EVIDENCE ON THE CARCINOGENICITY OF

Diisononyl Phthalate (DINP)

October 2013

Reproductive and Cancer Hazard Assessment Branch
Office of Environmental Health Hazard Assessment
California Environmental Protection Agency

The Office of Environmental Health Hazard Assessment's (OEHHA) Reproductive and Cancer Hazard Assessment Branch was responsible for the preparation of this document.

Primary Authors

Rajpal S. Tomar, Ph.D.
Staff Toxicologist
Cancer Toxicology and Epidemiology Section
Reproductive and Cancer Hazard Assessment Branch

John D. Budroe, Ph.D.
Chief, Cancer Toxicology and Epidemiology Section
Reproductive and Cancer Hazard Assessment Branch

Rose Cendak, M.S.
Research Scientist
Cancer Toxicology and Epidemiology Section
Reproductive and Cancer Hazard Assessment Branch

OEHHA Reviewers

Martha S. Sandy, Ph.D.
Chief, Reproductive and Cancer Hazard Assessment Branch

Lauren Zeise, Ph.D.
Deputy Director for Scientific Affairs

Director

George A. Alexeeff, Ph.D., D.A.B.T.

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 diisononyl 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 diisononyl phthalate. OEHHA reviewed and considered those submissions in preparing this document.

OEHHA developed this document with information on the possible carcinogenicity of diisononyl 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 DINP 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.

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

Diisononyl phthalate (DINP) is a phthalate ester primarily used as a general purpose plasticizer. Plasticizers increase the softness and flexibility of polymeric materials. Over 90% of the DINP produced is used to improve the flexibility, pliability and elasticity of a variety of polyvinyl chloride (PVC) products, including vinyl flooring, wire and cable insulation, stationery, coated fabrics, gloves, toys, tubing, garden hoses, artificial leather, footwear, automobile undercoatings, and roofing materials. Non-PVC products containing DINP include rubbers, inks, pigments, paints, lacquers, adhesives, and sealants. Exposure may occur to the general public through uses of PVC or non-PVC consumer products containing DINP, and to workers in occupational settings.

No epidemiology studies were identified that investigated the risk of cancer associated with documented exposure to DINP.

Animal Carcinogenicity

DINP has been tested in twelve dietary carcinogenicity animal studies: six studies in Fischer 344 (F344) rats, two studies in Sprague-Dawley (SD) rats and four studies in $B6C3F_1$ mice. Statistically significant increases in the following tumor types were observed: hepatocellular tumors (malignant and combined malignant and benign), mononuclear cell leukemia of the spleen, and renal tubule cell carcinomas. In addition, the following malignant tumors considered either rare or uncommon in the tissue and species of origin were observed in DINP-treated animals: renal transitional cell carcinoma, renal tubular cell carcinoma, pancreatic acinar cell carcinoma, testicular interstitial (Leydig) cell carcinoma, and uterine adenocarcinoma. The specific tumor findings were as follows:

Liver tumors

- In male F344 rats, the incidences of hepatocellular carcinomas and combined adenomas and carcinomas were significantly increased with significant dose-response trends in one study (Moore, 1998a as reviewed by CPSC, 2001). A significant trend in hepatocellular carcinoma was seen in a second study in male F344 rats (Lington *et al.*, 1997).
- In female F344 rats, the incidence of combined hepatocellular adenomas and carcinomas was significantly increased with a significant dose-response trend in one study (Moore, 1998a as reviewed by CPSC, 2001).
- In female SD rats, the incidence of hepatocellular carcinomas was significantly increased with a significant dose-response trend in one study (Bio\dynamics, 1986 as reviewed by CPSC, 2001).

- In male $B6C3F_1$ mice, the incidences of hepatocellular carcinomas and combined adenomas and carcinomas were significantly increased with significant dose-response trends in one study. A significant increase in combined hepatocellular adenoma and carcinoma was observed in a second study in male $B6C3F_1$ mice treated with DINP for 78 weeks followed by a 26 week recovery period (Moore, 1998b, as reviewed by CPSC, 2001).
- In female $B6C3F_1$ mice, the incidences of hepatocellular adenomas, carcinomas, and combined adenomas and carcinomas were significantly increased with significant dose-response trends in one study. A significant increase in hepatocellular carcinoma and combined adenoma and carcinoma was observed in a second study in female $B6C3F_1$ mice treated with DINP for 78 weeks followed by a 26 week recovery period (Moore, 1998b, as reviewed by CPSC, 2001).

Mononuclear cell leukemia (Spleen)

- In male F344 rats, the incidence of mononuclear cell leukemia was significantly increased with significant dose-response trends in two studies (Lington *et al.*, 1997; Moore, 1998a as reviewed by CPSC, 2001) and in a third study in male F344 rats treated with DINP for 78 weeks followed by a 26 week recovery period (Moore, 1998a as reviewed by CPSC, 2001).
- In female F344 rats, the incidence of mononuclear cell leukemia was significantly increased with significant dose-response trends in two studies (Lington *et al.*, 1997; Moore, 1998a as reviewed by CPSC, 2001) and in a third study (Moore, 1998a as reviewed by CPSC, 2001) in female F344 rats treated with DINP for 78 weeks followed by a 26 week recovery period.

Kidney tumors

- Renal tubular cell carcinoma incidence was significantly increased in one study in male F344 rats treated with DINP for 78 weeks followed by a 26 week recovery period (Moore, 1998a as reviewed by CPSC, 2001). These tumors are considered rare or uncommon in untreated male F344 rats.
- Renal tubular cell carcinoma incidence was increased in male F344 rats in two studies (Lington *et al.*, 1997; Moore, 1998a as reviewed by CPSC, 2001). The increases did not reach statistical significance; however, these tumors are considered rare or uncommon in untreated male F344 rats.
- Renal transitional cell carcinoma incidence was increased in male F344 rats in two studies (Lington *et al.*, 1997; Moore, 1998a as reviewed by CPSC, 2001). The increases did not reach statistical significance; however, these tumors are rare in untreated male F344 rats.

Pancreatic islet cell tumors

- In male SD rats, the incidence of pancreatic islet cell carcinomas was increased in one study (Bio\dynamics, 1986 as reviewed by CPSC, 2001). The increase did not reach statistical significance; however, these tumors are considered rare in untreated male SD rats.
- In female B6C3F_1 mice, the incidence of pancreatic islet cell carcinomas was increased in one study (Moore, 1998b as reviewed by CPSC, 2001). The increase did not reach statistical significance; however, these tumors are considered rare in untreated female B6C3F_1 mice.

Leydig cell tumors

- In male SD rats, the incidence of testicular interstitial (Leydig) cell carcinomas was increased in one study (Bio\dynamics, 1986 as reviewed by CPSC, 2001). The increase did not reach statistical significance; however, these tumors are considered uncommon in untreated male SD rats.

Uterine tumors

- In female SD rats, the incidence of endometrial adenocarcinoma was increased in one study (Bio\dynamics, 1986 as reviewed by CPSC, 2001). The increase did not reach statistical significance; however, these tumors are rare in untreated female SD rats.

Mechanism and Other Relevant Data

Following oral exposure, DINP is readily absorbed, metabolized, and excreted in urine and feces. DINP is hydrolyzed in the gastrointestinal tract to monoisononyl phthalate (MINP), which is then oxidized in the liver to form secondary metabolites with hydroxyl, oxo, and carboxyl functional groups. Excretion is biphasic. The majority of metabolites are excreted within the first 24 hours of consumption with the remaining metabolites excreted in the 24-48 hours subsequent. The estimated elimination half-life of these metabolites is four to eight hours. At high doses (~500 mg/kg bw/day), absorption is incomplete and the majority is excreted via feces as the diester and other metabolites. Dermal absorption of DINP is less than two percent.

DINP was not genotoxic in the limited number of assays in which it was tested. These consist of the *Salmonella* reverse mutation assay, the mouse lymphoma cell mutation assay, the Chinese hamster ovary (CHO) cell chromosomal aberration assay, and the *in vivo* micronucleus assay in rats and mice.

In *in vitro* transformation studies of Balb/c 3T3 cells, DINP was shown to be positive (without metabolic activation) in one of eight studies.

DINP is a peroxisome proliferation-activated receptor (PPAR) agonist in both humans and rodents, and activates both PPARα and PPARγ. DINP has also been shown to activate human constitutive activated receptor (CAR) and pregnane X receptor (PXR).

Evidence indicates DINP is antiandrogenic in male rats exposed *in utero*. DINP reduced testosterone production, mRNA production of steroidogenic acute protein (StAR), insulin-like-3 (*Insl*-3), steroid synthesis gene (CYP11a), and anogenital distance (AGD) in male rat offspring. DINP increased retention of areolas/nipple and plasma luteinizing hormone (LH) levels. In humans, early life disturbances of testosterone production induce testicular dysgenesis syndrome (TDS) which is associated with testicular germ cell cancer. These effects are independent of PPARα agonism.

Tumor necrosis factor α (TNF-α) can participate in a number of cell signaling pathways involved in inflammation, cell proliferation, and apoptosis. Alterations in TNF-α function may contribute to carcinogenesis. DINP has been shown to increase TNF-α levels and reduce phagocytosis in a human promonocyte cell line.

The inhibition of gap junction intercellular communication (GJIC) has been proposed as a non-genotoxic carcinogenic mechanism. DINP inhibited hepatic GJIC (measured by dye transfer) in rats and mice dosed via diet for two or four weeks.

The structurally-related phthalates, diethylhexyl phthalate (DEHP) and butyl benzyl phthalate (BBP), also have positive carcinogenicity data for multiple sites in animals. DEHP is listed under Proposition 65 as a carcinogen which produces liver, pancreatic, and Leydig cell tumors in rats. The International Agency for Research on Cancer (IARC, 2013) and the U.S. Environmental Protection Agency (U.S. EPA, 1993) classify DEHP as a possible human carcinogen (Group 2B and Class B2, respectively). BBP will be considered for possible listing as a carcinogen under Proposition 65 by the Carcinogen Identification Committee (CIC) in 2013. The U.S. EPA classified BBP as a Class C “possible human carcinogen” in 1993; and this classification is currently under reassessment (U.S. EPA, 2012a). In 2000, 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 in Group 3 “Not classifiable as to its carcinogenicity to humans” (IARC, 2000).

DINP has not been classified as to its potential carcinogenicity by the U.S. EPA, the U.S. Food and Drug Administration, the National Toxicology Program, the National Institute for Occupational Safety and Health, or IARC.

2. INTRODUCTION

2.1 Identity of Diisononyl Phthalate (DINP)

Diisononyl phthalate (DINP) is a substance comprised of isomeric compounds that are C9 (nine-carbon) rich, branched chain alkyl di-esters of *o*-phthalic acid (also known as 1,2-benzenedicarboxylic acid) (Figure 1) (U.S. EPA, 2005a; Consumer Product Safety Commission (CPSC), 2010). Isomers share the same molecular formula, but differ in the arrangement of their atoms. In this case, the alkyl esters of DINP are branched (rather than straight) carbon chains, and the isomers differ in the structure of these carbon chains. The number of carbons in each alkyl ester chain is most often nine, but some molecules in a DINP mixture may contain alkyl ester chains with eight or ten carbons.

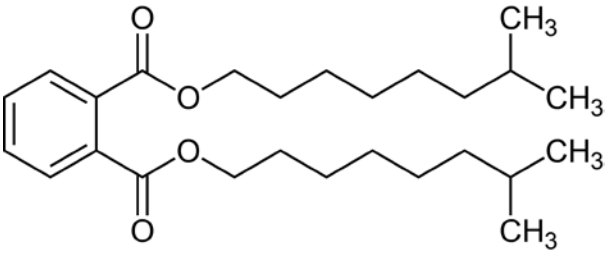


Figure 1. Chemical Structure of DINP[3]

Different DINP production processes may yield isomeric mixtures with differing proportions of nine-, eight-, and ten-carbon alkyl ester chains, containing differing amounts of straight chain impurities. Multiple Chemical Abstract Service (CAS) Registry numbers are associated with DINP. CAS number 68515-48-0 commonly refers to DINP-1, which is manufactured from propylene or butene by the "polygas" process and consists of a mixture of isomeric compounds with branched alkyl di-esters of either nine, eight, or ten carbons, with the bulk of the alkyl ester chains containing nine carbons (approximately 70% by weight) (U.S. EPA, 2005a). CAS number 28553-12-0 may refer to either DINP-2 or DINP-3; both are isomeric mixtures of branched 9-carbon alkyl ester chains. DINP-3 is no longer produced (EC JRC, 2003). CAS number 71549-78-5 refers to DINP-A, also known as Santicizer 900; it is similar to DINP-2, and has never been produced on a commercial scale. CAS numbers 14103-61-8, 20548-62-3, and 58033-90-2 all refer to a single DINP isomer, bis(3,5,5-trimethylhexyl) phthalate.

Some physical and chemical properties of DINP are detailed in Table 1.

3 This is a general structural representation. Branching can occur at various carbons of the alkyl side chains.

Table 1. Some physical and chemical properties of DINP (CPSC, 2010)

Chemical Class:	dialkyl phthalate esters
IUPAC name:	1,2-Benzenedicarboxylic acid, di-C8-10 branched alkyl esters, C9-rich; Di-isononyl phthalate
Molecular Formula:	$C_{26}H_{42}O_4$
Molecular Weight:	418.62
Appearance:	oily colorless liquid
Melting Point:	-48°C
Vapor Pressure	$6x10^{-8}$ - 3.8×10^{-5} KPa (20°C)[1,2]
Boiling Point:	370 °C
Water Solubility:	<0.001mg/L (insoluble)
Octanol-water partition coefficient (log K_{ow}):	~9

[1]Depending upon the method used for calculation
[2]5.4×10^{-7} mm Hg (25°C) (U.S. EPA, 2005a)

2.2 Occurrence, Use and Exposure

2.2.1 Production

DINP is among the primary phthalate esters manufactured worldwide for industrial applications. DINP production and consumption has been constantly increasing since 1994 (ECHA, 2010). At a global level, overall consumption of DINP and diisodecyl phthalate (DIDP) represent approximately 30 percent of the total consumption of plasticizers (ECHA, 2010). The American Chemistry Council (ACC, 2012) estimated annual world production of DINP to be 1.3 million metric tons in 2008, and projected annual world production of DINP to be 1.5 million metric tons in 2013, assuming 2.5 percent annual production growth in that time period.

Annual U.S. production volume information is available for DINP-1 and DINP-2, which are considered commercially interchangeable (CPSC, 2010). DINP represented approximately 10-15 percent of total phthalate plasticizer production in 1998 (CPSC, 2010). According to the 2006 TSCA Inventory Update Reporting (IUR) database, the annual production volume of DINP-1 (including imports) is in the range of 100 to <500 million pounds (U.S. EPA, 2005a). DINP production in the U.S. currently exceeds that of diethylhexyl phthalate (DEHP) (CPSC, 2010), and DINP has been cited by U.S. EPA as having the highest production volume among the 10 individual phthalates (U.S. EPA, 2009).

2.2.2 Use and Occurrence

DINP is a general purpose plasticizer. Plasticizers increase the softness and flexibility of polymeric materials. Over 90 percent of the DINP produced is used to improve the flexibility, pliability, and elasticity of a variety of polyvinyl chloride (PVC) products, including vinyl flooring, wire and cable insulation, stationery, coated fabrics, gloves, toys, tubing, garden hoses, artificial leather, footwear, automobile undercoatings, and roofing materials (CPSC, 2010). Less than 10 percent of the DINP produced is used in the production of non-PVC products, such as rubbers, inks, pigments, paints, lacquers, adhesives, and sealants (EC JRC, 2003). DINP has limited use in food packaging and is not used in medical applications.

The manufacturing, sales, or distribution in the state of California of toys and child care articles which contain certain phthalates has been illegal since 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 DEHP, dibutyl phthalate (DBP), or benzyl butyl phthalate (BBP) in concentrations exceeding 0.1%; 2) toys and child care articles intended for use by a child under three years of age containing DINP, DIDP, or di-n-octyl phthalate (DNOP) in concentrations exceeding 0.1%, if the products can be placed in a child's mouth.

In February 2009, the U.S. enacted an interim ban on the uses of DINP in “any children’s toy that can be placed in a child’s mouth” or “child care article” containing concentrations of more than 0.1 percent of DINP. This interim ban was mandated by the Consumer Product Safety Improvement Act (CPSIA) of 2008. The CPSIA also directs CPSC to convene a Chronic Hazard Advisory Panel (CHAP) “to study the effects on children’s health of all phthalates and phthalate alternatives as used in children’s toys and child care articles.” The CHAP will recommend to the Commission whether any phthalates (including DINP) or phthalate alternatives other than those permanently banned should be declared banned hazardous substances (CPSC, 2010).

DINP has also been detected in water, household dust, food, household products, and other sources (EC JRC, 2003; U.S. EPA, 2009).

2.2.3 Exposure and Biomonitoring

The DINP metabolite monoisononyl phthalate (MINP) was the sole urinary biomarker of DINP assessed in human biomonitoring studies prior to 2006. Because commercial DINP contains DIDP (C10 side chains) and DNOP (C8 side chains) (EC JRC, 2003), exposure to commercial DINP could result in a mix of DINP, DNOP, and DIDP metabolites in the urine. The occurrence of some of these metabolites in the urine of rats exposed to commercial DINP has been confirmed (Silva *et al.*, 2006a).

Due to widespread use of DINP, several biomonitoring studies have been conducted to assess the exposure of the general population. Silva *et al.* (2006b) measured the urinary concentrations of MINP and the MINP oxidative metabolites mono(hydroxyisononyl) phthalate (MHINP), mono(oxoisononyl) phthalate (MOINP), and mono(carboxyisooctyl) phthalate (MCIOP) in 129 adults with no known occupational or other exposure to DINP. MHINP, MOINP, and MCIOP were detected in 100, 97, and 87 percent of the samples, respectively, at geometric mean levels of 11.4, 1.2, and 8.6 µg/L, respectively. MINP concentrations were below detection limits.

