dominant; this could be due to their higher bioaccumulation

potential. A similar distribution pattern between BDE congeners in breast milk and their commercial mixtures has been observed (Darnerud *et al*., 2001; Law *et al*., 2006a; Lilienthal *et al*., 2006). Experimental studies in rodents treated with PBDEs such as BDE-47, -99, -100, and -153 have observed similar tissue distribution. It appears that all lower brominated congeners accumulate in lipid-rich tissues such as adipose and liver, and to a lesser extent in gastrointestinal tract, skin and adrenals (Hakk *et al*., 2002; 2006; 2009). It has been reported that BDE-99 metabolizes at a greater level among all three lower brominated congeners; and among the different metabolites are hydroxyl and phenolic derivatives (Sanders *et al*., 2006). These metabolites were also detected in wild life and human samples.

Calculations of half-life values of PBDEs indicate that congeners with 4-6 bromine atoms have higher values; and are in the range of 37-671 days (Jakobsson *et al*., 2003; Sjödin *et al*., 1999; Meyerinck *et al*., 1990). These values are very greater than the well-studied dioxins (eg. half-life for 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin in rodents was around 20 days). A similar trend (increased half-life with decreasing bromination) was observed in human serum samples collected from general population as well as occupational workers (Thuresson *et al*., 2006).

4. Adverse Health Effects

Polybrominated diphenylethers (PBDEs) are reported to cause adverse reproductive system effects (Hamers *et al*., 2006; Meerts *et al*., 2001), in thyroid (Darnerud *et al*., 2007; Ellis-Hutchings *et al*., 2006; Zhou *et al*., 2002), and in liver (Birnbaum and Cohen Hubal, 2006; Sanders *et al*., 2005; Zhou *et al*., 2002). Key

observed health effects as reported by these studies include: disruption of thyroid hormone pathways; endocrine hormone mimicking; fatty liver, change in BMI levels; neurotoxicity; and developmental defects (skeletal malformations). These adverse health effects are believed to contribute to disease development after long term exposures. The purpose of this study is to describe PBDE induced effects in liver, because of two key reasons: 1) Higher levels of PBDE are detected in liver and liver is most sensitive organ 2) long term exposures can be carcinogenic in livers. Since the liver toxicity is the primary focus of this study, only liver associated adverse health outcomes are described in the following sections.

Liver has been a target organ for accumulation of PBDEs and manifestation of the adverse health effects. Higher concentrations of total PBDEs have been reported in liver and adipose tissue, especially in children and newborns. This is mainly due to *in utero* exposure (placental transfer from mother to the baby) or neonatal exposure (through breast feeding). Young children are reported to have a higher exposure to these chemicals and/or a lower capacity to eliminate them, leading to increased burden and subsequent developmental effects (Tickner and Hoppin 2000; Flyn et al., 2000).

5. Observed health effects in humans

The ubiquitous exposure to persistent organic pollutants (POPs) including polybrominated diphenyl ethers (PBDEs) has been linked to obesity and diabetes (Sharp D 2009; Turyk M *et al*., 2009). Canadian aboriginals and Great Lakes sport fishermen both have higher rates of diabetes, which among other factors may be linked to eating POP contaminated seafood (Turyk M *et al*., 2009). In a longitudinal study conducted

between 1998-2006 in Greater Montreal, Quebec, Doucet *et al*., (2009) observed a huge increase in total PBDEs in foetal liver over time (mean ± SE: 1998, 284.4 ± 229.8 ng/g lipid; 2006, 1,607.7 ± 605.9; $p < 0.03$) and the tissue PBDE congener profile was similar to that of DE-71. Schecter *et al*., (2007) measured total PBDE levels in liver of new born and human foetuses, and observed a mean level of 23.1 parts per billion and a median level of 15.2 parts per billion. An inverse association (U-shaped) between PBDEs, specifically BDE-123, and body mass index (BMI) has been reported in human serum samples collected from US population (Lim *et al*., 2008). Similar results were observed by Daniels *et al* (2010) in US women and by Frederiksen *et al* (2009) in Denmark women where high BMI levels correlated with high levels of PBDEs.

These results suggest a correlation with diabetes, obesity and other liver related metabolic syndromes; which might be due to the potential of xenobiotics to disrupt glucose and lipid/fat metabolism in mammals. In summary, overall epidemiological data did not indicate any significant association between exposure to PBDEs and the risk of cancer (such as breast, testicular, pancreatic and thyroid cancers). For example, Hardell et al (1998) has reported a non-significant association between non-Hodgkin's lymphoma and adipose levels of BDE-47. Similarly an increased risk of testicular cancer in children was associated with maternal serum PBDE levels (Hardell et al 2006)Although a number of studies on the relationship between BMI levels and metabolic syndrome and diabetes or thyroid hormone status and PBDE levels are available; these studies do not suggest a consistent relationship (EFSA 2011).

6. Animal studies

There is shortage of information on acute toxicity in experimental animals on PBDE exposures. However, existing information depicted PentaBDE mixture with very low acute toxicity in oral or dermal exposure studies (EFSA 2011). The most common toxicity endpoints induced by PBDEs in liver are hepatocellular hypertrophy and induction of xenobiotic metabolism enzymes. Zhou *et al*. (2001) treated female Long-Evans rats (28d old) with technical mixtures DE-71, DE-79 or DE-83R up to 4days. DE-71 and DE-79 induced liver enlargement, hepatic ethoxyresorufin-O-deethylase (EROD), pentoxyresorufin-O-deethylase (PROD), and UDP-glucuronosyltransferase (UGT) activities. The observed increase in UGT enzyme activities indicates the formation glucorinated PBDE products. Another short term exposure treatment with DE-71 mixture or individual congeners (BDE-47, -99 and -153) up to three days in adult male F344 rats resulted in a weak up-regulation of Cyp1A1 mRNA; however later these results were ascribed to the dioxin-like impurities in the technical mixture.

A number of long-term exposure studies have described chronic effects of DE-71 mixtures and their congeners in rodents. Wistar rats were orally treated with a commercial PentaBDE mixture (containing approximately 45 % pentaBDE congeners), for over 28 days. Results indicated increased liver weight, centrilobular hepatocellular hypertrophy, decrease in apolar hepatic retinoids, and induction of hepatic CYP1A and CYP2B enzymes. Authors suggested that most observed effects in the liver were mediated by activation of the nuclear receptors PXR and CAR; and these effects were more sensitive in males than females (Van der Ven *et al*., 2008a). Dunnick and Nyska, (2009) conducted chronic toxicity studies in F344/N rats and B6C3F1 mice by oral exposure to DE-71 over 13 weeks. Liver was the primary target organ and the most

sensitive parameter was the increase in liver weights. Treatment-related increases in liver weights, liver cytochrome P450 (1A1, 1A2, 2B), UDPGT (rats only) levels, and liver lesions were seen both in rats and mice. The authors concluded that the observed effects were indicative of carcinogenic potential of DE-71 mixture after long-term exposure in rodents; and possible mode of action could be through induction of oxidative stress. Similar results have been described in the female rats exposed to DE-71 (Bruchajzer *et al*., 2010); male Sprague-Dawley rats exposed to single congener BDE-99 (Albina *et al*., 2010) and the rats exposed to Bromkal 70-5DE mixture (Öberg *et al*., 2010). Main observed effects were lipid peroxidation, symptoms of fatty liver (increase in relative liver mass), hepatic steatosis, induction of Cyp450 (CYP1A1 and 2B), catalase and SOD enzyme activities.

7. *In vitro* Studies

There is limited literature on *in vitro* toxic effects and none of these studies evaluated time- and dose- dependent effects of PBDEs. Stepleton *et al* (2009) exposed human hepatocytes to BDE-99 and BDE-209 and observed up-regulation of CYP1A2, CYP3A4, DI1, and GSTM1 in BDE-99–exposed hepatocytes (2.1-, 2.2-, 1.6-, and 1.6-fold, respectively), and a similar up-regulation in BDE-209–exposed hepatocytes. Also *in vitro* DE-71 exposures conducted by Fery *et al*., (2009) in three different liver cell lines (Primary hepatocytes, HepG2 and H4IIE), resulted in induction of cytochrome P450 (CYP3A1 and CYP3A4) enzymes. Shao *et al* (2007) exposed liver hematopoietic stem cells to single congener BDE-47 and observed a loss of mitochondrial membrane potential and initiation of apoptosis at low micromolar range. Similar results were observed in rainbow trout gill cells exposed to BDE47 by Shao *et al* (2010). These

researchers have shown induction of apoptosis likely originating from disruption of cellular redox status and mitochondrial oxidative injury (Shao *et al* 2010).

A per the current literature PBDEs are not genotoxic or mutagenic; however, they can induce mutations or cause DNA damage by induction of reactive oxygen species. A recent study investigated the formation of DNA adducts *in vitro*. The results suggested that PBDEs can form DNA adducts through Michael addition between deoxy-guanine of DNA and quinone metabolites of PBDEs. They also indicated that lower brominated congeners have higher potential for adduct formation (Lai *et al*., 2011).

8. Mode of Action of liver toxicity

Metabolism

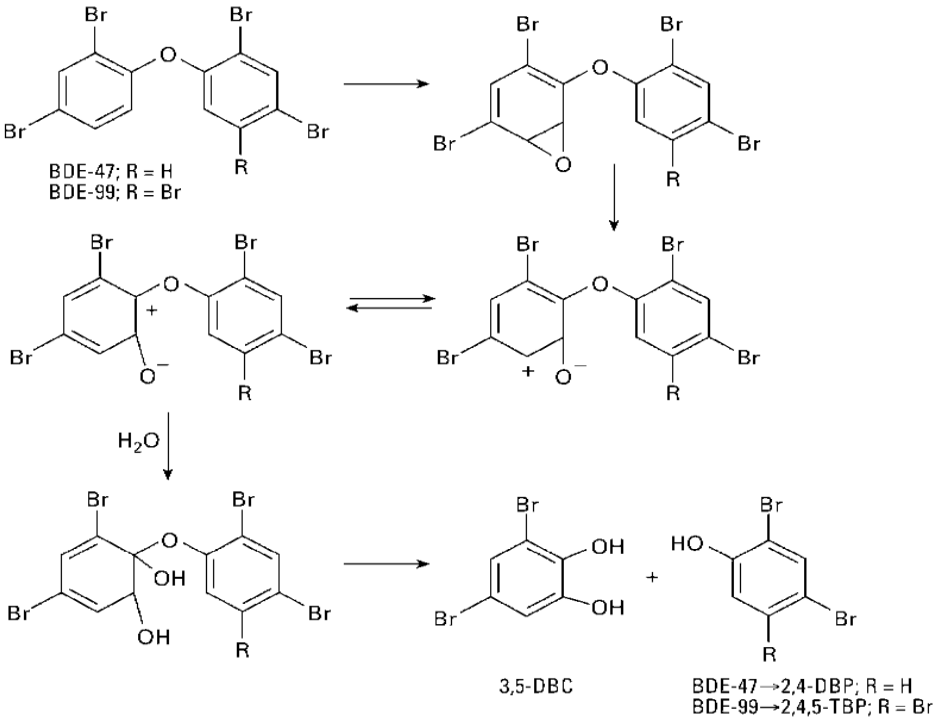


Figure 3. Proposed metabolic pathways of lower brominated PBDEs (Feliciano et al., 2008; Sanders et al., 2006)

Experiments conducted in rodents to investigate the metabolism of major PBDE congeners, such as BDE-47, -99 and -209 indicated that PBDEs undergo biotransformation, producing fully brominated congener, BDE-209. Three main metabolic pathways have been suggested on the basis of observations made in rodents as well as in human; these pathways include: bond cleavage, debromination, and hydroxylation (oxidative pathway). Primary metabolites include bromophenols, lower brominated congeners and their hydroxylated products (Sanders et al., 2006) (Fig. 3).

Metabolic studies performed in rats and mice treated with radio-labelled BDE-47, -99, and -100 resulted in detection of different hydroxylated (-OH) metabolites of tetra- and tri- brominated congeners (Sanders *et al*., 2006, Hakk and Letcher,2003, Marsh *et al*., 2006). Malmberg *et al*., (2005) identified more than 16 hydroxylated PBDEs in rats treated with seven environmentally relevant PBDEs. In some experiments researchers observed non-extractable radioactivity in various organs/tissues of PBDE treated rodents. They also observed formation of two glutathione conjugates, 5-(glutathion-S-yl)-2,2',4,4'-tetraBDE and 6-(glutathion-S-yl)-2,2',4,4'-tetraBDE, through an arene oxide intermediate (Sanders *et al*., 2006, Örn and Klasson-Wehler, 1998). These studies suggest the formation of highly reactive intermediates, which are known for greater toxic potential.

