EVIDENCE ON THE CARCINOGENICITY OF

BUTYL BENZYL PHTHALATE

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Reproductive and Cancer Hazard Assessment Branch
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PREFACE

Proposition 65[1] requires the publication of a list of chemicals "known to the state" to cause cancer or reproductive toxicity. It specifies that "a chemical is known to the state to cause cancer … if in the opinion of the state's qualified experts the chemical has been clearly shown through scientifically valid testing according to generally accepted principles to cause cancer ...." The "state's qualified experts" regarding findings of carcinogenicity are the members of the Carcinogen Identification Committee (CIC) of the Science Advisory Board[2].

The lead agency for implementing Proposition 65 is the Office of Environmental Health Hazard Assessment (OEHHA) of the California Environmental Protection Agency. After consultation with the CIC, OEHHA selected butyl benzyl phthalate as a chemical for consideration for listing by the CIC. Upon selection, the public was given the opportunity to submit information relevant to the assessment of the evidence on the carcinogenicity of butyl benzyl phthalate. OEHHA reviewed and considered those submissions in preparing this document.

OEHHA developed this document with information on the possible carcinogenicity of butyl benzyl phthalate to assist the CIC in its deliberations on whether or not the chemical should be listed under Proposition 65. The original papers discussed in the document are also provided to the CIC as part of the hazard identification materials. In addition, comments on this hazard identification document received during the public comment period also form part of the hazard identification materials, and are provided to the CIC members prior to their formal deliberations.

On December 5, 2013, the CIC is scheduled to deliberate on the carcinogenicity of butyl benzyl phthalate (BBP) and determine whether the chemical has been clearly shown through scientifically valid testing according to generally accepted principles to cause cancer. A transcript of the meeting will be available at www.oehha.ca.gov after the meeting.

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[1] The Safe Drinking Water and Toxic Enforcement Act of 1986 (California Health and Safety Code 25249.5 *et seq*.)
[2] Title 27 Cal. Code of Regs. §25302

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. EXECUTIVE SUMMARY

The phthalate ester butyl benzyl phthalate (BBP) is a high-production volume chemical that is primarily used as a plasticizer to add flexibility in plastics such as polyvinyl chloride (PVC) polymers. Examples of uses of BBP are vinyl tiles, paints, inks, food packaging, medical products packaging, and personal products such as cosmetics and fragrances.

In the U.S., BBP has been detected in air (both indoor and outdoor), indoor dust, and water (both surface water and water from sewage treatment plants). In Europe, BBP has also been detected in organisms such as fish.

Monobenzyl phthalate (MBzP) and monobutyl phthalate (MBuP), the two major BBP metabolites in humans, have been routinely detected in human urine samples from the U.S. Biomonitoring studies have found that the levels of MBzP in urine from pregnant women from California to be slightly higher than the levels in U.S. females in general.

Two cancer epidemiology studies have reported results for BBP or its metabolite MBzP. Both were case-control studies of breast cancer. Neither study reported a positive association, but both studies had limitations that may have prevented them from detecting excess risk. Thus, at this time, there is very little human cancer epidemiology evidence available.

BBP has been tested in several long-term carcinogenicity studies using rats and mice exposed via feed. Treatment-related increases in tumors were observed in two-year studies conducted in male and female F344 rats. Non-significant increases in tumor types considered rare in the tissue and species of origin were observed in two-year and 32-month studies in female rats and in 30-month studies in male rats. Specifically, the following increases in tumors were observed:

Pancreatic acinar cell tumors

- In male F344 rats, the incidences of pancreatic acinar cell adenoma and combined adenoma and carcinoma were significantly increased and with significant dose-response trends in one study (NTP, 1997a). The increases in adenoma and combined adenoma and carcinoma in the high-dose group in NTP (1997a) were also statistically significant when compared to weight-matched controls (NTP, 1997b). Pancreatic acinar cell carcinomas are considered rare in untreated male F344 rats.
- In female F344 rats, the incidence of pancreatic acinar cell adenoma in one study was significantly increased (NTP, 1997a). The increase did not reach statistical

significance; however, these tumors are considered rare in untreated female F344 rats.

- In male F344 rats, the incidence of pancreatic acinar cell adenomas in a 30-month restricted feed study were increased (NTP, 1997b), though the incidence did not achieve statistical significance. The tumors were observed late in the study, and when there were reduced numbers of animals at risk for later occurring tumors.

Mononuclear cell leukemia

- In female F344 rats, the incidences of mononuclear cell leukemia and combined leukemia and lymphoma were significantly increased and with significant dose-response trends in one study (NTP, 1982).
- In male F344 rats, the incidence of mononuclear cell leukemia in the high-dose group was significantly increased compared with weight-matched controls (NTP, 1997a; 1997b).

Urinary bladder tumors and hyperplasia

- In female F344 rats, the incidence of urinary bladder transitional epithelium carcinoma (4 cases) and papilloma (2 cases) were increased as well as the incidence of urinary bladder transitional epithelium hyperplasia in a 32-month feed restriction study (NTP, 1997b). The increase in tumors did not reach statistical significance; however, these tumors are considered rare in untreated female F344 rats.
- In a second study treated F344 rats, the incidence of urinary bladder transitional epithelium papilloma was slightly increased, while the incidence of urinary bladder transitional epithelium hyperplasia was significantly increased and with a significant dose-response trend in one study (NTP, 1997a). The increase in tumors did not reach statistical significance when compared with concurrent controls; however, these tumors are considered rare in untreated female F344 rats.

Adrenal medulla tumors

- In male F344 rats, the incidence of combined benign and malignant pheochromocytoma of the adrenal medulla in the high-dose group was significantly increased compared with weight-matched controls (NTP, 1997a; 1997b).

BBP is genotoxic in multiple *in vitro* and *in vivo* studies using mammalian cells:

- DNA base lesions in mouse osteoblast cell line and primary calvarial osteoblast cells.

- DNA single strand breaks in HepG2 human hepatocellular carcinoma cells.
- DNA-protein crosslinks in rat hepatic cell homogenate.
- Mouse bone marrow cell sister chromatid exchanges (SCEs) and chromosomal abberations (CA) *in vivo*.
- DNA-protein crosslinks in mice hepatocytes *in vivo*.

BBP induced malignant cell transformation of Syrian hamster embryo cells *in vitro*. Overall, BBP was not mutagenic in *S. typhimurium* or *E. coli*, and did not induce reverse or forward mutations, SCEs, or CAs *in vitro*.

BBP has been demonstrated to be both an aryl hydrocarbon receptor (AhR) agonist and an estrogen receptor (ER) agonist, increasing cell proliferation and tumorigenicity associated with the pathways induced by those receptors, including genomic and non-genomic signal transduction pathways.

BBP disrupts male rat testicular development *in utero* through steroidogenesis perturbation and possesses antiandrogenic activity; there is a potential increased risk of testicular tumors through induction of testicular dysgenesis syndrome in humans.

BBP increased expression of several genes (e.g. *VEGF*, *MYC*, *WT1*) associated with potential carcinogenic mechanisms in human breast epithelial cells and cancer cells *in vitro*, and in mammary glands from female SD rats exposed *in utero* and during the neonatal/prepuberty period.

BBP induced cell proliferation in human breast epithelial cells and cancer cells *in vitro* and in the epithelial structures of mammary glands from female SD rats exposed *in utero* and during the neonatal/prepuberty period, suggesting potential effects on tumor growth promotion and progression.

BBP increased angiogenesis, epithelial-mesenchymal transition, migration and invasiveness in human breast epithelial cells and cancer cells *in vitro*, and tumor formation by human breast epithelial cells and cancer cells in athymic nude mice *in vivo*, suggesting potential effects on tumor growth promotion and progression, and metastasis.

2. INTRODUCTION

2.1 Identity of Butyl Benzyl Phthalate (BBP)

Butyl benzyl phthalate (BBP) is a clear, oily liquid with a slight odor and a bitter taste. It is soluble in most organic solvents, as well as dimethyl sulfoxide, acetone, and 95% ethanol (NTP, 1997a). The structure of BBP is shown in Figure 1. Some physical and chemical properties of BBP are given below.

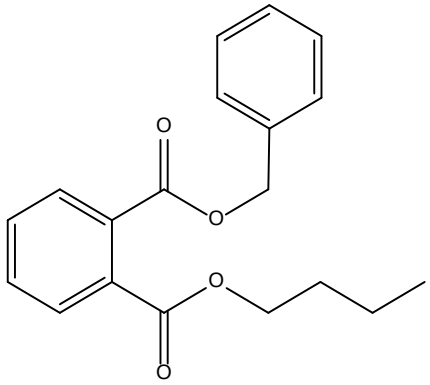


Figure 1. Chemical Structure of BBP

Molecular Formula:	$C_{19}H_{20}O_4$
Molecular Weight:	312.3597
CAS Registry Number:	85-68-7
IUPAC Name:	2-O-benzyl 1-O-butyl benzene-1,2-dicarboxylate
Synonyms:	1,2-Benzenedicarboxylic acid; butyl phenylmethyl ester; benzyl-n-butyl phthalate; phthalic acid, butyl benzyl ester; Santicizer 160; Sicol 160; Unimoll BB
Chemical Class:	Phthalate Ester
Density:	1.1 g/cm^3 (at 25°C)
Boiling Point:	370°C (at 760 mmHg)
Flash Point:	198°C
Melting Point:	<-35°C
Water Solubility:	2.8 mg/L (at 20-25°C)
Vapor Pressure:	8.55 x 10^{-6} mmHg (at 20°C)
Henry's Law Constant:	0.176 Pa-m^3/mole (at 25°C)
Log P (Octanol-water coefficient):	4.91

2.2 Occurrence, Use and Exposure

2.2.1 Production

BBP is synthesized by the reaction of the monobutyl ester of phthalic acid (PA) with benzyl chloride (Rittfeldt *et al.*, 1983). BBP is on the list of high production volume chemicals with production exceeding 1 million pounds annually in the U.S. (U.S. EPA, 1990). According to the Hazardous Substance Data Bank (HSDB) by the National Institute of Medicine (NIM), the production volume of BBP was 50-100 million pounds in 2002 (HSDB, 2009).

2.2.2 Use and Occurrence

BBP is a solvent and additive used in a number of industrial and household products, and a general purpose plasticizer. Plasticizers increase the softness and flexibility of polymeric materials. More than 70% of BBP is used as a plasticizer, mainly for polyvinyl chloride (PVC) in vinyl floor tiles, vinyl foam, carpet backing, and cellulosic resins (IARC, 1999; ECHA, 2009). Examples of other plasticizer uses of BBP include coatings for leather and textiles, calendering films, packaging medical products, and in printing inks, adhesives, sealants, and paints. Examples of non-plasticizer uses of BBP include uses as pesticide carriers, and uses in cosmetics, fragrances, munitions, industrial oils, and insect repellants (Graham, 1973; ECHA, 2009). The use of BBP in consumer products, such as food wraps, food packaging, and cosmetics has declined in recent years (ECB, 2007). As of 2007, the concentrations of BBP in baby products and children's toys were low and probably occurred as byproducts/impurities (ECB, 2007).

The manufacturing, sales or distribution in the state of California of toys and child care articles which contain certain phthalates has been illegal since January 1, 2009. The California phthalates ban, Assembly Bill 1108 (codified in California Health and Safety Code section 108935 *et seq.*), prohibits the manufacture, sale or distribution in commerce of: 1) toys and child care articles containing di(2-ethylhexyl)phthalate (DEHP), dibutyl phthalate (DBP) or BBP in concentrations exceeding 0.1%; 2) toys and child care articles intended for use by a child under three years of age containing diisononyl phthalate (DINP), diisodecyl phthalate (DIDP) or di-n-octyl phthalate (DnOP) in concentrations exceeding 0.1%, if the products can be placed in a child's mouth.

The Consumer Product Safety Improvement Act of 2008 (CPSIA) prohibited the sale in the U.S. of any "children's toy or child care article" individually containing concentrations of more than 0.1 percent of BBP as of February 10, 2009.

Throughout the stages of production, distribution and disposal, BBP can be released into air (indoor and outdoor), water, and soil (ECB, 2007). According to the California Toxic Release Inventory Program, 9 tons of BBP was emitted into the air in California in 2006 (California Air Resources Board, 2006). Measurements of BBP in outdoor air taken 100 meters from a PVC flooring factory have been reported to be as high as 400 ng/m^3 (ECB, 2007).

In the U.S., BBP has been detected in surface waters, such as the Delaware River, Mississippi River and Lake Michigan, at levels ranging from 0.2-4 µg/L (ECB, 2007). One study reported that while BBP was not detected in 5 sites of the San Francisco Bay, the concentration of BBP in the Mississippi River south of St. Louis was up to 2.4 µg/L (Gledhill *et al.*, 1980). In a study of U.S. publicly owned wastewater treatment works, BBP was detected in 34 of the 302 effluent samples, with concentrations ranging from 1 to 34 µg/L (U.S. EPA, 1982). BBP has also been detected in raw and treated drinking water (U.S. EPA, 2009).

BBP has been detected in marine sediments, soil, and biota such as some fish and invertebrates (IPCS, 1999; ECB, 2007; HSDB, 2009). The concentrations of BBP in fish and other aquatic organisms ranged from 1.47 - 2.9 ng/g lipid (HSDB, 2009).

BBP has been detected in foods, including milk (mean concentration 1.2 ng/g) (HSDB, 2009), and spices, eggs, and breakfast cereals, with levels up to 0.5 mg/kg (Wormuth *et al.*, 2006). Sources of BBP in foods include migration into the foods from its use as a plasticizer in food packaging materials.

BBP has also been detected as a contaminant of indoor dust. For example, Guo and Kannan (2011) measured the level of BBP in indoor dust from Albany, New York, and compared it to the levels in indoor dust from six Chinese cities. The median level of BBP in the dust from New York was 21.1 µg/g dry weight, compared to the median levels in the dust from the Chinese cities of 0.1-0.6 µg/g dry weight.

2.2.2 Exposure and Biomonitoring

Occupational exposure to BBP may occur during the production of BBP, the processing of BBP-containing PVC, including coatings and films, and the use of intermediate and end products (ECB, 2007). Some occupations with high exposure are auto repair shops and general building contractors. The most common routes of occupational exposure are inhalation and dermal. The National Institute for Occupational Safety and Health (NIOSH) estimated in the National Occupational Exposure Survey (NOES) (1981-1983) that approximately 331,000 workers in the U.S. were exposed to BBP (NIOSH, 1998).

For the general population, exposure to BBP and its metabolites can come from consumer products such as food and food packaging, cosmetics such as nail polish, fragrances, medical devices, baby products, children's toys (ECB, 2007) as well as from indoor dust (Guo and Kannan, 2011). Based on these data and the total daily BBP intake calculated from urinary levels of monobenzyl phthalate (MBzP), the principal BBP metabolite in humans, estimates were made of the contributions for different exposure routes to the total daily intake. Dietary intake was estimated to be the major source of BBP exposure in the U.S. population in all age groups, accounting for over 58% of exposure from all routes. Dermal absorption of BBP-containing dust was estimated to be another important route of BBP exposure in infants and toddlers (approximately 40% contribution).

Several biomonitoring studies conducted in the U.S. have assessed exposure to BBP in the general population as well as in specific populations, such as infants, pregnant women and firefighters (Table 1). Urinary levels of the BBP metabolite MBzP have been used as a biomarker for BBP in biomonitoring studies. Table 1 presents the results from a number of biomonitoring studies.

Table 1. BBP Biomonitoring studies in the U.S.

Groups Studied	Sample Collection Year	Gender	Age	Urinary MBzP Concentration[1]		Sample Size	References
				µg/L	µg/g creatinine		
U.S. population from NHANES[2]	2009-2010	F, M	6-11	11.6[a]	15.1	415	CDC, 2013
		F, M	12-19	10.6[a]	8.54	420	
		F, M	>20	5.61[a]	5.94	1914	
		F	All	6.04[a]	7.29	1350	
		M	All	6.93[a]	6.21	1399	
		F, M	All	6.46[a]	6.74	2749	
Infants/toddlers from Imperial County, CA	2000	F, M	12-18 mo	20.2[b] (range 5.4-316)	NA	19[3]	Brock *et al.*, 2002
Infants from NICUs	2005	F, M	neonates	43[a]	NA	54	Weuve *et al.*, 2006
Men from Boston	1999-2003	M	18-54	7.5[a,4]	NA	295	Duty *et al.*, 2005
Pregnant women[5] Various US locations	1999-2002	F	NA	9.3[b] (maximum 436)	11.7 (maximum 364)	214	Marsee *et al.*, 2006
Pregnant women from New York[6]	1999-2006	F	18-35	19.0[a] (range <LOD-1110)	NA	319	Whyatt *et al.*, 2012
Pregnant women from New Jersey	2009	F	33[7]	9.2[b] (range 1.0-181.3)	NA	34	Yan *et al.*, 2009
California firefighters[8]	2010-2011	NA	NA	8.18[a]	NA	101	Biomonitoring California, 2013a
Pregnant women from San Francisco area[9]	2010-2011	F	NA	7.73[a]	NA	89	Biomonitoring California, 2013b

F, female. M, Male. NA, not available.
[1] Geometric mean (a) or median (b). Some values are presented as µg/g corrected for creatinine.
[2] National Health and Nutrition Examination Survey (NHANES).
[3] Twelve children had two urine samples for analysis, and seven children had one sample.
[4] Adjusted for specific gravity.
[5] Recruited from Los Angeles, California; Minneapolis, Minnesota; and Columbia, Missouri.
[6] Part of the Columbia Center for Children's Environmental Health (CCCEH) cohort.
[7] Median. 25th percentile, 30; 75th percentile, 35.
[8] Firefighters Occupational Exposures (FOX) Project, conducted by UC Irvine and a Southern California fire department.
[9] Part of the Maternal and Infant Environmental Exposure Project (MIEEP).

Koo *et al.* (2002) estimated daily exposures to BBP in the U.S. from urinary MBzP based on an assumption that 36% of total absorbed BBP is excreted in the urine as MBzP. Using this methodology, these authors estimated that the median daily level of exposure to BBP in the general U.S. population is 0.85 µg/kg/day.

As discussed by Koo *et al.* (2002), exposure to BBP in the U.S. has been shown to be related to socio-economic factors. Families with monthly income less than $1500 had higher levels of BBP compared to families with higher incomes; families with high school education or less had higher levels of BBP than families with education beyond high school.

Figure 2 presents information on the trends over time in BBP exposure of U.S. males and females from 1999-2010, based on the U.S. Centers for Disease Control and Prevention's National Health and Nutrition Examination Survey (NHANES) biomonitoring measurements of urinary MBzP (CDC, 2013).

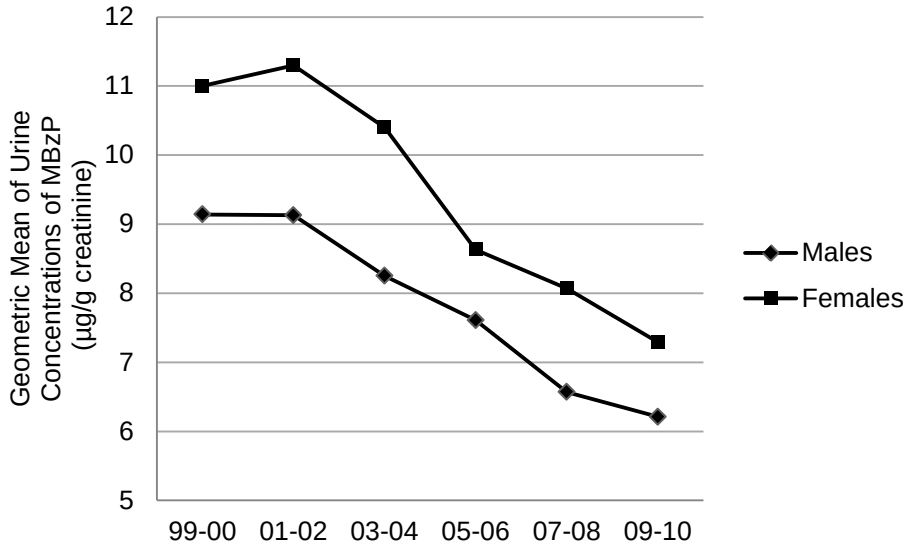


Figure 2. Geometric means of urine concentrations of MBzP (creatinine corrected) in the U. S. population from 1999 to 2010. (N > 1200) Data adapted from (CDC, 2013).

3. DATA ON CARCINOGENICITY

3.1 Carcinogenicity Studies in Humans

Two articles were identified as having cancer epidemiology study results for BBP or its metabolite MBzP (Aschengrau *et al.*, 1998; Lopez-Carrillo *et al.*, 2010)[3].

Aschengrau et al., 1998

The stated purpose of the Aschengrau *et al.* (1998) study was to describe the relationship between occupational exposure to estrogenic chemicals and breast cancer incidence in Cape Cod, Massachusetts. The investigators conducted a population-based, case-control study that estimated past occupational exposures to BBP and 17 other xenoestrogens based on lifetime work histories obtained by interview. The investigators did not estimate non-occupational exposures to the chemicals, as the analysis was exploratory. The 18 chemicals were selected for study because of 1) positive results of the chemical in an E-screen assay for estrogenicity (Soto *et al*., 1995), and 2) exposure data for the chemical were available in the NOES 1981–1983 database of potential occupational exposures.

Cases were women with incident breast cancer diagnosed from 1983 through 1986 (n=261), and controls were selected from the general population (n=753). Lifetime work histories for the women (or their next-of-kin if deceased) were obtained by interview and were converted to standard occupation codes for linkage to the NOES database. All exposure assessments were performed by an industrial hygienist without knowledge of case/control status. Exposure estimates were further refined by the industrial hygienist with additional data on production of the chemicals over time and the types of commercial products containing them. BBP was found to be associated with 51 job titles among the subjects.

Odds ratio results for probable past occupational BBP exposure, summarized in Table 2 below, indicated that there were no significant associations with breast cancer risk. The investigators additionally stated that "*no dose-response trend was seen with duration of exposure to butyl benzyl phthalate (data not shown)*."

[3] Articles were identified in the PubMed database with the following search string: "butyl benzyl phthalate" OR "butylbenzyl phthalate" OR butylbenzylphthalate OR bbp OR bbzp OR "mono benzyl phthalate" OR "monobenzyl phthalate" OR monobenzylphthalate OR mbzp OR "mono-n-butylphthalate" AND (epidemiology OR epidemiologic* OR cohort* OR control OR controls OR mortality OR incidence OR rate OR rates OR hazard OR odds OR risk OR ratio OR ratios) AND (cancer OR cancers OR malignancy*).

Table 2. Odds ratio estimates for past occupational exposure to BBP and breast cancer in Aschengrau *et al.* (1998)

Exposure Definition	Cases (n=261) exposed (%)	Controls (n=753) exposed (%)	Odds Ratio* (95% CI)
Any probable occupational exposure to BBP	26 (10.0%)	100 (13.3%)	0.7 (0.4–1.2)
BBP the only probable xenoestrogen	4 (1.5%)	14 (1.9%)	0.9 (0.3–2.9)
BBP plus other probable xenoestrogens	22 (8.4%)	86 (11.4%)	0.7 (0.4–1.2)

*Calculated with unconditional logistic regression. Adjusted for age at diagnosis, vital status at interview, family history of breast cancer, age at first birth, and personal history of prior breast cancer or benign breast disease.

The investigators acknowledged that the study was exploratory and that a limitation was lack of data on non-occupational exposures to the chemicals. Another limitation was that the study included deceased cases and corresponding deceased controls that had less accurate next-of-kin interviews. It is difficult to assess the extent of this limitation because the article did not provide the number or percent of interviews that were with next-of-kin. It did say that *"many cases were deceased or elderly at the start of the study,"* and that controls for deceased cases "*were selected randomly from a roster of all deaths among town residents.*"

Lopez-Carillo et al., 2010

Lopez-Carillo *et al.* (2010) conducted a hospital-based case-control study of breast cancer occurring in 2007 and 2008 at 25 tertiary hospital units in Northern Mexico, which were said to cover 90% of the study area's population. The investigators included 233 patients with histopathologically confirmed breast cancer, age 18+, without other cancer history, and with a residency period of >1 year in the study area. Controls were 221 healthy women matched by age (± 5 years) and place of residency that were identified through a master sample framework used in national health surveys, probabilistic selection of homes, and choosing one participant randomly when more than one eligible woman was available in a home.

First morning void urine samples from the cases were obtained after diagnosis but before any kind of cancer treatment (including surgery and radiation therapy) was performed. The samples were analyzed for MBzP and other phthalates.

The odds ratios in the study (shown in Table 3) showed significant negative associations with levels of MBzP and breast cancer among all subjects and among subjects who were premenopausal at the time of diagnosis.

Table 3. Adjusted odds ratios for MBzP urinary concentrations and breast cancer by menopausal status in Lopez-Carillo *et al.* (2010)

MBzP tertile (µg/g creatinine)	**Menopause Status at Time of Breast Cancer Diagnosis**					
	All		**Premenopause**		**Postmenopause**	
	Cases/ controls	**OR* (95% CI)**	**Cases/ controls**	**OR* (95% CI)**	**Cases/ controls**	**OR* (95% CI)**
0–5.18	113/74	Reference	44/21	Reference	69/53	Reference
5.19–10.79	68/74	0.60 (0.37–0.98)	26/27	0.36 (0.15–0.89)	42/47	0.71 (0.38–1.30)
10.80–58.62	50/73	0.46 (0.27–0.79)	17/26	0.22 (0.08–0.61)	33/47	0.61 (0.31–1.19)
Trend test p-value		0.008		0.006		0.169

*Calculated with unconditional logistic regression. Adjusted for current age, age of menarche, parity, and other phthalate metabolites

The main limitation of the Lopez-Carillo *et al.* study, which was acknowledged by the investigators, was that a single urine sample after cancer diagnosis does not allow evaluation of past exposures. The investigators said that the odds ratios assume that the urinary phthalate concentrations observed predicted their steady-state concentrations over time if the use of personal care products was fairly constant over time.