Becker *et al.* (2009) analyzed DINP metabolites in urine samples of 599 children from Germany aged between 3 and 14 years and collected in the time period May 2007 - February 2008. The median urinary concentrations of MHINP, MOINP, and MCIOP in those children were 11.0, 5.4, and 12.7 µg/L, respectively. Concentrations of those metabolites were significantly higher in toddlers (3-5 years) compared to children in older age groups (6-8 years; 9-11 years; 12-14 years).

Lin *et al.* (2011) measured phthalate metabolites in the urine, cord serum, and breast milk of pregnant central Taiwanese women after delivery in the time period December 2001 - November 2002. Phthalate metabolites were also measured in the urine of the children who were newborns at the start of the study in the age periods 2 - 3 years and 5 - 6 years. The DINP metabolites measured were MHINP, MOINP and MCIOP. The proportion of DINP metabolites in the total phthalate metabolite pool was higher in urine from the children (4.39 and 8.31 percent, for ages 2 - 3 and 5 - 6, respectively) than from the mothers (0.83 percent).

Calafat *et al.* (2011) assessed exposure to DINP in persons ≥ 6 years of age in the general U.S. population using data from the 2005-2006 National Health and Nutrition Examination Survey (NHANES). The authors reported that MCIOP was present in 89.9 percent of people sampled, with median concentrations of 5.1 µg/L. The results from the 2005-2006 NHANES survey also suggested concentrations of MCIOP have an inverse

relationship with the age of the study participant, with higher concentrations in children than in adults.

Koch *et al.* (2011a) studied the DINP exposure of German children aged 5.6 to 6.7 years by evaluating metabolite concentrations in urinary samples collected in February - March 2007. The authors found that 96, 78, and 77 percent of the samples had detectable concentrations of MHINP, MOINP, and MCIOP, respectively. The median concentrations of MHINP, MOINP, and MCIOP were 7.0, 4.2, and 13.4 µg/L, respectively. The 95th percentile concentrations of MHINP, MOINP, and MCIOP were 25.5, 12.5, and 45.5 µg/L, respectively.

Zeman *et al.* (2013) evaluated the phthalate exposure of a group of pregnant French women in October 2007. A total of 279 urine samples were collected. Mean and median MINP urinary concentrations were below the level of quantification (4 µg/L). Mean MHINP, MOINP, and MCIOP urinary concentrations were 7.6, 6.1, and 16.0 µg/L, respectively. Median MHINP, MOINP, and MCIOP urinary concentrations were 3.1, 2.2, and 6.1 µg/L, respectively. The authors estimated daily median and 95th percentile dietary DINP intakes of 1.1 and 7.6 µg/kg-day, respectively, from the median combined metabolite urinary concentrations.

Biomonitoring studies have also been conducted in workers exposed to DINP. For example, Koch *et al.* (2011b) obtained data from individuals with occupational exposure to DINP. Urine samples were obtained before and after the shifts of five car manufacturing employees engaged in seam sealing with DINP-based plastisol. Urine samples were also obtained from 10 other employees from the same plant for comparison. The comparison group's DINP exposure values were similar to the general population, but the post-shift DINP exposure values for all plastisol workers were greater than the comparison group (Table 2). Substantially higher DINP exposure levels were also reported in PVC film manufacturing workers compared to unexposed controls (Hines *et al.*, 2011).

Table 2. Median urinary DINP metabolite levels (µg/L) in the general population, exposed workers (n=5) and workers not occupationally exposed to DINP (n=10) (Koch *et al.*, 2011b)

Metabolite	General German population	Plastisol comparison group[1]		Plastisol exposed group[2]	
		Pre-shift	Post-shift	Pre-shift	Post-shift
MHINP	4.7 (0.4-31.1)[3]	5.7 (1.1-52.9)	6.2 (0.6-33)	26.0 (13.9-164)	117 (59.3-443)
MOINP	1.7 (0.3-8.7)	3.0 (0.4-11.5)	2.8 (0-16.3)	12.9 (6.0-68.6)	44.3 (10.7-175)
MCIOP	5.3 (0.7-16.7)	6.1 (1.2-69)	6.5 (0.3-31.1)	32.3 (9.7-103)	57.8 (24.7-286)

[1]Not occupationally exposed to DINP
[2]DINP-exposed workers
[3]Range of values indicated in parentheses.

DINP is one of the chemicals currently being measured in studies conducted by Biomonitoring California (a joint program of the California Department of Public Health, the Department of Toxic Substances Control and OEHHA).

3. DATA ON CARCINOGENICITY

3.1 Carcinogenicity Studies in Humans

No carcinogenicity studies in humans were found in the published literature or referenced in government documents.

3.2 Carcinogenicity Studies in Animals

Twelve animal carcinogenicity studies, consisting of six dietary studies in Fischer 344 (F344) rats (Lington *et al.*, 1997; Moore, 1998a), two dietary studies in Sprague-Dawley (SD) rats (Bio\dynamics *et al.*, 1986 as reviewed by CPSC, 2001) and four dietary studies in B6C3F_1 mice (Moore, 1998b, as reviewed by CPSC, 2001), were identified from the literature.

3.2.1 Studies in Rats

Two-year feeding studies in F344 rats (Lington *et al.*, 1997)

Lington *et al.* (1997) conducted two long-term carcinogenesis studies of DINP, one in male rats, and one in female rats. Technical grade DINP (CAS number 68515-48-0, purity 99%) was administered in the diet to groups of 110 male and female F344 rats at

dietary concentrations of 0, 300, 3000, or 6000 ppm for up to 24 months. Animals were six weeks old at the start of the studies. The mean daily intakes based on body weight and food consumption were 0, 15, 152, and 307 mg/kg/day for control, low-, mid-, and high-dose animals in the male rat study and 0, 18, 184, and 375 mg/kg/day for control, low-, mid-, and high-dose animals in the female rat study. Ten rats/sex/dose were sacrificed after 6, 12, and 18 months and the remaining rats were sacrificed at 24 months. These studies were conducted according to U.S. EPA good laboratory practice (GLP) standards.

Animals in each study were subjected to complete gross necropsy at interim sacrifice and study termination. Microscopic examination was performed on tissues from all major organs in the high-dose and control rats in each study. Gross lesions and target tissues (liver and kidney) from the mid- and low-dose groups were also examined microscopically in each study. Morphological examination of the liver was conducted from livers of two rats/sex/group by electron microscopy for peroxisome proliferation at termination of these studies.

Overall survival at 24 months was more than 60 percent in males and females of all dose groups. The most common causes of death for both studies were mononuclear cell leukemia (MNCL) and pituitary neoplasia. The authors did not report pituitary neoplasia incidence at terminal sacrifice. There was a significant decrease in body weight and food consumption in high-dose males after twelve months of exposure. In females, there was no difference in body weight as compared to controls.

The tumor incidence in male and female rats is given in Table 3. In males, there was an increased incidence of hepatocellular carcinoma in the high-dose group and a positive dose-response trend for hepatocellular carcinoma ($p=0.015$), but the increased high-dose incidence was not significant by pairwise comparison. Also, kidney tumors were observed in three mid-dose and two high-dose male rats. In the three mid-dose male rats, the neoplasms were transitional cell carcinomas arising from the urothelium. In the two high-dose male rats, the neoplasms were tubular cell carcinomas. MNCL was significantly increased in the mid- ($p<0.01$) and high-dose groups ($p<0.01$) and a positive dose-response trend for MNCL was observed ($p<0.001$).

In female rats, a dose related increase in the incidence of MNCL was observed which was significant by trend test ($p<0.001$), and by pairwise comparison with controls for the high-dose group ($p<0.001$).

The EC JRC risk assessment report on DINP (2003) stated that primary brain neoplasms (astrocytomas/oligodendrogliomas) were observed in these studies in two

high-dose male and two mid-dose female rats among those found dead or sacrificed due to morbidity, compared to none in the respective control groups. These tumor types are comparatively rare in F344 rats (Haseman *et al.*, 1998; Dinse *et al.*, 2010).

Table 3. Incidence[1] of treatment-related lesions in Fischer 344 rats administered DINP in feed for 24 months (Lington *et al.*, 1997)

Organ	Tumor	DINP dietary concentrations (ppm)				Trend test p-value[2]
		0	300	3000	6000	
Male Rats						
Liver	Neoplastic nodules[3]	3/81	1/80	1/80	1/80	NS
	Hepatocellular carcinoma	0/81	0/81	0/80	3/80	p=0.015
	Combined	3/81	1/80	1/80	4/80	NS
Kidney	Transitional cell carcinoma	0/81	0/80	3/80	0/80	NS
	Tubular cell carcinoma	0/80	1/81	0/80	2/80	NS
Leukemia	Mononuclear cell leukemia	33/81	28/80	48/80**	51/80**	p<0.001
Female rats						
Liver	Neoplastic nodules[3]	0/81	2/81	0/81	1/81	ND
	Hepatocellular carcinoma	1/81	0/81	0/80	1/80	ND
	Combined	1/81	2/81	0/80	2/80	NS
Leukemia	Mononuclear cell leukemia	22/81	20/81	30/80	43/80***	p<0.001

NS = not significant; ND = not done
Fisher pairwise comparison with controls: ** p < 0.01, *** p < 0.001
[1] Number of tumor-bearing animals/number of animals examined.
[2] p-values from exact trend test conducted by OEHHA.
[3] Since 1986, NTP refers to these tumors as "hepatocellular adenomas". They are benign hepatoproliferative lesions in rats.

Non-neoplastic findings

There was a significant increase in absolute and relative liver and kidney weights in both males and females for the mid- and high-dose groups for most of the treatment period. Absolute liver and kidney weights were increased in high-dose males (liver, 20%; kidney, 8%) and females (liver, 27%; kidney, 9%) at termination of the studies. Additionally, the spleen weights were significantly increased in both males and females at termination of the studies.

No treatment-related effects were observed in these studies in animals sacrificed at 6 and 12 months of treatment. At the 18-month sacrifice, minimal to slight centrilobular to midzonal hepatocellular enlargement was observed in 9 out of 10 males and 10 of 10 females. At the 24-month sacrifice, there was a slight centrilobular to midzonal hepatocellular enlargement in a small number of rats (exact number not stated) similar to the observations at the 18-month sacrifice.

Additional non-neoplastic findings were reported in the male rat study. These included a dose-related increase in serum alkaline phosphatase (AP), aspartate aminotransaminase (AST), and alanine aminotransaminase (ALT) in mid- and high-dose males at most time intervals. At termination, a dose-related increase in spongiosis hepatis (cystic degeneration), focal necrosis, and sinusoid ectasia (a synonym for sinusoidal peliosis) was noted in males. Ultrastructure examination of the liver did not reveal treatment-related peroxisome proliferation.

In the male rat study, the increase in kidney weights observed in treated rats was associated with some changes in histopathology and urinalysis parameters. Specifically, a decrease in osmolarity and an increase in urine volume and glucose levels was observed in treated male rats. Additionally, there was an increase in tubular cell pigmentation in high-dose male rats at the 18-month sacrifice.

Two-year feeding studies in F344 rats (Moore, 1998a as reviewed by CPSC, 2001)

Moore (1998a as reviewed by CPSC, 2001) conducted four long-term carcinogenesis studies of DINP: one two-year exposure study in male rats, one 78-week exposure + 26-week recovery study in male rats, one two-year exposure study in female rats, and one 78-week exposure + 26-week recovery study in female rats.

In the two-year exposure studies, DINP (CAS number 68515-48-0, purity 99%) was administered in the diet to groups of 70-85 male and female F344 rats at concentrations of 0, 500, 1500, 6000, and 12,000 ppm for 104 weeks. The average daily intake based on food consumption was 29.2, 88.3, 358.7, and 733.2 mg/kg/day for males and 36.4, 108.6, 442.2, and 885.4 mg/kg/day for females. A positive control group (n = 15) was included in the two-year male rat exposure study, in which animals were fed a diet containing 1000 ppm of WY-14,643 ([4-chloro-6-(2, 3 xylidino)-2-pyrimidinylthio]acetic acid), a peroxisome proliferator-activated receptor alpha (PPARα) agonist, for 13 weeks, followed by control diet for 91 weeks.

In the recovery studies, groups of 55 male and female rats were administered either a control diet or 12,000 ppm DINP in the diet for 78 weeks followed by a 26-week

recovery period during which the animals were fed a control diet. In the treated groups of each study, DINP intake during the 78 weeks was 637 and 773 mg/kg/day in the males and females, respectively.

Body weight and food consumption data were recorded at the beginning of the studies, at weekly intervals from week 1 to week 17, and at monthly intervals thereafter. Standard hematology and clinical chemistry parameters were assessed in 10 rats/sex/group at weeks 26, 52, 78, and 104. Gross examination was performed on all animals found dead, those killed in moribund condition, and those that survived until the end of the studies. All tissues from the controls, the high-dose groups, and from animals found dead or sacrificed moribund were examined microscopically. Hepatocellular proliferation rates were assessed in the two-year exposure studies for 5-15 rats/group at weeks 1, 2, 13, 79, and 104. Tissue samples from three livers and the duodenum were evaluated from each study for protein concentration, cyanide-insensitive palmitoyl-CoA oxidase (PCoA) activity, and DNA concentration.

Survival was similar in all dose groups in each study until week 78; however, by the end of the studies, a dose-related decrease in survival was observed in the 6000 and 12,000 ppm groups in the two-year exposure studies, as well as in the treated groups in the recovery studies. The decrease in survival in males from the 12,000 ppm group in the two-year exposure study was statistically significant.

In the two-year male rat exposure study, there was a significant dose-dependent increase in hepatocellular carcinoma ($p<0.001$, exact trend test) and combined hepatocellular adenoma and carcinoma ($p<0.001$, exact trend test) with carcinoma incidence ($p<0.001$) and combined adenoma and carcinoma incidence ($p<0.001$) statistically significantly different from controls by pairwise comparison at the high dose (Table 4). Hepatocellular carcinoma was first observed in high-dose males at the interim sacrifice and in other dose groups at the terminal sacrifice. A renal transitional cell carcinoma was observed in the 6000 ppm dose group and two renal tubular carcinomas were observed in the 12,000 ppm dose group. Also, there was a significant increase in MNCL in the 6000 ppm and 12,000 ppm dose groups ($p<0.05$) with a significantly positive dose-response trend ($p<0.01$).

In the two-year female rat exposure study, there was a significant increase in combined hepatocellular adenoma and carcinoma ($p<0.05$) in high-dose animals with a significantly positive dose-response trend for both carcinomas ($p<0.01$) and adenomas and carcinomas combined ($p<0.001$) (Table 4). Also, there were significant increases in MNCL at the two highest doses ($p<0.05$) with a significantly positive dose-response trend ($p<0.001$).

Table 4. Incidence[1] of treatment-related lesions in Fischer 344 rats administered DINP in feed for two years (Moore, 1998a as reviewed by CPSC, 2001)

Organ	Tumor	DINP dietary concentration (ppm)					Trend test p-value[2]
		0	500	1500	6000	12,000	
Male rats							
Liver	Hepatocellular adenoma	4/65	4/50	2/50	6/65	10/65	$p < 0.05$
	Hepatocellular carcinoma	1/65	0/50	0/50	1/65	12/65***	$p < 0.001$
	Hepatocellular carcinoma or adenoma	5/65	4/50	2/50	7/65	17/65***	$p < 0.001$
Kidney	Transitional cell carcinoma	0/65	0/55	0/55	1/65	0/65	NS
	Renal tubular carcinoma	0/65	0/55	0/55	0/65	2/65	NS
Spleen	Mononuclear cell leukemia[3]	22/65	23/55	21/55	32/65*	30/65*	$p < 0.01$
Female rats							
Liver	Hepatocellular adenoma	0/65	1/50	0/50	1/65	3/65	$p < 0.05$
	Hepatocellular Carcinoma	1/65	0/50	0/50	1/65	5/65	$p < 0.01$
	Hepatocellular carcinoma or adenoma	1/65	1/50	0/50	2/65	8/65*	$p < 0.001$
Spleen	Mononuclear cell leukemia[3]	17/65	16/49	9/50	29/65*	30/65*	$p < 0.001$

p-values associated with pairwise comparisons are based on logistic regression (from CPSC, 2001)
NS = not significant
Fisher pairwise comparison with controls: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; tests conducted by OEHHA unless otherwise noted
[1] Number of tumor-bearing animals/number of animals examined.
[2] p-values from exact trend test conducted by OEHHA.
[3] p-values associated with mononuclear cell leukemia are based on life table analysis, in which tumors in animals that die prior to terminal sacrifice are regarded as being (directly or indirectly) the cause of death (CPSC, 2001).

In the recovery studies, MNCL was significantly increased in both male and female rats compared to corresponding controls ($p<0.001$ and $p<0.01$, respectively) (Table 5). There was also a significant increase in renal tubular carcinomas in male rats ($p<0.05$). No statistically significant increases in liver tumors were observed in either the male or the female rat recovery studies. All tumors in both the male rat and female rat recovery studies were observed after 78 weeks of exposure.

Table 5. Incidence[1] of treatment-related lesions in Fischer 344 rats administered DINP in feed for 78 weeks and observed for an additional 26 weeks (Recovery Studies)[2] (Moore, 1998a as reviewed by CPSC, 2001)

Organ	Tumors	DINP Dietary Concentration (ppm)	
		0	Recovery group 12,000 ppm
Male rats			
Liver	Hepatocellular carcinoma	1/65	2/50
	Hepatocellular adenoma or carcinoma	5/65	7/50
Kidney	Renal tubular carcinoma	0/65	4/50*
Spleen	Mononuclear cell leukemia	22/65	31/50***
Female rats			
Liver	Hepatocellular carcinoma	1/65	1/50
	Hepatocellular adenoma or carcinoma	1/65	2/50
Spleen	Mononuclear cell leukemia	17/65	24/50**

Statistical tests conducted by OEHHA; statistical analyses of data from this study were not reported by CPSC, 2001.
Fisher pairwise comparison with controls: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$
[1] Number of tumor-bearing animals/number of animals examined.
[2] Treatment recovery group received 12,000 ppm for 78 weeks followed by a basal diet for 26 weeks.