In another experiment, BDE-99 treatment resulted in glucorinide and sulphonate conjugates in addition to monohydroxylated metabolites of tetra- and pentaBDEs. These results indicate that BDE-99 undergoes extensive metabolism among all PBDE congeners (Chen *et al*., 2006b, Hakk *et al*., 2006; 2009). Comparable results were observed upon

treatment of human hepatocytes *in vitro* with 10μM BDE-99 solution up to 72 hours (Stapleton *et al*., 2009). These exposures resulted in the formation of 2, 4, 5-tribromophenol, two monohydroxylated pentaBDE metabolites and an unknown tetraBDE. Most importantly all these metabolites, such as hydroxyl, methoxy, phenolic derivatives of PBDE congeners were detected in blood samples of wild animals (fish, birds) and mammals including humans. These results suggest that formation of highly reactive intermediates or metabolites is one of the significant toxic modes of action of PBDEs.

Nuclear Receptors and Xenobiotic metabolism enzymes

Xenobiotic metabolism enzymes (XMEs) play a significant role in metabolism, removal and detoxification of various foreign substances including environmental chemicals such as PBDEs and PCBs from human body. Most of the tissues particularly liver contain different XMEs known as phase I, phase II metabolizing enzymes and phase III transporters. In general these enzymes are in unstimulated state or at basal expression level and are activated upon exposure to xenobiotic chemicals. Phase-I are an important set of enzymes involved in hydrolysis, reduction and oxidation of xenobiotic chemicals, and most of these enzymes belong to the cytochrome P450 superfamily (CYP P450). Cyp450 enzymes are mainly responsible for oxidative metabolism of a wide range of both exogenous and endogenous xenobiotics. Through oxidative process, cyp450 enzymes convert these xenobiotics into hydrophilic molecules in order to excrete them from body more easily. Additionally in the process of xenobiotic metabolism, some chemicals such as poly aromatic hydrocarbons (PBDEs, PCBs, and Dioxins) can undergo

bioactivation into highly reactive intermediates, which can cause genotoxicity and carcinogenesis.

Since liver and intestine are the main target sites for several exogenous and endogenous xenobiotics, higher level of Cyp450s has been detected in these tissues. These enzymes stay active as long as the stimulus is present, and will return to the basal level upon removal/clearance of stimulus. Cyp1A (Aryl hydrocarbon Receptor – dependent), a major class of Cyp450s, is mainly involved in removal/detoxification of structurally similar (planar) lipophilic compounds which can accumulate to a toxic level otherwise. TCDD is a strong inducer of Cyp1A1 and known to metabolise at a slower rate, leading to continuous expression of Cyp1A1 and subsequent potent toxic and biological effects. For this reason Cyp1A1 and CYP1A1-dependent 7-ethoxyresorufin-O-deethylase (EROD) gene/protein levels are considered as sensitive biomarkers and applied in human risk assessment of dioxin-like compounds. In contrast to Cyp1A, another family of Cyp450s exist, known as Cyp3A, that mainly metabolizes non-planar, structurally diverse xenobiotic chemicals such as phenobarbital (PB)-like compounds. Cyp3A4s are regulated by nuclear receptors, like pregnane X receptor (PXR) or constitutive androstane receptor (CAR), and chemical to receptor interaction studies are significant for investigating mode of action of toxicity (Moore and Kliewer 2000).

AhR – dependent mechanism

Some *in vitro* studies conducted in rat hepatoma (H4IIE) and human colon cells, authors suggest that PBDEs may contain AhR- agonist or AhR-dependent EROD activity (Hamers *et al*., 2006, Chen *et al*., 2001). On the contrary Peters *et al*., indicated no significant AhR-dependent induction of Cyp1A1 activity in MCF-7 and HepG2 cells

(Peters *et al*., 2004). Some rodent studies have indicated an AhR-dependent activity (Peters *et al*., 2004; Chen and Bunce, 2003); however, studies conducted by Sanders *et al*., confirmed that the agonistic activity might be due to dioxin-like impurities present in the technical mixtures such as DE-71; and PBDEs alone may not induce Cyp1A1 enzymes (Sanders *et al*., 2005). Also molecular structure calculations specify that PBDEs are non-coplanar in structure, demonstrating no binding potential to AhR (Hu *et al*. 1994; Wang *et al*. 2005).

PXR and CAR – dependent mechanism

Pregnane X receptor is a transcription factor and highly expressed in liver, intestine and colon, and its main target gene is Cyp3A4. Due to its ambiguous and spherical shaped ligand binding pocket, it can bind to a wide-range of structurally different substrates, such as antibiotics, glucocorticoids, phenobarbitals and so many other pharmaceutical drugs. In mechanisms based on ligand activation/binding, PXR attaches to the 9-cis retinoic acid receptor (RXR) and forms a heterodimer, which in turn translocates to nucleus and binds to hormone responsive elements (HREs) at the 5'-promotor region of the CYP3A4 gene (Giguère 1999). Constitutive androstane receptor (CAR) has considerable similarity in ligand specificity with PXR, and needs RXR to form heterodimer upon ligand binding; the target genes belong to Cyp2B family. PXR and CAR are also responsible for induction of phase II enzymes, UDPGTs, sulfotransferases, and GSTs; and transport proteins MDR1, MRP2, MRP3, and Oatp2 (Kretschmer and Baldwin 2005). In a manner similar to AhR, bioactivation of certain xenobiotic substances can lead to toxicity and disease outcomes. Both have considerable cross-talk and known to induce Phenobarbital (PB)-like effects such as liver

carcinogenesis, thyroid homeostasis and thyroid neoplasia. Comparable results were observed in rodents treated with non-coplanar PCBs and PentaBDE mixtures (Eriksson *et al*., 2001, 2002; Meerts *et al*., 2000), indicating that PBDEs follow a similar mode of action (PXR-dependent) to that of Phenobarbitals.

In humans, Steroid X Receptor is analogous to PXR and known to have high substrate binding potential than in rodents, leading to more adverse/different health outcomes (Tabb *et al*. 2004). Due to their structural similarity with non-coplanar PCBs, it's highly probable that PBDEs may have binding affinity to PXR and CAR nuclear receptors. In several *in vitro* experiments using murine material, PBDEs have been reported to induce PXR- and CAR- mediated gene expression (Pacyniak *et al*., 2007; Wahl *et al*., 2008; Fery *et al*., 2009). Similar results were observed in rodents treated with DE-71 mixture as well as individual congeners BDE-47 and -99 (Sanders *et al*., 2005; Fery *et al*., 2009; Szabo *et al*., 2009). But there were no reports on AhR- dependent induction (Pacyniak *et al*., 2007). Additionally reporter gene assays conducted in human liver cells transfected with murine PXR (mPXR) or human PXR (called steroid X receptor, SXR) resulted in significantly higher induction of PXR-dependent gene expression in SXR transfected cells than mPXR transfected cells (Pacyniak *et al*., 2007). These results suggest that PBDEs, particularly components of DE-71 mixture are potent inducers of PXR- dependent genes such as Cyp3A4 and can cause higher toxicity in humans than in rodents. Literature till date indicates that PBDEs, predominantly lower brominated congeners, can induce toxicity and main mode of action is through PXR/CAR- dependent mechanism.

9. Oxidative Stress

Reactive Oxygen species (ROS) can be generated from a variety of sources classified as exogenous and endogenous; UV irradiation, ozone and polyaromatic hydrocarbons (PAH) are key examples for exogenous sources. There are multiple sources considered as endogenous such as mitochondrial oxidative phosphorylation, xenobiotic metabolism, active peroxisomes and inflammation. Reactions catalyzed by enzymes such as NADPH Oxidases (NOX1, 2 , 3, 4, 5, DUOX1 and DUOX2), CYP 450s, lipoxygenases and cytochrome P450 reductases will result in ROS production. It is understood that Cytochorme P450 mediated xenobiotic metabolism is a significant source of ROS; metabolism of ethanol, dioxin-like and phenobarbital-like compounds (Czekaj P 2004; Vrzal *et al*., 20004). In numerous studies these proto-type chemicals induced high levels of reactive intermediates resulting in increased oxidative stress in liver and also cancer induction, in some cases.

Oxidative stress can be either direct or indirect depending on the mode of action of the toxic chemical. Chemicals such as dioxins, PCBs, phenobarbitals act through an indirect method to promote ROS generation, mainly through enzyme induction. These chemicals are potent inducers of xenobiotic metabolism enzymes (Cyp1A, 2B, 3A etc.) and ROS can be generated by these enzymes during oxidation/reduction cycles (Schlezinger *et al*., 2006, Shertzer *et al*., 2004). In general, these chemicals upon binding to receptors like AhR, CAR or PXR induce Cyp450s, and form a triple complex of enzyme−substrate (chemical) −superoxide. Breakdown of this triple complex yields an oxidized/reduced substrate along with free superoxide as by-product, which in turn is converted into hydrogen peroxide under the catalysis of superoxide dismutase (SOD) (Schlezinger et al., 2006). Additionally, there is a high probability of formation of a wide

range of highly reactive intermediates such as arene epoxides, peroxides, quinones etc. Enzyme induction increases with increased exposure of substrate/toxic chemical, hence excess release of reactive oxygen species. These reactive oxygen species/free radicals can damage proteins (protein oxidation), lipids (lipid peroxidation) or DNA (oxidative DNA damage), which in turn release a variety of reactive species to enter a feedback loop of ROS generation.

In mammalian systems numerous defense systems have been reported to protect against accumulation of ROS; however in several abnormal/unhealthy conditions these systems fail or become ineffective. Oxidative stress (due to acute exposure of toxicant/toxic metabolites) can lead to apoptosis induction; on the other hand chronic low levels of ROS can activate specific signalling pathways; or promote diseases initiation and development and in some cases carcinogenesis (Finkel and Holbrook, 2000).

Several studies strongly indicate that ROS plays a significant role in adaptation of cell to its environment, either pushing towards cell proliferation, adapting to hypoxia, or apoptosis or carcinogenesis towards maintaining redox homeostasis. Cell fate highly depends on the levels of ROS, for they act as secondary messengers in regulating specific signalling pathways (Fig. 4). Low levels considered as physiological concentrations of ROS are very important in regulation of several cellular events including cell cycle. On contrary elevated levels result in apoptosis, irreversible damage to proteins, DNA and lipids, finally towards massive cell death via necrosis (Droge W., 2002). Cells exposed to chronic levels of TCDD, tend to adopt a carcinogenesis pathway in an attempt to inhibit the cell damage due to apoptosis (inhibition of apoptosis) (Bock and Kohle 2005).

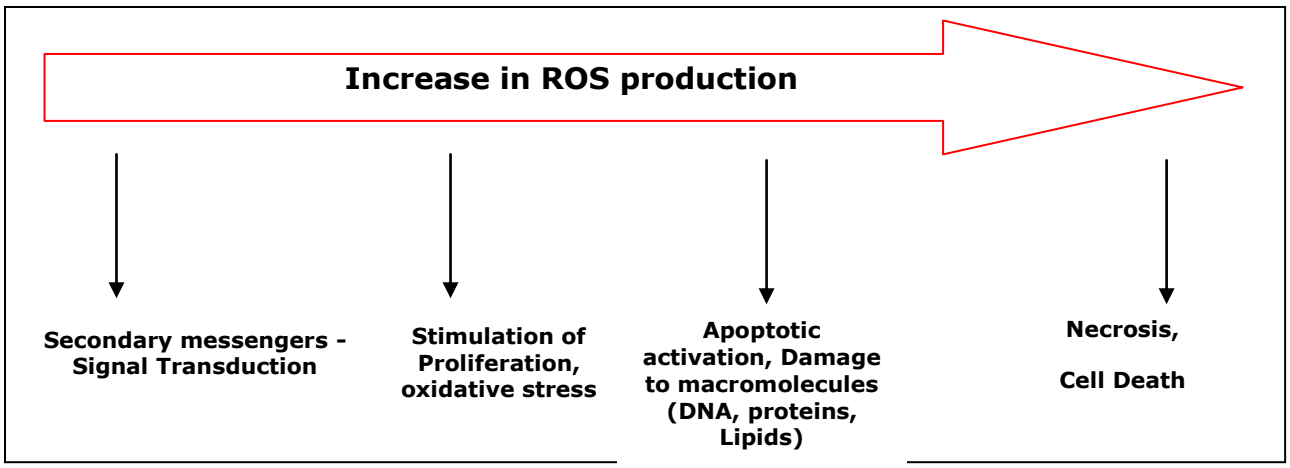


Figure 4. Effects of reactive oxygen species (ROS) on cell functions with varying magnitude of ROS production.