In summary, neither of the two studies that directly considered BBP exposure (Aschengrau *et al.*, 1998; Lopez-Carillo *et al.*, 2010) reported a statistically significant positive association with cancer risk. One of the studies (Lopez-Carillo *et al.*, 2010) reported a statistically significant negative dose-response trend between urinary MBzP levels and breast cancer risk.

3.2 Carcinogenicity Studies in Animals

A review of the carcinogenicity of BBP in experimental animals identified six dietary studies in Fischer 344 (F344) rats, two dietary studies in $B6C3F_1$ mice, one intraperitoneal (i.p.) injection study in Strain A mice, and two co-carcinogenicity studies in rats (one in Sprague-Dawley (SD) rats and one in F344 rats). These studies are listed in Table 4 by species, strain, and route of administration, and each is presented briefly below. Statistically significant or biologically important tumor findings are summarized in the text.

Table 4. Overview of BBP animal carcinogenicity studies

Study No.	Species	Strain	Sex (M, F)	Route of Administration	Reference
1	Rat	F344/N	M	feed	(NTP, 1982)
2			F		
3	Rat	F344/N	M	feed	(NTP, 1997a)
4			F		
5	Rat	F344/N	M	feed (with diet restrictions)	(NTP, 1997b)
6			F		
7	Mouse	$B6C3F_1$	M	feed	(NTP,1982)
8			F		
9	Mouse	Strain A	M	i.p. injection	(Theiss *et al.*, 1977)
10	Rat	SD	F	gavage	(Singletary *et al.*, 1997)[1]
11	Rat	F344	M	feed	(Kohno *et al.*, 2004)[1]

[1]Co-carcinogenicity study with a known carcinogen.

3.2.1 Studies in Rats

<u>Two-year feeding studies in F344 rats (NTP, 1982)</u>

Male and female F344/N rats (50/sex/group) were fed diets (Wayne Lab Blox Anima Feed) containing BPP (97.2 ± 1.1% purity for weeks 1-34, and 98.2% purity for weeks 35-105) at concentrations of 0, 6000, or 12,000 parts per million (ppm). These were equivalent to doses of 0, 240, or 500 mg/kg body weight for male rats, and 0, 300, or 600 mg/kg body weight for female rats. The feed was available to the animals *ad libitum* and changed once per week.

The male rat study was terminated at 30 weeks due to early deaths caused by unexplained internal hemorrhaging. Because of the early termination of the study, no histopathology was conducted on the animals. This study will not be considered further.

The survival rates in the female rats were 31/50 (62%) in the control group, 29/50 (58%) in the low-dose group, and 32/50 (64%) in the high-dose group by the end of the study. Mean body weights and feed consumption rates were somewhat reduced in BBP treated females (e.g., mean body weights in the low- and high-dose groups were 7.0% and 8.2% lower, respectively, than controls at 52 weeks, and 5.5% and 4.3% lower, respectively, at study termination).

In the study in female rats, both benign and malignant tumors were observed. As shown in Table 5, the incidence of mononuclear cell leukemia (MNCL) in female rats receiving 12,000 ppm BBP was significantly higher than in the control group ($p = 0.017$) and there was a significant dose-related trend. The incidence of MNCL was also significantly higher ($p = 0.008$) than the test laboratory historical control rate for "all leukemias" of 19% (range 12-24%; 1977 - 1982).

Increases in liver neoplastic nodules (now referred to as hepatocellular adenomas by NTP), hepatocellular adenomas and carcinomas (combined), and pituitary adenomas were also seen in BBP-treated female rats, although these increases did not reach statistical significance (Table 5).

Table 5. Tumor incidence[1] in female F344/N rats fed diets containing BBP and observed for 103 weeks (NTP, 1982)

Tumor Site & Type		Dose (ppm)			Trend test[2]
		0	6000	12,000	
Hematopoietic System	Mononuclear Cell Leukemia (MNCL)	7/44 (15.9%)	7/46 (15.2%)	18/48* (37.5%)	p < 0.01
	Leukemia or Lymphoma	7/44 (15.9%)	7/46 (15.2%)	19/48* (39.6%)	p < 0.01
Liver	Hepatocellular Adenoma	1/34 (2.9%)	1/34 (2.9%)	3/38 (7.9%)	NS
	Hepatocellular Adenoma or Carcinoma	1/34 (2.9%)	1/34 (2.9%)	4/38 (10.5%)	NS
Pituitary	Adenoma	20/41 (48.8%)	21/39 (53.8%)	26/39 (66.7%)	NS

Fisher pairwise comparison with controls: * $p < 0.05$

[1] Tumor incidence is shown as number of animals with tumor per number of animals alive at time of first occurrence of tumor.

[2] p-values from exact trend test conducted by OEHHA. NS, not significant ($p > 0.05$)

Two-year feeding studies in F344 rats (NTP, 1997a)

Male and female F344/N rats (50/sex/group) were fed NIH-07 diet containing BBP (purity ≥ 99%) at concentrations of 0, 3000, 6000, and 12,000 ppm (equivalent to 0, 120, 240, and 500 mg/kg body weight) for male rats and 0, 6000, 12,000, and 24,000 ppm (equivalent to 0, 300, 600, and 1200 mg/kg body weight) for female rats. Feed and water were available *ad libitum* and dosing lasted 104 weeks for male rats and 105 weeks for female rats.

The survival rates of the treated groups and the control groups were similar in both studies. The mean body weight for male rats in the high-dose group was 6% lower than that of the controls at 10 weeks, 8% lower at 57 weeks, and 6% lower than controls at 101 weeks. The mean body weight for female rats in the high-dose group was 5% lower than controls at 13 weeks, 22% lower at 57 weeks, and 27% lower at 101 weeks. The other dosed groups showed similar mean body weights as compared to their respective control groups.

Both benign and malignant tumors of the pancreas were seen among male rats treated with BBP (Table 6). There were statistically significant increases in the incidences of pancreatic acinar cell adenoma and of pancreatic acinar cell adenoma and carcinoma (combined) in male rats receiving 12,000 ppm BBP, compared to the controls. Significant dose-related trends in incidence were also observed ($p < 0.01$). Moreover, the incidence of pancreatic acinar cell adenoma and carcinoma (combined) in the 12,000 ppm dose group (11/44, 25%) was well above the range of historical control incidence for pancreatic acinar cell adenoma in F344 rats in NTP feed studies (1.6%, range 0-10%). Furthermore, pancreatic acinar cell carcinoma is rare in F344 male rats, having never been observed in untreated male F344 rats in NTP studies (Haseman *et al.*, 1990; Haseman *et al.*, 1998). A dose-related increase ($p < 0.05$) in pancreatic acinar cell hyperplasia was also observed in treated males (4/50, 7/49, 9/50, 12/50 for control, low-, mid-, and high-dose groups, respectively).

A slight increase in the incidence of hepatocellular adenoma and carcinoma (combined) was seen in high-dose males (4/48 versus 2/47 in controls); this increase did not reach statistical significance.

Table 6. Tumor incidence[1] in male F344/N rats fed diets containing BBP and observed for two years (NTP, 1997a)

Tumor Site & Type		Dose (ppm)				Trend test[2]
		0	3000	6000	12,000	
Pancreas	Acinar Cell Adenoma	3/41 (7.3%)	2/40 (5.0%)	3/46 (6.5%)	10/44* (22.7%)	p < 0.01
	Acinar Cell Adenoma or Carcinoma	3/41 (7.3%)	2/40 (5.0%)	3/46 (6.5%)	11/44* (25.0%)	p < 0.01

Fisher pairwise comparison with controls: * p < 0.05

[1] Tumor incidence is shown as number of animals with tumor per number of animals alive at time of first occurrence of tumor.

[2] p-values from exact trend test conducted by OEHHA.

Among the female rats treated with BBP, increases in benign tumors of the pancreas and urinary bladder were observed (Table 7). Pancreatic acinar cell adenoma is rare in female F344 rats, and the increased incidence of these tumors in females receiving 24,000 ppm BBP, although not statistically significantly different from controls, was considered to be treatment-related by NTP. NTP (1997a) reported the historical control incidence for pancreatic acinar cell adenoma and combined adenoma and carcinoma in female F344 rats in two-year feed studies as 0.2% (range 0 - 4%) and 0.3% (range 0 - 4%), respectively. In comparing the pancreatic tumor response to BBP in the female rat study with the stronger response seen in the male rat study, NTP (1997a) stated that the observed gender difference in susceptibility appeared to be consistent with results of studies by Lhoste *et al.* (1987a), Lhoste *et al.* (1987b) and Longnecker and Sumi (1990) which showed that testosterone is stimulatory and estrogen is inhibitory for growth of pancreatic acinar cell neoplasms in rat models.

A statistically significant dose-response trend ($p < 0.01$) in the incidence of urinary bladder transitional epithelium hyperplasia was observed in the female rats (Table 7). The severity of these lesions was greater in the high-dose group (mild to moderate) compared to controls (minimal to mild). The 4.1% incidence rate of transitional epithelium papilloma in the high-dose group exceeded the historical control range (0 – 2%) for NTP two-year F344 rat feed studies. Hyperplasia and papilloma of the urinary bladder represent a morphological and biological continuum in the progression of the proliferative lesions. Studies have shown that in the urinary bladder, papillary or nodular hyperplasia precedes the development of papilloma and carcinoma, and is considered a pre-neoplastic lesion (Fukushima *et al.*, 1982; Fukushima and Wanibuchi, 2000). Most urinary bladder hyperplasias and papillomas do not progress to highly malignant carcinomas within two years, the standard duration of NTP carcinogenicity bioassays (Jokinen, 1990).

A small increase in combined hepatocellular adenoma and carcinoma was observed in high-dose females (2/29 compared to 0/25 in the control group), which did not reach statistical significance. The increased incidence (6.8%) was only slightly above the range of historical control incidence for these tumors in female F344 rats in two-year feed studies (0.7%, 0 – 6%) (Haseman *et al.*, 1998).

A negative trend of mammary gland fibroadenoma ($p < 0.001$) and a significantly lower incidence in the high-dose group ($p < 0.001$) were observed in BBP-exposed female rats. NTP attributed the decrease to the lower body weights in the exposed groups.

Table 7. Tumor incidence[1] in female F344/N rats fed diets containing BBP and observed for two years (NTP, 1997a)

Tumor Site & Type		Dose (ppm)				Trend test[2]
		0	6000	12000	24,000	
Pancreas	Acinar Cell Adenoma[3]	0/37 (0)	0/37 (0)	0/37 (0)	2/35 (5.7%)	NS
Urinary bladder	Transitional Epithelium Hyperplasia[4]	4/49 (8.2%)	0/49 (0)	1/49 (2.0%)	10/50 (20.0%)	$p < 0.01$
	Transitional Epithelium Papilloma	1/48 (2.1%)	0/47 (0)	0/49 (0)	2/49 (4.1%)	NS

[1] Tumor incidence is shown as number of animals with tumor per number of animals alive at time of first occurrence of tumor.

[2] p-values from exact trend test conducted by OEHHA. NS, not significant ($p > 0.05$)

[3] Though not statistically significant, these tumors, which are rare in female rats, were considered to be treatment-related by NTP.

[4] Number of animals with hyperplasia/number of animals examined at the site.

Non-neoplastic findings

At the 15-month interim evaluation relative liver and kidney weights were significantly increased ($p < 0.01$) in the high-dose groups of both the male and female rat studies.

Two-year and three-year feeding studies with dietary restrictions in F344 rats (NTP, 1997b)

These studies were designed to examine the effect of dietary restriction on the toxicity and carcinogenicity of BBP in F344/N rats, and to evaluate the sensitivity of the bioassay with the use of weight-matched controls. Notably, these studies shared some of the groups of rats from the NTP (1997a) two-year feeding studies.

Male and female F344/N rats (60 per dose group) were fed with NIH-07 open formula containing BBP (purity ≥ 99%) at different concentrations. Feed was given either *ad libitum* (data presented in NTP, 1997a) or in restricted quantities. Ten animals from each group were sacrificed and evaluated at 15 months. The rest were kept on their studies for either two years, or three years (or sacrificed when survival of any group was reduced to 20%). The general design of the feed studies is shown in Figure 3 for male rats and Figure 4 for female rats. Four protocols were used for each gender, and four comparisons were made between the high-dose group and the corresponding control group under that protocol.

The results from studies in males and females conducted using the first protocol, where control and treated animals were fed *ad libitum*, were presented in NTP (1997a), have been discussed above (See Tables 6 and 7), and are referred to in Figures 3 and 4 as comparison 1.

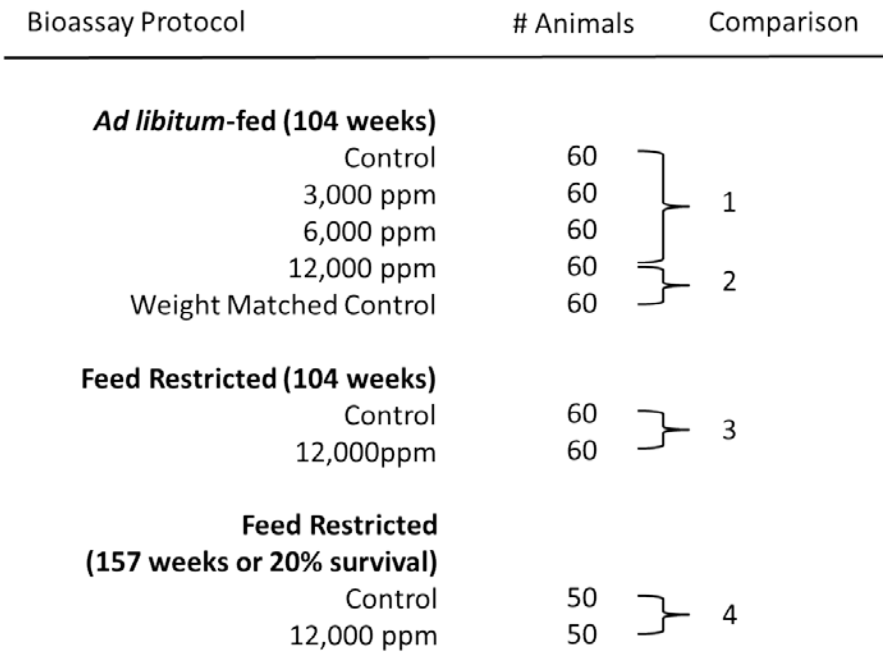
In the studies in males and females conducted under the second protocol, weight-matched controls were given feed in restricted quantities so that their average body weights matched those of the high-dose group in the respective study (i.e., male, female). For each study, comparisons were made between the tumor incidence observed in the weight-matched control groups and the high-dose group (weight-matched comparison, referred to in Figures 3 and 4 as comparison 2).

In the studies in males and females conducted under the third protocol, one control and one BBP-treated (high-dose) group were offered a restricted amount of feed that limited the mean body weight of the control group to approximately 85% that of the *ad libitum*-fed controls under the first protocol. These animals were on test for 2 years.

In the studies in males and females conducted under the fourth protocol, one control and one BBP-treated (high-dose) group were offered a restricted amount of feed, as under the third protocol, but the animals were on test for 3 years or until survival in either group was reduced to 20%.

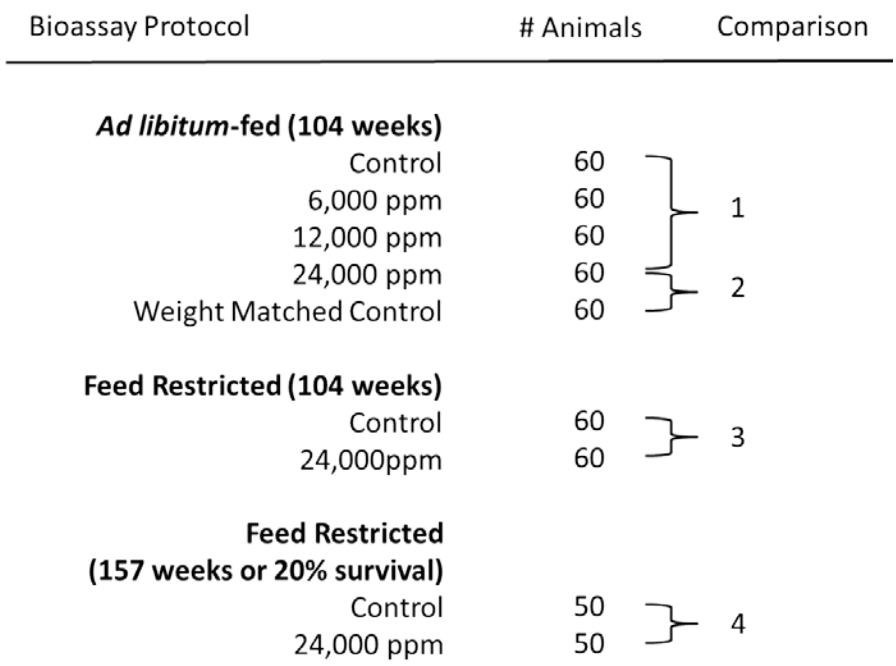
The mean body weights of the control and high dose groups in these series of studies are summarized in Table 8.

Figure 3. Design of feed restriction studies in male F344/N rats (adapted from NTP, 1997b)



Note: The male rats from the *ad libitum*-fed control and 12000 ppm groups were the same animals as those in NTP (1997a). The final number of animals/group was 50. Ten male rats from each group of comparisons 1, 2, and 3 were sacrificed and evaluated at 15 months.

Figure 4. Design of feed restriction studies in female F344/N rats (adapted from NTP, 1997b)



Note: The female rats from the *ad libitum*-fed control and 24000 ppm groups were the same animals as those in NTP (1997a). The final number of animals/group was 50. Ten female rats from each group of comparisons 1, 2, and 3 were sacrificed and evaluated at 15 months.

Table 8. Summary of average animal body weights in control and high-dose groups from BBP feed-restriction studies in F344 rats (NTP, 1997b)

Male rats	**NTP (1997a) controls, weight-matched controls, and NTP (1997a) high-dose groups (Comparisons 1 and 2)**			**2-Year Restricted Feed (Comparison 3)**		**30-Month[1] Restricted Feed (Comparison 4)**	
	Ad libitum-fed control	Weight-matched control	12,000 ppm BBP	Control	12,000 ppm BBP	Control	12,000 ppm BBP
Body weights (g)[2]	417	377	379	355	336	363	340
Female rats	**NTP (1997a) controls, weight-matched controls, and NTP (1997a) high-dose groups (Comparisons 1 and 2)**			**2-Year Restricted feed (Comparison 3)**		**32-Month[1] Restricted Feed, (Comparison 4)**	
	Ad libitum-fed control	Weight-matched control	24,000 ppm BBP	Control	24,000 ppm BBP	Control	24,000 ppm BBP
Body weights (g)[2]	225	203	199	187	175	189	175

[1] Survival fell to 20% at 30 months (males) and 32 months (females)

[2] Average weekly mean body weight for weeks 14 through 52

As discussed earlier, the survival rates of the BBP-treated groups (low-, mid-, and high-dose) and the *ad libitum*-fed control groups were similar in the male and female rat studies reported in NTP (1997a). However, when compared to the survival of the male and female weight-matched controls in NTP (1997b), the survival rates of the NTP (1997a) high-dose males and females were significantly lower. Mean body weights in the high-dose groups and the weight-matched control groups were about 10% lower than those of the *ad libitum*-fed control groups for both sexes (Table 8).

In the 2-year restricted feed studies and the 30- and 32-month restricted feed studies, survival rates were similar in both male and female rats in the respective BBP-treated and control groups. In the 2-year and the 30-month restricted feed studies in males, the mean body weights of the treated animals were lower than the respective controls (5.4% and 6.3% lower, respectively). Similarly, in the 2-year and the 32-month

restricted feed studies in females, the mean body weights of the treated animals were lower than the respective controls (6.4% and 7.4% lower, respectively).

When the incidences of pancreatic acinar cell adenoma and combined adenoma and carcinoma in the NTP (1997a) BBP high-dose males were compared with the incidences in weight-matched controls NTP (1997b), the increases were statistically significant (Table 9). The absence of pancreatic acinar cell tumors in the weight-matched controls is consistent with observations by NTP that the incidence of these tumors in untreated F344 rats is associated with body weight (increasing with higher body weight) (Haseman and Rao, 1992). Research has shown that calorie restriction significantly inhibits post-initiation pancreatic carcinogenesis in rats (Roebuck *et al.*, 1993). The incidence of pancreatic acinar cell hyperplasia in the NTP (1997a) BBP high-dose males was also increased compared to the weight-matched controls ($p < 0.01$) (NTP, 1997b).

Additional findings made when comparing tumor incidences in the NTP (1997a) BBP high-dose males with the incidences in weight-matched controls NTP (1997b), but not observed in comparisons with the *ad libitum*-fed controls (NTP, 1997a), include the following:

- When the incidences of combined benign and malignant pheochromocytoma of the adrenal medulla in the NTP (1997a) BBP high-dose males were compared with the incidences in weight-matched controls NTP (1997b), the increases were statistically significant ($p < 0.05$) (Table 9). However, the incidence was considerably lower than the NTP historical incidence in untreated controls, which is 33.7% (range 12-63%). For this reason, NTP concluded that this increased incidence of combined benign and malignant pheochromocytoma of the adrenal gland was not likely to be treatment-related.
- When the incidences of mononuclear cell leukemia in the NTP (1997a) BBP high-dose males were compared with the incidences in weight-matched controls NTP (1997b), the increases were statistically significant ($p < 0.01$) (Table 9).

Table 9. Tumor incidence[1] in male F344/N rats fed diets containing BBP for two years (NTP, 1997a): Comparisons with *ad libitum*-fed (NTP, 1997a) and weight-matched controls (NTP, 1997b)

Tumor Site & Type		Dose (ppm)		
		***Ad Libitum*-fed Control[2]**	**Weight-Matched Control**	**12,000 ppm[3]**
Pancreas	Acinar Cell Adenoma	3/41* (7.3%)	0/44*** (0)	10/44 (22.7%)
	Acinar Cell Adenoma or Carcinoma	3/41* (7.3%)	1/45** (2.2%)	11/44 (25.0%)
Adrenal Medulla	Benign or Malignant Pheochromocytoma	10/44 (22.7%)	3/45* (6.7%)	10/45 (22.2%)
Hematopoietic System	Mononuclear Cell Leukemia (MNCL)	31/50 (62.0%)	15/50** (30.0%)	30/50 (60.0%)

Fisher pairwise comparison between the 12,000 ppm group and either *ad libitum*-fed control or weight-matched control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

p-values for pairwise comparison with controls are indicated in the control columns.

[1] Tumor incidence is shown as number of animals with tumor per number of animals alive at time of first occurrence of tumor in any of the treatment or control groups.

[2] Same animals as presented in Table 6 (NTP, 1997a).

[3] Same animals as presented in Table 6 (NTP, 1997a).

In the male rats in the two-year restricted feed study, no treatment-related increases in tumors were observed; however, three cases of pancreatic acinar cell hyperplasia occurred in the 12,000 ppm BBP group versus none in the controls.

In the male rats in the 30-month restricted feed study, there were three pancreatic acinar cell adenoma and two pancreatic acinar cell hyperplasia cases in the 12,000 ppm BBP group, and no cases of either hyperplasia or adenoma in the controls (Table 10).

Table 10. Tumor incidence[1] in male rats fed diets containing BBP for 30 months: feed restriction protocol (NTP, 1997b)

Tumor site	Tumor type	Results[2]	
		Control	**12,000 ppm**
Pancreas	Acinar Cell Adenoma	0/10 (0)	3/13 (23.1%)

[1] Tumor incidence is shown as number of animals with tumor per number of animals alive at time of first occurrence of tumor.

[2] The denominators are relatively small because tumors were observed late in the study (study day 897).

When the incidences of tumors in the NTP (1997a) BBP high-dose females were compared with the incidences in weight-matched controls NTP (1997b), no treatment-related increases in tumors were observed. However, an increased incidence of hyperplasia of the urinary bladder transitional epithelium was observed in the NTP (1997a) BBP high-dose females (10/50) as compared to the weight-matched controls (0/50) (NTP, 1997b).

In the female rats in the two-year restricted feed study, two exposed females and no control females developed urinary bladder transitional cell papilloma. Transitional epithelium hyperplasia was statistically significantly increased in treated females (14/50) as compared to controls (0/50) ($p < 0.01$).

In the female rats in the 32-month feed restricted study, there were increases of urinary bladder papilloma or carcinoma (Table 11) and although the increases did not reach statistical significance, NTP considered these biologically important because urinary bladder transitional papilloma is generally considered to be rare in untreated F344 rats and no carcinomas had been observed in untreated female F344 historical controls. A treatment-related increase in transitional epithelium hyperplasia ($p < 0.001$) was also observed.

Table 11. Tumor incidence[1] in female F344/N rats fed diets containing BBP for 32 months: feed restriction protocol (NTP, 1997b)

Tumor Site & Type		Results	
		Control	24,000 ppm
Urinary bladder	Transitional Epithelium Hyperplasia[2]	0/50 (0)	16/50*** (32.0%)
	Transitional Epithelium Papilloma or Carcinoma	1/39 (2.6%)	6/45[3] (13.3%)

Fisher pairwise comparison: *** $p < 0.001$

[1] Tumor incidence is shown as number of animals with tumor per number of animals alive at time of first occurrence of tumor.

[2] Number of animals with hyperplasia/number of animals examined.

[3] Two papillomas and four carcinomas.

Non-neoplastic findings

In the two-year feed restriction studies in male and female rats, relative liver and kidney weights were significantly higher at the 15-month interim evaluation in the BBP treated groups compared to the respective controls. In addition, absolute mean kidney weight was significantly increased in BBP treated males as compared to controls in the two-year feed restriction studies.