Non-neoplastic findings

In the two-year exposure studies, significant increases in relative liver weights were observed in both males and females in the 6,000 and 12,000 ppm DINP exposure groups at all interim sacrifices and at study termination. Blood levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly increased at the two highest doses at weeks 52, 78, and 104 in both the male and female studies. Elevated blood levels of these enzymes are considered to be indicative of liver damage.

After one week of treatment, significant increases in mitotic cell number, mean labeling index for hepatocytes and PCoA activity were observed in the livers of all 5 high-dose males and 5 high-dose females assessed. After 2, 13, and 79 weeks of treatment, hepatic hypertrophy and significantly increased PCoA activity were evident in the livers of all high-dose males and females assessed. The number of mitotic cells and labeling indices in the liver were no longer increased compared to controls at the three latter time points, indicating that peroxisome proliferation, but not cell proliferation, was still occurring in the high-dose males and females in these two-year exposure studies.

At termination of the two-year exposure studies (week 104), hepatic hypertrophy was observed only in high-dose males (14/32, mean severity = 0.9) and females (27/37; mean severity = 1.5) and PCoA activity was again significantly elevated in these groups and in the 6000 ppm dose group in the female study. Large increases in hepatic PCoA activity were observed in the positive control animals at week 1 in the male rat study, and the EC JRC report (2003) stated that "questionable responses were observed at weeks 2 and 13".

Enlarged and granulated/rough livers were noted at necropsy in the mid- and high-dose male and female rats. There was also increased pigmentation in Kupffer cells/bile canaliculi in males and females at week 79 as well as at the study termination in the 6000 ppm, 12,000 ppm, and both the male and female recovery groups. Spongiosis hepatis was significantly increased in male rats in the two highest dose groups.

There were significant increases in absolute and relative kidney weights in both sexes in the two highest dose groups. Urinalysis at termination of the studies indicated decreased urine osmolarity, increased urine volume output, decreased electrolytes, and decreased creatinine in male rats, suggesting a compromised ability of the kidney tubule to concentrate urine. Kidney toxicity was further supported by a significant increase in blood urea nitrogen (BUN) in both male and female rats at the two highest dose groups at 26, 52, and 78 weeks, and in males only at week 104. The male rat recovery group kidney weights returned to normal by study termination, but the BUN mean value for recovery group males at study termination remained elevated and was comparable to the 12,000 ppm dose group males in the non-recovery study.

In the two-year exposure studies, histopathological changes observed in the kidney at 104 weeks included increased incidence and severity of mineralization of the renal papilla in males in the 6000 and 12,000 ppm dose groups. Increased renal tubule pigmentation was observed in both males and females in the 6000 and 12,000 ppm dose groups.

In the recovery studies, relative liver weights were not increased at study termination in either DINP-treated males or females, indicating that treatment-related effects on liver weight were reversible. However, blood levels of AST and ALT were significantly increased in the DINP-treated groups at study termination in both males and females, indicating that treatment-related changes in serum enzyme activity were not reversible. Liver PCoA activity was not evaluated in the recovery studies.

In the recovery studies, increased incidence and severity of mineralization of the renal papilla was observed in treated males at 104 weeks, as compared to controls.

Two-year feeding studies in Sprague-Dawley rats (Bio\dynamics, 1986 as reviewed by CPSC, 2001)

Bio\dynamics (1986, as reviewed by CPSC, 2001) conducted two long-term carcinogenesis studies of DINP-A: one in male rats, and one in female rats. DINP-A (CAS number 71549-78-5, trade name Saniticizer 900, purity 99.9%) was administered in the diet to groups of 70 male and female SD rats/group at concentrations of 0, 500, 5000, or 10,000 ppm for 104 weeks. The average daily intake based on food consumption was 0, 27, 271, and 553 mg/kg/day for males and 36, 33, 331, and 672 mg/kg/day for females.

In these studies, ten animals/group were sacrificed after one year, and the remaining animals were sacrificed at two years. Histopathological examination of the tissues from all major organs was conducted on the control and the high-dose animals in each study. Tumor incidence data for both studies are given in Table 6. In the male study there was an increased incidence of hepatocellular neoplastic nodules in the mid-dose group, though the increase was not statistically significant. However, the incidence of hepatocellular nodules was outside the historical control (historical controls, 8.4% vs. treatment, 8.7%) for chronic studies conducted between December 1979 and April 1989 in SD rats by Charles River. Similarly, the hepatocellular carcinoma incidence was outside the historical control range (control, 3.1% vs. treated, 8.7%), and the incidence was statistically significant compared to historical controls ($p<0.05$).

There was an increased incidence of testicular interstitial cell carcinoma. The increase was not significantly different compared to controls, but the percent tumor incidence was outside the range of historical controls (9.8% vs. treated, 11.67%). Because of the high degree of interstitial hyperplasia, these tumors were considered treatment-related by CPSC in their review of DINP in 2001 (CPSC, 2001). Testicular interstitial carcinomas are uncommon in SD rats (Chandra *et al*., 1992).

In their review, CPSC (2001) indicated that in the high-dose males there was an increase in incidence of pancreatic islet cell carcinoma (4/70 vs. 1/70 in controls); historical values for this tumor were not given in the document. However, in one study the spontaneous incidence of these tumors was 1/1340 in male SD rats (Chandra *et al*., 1992), suggesting that pancreatic islet cell carcinomas are a rare tumor in male SD rats.

In the female study, there was an increased incidence of hepatocellular nodules in the mid-dose group. Also, there was a significant increase in hepatocellular carcinoma at the middle ($p<0.05$) and high ($p<0.01$) doses with a strong positive trend ($p<0.001$).

These values were outside the range of historical controls of chronic studies conducted between December 1979 and April 1981 in SD rats by Charles River.

There was also an increased incidence of endometrial adenocarcinoma. These spontaneous uterine neoplasms are rare in SD rats (Anisimov and Nikonov, 1990; Chandra *et al*., 1992).

Table 6. Incidence[1] of treatment-related lesions in Sprague-Dawley rats administered DINP-A in feed for two years (Bio\dynamics, 1986 as reviewed by CPSC, 2001)

Organ	Tumor	Dinonylphthalate dietary concentrations (ppm)				Trend test p-value
		0	500	5000	10,000	
Male Rats						
Liver	Hepatocellular neoplastic nodules	2/70	5/69	6/69	5/70	NS
	Hepatocellular carcinoma	2/70	2/69	6/69	4/70	NS
Testis	Interstitial cell carcinoma	2/59	-[3]	-	7/60	-
Pancreas	Islet cell carcinoma	1/70	-	-	4/70	-
Female rats						
Liver	Neoplastic nodules	1/70	1/70	5/70	2/70	NS
	Hepatocellular carcinoma	0/70	0/70	5/70*	7/70**	p<0.001[2]
Uterus	Endometrial adenocarcinoma	0/70	-	-	2/69	-

NS= not significant
Fisher pairwise comparison with controls: * p < 0.05, ** p < 0.01, *** p < 0.001
[1] Number of tumor-bearing animals/number of animals examined.
[2] p-value from exact trend test conducted by OEHHA.
[3] No incidence data available

Non–neoplastic findings

In the male rat study, spongiosis hepatis incidence was significantly increased in mid- and high-dose rats compared to controls (p<0.01 for both groups). Mineral deposits in renal medullae were commonly observed in high-dose males as compared to controls (25/70 and 3/70, respectively; p<0.001). Testicular interstitial cell hyperplasia was significantly increased in treated male rats, occurring in 36.6% of the high-dose animals. The historical control data for testicular interstitial hyperplasia was compiled by Charles

River from 14 chronic studies, and ranged from 0% to 7.1%. Thus, the increase was well above the historical control range. As noted above, because of the clear treatment-related increase in interstitial cell hyperplasia, CPSC (2001) considered the increased incidence of testicular interstitial cell carcinomas to be treatment-related.

In the female rat study, spongiosis hepatis incidence was significantly increased in the high-dose rats compared to controls ($p<0.05$). The incidence of endometrial hyperplasia was greater in treated female rats as compared to controls (13/69 and 2/70, respectively; $p<0.01$). Historical control values were not provided in the report.

3.2.2 Studies in Mice

Two-year feeding studies in B6C3F$_1$ mice (Moore, 1998b as reviewed by CPSC, 2001)

Moore (1998b,) conducted four long-term carcinogenesis studies of DINP: one two-year exposure study in male mice, one 78-week exposure + 26-week recovery study in male mice, one two-year exposure study in female mice, and one 78-week exposure + 26-week recovery study in female mice. The studies were conducted according to U.S. EPA's GLP standard.

In the two-year exposure studies, DINP (CAS number 68515-48-0, purity 99 %) was administered to groups of 70 male and female B6C3F$_1$/Crl BR mice for 104 weeks at concentrations of 0, 500, 1500, 4000, and 8000 ppm in the diet. The mean daily dietary consumption of DINP was 0, 90.3, 275.6, 741.8 and 1560.2 mg/kg in males and 0, 112, 335.6, 910.3 and 1887.6 mg/kg in females.

In the recovery studies, groups of 55 male and female mice were administered either control diet or 8000 ppm DINP in the diet for 78 weeks, followed by a 26-week recovery period. The mean dietary consumption of DINP in the treatment groups was 1377 and 1501 mg/kg/day in the male and female recovery studies, respectively.

Body weight and food consumption were recorded prior to initiation of the studies, at weekly intervals from week 1 to week 17 and at monthly intervals thereafter. Mortality and clinical signs of toxicity were recorded daily. Laboratory analyses (e.g., hematology, clinical chemistry) were carried out on 10 mice/sex/group during weeks 26, 52, 78, and 104. Necropsy was performed on all animals found dead or sacrificed after week 78. After week 79, microscopic evaluation was done on a complete set of tissues from 10 mice in the control and 8000 ppm groups in the two-year exposure studies. Also at this time microscopic evaluation of the liver, testes with epididymis (males), uterus (females), spleen, kidneys, and any gross lesions was performed on 10 mice from each of the 500, 1500, and 4000 ppm dose groups in the two-year exposure

studies. Tissue samples from three liver lobes and the duodenum from 5 mice in the control and high-dose groups of each study were processed for protein concentration, PCoA activity, and DNA concentration at weeks 79 and 104.

In the two-year exposure studies, there was a significant decrease in survival among high-dose males (63% survival as compared to 87% in the control group). Also, there was a dose-related decrease in body weight gain at the two highest doses in both the male and female studies.

In the two-year male mouse exposure study, the incidence of hepatocellular carcinoma was significantly increased ($p<0.01$) at the highest dose, and the incidence of combined adenoma and carcinoma was increased at the two highest doses ($p<0.01$) with a positive trend for both cases ($p<0.001$) (Table 7).

In the two-year female mouse exposure study, the incidence of hepatocellular adenoma was significantly increased at the 8000 ppm ($p<0.001$) dose group, and the incidence of carcinoma was significantly increased at the 4000 ppm ($p<0.05$) and 8000 ppm ($p<0.001$) dose groups with a positive trend ($p<0.001$) (Table 7). Similarly, there was a significant increase in the combined adenoma and carcinoma at the three highest dose groups ($p<0.05$, $p<0.01$, $p<0.001$, respectively) compared to controls, with a positive dose-response trend observed ($p<0.001$). Two 8000 ppm dose group females were observed with pancreatic islet cell carcinoma. None of the corresponding control animals demonstrated this tumor.

Table 7. Incidence[1] of treatment-related lesions in B6C3F$_1$ mice administered DINP in feed for two years (Moore, 1998b as reviewed by CPSC, 2001)

Organ	Tumor	DINP dietary concentrations (ppm)					Trend test p-value[2]
		0	500	1500	4000	8000	
Male mice							
Liver	Hepatocellular adenoma	10/70	7/67	8/66	15/65	13/70	p<0.05
	Hepatocellular carcinoma	10/70	8/67	10/66	17/65	20/70**	p<0.001
	Hepatocellular adenoma or carcinoma	16/70	13/67	18/66	28/65**	31/70**	p<0.001
Female mice							
Liver	Hepatocellular adenoma	2/70	4/68	5/68	4/67	18/70***	p<0.001
	Hepatocellular carcinoma	1/70	2/68	5/68	7/67*	19/70***	p<0.001
	Hepatocellular adenoma or carcinoma	3/70	5/68	10/68*	11/67**	33/70***	p<0.001
Pancreas	Islet cell carcinoma	0/70	-	-	-	2/70	-

p-values associated with pairwise comparisons are based on logistic regression (from CPSC, 2001)
Fisher pairwise comparison with controls: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$
[1] Number of tumor-bearing animals/number of animals examined.
[2] p-value from exact trend test conducted by OEHHA.

In the male mouse recovery study, there was a significant increase in combined adenoma and carcinoma incidence in DINP-treated males compared to controls (Table 8). In the female mouse recovery study, significant increases were observed for hepatocellular carcinoma and combined adenoma and carcinoma ($p<0.01$) as compared to the controls (Table 8).

Table 8. Incidence[1] of treatment related lesion in B6C3F$_1$ mice administered DINP in feed for 78 weeks and observed for an additional 26 weeks (Recovery Studies)[2] (Moore, 1998b as reviewed by CPSC, 2001)

Organ	Tumors	DINP Dietary concentrations (ppm)	
		0	Recovery group 8000 ppm
Male Mice			
Liver	Hepatocellular adenoma	10/70	8/50
	Hepatocellular carcinoma	10/70	12/50
	Hepatocellular adenoma or carcinoma	16/70	19/50***
Female Mice			
Liver	Hepatocellular adenoma	2/70	8/50
	Hepatocellular carcinoma	1/70	13/50**
	Hepatocellular adenoma or carcinoma	3/70	18/50**

Statistical tests conducted by OEHHA; statistical analyses of data from this study were not reported by CPSC, 2001.
Fisher pairwise comparison with controls: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$
[1] Number of tumor-bearing animals/number of animals examined.
[2] Treatment recovery group received 8000 ppm for 78 weeks followed by a basal diet for 26 weeks.

Non-neoplastic findings

In the two-year exposure studies, liver masses were observed in both males and females at study termination in the 4000 and 8000 ppm dose groups. Focal necrosis, diffuse hepatocellular enlargement and cytoplasmic eosinophils were significantly elevated in the 8000 ppm dose groups in both the male and female studies.

There was no evidence of sustained cell proliferation in the livers of treated male or female mice in these two-year exposure studies. After 78 and 104 weeks, a significant increase in PCoA activity was observed in the 8000 ppm dose groups in both the male and female studies, suggesting the induction of peroxisome proliferation in these animals.

Mean and absolute kidney weights were decreased in the 1500, 4000, and 8000 ppm dose group males at week 79. Distended urinary bladders were commonly observed in the two high dose groups. Significant increases in urine output and in mean urine osmolarity, sodium, potassium, and chloride levels were observed in high dose male and female mice at weeks 26, 52, 78, and 104.

In the two-year female mouse exposure study, an increased incidence and severity of chronic progressive nephropathy was observed in the 8000 ppm dose group. When these animals were necropsied, the kidneys appeared granulated and/or pitted, with a rough appearance. CPSC (2001) noted the study author concluded that there may have been a treatment-related alteration in the concentrating ability of the renal tubule epithelium in the DINP-exposed mice, possibly due to chronic progressive nephropathy.

In the recovery studies, liver masses were observed in both males and females at study termination in the DINP-treated groups. No increases in focal necrosis of the liver were observed in the DINP-treated groups of either study at study termination.

3.3 Other Relevant Data

3.3.1 Pharmacokinetics and Metabolism

Human studies

Koch and Angerer (2007) administered 1.27 mg/kg of deuterium (D4)-labeled DINP-2 (CAS number 28553-12-0) orally in bread to a 63 year old man weighing 77 kg (98.51 mg total). Urine samples were taken prior to dosing and throughout the following 48 hours. Only 2 percent of the administered dose was excreted in urine as MINP. The major metabolites extracted in urine were: MHINP (20% of the administered dose), MOINP (10%), and MCIOP (10%). However, due to the difference in the elimination kinetics, 5.9% of the MOINP, 8.8% of the MHINP and 15% of the MCIOP were excreted the second day after administration. These four metabolites accounted for 43.6% of the oral DINP dose in urine within 48 hours after dosing. There was a multiphase elimination pattern: the elimination half-life was three hours in the first phase for the MINP and five hours for the oxidized metabolites. The elimination half-life for the second phase starting at 24 hours post-dosing is roughly 12 hours and 18 hours for the MINP and oxidized metabolites, respectively.

Anderson *et al*. (2011) assessed the variability of DINP elimination kinetics due to difference in the ingested dose and gender. The study was carried out in ten male and ten female subjects. Deuterated DINP (D4-DINP) (chemical purity > 99%) was administered orally at 0.012 mg/kg body weight (0.78 mg total) and 0.121 mg/kg body weight (7.3 mg total) in a single oral dose in two study periods. The urine samples were collected at intervals up to 48 hours. Liquid chromatography-mass spectrometry (LC-MS/MS) was used to measure metabolite concentrations. More than 90 percent of the metabolites were excreted in the first 24 hours of urine collection and the remainder in the second 24 hours. The total metabolites were 32.0±6.4 percent of the administered dose. The fractional excretion of the primary and secondary metabolites was 3.1%,

6.6%, 12.3%, and 10.9% for MINP, 7-MOINP, 7-MHINP, and 7-MCIOP, respectively. The estimated elimination half-lives of the metabolites were between four and eight hours. The authors stated that gender did not have a statistically significant effect on the excretion of DINP metabolites.