Among the numerous signalling pathways activated upon exposure to toxic chemical, genes encoding for detoxifying enzymes and antioxidant defense are the first to upregulate and induce a stress response (known as adaptive stress response pathways) (Crawford and Davies 1994); these are mainly regulated by transcription factor Nrf2. Other oxidative stress triggered signal pathways include, but not limited to, members of the mitogen-activated protein kinases (MAPKs), phosphatidylinositol-3-kinase (PI3K)/Akt pathway, phospholipase C-g1 (PLCg1) signaling, protein kinase C, p53 signaling, ataxia-telangiectasia-mutated (ATM) kinase, nuclear factor-kappaB (NF-kB) signaling, and Jak/Stat pathway (Benhar *et al*., 2002; Martindale and Holbrook, 2002; Leonard *et al*., 2004; Poli *et al*., 2004). The outcome from these pathways range from maintenance of redox homeostasis to enhancing viability, or promoting cell death or transformation of pro-oncogenes.

10. Apoptosis

Cell death can occur in two different ways, either by necrosis (known as swelling or accidental cell death) or by apoptosis (also known as suicidal or programmed cell death). Unlike necrosis, cells undergoing apoptosis show very distinct morphology including cytoplasmic shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation and formation of small membrane-bound apoptotic bodies (Elmore 2007). Apoptosis plays a crucial role in maintaining liver health by removing the undesirable cells that are undergoing senescence or damaged or infected due to toxic insult. In general this mechanism regulates the balance between number of cells created through mitosis and excluded by apoptosis (Elmore 2007). However this balance is disturbed in several pathophysiological conditions such as alcoholic liver disease, liver cholestasis, non-alcoholic steatohepatitis and viral hepatitis (Garcıa-Mediavilla *et al*., 2005). On the other hand "partial" apoptosis (low level and chronic apoptosis) resulting in incomplete exclusion of genetically damaged cells (carrying mutations) and their entry into cell cycle may lead to liver carcinogenesis (Guicciardi and Gores 2005).

There are two distinct apoptotic signalling pathways, with overlapping events, known as extrinsic and intrinsic pathway (Elmore 2007). Intrinsic pathway predominates in most liver abnormalities and is activated in response to various stimuli such as oxidative stress, endoplasmic reticulum stress and mitochondrial damage; whereas extrinsic pathway is triggered by activated cell surface receptors like Fas and TNF-α receptor-1. There is considerable overlap between these two pathways and they ultimately lead to downstream activation of several endonucleases. Among various caspases, caspase-3 and caspase-9 plays significant role leading to DNA fragmentation

and cell death. These caspases belongs to a family of cysteine proteases and contains a cysteine moiety in its catalytic site. Hence redox state of the cellular microenvironment regulates the activation of caspases (Fig 5). In several studies ROS levels were reported to actively regulate these caspase pathways (Porter *et al* 1999). As a consequence magnitude of oxidative stress (ROS levels) regulates the apoptotic phenotype in the liver. Supporting evidence has been reported by several researchers where prolonged or over activation of the apoptotic process resulted in hepatocellular damage either in animals or in mammalian cells upon treatment with toxic chemicals, such as PCBs (PCB-77, -126, -153) (S De *et al*., 2010, Tharappel *et al*., 2002, Lai *et al*., 2011). Also Howard *et al* (2003) demonstrated that increased production of ROS resulted in induction of apoptosis in hippocampal neural cultures exposed to non-coplanar PCBs.

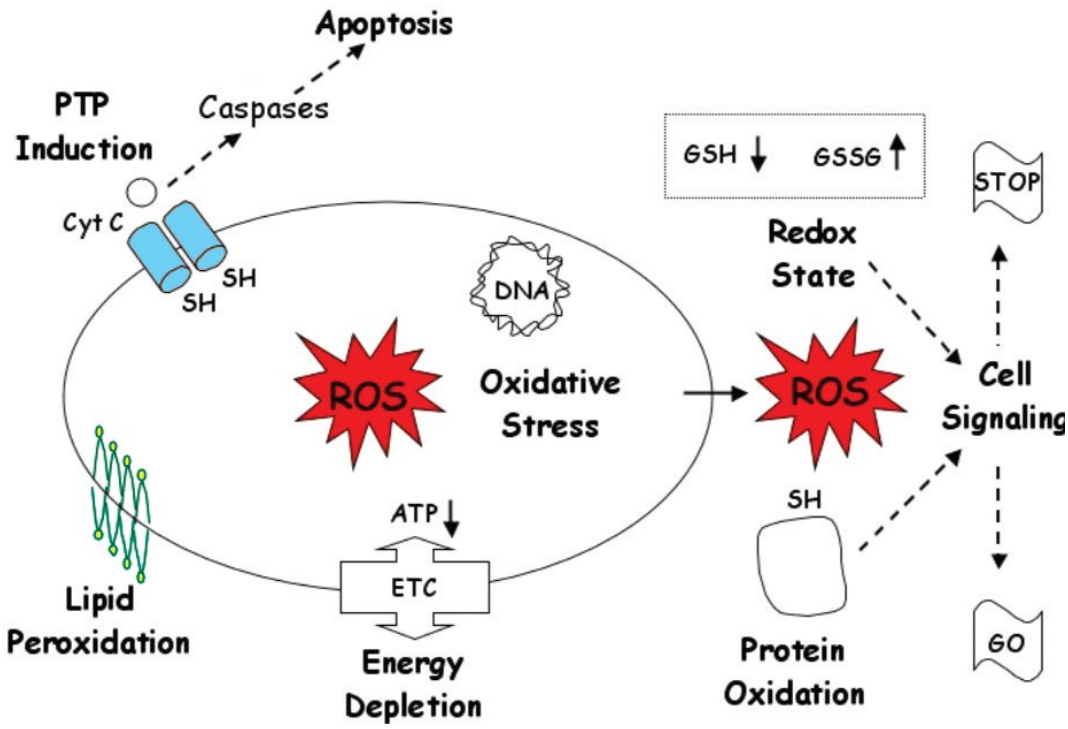


Figure 5. ROS mediated induction of oxidative, apoptosis and cell signalling (Batandier et al 2002).

11. Rationale

The following key points can be derived from the literature review and are considered as the basis for the hypothesis, aim and study objectives of this project.

- ✓ A temporal increase in BDE-47, -99 and other main congeners of pentaBDE mixture (DE-71) was observed; which is comparable to accumulation of polychlorinated biphenyls (PCBs) occurred few decades ago.
- ✓ PBDEs, due to their highly lipophilic nature, can target lipid-rich tissues such as adipose and liver. Also with their longer half-life values, they are presumed to accumulate over years and show their toxicological effects in exposed individuals.
- ✓ Key target organ of PBDEs is liver and most sensitive parameters are hepatocellular hypertrophy and induction of xenobiotic metabolism enzymes (Cyp450s).
- ✓ PBDEs are potent inducers of Cyp2B and 3A, and undergo oxidative metabolism suggesting the formation of hydroxylated metabolites and other reactive intermediates such as ROS, arene epoxides etc.
- ✓ PBDEs are agonists of PXR and CAR nuclear receptors and didn't show any binding potential to AhR receptor; comparable to behaviour of non-coplanar PCBs and Phenobarbitals.
- ✓ Receptor based studies indicated that PBDEs can exert greater toxicity in humans than in rodents.
- ✓ Most effects were mediated by PXR/CAR receptors and this pattern is comparable with other rodent tumour promoters, hence it is possible to assume that PBDEs can promote liver carcinogenesis from chronic exposure.

12. Hypothesis

The key hypothesis in this study is that the main mode of action of PBDE is through induction of oxidative stress; and the oxidative stress can lead to apoptosis in human liver upon long-term exposure.

13. Aim

The aim of this study is to investigate the mode of action of DE-71 mixture by measuring the key events resulting from time- and dose-dependent exposures in human liver cells.

14. Specific objectives

The experimental set up in this study was that HepG2 cells were exposed in a time – and dose – dependent manner to DE-71. To achieve the aim of this study the following objectives were identified:

- ✓ To examine cell viability of the exposed human liver cells by using colorimetric MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay
- ✓ To evaluate the release of intracellular reactive oxygen species (as an estimate of induced oxidative stress) in exposed cells by measuring the fluorescence produced by 2',7'-dichlorfluorescein-diacetate
- ✓ To determine the level of apoptosis in the exposed cells by measuring the fluorescence product formed due to caspase-3 enzyme activity and by DNA laddering assay

15. Significance

PentaBDE mixture chosen for this study contains congeners that are detected predominantly in human biological samples. There is appreciable agreement between observations from rodent studies and respective in vitro models; however there is a very limited data in human models. Since PBDEs have shown greater binding potential to human PXR than rodent PXR, studies involving human models are very important in extrapolating these results for risk assessment in humans. Additionally, in this project, the cells are exposed to DE-71 mixture, and not to individual congeners. This is important in the perspective of recent regulation to include mixtures of congeners/chemicals exhibiting similar mechanistic features (USEPA 2001). Also this study is aimed at understanding the dose and temporal relationships between various steps (oxidative stress and apoptosis) leading to cytotoxicity.

II. Methodology

1. HepG2 Cell culture

The HepG2 (human hepatocarcinoma) cell line is an immortalized human liver cell line derived from hepatocarcinoma patients (Aden *et al*., 1979). The preferred order in drug metabolizing activity, among various human *in vitro* liver models is: primary hepatocytes > cryopreserved hepatocytes > HepG2 cell (Xu *et al*. 2004). However, primary human hepatocytes have obstacles when it comes to their usage; these include availability, hard-to-handle, unable to proliferate and very expensive maintenance. Whereas HepG2 cells are relatively easy-to-handle, immortalized so can be cultured in the laboratory and are an effective tool to investigate metabolic activation by xenobiotic metabolism enzymes (Westerink and Schoonen, 2007). Also these cells were extensively used in a wide-range of experiments to test the toxicity of various compounds for the past ten years. They are used in *in vitro* toxicity testing due to their sensitivity with range of genotoxic chemicals such as polycyclic aromatic hydrocarbons (Knasmüller *et al*. 2004). Also these cells are particularly easy to use experiments requiring monolayer growth of cells, such as DCFH assay, MTT assay etc., which seem to be difficult with primary hepatocytes (Lautraite *et al*., 2003).

Methodology: HepG2 cells were obtained from ATCC (American type Culture Collection, Manassas, VA) and were maintained in complete growth medium containing Minimum Essential Medium (MEM, Gibco), 10% fetal bovine serum (FBS, Sigma), and 1.0mM sodium pyruvate with phenol-red and without any antibiotics. Cells were seeded at a density of 30,000 cells/cm^2 and grown in monolayer. Medium was replaced with

fresh medium every 2-3 days. Cells were passaged or split by 1:4 upon reaching ~80% confluence (after 5 days). For passage, cell monolayer was gently rinsed with 1X phosphate buffered saline (PBS, pH7.4) twice; 0.05% Trypsin – 0.053mM EDTA solution was added and incubated for 5 – 7 minutes at 37°C; cells were detached by gentle agitation or rocking; trypsin-EDTA was inactivated by adding 2 volumes of complete growth medium, and centrifuged at 1000rpm for 3min; thus the obtained cell pellet was resuspended in fresh complete growth medium, counted and seeded appropriately into growth vessels/dishes; incubated in a humidified chamber maintained at 37°C temperature and 5% CO_2.

2. DE-71 mixture and dose – and time – dependent exposures

As described previously, DE-71 mixture was chosen as a model compound to investigate the PBDE induced toxicity and risk assessment. The PBDE congener mixture DE-71 (lot no. 05500F16P) was purchased from Wellington Laboratories (Ontario, Canada); the congener composition is reported in Appendix-I. No dioxins or dioxin-like or other non-PBDE compounds were reported to be present in the congener by the manufacturer. The PBDE toxicity study conducted in HepG2 cells by Pacyniak *et al*., (2007) has been chosen as a basis for selection of range of a treatment doses and duration. In this experiment Pacyniak and colleagues treated a modified HepG2 cell line up to 24 hours with 0 – 100 μM of PBDEs (BDE-47 or -99 or -209), in order to study the PBDE mediated activation of human PXR (or steroid X receptor).