Short-term co-carcinogenicity gavage study in female SD rats (Singletary *et al*., 1997)

This study examined the effect of BBP on mammary gland carcinogenesis in female SD rats induced by 7,12-dimethylbenz[a]anthracene (DMBA). Female SD rats (27/group) were given BBP by gavage (in corn oil, at 43 days of age) at doses of 250 or 500 mg/kg/day for seven days before gavage administration of 31 mg/kg DMBA. Controls were given corn oil instead of BBP prior to DMBA. There was no significant difference in the mean body weights between groups. The animals were sacrificed at week 15 and examined for mammary tumors. The incidences of palpable mammary tumors per rat, and mammary adenocarcinomas per rat were all significantly reduced in the female rats exposed to BBP and DMBA, as compared to rats exposed solely to DMBA. It is worth noting that the rats were sexually mature and past the rapid-growth phase of mammary gland development when exposed to BBP.

Co-carcinogenicity feeding study (40 weeks BBP-containing feed + 20 weeks control feed) in male F344 rats (Kohno *et al.*, 2004)

This study examined the effect of BBP on prostate carcinogenesis in male rats induced by 3,2'-dimethyl-4-aminobiphenol (DMAB). Five-week old male F344 rats (16/group) were given subcutaneous injections of 25 mg/kg DMAB every other week for 20 weeks. Starting one week after the first injection, two groups of rats were given feed containing either 10 ppm or 100 ppm BBP for 40 weeks. The control group was given injections of DMAB and received regular feed throughout the study. All animals were sacrificed at week 60. No change in the daily food intake was observed in any of the groups and the mean body weights in the BBP treated groups were similar to those of the controls. The incidence of prostate intraepithelial neoplasm (PIN) for the rats treated with both DMAB and 10 ppm or 100 ppm BBP was lower than that for the rats treated with DMAB only. The incidences of prostate adenocarcinoma were the same in all three groups of rats. Overall, dietary administration of BBP for 40 weeks did not increase the incidence of prostate adenocarcinoma or the pre-neoplastic lesion PIN.

3.2.2 Studies in Mice

Two-year feeding studies in B6C3F$_1$ mice (NTP, 1982)

Male and female B6C3F$_1$ mice (50/sex/group) were fed diets containing BPP (97.2 ± 1.1% purity for weeks 1-34, and 98.2% purity for weeks 35-105) at concentrations of 0, 6000, or 12000 ppm for 105 weeks. Feed was available *ad libitum* and changed weekly. Dose-related decreases in mean body weights of male and female mice were observed throughout the study. The mean body weights in the low- and high-dose males were 4.9% and 16.2% lower, respectively, than controls at 52 weeks, and 2.6% and 14.4% lower, respectively, at study termination. For female mice, the mean body weights in the low- and high-dose groups were 8.6% and 19.7% lower, respectively, than controls at 52 weeks, and 7.5% and 22.6% lower, respectively, at study termination.

No statistically significant increases in tumor incidence were observed in either the male mouse or the female mouse study. In female mice marginal increases of hepatocellular adenoma and carcinoma of the liver were observed in the 6000 ppm and 12,000 ppm groups (5/38 and 6/37, respectively) compared to the controls (2/36).

Short-term i.p. injection study in male Strain A mice (Theiss *et al.*, 1977)

Male Strain A mice (20/group) 6 to 8 weeks old were given i.p. injections of BBP three times per week over 8 weeks. The control group was given the vehicle tricaprylin, and the exposed groups were given 160, 400, or 800 mg/kg/injection. All 60 mice survived until the end of the study and were sacrificed 24 weeks after the first injection. Forty-eight hours after sacrifice, the lungs were examined under a dissecting microscope and the number of lung tumors per mouse assessed. No treatment-related increase of pulmonary adenomas was observed. No tissues at other sites were examined.

Summary of animal carcinogenicity study findings

The following treatment-related tumors were observed:

- Pancreatic acinar cell adenoma or carcinoma in studies in male rats in comparison to *ad libitum* (NTP, 1997a) and weight-matched controls (NTP, 1997b)
- Pancreatic acinar cell adenoma in female rats (NTP, 1997a)
- Mononuclear cell leukemia in one study in female rats (NTP, 1982)

- Mononuclear cell leukemia in one study in male rats in comparison to weight-matched controls (NTP, 1997b)
- Urinary bladder papillomas and carcinomas of the transitional epithelium in one study in female rats (NTP, 1997b: 32-month feed restriction study)
- Benign and malignant pheochromocytoma (combined) of the adrenal medulla in one study in male rats in comparison to weight-matched controls (NTP, 1997b)

Pancreatic acinar cell carcinoma is rare in male F344 rats, and pancreatic acinar cell adenoma and urinary bladder transitional epithelium carcinoma are rare in female F344 rats.

Treatment-related increases in pancreatic acinar cell hyperplasia were observed in multiple studies in male rats (NTP, 1997a; 1997b: in comparison to weight-matched controls and in 2-year and 32-month feed restriction studies). Treatment-related increases in urinary bladder transitional epithelium hyperplasia were observed in multiple studies in female rats (NTP, 1997a; 1997b: in comparison to weight-matched controls and in 2-year and 32-month feed restriction studies).

3.3 Other Relevant Data

3.3.1 Pharmacokinetics and Metabolism

The disposition of BBP has been studied in humans and rodents. Absorption via the oral route and subsequent metabolism appears to be rapid in both humans and animals, although the proportion of metabolites may differ. The metabolic scheme presented in Figure 5 below is based on studies in rats.

3.3.1.1 Human studies

Absorption of BBP following oral exposure in humans appears to be rapid and fairly complete. In a study to determine quantitative biomarkers of phthalate exposure in humans (Anderson *et al.*, 2001), volunteers were exposed to a mixture of phthalates at relatively low doses. The deuterium-labeled BBP (d_4-BBP; deuterium labeling on the aromatic ring) was mixed into margarine and administered on toast to 24 volunteers (eight per dose group: 0, 253 µg, and 506 µg labeled BBP) along with other phthalates. Urine was collected from each subject at 24 hrs, 2 days and 6 days post-dosing. MBzP was the major metabolite of BBP excreted in urine, along with relatively small amounts of monobutyl phthalate (MBuP). Approximately 67 and 73% of d_4-BBP was metabolized to d_4-MBzP in the low- and high-dose subjects, respectively, and about 6% was metabolized to d_4-MBuP for both dosage groups within 24 hours. Thus, it appears

that absorption of BBP is rapid and metabolism and elimination is fairly complete in the first 24 hours. This study was limited by small sample size (eight volunteers per dose group). The investigators reported inter-individual variability in MBzP excretion yield with a relative standard deviation (RSD) of 26 and 39% for the low and high dose, respectively. The investigators considered this acceptable. A relatively higher RSD (59%) was reported for MBuP excretion, in part because this pathway represented a small fraction of total metabolism. Further studies of BBP metabolism in humans could provide additional information to more accurately interpret analytical data from human urine analysis.

One supporting study was found that evaluated metabolism of BBP in a human-derived cell line. Picard *et al.* (2001) investigated BBP metabolism using human breast cancer cells (MCF-7 cells) incubated in the presence of radiolabelled BBP. The major metabolites detected were the BBP monoesters, MBuP and MBzP. Benzoic acid (BA) and phthalic acid (PA) were also identified but in small quantities. Forty-eight hours after treatment, BBP represented only 10% of the initial radioactivity; MBzP represented 48% of the initial radioactivity, MBuP 25%, PA 6%, and BA 3%. The metabolite profile in these cells is somewhat similar to that seen in the human volunteer study of Anderson *et al*. (2001).

3.3.1.2 Animal studies

Absorption

Oral Administration

The absorption kinetics of BBP in male F344 rats after oral administration is dose-dependent. At doses of 2-200 mg/kg body weight, the majority of BBP (up to 74%) is systemically absorbed and excreted in the urine after 24 hours. However, after administration of 2000 mg/kg body weight, only 16% is excreted in urine with the majority in feces, indicating incomplete absorption of BBP or its metabolites during enterohepatic circulation (Eigenberg *et al.*, 1986).

Dermal and Subcutaneous Administration

Dermal absorption occurs at a much slower rate than oral absorption. Investigators applied 157 µmol/kg body weight ^{14}C-BBP to the shaved skin of male F344 rats, and then covered the area with a perforated cap, yielding an applied dermal dose of 5-8 mg/cm^2 (Elsisi *et al.*, 1989). After seven days, approximately 30-40% of the applied dose was excreted in the urine or feces, and 45% was found at the area of application.

Following a single subcutaneous injection (100 mg/kg) of BBP given to immature (20 - 22 days old on receipt) female Alpk:APfSD (Wistar-derived) rats, peak plasma concentrations of the metabolites MBuP and MBzP occurred within 1 hour of administration (Brady *et al.*, 2000). However, the area under the curve (AUC) values of the two metabolites were much lower following subcutaneous injection as compared to the same dose given by oral administration (Brady *et al.*, 2000). The data indicate that BBP is less efficiently absorbed into the systemic circulation by the subcutaneous route, as compared to the oral route.

No data on absorption of inhaled BBP are available.

Distribution

To determine the excretion and tissue distribution of BBP, male Sprague-Dawley rats were treated by gavage with 16, 160, or 1600 mg/kg body weight ^{14}C-BBP and the urine and feces were collected for five days (ECB, 2007). Levels of radioactivity remaining in the animals at sacrifice on day 5 were minimal. The largest amounts of radioactive ^{14}C residues were detected in the liver, kidney, small intestine and total gut contents. BBP did not accumulate in any tissue. Excretion of ^{14}C label in urine was rapid and appeared to be largely independent of the dose of ^{14}C-BBP administered. More than 80% of the administered dose was excreted in the urine within five days, with most of the remaining dose (all but the small amount of radiolabel detected in the tissues) excreted in the feces.

Elsisi *et al.* (1989) reported a small fraction of radiolabel following dermal exposure of male F344 rats to ^{14}C-BBP is found in the muscle (4.5%), brain, spinal cord and testis (0.5%).

There was no evidence in these studies that tissue accumulation of ^{14}C-BBP occurs.

Metabolism

Figure 5 illustrates the proposed routes of BBP metabolism, based on studies in female Wistar rats. Metabolism is expected to be qualitatively similar in humans. BBP is hydrolyzed by lipases (*e.g.*, liver, gastrointestinal and pancreatic lipases) to its monoester phthalate metabolites, which either go through phase II metabolic conjugation (e.g. glucuronides) or are further metabolized to other breakdown products. Nativelle *et al.* (1999) dosed female Wistar rats with BBP (150, 475, 780, and 1500 mg/kg body weight/ day) by gavage for three consecutive days. Six metabolites, MBuP

(29 - 34%), MBzP (7 - 12%), hippuric acid (51 - 56%), PA(2 - 3%), an ω-oxidized metabolite of MBuP (1 - 2%), and BA (less than 1%) were identified in urine after 24 hours. The parent BBP was not present. MBuP was identified as the major urinary metabolite of BBP in female Wistar rats (Nativelle *et al.*, 1999). This is in contrast to the human study cited above (Anderson *et al.*, 2001) where MBuP was a minor metabolite and MBzP was a major metabolite.

In an excretion and metabolism study, male F344 rats were dosed by gavage with ring-labeled ^{14}C-BBP at 2, 20, 200, or 2000 mg/kg body weight, or administered 20 mg/kg body weight intravenously (Eigenberg *et al.*, 1986). Urinary ^{14}C was composed of monoester phthalate derivatives (10 - 42% of the dose) and glucuronides of these derivatives (2 - 12% of the dose). At four hours after intravenous administration of 20 mg/kg ^{14}C BBP, 53-58% of the radiolabel was excreted in the bile. No parent compound was found in bile. The metabolites found in the bile were as follows: MBuP-glucuronide and MBzP-glucuronide (26% and 13% of the dose, respectively), trace amounts of free monoesters (2% of the dose), and unidentified metabolites (14% of the dose). Similar to the observations by Nativelle *et al.* (1999), larger quantities of MBuP (44%) than of MBzP (16%) were formed during rat metabolism, in contrast to the one study conducted in humans. In another metabolism study in male F344 rats, BBP was reported to be partially hydrolyzed by intestinal esterases, primarily to MBuP and benzyl alcohol, with MBzP and n-butanol as minor products of hydrolysis. There was a preference for hydrolysis of benzyl ester, resulting in a predominance (around 3:1) of MBuP in the urine compared to MBzP (Agarwal *et al.*, 1985).

Following a single oral dose of 100 mg BBP/kg to immature (20 - 22 days old on receipt) female Alpk:APfSD (Wistar-derived) rats, Brady *et al.* (2000) found a total of 26.6% of the calculated administered dose of BBP was excreted as mono-phthalates (free and glucuronidated) in the urine during the 24-hour collection period. The major metabolite present in the urine was MBuP (free and glucuronidated), which accounted for 19.7% of the dose. Peak concentrations of MBzP in plasma occurred within 1 hour of dosing, while plasma concentrations of MBuP did not peak until 8 hours after dosing. When the same dose was given subcutaneously, the corresponding total amount excreted in the urine as mono-phthalates (free and glucuronidated) during the first 24 hours was only 4.6%, consisting of similar amounts of MBuP (2.5%) and MBzP (2.1%).

Excretion

In the study in male F344 rats receiving a single oral dose of 2-200 mg/kg ^{14}C BBP by Eigenberg *et al.* (1986) described above, at 24 hours, 61-72% of the oral dose was excreted in the urine and 13-15% in feces. At 2000 mg/kg, 16% of the ^{14}C was excreted in the urine and 65% was excreted in feces 24 hours following dosing. The increased fecal elimination at 2000 mg/kg body weight may have been due to incomplete absorption of administered BBP or of its metabolites during enterohepatic circulation. The half-lives of the parent BBP and the monoesters were approximately six hours in all tissues in rats (Eigenberg *et al.*, 1986). After 96 hours, excretion of radiolabel in the urine was between 70-80% of the administered label in the doses ranging from 2-200 mg/kg body weight; whereas, 96 hours after oral administration of 2000 mg/kg body weight in rats, 22% of the label appeared in urine and 72% in feces. The excretion of radioactivity in the feces was 20% after intravenous administration.

Brady *et al.* (2000) reported that the terminal half-lives of BBP's two primary metabolites were similar: 7 hours and 4 hours for MBuP and MBzP, respectively, following a single gavage dose (100 mg/kg body weight) of BBP given to immature female Alpk:APfSD (Wistar-derived) rats. When the same dose was administered subcutaneously, the terminal half-lives for both metabolites were approximately 15 hours. Thus the available animal and human data suggest the half-lives of BBP and its metabolites following oral or subcutaneous exposure are less than 24 hours.

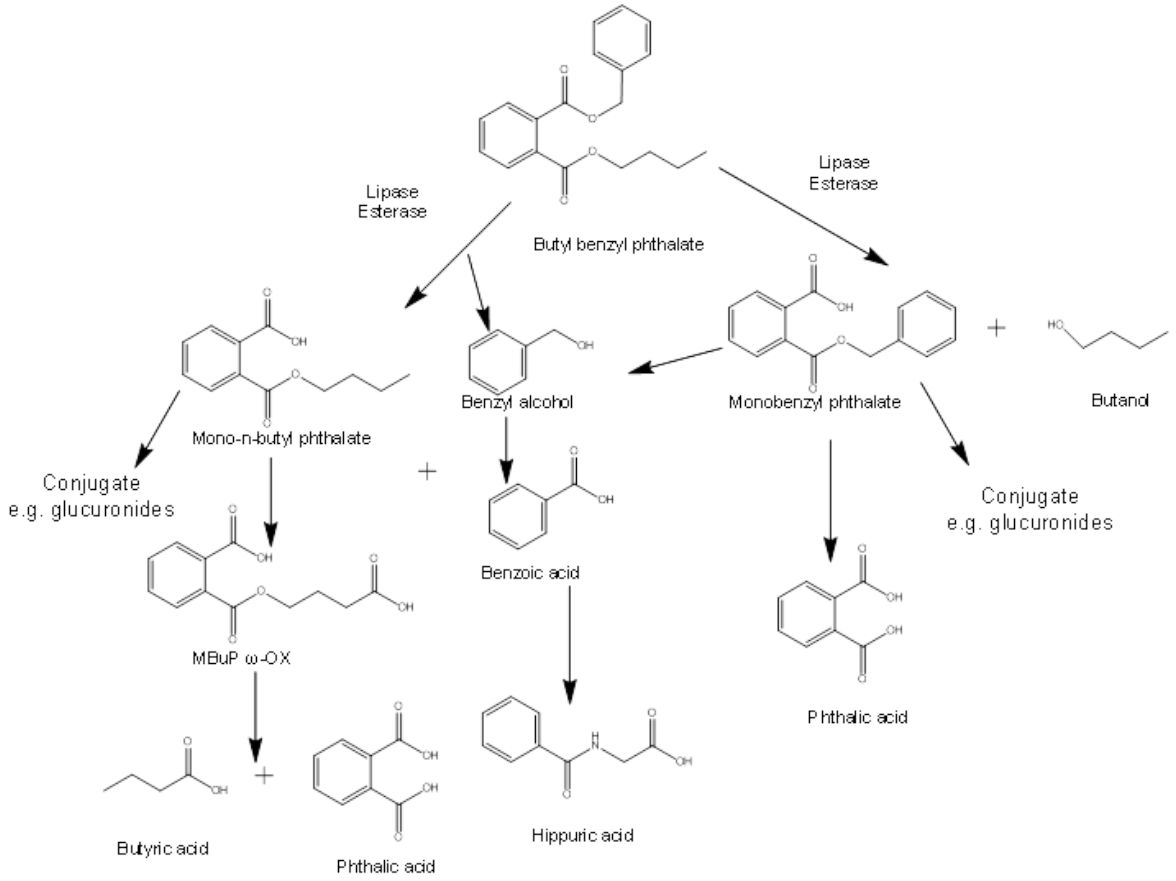


Figure 5. Proposed routes of butyl benzyl phthalate metabolism in female Wistar rats (adapted from Nativelle *et al.*, 1999)

3.3.2 Genotoxicity

In vitro in bacteria

The genotoxicity of BBP has been investigated *in vitro* in reverse mutation assays in bacteria, forward mutation assays at the *Tk* locus in L5178Y mouse lymphoma cells, DNA repair assays in bacteria, sister chromatid exchange (SCE) and chromosomal aberration (CA) assays in Chinese hamster ovary (CHO) cells, and DNA-protein crosslink (DPC) assays in rat hepatic cells. *In vivo*, BBP has been tested using the sex-linked recessive lethal mutation assay in *Drosophila melanogaster*, SCE and CA assays in mouse bone marrow cells, micronucleus assay in mice and rats, and DPC assay in mice. The findings are presented in Tables 12, 13, and 14 below.

BBP's ability to induce reverse mutations was examined in *Escherichia coli* and *Salmonella typhimurium* in the presence and absence of metabolic activation systems. In wild-type and uvrA- strains of *E. coli*, treatment with 30 mg/plate BBP did not induce mutations (Kurata, 1975, as cited by Omori, 1976). In the TA98 and TA100 strains of *S. typhimurium*, 30 mg/plate BBP did not induce mutations with rat liver S9 (Kurata, 1975, as cited by Omori, 1976). Two studies from the Monsanto Company (1976, as cited by

ECB, 2000), showed negative results with concentrations of BBP up to 10 µl/plate across different strains of *S. typhimurium*. In another study, levels of BBP up to 1 mg/plate did not induce mutations in the TA98 or TA100 strains of *S. typhimurium* with or without rat liver S9 (Kozumbo *et al.*, 1982). In one of the TA100 mutagenicity tests without S9 performed by NTP (1997a), the first trial was marginally positive and the second trial was negative. A repeated test by the same group and a similar assay performed by a different laboratory showed negative results for TA100. Overall, BBP was not mutagenic in *S. typhimurium* or *E. coli*.

The bactericidal effects of BBP on DNA repair-deficient bacteria were examined by Kurata (1975, as cited by Omori, 1976) with different strains of *Bacillus subtilis* (*B. subtilis*) (recA-) and *E. coli* (uvrA-, PolA-, or recA-). No effect was observed with 30 mg/plate BBP, indicating that BBP did not induce any DNA damage in these bacteria.

In vitro in mammalian cells

In an assay performed by NTP (1997a) with L5178Y mouse lymphoma cells, BBP treatment without rat liver S9 fraction induced significant increases of mutant fractions at the *Tk* locus in one trial, but the response was not repeated in the other three trials. BBP did not induce mutations in L5178Y mouse lymphoma cells with rat liver S9 fraction.

Two observations of increased mutant frequency with BBP treatment at 0.015 µl/ml were noted in L5178 mouse lymphoma cells in the absence of S9 fraction (Barber *et al.*, 2000). In the same assay with rat liver S9 fraction, there were positive increases of mutant fractions with 0.6-1.2 µl/ml BBP, with the range for relative growth at these doses being 1-10.4%. Significantly increased mutant fractions were noted for the 0.60 µl/ml dose group. Relative growth values for the two replicate assays were 9.1 and 10.4%. The positive assay data for BBP was considered by the authors to be unreliable due to toxicity, and they determined that BBP was non-mutagenic in L5178Y mouse lymphoma cells in this study.

The ability of BBP to induce DNA base lesions was tested in the mouse osteoblast cell line MC3T3-E1 and mouse primary calvarial osteoblast cultures (Sabbieti *et al.*, 2009). DNA base lesions were measured by the level of apurinic/apyrimidinic (AP) sites (a location in DNA that has neither a purine nor a pyrimidine base, either spontaneously or due to DNA damage). In a normal cell about 2×10^5 spontaneous AP base lesions are generated every day. BBP induced statistically significant increases ($p < 0.05$) of greater than 30×10^5 AP sites per cell in 24 hours in both MC3T3-E1 and mouse primary calvarial osteoblasts.

HepG2 human hepatocellular carcinoma cells were treated with BBP for 24 or 48 hours and tested for DNA single strand breaks with the comet assay. BBP treatment for either 24 or 48 hours caused dose-dependent increases of the Olive tail moment at both time points (Choi *et al.*, 2010). A dose as low as 2.5 µM induced a statistically significant increase of DNA damage; the amount of damage was higher in cells treated with BBP for 48 hours compared to 24 hours.

BBP induced a significant trend ($p = 0.004$) of SCE induction in CHO cells, without any significant increase at any one dose level (NTP, 1997a). A repeat test showed clearly negative results across all dose groups and no significant trend was observed. BBP did not induce any CAs in CHO cells with or without rat liver S9.

DPC is a covalent crosslinking of DNA and protein, and can impair normal DNA metabolic processes, such as replication, transcription, and recombination (Barker *et al.*, 2005). The mutagenic effects of DPCs are not well understood. DPCs induced by some chemicals have been correlated with SCEs or DNA single-strand breaks (Fornace, 1982), indicating that DPCs might contribute to the overall carcinogenicity of the chemicals. DPCs were measured in a Wistar rat liver cell suspension after treatment with BBP for one hour (Qin *et al.*, 2010). BBP at 80 µM significantly increased the formation of DPCs in the liver cells by 32.5%, as compared to the control ($p < 0.01$). Doses of 5 and 20 µM did not have any effect on DPC formation.

In summary, BBP did not induce any reverse or forward mutations, SCEs, or CAs *in vitro*. BBP did induce DNA base lesions in a mouse MC3T3-E1 osteoblast cell line and mouse primary calvarial osteoblast cells, and DNA single strand breaks in HepG2 human hepatocellular carcinoma cells, and DPCs in rat hepatic cell homogenate.

Table 12. *In vitro* Genotoxicity Studies of BBP in Bacteria

Assay System (Gene Mutation)	Conc. Tested	Results		Activation System	References
		- S9	+ S9		
E. coli (Wild-type, uvrA-)	30 mg/plate	-	NT	NA	Kurata, 1975, as cited by Omori, 1976
B. Subtilis (recA-)	30 mg/plate	-	NT	NA	Kurata, 1975, as cited by Omori, 1976
S. typhimurium (TA98, TA100)	30 mg/plate	NT	-	S9[1]	Kurata, 1975, as cited by Omori, 1976
S. typhimurium (TA99, TA100, TA1535, TA1537, TA1538)	0.001-10 µl/plate	-	-	S9[1]	Monsanto study BO-76-017, as cited by ECB, 2000
S. typhimurium (TA99, TA100, TA1535, TA1537, TA1538)	0.1-10 µl/plate	-	-	S9[1]	Monsanto study LF-76-124C, as cited by ECB, 2000
S. typhimurium (TA98, TA100)	0-1 mg/plate	-	-	Rat liver S9	Kozumbo *et al.*, 1982
S. typhimurium (TA98, TA100, TA1535, TA1537)	0.33-11.55 mg/plate	-	-	Rat liver S9, Hamster liver S9	NTP, 1997a (performed at Case Western Reserve University)
S. typhimurium (TA98, TA100, TA1535, TA1537)	0.1-10 mg/plate	-	-	Rat liver S9, Hamster liver S9	NTP, 1997a (performed at EG&G Mason Research, Inc.)
E. coli (uvrA-, PolA-, recA-)	30 mg/plate	-	NT	NA	Kurata, 1975, as cited by Omori, 1976

NT, not tested; NA, not applicable
[1] Origin of S9 fraction not given in the references

Table 13. *In vitro* Genotoxicity Studies of BBP in Mammalian Cells

Endpoint	Assay System	Conc. Tested	Results		Activation System	References
			- S9	+ S9		
DNA-protein crosslinks	male Wistar rat liver cells	5, 20, 80 µM	+	NT	NA	Qin *et al.*, 2010
DNA base lesions (AP sites)	MT3T3-E1 mouse osteoblast cell line	1 µM	+	NT	NA	Sabbieti *et al.*, 2009
	Mouse primary calvarial osteoblasts, from newborn Harlan Sprague–Dawley ICR (CD-1) male mice	1 µM	+	NT	NA	
DNA single strand breaks (comet assay)	HepG2 hepatocellular carcinoma cell line	2.5-250µM	+	NT	NA	Choi *et al.*, 2010
Forward Mutation	L5178Y mouse lymphoma cells (*Tk* locus)	0.005-0.06 µl/ml	-	-	Aroclor 1254-induced rat liver S9	NTP, 1997a
		0.015-1.2 µl/ml	-	-	Aroclor 1254-induced rat liver S9	Barber *et al.*, 2000
Sister Chromatid Exchanges	Chinese hamster ovary (CHO) cells	0.4-12.50 µg/ml	-	NT	NA	NTP, 1997a
		125-1250 µg/ml	NT	-	Aroclor 1254-induced rat liver S9	
Chromosomal Aberrations	CHO cells	125-1250 µg/ml	-	-	Aroclor 1254-induced rat liver S9	NTP, 1997a

NT, not tested; NA, not applicable

In vivo genotoxicity studies

In vivo studies on the genotoxicity of BBP are summarized in Table 14.