A number of other studies reviewed in Section 2.2.3 have reported detection of the DINP metabolites MINP, 7-MOINP, 7-MHINP, and 7-MCIOP.

Animal studies

Oral Absorption

Oral absorption of ^{14}C-DINP (CAS number 68515-48-0) was studied in male albino rats either untreated or pretreated with non-labeled DINP for a period of five days (Hazelton, 1972 as reviewed in CPSC, 2001; EC JRC, 2003). On the sixth day, 2500 mg/kg/day of ^{14}C-DINP was orally administered in 0.5 ml volume. Expired gases and fecal and urinary excreta were collected at 12-hour intervals during a 72-hour period. The heart, liver, kidneys, intestines, stomach, and aliquots of fat and muscles were removed and weighed. Within 72 hours, 85 percent of the ^{14}C-DINP dose was excreted in feces, most within the first 24 hours. The remaining dose was excreted in urine (average 12%), most within the first 12 hours. Trace amounts remained in the tissues.

In a study by the Midwest Research Institute (MRI) (1983, as reviewed by EC JRC, 2003), DINP (CAS number 68515-48-0, purity 97-98%) was administered orally to male and female F344 rats in a single oral dose of 50 or 500 mg/kg. Additionally, groups of male rats were administered 5 daily doses of 50, 150, or 500 mg/kg/day. The radioactivity in urine and feces was examined for up to 72 hours after the last dose. Blood and tissue levels of radioactivity were determined in males sacrificed at 1, 4, 8, 24, and 72 hours, and in females up to 24 hours.

In male and female rats given a single dose of radioactive DINP, radioactivity was eliminated in urine and feces within 72 hours after dosing, with the majority being eliminated within the first 24 hours. About 49 percent of the dose was absorbed at the low dose of 50 mg/kg, compared to 39 percent at the high dose of 500 mg/kg, suggesting that saturation occurred at the high dose. At the low dose, an equal amount of radioactivity was recovered in urine and feces, but at the high dose more radioactivity was recovered in feces.

These data indicate that a single low dose of DINP is readily absorbed by rats, but high or repeated doses are less well absorbed. DINP elimination is fairly rapid and DINP and its metabolites do not bioaccumulate in rats.

Dermal absorption

Dermal absorption of ^{14}C-DINP was studied in conditioned and non-conditioned male F344 rats (MRI, 1983b as reviewed by EC JRC, 2003). Preconditioning was done by applying non-labeled DINP to the skin for 3 days. Neat ^{14}C- labeled DINP (CAS number 68515-48-0) was applied in a volume of 0.2 ml to a 12 cm^2 area of the skin. ^{14}C- labeled DINP remained on the skin for up to seven days in non-conditioned rats and for up to three days on conditioned rats. An additional non-conditioned group received a single dermal application of 0.1 ml.

Urine and feces were collected between 0-1, 1-2, and 3-7 days following the single dose application. Two rats from each group receiving a 0.2 ml dose and one rat from the group receiving a 0.1 ml dose were sacrificed on days 1, 3, and 7. Radioactivity was determined from the excreta, gastrointestinal (GI) tract, blood, and non-treated areas of skin as well as selected tissues (liver, kidneys, testes, fat, and muscles). At 24 hours after the start of treatment, 0.3 percent of the applied dose was recovered from the urine, feces, GI tract, and tissues. The total absorption after seven days was 4 percent at the high dose. Most of the applied dose was recovered from the application areas. Skin from the non-treated area contained a high level of radioactivity compared to other tissues. The EC JRC review (2003) concluded that dermal absorption in adult male F344 rats is slow, and the total amount absorbed is low.

Biotransformation

DINP is metabolized by hydrolysis of phthalic acid or side chain oxidation of the ester group. Saravanabhavan and Murray (2012) have proposed a metabolic transformation pathway for DINP. That metabolic transformation pathway is given in Figure 2.

Figure 2. Proposed Metabolism of DINP (adapted from Saravanabhavan and Murray, 2012)

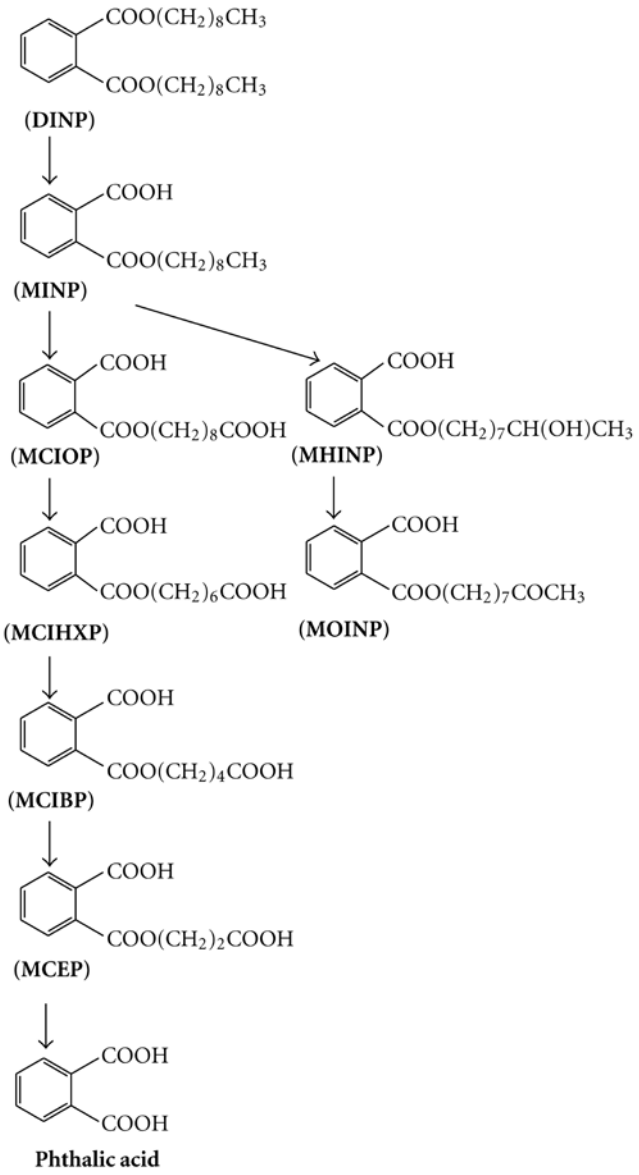


Figure legend: DINP: Diisononyl phthalate; MINP: monoisononyl phthalate; MCIOP: monocarboxy isooctyl phthalate; MHINP: monohydroxy isononyl phthalate; MCIHXP: monocarboxy isohexyl phthalate; MOINP: monooxoisononyl phthalate; MCIBP: monocarboxy isobutyl phthalate; MCEP: monocarboxyethyl phthalate.

In the MRI (1983) study of F344 rats (as reviewed by CPSC, 2001), most of the radioactivity collected in urine was in the form of phthalic acid (PA), with a minor amount

identified as side chain oxidation products of the monoester. In feces, 8 percent and 41 percent of the radioactivity was associated with the diester parent compound following a single low or high dose, respectively. The remainder eluted in the areas of MINP and MINP oxides (MOINP). PA was absent in high-dose samples and minor amounts were present in low doses.

In the liver, the major metabolites were MINP and MOINP at both the low and high doses. A small amount of PA was present in the liver at the low dose. From one hour to 72 hours, a progressive decrease of MINP and PA was observed with a concurrent increase in MOINP. In the testis, the major metabolites recovered were MINP, its oxidation products, and PA. A small amount of polar components were also present. At the high dose, slightly higher amounts of MINP and lower amounts of PA were recovered.

Silva *et al*. (2006a) dosed groups of two 75-day old female SD rats with a single oral dose of 300 mg/kg DINP-1 (CAS number 68515-48-00) or DINP-2 (CAS number 28553-12-0). For each group of rats, 24-hour urine samples were collected one day before and three subsequent days after dosing. The metabolites were extracted from urine, resolved with high performance liquid chromatography, and analyzed by mass spectrometry. Urine samples were spiked with ^{13}C-labeled internal standards.

The authors reported mean urinary levels of DINP metabolites in the four rats dosed with the two commercial DINP preparations. In this study, MCIOP was the major metabolite of DINP (82%). MHINP (7.6 percent) and MOINP (3.1 percent) were also detected. The authors state that the relative amounts of the metabolites in rats dosed with two DINP products differed, though the same metabolites were identified irrespective of the technical mixture used.

Distribution

In male albino rats given 0.5 ml ^{14}C-DINP orally after five days of dosing with unlabeled DINP, the liver and GI tract contained the most radioactivity (Hazleton, 1972 as reviewed by EC JRC, 2003). Most of the radioactivity retained by the animals was found in the carcass and remaining viscera and none was recovered in the expired air.

In male and female F344 rats given single or repeated oral doses of ^{14}C-DINP, radioactivity was cleared rapidly from the tissues (MRI, 1983 as reviewed by EC JRC, 2003). The radioactivity present in the blood and most tissues was highest one hour following administration of both the low and high doses. The highest levels were observed in the liver (4.7 percent of the administered dose) followed by the kidneys

(0.31 percent), and the blood (1.62 percent). The radioactivity in the liver was localized to the cytosol. Most of the radioactivity in blood was recovered in plasma. In animals given five daily doses, blood and tissue radioactivity levels were highest one hour following the last dose and overall radioactivity levels were highest in the liver, followed by the kidney, blood, and skin.

Smith *et al*. (2000) studied DINP and its metabolites MINP and PA in F344 rats administered 0, 0.1, or 0.2 percent DINP and in B6C3F_1 mice administered 0, 0.05 or 0.6 percent DINP in the feed for four weeks. DINP, MINP, and PA were measured in the liver and serum at two weeks and four weeks. DINP levels in the livers of both rats and mice were greater at the high dose at two weeks, but not at four weeks while levels of MINP in the liver and serum increased over time and were greater than DINP and PA levels. In rats, PA increased with time in the liver and serum. In mice, PA increased with time at the low dose, but not at the high dose. PA concentrations were not dose-dependent in the liver or serum of rats or mice.

Excretion

In the MRI study (1983, as reviewed by EC JRC, 2003) described above, almost all radioactivity was excreted in urine and feces within the first 48 hours. The excretion in urine was higher than in feces at the low doses, but more was excreted in feces at the high dose (MRI, 1983 as reviewed by EC JRC, 2003). Excretion toxicokinetics of the DINP metabolites in urine followed a biphasic pattern with varying elimination half-lives (Silva *et al.*, 2006a). During the first phase of elimination, MCIOP, MHINP, and MOINP were excreted with a $t_{1/2}$ of 7.6, 8.5, and 8.3, respectively. The urinary metabolites of the three oxidative metabolites in the second 24 hours of collection were 83, 58, and 76 percent lower than the levels in the first 24-hour collection. Repeated dosing appeared not to cause accumulation of DINP or its metabolites.

As noted above, when male albino rats were given 0.5 ml ^{14}C-DINP orally after five days of dosing with unlabeled DINP, no radioactivity was recovered in the expired air (Hazleton, 1972 as reviewed by EC JRC, 2003).

Synopsis

The studies described above indicate that orally administered DINP is readily absorbed, metabolized and distributed to various tissues, though mainly to the liver, kidneys and blood. Following a single high dose or repeated dosing, the absorption is incomplete. DINP is hydrolyzed, forming monoesters. The hydrolytic monoesters undergo oxidative

metabolism to form secondary metabolites with hydroxy, oxo and carboxyl functional groups.

3.3.2 Genotoxicity

The genotoxicity of DINP has been investigated in a limited number of assay systems. These consist of the *Salmonella* reverse mutation assay, the mouse lymphoma cell mutation assay, the Chinese hamster ovary (CHO) cell chromosomal aberration assay, and the *in vivo* micronucleus assay in rats and mice. The findings are presented below.

- DINP (CAS number 68515-48-0 and 28553-12-0) has been tested for the ability to induce reverse gene mutations in *Salmonella typhimurium* (test strains TA 98, TA 100, TA 1535, TA 1537 and TA 1538) with and without metabolic activation. No positive response was observed in the presence or absence of metabolic activation (as reviewed in EC JRC, 2003).
- DINP did not induce forward mutations in L5178Y TK +/- mouse lymphoma cells in studies conducted with and without activation by a rat liver S9 fraction (Hazleton, 1986b as reviewed by EC JRC, 2003; Barber *et al.*, 2000).
- DINP was negative for chromosomal aberrations in CHO cells *in vitro* in the presence or absence of an exogenous metabolic system (Aroclor-induced SD rat liver S9) (Exxon Biomedical Sciences, 1996, as reviewed in EC JRC, 2003; McKee *et al.*, 2000).
- DINP was negative in *in vivo* micronucleus assays in male CD-1 mice dosed at 500, 1000, and 2000 mg/kg for two days (McKee *et al.*, 2000) and in F344 rats given 0.5, 1.7, and 5 mg/kg/day DINP in corn oil for five days (as reviewed in U.S. EPA, 2005a).

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. EC JRC (2003) reviewed seven *in vitro* cell transformation studies conducted in Balb/c 3T3 A31 mouse cells during the early 1980s (Table 9). All those reviewed studies were conducted in the absence of metabolic activation. The three studies conducted by Litton Bionetics were negative. One of the four studies conducted by Microbiological Associates was positive for the induction of type II (transformed foci with a more ordered and defined edge than type III foci) and type III (transformed foci characterized as dense multilayered with random orientation at the focus edge and invasion into surrounding monolayer) foci, when compared to acetone negative controls. In this study, three, six, and twelve type III foci were observed at doses of 0.03, 0.3, and

1.0 µl/ml, respectively. These increases were statistically significant and thought to be dose-related. A more recent study by Barber *et al*. (2000) also conducted in Balb/c 3T3 A31 mouse cells without metabolic activation was negative for cell transformation (Table 9).

DINP has not been tested for cell transformation induction in other cell types, such as Syrian hamster embryo (SHE) or C3H/10T1/2 cells.

Table 9. *In vitro* cell transformation studies using Balb/c-3T3 A31 mouse cells (clone 1-13)

Doses (µg/ml)	**Results**	**References**
2.5 - 254.5	Negative	Litton Bionetics (1981b)[1]
0.2 - 3260	Negative	Litton Bionetics (1981c)[1]
0.125 - 3.75	Negative	Litton Bionetics (1985b)[1]
0.1 - 1	Increased foci, not statistically significant	Microbiological Associates (1981a)[1]
0.1 - 1	Increased foci, not statistically significant	Microbiological Associates (1981b)[1]
0.03 - 1	Positive	Microbiological Associates (1981c)[1]
0.03 - 1	Increased foci, not statistically significant	Microbiological Associates (1982)[1]
0.125 - 3.75	Negative	Barber *et al*. (2000)

[1] As reviewed by and reported in EC JRC (2003).

3.3.4 Animal Tumor Pathology

DINP significantly increased the incidence of hepatocellular tumors in male and female F344 rats, female SD rats and male and female B6C3F$_1$ mice. DINP significantly increased the incidence of renal tubular cell carcinomas in male F344 rats and MNCL in male and female F344 rats. This section describes the relevant pathology details for the MNCL, liver and kidney tumors observed[4].

4 As discussed in Section 3.2.1, additional malignant tumors considered either rare or uncommon in the tissue and species or origin were observed in DINP-treated animals: renal transitional cell carcinoma, testicular interstitial (Leydig) cell carcinoma, and endometrial adenocarcinoma.

Liver

The hepatocellular tumors observed in treated male and female F344 rats and B6C3F_1 mice were classified as adenomas and carcinomas; in SD rats they were referred to as liver neoplastic nodules and carcinomas. The pathology term liver "neoplastic nodule" was generally superseded by the term "hepatocellular adenoma" beginning in the 1980's (Maronpot *et al.*, 1986). Hepatocellular adenomas and carcinomas arise from the same cell type and hepatocellular adenomas can progress to hepatocellular carcinomas so the two tumor phenotypes are aggregated when evaluating study results (Maronpot *et al.*, 1986; Thoolen *et al.*, 2010).

Kidney

Renal neoplasia was observed in three mid-dose (transitional cell carcinoma) and two high-dose (tubular cell carcinoma) rats in the Lington *et al*. (1997) male F344 rat study. Further, two tubular cell carcinomas occurred in the high-dose group in the male F344 rat study by Moore (1998a, as reviewed by CPSC, 2001).

The Lington *et al.* (1997) male F344 rat study and the study review by EC JRC (2003) did not include a description of the renal transitional cell carcinoma pathology. In general, transitional cell carcinoma arises from the transitional cell lining (urothelium) of the renal pelvis and ureter and tends to fill the pelvis and invade kidney parenchyma. The typical transitional cell carcinoma has a lobular pattern showing little cellular atypia. The cellular changes include nuclear enlargement, hyperchromatism, and disorderly orientation. Mitotic activities are common (IARC, 1992; Montgomery Jr. and Seely, 1990).

Laboratory historical control data were not available for the two renal tumor types described above. However, renal tubular cell carcinomas and renal transitional cell carcinomas have been described as uncommon (Montgomery Jr. and Seely, 1990) and rare (Chandra *et al.*, 1992), respectively. For comparison, the control incidences of male F344 rat renal tubular cell adenomas/carcinomas and transitional cell adenomas/carcinomas observed in NTP studies using dosing in NIH-07 feed are 8/1002 and 1/1002, respectively (NTP, 2013).

Mononuclear cell leukemia (MNCL)

A statistically significant treatment-related increase in MNCL incidence was observed in both male and female F344 rats in the Lington *et al*. (1997) and Moore (1998a, as reviewed by CPSC, 2001) studies.