Methodology: To prepare stock solution, DE-71 mixture (obtained as oil mixture) was completely dissolved in 100% DMSO and stored at $<4^0$C. Though the DE-71 stock

appeared as a clear solution, prior to use in treatment it was vigorously vortexed for 20-30s. Working stocks were prepared by diluting the stock solutions appropriately with 100% DMSO. Always a 500X working stock that of final treatment dose was prepared to maintain the final DMSO concentration at minimal toxicity levels (0.002%). A range of doses 1, 5, 10, 25, 50, 100 μM of DE-71 mixture or 0.002% DMSO and a range of duration points 24, 48, 72 hours were used to conduct dose- and time- dependent treatments. Cells that were passaged between five and ten were used for treatments. After 24H, cells were observed under microscope for proper monolayer growth prior to treatment. Treatment medium was prepared by adding appropriate volume of DE-71 working stock to the complete growth medium and vigorously mixed by vortexing. After 24 hours, medium was gently aspirated and equal volume of treatment medium was added to the cells. Cells were processed at completion of set exposure time (24 or 48 or 72 hours) according to the assay protocol.

3. Measurement of cell viability and IC_{50} in human hepatoma cells

In majority of the *in vitro* experiments, measurement of cell viability or proliferation has been extensively used as an indicator for cytotoxic effects due to chemical exposure. One of the reliable and widely accepted cell viability assays is through colorimetric quantification of reduced tetrazolium salts by viable cells. The yellow salt of MTT [3-(4, 5-dimethylthiazolyl-2)-2, 5- diphenyltetrazolium bromide] is cell permeable and can be reduced into purple colour salts by metabolically active cells through the catalytic activity of dehydrogenase enzymes. These intracellular purple colour crystals (formazan) are dissolved using solvents such as DMSO or isopropanol

then absorbance at 650nm is quantified using a spectrophotometer (Ferrari *et al*., 1990). These absorbance values can be used for calculating the number of viable cells.

Methodology: Cells were seeded at a density of 30,000 cells/cm^2 in 96 well plates. After 24h, time and dose dependent treatments were conducted as per the experimental setup. At the completion of treatment, MTT assay was conducted using Vybrant® MTT Cell Proliferation Assay Kit (In vitrogen, Burlington, ON), as per manufacturer's instructions. Briefly, growth medium was gently aspirated by pipetting at the end of exposure; equal volume of phenol-red free complete growth medium supplemented with 10% 12mM MTT salt was added to each well and incubated at 37^0C; after 4 hours, equal volume of 10% triton-x-100 dissolved in 0.1N HCl in isopropanol solution was added to each well; later crystals were suspended by pipetting and the entire plate was placed on an orbital shaker for 15min at 120rpm; absorbance of final solution was measured spectrophotometrically at 570 and 690nm on a multi-well plate reader. Percent cell viability compared to control was calculated as per the following formula:

% cell viability = ("Sample Well Absorbance" / "Control Well Absorbance") x 100

4. Detection of HepG2 cells expressing high levels of intracellular reactive oxygen species using flow cytometry

ROS assay can measure reactive oxygen species containing hydroxyl or peroxyl groups, such as H_2O_2. This assay is mainly based on the cell-permeable fluorogenic probe 2', 7' –dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA can diffuse into cells, and undergo deacetylation by cellular esterases into a non-fluorescent DCFH. In the presence of reactive oxygen species, DCFH can be oxidized into a highly fluorescent

DCF product, which can be measured by any fluorescent reader at 485 nm (excitation) and 530 nm (emission).

Methodology: For the ROS assay, cells were seeded at a density of 30,000 cells/cm^2 in 25cm^2 flasks. After 24h, time and dose dependent treatments were conducted as per the experimental set up. Following the treatment of the cells, ROS assay was conducted using H_2DCFDA kit (In vitrogen, Burlington, ON), as per manufacturer's instructions (all steps were performed in dark or minimum light). Briefly, at the completion of set exposure, medium was aspirated; equal volume of 1X HBSS buffer supplemented with 5μM H_2DCFDA salt was added and incubated at 37^0C; after 30min, the buffer was aspirated and cells were washed twice with fresh 1X HBSS buffer; cells were collected by trypsinization followed by centrifugation; the cell pellet was suspended in 500μL 1X PBS buffer and placed on ice until measurement. A total of 20,000 cells (counts) per sample were measured on a Flow Cytometer (Becton-Dickinson) equipped with an argon laser lamp (FL-1: emission, 480 nm; band pass filter, 510-550 nm) using the CXP data acquisition software on list mode. Each measurement obtained a histogram with cell counts on vertical axis and DCF fluorescence intensity on horizontal axis. Cells with signal strength greater than one (10^0) were gated as DCFH-DA positive population. Each experiment was validated by running the unstained control which always resulted in negligible positive population. Also in order to validate the method, an initial experiment was conducted on cells treated with 0.5mM H_2O_2 for 6 hours (positive control).

5. Determination of apoptosis in human hepatoma cells

Apoptosis can be mainly activated via two distinct pathways: extrinsic and intrinsic pathways (Ashkenazi 2008; Mayer and Oberbauer, 2003). Though triggered by different activators, both pathways lead to activation of cysteine-dependent aspartic acid specific proteases, known as "caspases" (Shi Y 2002). These caspases play vital role in inducing typical morphological and biochemical changes that are characteristic to apoptosis. Among all caspases, Caspase-3 (CPP32/apopain) is considered as key enzyme and detectable in a wide range of cell lines. It can cleave any substrates/proteins containing specific amino acid sequence Asp-Glu-Val-Asp (DEVD) such as poly (ADP-ribose) polymerase (PARP), DNA-dependent protein kinase, protein kinase Cδ and actin. Cell lysate collected from treatments can be tested for apoptosis by measuring the activity of caspase-3 enzyme. This can be performed by incubating cell lysate with any caspase-3 substrate such as 7-amino-4-trifluoromethyl coumarin (AFC), which forms a fluorescent product upon cleavage by capsase-3. Hence the level of caspase-3 enzyme (apoptosis levels) is directly proportional to the fluorescence signal detected. Additionally, in the majority of the cases, activation of caspases leads to various biochemical and cellular changes such as DNA fragmentation (Ucker *et al*., 1992). DNA fragmentation is mainly due to caspase mediated activation of an endonuclease, known as CAD (caspase-activated DNase), which can cause DNA fragmentation at linker regions between nucleosomes (Sakahira *et al*., 1998). Although apoptosis can occur without DNA fragmentation in some cell types, at large, caspase activation can be confirmed by DNA fragmentation.

Methodology: Cells were seeded at a density of 30,000 cells/cm^2 in 25cm^2 flasks. After 24h, time and dose dependent treatments were conducted as per the experimental

setup. At the completion of treatment, caspase assay was conducted using EnzChek® Caspase-3 Assay Kit (Invitrogen, Burlington, ON), as per manufacturer's instructions. Briefly, cells were trypsinized and centrifuged; cell pellet was washed with 1X PBS buffer and suspended in 1X cell lysis buffer (10mM Tris pH 7.4, 100mM NaCl, 1mm EDTA, 0.015 triton-X-100); mixed well by vortexing and incubated on ice for 30min; centrifuged at 5000rpm for 5min; a fraction of the supernatant was used for protein determination; the remaining supernatant was added to 96-well plate; to each well containing a test sample supernatant, equal volume of 2X reaction buffer supplemented with 1% DEVD substrate was added; mixed well by pipetting and incubated at room temperature in dark for 30min; fluorescence was measured at excitation 360±40nm and emission 460±40nm on a multiwell plate reader. For protein determination bicinchoninic acid assay (BCA assay) was performed. Background controls included were, 1X cell lysis buffer, untreated cell lysate. Briefly, a sample of cell lysate was added to 96-well plate; 8 volumes of the BCA mixture (BCA: $CuSO_4$ = 50:1) was added to each well containing the cell lysate, mixed by pipetting and incubated at 37^0C; after 30min absorbance was measured at 562nm on a multi-well plate reader; protein concentration was determined from the linear regression curve developed using BSA standard curve. Total protein normalized caspase activity was calculated by the following formula:

Fold change in caspase activity (compared to untreated control) =

{"Test Sample" fluorescence / total protein of "test sample"} /

{"Control" fluorescence / total protein of "control"}

Additionally, in order to analyze DNA fragmentation, total DNA was extracted from exposed cells. In brief, at the end of the exposure, floating cells (dead cells), if any, in the medium were collected; adherent cells were detached by trypsinization and a cell pellet was obtained upon centrifugation; both cell pellets were lysed in lysis buffer (10mM Tris pH 7.4, 10mM EDTA, 0.5% Triton-X-100) by incubating on ice for 30min, followed by centrifugation; the supernatant was treated for RNA and protein digestion with RNAse for an hour and Proteinase K for 2 hours respectively; DNA was precipitated by adding 0.5 volumes of 3M sodium acetate and 2.5 volumes of ice-cold absolute ethanol and maintained at -80^0C for an hour followed by centrifugation at maximum speed; DNA pellet was washed with 80% ethanol and air dried for less than 5min; dry pellet was completely suspended in TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) and analyzed on a 2% agarose gel by running at 35V for 4-5 hours.

6. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (La Jolla, CA) for Windows. Each experiment was repeated three times, and each exposure group within an experiment was assayed in duplicate or triplicate. Data was presented as mean ± S.E.M of replicate groups. One-way ANOVA (analysis of variance) and Student's t-test were used to compare differences in mean values among multiple groups (all treatment groups are compared with the vehicle control group) and to calculate statistical significance. The differences between the mean values were considered statistically significant when $P < 0.05$. The results of this statistical analysis were reported in detail, in appendix – II (tables 1, 2, and 3).

III. Results

1. Cell viability and IC_{50} of DE-71 in human hepatoma cells

To investigate cell proliferation or cytotoxicity, HepG2 cells were treated with DE-71 mixture in a time- and dose- dependent manner. Results showed that human liver cells are sensitive to DE-71 and exhibited a dose- and time- dependent response in *in vitro* cultures. MTT assay results, as shown in Figure 6 indicated that, at lower doses, DE-71 was able to slightly increase cytotoxicity. Statistically significant increase in MTT readings was observed at doses ranging from 10 – 50μM ($p<0.01$) compared to vehicle control (DMSO). The maximum increase was observed in cells treated with 50μM and 25μM DE-71(120%) up to 24 hours or 72 hours respectively. Statistically significant inhibition in cell growth, 68% and 46% compared to vehicle control ($p<0.001$), was observed at the highest dose (100μM). The IC_{50} (treatment dose causing 50% inhibition in cell growth) value was observed at 100μM dosage for 72 hours exposure. Importantly, DE-71 showed an apparent effect on cell viability between all doses tested (0 – 100μM) at 72 hours, and all effects observed at this dose- and time- points were statistically significant ($p<0.05$). These observations indicated that cytotoxic effects are very prominent at longer treatment times. Vehicle control, 0.002% DMSO did not induce any changes in cell viability and had minimal effect on overall cell growth. Overall, DE-71 has no lethal effects at acute exposure levels; however, it can be significantly cytotoxic at chronic exposure levels. The cell death might be due to apoptosis or necrosis, which was further investigated in this study.