DPCs were measured in male mouse liver homogenates after daily intraperitoneal injections of 500 or 1000 mg/kg/day BBP for 14 days (Guo *et al.*, 2009). At both doses, levels of DPC and malondialdehyde (indicative of lipid peroxidation and possible oxidative DNA damage) were significantly increased ($p < 0.01$) in the treated mouse livers as compared to the levels in the control group.

SCEs and CAs were measured in the bone marrow cells of male $B6C3F_1$ mice after i.p. injections of BBP over a dose range of 1250-5000 mg/kg body weight (NTP, 1997a). After 23 hours of BBP treatment, there was a reduction of response in the 5000 mg/kg group compared to the two lower dose groups. It is possible that the response at 5000 mg/kg was an outlier, and NTP did trend analysis after removing this dose; the trend was significant ($p = 0.0067$) when the 5000 mg/kg group was excluded. There was a statistically significant dose-related trend ($p = 0.025$) of the mean SCEs/cell with 42 hours of BBP treatment.

BBP treatment for 17 hours induced a statistically significant trend ($p = 0.003$) of CAs in mouse bone marrow cells. The repeated trial confirmed this trend. In both trials 5000 mg/kg BBP induced a statistically significant increase compared to vehicle control. The number of cells with CAs increased more than four times with 5000 mg/kg BBP treatment.

The ability of BBP to induce micronucleated polychromatic erythrocytes was examined in mice and rats. BBP did not induce any increase of micronucleus in the peripheral erythrocytes of B6C3F1 mice (NTP, 1995). In the rat study, female Alpk:AP_fSD rats were given BBP in drinking water throughout pregnancy and lactation until postnatal day 90, and the bone marrow erythrocytes of the dams were examined with the micronucleus assay (Ashby *et al.*, 1997). There was no increase of micronuclei in the erythrocytes from the bone marrow of the exposed rats compared to the controls.

Male adult Canton-S *D.melanogaster* were exposed to BBP in feed (10000 and 50000 ppm) or by injection (500 ppm) to test for sex-linked recessive lethal mutations (NTP, 1997a). The numbers of lethal mutations/number of X chromosomes over for three mating trials were analyzed. There was a 15% increase of the lethal mutations/number of X chromosomes tested in the group treated with 10000 ppm BBP in feed. No increase was found in the group treated with 50000 ppm BBP in feed or in the group treated with 500 ppm BBP by injection.

In a dominant lethal assay, BBP was given three times subcutaneously to male $B6C3F_1$ and CD-1 mice up to 4560 mg/kg/day, and the mice were mated with untreated female virgin mice of the same strain (Bishop *et al.*, 1987). The female mice were sacrificed 17 days after the beginning of the mating period and the fetal death rate was examined. BBP did not induce any change in the number of viable fetuses.

Table 14. *In vivo* Genotoxicity Studies of BBP

Endpoint	Species/ Strain/Sex	Tissue Analyzed	Dose Tested	Route	Results	References
DNA-protein crosslinks	Kunming mice, male	Mouse hepatic cells	125-1000 mg/kg	i.p. injection	+[1]	Guo *et al.*, 2009
Sister Chromatid Exchanges	$B6C3F_1$ mice, male	Mouse bone marrow cells	1250-5000 mg/kg	i.p. injection	+[2]	NTP, 1997a
					+[3]	
Micronucleus	$B6C3F_1$ mice, male	Mouse bone marrow polychromatic erythrocytes	312.5-2500 mg/kg	i.p. injection	-[4]	NTP, 1995
	Alpk:AP_fSD rats, female, pregnant/ lactating	Mouse bone marrow polychromatic erythrocytes	1 mg/L	in drinking water	-[5]	Ashby *et al.*, 1997
Chromosomal Aberrations	$B6C3F_1$ mice, male	Mouse bone marrow cells	1250-5000 mg/kg	i.p. injection	+[6]	NTP, 1997a
					-[7]	
Sex-linked Recessive lethal mutations	*D. melanogaster*	Germ cells	10,000 ppm	feed	+	NTP, 1997a
			50,000 ppm	feed	-	
			500 ppm	injection	-	
Mouse Dominant Lethal Assay	$B6C3F_1$ and CD-1 mice	Mouse fetuses	400-4,560 mg/kg/ day	s.c. injection[8]	-	Bishop *et al.*, 1987

[1] Five animals in each group, $p < 0.01$ for 500 and 1000 mg/kg doses compared to controls.
[2] Positive trend when excluding the highest dose from the analysis (p=0.0067); harvest at 23 hours.
[3] Positive trend (p=0.025); harvest at 42 hours.
[4] Cells were collected at twenty-four hours after three treatments.
[5] Dams were exposed starting on day 1 of pregnancy (PCD 1) pregnant and terminated on PCD 45.
[6] This experiment was positive in two trials; harvest at 17 hours post-treatment (standard harvest protocol).
[7] Harvest at 36 hours post-treatment (non-standard harvest protocol).
[8] Male mice received three subcutaneous injections of BBP and then were mated with untreated female mice of the same strain. Fetuses were examined seventeen days after the beginning of the mating period.

In summary, *in* vivo studies of BBP did not induce dominant lethality in mouse fetuses, sex-linked recessive lethal mutations in *D. melanogaster*, or micronucleated polychromatic erythrocytes in male mice or female rats. BBP induced SCEs in mouse bone marrow cells in a single experiment and CA in repeated experiments. BBP also induced DPCs in mouse hepatic cells.

Overall, BBP caused the following genotoxicity effects:

- DNA base lesions in mice
- DNA single strand breaks in human HepG2 cells
- DPCs in rat hepatic cell homogenate (*in vitro*) and mouse hepatic cells (*in vivo*)
- SCEs and CA in mice *in vivo*

BBP did not induce any reverse or forward mutations, SCEs, or CAs *in vitro*, or dominant lethal mutations, sex-linked recessive lethal mutations, or micronuclei *in vivo*.

3.3.3 In vitro Cell Transformation Studies

Cell transformation assays are designed to detect a change in growth pattern of cells that is indicative of loss of contact inhibition, a phenotype that is characteristic of cancer cells. BBP has been tested for the ability to induce cell transformation in Balb/c 3T3 A-31 mouse embryonic fibroblast cells (Barber *et al.*, 2000) and Syrian hamster embryo (SHE) cells (LeBoeuf *et al.*, 1996).

In the Balb/c 3T3 A-31 mouse cell assay, cells were incubated with five different concentrations of BBP for three days, and then in fresh media for four weeks before staining (Barber *et al.*, 2000). No supplemental metabolic activation system was included in this assay. Colonies that survived treatment were fixed, stained, and counted. BBP did not induce cell transformation of Balb/c 3T3 A-31 mouse cells in this study (Table 15).

In the SHE cell transformation studies by LeBoeuf *et al.* (1996), cells were exposed to BBP either at doses of 25-250 µg/ml for 24 hours, or at doses of 1-20 µg/ml for 7 days. The authors reported that globular precipitate was seen in the medium at all doses above 25 µg/ml in the 24-hour exposure studies. The authors did not comment on whether globular precipitate was seen in the 7-day exposure experiment at the 20 µg/ml dose. No increase in morphological cell transformation was observed in SHE cells after exposure to BBP for 24 hours. After 7-day exposure to BBP at doses of 1-20 µg/ml, statistically significant ($p < 0.05$) increases in morphological cell transformation were seen with 2, 5, and 10 µg/ml BBP treatment, but not 20 µg/ml. The authors concluded that BBP was positive in the SHE morphological transformation assay (Table 15).

Table 15. *In vitro* Cell Transformation Studies of BBP

Assay System	Concentration Tested	Results	Reference
Mouse Balb/3T3 A-31 embryonic fibroblast clones	0.01-0.16 µl/ml	-	Barber *et al*., 2000
Syrian hamster embryo cells	25-250 µg/ml (24-hour exposure)	-	LeBoeuf *et al*., 1996
	1-20 µg/ml (7-day exposure)	+	

3.3.4 Animal Tumor Pathology

Among male and female rats treated with BBP, tumors of the hematopoietic system, pancreatic acinar cells, urinary bladder, and adrenal medulla were observed. Increases of pancreatic acinar cell focal hyperplasia and urinary bladder transitional cell hyperplasia, two pre-neoplastic lesions, were observed in male rats and female rats, respectively.

Mononuclear Cell Leukemia (MNCL)

BBP caused increased mononuclear cell leukemia (MNCL) in one study in female rats (NTP, 1982) and one study in male rats (NTP, 1997b: comparison 2 [high dose group compared to weight-matched controls]).

MNCL is one of the most common neoplasms in aging F344 rats, and is morphologically characterized by cells that resemble large granular lymphocytes. Over time, the incidence of MNCL in untreated F344 rats from NTP two-year studies has increased significantly. The incidence of MNCL was 28.5% in untreated male F344 rats and 19.6% in female F344 rats during 1970-1979, compared to 50.5% (male) and 28.1% (female) during 1990-1996 (Haseman *et al.*, 1998; Caldwell, 1999). MNCL can spread to multiple organs including the liver, lungs, and spleen, with splenic infiltration by tumor cells being the most consistent hallmark (Stromberg and Vogtsberger, 1983). The MNCL observed in the NTP studies on BBP was generally characterized by splenomegaly and often by hepatomegaly. No differences in tissue distribution or cytological characteristics were seen between the control animals and the treated ones. Based on a morphological similarity to granular lymphocytes, MNCL is also called LGL leukemia (large granular lymphocytic leukemia) or Tγ lymphocyte leukemia. While LGL

bear some resemblance to monocytes, they lack certain monocyte characteristics, such as esterase staining, adherence to plastics or nylon wool and phagocytosis (Reynolds *et al.*, 1981). Caldwell (1999) reported no human counterpart to rat LGL leukemia. More recently, U.S. EPA (2012b) has noted that several authors have concluded that rat MNCL is similar to human natural killer cell (NK) LGL leukemia (Stromberg, 1985; Ishmael and Dugard, 2006; Thomas *et al.*, 2007).

Exocrine Pancreas

Increased incidences of pancreatic acinar cell adenoma and carcinoma were seen in BBP-treated male rats (NTP, 1997a; 1997b: comparison 2 [high dose group compared to weight-matched controls]), with a significant dose-related trend observed in the NTP (1997a) study. Treatment-related increases in pancreatic acinar cell hyperplasia were observed in multiple studies in male rats (NTP, 1997a; 1997b: comparisons 2 [high dose group compared to weight-matched controls], 3 [feed restricted study] and 4 [feed restricted study, 157 weeks]).

Spontaneous pancreatic acinar cell neoplasms are rare in rats (Milman *et al.*, 1978). NTP stated that no pancreatic acinar carcinomas had been observed in historical control F344 rats in feed studies (NTP, 1997a). Pancreatic acinar cell hyperplasia, adenoma and carcinoma represent a continuous progression of the lesion (Boorman and Eustis, 1984; Eustis *et al.*, 1990). Pancreatic acinar cell adenoma is distinguished from hyperplasia mainly by size of the lesion (adenomas are >3 mm in diameter) (Boorman and Eustis, 1984). Pancreatic acinar cell carcinomas are distinguished from adenomas with a heterogeneous growth pattern, highly vascular areas, fibroplasia, glandular patterns, and local invasion (Boorman and Eustis, 1984; Eustis *et al.*, 1990). Even poorly differentiated pancreatic acinar cell carcinomas retain areas of well-differentiated acini with zymogen granules (Hansen *et al.*, 1995).

Adrenal Medulla

BBP induced benign and malignant pheochromocytoma (combined) of the adrenal medulla in one study in male rats (NTP, 1997b: comparison 2 [high dose group compared to weight-matched controls]).

Pheochromocytoma of the adrenal medulla is not a rare disease in untreated aging F344 rats and is more common in males than females (Hamlin II and Banas, 1990). The NTP historical incidence of this lesion was 25.5% (489/1915, range 6-65%) in untreated male F344 rats (Haseman *et al.*, 1990). The incidences of benign and malignant pheochromocytomas were combined because they represent a biological

continuum. Morphologically there are no clear-cut features that distinguish benign and malignant pheochromocytomas (Hamlin II and Banas, 1990). Malignant pheochromocytomas are diagnosed when there is evidence of capsular and extracapsular soft tissue invasion or metastasis.

Urinary Bladder

BBP induced four cases of urinary bladder carcinoma of the transitional epithelium in one study in female rats (NTP, 1997b: comparison 4 [feed restricted study, 157 weeks]). Treatment-related increases in urinary bladder transitional epithelium hyperplasia were observed in multiple studies in female rats (NTP, 1997a; 1997b: comparisons 1 [all groups *ad libitum*-fed], 2 [high dose group compared to weight-matched controls], 3 [feed restricted study] and 4 [feed restricted study, 157 weeks]).

Transitional cell neoplasms of the urinary bladder are rare in untreated Fischer rats (Jokinen, 1990; Shirai and Takahashi, 1998). Papillary or nodular hyperplasia of the urinary bladder in rats is considered a pre-neoplastic lesion and can progress to papilloma or carcinoma (Fukushima *et al.*, 1982; Fukushima and Wanibuchi, 2000). The hyperplasia observed in the NTP (1997a) study was not simple hyperplasia, but focal to multi-focal thickening of the transitional epithelium. Fukushima *et al.* (1982) suggested that there are two types of urinary bladder transitional cell hyperplasia, namely reversible hyperplasia and irreversible hyperplasia. The irreversible type progresses to papilloma and eventually carcinoma, while the reversible type can revert back to normal epithelium. In rats, papilloma is distinguished from hyperplasia by the characteristics of the supportive core (Shirai and Takahashi, 1998). The papilloma has a complex fibrovascular core with secondary or tertiary papillae stalks, whereas the hyperplasia has a simple connective tissue core (Shirai and Takahashi, 1998).

In rats, most urinary bladder transitional cell carcinomas arise from papillomas and can be exophytic, endophytic, or both (Jokinen, 1990). The endophytic carcinomas can invade the bladder wall and are generally more malignant. The differentiation of transitional cell carcinoma from papilloma is based primarily on invasion, and then to a lesser extent on growth patterns and cell atypia (Jokinen, 1990).

3.3.5 Effects on Gene and Protein Expression

A number of studies have investigated the ability of BBP to alter gene or protein expression. These include two *in vivo* studies conducted in rats, focusing on gene expression in the mammary gland, *in vitro* gene expression studies in an immortalized human breast epithelial cell line and a human breast cancer cell line, and an *in vitro* protein expression study in a human liver cancer cell line. These types of studies can provide insights into the mechanisms of action of BBP at a molecular level. The findings from these studies, with an emphasis on data relevant to the carcinogenicity of BBP, are described below.

3.3.5.1 In vivo differential cDNA microarray analysis

Effects of neonatal/prepubertal lactational exposure to BBP on gene expression

In the study published by Moral *et al.* (2007), lactating Sprague-Dawley CD rats (10 dams per group) were gavaged five days per week with 500 mg BBP/kg/day or an equivalent volume of sesame oil on days 2–20 after giving birth. One female offspring from each litter was sacrificed on day 21, 35, 50, and 100. At sacrifice, the fourth pair of mammary glands from each animal was rapidly dissected and frozen in liquid nitrogen for subsequent microarray analysis.

The data collected from microarray analysis demonstrated that neonatal/prepubertal exposure to BBP via lactational transfer had an effect on the genomic expression profile of the mammary gland. On day 21, hundreds of genes, including a significant number related to proliferation and differentiation, communication, and signal transduction, were up-regulated in the mammary glands of the exposed animals. The number of BBP-modulated genes decreased gradually over time after weaning (i.e., day 21). Moral *et al.* (2007) performed functional analysis on the genes that were up-regulated by BBP and reported that the following functions were significantly over-represented ($p < 0.05$): cell proliferation, signal transduction (transforming growth factor-beta [TGF-β] signaling pathway, metabotropic glutamate receptor group I pathway, PI3 kinase pathway, endothelia-signaling pathway, and interleukin-signaling pathway) and cell communication (neuronal activities and anterior/posterior patterning).

The investigators selected nine genes associated with potential carcinogenic mechanisms for validation by quantitative real time polymerase chain reaction (qRT-PCR); eight of the nine were significantly up-regulated and one was significantly down-regulated. These results are summarized in Table 16. Among the eight up-regulated genes, two are highly relevant to carcinogenesis: aryl hydrocarbon receptor (*Ahr*) and Wilms tumor 1 (*Wt1*). *Ahr* was up-regulated 2-fold compared to the

control. *Ahr* is a ligand-activated transcription factor and is known to interact with signaling pathways that are mediated by estrogen receptors (ERs) and other hormone receptors. It controls important physiological processes, such as xenobiotic metabolism and hormone signaling, and it may play a role in mammary gland development (Guo *et al.*, 2003). *Wt1* was up-regulated 2.77-fold compared to the control ($p < 0.05$). The expression of *Wt1* gene expression patterns are indicative of developmental status in normal mammary glands, and are often altered in breast cancers and breast cell lines (Silberstein *et al.*, 1997).

Glutamate decarboxylase 1 (*Gad1*) was significantly down-regulated (2.69- to 3.65-fold between days 21 to 50). *Gad1* codes for the protein Gad67, which catalyzes the production of the neurotransmitter γ-aminobutyric acid (GABA). The GABA-ergic system has been found to be involved in hormonal regulation and pathogenesis of breast cancer in humans and mice (Opolski *et al.*, 2000). *Gad1* can be regulated by estrogens in the rat brain (Nakamura *et al.*, 2004).

Among the nine genes in Table 16, *Tsn* was the only one with differential expression (i.e., up-regulation) at day 100. *Tsn* encodes a DNA-binding protein, namely translin, in humans (Kasai *et al.*, 1994; Aoki *et al.*, 1997). Translin recognizes conserved target sequences at the breakpoint junction of chromosomal translocations. Translin DNA-binding complexes are present at recombination hotspots and may serve as indicators of breakpoints in genes which are fused by translocations. These binding activities may play a crucial role in chromosomal translocation in lymphoid neoplasms, such as MNCL (Kasai *et al.*, 1994; Aoki *et al.*, 1997).

Table 16. Gene expression changes in nine selected genes[1] in the mammary glands of rats exposed to BBP via lactational transfer (Moral *et al.*, 2007)

Functional group	Symbol	Gene description	Gene expression[2]	Days[3]
Morphogensis, organogenesis, and cell differentiation, organization	*Foxg1*	Forkhead box G1	++	21
	Wt1	Wilms tumor 1	+++	21
	Fabp3	Fatty acid binding protein 3	++	21
	Cdh8	Cadherin-8	+++	21
	Gad1	Glutamate decarboxylase 1	----	35
			---	50
Transcription factors	*Foxg1*	Forkhead box G1	++	21
	Hfh1	HNF-3/forkhead homolog-1	++	21
	Ahr	Aryl hydrocarbon receptor	++	21
	Nfyc	Nuclear transcription factor-Y gamma	+	21
Response to stress	*Ahr*	Aryl hydrocarbon receptor	++	21
GABA and hypoxia response	*Gad1*	Glutamate decarboxylase 1	----	35
			---	50
Metabolism	*Fabp3*	Fatty acid binding protein 3	++	21
Chromosomal translocation	*Tsn*	Translin	+	100

[1]Validated by qRT-PCR

[2]Presented as the fold change of BBP-induced gene expression compared to control. +, ≥ 1 fold, < 2 fold; ++, ≥ 2 fold, < 3 fold; +++, ≥ 3 fold, < 4 fold; ---, ≥ -4 fold < -3 fold; ----, ≥ -5 fold < -4 fold

[3]Mammary glands (4th pair) collected on days 21, 35, 50, and 100

Table 17 presents the findings for some additional genes up-regulated by BBP that may be potentially associated with tumorigenesis.

Table 17. Some additional genes with differential expression[1] at day 21 in the mammary glands of rats exposed to BBP via lactational transfer (Moral *et al.*, 2007)

Symbol	Gene description	Functions	Gene expression[2]
Hspb7	Heat shock 27 kD protein family, member 7	Response to stress	++
Hsf2	Heat shock factor 2	Response to stress	++
Gab2	Growth factor receptor bound protein 2-associated protein 2	Signal transduction	+
Ghrh	Growth hormone releasing hormone	Signal transduction	++
Prkar2a	Protein kinase, cAMP-dependent, regulatory, type 2, alpha	Signal transduction	++
Arhgef1	Rho guanine nucleotide exchange factor (GEF) 1	Signal transduction	++
Gjb5	Gap junction membrane channel protein beta 5	Cell-cell signaling	+
Prkcc	Protein kinase C, gamma	Metabolism, signal transduction	++
Slk	Serine/threonine kinase 2	Metabolism, signal transduction	+
Egf	Epidermal growth factor	Cell proliferation, cellular transport	++
Rab10	RAB10, member RAS oncogene family	Oncogene, cellular transport	++
Mel	RAB8A, member RAS oncogene family	Oncogene	++
p-450olf1	Olfactory-specific cytochrome P-450 IIG1	P-450-dependent oxidative metabolism	++
Cpa1	Carboxypeptidase A1 (pancreatic)	Proteolysis during cellular protein catabolic process	+[3]

[1]Based on microarray analysis.

[2]Presented as the fold change of BBP-induced gene expression compared to control. +, ≥ 1 fold, < 2 fold; ++, ≥ 2 fold, < 3 fold

[3]*Cpa1* was detected as one of up-regulated genes at day 50, but not at day 21.

Effects of *in utero* exposure to BBP on gene expression

In the study published by Moral *et al.* (2011), pregnant Sprague-Dawley CD rats were administered 120 or 500 mg BBP/kg/day by gavage five days per week from day 10 post-conception to delivery. Female offspring were sacrificed at days 21, 35, 50, and 100, and the fourth pair of mammary glands from each animal was rapidly dissected and frozen for subsequent microarray analysis.

Microarray analysis demonstrated that *in utero* exposure to BBP alters the genomic expression profile of the mammary gland in a dose-dependent manner (Table 18). BBP exposure *in utero* down-modulated the expression of genes related to functions like immunity, apoptosis, stress, metabolism, and also differentiation markers of mammary glands. BBP exposure *in utero* up-modulated the expression of genes related to cellular signal transduction and communication. The authors suggested that BBP exposure *in utero* shifts the proliferation/apoptosis/differentiation balance toward proliferation in the mammary gland, rendering the tissue more susceptible to malignant transformation.

Results for the differential expression on day 35 of fourteen genes selected for validation by qRT-PCR are shown in Table 18. The expression level of four immune-related genes were decreased by high-dose BBP treatment, including *Cd5*, *Cd24*, Cathepsin E (*Ctse*), and *Cd45*, also known as protein tyrosine phosphatase receptor type C (*Ptprc*). Both the high and low doses of BBP decreased mammary differentiation markers, such as casein kappa (*Csn3*), milk fat globule-EGF factor 8 protein (*Mfge8*), lactalbumin alpha (*Lalba*), and whey acidic protein (*Wap*). Both the high and low doses of BBP increased expression of crystalline αb (*Cryab*), which has been described to have anti-apoptotic effects. Both the high and low doses of BBP decreased expression of *Bhlhb3*, a transcription factor that has been reported to repress the transcription of cyclin D1 and thus inhibit proliferation (Li *et al.*, 2011). Expression of genes involved in lipid and carbohydrate metabolism were also altered (Table 18) (Moral *et al.*, 2011).

Table 18. Gene expression changes[1] in fourteen selected genes[2] in the mammary glands of rats exposed to BBP *in utero* (Moral *et al.*, 2011)

Symbol	Gene description	Functions	BBP dose (mg/kg/d)	Gene expression[3]
Lalba	Lactalbumin alpha	Mammary gland differentiation, apoptosis	500	--
Mfge8	Milk fat globule-EGF factor 8 protein	Mammary gland differentiation, cell adhesion, transport	120	-
			500	--
Wap	Whey acidic protein	Mammary gland differentiation	120	--
			500	--
Cd5	Cd5 molecule	Immune system, cell adhesion, transport	500	--
Cd24	Cd24 molecule	Immune system	120	-
			500	--
Ctse	Cathepsin E	Immune system	500	--
Ptprc	Protein tyrosine phosphatase receptor type C (Cd45)	Immune system	120	--
			500	--
Thedc1[4]	Thioester hydrolase domain-containing protein 1	Lipid metabolism	500	NC(35 d)
				+*(50 d)
Bhlhb3	Class E basic helix-loop-helix protein 4	Transcription repressor, tumor suppressor gene, proliferation	120	--
			500	--
Slpi	Secretory leukocyte peptidase inhibitor	Immune system, other metabolic process	500	NC
Prkcq	Protein kinase C, theta	Signal transduction, apoptosis	500	-
Ddit4	DNA-damage-inducible transcript 4 (REDD-1)	Response to stress, apoptosis	120	--
			500	--
Cryab	crystallin, alpha B	Response to stress, apoptosis, other metabolic process	120	+*2 fold
			500	+*15 fold
Csn3	casein kappa	Mammary gland differentiation	120	--

[1]Assessed at day 35

[2]Validated by qRT-PCR

[3]Presented as the fold change of BBP-induced gene expression compared to control. NC = No change from response observed in controls; - indicates small decrease in response compared to controls (1 fold > - $\geq$ 0.5 fold); -- indicates greater decrease in response compared to controls (0.5 > -- $\geq$ 0 fold); +* indicates significantly increased compared to controls

[4]*Thedc1* was up-regulated genes at day 50, but not at day 35

3.3.5.2 In vitro differential cDNA microarray analysis

R2d Cells

Hsieh *et al.* (2012a) studied the effects of BBP on gene expression in a human breast stem cell line (R2d) that expresses functional ERs and stem cell markers ($CD44^+/CD24^-$). This non-tumorigenic, immortalized cell line was developed from normal human breast epithelial cells by infection with SV40 large T-antigen and X-ray irradiation, followed by culture in growth factor/hormone-deprived medium (Kang *et al.*, 1998; Wang *et al.*, 2010). In response to estrogen stimulation, R2d-like cells have been shown to gain cell growth activity, undergo epithelial-mesenchymal transition (EMT), promote angiogenesis (prostaglandin E2 [PGE2] pathways), and initiate tumor development (Al-Hajj *et al.*, 2003).