The most common background tumor in F344 rats is MNCL, which is generally observed after 18 months of age. Several organs may be infiltrated, and the liver and lungs are commonly involved. Well-differentiated MNCL cells resemble normal large granular lymphocytes (LGL), but in poorly differentiated cells granules may be detected only ultrastructurally (Ward *et al*., 1990).

Based on a morphological similarity to granular lymphocytes, MNCL is also called LGL leukemia or Tγ lymphocyte leukemia. While LGL bears 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 *et al.* (1999) reported no human counterpart to rat LGL leukemia. More recently, a U.S. EPA report (2012b) has noted that several authors have concluded that rat MNCL is similar to human natural killer cell (NK) LGL leukemia (Stromberg *et al.*, 1985; Ishmael and Dugard, 2006; Thomas *et al.*, 2007).

3.3.5 Effects on Gene Activation, Expression and Enzyme Activity

Several studies have investigated the ability of DINP or the metabolite MINP to elicit PPAR, constitutive androstane receptor (CAR) and pregnane X receptor (PXR) activation, and the effects resulting from activation of those receptors. These types of studies can provide insights into the mechanisms of action of DINP at a molecular level. The results of those studies are described below.

Valles *et al.* (2003)

Valles *et al*. (2003) studied the role of PPARα in response to DINP in wild-type and PPARα-null mice. Male and female wild-type B6C3F$_1$, SV129 wild-type, and SV129 PPARα-null mice were administered DINP either in feed or by gavage. The authors organized these activities into four studies. In Study 1, DINP (CAS number 68515-48-0, obtained from ExxonMobil Chemical Company, Houston TX) was administered in diet to groups of male and female B6C3F$_1$ mice (9-10 weeks old) at dietary concentrations of 0, 150, 500, 1500, 4000, and 8000 ppm for two weeks. In Study 2, male and female B6C3F$_1$, SV 129 and SV129 PPARα-null mice (26-30 weeks of age) were fed 0 or 8000 ppm of DINP for one or three weeks. The male mice were exposed to DINP obtained from Aristech (Pittsburgh, PA; CAS number 28553-12-0), and the female mice were exposed to DINP obtained from ExxonMobil (CAS number 68515-48-0). In Study 3, male and female B6C3F$_1$, SV 129 and SV129 PPARα-null mice (9 weeks old) were fed 0 or 8000 ppm DINP (CAS number 68515-48-0) for one week. In Study 4, male SV129 and SV129 PPARα-null mice (9 weeks old) were gavaged with either 0.1% methyl

cellulose (control) or 1 g/kg body weight/day DINP (CAS number 68515-48-0) in 0.1% methyl cellulose for one week.

The alterations of DINP-induced responses as a function of dose were examined in Study 1 and the role of PPARα in mediating PPARα responses in Studies 2 through 4. The study results are presented in Table 10.

Table 10. Changes in liver/body weight ratios, hepatocyte proliferation and enzyme induction in B6C3F$_1$, SV129 and SV129-PPARα mice exposed to DINP (adapted from Valles *et al.*, 2003)

Study	DINP (ppm)	Exposure (weeks)	Age of mice (weeks)	B6C3F$_1$ mice		SV129 mice		PPARα-null mice	
				Male	Female	Male	Female	Male	Female
1	0	2	9-10			ND			
	500	2	9-10	NC	NC				
	1500	2	9-10	NC	↑LBWR				
	4000	2	9-10	↑LBWR ↑CoA	↑LBWR ↑CoA				
	8000	2	9-10	↑LBWR ↑CoA	↑LBWR ↑CoA				
2	0	1	26-30						
	8000	1	26-30	↑CoA	↑CoA	↑LBWR ↑CoA	↑LBWR ↑CoA	NC	NC
	0	3	26-30						
	8000	3	26-30	↑CoA	↑CoA	↑LBWR ↑CoA	↑LBWR ↑CoA	NC	↑LBWR
3	0	1	9						
	8000	1	9	↑CoA	↑LBWR ↑CoA ↑LI	↑LBWR ↑CoA ↑E	↑LBWR ↑CoA ↑LI ↑E	↑E[1]	↑E[1]
4	0	1	9	ND	ND		ND		ND
	1 g/kg BW	1	9	ND	ND	NC	ND	NC	ND

LBWR: liver/body weight ratio; CoA: palmitoyl CoA oxidase activity; LI: labeling index (used to determine hepatocyte proliferation status); E: β/ω fatty acid oxidation enzymes (acetyl CoA oxidase, multifunctional protein II, thiolase, Cyp4a); BW: body weight; ND: Not done; ↑: significantly increased compared to control; NC: no change.

[1]A reduced but detectable induction noted for β/ω fatty acid oxidation enzymes, with the exception of Cyp4A.

As Table 10 illustrates, DINP induced statistically significant increases in liver-to-body weight ratio (LBWR) in male B6C3F$_1$ mice exposed to 4000 and 8000 ppm DINP and in females exposed to 1500 ppm or higher for two weeks (Study 1). DINP also induced significant increases in LBWR in male SV129 wild-type mice exposed to 8000 ppm for one or three weeks (Studies 2 and 3), but not in male PPARα-null mice. In females, there was an increase in LBWR in younger B6C3F$_1$ and SV129 mice, but not in younger PPARα-null mice (Study 3). However, DINP induced a significantly increased LBWR in older B6C3F$_1$ and SV129 females and older PPARα-null females. DINP given by gavage at 1 g/kg/day for one week did not cause an increase in LBWR for younger male wild-type or PPARα-null mice (Study 4).

Hepatocyte proliferation was measured in female mice from Study 3 by counting BrdU-labeled hepatocytes in three zones of the liver lobules. There was a two-fold increase in labeling index (LI) in zone three in wild-type SV129 females and in zones one and three in B6C3F$_1$ females. There was no increase in LI in any of the zones in PPARα-null female mice.

DINP significantly increased palmitoyl CoA oxidase activity in the liver of male and female B6C3F$_1$ mice exposed to 4000 and 8000 ppm in Studies 1 and 3 and in SV129 wild-type mice exposed to 8000 ppm for one or three weeks in Study 3. No effect on palmitoyl CoA oxidase activity was observed in PPARα-null mice treated with 8000 ppm for up to three weeks.

The protein levels of four enzymes involved in β and ω oxidation of fatty acids (acetyl CoA oxidase, multifunctional protein II, thiolase and Cyp4a) were increased in male and female SV129 wild-type mice exposed to 8000 ppm DINP in Study 3 compared to controls. In PPARα-null mice, the induction of Cyp4a was abolished in the liver. A reduced but detectable induction of the remaining enzymes described above was observed in PPARα-null mice. The authors suggested this may be due to induction via other PPAR subtypes.

In Study 2, PPARα-induced gene expression was assessed in female wild-type and PPARα null mice after three weeks of exposure to DINP. Genes involved in DNA repair and recombination (ATP-dependent helicase, endonuclease II homolog), metabolism (Cyp2a4), and protein trafficking (FKBP-1, FKBP-13) were induced in wild-type mice, but not in PPARα-null mice. The testosterone 15α-hydroxylase Cyp2a4 was up-regulated in wild-type SV129 mice but not PPARα-null mice. This contrasts with the gene expression of the testosterone 16α-hydroxylase Cyp2d9, which was down-regulated in wild-type SV129 mice but up-regulated in PPARα-null mice.

These data suggest that age at exposure and sex may have an effect on some liver parameters (LBWR, hepatocyte proliferation, palmitoyl CoA oxidase and β/ω fatty acid oxidation enzyme induction) thought to be under PPARα receptor control. Additionally, the increase in LBWR seen in DINP-exposed older female PPARα-null mice and the increases in β/ω fatty acid oxidation enzyme induction seen in DINP-exposed younger male and female PPARα-null mice suggest that the changes in these liver parameters caused by DINP may not be due to PPARα induction.

Bility *et al.* (2004)

Bility *et al*. (2004) studied MINP-induced activation of mouse and human peroxisome proliferator activated receptors (PPARα, PPARβ, and PPARγ), PPARα target genes and adipogenesis. PPARα, PPARβ, and PPARγ activation was assayed using a luciferase reporter plasmid-based transactivation assay in mouse 3T3-L1 fibroblasts containing chimeric mouse or human PPAR-ligand binding domain/Gal4-DNA binding domain. PPARα target gene expression was assayed using functional analysis of acetyl CoA oxidase and CYP4a mRNA induction in rat hepatoma FaO cells and human hepatoma HepG2 cells. Adipogenesis was assayed in mouse 3T3-L1 fibroblasts using a standard differentiation assay. Positive controls used were WY-14,643 (25 μM) for PPARα, tetradecylthioacetic acid (50 μM) for PPARβ, and troglitazone (3 μm) for PPARγ. MINP was used at concentrations of 3, 10, 30, 100, and 200 μM.

MINP caused a dose-dependent increase in PPARα activation at concentrations ≥ 3 μM for mouse PPARα and ≥ 10 μM for human PPARα with maximal fold-induction of 27.1 and 5.8 for mouse and human PPARα, respectively. MINP did not activate human or mouse PPARβ. MINP caused a dose-dependent increase in mouse and human PPARγ activation at concentrations of ≥ 3 μM and ≥ 30 μM, respectively, with maximal fold-induction of 14.1 and 9.3 for mouse and human PPARγ, respectively. MINP caused a dose-dependent increase in the induction of acetyl CoA oxidase and CYP4A mRNA in FaO cells but not in HepG2 cells. Strong induction of adipogenesis was observed with MINP at 50 μM.

These results suggest that under the conditions used, MINP activated both mouse and human PPARα and PPARγ receptor, but the degree of receptor activation was greater for mouse than for human receptor for both receptor types.

DeKeyser *et al.* (2011)

DeKeyser *et al*. (2011) studied the activation of the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR) by DINP (CAS number 68515-48-0) using

reporter transactivation assays and two-hybrid studies in COS-1 cells. COS-1 cells are an African green monkey kidney epithelial cell line which does not possess endogenous CAR expression or activity. The CAR gene expresses a number of splice variants during pre-mRNA processing, i.e., CAR1, CAR2 and CAR3. CAR2 encodes for a ligand-activated receptor. Approximately 30 percent of the reference transcript level for CAR in human liver tissues is the CAR2 transcript. The CAR2 transcript cannot be generated in rodents or marmosets (DeKeyser *et al.*, 2009).

In transactivation studies, COS-1 cells were transfected with either the CAR1, CAR2, or CAR3 transcript. DINP displayed potent activation of the 2B6-XREM-PBREM reporter (derived from the natural CYP2B6 gene promoter) compared with the positive control through CAR2 with non-activation of CAR1 and CAR3. DINP was a weak activator of PXR. The EC_{50} values for CAR and PXR were 0.34 and 3.6 µM, respectively.

Ex vivo studies also showed that DINP induced CYP2B6 and CYP3A4 in primary human hepatocytes. CYP2B6 metabolizes a number of substrates that are usually lipophilic with one or two hydrogen bond acceptors, on which it catalyses various oxidative reactions (Turpeinen *et al.*, 2006). CYP3A4 is the most abundant cytochrome P450 in human liver, and metabolizes a wide variety of substrates, including about 60 percent of currently known drugs (Zhou *et al.*, 2005).

3.3.6 Effects on Steroidogenesis

Traditionally, developmental and reproductive toxicity studies do not detect chemicals that induce effects during development and only appear post-natally. Therefore, NTP-CERHR (2003), after reviewing the human development and reproductive effects of DINP, recommended additional perinatal developmental studies in orally-exposed rats addressing the landmarks of sexual maturation, as fetuses were not exposed in available studies during this critical period of sexual development. The recent studies wherein dams were exposed to DINP during critical periods of sexual development are summarized here. These studies clearly indicate antiandrogenic effects of DINP in male offspring.

Hannas *et al*. (2011) orally dosed groups of Harlan SD timed pregnant rats (3-6/group) on gestation days (GD) 14-18 with 0, 500, 780, 1000, and 1500 mg/kg/day with DINP or DINP-2. At necropsy on day 18, there were 37 pregnant dams and 6, 3, 4, 6, and 6 litters treated with 0, 500, 750, 1000, and 1500 mg/kg DINP-2 and 3, 2, 1, 3, and 3 litters treated with 0, 500, 750, 1000, and 1500 mg/kg DINP. A single testis from the first three males was used to determine *ex vivo* fetal testosterone production and the remaining testes were used for gene expression. No effects were observed on body

weights, mortality, or litter size at any dose level. Both DINP formulations reduced the testosterone production in a dose-dependent manner at doses of 500 mg/kg ($p<0.05$) or higher ($p<0.001$) and there was no interaction between dose and DINP isoforms. DINP also reduced RNA expression levels of steroidogenic acute protein (StAR), insulin like-3 (*Insl-3*), steroid synthesis gene, and CYP11A levels.

In a follow up study, Hannas *et al*. (2012) tested the hypothesis that exposure to WY-14643, a PPARα agonist, would reduce the fetal testicular testosterone production in a manner similar to phthalates during sexual differentiation (GD14 -18). Using PCR assay data for key target genes representing sexual determination and differentiation, steroidogenesis, gubernaculum development, and androgen signaling pathway, the authors ranked the relative potency of several phthalates including DINP. The potent PPARα agonist, WY-14643, did not reduce testosterone production suggesting that the antiandrogenic activity of phthalates is not PPARα-mediated. No PPARα genes were activated in fetal testes by phthalates.

Boberg *et al*. (2011) studied the reproductive and behavioral effects of DINP-2. Groups of Wistar rats were orally dosed from GD7 to postnatal day (PND) 17 with corn oil (vehicle), 300, 600, 750, or 900 mg DINP/kg/day. In male offspring, DINP-2 caused increased nipple retention, reduced anogenital distance, reduced sperm motility and increased sperm count. Testicular pathology indicated multinucleated gonocytes and enlarged diameters of seminiferous tubules. DINP-2 also caused masculinization of DINP-2-exposed females, as indicated by an increased performance in the Morris Water Maze compared to female and male controls.

Lee and Koo (2007) studied the effects of DINP in the Hershberger assay. In this assay, juvenile castrated males are treated with testosterone propionate. The assay measures the ability of the test chemical to interfere with the testosterone-dependent growth of accessory sex tissues. Groups of six-week old SD rats were given 0.4 mg/kg/day testosterone propionate following castration. After one week of recovery, rats were orally gavaged with DINP at 0, 20, 100, or 500 mg/kg. The control group consisted of untreated animals and animals treated with testosterone propionate. No significant effects were observed in absolute body, liver, kidney, or adrenal weights compared to rats treated with testosterone. The absolute weights of the seminal vesicles and levator ani/bulbocavernosus were significantly reduced at all DINP doses as compared to the testosterone controls.

Borch *et al*. (2004) studied the hormonal effects of DINP-2 or a combination of DEHP and DINP-2 in male rat fetuses. Groups of eight pregnant Wistar rats were orally gavaged with peanut oil (controls) or 750 mg/kg/day DINP-2. Male fetuses were

sacrificed on GD21 and blood and testes were collected for hormonal analysis. The testicular testosterone contents and testicular testosterone production (*ex vivo)* were significantly reduced (26%) while plasma luteinizing hormone (LH) was significantly increased as compared to controls.

Masutomi *et al*. (2003) fed DINP-2 to pregnant SD rats at concentrations of 400, 4000, and 20,000 ppm from GD15 to PND10. Offspring were examined for anogenital distance, pubertal organ weights, onset of puberty, estrous cyclicity, organ weights, and histopathology of endocrine organs at adult age (week 11). Treatment with DINP-2 at 20,000 ppm resulted in a degeneration of meiotic spermatocytes and Sertoli cells in the testes and a decrease of corpora lutea in the ovary at week 11.

In a subsequent study, Masutomi *et al*. (2004) studied the expression of pituitary hormones in Wistar rats exposed to 2000 ppm DINP-2 in a soy-free diet from GD15 to PND10. DINP-2 exposure had no effect on the percentage of pituitary cells expressing the hormone in excised pituitary tissue at postnatal week 3.

Harris *et al.* (1997) tested a series of phthalates including DINP for estrogenic and mitogenic activity. Estrogenic activity was tested in a yeast screen assay, wherein a human estrogen receptor gene was integrated into the yeast genome and was expressed in a form capable of binding to an estrogen-response element and controlling the expression of the reporter gene Lac-Z. When the receptor was activated, the lac-Z was expressed resulting in production of the enzyme β-galactasidase. DINP was tested at concentrations of 10^{-3} M to 4.8×10^{-7} M, with 17β-estradiol used as a positive control. Mitogenic activity was tested in the human breast cancer cell lines MCF-7 and ZR-75. DINP was tested at concentrations of 10^{-3} M to 5×10^{-7} M, with 17β-estradiol used as a positive control. The estrogenic activity yeast screen assay data for DINP demonstrated reproducibility problems. A potency could not be calculated for DINP, and the maximum response was only 15 percent that of 17β-estradiol. In contrast, in the human breast cancer cell proliferation assays, DINP induced cell proliferation in both cell lines used, at concentrations ranging from 10^{-5} to 10^{-7} M.

Gray *et al*. (2000) dosed groups of six to eight pregnant SD rats by gavage with 0 or 750 mg/kg/day DINP from GD14 to PND 3. The sperm plug positive day was defined as GD1. There was no overt maternal toxicity or reduced litter size. At GD21, pregnancy weight gain was reduced by 14 grams in the DINP treatment group. A significant increase was observed in retained areolas/nipples in 22% of male pups. There were also some reproductive system malformations in 7.7% of male pups, including small and atrophic testes, fluid-filled testes, unilateral epididymal agenesis with hypospermatogenesis, and scrotal fluid-filled testes devoid of spermatids.