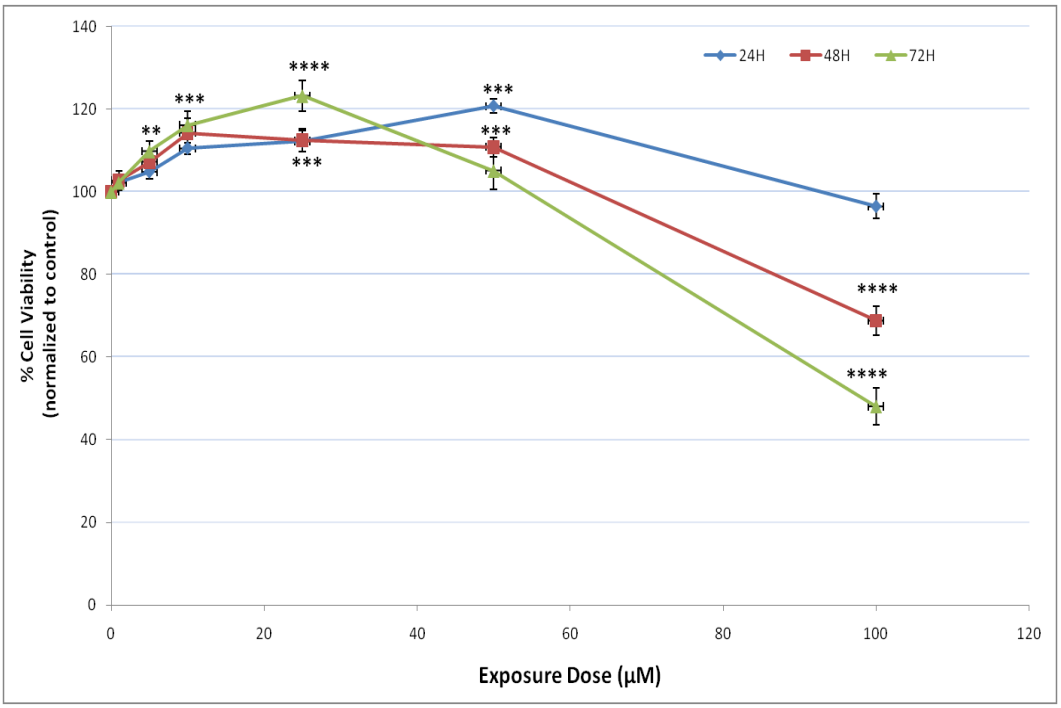


Figure 6: *Effects of DE-71 on HepG2 cell viability measured by MTT assay as described in methods. Cells were cultured for 72 hours in complete growth medium supplemented with either 0.002% DMSO (vehicle control), or range of DE-71 doses, 1 – 100µM in a dose- and time- dependent manner. Change in cell viability in a dose (6A)- and time (6B)- dependent manner are shown in above Figures. Data points represent mean ± SEM, obtained from three independent experiments, and each experiment contained four replicates. One way ANOVA and Student's t-test was conducted to calculate statistical significance.*

*Note: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$, ****: $p < 0.0001$; versus vehicle control (DMSO); exact p values (t-Test) are listed in Appendix-II.*

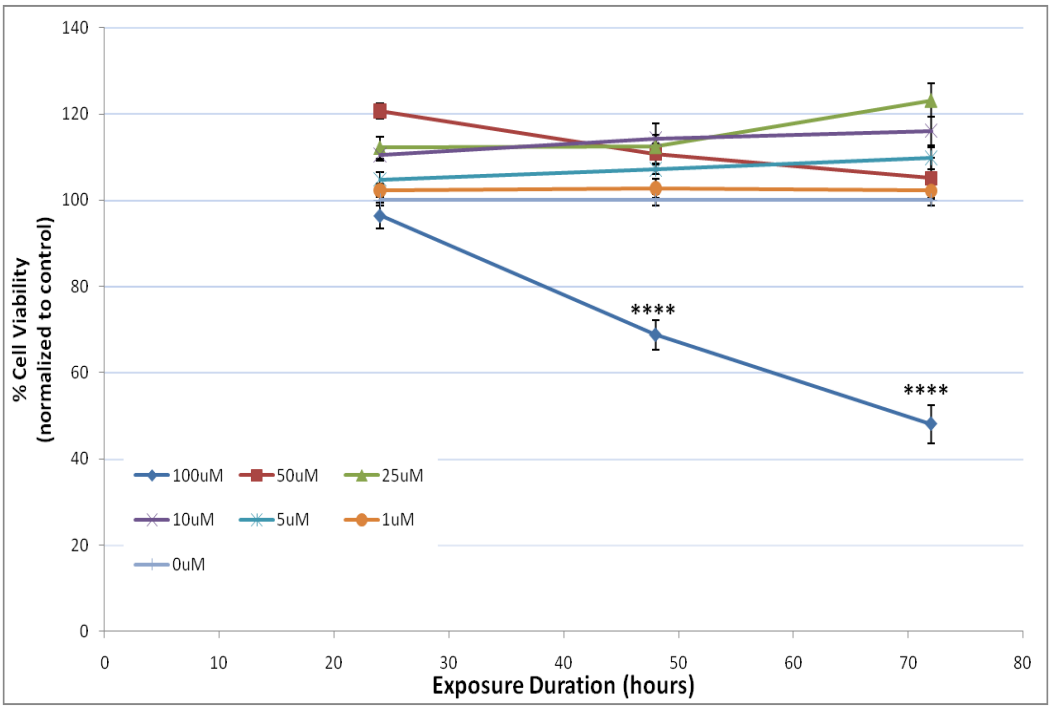
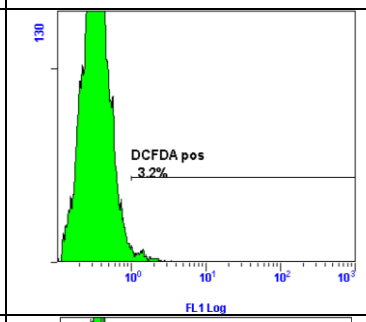
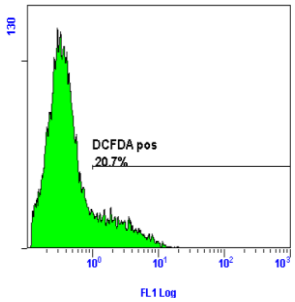
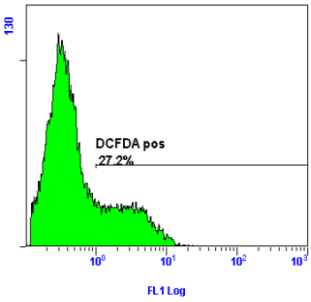
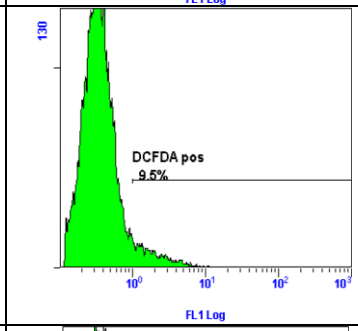
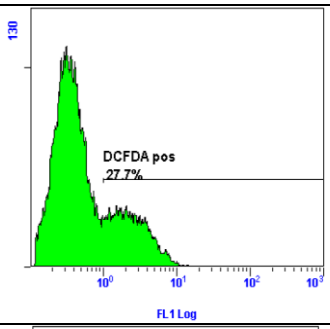
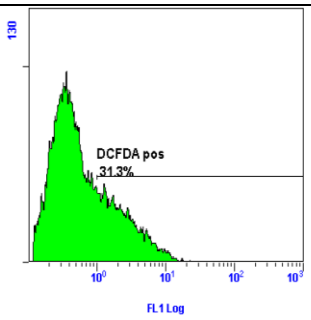
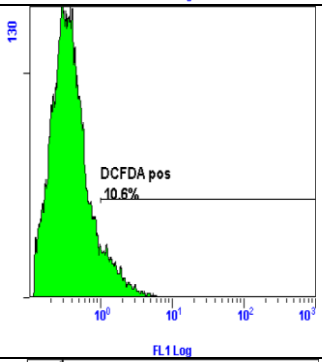
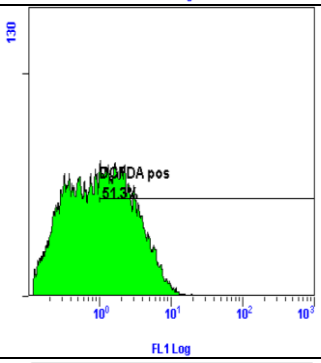
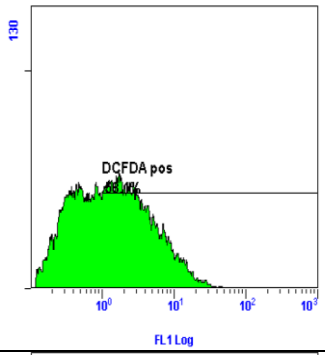
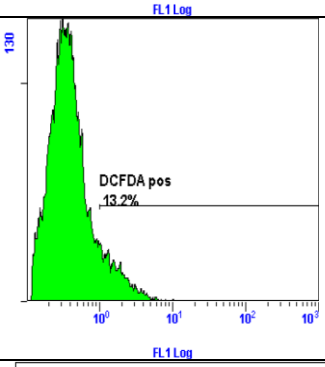
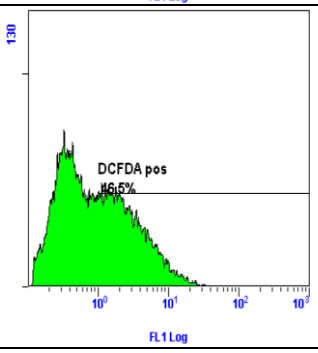
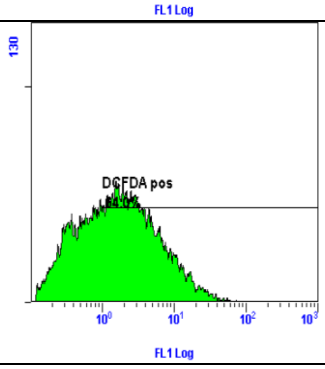
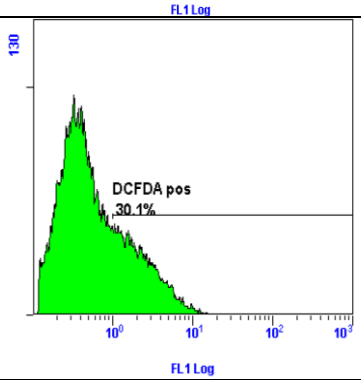
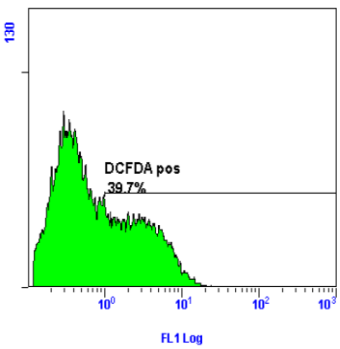
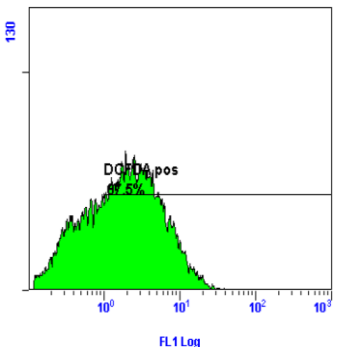


Figure 6: *Effects of DE-71 on HepG2 cell viability measured by MTT assay as described in methods. Cells were cultured for 72 hours in complete growth medium supplemented with either 0.002% DMSO (vehicle control), or range of DE-71 doses, 1 – 100μM in a dose- and time- dependent manner. Change in cell viability in a dose (6A)- and time (6B)- dependent manner are shown in above Figures. Data points represent mean ± SEM, obtained from three independent experiments, and each experiment contained four replicates. One way ANOVA and Student's t-test was conducted to calculate statistical significance.*

*Note: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$, ****: $p < 0.0001$; versus vehicle control (DMSO); exact p values (t-Test) are listed in Appendix-II.*

2. Detection of liver cells expressing high levels of intracellular reactive oxygen species

Using a fluorescent probe, DCFH-DA, liver cells expressing intracellular reactive oxygen species such as H_2O_2 were monitored. Overall results indicated that cells exposed to DE-71 had accumulated intracellular ROS. Flow cytometric analysis showed an increase in percentage of ROS positive population in a time-dependent manner ($p<0.05$) (Figures 7A and B). At 24 hours of incubation time, the percentage of ROS positive cells increased to 14%, 12%, and 29% in 10μM, 50μM, and 100μM DE-71 treatment groups, respectively, compared to the control group (2.5%). A similar pattern was observed at 72 hours, showing an increase to 57%, 59%, and 65% at 10uM, 50uM, and 100μM DE-71 treatment groups respectively, compared to control group (25.73%). However, at 48 hours these values initially increased to 45%, and 46% at 10uM and, 50μM DE-71 treatment groups respectively compared to control (21.08%), but later decreased to 38% in 100μM DE-71 treatment group (Figure 7B). All results were statistically significant, at $p<0.05$, compared to control except for the exposure at 5μM dose. Further, dose-wise comparison indicated that DE-71 mixture can trigger ROS release at doses greater than 10μM. At times, significant background ROS levels were observed in untreated or vehicle treated cells; the reasons for this is not known, but we speculate that this might be due to the fact that these cells are cancerous and proliferated profusely. Although the background ROS levels are high; the final results show that, when normalized to vehicle control, the readings for ROS are still significantly higher in DE-71 treated cells. Overall, ROS assay results indicate that chronic exposure of human liver cells to DE-71 mixture leads to an accumulation of intracellular ROS.