R2d cells were exposed to 1 µM BBP for 24 hours prior to collection for microarray analysis. Hsieh *et al.* (2012a) reported that BBP-treatment changed expression levels of 941 genes by more than ten-fold: 473 genes were up-regulated and 468 were down-regulated. Hsieh *et al.* (2012a) performed pathway analysis on the data, and concluded that the functional gene expression pattern induced by BBP is substantially similar to the pattern induced by estrogen (Al-Hajj *et al.*, 2003). Positively-regulated pathways involved in cell proliferation, some pathways involved in EMT in cancer cells, and some PGE2 pathways involved in cancer progression were selected were among the pathways altered by BBP. Eleven genes involved in these pathways were selected for validation by qRT-PCR; their pathway groups and patterns of differential expression are summarized in Table 19.

Among the genes presented in Table 19, teratocarcinoma-derived growth factor 1 (*CRITPO*), fibroblast growth factor (*FGF*), protein kinase, cAMP-dependent, regulatory, type I, beta (*PKA-cat*), vascular endothelial growth factor D (*VEGF-D*), and α platelet-derived growth factor receptor precursor (*PDGF-R-α*) are well-known mediators of cancer progression (Konno *et al.*, 2003; Carvalho *et al.*, 2005; Strizzi *et al.*, 2008; Leushacke *et al.*, 2011; Merkle and Hoffmann, 2011). Overexpression of FGF, VEGF-D, and PDGF-R-α is negatively correlated with cancer patient survival (Obermair *et al.*, 1997; Leushacke *et al.*, 2011). Moreover, the VEGF-D- and PKA-signaling pathway is associated with environmental hormones and mediates cancer cell proliferation and invasion (Buteau-Lozano *et al.*, 2008). LEF-1 is a known transcription factor involved in the Wnt/β-catenin signaling pathway (Love *et al.*, 1995), and a positive mediator of estrogen-induced mesenchymal transition in breast epithelial cells (Kim *et al.*, 2002).

Table 19. Gene expression changes in 11 selected genes[1] in R2d cells exposed to BBP (Hsieh *et al.*, 2012a)

Functional group	Symbol/Name	Gene description	Gene expression[2]
Proliferation (positive regulation)	*CRIPTO*	teratocarcinoma-derived growth factor 1	++++
	FGF	fibroblast growth factor	+++
	EDNRA	Endothelin receptor type A	+
	LMO1	LIM domain only 1 (rhombotin 1)	+
	VEGF-D	c-fos induced growth factor (vascular endothelial growth factor D)	+
	Galpha(i)-specfic peptide GPCRs	guanine nucleotide binding protein (G protein), q polypeptide	+
	PKA-reg (cAMP-dependent)(CAT)	protein kinase, cAMP-dependent, regulatory, type I, beta	++
	LEF-1	lymphoid enhancer-binding factor 1	+++
Some pathways of EMT in cancer cells	*PDGF* receptor	platelet-derived growth factor receptor	+++
	PDGF-R-α	α platelet-derived growth factor receptor precursor	++++
	EDNRA	Endothelin receptor type A	+
	LEF-1	lymphoid enhancer-binding factor 1	+++
PGE2 pathways in cancer cells	*LEF-1*	lymphoid enhancer-binding factor 1	+++
	ADCV	Adenylate cyclase	+++
	TCF (LEF-1)	transcription factor, T-cell specific	+
	PKA-reg (cAMP-dependent)(CAT)	protein kinase, cAMP-dependent, regulatory, type I, beta	++

[1]Validated by qRT-PCR

[2]Presented as the fold change of BBP-induced gene expression compared to control. +, ≥ 10 fold, < 20 fold; ++, ≥ 20 fold, < 30 fold; +++, ≥ 30 fold, < 40 fold; ++++, ≥ 40 fold.

MCF-7 Cells

Kim and colleagues (2011) studied the effect of BBP on gene expression profiles in a human breast cancer cell line (MCF-7 cells) using a cDNA microarray (Kim *et al.*, 2011). MCF-cells were treated with 100 µM BBP or 10 nM 17-β estradiol (E2) (as a positive control) for 24 hours. Total RNA was isolated from treated cells and then changes of gene expression were analyzed using the in-house designed cDNA microarray which included 416 endocrine related genes.

Table 20 summarizes the findings for some carcinogenesis-related genes which are differentially expressed by exposure to BBP or E2 in MCF-7 cells. Among these genes,

it is worth noting that *MYC* and *PGR* are ER target genes (Bourdeau *et al.*, 2008). *WT1* was also up-regulated in the mammary glands of rats exposed to BBP via lactation (Table 16, Moral *et al.*, 2007). BCL2-antagonist/killer 1 (*BAK1*) and BCL2-like 1 (*BCL2L1*) are apoptosis-related genes (Kroemer, 1997). *CYP1B1* is a target gene of AhR-mediated gene expression (Puga *et al.*, 2005; 2009), and *CYP17A1* is involved in steroidogenesis (Scott *et al.*, 2009).

Table 20. Differential expression of some carcinogenesis-related genes in BBP- and E2[1]- treated MCF-7 cells (Kim *et al.*, 2011)

Gene name	Gene symbol	Change of expression	
		BBP	E2
Cytochrome P450, family 1, subfamily B, polypeptide 1	*CYP1B1*	up	up
Myc myelocytomatosis viral oncogene homolog (avian)	*MYC*	up	up
Progesterone receptor	*PGR*	up	up
BCL2-antagonist/killer 1	*BAK1*	down	down
BCL2-like 1	*BCL2L1*	down	down
Cytochrome P450, family 17, subfamily A, polypeptide 1	*CYP17A1*	up	down
Wilms tumor 1	*WT1*	up	up

[1]17-β estradiol

3.3.5.3 Proteomic analysis

Choi *et al.* (2010) studied changes in proteins secreted by a human hepatocellular carcinoma cell line (HepG2 cells) induced by BBP. HepG2 cells were treated with BBP at concentrations of 0, 10, or 25 µM for 24 or 48 hours. The control and treated culture media were then collected and subjected to proteomic analysis using large-size isoelectric focusing two-dimensional SDS polyacrylamide gel electrophoresis (2D-SDS-PAGE). A number of proteins were found to be differentially expressed in cells treated with BBP. Table 21 presents findings for nine proteins.

Table 21. Some differentially expressed proteins secreted from HepG2 cells exposed to BBP (Choi *et al.*, 2010)

Functional group	Symbol/Name	Protein description	Changes in expression
Tumor progression and metastasis	DEK protein	Abundant chromatin protein	Down
	Gelsolin	Actin-binding protein	Up
	ITIH2	Inter-alpha-trypsin inhibitor heavy chain H2	Down
	RKIP	Raf kinase inhibitory protein	Up
	Rho-GDI	Rho GDP inhibitor	Up
Oxidative stress	Cystatin C	Cysteine protease inhibitor	Down
Energy metabolism	ETFB	Electron transfer flavoprotein subunit beta	Up
	TIM	Triose phosphate isomerase	Up
Cell structure	HRP	Haptoglobin-related protein	Down

Among the identified proteins, abundant chromatin protein (DEK), Rho GDP inhibitor (Rho-GDI), inter-alpha-trypsin inhibitor heavy chain H2 (ITIH2), Raf kinase inhibitory protein (RKIP), and gelsolin, are associated with tumor progression and metastasis.

DEK, a chromatin protein, induces positive supercoils into closed circular DNA in mammalian cells (Waldmann *et al.*, 2004). The association of DEK and human malignancies was identified by its chromosomal translocation outcomes in a subset of acute myeloid leukemia patients (Kappes *et al.*, 2008). In addition, DEK plays a role in repair of DNA strand breaks and protects cells from genotoxic agents (Kappes *et al.*, 2008). The reduction in DEK protein expression by BBP may be associated with the increased DNA damage observed in these cells, measured as a significantly increased Olive tail moment in the Comet assay (Choi *et al.*, 2010).

A dose-dependent increase in the expression of Rho-GDI, a regulator of Rho GTPase, was also observed. Rho-GDI is reported to play an essential role in malignant transformation and is up-regulated in metastasized colorectal cancer specimens (Zhao *et al.*, 2008).

ITIH2, a serine protease, was originally found to stabilize the extracellular matrix by covalent linkage to hyaluronan (Bost *et al.*, 1998). Down-regulation of ITIH2 in breast tumors suggests the association of ITIH2 with initiation and progression of tumor malignancy (Hamm *et al.*, 2008).

RKIP, a Raf kinase inhibitory protein, was identified to be associated with a metastasis suppressor gene (Fu *et al.*, 2002).

Gelsolin, an actin-binding protein, acts as an inhibitor of apoptosis by stabilization of mitochondria (Koya *et al.*, 2000; Wang *et al.*, 2009). In addition, gelsolin was reported to increase TNF-induced apoptosis in MCF-7 cells and facilitate migration and invasion of tumor cells (Li *et al.*, 2009).

Cystatin C, a cysteine protease inhibitor, is associated with protection of PC12 cells against various types of oxidative stress (Nishiyama *et al.*, 2005).

ETFB and TIM are involved in energy metabolism (Schiff *et al.*, 2006; Pretsch, 2009).

3.3.6 Effects on Cell Proliferation, Apoptosis and Necrosis

It has been established that the genes modulated by treatment with BBP are involved in cell proliferation, apoptosis, epithelial-mesenchymal transition, tumor cell migration and invasion, and tumor progression. This was shown through cDNA microarray analysis and proteomic analysis, followed by pathway analysis. Furthermore, the results of a number of cellular studies from several different independent research groups support the findings regarding the cDNA microarray and proteomic analysis data. Here, we summarize the major results according to their relevance to particular carcinogenic endpoints with emphasis on significant findings corresponding to the results from genomic and proteomic analysis.

3.3.6.1 Cell Proliferation

Breast cells

Rat mammary gland structures include terminal end buds (TEB), terminal ducts (TD), alveolar buds (AB), and type 1 lobules (LB1) (see Appendix A).

Moral and colleagues showed that neonatal/prepubertal exposure to BBP at 500 mg/kg/day maternal exposure increased the proliferative index as measured by BrdU incorporation into DNA in TEBs at day 35 and in LB1 at days 35 and 100 ($p < 0.05$ in all cases) in female SD rats (Moral *et al.*, 2007). In another study, Moral *et al.* reported that prenatal exposure to BBP (at 120 or 500 mg/kg/day maternal exposure) significantly increased the proliferative index of epithelial structures of mammary glands, including TEB, TD, and LB1, in female SD rat offspring (Moral *et al.*, 2011).

In vitro

R2d cells (description in Section 3.3.5) were treated with BBP (10 nM - 10 µM) for 24 hours in FBS-free medium (Hsieh *et al.*, 2012a). BBP significantly increased cell proliferation in a dose-dependent manner. Additionally, the estrogen inhibitors ICI182780 and tamoxifen (TAM) inhibited BBP-induced cell proliferation. These data demonstrate that BBP can mimic estrogen to promote cell proliferation in R2d cells.

The E-screen assay uses MCF-7 cells to test the estrogenic activity of environmental chemicals (Soto *et al.*, 1995). The *in vitro* E-screen assay method is one of the most sensitive assays for assessing the estrogenic activity of several phthalate esters. Several studies performed E-screen assays to assess the estrogenic potential of BBP in MCF-7 cells. The results are summarized in Table 22.

Most studies show that a concentration of 10 µM BBP is able to induce maximal cell proliferation in the E-screen assay. BBP is approximately 10^{-7} to 10^{-6} less potent than E2. At concentrations from 100 to 1000 µM, hippuric acid, which has been reported to be a metabolite of BBP in MCF-7 cells, was found to significantly increase MCF-7 cell proliferation up to 4-fold compared to untreated controls (Picard *et al.*, 2001).

Table 22. A partial list of MCF-7 and ZR-75 cell proliferation studies of BBP and hippuric acid

Compound	Concentration[a]	RPE, %[b]	RPP, %[c]	Reference
17β-estradiol[d]	30 pM	100	100	Soto *et al.*, 1995; Sonnenschein *et al.*, 1995; Okubo *et al.*, 2003
	10 nM	100	100	Harris *et al.*, 1997; Jobling *et al.*, 1995[g]; Picard *et al.*, 2001
BBP	10 µM	90	0.0003	Soto *et al.*, 1995; Sonnenschein *et al.*, 1995
	10 µM	80	0.01	Harris *et al.*, 1997
	10 µM[e]	75	-	Jobling *et al.*, 1995[g]; Parveen *et al.*, 2008
	10, 20 µM[e]	-	-	Hsieh *et al.*, 2012a
	0.4 µM[e]	-	-	Suzuki *et al.*, 2001
	100 µM[e]	80	0.001	Kim *et al.*, 2004
	1, 10, 100 µM[e]	-	-	Hashimoto *et al.*, 2003; Hong *et al.*, 2005
	5 µM[f]	-	0.01[f]	Anderson *et al.*, 1999[f]
	1 µM[f]	-	0.001[f]	
	1, 2.5, 10 µM[e]	-	-	Picard *et al.*, 2001
	250, 1000 µM[e]	50	-	Okubo *et al.*, 2003
Hippuric acid	100, 250, 500, 1000 µM[e]	-	-	Picard *et al.*, 2001

- , not reported

[a]The lowest concentration needed for maximal cell yield.

[b]The relative proliferative effect (RPE) is calculated as 100x(PE-1) of the test compound/(PE-1) of E2; a value of 100 indicates that the compound tested is a full agonist, a value of 0 indicates that the compound lacks estrogenicity at the doses tested, and intermediate values suggest that the xenobiotic is a partial agonist.

[c]Relative proliferative potency (RPP) is the ratio between E2 and xenobiotic doses needed to produce maximal cell yields x 100. All compounds designated as full or partial agonists increased cell yields significantly over the control ($p < 0.05$).

[d]17β-estradiol (E2) was used as positive control

[e]Indicates the concentration which significantly increased cell proliferation compared to the control in the study.

[f]The value indicates EC_{50} which expresses the concentration of BBP needed to produce 50% of the maximal response induced by 17β-estradiol at the concentration of 7 pM.

[g]The study used ZR-75 cells (see next section for details)

ZR-75 cells, which are an estrogen-responsive human breast cancer cell line with ER-positive immunostaining, have been shown to have a more estrogen-specific response compared to MCF-7 cells (Jobling *et al.*, 1995; Harris *et al.*, 1997). 10 µM BBP induced an approximately 2-fold increase in cell proliferation (Jobling *et al.*, 1995; Harris *et al.*, 1997). The growth responses to BBP in ZR-75 cells were all less than the maximal responses elicited by E2 (50 to 75% less).

Hsieh *et al.* (2012c) reported that 1 µM BBP induced cell proliferation in MDA-MB-231 cells (ER-negative human epithelial breast cancer cell line) at 48 and 72 hours of treatment in a dose-dependent manner.

Osteoblastic cells

Rat Py1a osteoblasts are an immortalized clonal cell line, isolated from rat bone (Raisz *et al.*, 1993). Agas *et al.* (2007) investigated the effects of BBP in modulating bone cell viability and proliferation by using rat Py1a osteoblasts. The results indicated that treatment with 1 µM BBP for 24 hours increased cell proliferation. BBP treatment also increased cyclin D3 levels. Cyclin D3 plays a critical role in G1 to S cell cycle stage transition in mammalian cells. The authors suggested that cyclin D3 acts as a putative proto-oncogene in BBP- treated osteoblastic cells, suggesting a possible effect of the phthalate endocrine disruptor in cancer processes.

In another study (Sabbieti *et al.*, 2009), MC3T3-E1 cells, a non-transformed cell line derived from newborn mouse calvariae displaying osteoblast-like characteristics, were used to investigate the effects of BBP on bone cell proliferation. 1 µM BBP induced cell proliferation in p53-knockdown MC3T3-E1 cells ($p < 0.05$) but not in wild-type MC3T3-E1 cells; p53 acts as a tumor suppressor in mammalian cells. Therefore, Sabbieti *et al.* (2009) suggested that the proliferative effects of BBP could be mediated by p53, either through inhibition of p53 activation or p53 mutation.

3.3.6.2 Apoptosis and Necrosis

Apoptosis is the process of programmed cell death that occurs in multicellular organisms. Apoptotic biochemical events lead to characteristic cellular morphological changes and death. Caspases play a central role in the transduction of apoptotic signals. Active effector caspases, such as caspase-3, proteolytically degrade intracellular proteins, eventually leading to apoptosis (Susin *et al.*, 2000). Therefore, caspase-3 is routinely used as an indicator of commitment of cells to apoptosis. In addition, a large family of apoptosis-regulating proteins has been identified, including cell death blockers (such as Bcl-2) and cell death promoters (such as Bax) (Kroemer, 1997). Bax counteracts the anti-apoptotic effects of Bcl-2 by forming a heterodimer with Bcl-2. The ratio of Bcl-2 to Bax, rather than the levels of the individual proteins, is considered to be critical in determining the survival or death of cells.

The effects of BBP on apoptosis have been studied in breast, bone, ovary, and immune systems (Marchetti *et al.*, 2002; Kim *et al.*, 2004; Agas *et al.*, 2007; Naarala and Korpi, 2009; Sabbieti *et al.*, 2009; Chen *et al.*, 2012; Mankidy *et al.*, 2013). The data are

conflicting: BBP was found to decrease human breast cancer cell apoptosis in one study (Kim *et al.*, 2004), increase human breast cancer cell and mouse osteoblasts apoptosis in two studies (Sabbieti *et al.*, 2009; Mankidy *et al.*, 2013), and had no observable effect on rat osteoblast, human granulosa cell and mouse macrophage apoptosis in other studies (Marchetti *et al.*, 2002; Agas *et al.*, 2007; Naarala and Korpi 2009; Chen *et al.*, 2012). The results of these studies are discussed in more detail below.

Breast cells

Kim *et al.* (2004) showed that 100 µM BBP inhibited TAM-induced apoptosis in MCF-7 cells. E2 also inhibits TAM-induced apoptosis in these cells. 100 µM BBP also significantly increased cell proliferation in MCF-7 (Table 22), but not in MDA-MB-231 cells. Expression of Bcl-2 was decreased while Bax expression was up-regulated by TAM. E2 and BBP demonstrated the ability to reverse the expression patterns of Bcl-2 and Bax induced by TAM back to normal. Therefore, the data suggest that the inhibitory effect of BBP on TAM-induced apoptosis involves an increase in the intracellular Bcl-2 to Bax ratio. Given that BBP is currently widely used, the findings that revealed the promoting effect of BBP on chemotherapeutic drug resistance to TAM in breast cancer may be of biological relevance to humans. BBP may decrease the survival rate of breast cancer patients who are taking TAM.

Conversely, 3.3 µM BBP induced caspase-3 activation in MVLN cells (derived from MCF-7 cells) (Mankidy *et al.*, 2013).

Bone osteoblastic cells

Sabbieti *et al.* (2009) reported that BBP at a concentration of 1 µM significantly increased pro-apoptotic Bax protein expression levels and activated caspase-3 protein expression levels in mouse MC3T3-E1 cells. In contrast, BBP had no effect on apoptotic outcomes in rat Py1a osteoblasts (Marchetti *et al.*, 2002; Agas *et al.*, 2007), and did not change the Bcl-2/Bax ratio (Agas *et al.*, 2007).

Ovary cells

Chen *et al.* (2012) reported that while apoptosis was not induced, necrosis was induced in HO23 cells (immortalized human granulosa cell line established by transfection of primary cells obtained from IVF patients with the SV40 genome, Ha-ras oncogene, and the p53 gene (Hosokawa *et al.*, 1998) after exposure to 1 µM BBP for 48 hours. Use of either CYP1B1 or AhR siRNA knockdown to block CYP1B1 or AhR activity reduced

necrosis, suggesting involvement of AhR- and CYP1B1-mediated pathways in BBP-induced HO23 cellular necrosis.

3.3.7 Effects on Angiogenesis, Epithelial-Mesenchymal Transition, Migration and Invasion

3.3.7.1 Angiogenesis

Angiogenesis is a fundamental step in the transition of tumors from a benign state to a malignant state (Folkman, 1971). In addition, vascular endothelial growth factor (VEGF) has been shown to be a key factor in angiogenesis and tumor progression that stimulates endothelial cell proliferation and migration *in vitro* and *in vivo* (Ferrara and Davis-Smyth, 1997).

Buteau-Lozano *et al.* (2008) reported BBP but not the metabolite MBuP induced a dose-dependent increase in VEGF levels in MELN and MCF-7 cells. MELN cells are derived from MCF-7 cells and express endogenous ERα and a ERE-coupled luciferase gene (Balaguer *et al.*, 2001).

Hsieh *et al.* (2012a) reported that BBP increased angiogenesis by MCF-7 cells. Both the tube formation ability of human umbilical vein endothelial cells (HUVECs*) in vitro* and a Matrigel plug/nude mouse assay *in vivo* were used to assay angiogenesis induction. BBP significantly increased both the *in vitro* tube formation of HUVECs and the production of functional blood vessels and induced hemoglobin expression in the Matrigel plug/nude mouse assay.

3.3.7.2 Epithelial-Mesenchymal Transition (EMT)

Excessive epithelial cell proliferation and angiogenesis are hallmarks of the initiation and early growth of primary epithelial cancers (Hanahan and Weinberg, 2000). The subsequent increase in invasiveness, initially established by invasion through the basement membrane, is thought to signal the onset of the last stages of the multi-step process that eventually leads to tumor metastasis. Therefore, activation of an EMT has been proposed as a critical mechanism for the achievement of malignant phenotypes by epithelial cancer cells (Thiery, 2002).

Figure 6 demonstrates the critical process of EMT and its reverse process, mesenchymal-epithelial transition (MET), in tumor progression. Progression from normal epithelium to invasive carcinoma goes through several stages as follows. First, the invasive carcinoma stage involves epithelial cells losing their polarity and detaching

from the basement membrane. Then, the composition of the basement membrane changes, followed by the alteration of cell-ECM (extracellular matrix) interactions and signaling networks. The next step involves EMT and an angiogenic switch, facilitating the malignant phase of tumor growth. Progression from this stage to metastatic cancer also involves EMTs, enabling cancer cells to enter the circulation and exit the blood stream at a remote site, where they may form micro- and macro-metastases, which may involve METs and thus a reversion to an epithelial phenotype (Kalluri and Weinberg, 2009).

EMT and MET are usually detected by changes between the ratio of mesenchymal markers, such as CD90 and vimentin, and epithelial markers, such as cytokeratin 7 or E-cadherin.

Hsieh *et al.* (2012b) reported that 1 µM BBP induced EMT in R2d cells. Western blotting analysis indicated BBP caused a significant increase in the level of vimentin and CD90, and reduced levels of cytokeratin 7 and E-cadherin. In addition, BBP changed the typical epithelial cell morphology and contiguous colony appearance of R2d cells into more spindle-like cells and scattered colonies, resembling mesenchymal cells. These morphological changes were observed by staining with Alexa 488–conjugated β-actin. To further verify whether BBP-treated cells changed their EMT associated with the alteration of their migration and invasive ability, wound healing and invasion assays were also performed (section 3.3.7.3).

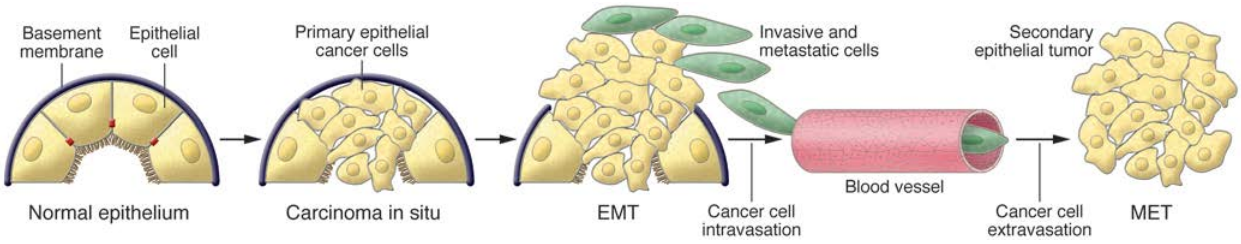


Figure 6. Contribution of epithelial-mesenchymal transition to cancer progression (from Kalluri and Weinberg, 2009)

3.3.7.3 Migration and Invasion

Cell motility, in particular migration and invasion, is an important feature of tumor progression following EMT (Thiery, 2002; Thompson *et al.*, 2005). It has been proposed that EMT is regulated by estrogenic pathways (Ding *et al.*, 2006) and pathways involving TGF-β (Valcourt *et al.*, 2005). The estrogenic pathways are discussed in more details in Section 4.4.

Hsieh *et al.* (2012a; 2012b) reported that both cell invasion and migration abilities were enhanced significantly by BBP treatment in both R2d and MCF-7 cells.

Treatment of MDA-MB-231 cells with BBP enhanced both migration and invasion activity (Hsieh *et al.*, 2012c).

To summarize, BBP has been shown to significantly enhance migration and invasion activity in three different cell lines.

3.3.8 Effects on Colony and Tumor Formation in vitro and in vivo

3.3.8.1 Colony Formation in vitro

One of the characteristics of cancer cells is the ability to grow and form colonies on soft agar plates (Aapro *et al.*, 1987). Therefore, the soft agar colony formation assay provides a fast, simple, and robust method for identification of tumor-specific agents in a biologically-relevant microenvironment.