In a follow-up study, Ostby *et al*. (2001) dosed pregnant SD female rats by gavage with DINP at concentrations of 0, 1000, or 1500 mg/kg from GD 14 through PND 3. In this study, the incidence of areolas on PND 13 was 55% at 1000 mg/kg, and 75% at 1500 mg/kg compared to 14% in controls.

Lee *et al.* (2006) studied the effects of perinatal exposure to DINP and other phthalates on *grn* and *p130* mRNA expression in the hypothalamus of the offspring of pregnant lactating Wistar rats. Maternal rats were fed a phytoestrogen-free diet containing DINP (0, 40, 400, 4000 or 20,000 ppm) from gestational day 15 to weaning (PND 21). DINP exposure during the perinatal period resulted in an increase in hypothalamic *grn* and *p130* mRNA levels at PND 7 in females and males, respectively. The lordosis quotient was decreased in females perinatally exposed to DINP and DBP, and DINP significantly reduced anogenital distance (AGD) in males at all doses used.

It has been hypothesized that testicular germ cell cancer, cryptorchidism, hypospadias, and low sperm count comprise a testicular dysgenesis syndrome (TDS) with a common origin in fetal life (Skakkebaek *et al*., 2001). Testosterone production plays a determining role during fetal and early postnatal life and thus disturbances of testosterone production in fetal life are important in leading to TDS. Testosterone may also regulate *Insl* 3 post-natally in mice thus increasing the risk of failure of testis descent. Lower testosterone concentrations also affect dihydrotestosterone (DHT)-induced development of the prostate and external genitalia. DHT is responsible for normal apoptosis of nipple anlagen in males which results in the lack of nipple development and growth of the perineum to produce normal male anogenital distance (reviewed in NRC, 2008). The potential end points of TDS syndrome are: compromised Leydig cell function in adulthood, increased LH drive to maintain testosterone level, reduced size of the prostate and seminal vesicle, and reduced anogenital distance (Sharpe and Skakkebaek, 2008).

DINP reduced testosterone production and mRNA expression levels of StAR and *Insl-3* (Hannas *et al.*, 2011, 2012), reduced anogenital distance, increased diameter of seminiferous tubule (Boberg *et al*., 2011), decreased seminal vesicle weight (Lee and Koo, 2007), and reduced testicular testosterone contents and *ex vivo* testosterone production (Borch *et al*., 2004). These alterations are remarkably similar to a testicular dysgenesis syndrome observed in humans (Skakkebaek *et al*., 2001), except for responses for which rats are dimorphic, such as retention of nipples. Additionally, the rats do not develop testicular germ cell cancer, the most common cancer in young men. Rats develop Leydig cell tumors of the testes, which occur spontaneously as well as following exposure to biologically active phthalates.

The effects of perinatal exposure to DINP on testosterone production and related parameters are summarized in Table 11.

Table 11. Effects of perinatal exposure to DINP on testosterone production and related parameters[1]

Test system	Doses	Exposure duration	Effects	Reference
Harlan SD timed pregnant rats	0, 500, 750, 1000 or 1500 mg/kg bw/day by gavage	GD 14 to 18	↓ ex *in vivo* testosterone at ≥ 500 mg/kg ↓ RNA expression of StAR, *Insl3*, CYP11a	Hannas *et al.* (2011)
Pregnant SD rats	0, 400, 4000, or 20000 ppm in feed	GD 15 to PND 10	Testicular atrophy and histopathology at 20000 ppm	Masutomi *et al.* (2003)
	0 or 20000 ppm in feed	GD 15 to PND 10	No effects on pituitary hormones	Masutomi *et al.* (2004)
	0, 1000 or 1500 ppm in feed	GD 14 to PND 3	↑ Areolas in males at PND 14	Ostby *et al.* (2001)
	0 or 750 mg/kg by gavage	GD 14 to PND 3	↑ Aerolas/nipples in 22 percent of male pups. Small atrophic testes	Gray *et al.* (2000)
Pregnant Wistar rats	0, 50, 100, or 200 mg Wy - 14643 mg/kg bw/day by gavage	GD 14 to 18	No PPAR related genes were affected in the fetal testis. No effect on testosterone	Hannas *et al.* (2012)
	0, 300, 600, 750, or 900 mg/kg bw/day by gavage	GD 17 to PND 17	↑ Nipple retention, diameter of seminiferous tubule ↓ AGD, sperm motility	Boberg *et al.* (2011)
	0 or 750 mg/kg bw/day by gavage	GD 7 to GD 21	↓ Testicular testosterone contents and *ex vivo* production	Borch *et al.* (2004)
Wistar rats	0, 40, 400, 4,000 or 20,000 ppm in feed	GD 15 to PND 21	↓ AGD in males ↑mRNA for grn in females ↑mRNA for p 130 in males ↓lordosis quotient (females	Lee *et al.* (2006)
SD rats Hershberger assay	0, 20, 100, or 1500 mg/kg bw/day by gavage	10 days	↓ Absolute weight of seminal vesicle at ≥ 20 mg/kg	Lee and Koo (2007)

AGD, anogenital distance; bw, body weight; GD, gestational day; grn, granular precursor; PND, postnatal day; CYP11a, steroidogenic P450 enzyme; *Insl3*, Insulin like-3; StAR, steroidogenic acute regulatory protein

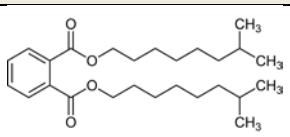
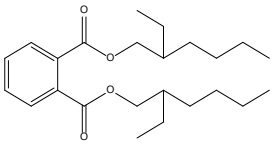
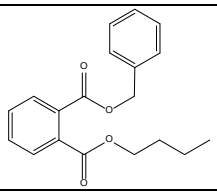
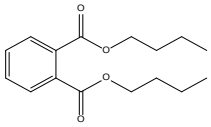
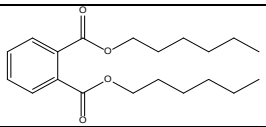
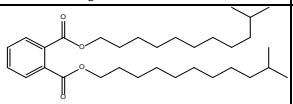
[1] ↑, increase compared to controls; ↓, decrease compared to controls.

3.3.7 Structure Activity Comparisons

DINP 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 DINP and 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, and as causing reproductive toxicity (male reproductive toxicity, developmental toxicity). DEHP is the only phthalate listed as a carcinogen under Proposition 65. Table 12 summarizes the structure and other information on DINP, DEHP, and four other phthalates, namely BBP, DBP, Di-n-hexyl phthalate, and DIDP, which are listed as causing reproductive toxicity under Proposition 65.

Table 12: Phthalates listed or being considered for listing under Proposition 65.

Name	Acronym	Structural formula	CAS number	Classification	Date listed
Diisononyl phthalate	DINP			Under listing consideration	---
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	DHP		84-75-3	Female and Male Reproductive	2005
Diisodecyl phthalate	DIDP		26761-40-0/ 68515-49-1	Developmental	2007

DINP and DEHP (OEHHA, 2003; IARC, 2013) have positive carcinogenicity data in rats and mice. BBP has positive carcinogenicity data in rats (OEHHA, 2013). DINP, DEHP and BBP all increase MNCL incidence in rats (DEHP in male rats only). DINP increases pancreatic islet cell tumor incidence in rats and mice, while BBP and DEHP both increase pancreatic acinar cell tumor incidence in rats. Both DEHP and DINP but not BBP increase liver tumor incidence in rats and mice and testicular interstitial cell tumor incidence in rats. DINP increases renal transitional cell tumor incidence and renal tubular carcinoma incidence in rats and BBP increases bladder transitional epithelial cell tumor incidence in rats. The tumor sites for DINP, DEHP and BBP are summarized in Table 13.

Table 13: DINP, DEHP and BBP 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
DINP	M F	M F	F[1]	M[1]		M		M F		M[3]		F[5]
DEHP[#]	M F	M F		M[2]		M		M				
BBP[&]				M[2]				M F		F[4]		

[#]OEHHA (2002); IARC (2013)
[&]OEHHA, (2013)
M = male, F = female
[1]Pancreatic islet cell carcinoma
[2]Pancreatic acinar cell adenoma or carcinoma
[3]Renal transitional epithelial cell carcinoma and renal tubular carcinoma
[4]Bladder transitional epithelium hyperplasia, papilloma and carcinoma
[5]Endometrial adenocarcinoma

DINP, BBP (OEHHA, 2013) and DEHP (Caldwell, 2012; IARC, 2013) 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.

DINP, DEHP (IARC, 2013) and BBP (OEHHA, 2013) have all been shown to activate PPARα, PPARγ, ER and PXR. Both BBP and DEHP but not DINP have been demonstrated to activate AhR. DEHP and DINP activate CAR and inhibit GJIC; no data are available for activation of CAR or inhibition of GJIC by BBP. DINP, DEHP and BBP 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 14.

Table 14: DINP, DEHP and BBP 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								
DINP	NE	-	-	+/-[1]	+	+	-	+	+	+	+
DEHP[#]	+	+	+	+	+	+	+	+	+	+	+
BBP[&]	+	-	+	+	+	+	+	+	NE	NE	+

NE: Not evaluated. [1]One of nine assays positive.
[#]Caldwell (2012); IARC (2013)
[&]OEHHA (2013)

To summarize, DINP demonstrates carcinogenicity similar to that of some other phthalates. DINP and DEHP have positive carcinogenicity data in rat and mouse studies, and BBP has positive carcinogenicity data in mouse studies, as shown in Table 13. DINP, DEHP, and BBP all induce tumors at multiple sites. DINP, DEHP and BBP all significantly induce MNCL; DINP induces pancreatic islet cell tumors, and BBP and DEHP induce pancreatic acinar cell tumors.

DINP has not been shown to be genotoxic, although it has been tested in a limited number of assay systems. DINP has positive results in cell transformation genotoxicity assays performed *in vitro*. DEHP has positive results in gene mutation, chromosomal damage, cell transformation, and DNA damage assays performed *in vitro* and *in vivo* (Caldwell, 2012; IARC, 2013). BBP has positive results in chromosomal damage assays performed *in vivo* and in DNA damage and cell transformation assays performed *in vitro* (OEHHA, 2013). 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 14).

DEHP is listed under Proposition 65 as causing cancer and is classified by IARC as a Group 2B carcinogen (IARC, 2013) and U.S. EPA as a Group B2 carcinogen (U.S. EPA, 1993a). BBP will be considered for possible listing as a carcinogen under Proposition 65 by the Carcinogen Identification Committee in 2013 (OEHHA, 2013). The U.S. EPA classified BBP as a Class C "possible human carcinogen" in 1993 (U.S. EPA, 1993b); this classification is currently under reassessment (U.S. EPA, 2012a). In 2000, 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 in Group 3 "Not classifiable as to its carcinogenicity to humans" (IARC, 2000).

4. MECHANISMS

DINP increased the incidence of MNCL in male and female F344 rats, liver tumors in male and female F344 rats, female SD rats, and male and female B6C3F1 mice, and renal tubular cell carcinomas in male Leydig interstitial cell carcinomas of the testes in male SD rats. In addition, rare or uncommon transitional cell carcinomas and tubular cell carcinomas of the kidney have been observed in DINP-treated male F344 rats. Additional malignant tumors considered either rare or uncommon in the tissue and species of origin that occurred in DINP-treated animals include renal transitional cell carcinomas in male F344 rats, and endometrial adenocarcinomas in female SD rats. The mechanisms by which DINP induces tumors are not known; however, several studies provide information on a number of possible mechanisms of action. These are discussed below. Also discussed below are hypotheses that have been put forward

suggesting the involvement of PPARα-agonism in DINP-induced mouse and rat liver tumorigenesis (Klaunig *et al.*, 2003), and α2u-globulin nephropathy in DINP-induced renal tubular cell tumorigenesis (Caldwell *et al.*, 1999).

4.1 Genotoxicity

DINP has been tested in a limited number of genotoxicity assays, and found to be negative in each. Specifically, DINP was negative in the *Salmonella* reverse mutation assay (TA 98, 100, 1535, 1537, and 1538), the mouse lymphoma cell mutation assay, the CHO cell chromosomal aberration assay, and *in vivo* micronucleus assays in rats and mice.

However, DINP has not been adequately tested for induction of oxidative DNA damage in assays such as the single cell gel electrophoresis, or comet assay, which detects DNA single strand breaks, or in *Salmonella* strains sensitive to oxidative DNA damage (e.g., TA 102 and TA 104). This is noteworthy since PPARα agonist-mediated response is associated with a marked increase in reactive oxygen species (ROS) (e.g. hydroxyl radicals, superoxide, hydrogen peroxide (H_2O_2)) generating enzymes. ROS (e.g., H_2O_2 and hydroxyl radicals) may interact with cellular macromolecules leading to oxidative stress with possible alterations in DNA, protein, or lipids (Yeldani *et al*., 2000). The structurally-related phthalates DEHP and BBP both induce DNA single strand breaks (see Section 3.3.7 and Table 10). In addition, both DEHP and its metabolite MEHP have been demonstrated to induce ROS in LNCaP human prostate adenocarcinoma cells (Erkekoglu *et al.*, 2011) and DNA damage in MA-10 mouse Leydig cell tumor cells (Erkekoglu *et al.*, 2010). It has also been noted that Kupffer cell (liver-resident macrophages) activation by peroxisome proliferators (PP) involves the generation of ROS (reviewed in Rusyn and Corton, 2012).

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 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 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 in 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.

DINP is a PPAR agonist and activates both PPARα and PPARγ but not PPARβ 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, PPARs 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, PPAR-binding protein (PBP) is a nuclear receptor co-activator required for PPARα-regulated gene expression in the 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 by 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 PPARα activation and liver tumor induction

Several chemicals that induce peroxisome proliferation in the liver of rats and mice (i.e., PPARα agonists) also induce liver tumors (Rao and Reddy, 1987; Reddy *et al.*, 1980). It has been hypothesized that liver tumor induction in rats and mice by PPARα agonists results from mechanisms associated with the activation of PPARα (Klaunig *et al.*, 2003). It has also been suggested that rat and mouse liver tumors induced by PPARα agonists are not relevant to human cancer risk assessment because of differences in activation characteristics between rodent and human PPARα. Evidence relevant to the evaluation of the PPARα mode of action (MOA) hypothesis for liver tumor induction is briefly discussed below, followed by a discussion of findings relevant to this hypothesis from studies of DINP in rodent liver.

Klaunig *et al.* (2003) presents a list of key events in the hypothesized pathway leading from PPARα agonist treatment to liver tumor development. The events identified as causal in the proposed tumor progression pathway are 1) activation of PPARα, 2) perturbation of cell proliferation and apoptosis, and 3) selective clonal expansion. Klaunig *et al.* (2003) noted that rats and mice are more responsive than humans to some effects of PPARα agonists in liver: peroxisome proliferation, induction of fatty acid β-oxidation metabolic pathways, minimum ligand concentration for receptor activation, maximum receptor activation. They suggested that the liver tumor induction observed by some PPARα agonists in rats and mice is not relevant to human cancer risk assessment.

The PPARα agonist rat/mouse liver tumor MOA hypothesis proposed by Klaunig *et al.* (2003) depended in part on studies by Peters *et al.* (1997) and Ward *et al.* (1998) using PPARα-null mice derived from wild-type SV129 mice. Peters *et al.* (1997) found that 11 months of treatment with the PPARα agonist Wy-14,643 did not induce liver tumors in nine PPARα-null mice but did induce multiple hepatocellular tumors in the corresponding wild-type mice. DEHP also did not induce peroxisome proliferation in PPARα-null mice after 24 weeks of exposure (Ward *et al.*, 1998). Klaunig *et al.* (2003) suggested that a long-term DEHP cancer bioassay in PPARα-null mice would also be negative. IARC changed the carcinogen classification of DEHP from 2B (possibly carcinogenic to humans; sufficient evidence in experimental animals) to 3 (not

classifiable as to its carcinogenicity to humans) in 2000, based in part on consideration of the Peters *et al.* (1997) and Ward *et al.* (1998) data.

A number of PPARα agonist studies in rats and mice which have generated data relevant to the proposed PPARα agonist tumor MOA have been performed since the publication of that MOA hypothesis. Much of that data has been reviewed by Guyton *et al.* (2009), Caldwell (2012) and Rusyn and Corton (2012).

Ito *et al.* (2007) found that exposure of SV129-derived PPARα-null mice to 100 or 500 ppm DEHP in the diet for up to 22 months resulted in the induction of liver tumors in those mice, with a significant trend for dose-response. These data indicate that PPARα agonists may induce liver tumor by a mechanism independent of PPARα activation.

Yang *et al.* (2007) developed LAP-VP16PPARα transgenic mice (from a wild-type SV129 mouse) transfected with a mouse PPARα receptor fused to the viral transcriptional activator VP16 under the control of the liver-enriched activator protein (LAP). These mice constitutively expressed activated PPARα in hepatocytes in a targeted manner. The authors then used these mice to study whether the activation of PPARα in hepatocytes only is sufficient to induce liver tumors. The LAP-VP16PPARα mice demonstrated peroxisome proliferation (including increased PCoA activity) and hepatocyte proliferation at 8 - 10 weeks of age. However, no liver tumors were noted in more than 20 LAP-VP16PPARα mice at 11 months of age. In contrast, wild-type mice fed 0.1% Wy-14,643 in the diet for 11 months developed hepatocellular carcinomas (exact number of tumor-bearing animals not provided by authors). These data indicate that PPARα activation by itself is not sufficient to induce hepatocarcinogenesis.

The National Research Council (2008) cited the Ito *et al.* (2007) data in a report on risk assessment and stated that it “called into question” the decision made by IARC in 2000 to reclassify DEHP from group 2B to group 3, based on the conclusion that the induction of liver tumors in rats and mice by the PPARα-agonist DEHP occurred via a mechanism that was not relevant to humans.