(A)	24H	48H	72H
DMSO			
5µM DE-71			
10µM DE-71			
50µM DE-71			
100µM DE-71			

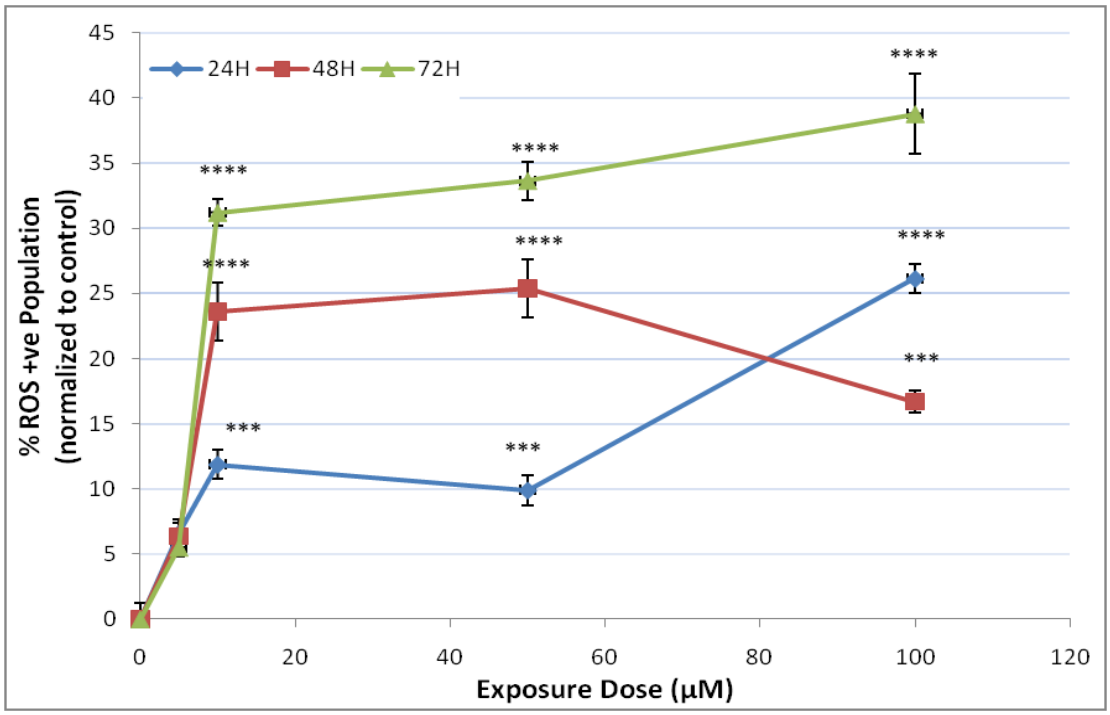


***Figure** 7a: Flow cytometric detection of ROS positive cells upon exposure to 0.02% DMSO (vehicle control) or 5, 10, 50 and 100µM DE-71 using fluorescent probe DCFH-DA. (A) Representative histograms obtained from flow cytometer for each cell population exposed in a time- (24-72 hours) and dose- (0-100µM) dependent manner. ROS production was indicated as a shift in peak fluorescence towards right; These results were, presented as mean ± SEM, obtained from three independent experiments and each experiment contained duplicates. One way ANOVA and Student's t-test were conducted to calculate statistical significance*

*Note: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$, ****: $p < 0.0001$; versus vehicle control (DMSO); exact p values (t-Test) are listed in Appendix-II.*

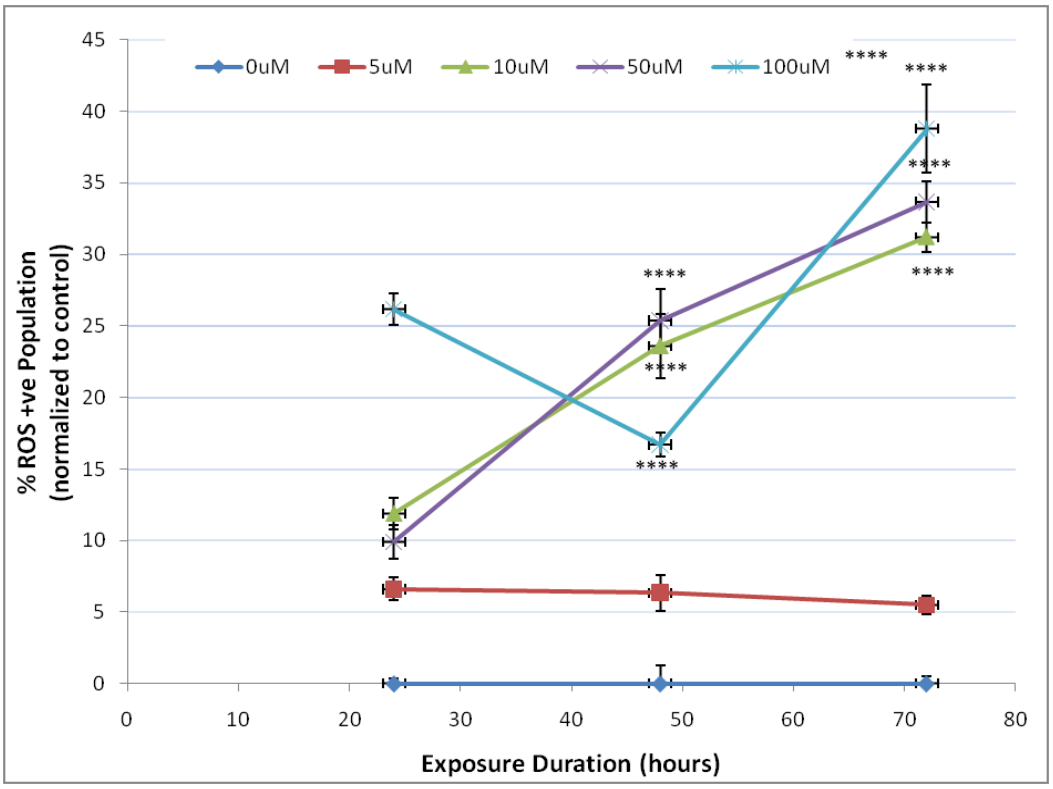


***Figure** 7b: Flow cytometric detection of ROS positive cells upon exposure to 0.02% DMSO (vehicle control) or 5, 10, 50 and 100µM DE-71 using fluorescent probe DCFH-DA. (B) Change in percentage of ROS +ve populations in a dose (B1)- and time (B2)- dependent manner are shown in above Figures. These results were, presented as mean ± SEM, obtained from three independent experiments and each experiment contained duplicates. One way ANOVA and Student's t-test were conducted to calculate statistical significance*

*Note: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$, ****: $p < 0.0001$; versus vehicle control (DMSO); exact p values (t-Test) are listed in Appendix-II.*

3. Apoptosis in human hepatoma cells

Caspase 3 is a critical mediator of apoptosis and highly correlates with apoptotic rates in various tissues. Therefore, the activation of caspase-3 is considered a reliable marker for cells undergoing apoptosis. In order to determine the induction of apoptosis from exposure to DE-71, caspase-3 enzyme levels were measured in HepG2 cells exposed to DE-71 in a time- and dose- dependent manner. The results showed an increase in caspase-3 activity at higher doses of DE-71 and longer incubation times (Fig. 8). Caspase-3 levels remained unchanged at 24 hours of incubation in all DE-71 treatment groups compared to the vehicle control. Likewise, no effect was observed at doses 10μM or lower, comparable to ROS assay results. Caspase-3 activity increased by 2.8 folds compared to vehicle control in cells exposed to 100μM DE-71 for 48 hours ($p<0.01$); however it didn't largely change upon further incubation (2.5 folds at 72 hours, $p<0.01$). Exposure to 50μM DE-71 induced a statistically significant 1.7 fold increase compared to vehicle control from the longest period of exposure (72 hours). Overall a time – dependent increase was observed with increased exposure. Since caspase-3 activity is a hallmark for apoptosis, change in caspase levels can be directly proportional to the quantity of cells undergoing apoptosis.

Activated caspases can play a major role in execution of apoptotic mediated cell death through selective cleavage of cellular substrates resulting in morphological changes; also they activate certain DNAses to induce DNA fragmentation. Therefore, to confirm the apoptotic mediated cell death, an assay for DNA fragmentation in DE-71 exposed HepG2 cells, was conducted. For this assay, only groups that were showing high (and statistically significant) caspase activity and/or apparent cell death (50μM at 72

hours, 100μM at 48 hours and 72 hours) were selected. Inverted microscope images of these groups were shown in Figure 9A. Most significantly, cells treated with 100μM DE-71 for 72 hours are showed distinguishable characteristics (compared to untreated) such as cell shrinkage, less confluence, dead cells (floating). No identifiable morphological changes were observed in other groups. DNA gel electrophoresis showed very slight fragmentation in groups exposed to 100μM for 48-72 hours (Figure 9B). A continuous smear (without apparent laddering) was observed in 50μM DE-71 treated group; No smearing or laddering was observed in DMSO treated or medium only (untreated) group. Over all results indicated that higher doses of DE-71 mixture contains the potential to induce Caspase mediated cell death in human liver cells, up on longer incubations.

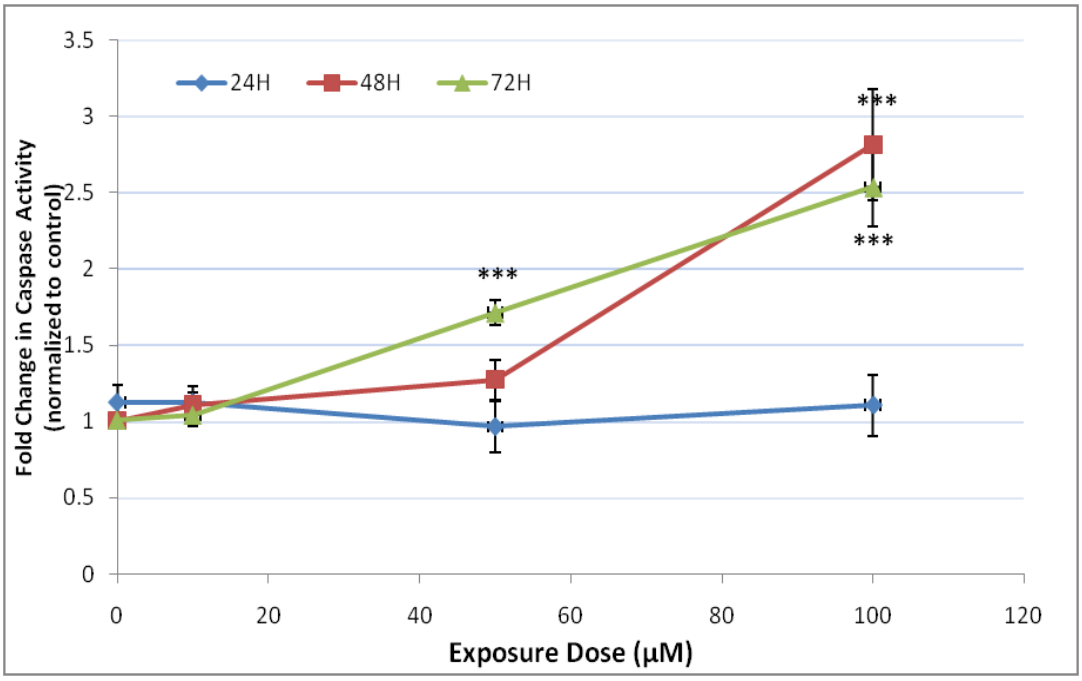


Figure 8*a: Fold change in caspase-3 activity in HepG2 cells exposed to 0.02% DMSO or 10, 50, 100µM DE-71 for 24, 48 and 72 hours. These values were normalized to total protein quantity, as described in methods section. Caspase activities in a dose (8A)- and time (8B)- dependent manner are shown in above Figures. Each value represents the mean ± SEM of three independent experiments, and each experiment contained duplicates. One way ANOVA and Student's t-test was conducted to calculate statistical significance*

*Note: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$, ****: $p < 0.0001$; versus vehicle control (DMSO); exact p values (t-Test) are listed in Appendix-II.*