Hsieh *et al.* (2012c) reported that MDA-MB-231 cells treated with BBP demonstrated increases in ability to form colonies on soft agar in a dose-dependent manner.

3.3.8.2 Tumor Formation in Vivo

Athymic nude mice are hairless, lack a normal thymus gland, and have a defective immune system because of a genetic mutation. Athymic nude mice are often used in cancer research because they do not reject tumor cells, regardless of whether the cells come from mice or other species.

Hsieh *et al.* (2012c) reported that BBP promotes human breast tumor growth on MDA-MB-231 cell xenografts in the female athymic nude mouse model. In the study, $2x10^6$ MDA-MB-231 cells stably expressing green fluorescent protein (GFP) were subcutaneously injected into the flanks of the nude mice. When the tumor size reached 30 mm^3, the mice were randomly divided into 2 groups, one administered normal saline and the other administered BBP. The animals received either a dose of 800 mg/kg/day BBP or vehicle control via intraperitoneal injection each day for 45 days. The growth of the xenograft tumors was monitored by whole-body bioluminescence and measured by a caliper to determine the tumor volumes (mm^3). BBP treatment significantly increased tumor growth.

In another study, Hsieh *et al.* (2012b) reported that BBP induced tumorigenesis and metastasis of R2d cells in the athymic nude mouse model. In this study, $2x10^5$ R2d-YFP (yellow fluorescent protein) cells were intravenously injected into nude mice. The mice were given a dose of 800 mg/kg/day BBP or vehicle control via intraperitoneal injection for 8 weeks, and demonstrated lung colonization of the R2d-YFP cells in 5 of 6 BBP-treated mice, compared to no lung colonization in controls. BBP treatment significantly increased R2d cell tumor growth and metastases *in vivo*.

3.3.9 Effects on Mammary Gland Development and Cancer Susceptibility

In utero and early life exposure to BBP in rats changes the structure of the mammary gland. A description of the structure of the mammary gland and its development in rats of both genders, as well as the basis for increased susceptibility of those changes to neoplastic transformation, is provided in Appendix A. Carcinogenesis studies with BBP dosing early in life have not been performed.

3.3.9.1 The Effects of In Utero Exposure to BBP on Female Offspring Rat Mammary Gland Development

In a study published by Moral *et al.* (2011), 120 mg (low-dose group) or 500 mg (high-dose group) BBP/kg body weight/day was administered by gavage to pregnant Sprague Dawley CD rats from day 10 post-conception to delivery. Female offspring were sacrificed at postnatal days (PND) 21, 35, 50, and 100 and their fourth mammary gland pair was dissected. The morphology and proliferative index of the mammary gland were studied using whole mount preparations and BrdU incorporation, respectively. The gene expression profile was assessed by microarray and genes with the pattern of differential expression were further validated by real time RT-PCR (Moral *et al.*, 2011).

Effects of *In Utero* Exposure to BBP on Mammary Gland Morphology

In utero exposure to the high dose of BBP resulted in modifications in mammary gland architecture, including increased TDs in comparison to the controls ($p = 0.003$) at PND 21 and an increased number of ABs in comparison with the control group ($p = 0.02$) at PND 35. Female rat offspring with increased numbers of undifferentiated TEBs may have greater susceptibility to breast carcinogen insults later in life compared to unexposed animals.

BBP exposure (both dose groups) also resulted in significant increases in the proliferative index of the mammary gland epithelial structures, including TEB, TD, and LB1.

As described in Appendix A, the TEB is the least-differentiated epithelial structure in the mammary gland and the most susceptible to malignant transformation. Therefore, alterations in number and proliferation of TEBs by prenatal exposure to BBP may increase susceptibility of the mammary gland of female offspring to breast cancer.

Effects of *In Utero* Exposure to BBP on Sexual Maturation

In utero exposure to the high dose of BBP significantly retarded the vaginal opening by two days (from PND 31 to PND 33). This finding demonstrates that prenatal exposure to BBP causes a significant delay in female sexual maturity which can lead to a delayed pubertal onset. A delayed pubertal onset leads to delayed TEB differentiation; therefore, it may increase the susceptibility of the mammary gland to malignant transformation (Rudel *et al.*, 2011; Institute of Medicine of the National Academies, 2012).

Effects of *In Utero* Exposure to BBP on Gene Expression

BBP exposure down-modulated the expression of genes related to functions like immunity, apoptosis, stress, metabolism, and also differentiation markers of mammary glands. On the other hand, BBP exposure up-modulated the expression of genes related to cellular signal transduction and communication. All the data suggest a proliferation/apoptosis/differentiation balance tilted to proliferation in the mammary glands of the BBP-exposed rats, and their TEBs may be more susceptible to malignant transformation. For example, high doses of BBP decreased expression of immune system-related genes, including *Cd*5 and *Cd*45 (Moral *et al.*, 2011). Both the high and low doses of BBP in this study decreased mammary differentiation markers, such as casein kappa (Csn3), milk fat globule-EGF factor 8 protein (Mfge8), lactalbumin alpha (Lalba), and whey acidic protein (Wap). Both the high and low doses of BBP in this study increased expression of *Cryab*, which has been described to have anti-apoptotic effects. These alterations in gene expression may lead to an increased susceptibility to carcinogenesis.

3.3.9.2 The Effects of Neonatal/Prepubertal Exposure to BBP on Female Rat Mammary Gland Development

Moral *et al.* (2007) gavaged lactating Sprague Dawley CD rats (10 dams per group) five days/week with 500 mg BBP/kg body weight/day or an equivalent volume of sesame oil on days 2–20 after giving birth. Female offspring were processed and observed at PND 21, 35, 50, and 100 for maturation parameters and for mammary gland architecture and morphology, proliferative index, and genomic profile of the mammary gland.

The results showed that neonatal/prepubertal exposure to BBP at 500 mg/kg/day significantly increased the uterine weight to body weight ratio at PND 21. Typically, increased uterine weight after chemical exposure indicates that the chemical is capable of inducing estrogenic effects through interaction with ERα.

Neonatal/prepubertal exposure to BBP at 500 mg/kg/day did not induce significant mammary gland morphological changes or change the number of epithelial structures, such as TEB, TD, and LB1, when compared to control groups. Between PND 21 and 100, the number of undifferentiated TEBs decreased and the number of TDs and LB1 increased in both control and BBP treated animals. However, BBP exposure did significantly increase the proliferative index in TEBs at PND 35 and in LB1 at PND 35 and 100.

In addition, neonatal/prepubertal exposure to BBP at 500 mg/kg/day had an effect on the genomic profile of the mammary gland, mostly at the end of the treatment (PND 20); the number of modulated genes subsequently decreased after treatment cessation. By PND 21, a significant number of genes related to proliferation and differentiation, communication, and signal transduction were up-regulated in the mammary glands of the exposed animals (e.g. *Ahr* and *Wt1*). The only down-regulated gene was glutamate decarboxylase 1 (*Gad1*). *Gad1* codes for the protein Gad67, which catalyzes the production of the neurotransmitter GABA. Since BBP has also shown an effect on the methylation status of ERα promoter-associated CpG islands in MCF-7 cells *in vitro* (Kang and Lee, 2005), it is possible that the observed changes in *Gad1* are due to an effect of BBP on the methylation of the *Gad1* promoter. The GABA-ergic system has been found to be involved in hormonal regulation and pathogenesis of breast cancer in humans and mice (Opolski *et al.*, 2000).

Overall, these data suggest that neonatal/prepubertal exposure to BBP increases the proliferative index of undifferentiated mammary gland epithelial structures, such as TEBs and LB1, and affects the gene expression profile of the mammary gland. The

effects of BBP may cause increased susceptibility of female rat mammary glands to malignant transformation later in life.

3.3.9.3 The Effects of In Utero Exposure to BBP on Male Offspring Rat Mammary Gland Development

Pregnant Sprague Dawley CD rats were exposed by gavage daily with BBP from gestation day 14 through gestation day 21 (Sumner *et al.*, 2009). Three dams per group were dosed with corn oil vehicle (control), 25 mg/kg/day BBP (low dose), or 750 mg/kg/day BBP (high dose). Seven of the 16 male pups from the maternal low-dose group retained areolae on PND 11. All six male pups in the maternal high-dose group retained areolae, and three of those animals retained nipples at PND 11 or 13. No abnormal findings were observed in the 17 male pups from the maternal control group. The results indicate *in utero* exposure to BBP has profound effects on male rat pup mammary development. BBP is known to disrupt reproductive development in male rats following *in utero* exposure by affecting fetal testosterone biosynthesis in the Leydig cells (Nagao *et al.*, 2000). Perturbations of male rat pup mammary development from *in utero* exposure to BBP may increase the susceptibility of male rats to breast cancer later in life (Latendresse *et al.*, 2009).

3.3.9.4 The Effects of BBP on Neoplastic Transformation of Human Breast Epithelial Cells in 3-Dimensional Culture in vitro

Fernandez and Russo (2010) treated MCF-10F cells with 10^{-3} - 10^{-6} M BBP continuously for two weeks, with fresh medium added every day. The MCF-10F human breast epithelial cell line is spontaneously immortalized, non-transformed/non-invasive, and does not grow in soft agar or form tumors in nude mice (Singhal *et al.*, 1999). At the end of ten passages following the end of treatment, the invasive capacity of the BBP-treated cells was evaluated using Boyden chambers. MCF-10F cells treated with BBP (10^{-5} M and 10^{-6} M) showed a higher invasive capacity compared to the control, and the differences were statistically significant ($p \leq 0.01$) (Fernandez and Russo, 2010). Furthermore, the invasion capacity of MCF-10F cells treated with 10^{-6} M BBP was similar to that of cells treated with 0.007 nM estradiol. Overall, the results show that BBP could induce neoplastic transformation of MCF-10F cells *in vitro*.

3.3.10 Effects on Endometriosis and Ovarian Cancer

Endometriosis has been associated with increased risk of ovarian cancer in humans, based on epidemiological data. BBP exposure has been proposed to increase endometriosis risk based on human epidemiological data (Reddy *et al.*, 2006; Rozati *et al.*, 2008; Huang *et al.*, 2010; Weuve *et al.*, 2010). BBP exposure could therefore potentially increase the risk for human ovarian cancer by increasing endometriosis incidence. This section briefly reviews the evidence for both associations.

Endometriosis is defined as the presence of endometrial tissue outside the uterine cavity, often occurring on the surface of the ovaries and the pelvic peritoneum. Endometriosis occurs in an estimated 5-10% of women of reproductive age, and because the extra-uterine endometrial tissue responds to cyclic changes in estrogen just like endometrial tissue lining the uterus, often presents with pelvic pain, inflammation, and infertility (Worley *et al.*, 2013). The etiology of endometriosis is not known, but is believed to involve genetic, hormonal, and immunological factors (Worley *et al.*, 2013).

Endometriosis behaves similarly in many ways to invasive cancer: it can attach to, invade, and damage affected tissues, and it can spread to distant tissues (e.g., bladder, intestine). Malignant transformation of endometriotic ovarian cysts to ovarian carcinoma occurs, and has been associated with hyper-estrogenism (Worley *et al*., 2013). Numerous epidemiology studies have reported that women with endometriosis have an increased risk of developing epithelial ovarian cancer, particularly of the endometrioid and clear cell subtypes (Brinton *et al.*, 2005; Melin *et al.*, 2006; Pearce *et al.*, 2012).

Melin *et al*. (2006) analyzed the records of 64,492 women hospitalized with endometriosis between years 1969 to 2000 in Sweden. The risk of ovarian cancer was elevated with standard incidence ratio (SIR) of 1.43 (95% confidence interval [CI]: 1.19-1.71), the risk was particularly elevated among women with long-standing ovarian endometriosis (SIR = 2.23, 95% CI: 1.36-3.44). Brinton *et al.* (2005) linked a national cohort of women with a history of endometriosis in Denmark in the period 1978-1998 with cancer registry data to assess cancer risk. They determined that women with endometriosis had a predisposition to ovarian cancer, and the association was restricted to endometrioid (relative risk [RR] = 2.53, 95% CI: 1.19-5.38) and clear cell (RR = 3.37, 95% CI: 1.24-9.14) malignancies.

Most recently, Pearce *et al.* (2012) reported that endometriosis was statistically significantly associated with risk of clear-cell (odds ratio [OR] = 3.05, 95% CI: 2.43-3.84), low-grade serous (OR = 2.11, 95% CI: 1.39-3.20), and endometrioid invasive (OR = 2.04, 95% CI: 1.67-2.48) ovarian cancers in an analysis of data pooled from 13 case-

control studies in six countries. On the other hand, endometriosis was not significantly associated with mucinous or high-grade serous invasive ovarian cancers, nor with borderline ovarian tumors of either subtype (serous or mucinous).

Table 23 summarizes several epidemiological studies on the potential association of BBP exposure and endometriosis (Reddy *et al.*, 2006; Itoh *et al.*, 2009; Roya *et al.*, 2009; Huang *et al.*, 2010; Weuve *et al.*, 2010). Two prospective case-control studies from India showed that BBP concentration in blood was strongly correlated with endometriosis severity, and that the blood concentrations of BBP in women with endometriosis were significantly higher compared to the women without endometriosis (Reddy *et al.*, 2006; Roya *et al.*, 2009).

The urinary concentration of MBuP, one of major urinary metabolites of both BBP and DBP, in women with endometriosis was significantly higher compared to the women without endometriosis in the U.S. and Taiwan (Huang *et al.*, 2010; Weuve *et al.*, 2010). Contrary to the studies in the U.S. and Taiwan, Itoh *et al.* (2009) reported finding no significant association between urinary MBuP concentrations in Japanese women with endometriosis. The urinary concentration of MBzP, the other major urinary monoester phthalate metabolite which is produced only from BBP, has not been significantly associated with endometriosis in the U.S., Taiwan, or Japan (Itoh *et al.*, 2009; Huang *et al.*, 2010; Weuve *et al.*, 2010).

Table 23. Epidemiological studies on BBP exposure and endometriosis (EN)

Country/ study type/year	Subjects	Samples/ Biomarkers	Results	References
U.S./ NHANES cross-section/ 1999-2004	87 women with history of EN (self-reported), 1,020 without EN	Urine/MBuP, MBzP	MBuP: EN: OR=1.36[1] (95% CI, 0.77-2.41) MBzP: no significant association with EN	Weuve *et al.*, 2010
Taiwan/ case-control/ 2005-7	Case: 28 women with EN Control: 29 age-matched women without EN	Urine/MBuP, MBzP	MBuP: EN women ↑MBuP (94.1 vs 58 µg/g, p<0.05) EN: OR=3.64[2] (95% CI,1.16-10.03;p<0.026) MBzP: no significant association with EN	Huang *et al.*, 2010
Japan/ case-control/ 2000-1	137 EN women: 80 control (stages 0-1); 57 case (stages 2-4)	Urine/MBuP, MBzP	No significant association between EN and MBuP, MBzP	Itoh *et al.*, 2009
India/ prospective case-control	Cases: 99 infertile women with EN. Controls: 135 age-matched infertile women without EN.	Serum/BBP	EN women ↑BBP (3.32±2.17 µg/ml vs 0.15±0.21µg/ml, p<0.0001) Correlation of BBP concentration and EN severity: r=+0.89, p<0.0001	Rozati *et al.*, 2008
India/ prospective case-control	Cases: 49 infertile women with EN. Control1: 38 age-matched infertile women without EN. Control2: 21 age-matched women with proven fertility, without EN.	Plasma/BBP	EN women ↑BBP (Case group, 0.66±0.61 µg/ml vs. control1, 0.12±0.2 µg/ml, p<0.05; case group, 0.66±0.61 µg/ml vs control2, 0.11±0.22 µg/ml, p<0.05) Correlation of BBP concentration and EN severity: r=+0.78, p<0.0001	Reddy *et al.*, 2006

[1]The OR represents the comparisons between the highest versus lowest three quartiles of urinary MBuP.
[2]The OR was calculated from the subjects with the level of urinary MBuP above median levels.

Although the underlying mechanisms and factors involved with the malignant transformation of endometriosis remain unclear, current molecular studies have linked endometriosis with endometriosis-associated ovarian carcinoma through pathways related to oxidative stress, inflammation, and hyper-estrogenism (Worley *et al.*, 2013). The phthalate DEHP has been found to induce oxidative stress and inflammation *in vivo* and *in vitro* (IARC, 2012). BBP has been shown to have estrogenic activity (Sections 3.3.6, 3.3.9, 4.4). In addition, female infertility patients with endometriosis had higher levels of PPARγ expression than women with other causes of infertility (Caserta *et al.*, 2013). BBP has been found to activate PPARα, β, and γ (Section 4.2), and PPARα, β, and γ are all found in human uterus and ovarian tissue (Komar, 2005; Knapp *et al.*, 2012).

To summarize, there is evidence that BBP is associated with increased risk of endometriosis and that endometriosis is associated with human ovarian cancer, leading

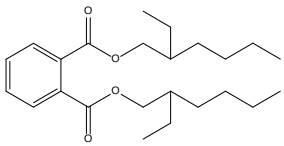
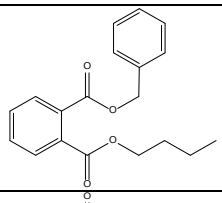
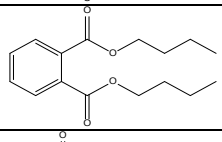
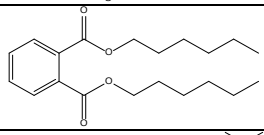
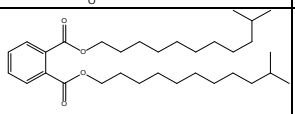
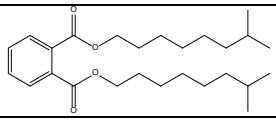
to the possibility that BBP exposure could potentially increase the risk for ovarian cancer by increasing endometriosis incidence.

3.3.11 Structure Activity Comparisons

BBP is a diester of phthalic acid. Phthalic acid is an aromatic dicarboxylic acid in which the two carboxylic acid groups are located on adjacent carbons (positions 1 and 2) in the benzene ring. The two carboxylic acid groups of phthalic acid can form ester links, generally with short- or long-chain alkanes. There are two general groups of phthalates, the first of which consists of high molecular weight phthalates with seven or more carbon atoms in their backbone, such as diisononyl phthalate (DINP) and diisododecyl phthalate (DIDP). The second group consists of low molecular weight phthalates with three to six carbon atoms in their backbone, such as DEHP, BBP and DBP.

DEHP is listed under Proposition 65 as a carcinogen, a male reproductive toxicant, and a developmental toxicant. DEHP is the only phthalate listed as a carcinogen under Proposition 65. Table 24 summarizes the structure and other information of four other phthalates, namely DBP, DNHP, and DIDP, which are listed as reproductive or developmental toxicants under Proposition 65, or are under listing consideration.

Table 24. Phthalates listed or being considered for listing under Proposition 65.

Name	Acronym	Structural formula	CAS number	Classification	Date listed
Di(2-ethylhexyl) phthalate	DEHP		117-81-7	Cancer Developmental, Male Reproductive	1988 2003
Butyl benzyl phthalate	BBP		85-68-7	Developmental	2005
Di-n-butyl phthalate	DBP		84-74-2	Developmental, Female and Male Reproductive	2005
Di-n-hexyl phthalate	DnHP		84-75-3	Female and Male Reproductive	2005
Diisododecyl phthalate	DIDP		68515-49-1 /26761-40-0	Developmental	2007
Diisononyl phthalate	DINP			Under listing consideration	----

BBP has positive carcinogenicity data in rats. DINP and DEHP have positive carcinogenicity data in rats and mice. BBP, DINP and DEHP all increase MNCL incidence in rats (DEHP in male rats only). BBP and DEHP both increase pancreatic acinar cell tumor incidence in rats, while DINP increases pancreatic islet cell tumor incidence in rats and mice. Both DEHP and DINP but not BBP increase liver tumor incidence in rats and mice and testicular interstitial cell tumor incidence in rats. BBP increases bladder transitional epithelial cell tumor incidence in rats, and DINP increases renal transitional cell tumor incidence and renal tubular carcinoma incidence in rats. The tumor sites for BBP, DEHP and DINP are summarized in Table 25.

Table 25. BBP, DEHP and DINP tumor sites in rats and mice

Chemical	Liver		Pancreas		Testicular interstitial cell		MNCL		Renal and Bladder		Uterine	
	Mice	Rats	Mice	Rats	Mice	Rats	Mice	Rats	Mice	Rats	Mice	Rats
BBP				M[1]				M F		F[3]		
DEHP*	M F	M F		M[1]		M		M				
DINP[&]	M F	M F	F[2]	M[2]		M		M F		M[4]		F[5]

M: Male; F: Female

*OEHHA (2002), IARC (2012)

&OEHHA (2013)

[1]Pancreatic acinar cell adenoma or carcinoma

[2]Pancreatic islet cell carcinoma

[3]Bladder transitional epithelium hyperplasia, papilloma and carcinoma

[4]Renal transitional epithelial cell carcinoma and renal tubular carcinoma

[5]Endometrial adenocarcinoma

BBP, DEHP and DINP have all demonstrated positive data in *in vitro* cell transformation assays. Both BBP and DEHP induce DNA and chromosomal damage; DINP has not been evaluated for induction of DNA damage. DEHP but not BBP or DINP has also been demonstrated to induce gene mutations.

BBP, DEHP and DINP have all been shown to activate PPARα, PPARγ, ER and pregnane X receptor (PXR). Both BBP and DEHP but not DINP have been demonstrated to activate AhR. DEHP and DINP activate constitutive androstane receptor (CAR) and inhibit gap junction intercellular communication (GJIC); no data are available for activation of CAR or inhibition of GJIC by BBP. BBP, DEHP and DINP have all demonstrated anti-androgenic and anti-steroidogenesis effects.

The genotoxicity, receptor activation and other data potentially related to mechanisms of action are summarized in Table 26.

Table 26. BBP, DEHP and DINP genotoxicity, receptor activation and other data related to mechanisms of action

Chemical	Genotoxicity			*In vitro* cell transformation	PPAR α+γ	ER	AhR	PXR	CAR	GJIC inhibition	Anti-androgenic and anti-steroidogenesis
	DNA damage	Gene mutations	Chromosomal damage								
BBP	+	-	+	+	+	+	+	+[2]	NE	NE	+
DEHP*	+	+	+	+	+	+	+	+	+	+	+
DINP[&]	NE	-	-	+/-[1]	+	+	-	+	+	+	+

NE: Not evaluated. PPAR: Peroxisome proliferator activated receptor. ER: Estrogen receptor. AhR: Aryl hydrocarbon receptor. PXR: Pregnane X receptor. CAR: Constitutive androstane receptor. GJIC: Gap junction intercellular communication.

*OEHHA (2002), IARC (2012)

&OEHHA (2013)

[1]One of nine assays positive. [2] The data are from Mnif *et al*., 2007.

To summarize, BBP, DEHP, and DINP have positive carcinogenicity data in rat and mouse studies, as shown in Table 25. Among these compounds, DEHP is listed under Proposition 65 as being known to the state of California to cause cancer and is classified by IARC as a Group 2B carcinogen and U.S. EPA as a Group B2 carcinogen. BBP, DEHP, and DINP all induce tumors at multiple sites. BBP, DEHP, and DINP all significantly induce MNCL; BBP and DEHP also induce pancreatic acinar tumors, and DINP induces pancreatic islet cell tumors. DEHP and DINP but not BBP induce liver tumors. DEHP has positive results in gene mutation, chromosomal damage, cell transformation, and DNA damage assays performed *in vitro* and *in vivo*. BBP has positive results in chromosomal damage assays performed *in vivo* and in DNA damage and cell transformation assays performed *in vitro*. DINP has positive results in cell transformation genotoxicity assays performed *in vitro*. BBP, DEHP and DINP activate PPARα, PPARγ, ER, and PXR and demonstrate anti-androgenic and anti-steroidogenesis effects, and BBP and DEHP activate AhR (Table 26).

4. MECHANISMS

BBP increased the incidence of pancreatic acinar cell adenoma and carcinoma in male rats, urinary bladder transitional epithelium hyperplasia, papilloma and carcinoma in female rats, MNCL in male and female rats, and benign and malignant pheochromocytoma of the adrenal medulla in male rats.

The mechanisms by which BBP induces tumors are not known; however, several studies provide information on a number of possible mechanisms of action. Several potential mechanisms of carcinogenicity (genotoxicity, DNA methylation alterations, androgenic pathway disruption, PPAR-mediated, AhR-mediated, ER-mediated mechanisms) are discussed below.

4.1 Genotoxicity

BBP tested positive in a variety of genotoxicity assays (described in Section 3.3.2 Genotoxicity). Evidence for genotoxicity includes positive tests for DNA base lesions in mice, DNA single strand breaks in human HepG2 cells, DPCs in rat hepatic cell homogenate (*in vitro*) and mouse hepatic cells (*in vivo*) and SCEs and CAs in mice *in vivo*. These findings suggest that genotoxic mechanisms may play a role in BBP tumorigenesis.

In vivo observations that BBP up-regulates expression of *TSN* (Moral *et al.*, 2007; described in Section 3.3.5.1), a gene encoding the DNA-binding protein translin, which is associated with DNA recombination breakpoints, further suggests a role for genotoxicity in BBP tumorigenesis. Translin DNA-binding complexes are present at recombination hotspots and may serve as indicators of breakpoints in genes which are fused by translocations (Kasai *et al.*, 1994; Aoki *et al.*, 1997). Translin activities have been suggested to play a crucial role in chromosomal translocation in lymphoid neoplasms, such as mononuclear cell leukemia (Kasai *et al.*, 1994; Aoki *et al.*, 1997), and in hepatitis C virus-induced hepatocellular carcinoma (Li *et al.*, 2003).