IARC reevaluated the carcinogenicity of DEHP in 2013, and based in part on the Ito *et al.* (2007) and Yang *et al.* (2007) data, changed its classification to group 2B. In its reevaluation of DEHP carcinogenicity, IARC stated “multiple molecular signals and pathways in several cell types in the liver, rather than a single molecular event, contribute to the induction of cancer in rats and mice. Thus, the relevance to human cancer of the molecular events that lead to cancer elicited by di(2-ethylhexyl) phthalate *[DEHP]* in several target tissues (e.g. the liver and testis) in rats and mice cannot be ruled out”.

PPARα activation by DINP

As discussed above and in section 3.3, DINP and its metabolite MINP are PPARα agonists. MINP has been demonstrated to transactivate both mouse and human PPARα (Bility *et al.*, 2004).

Many of the liver changes typically associated with PPARα activation have been reported in studies of rats and mice treated with DINP. As summarized below, this includes liver enlargement, increased liver to body weight ratio (LBWR), increased number and size of liver peroxisomes, and increased levels of fatty acid β-oxidation enzyme activity, such as PCoA and lauryl CoA oxidase (LCoA).

- Hüls (1992; reviewed in EC JRC, 2003) exposed female F344 rats to DINP, DINP-2 or DINP-3 at doses of 0, 25, 75, 150 or 1500 mg/kg-day by gavage for 14 days. LCoA activity was used as a marker for peroxisomal ß-oxidation. DINP, DINP-2 and DINP-3 all significantly induced hepatic LCoA activity in the high dose group.
- Smith *et al.* (2000) exposed male F344 rats and male B6C3F_1 mice to DINP or DINP-A in the diet for 2 or 4 weeks. Dose levels were 0, 1000 and 12,000 ppm, and 0, 500 and 6000 ppm for rats and mice, respectively. Both DINP and DINP-A significantly increased hepatic PCoA activity in rats and mice of both sexes compared to controls after either 2 or 4 weeks of treatment at the high dose but not the low dose.
- Male and female F344 rats were fed diets containing 0, 0.6, 1.2 or 2.5% DINP for 21-days (BIBRA, 1985; reviewed in EC JRC, 2003). Hepatic peroxisome proliferation (as evaluated by electron microscopy) and PCoA activity were significantly increased compared to controls at doses of 1.2% and higher.
- Male and female B6C3F_1 mice were fed diets containing 500, 1500, 4000 or 8000 ppm DINP for 4 weeks by Kaufmann *et al.* (2002). Both male and female mice had significantly increased hepatic peroxisome proliferation compared to controls at dose levels of 1500 ppm and greater. Male and female mice had significantly increased hepatic PCoA activity compared to controls at dose levels of 500 and 1500 ppm, respectively.
- In the Moore (1998a, as reviewed by CPSC, 2001) two-year DINP feeding studies in male and female F344 rats, hepatic hypertrophy and PCoA activity were significantly increased after 2, 13, 79 and 104 weeks in high dose males and females (12,000 ppm).
- In the Moore (1998b, as reviewed by CPSC, 2001) two-year DINP feeding studies in male and female B6C3F_1 mice, a significant increase in hepatic PCoA activity was observed in both males and females after either 78 or 104 weeks of

exposure to the highest dose used (8000 ppm). Hepatic PCoA activity was not assayed at the lower doses.

- In the Lington *et al.* (1997) two-year DINP feeding studies in male and female F344 rats, slight hypertrophy of liver parenchymal cells was observed in the high dose (6000 ppm) males and females at 2 years. No treatment-related peroxisome proliferation was evident in the livers of these animals by ultrastructural examination.
- As described in section 3.3.5, Valles *et al.* (2003) exposed male and female $B6C3F_1$, SV 129 and SV129 PPARα-null mice (26-30 weeks of age) to 0 or 8000 ppm DINP in feed for one or three weeks (Study 2). The male mice were exposed to DINP-2 (CAS number 28553-12-0), and the female mice were exposed to DINP (CAS number 68515-48-0). Increases in LBWR were observed in male and female $B6C3F_1$ and SV129 wild-type mice, and in female PPARα-null mice exposed to DINP. No increase in LBWR was observed in DINP exposed male PPARα-null mice. The increased LBWR observed in PPARα-null female mice exposed to DINP at 26-30 weeks of age contrasts with the results of Study 3, where no increase in LBWR was observed in 9-week old female PPARα-null mice exposed to 8000 ppm for one week. These data indicate that some hepatic effects such as increased LBWR thought to be solely dependent on PPARα activation can occur independently of PPARα activation. These findings also indicate that responses to DINP differ with life stage of exposure.

To summarize, DINP consistently causes liver changes associated with PPARα in rats and mice. However, limited data exist indicating some liver changes (increased LBWR) in mice can occur independently of PPARα activation after DINP treatment.

The degree to which a PPARα agonist induces liver responses indicative of PPARα activation has not been found to correlate with the ability of the chemical to induce liver tumors. For example, in the F344 rat studies of Smith *et al.* (2000) described above, DIDP was found to be a more potent inducer of hepatic PCoA activity than DINP (by approximately 3-fold), yet no liver tumors were observed in studies of male and female F344 rats exposed to DIDP in the diet for two years (0, 400, 2000 or 8000 ppm).

Role of PPARα-activation in DINP induced liver tumors

The PPARα liver tumor MOA hypothesis proposed by Klaunig *et al.* (2003) identifies increased hepatic cell proliferation and perturbation of apoptosis as PPARα-dependent causal events leading to tumor formation. However, the data on short-term induction of hepatocellular proliferation in DINP-exposed $B6C3F_1$ mice are inconsistent, and long-

term hepatocellular proliferation has not been shown to occur in DINP-exposed rats. These studies are summarized below.

Cell proliferation

- In the Moore (1998a, as reviewed by CPSC, 2001) male and female F344 rat dietary DINP studies, after one week of treatment, significant increases in the mitotic cell number and mean labeling index for hepatocytes were observed in the livers of all 5 high-dose males and 5 high-dose females assessed. However, after 2, 13 and 79 weeks of treatment, the number of mitotic cells and labeling indices in the liver were no longer increased compared to controls. Thus the initial increase in hepatic cell proliferation was not sustained in these animals, despite sustained increases in markers of peroxisome proliferation increased hepatic hypertrophy and PCoA activity (as described earlier).
- Smith *et al.* (2000) found that 12,000 ppm DINP and DINP-A but not 1000 ppm in the diet induced periportal parenchymal cell proliferation (as measured by labeling index increases) in male F344 rats compared to controls after two weeks of exposure. After four weeks of exposure, hepatocellular proliferation was still increased compared to controls in the 12,000 ppm DINP-A males, but not the 12,000 ppm DINP males.
- Valles *et al.* (2003) fed female B6C3F_1, SV129 wild-type, and SV129 PPARα-null mice feed containing 0 or 8000 ppm DINP for one week. DINP treatment significantly increased hepatocellular proliferation compared to controls in liver zones 1 (periportal area) and 3 (centrilobular area) of the B6C3F_1 females and in liver zone 3 of the SV129 wild-type females. The one week exposure to DINP did not increase hepatocellular proliferation in SV129 PPARα-null females.
- Smith *et al.* (2000) observed hepatocellular proliferation in male B6C3F_1 mice exposed to 6000 ppm DINP in the diet at two weeks of exposure, but not at four weeks of exposure. No hepatocellular proliferation was observed in male B6C3F_1 mice exposed to 6000 ppm DINP-A after either two or four weeks of treatment.
- Kaufmann *et al.* (2002) reported significantly increased hepatocellular proliferation in liver zone 1 (portal tract) of B6C3F_1 male mice compared to controls after four weeks of exposure to DINP in the diet at exposure levels of 1500 ppm (282% increase) and 8000 ppm (1009% increase) but not at 4000 ppm. However, hepatocellular proliferation was not significantly increased in liver zone 1 of female mice after four weeks of exposure to DINP at any concentration.
- In the Moore (1998b, as reviewed by CPSC, 2001) male and female B6C3F_1 mouse dietary studies, there was no evidence of increased hepatic cell

proliferation as measured by mean labeling index at the 8000 ppm dietary dose level after either 78 or 104 weeks of treatment compared to controls.
- In isolated primary F344 rat hepatocytes exposed *in vitro* to the DINP metabolite MINP at concentrations ranging from 150 to 250 µM, DNA replicative synthesis was significantly induced (Shaw *et al.*, 2002).

Perturbation of apoptosis
- Kaufmann *et al*. (2002) studied apoptosis (as measured by TUNEL stain labeling) in male and female $B6C3F_1$ mice exposed to DINP in the diet at doses of 500, 1500, 4000 or 8000 ppm for either one or four weeks of exposure. Concurrent controls were provided for the four week exposure groups but not the one week exposure groups, making interpretation of the one week exposure group results problematic. The rates of hepatocellular apoptosis (assessed in zones 1, 2 and 3) were not significantly different from controls in males and females at all DINP dose levels after four weeks of DINP exposure.
- In isolated primary F344 rat hepatocytes exposed *in vitro* to the DINP metabolite MINP at concentrations ranging from 150 to 250 µM, transforming growth factor β1-induced apoptosis was significantly suppressed (Shaw *et al.*, 2002).

Synopsis

The inconsistency of the short-term hepatocellular proliferation in DINP-exposed rats and mice and the lack of sustained long-term hepatocellular proliferation in DINP-exposed rats suggests that PPARα activation may not be causally related to DINP-induced liver tumors in rats and mice.

4.2.2 PPARγ activation

As discussed above and in section 3.3.6, the DINP metabolite MINP 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 is also available which indicates that an increased risk of bladder cancer exists in type-2 diabetics using pioglitazone as an anti-diabetic therapeutic (Mamtani *et al.*, 2012; Azoulay *et al.*, 2012).

Urothelium lines the renal pelvis and ureter, as well as the bladder. As noted previously, DINP also induced urothelial tumors in the rat urothelium, specifically, transitional cell carcinomas of the kidney, in two chronic exposure studies conducted in male F344 rats (Lington *et al.*, 1997; Moore, 1998a, as reviewed by CPSC, 2001). The increase in transitional cell carcinoma incidence in those two studies was not statistically significant, but those tumors are considered rare in untreated male F344 rats.

4.3 Activation of CAR and PXR

DINP activates the human constitutive androstane receptor (CAR) and pregnane X receptor (PXR), two closely related liver-enriched nuclear hormone receptors which regulate the transcription of a number of drug-metabolizing enzymes and transporters. For example, activation of CAR and PXR results in the induction of a number of Cyp2b, Cyp2c, and Cyp3a isozymes (Hester *et al.*, 2012).

DeKeyser *et al*. (2011) studied the activation of CAR and PXR by DINP using reporter transactivation assays and two-hybrid studies in COS-1 cells (studies described in Section 3.3.5). In transactivation studies, COS-1 cells were transfected with the human CAR2 transcript or the human PXR transcript. DINP displayed potent activation of the 2B6-XREM-PBREM reporter (derived from the natural CYP2B6 gene promoter) compared with the positive control through human CAR2, indicating that DINP induces CYP2B6 through CAR. DINP was a weak activator of human PXR in this system. *Ex vivo* studies showed that DINP treatment of primary human hepatocytes resulted in increased protein expression of the CAR and PXR inducible enzymes CYP2B6 and CYP3A4 (DeKeyser *et al*., 2011). Interestingly, testosterone is a substrate for both CYP2B6 and CYP3A4 (Imaoka *et al.*, 1996).

PPARα-humanized (hPPARα) mice that express human PPARα only in the livers of PPARα-null mice were used by Ito *et al.* (2012) to study the differences between hepatic mouse and human PPARα and CAR activation in response to the phthalates DBP and DEHP. Twelve-week old hPPARα and wild-type mice (mPPARα; SV129 genetic background) were exposed by gavage to 0, 2.5 or 5 mmol/kg body weight DBP or DEHP in corn oil daily for two weeks. Both phthalates tested activated both PPARα and CAR in both hPPARα and mPPARα mice. However, PPARα activation was stronger in mPPARα mice than in hPPARα mice, while CAR activation was stronger in hPPARα mice compared to mPPARα mice.

It is not known whether the ablity of DINP to activate CAR and PXR, and the resulting increase in expression of several transporters and enzymes, including enzymes involved in testosterone metabolism, could be involved in tumorigenesis.

4.4 Effects on steroidogenesis and androgen-responsive tissues

As discussed in Section 3.3.6, male rats exposed *in utero* to DINP during critical periods of sexual development exhibited a number of effects that are consistent with exposure to either an antiandrogenic agent or an estrogenic agent. These include reduced AGD, reduced seminal vesicle volume, multinucleated gonocytes and enlarged seminiferous tubules. These effects were independent of PPARα activation (Hannas *et al*., 2012).

Lee and Koo (2007) studied the potential antiandrogenic effect of DINP in castrated immature male SD rats using a Hershberger assay procedure. DINP demonstrated antiandrogenic effects in the assay, including reduced seminal vesicle and levator ani/bulbocavernosus weights in the exposed rats.

Other phthalates have been shown to interfere with steroidogenesis, and studies of DINP suggest that it acts similarly. Specifically, DINP was found to reduce the mRNA expression levels of StAR, *Insl*-3 and the steroid synthesis gene, CYP11A, in Harlan SD timed pregnant rats (3-6/group) exposed on GDs 14-18 to either of two DINP formulations (CAS 28553-12-0; 68515-48-0) at doses of 0, 500, 780, 1000 or 1500 mg/kg/day (Hannas *et al*., 2011). As reviewed by Scott *et al.* (2009), several of the key genes involved in steroidogenesis are down-regulated after *in utero* exposure to either the DINP metabolite MINP, or the DEHP metabolite MEHP. These genes are StAR, HMG-CoA synthetase, and SRB1 (all involved in cholesterol uptake/transport) and the steroidogenic enzymes CYP11a, 3β-Hsd, and CYP17.

The study of Lee and Koo (2007) indicates that DINP inhibited the effect of exogenously added testosterone in this castrated immature male rat model, although the exact mechanism by which DINP induces these effects (e.g., AR antagonism, 5α reductase inhibition) has not been determined. The study of Hannas *et al.* (2011) and those reviewed by Scott *et al.* (2009) indicate that DINP interferes with the expression of a number genes involved in testosterone production. Taken together, these studies suggest that DINP may have multiple effects on androgen hormonal signaling pathways, and raise the possibility that these effects may be involved in tumorigenesis. For example, DINP's suppression of androgenic hormonal activity may be involved in the induction of Leydig cell hyperplasia and tumors in rats, given that the primary function of these cells in the mammalian testis is testosterone production.

Other mechanisms may be operative when exposure to DINP occurs early in life. For example, it has been hypothesized that reproductive disorders of human newborns (cryptorchidism, hypospadias) and young adults (testicular germ cell cancer, low sperm

counts) may comprise a testicular dysgenesis syndrome (TDS) with a common origin in fetal life. TDS has been associated with deficient androgen production during fetal testis development (Sharpe and Skakkebaek, 2008).

4.5 Tumor necrosis factor-α (TNF-α) induction

TNF-α is a cytokine produced by a number of cell types, including macrophages, natural killer (NK) cells, and CD4+ lymphocytes. TNF-α can participate in multiple cell signaling pathways involved in inflammation, cell proliferation, and apoptosis. As discussed below, there is some information that suggests that DINP may induce the release of TNF-α by macrophages (i.e., Bennasroune *et al.*, 2012). In addition, the structurally related phthalate DEHP has been shown to activate Kupffer cells (resident macrophages in the liver) to release TNF-α and ROS (reviewed in Rusyn *et al.*, 2000); the response of Kupffer cells to DINP has not been studied. In some cell types, such as the liver, TNF-α induces cell proliferation and decreases apoptosis (Diehl, 2000). Such effects would likely favor tumor development. On the other hand, in other tissues, such as the bone marrow, TNF-α suppresses cell proliferation (Bachegowda *et al.*, 2013). Such effects on the immune system could also favor tumor development as a result of impaired immuno-surveillance. Additionally, excessive inhibitory cytokine (including TNF-α) signaling has been observed in myelodysplastic syndromes (MDS), which can progress to leukemias (Bachegowda *et al.*, 2013).

Bennasroune *et al.* (2012) studied the effects of DINP alone or in combination with 4-n-nonylphenol (NP) on various immune system parameters using the human promonocyte cell line THP-1 (a macrophage-like model). The various parameters studied were: cytokine secretion (IL-1, IL-8, TNF-α), phagocytosis, and mitogen-activated protein kinase (MAPK) activation. Exposure of THP-1 cells to DINP (0, 0.2, 2, 5, and 10 µM) reduced phagocytosis in a dose dependent manner. No effects were observed on IL-1 and IL-8 levels. TNF-α levels were increased with exposure to 10 µM DINP alone, and by a combination of DINP and NP at all dose levels. Exposure of THP-1 cells to 2 µM DINP alone or in combination with NP decreased MAPK/ERK1/2 phosphorylation levels. This effect on phosphorylation was abolished by treatment with the estrogen receptor antagonist ICI-182780, suggesting this response was mediated through an estrogen receptor-dependent pathway.

4.6 Gap Junction Intercellular Communication (GJIC)

Gap junction intercellular communication (GJIC) is a process by which cells exchange small molecules to maintain homeostasis. The inhibition of GJIC has been proposed as a non-genotoxic carcinogenic mechanism (Klauing, *et al*., 2003). Several tumor types,

including hepatocellular carcinomas, have been shown to demonstrate inhibited GJIC (Trosko *et al*., 1990).