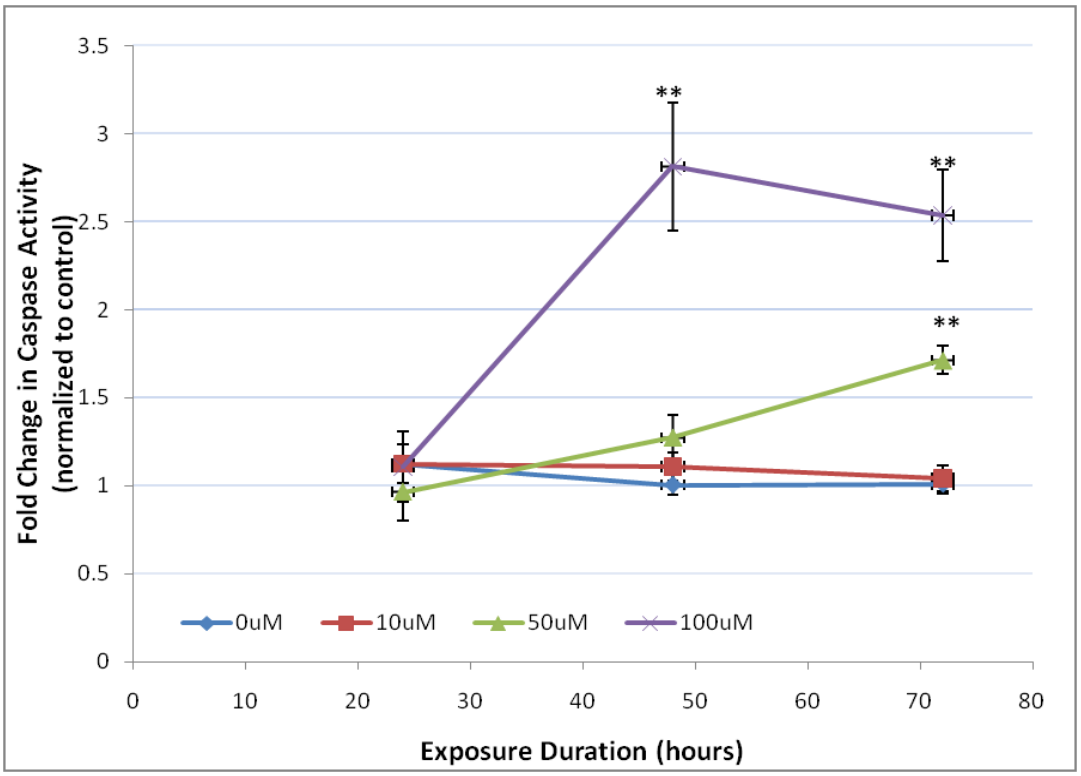


Figure 8b*: Fold change in caspase-3 activity in HepG2 cells exposed to 0.02% DMSO or 10, 50, 100µM DE-71 for 24, 48 and 72 hours. These values were normalized to total protein quantity, as described in methods section. Caspase activities in a dose (8A)- and time (8B)- dependent manner are shown in above Figures. Each value represents the mean ± SEM of three independent experiments, and each experiment contained duplicates. One way ANOVA and Student's t-test was conducted to calculate statistical significance*

*Note: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$, ****: $p < 0.0001$; versus vehicle control (DMSO); exact p values (t-Test) are listed in Appendix-II.*

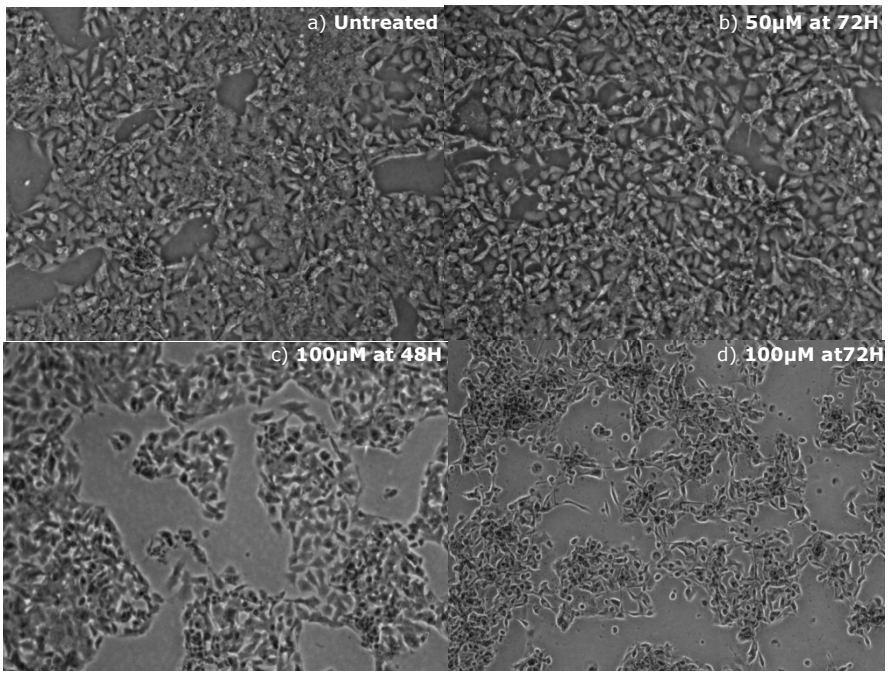


Figure 9A*: Microscope images obtained under 10X objective (without any staining) of the groups either (a) untreated or treated with (b) 50µM (for 72H) or (c and d) 100µM DE-71 (for 48H, 72H respectively).*

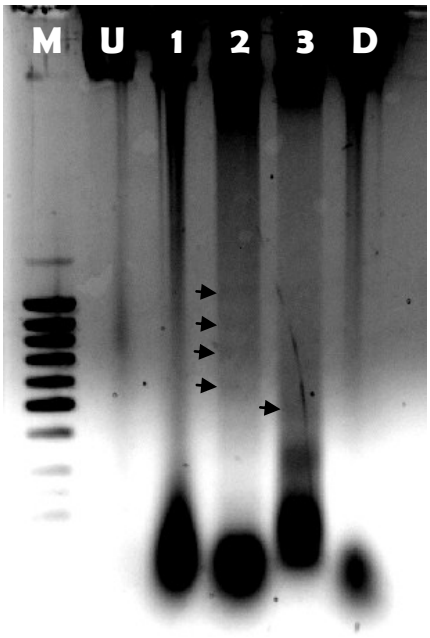


***Figure 9B**: DNA fragmentation in HepG2 cells treated with or without DE-71 analyzed by agarose gel electrophoresis. Lane M: low range DNA ladder, U: Untreated control, 1: 50µM DE-71 for 72H, 2: 100µM DE-71 for 48 hours, 3: 100µM DE-71 for 72 hours, D: DMSO treated control. Arrows indicate fragmented DNA bands.*

IV. Discussion

PBDEs, most importantly lower brominated congeners are predominantly detected in liver as documented in several epidemiological as well as experimental studies. However there is a paucity of information on PBDE induced liver toxicity. Also PBDEs are reported to show species specific differences in accumulation, biotransformation and metabolism. Laboratory studies suggest that PBDEs may cause greater toxicities in humans compared to fish and rodents due to increased metabolism (Chen *et al*. 2006b; Qiu *et al*. 2007; Stapleton *et al*, 2009). Additionally, since PBDEs are detected as mixtures in human tissues, studies highlighting the effects of PBDE mixtures are significant. In this study we attempted to investigate liver toxicity caused by the DE-71 mixture in a dose- and time- dependent manner, and its potential mode of action. HepG2 cell line was selected as a human *in vitro* liver model; these cells are extensively used for liver toxicity studies and reported to show significant inducible levels of biotransformation enzymes (phase-I and -II enzymes, and xenobiotic transporters) (Brandon *et al*., 2006; Hewitt and Hewitt, 2004).

DE-71 caused dose- and time- dependent inhibition in cell viability, as measured in terms of overall mitochondrial dehydrogenase activity in both proliferating and non-proliferating cells. Statistically significant inhibition of cell viability was observed only at higher doses (100μM) and longer incubation times, 48-72 hours ($p<0.05$). Lower doses (1-50μM) did not induce any reduction in cell viability compared to untreated or vehicle treated groups. These results are in agreement with scientific literature that reported on PBDE toxicity in human *in vitro* models and rodents, exposed to lower doses. Individual

BDE congeners (BDE-47, -77, -99, -100, -153, -154, -183, and -209) did not induce any inhibition in cell viability (MTT assay) in human *in vitro* models (MCF-7, HepG2, and H4IIE cells) up to 0.01-10μM doses for 72 hours (Peters *et al*., 2004).

Similar results (no cytotoxicity) were obtained in human adenocarcinoma cells, following exposure to 10μM of BDE-47, or -99, or 100, or -183 (Canton *et al*., 2006). Also in another study, carp hepatocytes were exposed to individual PBDE congeners (BDE-47, -99, -100, -153 and 6-OH-BDE-47) and commercial DE-71 mixture at doses 0-7.5μM for 5 days. None of the BDEs or DE-71 mixture induced cytotoxicity at the test doses except, BDE-99 and -153 that showed decreases in cell viability at 7.5μM dose. Also an increase of 200% in MTT values compared to controls was observed in several treatment groups (Kuiper *et al*., 2004).

Our results indicated an IC_{50} (dose causing 50% inhibition in cell viability) value at 100μM for 72 hours. DE-71 showed a dose-response effect causing cytotoxicity at doses >25μM in MCF-7 cells (Feliciano and Bigsby, 2008). Also individual BDE congeners (BDE-47 and -209) reduced cell viability up to 40-50% at doses of 100μM and 72H incubation time in RTG-2 cells (Jin *et al*., 2010). DE-71 was also able to cause cytotoxicity in other human in vitro models such as neuronal cells (astrocytomas, human neuroblastoma cells etc) at much lower concentrations (IC_{50} ~ 10-15μM) (He *et al*., 2008a, b; Giordano *et al*., 2008; Shao *et al*., 2008). This might be due to differences in signalling pathways of cytotoxic effects or variation in degree of biotransformation of PBDEs. The observed cytotoxic effects only of longer incubations suggest the accumulation of potent toxic metabolites in liver cells. Since liver has greater biotransformation ability compared to other tissues, long-term exposures are likely to

cause an increase or accumulation of metabolites. Canton *et al*., (2006) observed a significant induction in cell viability due to hyroxylated PBDEs in human adenocarcinoma cells, on the contrary parent PBDEs or methylated PBDEs did not show any toxicity at similar doses (Canton *et al*., 2006). Likewise hydroxylated metabolites were detected in primary human cells exposed to BDE-99 *in vitro* (Stapleton *et al*., 2009) and authors suggest that these metabolites are more toxic than their parent PBDEs. Hydroxylated metabolites were also detected in rodents (Chen *et al*., 2006, Huwe and Smith 2007), fish (Benedict *et al*. 2007; Stapleton *et al*. 2004) and humans (Athanasiadou *et al*. 2008; Qiu *et al*. 2009; Thuresson *et al*. 2005, 2006). Cell viability results from this study and other relevant published results indicate that PBDEs upon long term exposure can result in cytotoxicity of liver cells.

On the contrary at lower doses, 10 – 50μM, DE-71 induced 20% statistically significant increase in MTT results above vehicle control ($p<0.01$). Increase in the mitochondrial dehydrogenase activity can be linked to various cellular processes, such as an increase in a number of viable cells or active cellular proliferation or increase in oxidative metabolism. Long term treatment (10 days), DE-71 was reported to produce a biphasic response curve in human breast carcinoma cells, suggesting a statistically significant increase in cell number at lower concentrations followed by a cytotoxic response at doses above 25μM (MTT assay) (Feliciano and Brigsby, 2008). This might be mainly due to internal reduced environment (increased ratios of NADPH/NADP, FADH/FAD, FMNH/FMN, and NADH/NAD) favouring the cellular proliferation process. Similar, results from PC12 cells and zebrafish exposed to low dose hydroxylated PBDEs indicate that these metabolites have the potential to increase mitochondrial

activity above control in a dose-dependent manner (Dingemans *et al*., 2008, van Boxtel *et al*. 2008). The suggested mechanism is by mitochondrial uncoupling induced by metabolites such as 6-OH-BDE-47, 6′-OH-BDE-49, and 3-OH-BDE-47. Furthermore redox-active products such as phenols/phenolic intermediates were found to increase MTT assay results by interfering with mitochondrial respiration process (Longpre and Loo 2008; Peng *et al*., 2005; Wisman *et al*., 2008). Collectively cell viability results indicate that DE-71 can cause cytotoxicity at high doses upon long term incubations, and the observed cytotoxicity could be ascribed to accumulation of oxidative metabolites over time. Low doses were responsible either for creating favourable conditions towards cellular proliferation or to produce redox metabolites by interfering with mitochondrial respiration.

Oxidative stress has been well studied largely because of human health issues linked with chronic oxidative stress. It is believed that oxidative stress leads to lipid peroxidation, oxidative damage to DNA and proteins, various metabolic diseases and carcinogenesis. Reactive intermediates resulting from oxidative stress can be formed in any tissue or organ depending on the toxicant. Lipophilic chemicals like PCBs, dioxins and PBDEs are reported to accumulate in liver. Since liver is the key toxin metabolizing site it is also vulnerable to damage. Liver toxicity is believed to be related to liver diseases as has been observed in exposed individuals. For example, severe oxidative stress is a key factor in pathogenesis of non-alcoholic fatty liver disease and obesity. Additionally lack of nutrients can aggravate this condition leading to progression of steatohepatitis (Baumgardner *et al*., 2008; Sass *et al*., 2005). Factors like induced metabolism of PBDEs, their metabolites, and reactive intermediates formed during

metabolism can cumulatively/synergistically contribute to increased release of ROS and subsequent oxidative stress.