Treatment of HepG2 cells (a human hepatocellular carcinoma-derived cell line) with BBP resulted in the reduction of DEK protein expression and induction of DNA damage in a dose-dependent manner (Choi *et al.*, 2010) (described in section 3.3.5.3, Proteomic analysis). DEK, a chromatin protein, induces positive supercoils into closed circular DNA in mammalian cells (Waldmann *et al.*, 2004). The association of DEK and human malignancies was identified by its chromosomal translocation outcomes in a subset of acute myeloid leukemia patients (Kappes *et al.*, 2008). In addition, DEK plays a role in repair of DNA strand breaks and protects cells from genotoxic agents (Kappes *et al.*,

2008). This suggests that the BBP-induced loss of DEK activity could interact with BBP genotoxicity to contribute to the carcinogenicity of BBP.

4.2 Activation of Peroxisome Proliferator Activated Receptor (PPAR)

Peroxisomes are single membrane-bound cytoplasmic organelles present in plants and animals. Peroxisomes possess at least one hydrogen peroxide (H_2O_2)-generating flavin oxidase together with the antioxidant enzymes superoxide dismutase and catalase. A group of structurally diverse chemicals such as hypolipidemic drugs (clofibrate, nafenopin, ciprofibrate, Wy-14, 643), plasticizers (DEHP, DBP, DINP), leukotrienes (LTP4), fatty acids and eicosanoids may increase peroxisome number and size (Reddy *et al.*, 1982; Sharma *et al.*, 1988; Kliewer *et al.*, 1997). Chemicals having this effect have become collectively identified as peroxisome proliferators (PPs). A PP receptor was identified and cloned (Issemann and Green, 1990), and has become known as peroxisome proliferator activated receptor α (PPARα). Since then two more PPARs have been identified and designated PPARβ/δ and PPARγ (reviewed by Pyper *et al.*, 2010), respectively. All three members of the PPAR family control metabolic pathways involved in lipid and energy metabolism (Chawla *et al.*, 2001) and they are involved in metabolic disorders such as atherosclerosis, hyperlipidemia, and obesity.

BBP is a PPAR agonist and activates PPARα, PPARβ and PPARγ receptors.

PPARα, PPARβ/δ, and PPARγ are present in a number of human tissues, including intestine, lung, heart, kidney, adrenal, thymus, stomach, and spleen. Expression varies by tissue and life stage. While relative levels of mRNA and protein expression of the PPAR subtypes varies by tissue in the fetus, in general, the level of mRNA expressed during the fetal period is comparable to or higher than that of the adult (Abbott *et al.*, 2010). High levels of PPARα expression are found in tissues with active fatty acid catabolism.

Similar tissue expression profiles of PPARα have been found in rodents and humans (Bookout *et al.*, 2006). In the human liver, PPARα levels may vary significantly among individuals but are considered to be lower than in the rodent liver (Palmer *et al.*, 1998). A recent study using more advanced techniques shows similar PPARα expression as measured by mRNA transcription in the hepatocytes and livers of mice and humans (Rakhshandehroo *et al.*, 2009). PPARα regulates target genes that modulate fatty acid oxidation, lipoprotein, glucose/glycerol, cholesterol, bile acid, xenobiotics and amino acids (Rakhshandehroo *et al.*, 2010). PPARβ regulates genes that modulate glucose homeostasis. PPARγ regulates genes for adipogenesis and fatty acid metabolism in skeletal muscle (Berger and Moller, 2002).

Following ligand binding, PPAR heterodimerize with the 9-cis retinoic acid receptor (RXR). This complex then binds to the PPAR-response element (PPRE) in the promoter region of the target gene. The ligand binding to PPARs leads to conformational changes that release co-repressors and recruits a co-activator. Transcriptional events mediated by co-activators could also represent a level of regulation for modulating PPARα gene expression. For example, the co-activator proliferator-activated receptor-binding protein (PBP) is required for PPARα regulated gene expression in liver (Jia *et al.*, 2004).

The major effects of peroxisome proliferation are increases in the size and number of peroxisomes in liver parenchymal cells and changes in peroxisomal and microsomal fatty acid β-oxidation enzyme levels. The evaluation of peroxisome proliferation is determined either by monitoring the peroxisome number or volume by electron microscopy or assaying for the activity of related peroxisomal and microsomal fatty acid oxidases. The induction of cyanide-insensitive palmitoyl-CoA oxidase (PCoA) activity is considered to be the primary biochemical indicator of peroxisome proliferation.

4.2.1 PPAR binding and activation by BBP and its metabolites

BBP and its metabolite MBzP activate all three types of PPAR in mice and humans (Hurst and Waxman, 2003; Lampen *et al.*, 2003; Bility *et al.*, 2004; Corton and Lapinskas, 2005; Lapinskas *et al.*, 2005) (Table 27). MBuP has not demonstrated binding to rodent or human PPARβ or human PPARγ, and data on the binding of MBuP to human and rodent PPARα and rodent PPARγ is conflicting (Table 27).

Table 27. Activation of PPARs by BBP and its major metabolites (adapted from Corton and Lapinskas, 2005)

Chemical Name	PPARα		PPARγ		PPARβ	
	Rodent	Human	Rodent	Human	Rodent	Human
BBP	++	++	++	++	+	+
Major metabolites						
MBuP	-/+	-/+	-/+	-	-	-
MBzP	++	+	++	+	++	+

4.2.1.1 PPARα activation by BBP

Liver changes typically associated with PPARα activation (e.g., liver enlargement, increased liver to body weight ratio, increased number and size of liver peroxisomes, and increased levels of fatty acid β-oxidation enzyme activity, such as palmitoyl-CoA oxidase) have been reported in studies of rats and mice treated with BBP, and are summarized below.

- Barber *et al.* (1987) exposed male and female F344 rats to BBP in feed for a period of 21 days at levels of 2.5%, 1.2% and either 0.6% or 0.3% (low BBP dose not specified). Hepatic peroxisome proliferation was judged to have increased in the 2.5% dose group in both males and females (2 animals evaluated/dose group/sex by subjective electron microscopy inspection). BBP weakly induced PCoA oxidase activity in both male and female rats.
- The ability of BBP to induce hepatic peroxisome proliferation was assessed in female F344 rats in collaborative studies conducted at the same time, in the same laboratory, and with the same dose levels as the NTP (1997a) two-year BBP carcinogenicity studies (Monsanto 1994, as reviewed by NTP, 1997a). Liver enzyme induction was assessed after one month and one year. Induction of hepatic PCoA oxidase was observed at both time points in mid- (12,000 ppm) and high-dose (24,000 ppm) females. Induction of hepatic carnitine acetyl transferase was observed at both time points in all three dose groups.
- Male rat relative liver weights were significantly increased ($p < 0.01$) after 26 weeks of exposure to 8300 ppm or 25,000 ppm BBP in feed (NTP, 1997a).

- In the NTP (1997b) two-year carcinogenicity studies (described in Section 3.2.1), relative liver weights in high-dose males and females were significantly increased at the 15-month interim evaluation ($p < 0.01$) when compared to their respective *ad libidum* or weight-matched controls.
- In the two-year feed restriction studies in male and female rats, relative liver weights were significantly higher at the 15-month interim evaluation in the BBP-treated groups compared to the respective controls (NTP, 1997b) (described in Section 3.2.1).

4.2.1.2 The potential role of PPARα activation in BBP-induced tumors

The classical tumor target of PPARα agonists is liver, but BBP does not induce liver tumors in rats or mice. Pancreatic acinar cells are a target tumor site of some PPARα agonists, including BBP. A PPARα-dependent mode of action (MOA) for has been proposed for the induction of pancreatic acinar cell tumors (Klaunig *et al.*, 2003). The proposed causal events are 1) induction of hepatic PPARα, resulting in decreased cholesterol 7α-hydroxylase transcription, which was stated to be the rate-limiting step of bile acid synthesis; 2) a reduction in bile acid flow, alteration in bile acid composition, or induction of cholestasis (a blockage of bile flow from the liver to the duodenum); 3) increased secretion of cholecystokinin (CCK), resulting in increased pancreatic acinar cell proliferation, which leads to increased pancreatic acinar cell tumor incidence. However, substantial data gaps exist for the proposed steps in the postulated MOA for pancreatic acinar cell tumors. Moreover, no data are available indicating that BBP alters bile acid flow or composition, induces cholestasis, or affects CCK production. Thus, there are no data to suggest that PPARα is involved in the induction of pancreatic acinar cell tumors by BBP, or in BBP tumorigenesis in general.

4.2.2 PPARγ activation

As discussed above, the BBP metabolite MBzP is capable of PPARγ transactivation (Bility *et al.*, 2004), activating both mouse and human PPARγ. Data suggesting that PPARγ activation may play a role in the mechanism of induction of transitional cell carcinomas comes from studies of the anti-diabetic drug and PPARγ agonist pioglitazone. Pioglitazone has been demonstrated to induce PPARγ in rat and human urothelial tissue (Chopra *et al.*, 2008) and to induce rat urothelial (transitional cell) bladder tumors (Tseng and Tseng, 2012). Positive human epidemiological data are also available which indicates an increased risk of bladder cancer exists in type-2 diabetics using pioglitazone as an anti-diabetic therapeutic (Azoulay *et al.*, 2012; Mamtani *et al.*, 2012).

As noted previously, BBP induces urinary bladder transitional epithelium hyperplasia, papilloma and carcinoma in female rats (NTP 1997a; 1997b).

4.3 Aryl Hydrocarbon Receptor (AhR)

4.3.1 General Background

AhR is a ligand-activated transcription factor belonging to the bHLH receptor family. In resting cells, AhR is sequestered by chaperone proteins in the cytoplasm. While binding with its stimulating ligands, such as phthalates and dioxins, AhR undergoes conformational change and is released from its cytoplasmic chaperones. The activated AhR regulates gene expression through both nongenomic and genomic mechanisms. The genomic mechanism involves the nuclear translocation of AhR from the cytoplasm, formation of a heterodimer with the AhR nuclear translocator (ARNT), and binding to AhR response elements in the promoter regions of target genes. In the nongenomic mechanism, AhR is translocated to the cytoplasmic membrane, where it triggers a rapid signaling response through induction of intracellular calcium and cyclic AMP, which eventually leads to transcriptional activation in the nucleus. A number of genes are regulated by AhR, including CYP1A1, CYP1A2, CYP1B1, JUN, FOS, GSTM, UTG1A, ALDH1, and TNF-α (Puga *et al.*, 2005; Puga *et al.*, 2009).

AhR is expressed in many human and rodent tissues, including lung, kidney, esophagus, pancreas, liver, testis, breast, thymus gland, immune system, and bladder, mainly in epithelial cells (Carver *et al.*, 1994; Jiang *et al.*, 2010).

The AhR can be activated by numerous chemicals, such as phthalates and indoles, which have little structural or physiochemical similarity with classical AhR agonists like the polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls, and TCDD (Denison and Nagy, 2003; Long *et al.*, 2003). Studies have also shown that cross-talk exists between the AhR, ER, androgen receptor (AR), and other nuclear receptors (Pocar *et al.*, 2005).

4.3.2 AhR and Carcinogenesis

Experimental evidence suggests that AhR plays an important role in cell proliferation, differentiation, hepatic and immune system homeostasis, and tumor development (Guo *et al.*, 2003).

The best-characterized gene/protein target through which AhR contributes to carcinogenesis is CYP1B1. Transcriptional activation of CYP1B1 by AhR has been observed in various types of human cancers, including cancers of the breast, GI tract, lung, skin, immune system, brain, and testes (Murray *et al.*, 2001). CYP1B1 encodes a monooxygenase that is capable of metabolizing xenobiotics, such as PAHs, as well as endogenous estrogen. With regard to estrogen metabolism, CYP1B1 hydroxylizes 17β-estradiol to 4-hydroxy estradiol (4-OHE2), which is a more potent carcinogen than 17β-estradiol. 4-OHE2 is further oxidized to estrogen-3,4- semiquinone and quinine, accompanied by the generation of reactive oxygen species and DNA adducts (Belous *et al.*, 2007).

4.3.3 BBP and AhR Interaction

Krüger *et al.* (2008) used a CALUX assay to determine the AhR activity of BBP. This assay system consists of mouse hepatoma Hepa1.12cR cells that are stably transfected with a PAH/HAH-inducible luciferase expression vector pGudLuc1.1 which is capable of responding in a time-, dose-, and AhR-dependent manner (Garrison *et al.*, 1996). Krüger *et al.* (2008) reported a competitive response for BBP in the presence of TCDD in the AhR-CALUX assay, with an effective concentration (EC_{50}) of 8.4×10^{-5} M. BBP alone did not show significant agonistic AhR effects, but did enhance TCDD-induced AhR activity in a dose-dependent manner.

Krüger *et al.* (2008) noted that the AhR agonist TCDD has been reported to reduce testosterone levels in animals (Choi *et al.*, 2008), and to modulate levels of cytochrome P450 enzymes involved in the steroid biosynthetic cascade, possibly through an AhR interaction with dioxin-responsive elements present in the promoter regions of these cytochrome P450 genes (Dasmahapatra *et al.*, 2000). Krüger *et al.* (2008) proposed that the weak agonistic AhR activity of BBP and other phthalates (DEHP and DBP) may result in effects on steroidogenesis and testosterone levels, similar to those seen with TCDD, and that these effects could be involved in the altered reproductive development observed in male rats exposed to BBP, DEHP, and DBP *in utero*.

Mankidy *et al.* (2013) reported that BBP at concentrations of 0.01, 0.1 and 1 mg/L exhibited weak AhR agonist potency in a CALUX assay using rat hepatoma (H4IIE) cells. The potency corresponded to $(1.5 – 2)\times10^{-7}$ mg/L TCDD-equivalents. The effects were significant at the lowest concentration of 0.01 mg/L ($p < 0.01$), and demonstrated no additional increase in activity at the higher concentrations of BBP. DBP and DEHP also exhibited weak potency as AhR agonists. The order of potency of these three phthalates, from most to least potent, is DEHP > BBP > DBP, which is consistent with the data reported by Krüger *et al.* (2008).

4.3.4 BBP and AhR-Mediated Nongenomic Mechanisms

Hsieh *et al.* (2012c) reported that treatment of estrogen receptor (ER)-negative breast cancer cells (MDA-MB-231) with BBP and DBP at 1 µM induced cell proliferation (3.2-fold), migration (2.6-fold), invasion (BBP, 2.7-fold; DBP, 3.1-fold), and tumor formation (EC_{50}: BBP, 0.12 µM; DBP, 0.22 µM). These effects were measured by cell counts and BrdU incorporation, migration of cells across a scratched area in a confluent cell monolayer, invasion through a layer of matrigel, and xenograft tumor growth of cells implanted in nude mice following i.p. injection of BBP or DBP.

Hsieh *et al.* (2012c) proposed that these effects of BBP are mediated by the non-genomic AhR-mediated mechanism depicted in Figure 7.

Additionally, the authors' data showed that BBP stimulated the cell surface AhR and triggered the downstream cyclic AMP (cAMP)-PKA CREB1 (CAMP responsive element binding protein 1) signaling cascade. This AhR mediated nongenomic pathway led to increased expression of histone deacetylase 6 gene (HDAC6), which facilitated nuclear assembly of the β-catenin-LEF1/TCF4 (Lymphoid enhancer-binding factor 1/transcription factor 4) transcriptional complex and transactivation of the c-Myc oncogene. Taken together, these results suggest that BBP could promote tumor development of breast cancer through a signaling cascade to enhance tumor expression of two oncogenes, HDAC6 and c-Myc.

Expression of *MYC* gene was also shown to increase in BBP-treated human breast adenocarcinoma MCF-7 cells as evaluated by cDNA microarray analysis (Kim *et al.*, 2011) (refer to Section 3.3.5).

Figure 7 outlines the potential downstream pathways of nongenomic AhR activation leading to BBP-induced tumor progression via activation of c-Myc expression.

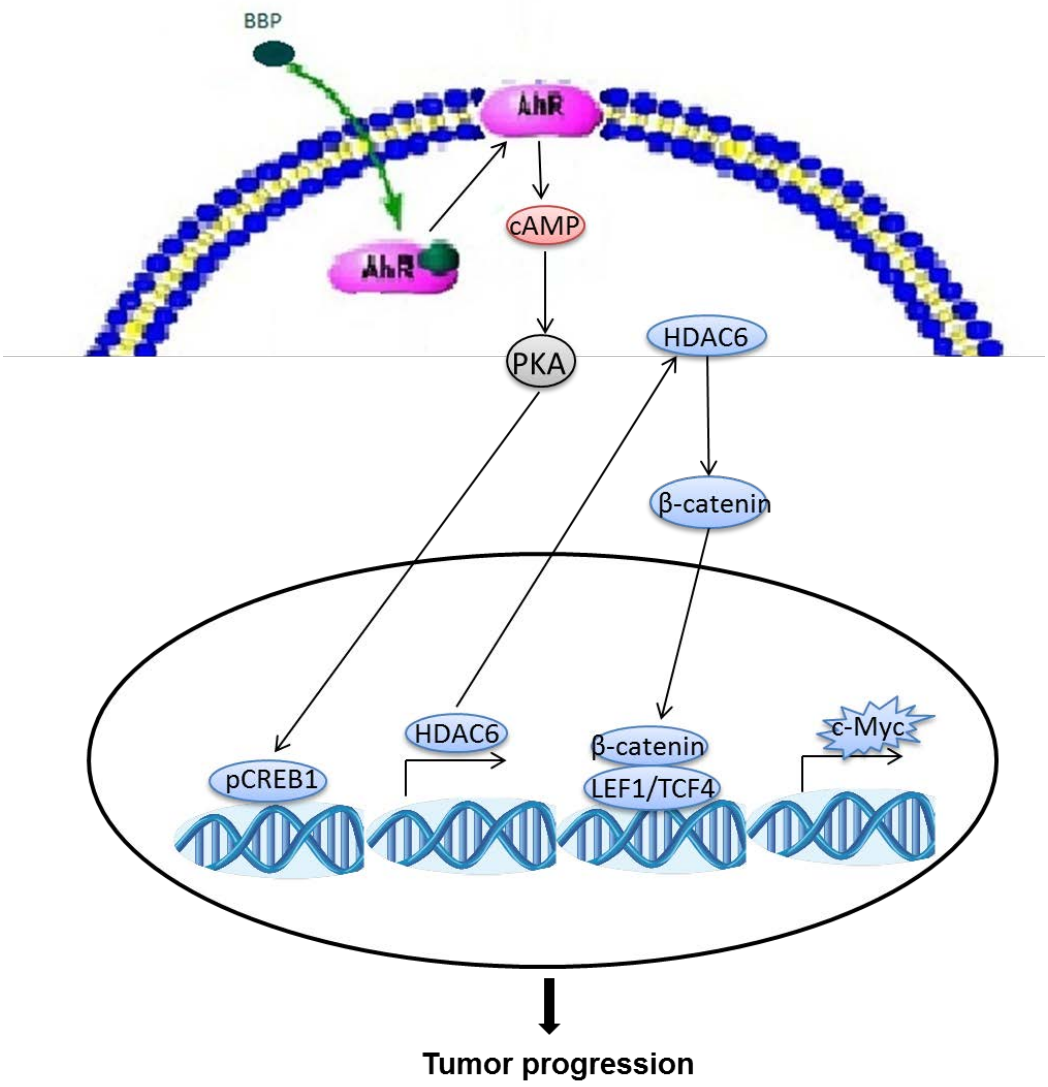


Figure 7. Schematic diagram of working hypothesis: nongenomic AhR mediated mechanism of BBP induced tumor progression. The downstream pathways of nongenomic AhR activation leading to the activation of c-Myc expression.

4.3.5 The Effect of BBP on AhR-Mediated Genomic Mechanisms in Human Granulosa Cells In vitro

Chen *et al.* (2012) reported BBP at a concentration of 1 µM significantly increased the mRNA levels of AhR, ARNT, and CYP1B1 in HO23 cells (immortalized human granulosa cells) (hGC) (Chen *et al.*, 2012). The data indicated that BBP works through an AhR-mediated genomic mechanism to induce the AhR GFP-tag protein to localize and accumulate around the nucleus. Subsequently, AhR GFP-tag protein heterodimerization with ARNT, the transcriptional complex was observed in the nucleus

by fluorescence microscopy live imaging and also detected by immunoprecipitation analysis. Chromatin immunoprecipitation confirmed that BBP increased the binding of AhR-ARNT transcriptional complex to the promoter region of CYP1B1. In addition, CYP1B1 reporter assay revealed that BBP increased luciferase reporter gene expression.

These data suggest that BBP-induced HO23 cell toxicity is AhR- and CYP1B1-dependent and BBP may be involved in the AhR-mediated genomic mechanism in human granulosa cells. CYP1B1 is involved in the oxidative metabolism of estrogens to form the catechol 4-OHE_2, leading to DNA adduct formation, such as 4-OHE_2-N7-G and 4-OH_2-N3-A (Belous *et al.*, 2007). The oxidative metabolism of estrogens has been implicated in the development of breast cancer (Belous *et al.*, 2007). Therefore, the induction of CYP1B1 by BBP could result in increased cancer risk.

4.4 Estrogen Receptor (ER)

4.4.1 General Background

ER, a transcription factor and member of the nuclear receptor family, binds estrogen and regulates gene transcription. ER had been assumed to exist as a single species until a novel estrogen receptor was isolated in 1996 (Mosselman *et al.*, 1996). The classical ER was renamed ERα and the newly identified ER was named ERβ. In general, ERα promotes proliferation whereas ERβ has pro-apoptotic and pro-differentiating functions.

ERα and ERβ share high homology in their DNA-binding domains (95%) but less homology in their ligand-binding domains (53%) (Younes and Honma, 2011). Both the distribution and ligand-binding specificity of ERα and ERβ are different in various tissues. The relative levels of ERα and ERβ in a given cell are important determinants of its response to estrogens and other estrogenic chemicals, such as BBP.

In certain organs, ERα and ERβ are expressed at comparable levels, sometimes in different cell types within the same organ, whereas in others one or the other predominates. ERα is predominantly expressed in the uterus, prostate (stroma), ovary (theca cells), testes (Leydig cells), epididymis, bone, breast, liver, kidney, white adipose tissue, and various regions of the brain. ERβ is mainly expressed in the colon, prostate (epithelium), testis, ovary (granulosa cells), bone marrow, salivary gland, vascular endothelium, lung, bladder, and other regions of the brain (Nilsson and Gustafsson, 2011).

The classic genomic mechanism through which ERs modulate gene expression is by binding of the activated ER to estrogen response elements (ERE) on DNA, e.g., promoter regions of estrogen-responsive genes, as either homodimers (ERα/ERα or ERβ/ERβ) or heterodimers (ERα/ERβ). Binding of the activated ER to EREs, in association with basal transcription factors, coactivators, and corepressors (which may be ligand-, life stage-, and tissue-specific), alters ER-target gene expression.

Other ER-mediated mechanisms involve non-genomic signaling pathways. The pathways which affect cell proliferation and apoptosis include plasma membrane-bound ER–mediated activation of second-messengers and protein-kinase signaling, such as the cAMP and MAPK families (Yager and Davidson, 2006). Some of the target genes of ER have been identified as pS2, progesterone receptor (PR), insulin growth factor binding protein 4 (IGFBP4), Cyclin D1, and c-Myc (Berry *et al.*, 1989; Bourdeau *et al.*, 2008).

4.4.2 BBP and ER Interaction

BBP can bind to both ERα and ERβ; however, BBP stimulates the transcriptional activity of ERα but not ERβ (Fujita *et al.*, 2003). Unlike estradiol and tamoxifen, BBP acts as an ERα-selective agonist.

Takeuchi *et al.* (2005) suggested that phthalate esters with alkyl side chains ranging from three to six carbons in length exhibit strong estrogenic activity, while esters with shorter (C2) or longer side chains show weaker estrogenic activity. In general, estrogenic activity is attributed to the hydroxyl group attached to benzene or its derivatives, the length and complexity of side chains, as well as the presence of a phenolic ring (Takeuchi *et al.*, 2005). BBP, which has a phenolic ring, showed the highest estrogenic activity among phthalate esters (See 4.4.3.1 below).

4.4.3 Estrogenic Effects of BBP

4.4.3.1 Classical estrogenic effects (via ER-ERE genomic mechanism)

BBP has been shown to increase the expression of ER target genes, such as pS2, PR, and cyclin D3 (Picard *et al.*, 2001; Agas *et al.*, 2007), each of which may be involved in ER-mediated cell proliferation. For example, the pS2 gene product is a small, secreted polypeptide currently of unknown function, but with structural features similar to some growth factors (Berry *et al.*, 1989), and cyclin D3 is involved in cell cycle control, regulating G1 progression to S phase (Caporali *et al.*, 2003).

BBP acts as a xenoestrogen *in vitro*, increasing cell proliferation of MCF-7, MELN, and ZR-75 cells in culture (Sonnenschein *et al.*, 1995; Soto *et al.*, 1995; Harris *et al.*, 1997; Andersen *et al.*, 1999; Picard *et al.*, 2001; Suzuki *et al.*, 2001; Hashimoto *et al.*, 2003; Okubo *et al.*, 2003; Kim *et al.*, 2004; Hong *et al.*, 2005; Buteau-Lozano *et al.*, 2008; Parveen *et al.*, 2008; Hsieh *et al.*, 2012a). In these studies BBP has the highest estrogenic activity among the phthalate esters tested. The list of relative potencies is: BBP > DBP > DEHP > DINP, with the potencies ranging from approximately $1x10^6$ to $5x10^7$ times less than that of 17β–estradiol.

4.4.3.2 Effects on ERα-mediated signal transduction

BBP → ERα → MEK/ERK1/2, p38 kinase, PI3-K/Akt →VEGF

As discussed in Section 3.3.7.1, BBP has been shown to induce the expression and secretion of VEGF (vascular endothelial growth factor, involved in angiogenesis), in *in vitro* studies with human breast MELN cells (Buteau-Lozano *et al*., 2008). These studies demonstrated that the effects of BBP on VEGF is mainly ERα-dependent in MELN cells. This work also provided evidence for the involvement of three specific kinase signaling pathways in mediating these effects, namely, a MEK/ERK1/2 pathway, a p38 kinase pathway, and a PI3-K/Akt pathway (Buteau-Lozano *et al*., 2008). All three pathways can stimulate cell proliferation. Thus these studies suggest a number of ways in which ERα-mediated signal transduction may be involved in BBP tumorigenesis.