To investigate oxidative stress induced by DE-71 mixture, we performed an assay for intracellular ROS detection through flow cytometry. This assay indicates the percent of liver cell population over-expressing intracellular ROS compared to vehicle or untreated controls. Flow cytometry results showed that DE-71 induced intracellular ROS in 10 – 100μM treatment groups over time (24-72 hours), except there was a reduction at 100μM dose for 48 hours duration. All these values were statistically significant ($p<0.01$) compared to vehicle control and maximum induction was observed in 100μM treatment group at 72 hours (39% over vehicle control).

These results were consistent with results obtained in other *in vitro* models using cells of human origin. PBDE-47 caused excess release (up to 3 fold) of ROS in jurkat cells (human T lymphocytes) between 25-100μM doses at 24-72 hour incubation time (Yan *et al*., 2011). Also PBDE-47 stimulated ROS production over time in human fetal liver hematopoietic stem cells (Shao *et al* 2007). Other sensitive cell lines of neuronal origin have also showed similar results at lower doses and incubation times (Huang *et al*., 2010; He *et al* 2008, Reistad and Mariussen, 2005). Xenobiotic metabolism of PBDEs could be the basis for the observed excess release of ROS. Metabolism studies performed in rodents showed that the liver is the target organ for accumulation of metabolites, and metabolism occurs through oxidative pathways. In addition, rats and mice treated with various lower brominated congeners resulted in detection of different hydroxylated (-OH) metabolites of tetra- and tri- brominated congeners (Sanders *et al*., 2006, Hakk and Letcher, 2003, Hakk *et al*., 2002, 2006, Marsh *et al*., 2006; Malmberg *et al*., 2005).

Recent results suggest that Cytochrome P450 mediated xenobiotic metabolism is a significant source of ROS. PBDE exposure resulted in induction of phase-I metabolism genes in various *in vitro* and rodent studies (Pacyniak *et al*., 2007; Wahl *et al*., 2008; Richardson *et al*., 2008; Szabo *et al*., 2009; Raldua *et al*., 2008). ROS assay results confirmed our hypothesis that PBDE mediated induction of xenobiotic metabolism is responsible for excess release of reactive oxygen species, and oxidative stress. Collectively the most possible mode of action of toxicity is through Phenobarbital-like interaction with PXR/CAR receptor. PBDEs can bind to PXR/CAR receptors and modulate expression of several enzymes such as Cyp3A4, Cyp2B1, UDPGTs, sulfotransferases, and GSTs. Elevated induction of these enzymes can lead to excess release of highly reactive intermediates, particularly ROS. A deficient anti-oxidant defense system inside the cell, failing in removal of excess ROS can lead to oxidative stress and related toxic effects. For example, treatment with tetra-BDEs and OH-tetra-BDEs resulted in DNA damage through ROS leading to replication blockage and subsequent chromosomal breaks in genetically modified chicken DT40 cells (Ji *et al*., 2011). The extent or severity of oxidative stress may vary from chicken to human due to the differences in genetic make-up between these two biological systems.

Many studies suggest that one of the downstream effects of oxidative stress is induction of cytotoxicity by activating apoptotic pathways (Su *et al*., 2006; Simbula *et al*, 2007; Reinecke *et al*., 2006; Zhang *et al*., 2009; De *et al*., 2010). The induction of apoptosis in DE-71 treated HepG2 cells was measured to investigate the cytotoxicity caused by apoptosis. ROS release was also measured as a measure of apoptosis produced by the exposure. The characteristic features of apoptosis include, cell shrinkage,

chromatin condensation, DNA fragmentation and the activation of cysteine specific proteases "caspases" (Lee *et al*., 2005). We have analyzed the activation of caspases and DNA fragmentation in DE-71 treated HepG2 cells. Caspase-3 enzymes play an active role in cleavage of a number of structural proteins involved in maintaining the cytoplasmic and nuclear architecture and integrity, eventually leading to cell death known as "apoptosis" (Kothakota *et al*., 1997, Rudel and Bokoch 1997). Caspase assay results showed that DE-71 significantly increased expression of caspases, up to 3-folds at 100μM ($p<0.01$). No significant increase above control was observed at doses below 50μM, except that 50μM induced a 1.7 fold increase at 48 hours incubation time. Using 50μM treatment dose, both ROS and caspase levels increased with time (24-72 hours), but didn't induce any inhibition in cell growth; however a slight decrease in mitochondrial activity (from 120% to 105%) was observed. These results show that PBDE at 100μM dose for 72 hours induced ROS activity, caspase levels and cell death. Additionally, although ROS levels increased at the beginning of this time course study, they could induce high levels of caspase activity (and apoptotic cell death) only at longer incubation times. This indicates that at longer exposure duration, cells were not able to succumb to the effects of oxidative stress and lead the cells towards apoptotic cell death. On the contrary, lower doses of oxidative stress may create conditions (such as activation of relevant signalling pathways) favourable for increased cell proliferation.

Results from MTT assay, ROS assay combined with caspase assay, suggests that the production of high levels of ROS over time induced high caspase levels that resulted in apoptotic cell death. Changes induced by low doses at any times or higher doses at shorter incubations couldn't result in cell death. We have also performed microscopic

observation of HepG2 cells exposed to highest doses and time points to study the growth patterns. Particularly cells exposed to 100μM showed different morphological features compared to control cells. Cells at 48 hours also showed inhibition in growth (isolated cells without any networks and less confluent), whereas at 72 hours, cell shrinkage and severe cell death was observed. In addition, in order to further confirm that caspases lead to apoptosis, we analyzed the DNA pattern on gel electrophoresis. Caspase-3 has an ability to cleave an inhibitor that protects DNA endonuclease responsible for cleaving chromosomal DNA specifically during apoptosis (Sakahira *et al*., 1998; Enari *et al*., 1998). The severity of DNA fragmentation is cell type specific and it may occur some times without caspase-3 activation. Only 100μM treatment group showed an apparent DNA laddering, and controls groups showed intact DNA.

V. Conclusion

This study demonstrated that DE-71 mixture has a potential to induce cytotoxicity in human liver cells through initiation of apoptotic events. The apoptotic death is possibly due to induction of ROS followed by activation of caspase pathway and DNA damage. Also our results are consistent with mechanisms suggested in the scientific literature from PBDE metabolism studies in rodents. These studies suggest that, PBDEs interact with PXR/CAR receptors and modulate expression of various CYP enzymes involved in oxidative metabolism; these metabolites can result in oxidative stress response (Dunnick and Nyska *et al*., 2009; Fery *et al*., 2009; Wahl *et al*, 2008; Bruchajzer *et al*., 2010; Albina *et al*., 2010). Epidemiological studies and experimental studies established that liver is the target organ; and PBDEs or their metabolites are predominantly accumulated in liver tissues. Increased and long-term exposure to PBDEs can lead to accumulation of

toxic metabolites such as hydroxylated metabolites, phenolic intermediates and arene epoxides. This accumulation of toxic metabolites could burden the liver cells and increases the risk for various oxidative stress and apoptosis related diseases. Since PBDEs exposure occurs throughout the life; the toxic metabolites can accumulate in human body at various life stages, from childhood to adulthood. This type of continuous and sustained exposure during a lifetime could increase the risk of increases adult disease susceptibility.

VI. Future considerations

Several scientific studies suggested that PBDEs, unlike dioxins, modulate PXR/CAR nuclear receptors. However, molecular mechanism studies elucidating this binding interaction between PBDEs and receptors are lacking. It is important and necessary to understand the differences between binding potentials of PBDEs versus their metabolites, because of the perceived risk of human toxicity from exposure to PBDE. This is, additionally, significant since some of these metabolites exert greater toxicity than parent compounds. The mechanism of toxicity and the pathway is through PXR/CARs and are believed to induce physiological changes including cholesterol metabolism, lipid metabolism, thyroid metabolism. Agonists of PXRs have affinity towards Liver X Receptor (LXR), and these receptors manage transcription of genes involved in cholesterol, bile acid, lipid, and carbohydrate homeostasis (Kalaany and Mangelsdorf 2006). In several human liver samples tested, levels of PXR and Cyp3A4 correlated with Hepatocyte Nuclear Factor 3 γ (FOXA3) (Lamba *et al*., 2010). Lowering or absence of FoxA3 levels was reported to cause decrease in glucose levels and activation of gluconegenesis in liver cells (Shen *et al*., 2001). Also agonists of FoxA3

receptors show structural similarities with that of LXR. Recent human and experimental animal studies suggested that PBDE exposure is positively correlated with various metabolic indexes such as BMI, serum cholesterol and lipolysis (Sharp D 2009; Turyk M *et al*., 2009). Hence the evaluation of LXR/FXR expression levels in treated human hepatic cells could provide further information on the role of PBDEs in modulation of hepatic metabolism and impact on metabolic diseases. Also a population based study to determine the levels of various factors including PBDE metabolites in serum, oxidative stress biomarkers and caspase activities could provide significant and relevant information. Linking this information to lifestyle and demographics will certainly help in identifying the susceptible populations who are at greater risk due to PBDE mediated adverse health effects. Further research in *in-vitro* methods and population based studies are suggested and strongly recommended to better understand the toxicity and the pathway of action of PBDE and its metabolites.

VII. References

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APPENDIX-II

Table 1: Results obtained from statistical analysis performed on the MTT Assay results (for all time and dose dependent treatments)

Mean±SEM	0µM	1µM	5µM	10µM	25µM	50µM	100µM
24H	100.0 ± 1.225	102.3 ± 1.516	104.8 ± 1.664	110.5 ± 1.356	112.2 ± 2.517	120.7 ± 1.745	96.45 ± 3.001
48H	100.0 ± 1.343	102.7 ± 2.241	107.2 ± 1.064	114.2 ± 3.495	112.4 ± 2.768	110.8 ± 2.366	68.79 ± 3.494
72H	100.0 ± 1.217	102.2 ± 1.878	109.8 ± 2.545	116.1 ± 3.315	123.2 ± 3.801	105.1 ± 4.641	48.10 ± 4.472

p-Values vs. 0µM	1µM	5µM	10µM	25µM	50µM	100µM
24H	0.2415	0.0314	<0.0001	0.0002	<0.0001	0.2852
48H	0.3153	0.0003	0.001	0.0006	0.0006	< 0.0001
72H	0.3443	0.0017	0.0001	< 0.0001	0.2991	< 0.0001

p-Values vs. 24H	48H	72H
1µM	0.9016	0.9198
5µM	0.2193	0.0494
10µM	0.3374	0.171
25µM	0.9672	0.0587
50µM	0.0027	0.0018
100µM	< 0.0001	< 0.0001

Table 2: Results obtained from statistical analysis performed on the ROS Assay results (for all time and dose dependent treatments)

Mean±SEM	0µM	5µM	10µM	50µM	100µM
24H	2.550 ± 0.3547	9.150 ± 0.7953	14.43 ± 1.086	12.45 ± 1.153	28.70 ± 1.105
48H	21.08 ± 1.277	27.43 ± 1.258	44.67 ± 2.237	46.44 ± 2.224	37.77 ± 0.8369
72H	25.73 ± 0.4973	31.23 ± 0.6613	56.93 ± 1.036	59.38 ± 1.464	64.50 ± 3.057

p-Values vs. 0µM	5µM	10µM	50µM	100µM
24H	0.1	< 0.0001	< 0.0001	< 0.0001
48H	0.0102	< 0.0001	< 0.0001	< 0.0001
72H	0.06	< 0.0001	< 0.0001	< 0.0001

p-Values vs. 24H	48H	72H
5µM	0.0213	0.6857
10µM	< 0.0001	< 0.0001
25µM	< 0.0001	< 0.0001
50µM	< 0.0001	< 0.0001
100µM	< 0.0001	< 0.0001

Table 3: Results obtained from statistical analysis performed on the Caspase Assay results (for all time and dose dependent treatments)

Mean±SEM	0µM	10µM	50µM	100µM
24H	1.125 ± 0.1118	1.125 ± 0.1091	0.9651± 0.1650	1.107 ± 0.2004
48H	1.005 ± 0.05502	1.109 ± 0.08329	1.274 ± 0.1310	2.814 ± 0.3649
72H	1.010 ± 0.05196	1.043 ± 0.07362	1.713 ± 0.07963	2.537 ± 0.2621

p-Values vs. 0µM	10µM	50µM	100µM
24H	1	0.4516	0.9374
48H	0.3209	0.0871	0.0006
72H	0.722	< 0.0001	0.0002

p-Values vs. 24H	48H	72H
10µM	0.5627	0.2472
50µM	0.214	0.0014
100µM	0.0035	0.0013