BBP → ERα→EGFR/PKA/AP-2a → HDAC6/Akt/PP1→β-catenin → Vimentin

As discussed in Section 3.3.7.2, BBP has been shown to induce an EMT (epithelial-mesenchymal transition) in human breast transitional epithelial R2d cells, as assessed by an increase in the ratio of mesenchymal (vimentin) to epithelial (cytokeratin 7) cell markers (Hsieh *et al.*, 2012b). EMT is one of the steps required for progression of an epithelial neoplastic lesion to malignancy and metastatic cancer (see Section 3.3.7.2 and Figure 6).

Hsieh *et al.* (2012b) concluded from their studies that BBP's induction of EMT in R2d cells was mediated through ERα, since addition of an ERα antagonist blocked events upstream to EMT induction. Figure 10 presents the ERα-mediated pathway proposed by Hsieh *et al.* (2012b) for EMT induction by BBP, based on their study findings. Briefly, the authors reported that BBP stimulated ERα and triggered the downstream EGFR–PKA signaling cascade, leading to increased expression of the transcription factor AP-2a in the nucleus of R2d cells. These investigators also found that BBP-induced transcription factor AP-2a activation plays a novel role in regulating the HDAC6 (histone

deacetylase 6) promoter, leading to increased expression of the protein phosphatase-1(PP1)/HDAC6 complex. They also reported that BBP-increased expression of the PP1/HDAC6 complex caused Akt activation and GSK3β inactivation, leading to transcriptional activation of vimentin through the β-catenin–TCF-4/LEF1 pathway.

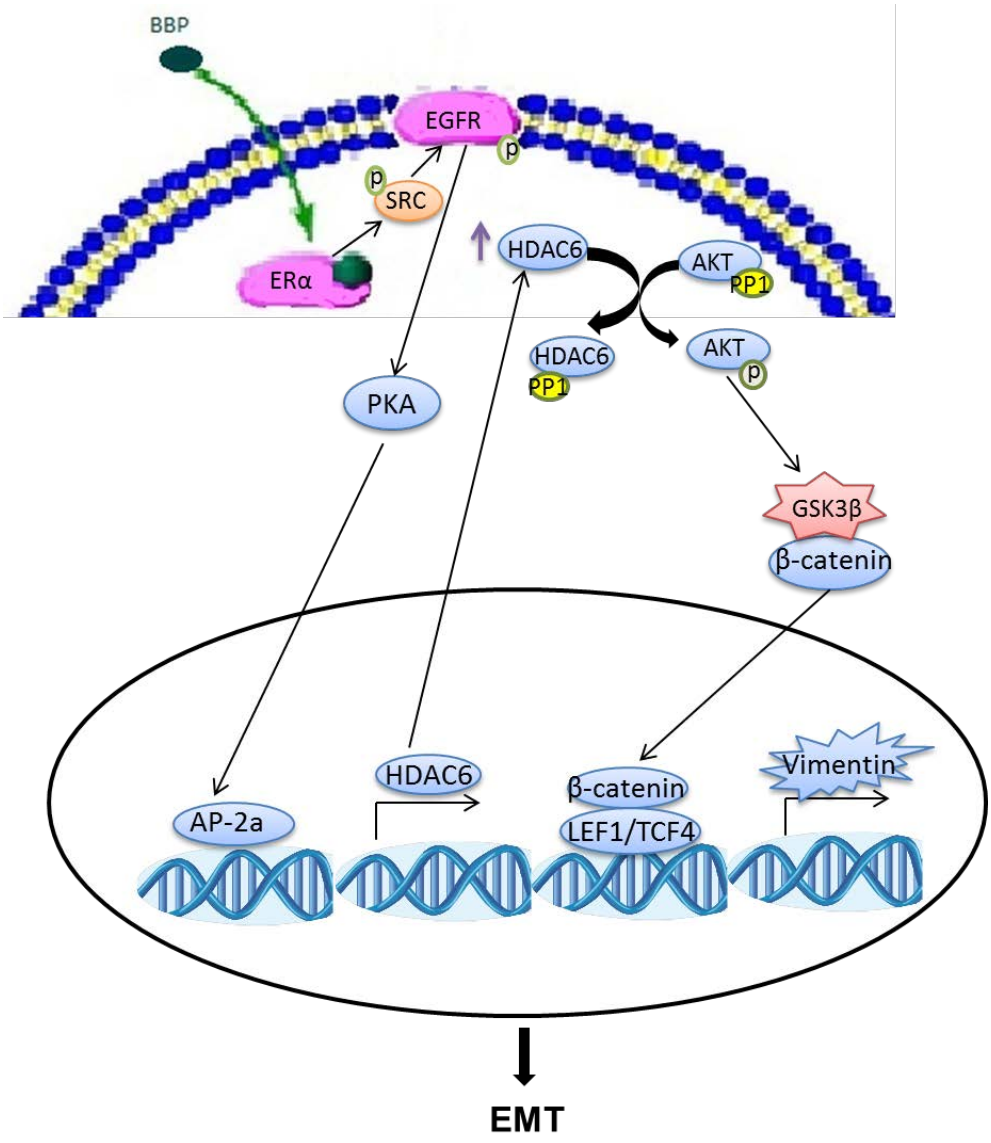


Figure 8. ER-mediated epithelial-mesenchymal transition induced by BBP (adapted from Hsieh *et al.*, 2012b). Schematic diagram of the increase in HDAC6 and the downstream pathway leading to activation of EMT by BBP.

4.5 Androgen receptor (AR) and steroidogenesis

Male rats exposed *in utero* to BBP during critical periods of sexual development have been shown to exhibit a number of effects that are consistent with exposure to either an antiandrogenic agent, or an estrogenic agent. These include reduced anogenital distance (AGD), reduced testes weight, multinucleated gonocytes, and retained areolas (Gray *et al.*, 2000; Hotchkiss *et al.*, 2004; David, 2006; Sumner *et al.*, 2009). These effects were independent of PPARα activation (Ward *et al.*, 1998).

The androgen receptor (AR) is a member of the nuclear receptor superfamily (Mangelsdorf *et al.*, 1995). When an androgen binds to the ligand binding domain of AR, AR rapidly translocates to the nucleus, where it directly interacts with DNA as a homodimer, at androgen response elements found in the regulatory regions of target genes. This complex can subsequently recruit coactivators through the ligand-dependent transactivation function (AF-2) located in the ligand binding domain and hence control transcription of specific genes (Jenster, 1998).

A number of *in vitro* studies suggest that BBP is an AR antagonist. BBP inhibited the activation of human AR (hAR) by dihydrotestosterone (DHT) in a yeast assay (Sohoni and Sumpter, 1998), in CHO cells (Takeuchi *et al.*, 2005), in the AR CALUX assay in CHO-K1 cells (Krüger *et al.*, 2008), and in human breast cancer MDA-kb2 cells (Christen *et al.*, 2010). On the other hand, BBP was negative in the Hershberger assay for antiandrogenic effects in castrated immature male SD rats (Lee and Koo, 2007). Additional evidence suggesting that BBP is an AR antagonist comes from observations that BBP up-regulates AR mRNA expression 2-fold in human breast cancer MDA-kb2 cells (Christen *et al.*, 2010) and in fathead minnow embryos (Mankidy *et al.*, 2013). This up-regulation of AR is postulated by Mankidy *et al.* (2013) to be a compensatory response to BBP's antiandrogenic effects.

No activation of the AR was observed with BBP the yeast hAR screening assay (Sohoni and Sumpter, 1998) or in the AR CALUX assay (Krüger *et al.*, 2008).

BBP, like several other phthalates, has been shown to lower testosterone levels in male rats following exposure *in utero* (Hotchkiss *et al.*, 2004; Howdeshell *et al.*, 2008). This effect is thought to be a result of interference with steroidogenesis. Some phthalates, including DEHP and DINP, have been shown to down-regulate several genes involved in steroidogenesis, including steroidogenic acute regulatory protein (StAR) and the steroid synthesis gene CYP11a (Scott *et al.*, 2009; Hannas *et al.*, 2011). While the ability of BBP to down-regulate genes involved in steroidogenesis has not been

investigated, BBP's effects on the developing male reproductive system and on fetal testosterone levels are consistent with such activity.

These studies suggest that BBP may have multiple effects on androgen hormone signaling pathways, and raise the possibility that these effects may be involved in tumorigenesis. For example, deficient androgen production during fetal testis development has been associated with testicular dysgenesis syndrome (TDS) (Sharpe and Skakkebaek, 2008). Disorders of the male reproductive system that comprise TDS include testicular germ cell cancer, low sperm counts, cryptorchidism, and hypospadias (Sharpe and Skakkebaek, 2008).

4.6 DNA methylation

Kang and Lee (2005) reported that MCF-7 cells treated with BBP at a concentration of 10 µM led to demethylation of CpG islands in the 5′ upstream promoter region of the ERα gene. In general, demethylation of promoter CpG islands up-regulates gene expression. The extent to which BBP alters DNA methylation in other genes is not known, nor is it clear whether the induction of ERα expression by BBP in a yeast-based ER transcription assay is mediated through DNA demethylation (Kang and Lee, 2005).

5. REVIEWS BY OTHER AGENCIES

Data on the carcinogenicity of BBP has been reviewed by the U.S. EPA (2003), the International Agency for Research on Cancer (IARC, 1999), the U. S. Consumer Product Safety Commission (CPSC, 2010), and the European Chemicals Bureau (ECB, 2007).

The Integrated Risk Information System (IRIS) of the U.S. EPA provides a webpage for BBP which includes a BBP carcinogenicity assessment. The website was last updated in 2003 (U.S. EPA, 2003), but the BBP carcinogenicity data has not been changed since 1993. The evidence of carcinogenicity of BBP in animals was summarized as follows: 1) a statistically significant increase in mononuclear cell leukemia has been seen in female rats; 2) the response in male rats was inconclusive; 3) there was no carcinogenic response in mice. IRIS classified BBP as a class C chemical: "possible human carcinogen". A quantitative estimate of carcinogenic risk of BBP from oral exposure to humans was not available. BBP is currently under reassessment by IRIS (U.S. EPA, 2012a). BBP was also included in the first group of 67 chemicals in the Endocrine Disruptor Screening Program by the U.S. EPA.

IARC determined that the evidence of the carcinogenicity of BBP in humans was inadequate, and the evidence in experimental animals was limited, and classified BBP as a Group 3 “Not classifiable as to its carcinogenicity to humans” (IARC, 1999).

In a hazard identification report, the CPSC reviewed the genotoxicity/carcinogenicity data on BBP and concluded that there is strong evidence that BBP can alter the expression of genes and that it has carcinogenic activity. However, because it found the carcinogenicity was only seen in one species and sex, it did not classify BBP as carcinogenic under the Federal Hazardous Substances Act (FHSA) (CPSC, 2010).

The Institute for Health and Consumer Protection (IHCP) of the ECB published a risk assessment report on BBP (ECB, 2007). Based on the data available, ECB concluded that BBP was not mutagenic, and no classification for carcinogenicity was given.

6. SUMMARY AND CONCLUSION

6.1 Summary of Evidence

Two cancer epidemiology studies have reported results for BBP or its metabolite MBzP. Neither study found a positive association. A case-control study of breast cancer in Mexico reported a significant negative association with urinary MBzP concentration, but had the major limitation of assessing exposure with a single urine sample after cancer diagnosis and could not evaluate past exposures. A case-control study of breast cancer in the U.S reported no significant association with past occupational exposure to BBP as estimated from questionnaire data, but had the major limitation of estimating only occupational exposures. Thus both studies were limited in their ability to detect excess risk and there is little human cancer evidence available at this time.

BBP is associated with an increased risk of endometriosis and endometriosis is associated with human ovarian cancer, leading to the possibility that BBP exposure could potentially increase the risk for ovarian cancer by increasing endometriosis incidence.

BBP has been tested in several long-term carcinogenicity studies using rats and mice exposed via feed. Treatment-related increases in tumors were observed in two-year studies conducted in male and female F344 rats. Non-significant increases in tumor types considered rare in the tissue and species of origin were observed in two-year and 32-month studies in female rats and in 30-month studies in male rats. Specifically, the following increases in tumors were observed:

Pancreatic acinar cell tumors

- In male F344 rats, the incidences of pancreatic acinar cell adenoma and combined adenoma and carcinoma were significantly increased and with significant dose-response trends in one study (NTP, 1997a). The increases in adenoma and combined adenoma and carcinoma in the high-dose group in NTP (1997a) were also statistically significant when compared to weight-matched controls (NTP, 1997b). Pancreatic acinar cell carcinomas are considered rare in untreated male F344 rats.
- In female F344 rats, the incidence of pancreatic acinar cell adenoma in one study was significantly increased (NTP, 1997a). The increase did not reach statistical significance; however, these tumors are considered rare in untreated female F344 rats.
- In male F344 rats, the incidence of pancreatic acinar cell adenomas in a 30-month restricted feed study were increased (NTP, 1997b), though the incidence did not achieve statistical significance. The tumors were observed late in the study, and when there were reduced numbers of animals at risk for later occurring tumors.

Mononuclear cell leukemia

- In treated F344 rats, the incidences of mononuclear cell leukemia and combined leukemia and lymphoma were significantly increased and with significant dose-response trends in one study (NTP, 1982).
- In male F344 rats, the incidence of mononuclear cell leukemia in the high-dose group was significantly increased compared with weight-matched controls (NTP, 1997a; 1997b).

Urinary bladder tumors and hyperplasia

- In female F344 rats, the incidence of urinary bladder transitional epithelium carcinoma (4 cases) and papilloma (2 cases) were increased as well as the incidence of urinary bladder transitional epithelium hyperplasia in a 32-month feed restriction study (NTP, 1997b). The increase in tumors did not reach statistical significance; however, these tumors are considered rare in untreated female F344 rats.
- In a second study female F344 rats, the incidence of urinary bladder transitional epithelium papilloma was slightly increased, while the incidence of urinary bladder transitional epithelium hyperplasia was significantly increased and with a significant dose-response trend in one study (NTP, 1997a). The increase in tumors did not reach statistical significance when compared with concurrent

controls; however, these tumors are considered rare in untreated female F344 rats.

Adrenal medulla tumors

- In male F344 rats, the incidence of combined benign and malignant pheochromocytoma of the adrenal medulla in the high-dose group was significantly increased compared with weight-matched controls (NTP, 1997a; 1997b).

BBP is genotoxic in multiple *in vitro* and *in vivo* studies using mammalian cells:

- DNA base lesions in mouse osteoblast cell line and primary calvarial osteoblast cells.
- DNA single strand breaks in HepG2 human hepatocellular carcinoma cells.
- DNA-protein crosslinks in rat hepatic cell homogenate.
- Mouse bone marrow cell sister chromatid exchanges *in vivo*.
- Chromosomal aberrations in mouse bone marrow cells *in vivo*.
- DNA-protein crosslinks in mice hepatocytes *in vivo*.

Overall, BBP was not mutagenic in *S. typhimurium* or *E. coli*, and did not induce reverse or forward mutations, SCEs, or CAs *in vitro*.

BBP induced malignant cell transformation of Syrian hamster embryo cells *in vitro*.

BBP has been demonstrated to be both an AhR agonist and an ER agonist, increasing cell proliferation and tumorigenicity associated with the pathways induced by those receptors, including genomic and non-genomic signal transduction pathways.

BBP disrupts male rat testicular development *in utero* through steroidogenesis perturbation and possesses antiandrogenic activity; there is a potential increased risk of testicular tumors through induction of testicular dysgenesis syndrome in humans.

BBP increased expression of several genes (e.g. *VEGF*, *MYC*, *WT1*) associated with potential carcinogenic mechanisms in human breast epithelial cells and cancer cells *in vitro*, and in mammary glands from female SD rats exposed *in utero* and during the neonatal/prepuberty period.

BBP induced cell proliferation in human breast epithelial cells and cancer cells *in vitro* and in the epithelial structures of mammary glands from female SD rats exposed *in utero* and during the neonatal/prepuberty period, suggesting potential effects on tumor growth promotion and progression.

BBP increased angiogenesis, epithelial-mesenchymal transition, migration and invasiveness in human breast epithelial cells and cancer cells *in vitro*, and tumor formation by human breast epithelial cells and cancer cells in athymic nude mice *in vivo*, suggesting potential effects on tumor growth promotion and progression, and metastasis.

6.2 Conclusion

The evidence for carcinogenicity of BBP comes from:

- Multiple carcinogenicity studies in F344 rats
    - Pancreatic acinar cell tumors in male rats (including carcinomas, which are rare) with a strong positive dose-response trend (and in comparison with weight-matched controls).
    - Observations of pancreatic acinar cell adenomas in female rats, which are rare, and in male rats in a 30-month feed restriction study.
    - Mononuclear cell leukemia in female rats, and in male rats in comparison with weight-matched controls.
    - Observations of urinary bladder transitional epithelium tumors, including four carcinomas, which are rare in one study and papilloma in a second study in female rats, and hyperplasia of the urinary bladder transitional epithelium in both studies.
    - Adrenal medulla tumors in male rats in comparison with weight-matched controls.

- Multiple genotoxicity studies in mammalian cells
    - Base lesions in mouse osteoblast cell line and primary osteoblasts.
    - DNA single strand breaks in human hepatocellular carcinoma cell line.
    - DNA-protein crosslinks in rat hepatic cell homogenate (*in vitro*) and in mouse hepatocytes (*in vivo*).
    - Sister chromatid exchange in mouse bone marrow cells.
- Malignant cell transformation of SHE cells *in vitro.*
- AhR and ER agonism.
- Anti-androgenic activity and steroidogenesis disruption
- Increased expression of several genes (e.g. *VEGF, MYC, WT1*) associated with potential carcinogenic mechanisms and cell proliferation in human breast epithelial cells and cancer cells *in vitro*, and in mammary glands from female offspring SD rats exposed *in vivo*.
- Increased angiogenesis, epithelial-mesenchymal transition, migration and invasiveness in human breast epithelial cells and cancer cells *in vitro*, and tumor

formation by human breast epithelial cells and cancer cells in athymic nude mice *in vivo*.

- Structure-activity similarities with other carcinogenic phthalates, including DEHP, which has been identified by IARC as a Group 2B carcinogen and listed under Proposition 65 as a carcinogen.

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APPENDIX A

In utero and early life exposure to BBP in rats changes the structure of the mammary gland. This appendix provides a description of the structure of the mammary gland and its development in rats of both genders, as well as the basis for increased susceptibility of those changes to neoplastic transformation.

Introduction to Female Rat Mammary Gland Anatomy and Development

The basic architecture of the female rat mammary gland has been described as a complicated structure comprised of parenchyma and stroma. The mammary stroma consists of the epithelial extracellular matrix, adipocytes, fibroblasts, inflammatory cells and other cells. The parenchyma consists of one or two major lactiferous ducts that grow from the nipple into the surrounding fat pad. The stroma maintains the correct polarized morphology of the lactiferous duct tree, and also serves as a communicating bridge between mammary epithelia and their local environment throughout the mammary gland development. The mammary ducts are tubular structures with walls composed of two main cell types, an internal layer of epithelial cells lining the lumen (also known as luminal epithelial cells) and an external and discontinuous layer of myoepithelial cells resting on the basement membrane. During puberty, these mammary ducts swell into terminal end buds (TEBs) that consist of multiple layers of body cells (which have reduced polarity and undergo dynamic rearrangements) and a single layer of cap cells at the leading edge. The cap cells are believed to represent a pluripotent stem cell population, capable of differentiating into both mammary ductal and mammary myoepithelial cell types. TEBs occur on the mature duct tree which reaches the end of the fat pad of the mammary gland. These mature duct trees with TEBs develop through bifurcation of the duct, and are secondary branches sprouting from primary ducts. TEBs are teardrop-shaped ducts with a diameter of about 100 µm in the rat. TEBs are considered the most vulnerable mammary gland target structure for carcinogen exposure (Russo and Russo, 1996; Medina, 2007).

In female rats, the mammary ductal development in the first two to three weeks of life up to just before puberty is characterized by formation of TEBs. Just before puberty, TEBs begin to cleave into three to five smaller buds or alveolar buds (ABs). The progressive differentiation of TEBs into ABs is accentuated by each estrous cycle, which starts when rats are four to six weeks old. ABs further progress and differentiate to lobules (LB). LBs in the virgin female rat are called type 1 lobules (LB1). During pregnancy and lactation, the LBs increase significantly in size and in number of component alveoli and gradually differentiate to type 2 (LB2) and type 3 lobules (LB3). A large number of TEBs in virgin female rats never differentiate to ABs; they become progressively smaller

and finger-shaped; at this stage, they are characterized as terminal ducts (TDs). The degree of susceptibility to carcinogenic insults is associated with the degree of gland differentiation. Therefore, the order of vulnerability of mammary gland target structures from high to low are TEBs, TDs, LB1, LB2, and LB3 (Russo, 1983).

In general, the mammary gland development is regulated by hormones, growth factors, and stromal factors. The female mammary gland undergoes most of its development post-natally from TEBs to ABs, as described in previous paragraph, and then achieves a fully-differentiated state (LB3) late in pregnancy. This process includes some critical events that can be disrupted by exposure to certain endocrine-disrupting chemicals (EDCs) such as BBP. During the development, the number of TEBs and the life stage of the animal play an important role in the susceptibility and the sensitivity of the gland to exogenous EDCs or endogenous perturbations (changes in hormones/receptors of the dam or offspring) (Fenton, 2006). It has been reported by Fenton (2006) that gestation, puberty, and pregnancy are the critical windows during which EDC exposure may most affect mammary gland development and breast cancer susceptibility. Critical events include mammary bud and ductal tree development in the fetus, exponential epithelial outgrowth during puberty, and the rapid transition to lactational competency that occurs during late pregnancy. These critical events involve various degrees of rapid cell proliferation and differentiation within the mammary bud and ductal structures (like TEBs and TDs), which provides biological bases for the specific windows of susceptibility of the mammary gland target structures to EDC effects.

Biological and Cellular Bases of the Susceptibility of the Female Rat Mammary Gland to Carcinogenesis

Cell proliferation and differentiation

The higher susceptibility of the TEBs to neoplastic transformation is attributed to the fact that the TEBs are composed of an actively proliferating epithelium, as determined by the mitotic and DNA-labeling indices (Russo and Russo, 1996). The shortest length of time of the cell cycle G1 phase in the mammary gland is observed in intermediate cells (also known as body cells or cap cells) located in TEBs. TEBs are also characterized as having the highest growth fraction, which progressively diminishes in the more differentiated ABs and lobules. The TEB is not only the structure with the highest proliferative ratio but also the lowest percentage of cell loss in comparison with other parenchymal structures, such as TDs and LB1-3. Overall, the evidence indicates that intermediate cells in TEBs with shorter G1 growth phases and less DNA repair time are more prone to form mutations in comparison with other cell types.

In addition, Russo and Russo (1996) reported that during differentiation of the female rat mammary gland there is the potential for modifications to a number of events, for example: changes to cell kinetics, including decreased growth fraction and lengthened G1 phase; changes to gland structure; decreased formation of polar metabolites and increased formation of phenolic metabolites; and decreased binding of carcinogens to DNA. When assessing and identifying chemicals for breast cancer risk, all the events listed above, which have been shown to affect the susceptibility of the mammary gland to carcinogenesis, should be taken into account.

In conclusion, cell proliferation is important in breast cancer initiation, whereas cell differentiation inhibits breast cancer development.

Mammary gland stem cells

It has been reported that stem cells located in the cap region of TEBs are responsible for the growth that drives ductal extension during branching of the mammary gland (Gjorevski and Nelson, 2011). Russo and colleagues believe that stem cells are the cell origin of mammary ductal carcinomas (Russo and Russo, 1999). They hypothesized that these target cells could eventually become the stem cells of the neoplastic event, depending upon: (a) topographic location within the mammary gland tree (TEB), (b) age of the host at exposure to a genotoxic agent, and (c) reproductive history (hormone levels) of the host. Both epidemiological findings and rodent carcinogenesis data support their hypothesis. Women who have been shown to have higher numbers of stem cells and who have been exposed to potential breast carcinogens at a young age may have an increased risk of developing breast cancer. This may explain the higher incidence of breast cancer that has been observed in nulliparous women and in women having an early menarche. In addition, the higher cancer incidence elicited by carcinogens in rodents when exposure occurs at a young age also supports the hypothesis.

The window of susceptibility

At early life stages, including *in utero*, neonatal and pre-puberty stages, the breast is more susceptible to carcinogenesis, which is consistent with the data indicating that cell replication and the concentrations of estrogen receptor type α (ER α) in TEBs are at their peak during these life stages. The increased mammary gland tumor response from carcinogen exposure early in life is attributed to the presence of proliferating and undifferentiated structures such as TEBs, which are present during the pubertal mammary epithelial expansion and display elevated DNA synthesis and active cell proliferation compared with other mammary gland structures (Kleinberg *et al.*, 2009).

Male Rat Mammary Gland Development

At birth, the mammary glands of male and female rats are similar. Mammary epithelial growth occurs in male rats and men, but male mice lack mammary epithelium and therefore mammary gland growth cannot occur. At birth, male rat mammary glands are composed of fat with some glandular tissues, such as ducts and lobules. However, male rats do not normally possess nipples because androgens during gestation induce regression of that tissue. Retained nipples and areolae in male rats are the characteristic effects of prenatal anti-androgen exposure (Foley *et al.*, 2001). The few studies that have evaluated the effects of ECDs on male mammary glands have indicated that male rats could be more sensitive than female rats. For example, after *in utero* and lactational genistein (phytoestrogen) exposure, You *et al.* (2002) observed enhanced mammary glandular differentiation at weaning, and male pups were more susceptible than female pups (You *et al.*, 2002). In addition, Latendresse and colleagues (Latendresse *et al.*, 2009) conducted a multigenerational study in rats and reported that mammary gland hyperplasia in male rats is one of the most sensitive markers of estrogenic endocrine disruption by ECDs such as genistein and ethinyl estradiol.