Male F344 rats were fed a diet containing 0, 1000, or 12,000 ppm DINP or DINP-A and male B6C3F_1 mice were fed a diet containing 0, 500 or 6000 ppm DINP or DINP-A for two or four weeks by Smith *et al*. (2000). Hepatic GJIC (measured by dye transfer) was inhibited in the rats after two weeks of treatment with 12,000 ppm but not 1000 ppm DINP and DINP-1. After four weeks of treatment, hepatic GJIC was inhibited in the rats exposed to 12,000 ppm DINP-A, but not in the rats exposed to 1000 ppm DINP-A or DINP, or 12,000 ppm DINP. In the mice, GJIC was significantly inhibited by 6000 ppm but not 500 ppm DINP-A after both two and four weeks of treatment. GJIC was not significantly inhibited by 500 or 6000 ppm DINP in the mice after two weeks of treatment, but was significantly inhibited after four weeks of treatment by 6000 ppm but not 500 ppm DINP.

4.7 α2u-Globulin Nephropathy

Renal tubule tumors

An increased incidence of renal tubule cell carcinomas was observed in three male F344 rat DINP chronic exposure studies (Lington *et al.*, 1997; Moore *et al.*, 1998a, as reviewed by CPSC, 2001). The increased tumor incidence was statistically significant in one study ($p<0.05$) (Moore, 1998a, as reviewed by CPSC, 2001). Additionally, while an increased tumor incidence was observed only in male rats, increased kidney/body weight ratios and nephropathy were observed in both male and female rats.

Caldwell *et al*. (1999) used archived kidney tissue obtained at a 12-month interim sacrifice of the Lington *et al.* (1997) studies to investigate renal tubule cell proliferation and α2u-globulin accumulation. The protein α2u-globulin is specific to male rats, and some renal tubule cell tumors induced by agents that induce α2u-globulin accumulation in male rat renal tubules have been suggested to be not relevant to human cancer risk assessment. The authors chose to evaluate kidneys from the 12-month interim sacrifice animals because the 6-month 6000 ppm exposure group interim sacrifice animals did not demonstrate α2u globulin accumulation. The authors noted increased tubular regeneration at all dose groups, increased tubular epithelial hypertrophy at the 3000 and 6000 ppm dose groups as measured by visual inspection of proliferating cell nuclear antigen (PCNA) staining, and increased staining for renal tubule α2u-globulin in the 6000 ppm dose group. The authors concluded that α2u-globulin accumulation in male rat kidneys was a likely mechanism for the renal tubule carcinomas observed in DINP-exposed male F344 rats in the Lington *et al.* (1997) studies.

However, a quantitative evaluation of the PCNA proliferation index indicated that the reported increase in renal tubule cell proliferation was not statistically significant, and the increase in 6000 ppm males (125% of controls) was not substantially greater than the increase observed in 6000 ppm females (112%). The increases in tubular regeneration and tubular epithelial hypertrophy were also not statistically significant, all the increases in both parameters were scored as 1 (minimal) on a scale of 0 to 5, and tubular regeneration was noted in 1 of 10 of the 6000 ppm dose group female rats. Additionally, increased α2u-globulin was only noted in the male 6000 ppm rat group, while Lington *et al.* (1997) observed an increased incidence of renal tubule carcinomas in both the 300 and 6000 ppm exposure groups.

A criteria list for determining whether chemicals that induce male rat kidney tumors and may cause increased renal α2uglobulin production should be considered to be potential human carcinogens has been defined in an IARC publication (Swenberg and Lehman-McKeeman, 1999).

The criteria published by IARC (Swenberg and Lehman-McKeeman, 1999) are as follows:

1. Renal tumors occur only in male rats.
2. Acute exposure exacerbates hyaline droplet formation.
3. α2u-globulin accumulates in hyaline droplets.
4. Subchronic histopathological changes including granular cast formation and linear papillary mineralization.
5. Absence of hyaline droplets and characteristic histopathological changes in female rats and mice.
6. Negative for genotoxicity in a battery of tests.

Additional supporting evidence could include:

1. Reversible binding of chemical (or metabolites) to α2u-globulin.
2. Increased and sustained cell proliferation in P2 segment of proximal tubules in male rat kidneys.
3. Dose-response relationship between hyaline droplet severity and renal tumor incidence.

The above data for DINP does not meet IARC criteria items 2, 4 and 5. With regard to criteria 2 (exacerbation of hyaline droplet formation by acute exposure), DINP did not increase renal tubule α2u-globulin accumulation after six months of exposure; increases were not seen until 12 months of treatment. With respect to criteria 4, no data is available on subchronic histopathological changes such as granular cast formation and

linear papillary mineralization. With regard to criteria 5 (absence of hyaline droplets and characteristic histopathological changes in female rats and mice), renal tubular regeneration was noted in one 6000 ppm female rat. Additionally, the data for DINP do not meet any of the three supporting criteria. Reversible binding of DINP or its metabolites to α2u-globulin has not been shown to occur, DINP does not significantly increase cell proliferation in rat renal tubules, and a dose-response relationship has not been demonstrated between hyaline droplet severity and renal tumor incidence.

Thus, α2u-globulin accumulation in the renal tubules of male rats do not explain the renal tubule carcinomas observed in DINP-exposed rats.

5. REVIEWS BY OTHER AGENCIES

DINP has not been classified as to its potential carcinogenicity by the U.S. EPA, the U.S. Food and Drug Administration, the National Toxicology Program, the National Institute for Occupational Safety and Health, or IARC. IARC, however, reviewed another phthalate, DEHP, which had been classified as a Group 3 chemical, "not classifiable as to its carcinogenicity", based on the consideration that liver tumors in rats and mice were caused by a mechanism involving peroxisome proliferation that was considered to be not relevant to humans. Because of additional scientific information reported since which suggests that many molecular signal and pathways rather than a single mechanism contribute to DEHP induced-cancer, IARC reclassified DEHP as a group 2B carcinogen (possibly carcinogenic to humans) (IARC, 2013).

Data relating to the carcinogenicity of DINP have been reviewed by the CHAP of the CPSC, (CPSC, 2001), CPSC staff (CPSC, 2010), and the U.S. EPA (U.S. EPA, 2005a) in a technical review.

- The CPSC CHAP (2001) concluded that DINP is clearly carcinogenic to rodents, inducing hepatocellular carcinoma in rats and mice of both sexes. A majority of the CPSC CHAP considered the increased mononuclear cell leukemia observed in DINP-exposed male and female rats to be of questionable significance due to high and variable background and possible strain specificity. The CPSC CHAP considered the renal tubule tumors observed in DINP-exposed male F344 rats to be caused via a α2u-globulin mechanism of action, and therefore rat-specific.

- CPSC staff (2010) considered DINP to be possibly carcinogenic to humans based on limited evidence of carcinogenicity in experimental animals.

- The U.S. EPA conducted a technical review of DINP (2005a), but did not classify the compound as to its carcinogenic potential. For liver tumors, U.S. EPA (2005b) stated that "EPA reserves judgment on whether DINP can reasonably be anticipated to cause liver cancer in humans". For mononuclear cell leukemia, U.S. EPA stated that "EPA believes that it is therefore highly unlikely that these findings were unrelated to treatment", and "the biological relevance to human cancer risk remains uncertain and cannot be discounted."

6. SUMMARY AND CONCLUSION

6.1 Summary of Evidence

No epidemiology studies were identified that investigated the risk of cancer associated with documented exposure to DINP.

The bulk of the evidence for carcinogenicity of DINP comes from multiple long-term carcinogenicity studies in rats and mice exposed via feed. Treatment-related increases in tumors were observed at a number of sites. Non-significant increases in several tumor types considered either rare or uncommon in the tissue and species of origin were also observed in DINP-treated animals.

Specifically, the following increases in tumors were observed (all studies had a two-year treatment duration except where noted):

Liver tumors

- In male F344 rats, the incidences of hepatocellular carcinomas and combined adenomas and carcinomas were significantly increased with significant dose-response trends in one study (Moore 1998a as reviewed by CPSC, 2001). A significant trend in hepatocellular carcinoma was seen in a second study in male F344 rats (Lington *et al.*, 1997).
- In female F344 rats, the incidence of combined hepatocellular adenomas and carcinomas was significantly increased with a significant dose-response trend in one study (Moore, 1998a as reviewed by CPSC, 2001).
- In female SD rats, the incidence of hepatocellular carcinomas was significantly increased with a significant dose-response trend in one study (Bio\dynamics, 1986 as reviewed by CPSC, 2001).
- In male B6C3F_1 mice, the incidences of hepatocellular carcinomas and combined adenomas and carcinomas were significantly increased with significant dose-response trends in one study. A significant increase in combined hepatocellular

adenoma and carcinoma was observed in a second study in male B6C3F_1 mice treated with DINP for 78 weeks followed by a 26 week recovery period (Moore, 1998b, as reviewed by CPSC, 2001).

- In female B6C3F_1 mice, the incidences of hepatocellular adenomas, carcinomas and combined adenomas and carcinomas were significantly increased with significant dose-response trends in one study. A significant increase in hepatocellular carcinoma and combined adenoma and carcinoma was observed in a second study in female B6C3F_1 mice treated with DINP for 78 weeks followed by a 26 week recovery period (Moore, 1998b as reviewed by CPSC, 2001).

Mononuclear cell leukemia (Spleen)

- In male F344 rats, the incidence of mononuclear cell leukemia was significantly increased with significant dose-response trends in two studies (Lington *et al.*, 1997; Moore, 1998a as reviewed by CPSC, 2001) and in a third study in male F344 rats treated with DINP for 78 weeks followed by a 26 week recovery period (Moore, 1998a as reviewed by CPSC, 2001).
- In female F344 rats, the incidence of mononuclear cell leukemia was significantly increased with significant dose-response trends in two studies (Lington *et al.*, 1997; Moore, 1998a as reviewed by CPSC, 2001) and in a third study (Moore, 1998a as reviewed by CPSC, 2001) in female F344 rats treated with DINP for 78 weeks followed by a 26 week recovery period.

Kidney tumors

- Renal tubular cell carcinoma incidence was significantly increased in one study in male F344 rats treated with DINP for 78 weeks followed by a 26 week recovery period (Moore, 1998a as reviewed by CPSC, 2001). These tumors are considered rare or uncommon in untreated male F344 rats.
- Renal tubular cell carcinoma incidence was increased in male F344 rats in two studies (Lington *et al.*, 1997; Moore, 1998a as reviewed by CPSC, 2001). The increases did not reach statistical significance; however, these tumors are considered rare or uncommon in untreated male F344 rats.
- Renal transitional cell carcinoma incidence was increased in male F344 rats in two studies (Lington *et al.*, 1997; Moore, 1998a as reviewed by CPSC, 2001). The increases did not reach statistical significance; however, these tumors are rare in untreated male F344 rats.

Pancreatic islet cell tumors

- In male SD rats, the incidence of pancreatic islet cell carcinomas was increased in one study (Bio\dynamics, 1986 as reviewed by CPSC, 2001). The increase

did not reach statistical significance; however, these tumors are considered rare in untreated male SD rats.

- In female B6C3F_1 mice, the incidence of pancreatic islet cell carcinomas was increased in one study (Moore, 1998b as reviewed by CPSC, 2001). The increase did not reach statistical significance; however, these tumors are considered rare in untreated female B6C3F_1 mice.

Leydig cell tumors

- In male SD rats, the incidence of testicular interstitial (Leydig) cell carcinomas was increased in one study (Bio\dynamics, 1986 as reviewed by CPSC, 2001). The increase did not reach statistical significance; however, these tumors are considered uncommon in untreated male SD rats.

Uterine tumors

- In female SD rats, the incidence of endometrial adenocarcinoma was increased in one study (Bio\dynamics, 1986 as reviewed by CPSC, 2001). The increase did not reach statistical significance; however, these tumors are rare in untreated female SD rats.

DINP was negative in the limited number of genotoxicity assay systems it has been tested in: *Salmonella* reverse mutation assay, mouse lymphoma cell mutation assay, CHO cell chromosomal aberration assay, and the *in vivo* micronucleus assay in rats and mice.

DINP has been tested for the ability to induce *in vitro* cell transformation in eight studies, all conducted with Balb/c-3T3 A31 mouse cells, with conflicting results. DINP was clearly positive in one study, negative in four studies, and non-significant increases in transformed foci were observed in three studies.

DINP is a PPAR agonist and activates both rodent and human PPARα and PPARγ but not PPARβ receptors. In studies with DINP's monoester metabolite MINP, the metabolite activated both the mouse and human PPARα and PPARγ receptors, but the degree of PPARα and PPARγ activation was greater for the mouse receptor than for the human receptor for both receptor types.

DINP induces a number of liver changes in rodents consistent with PPARα activation. However, studies with PPARα-null mice indicate that DINP induces some of these liver changes independently of PPARα activation (e.g., increased LBWR associated with older age at exposure in female PPARα-null mice and increased β/ω fatty acid oxidation enzyme induction associated with younger age at exposure in male and female PPARα-

null mice). With regard to induction of cell proliferation and suppression of apoptosis by DINP in the liver, the findings on apoptosis have been inconsistent, the data on short-term induction of hepatocellular proliferation in DINP-exposed $B6C3F_1$ mice are inconsistent, and long-term hepatocellular proliferation does not occur in rats.

DINP has been shown to activate human CAR and PXR.

Prenatal exposure of male rats to DINP results in a number of effects consistent with exposure to an antiandrogenic agent, including the reduction of fetal testis testosterone production, reduced AGD, reduced seminal vesicle volume, multinucleated gonocytes and enlarged seminiferous tubules. DINP also has been shown to interfere with steroidogenesis, reducing mRNA expression levels of genes involved in steroid production, such as StaR, *Insl*-3, and CYP11A.

TNF-α can participate in a number of cell signaling pathways involved in inflammation, cell proliferation, and apoptosis. Alterations in TNF-α function may contribute to carcinogenesis. In studies conducted in a human promonocyte cell line, DINP reduced phagocytosis in a dose-dependent manner and increased TNF-α levels.

The inhibition of GJIC has been proposed as a non-genotoxic carcinogenic mechanism. DINP inhibited hepatic GJIC (measured by dye transfer) in rats and mice dosed via diet for two or four weeks.

DINP shares some structural similarity with DEHP and BBP. All three compounds have positive carcinogenicity data for rodents. DEHP has been classified by IARC as a Group 2B carcinogen, by U.S. EPA as a Class B2 carcinogen and is listed under Proposition 65 as causing cancer. U.S. EPA classified BBP as a Class C “possible human carcinogen” in 1993; this classification is currently under reassessment (U.S. EPA, 2012a). In 2000, 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 in Group 3 “Not classifiable as to its carcinogenicity to humans” (IARC, 2000).

6.2 Conclusion

The evidence for carcinogenicity of DINP comes from:

- Multiple studies in rats and mice
    - Liver tumors in male and female F344 rats.
    - Liver tumors in female SD rats.
    - Liver tumors in male and female B6C3F$_1$ mice.
    - Mononuclear cell leukemia in male and female F344 rats.
    - Renal tubular cell carcinomas, which are rare or uncommon, in male F344 rats.
    - Observations of renal transitional cell carcinomas, which are rare, in male F344 rats.
    - Observations of pancreatic islet cell carcinomas, which are rare, in male SD rats and female B6C3F$_1$ mice.
    - Observations of testicular interstitial (Leydig) cell carcinomas, which are uncommon, in male SD rats.
    - Observations of uterine adenocarcinomas, which are rare, in female SD rats.
- DINP induced *in vitro* cell transformation in Balb/c-3T3 A31 mouse cells in one study, but not others.
- DINP or the metabolite MINP activate the following nuclear receptors: mouse and human PPARα and PPARγ, and human CAR and PXR.
- *In utero* exposure to DINP interferes with steroidogenesis in male rats, reducing testicular mRNA levels of StAR, *Insl*-3, and CYP11A, and results in a number of effects on the developing male reproductive system consistent with exposure to an antiandrogen.
- DINP stimulated TNF-α production in a human promonocyte cell line and suppressed phagocytosis.
- DINP inhibited hepatic GJIC in rats and mice.
- Structure activity comparisons with other phthalates, including DEHP, which has been classified by IARC as a Group 2B carcinogen, by U.S. EPA as a Class B2 carcinogen, and is listed under Proposition 65 as being known to the state of California to cause cancer.

7. REFERENCES

Abbott BD, Wood CR, Watkins AM, Das KP, Lau CS (2010). Peroxisome proliferator-activated receptors alpha, beta, and gamma mRNA and protein expression in human fetal tissues. *PPAR Res* 2010*.* pii: 690907*,* 2010

American Chemistry Council (ACC) High Phthalates Panel (2012, July). Overview of Our Work and Review of Current Challenges. *Powerpoint presentation at the SPI Flexible Vinyl Products 23rd Annual Conference.* Available at: http://www.plasticsindustry.org/files/events/Eileen_Conneely%20Tuesday.pdf

Anisimov VN, Nikonov AA (1990). Tumors of the vagina, uterus and oviduct. In: *Pathology of Tumors in Laboratory Animals*. *Volume 1 - Tumors of the Rat.* V Turusov, U Mohr (Eds). Lyon, France: IARC Scientific Publication, pp. 445-458.

Anderson WA, Castle L, Hird S, Jeffery J, Scotter MJ (2011). A twenty-volunteer study using deuterium labelling to determine the kinetics and fractional excretion of primary and secondary urinary metabolites of di-2-ethylhexylphthalate and di-iso-nonylphthalate. *Food Chem Toxicol* **49**:2022-2029.

Azoulay L, Yin H, Filion KB, Assayag J, Majdan A, Pollak MN, Suissa S (2012). The use of pioglitazone and the risk of bladder cancer in people with type 2 diabetes: nested case-control study. *BMJ* **344**:e3645.

Bachegowda L, Gligich O, Mantzaris I, Schinke C, Wyville D, Carrillo T, Braunschweig I, Steidl U, Verma A (2013). Signal transduction inhibitors in treatment of myelodysplastic syndromes. *J Hematol Oncol* **6**:50.

Barber ED, Cifone M, Rundell J, Przygoda R, Astill BD, Moran E, Mulholland A, Robinson E, Schneider B (2000). Results of the L5178Y mouse lymphoma assay and the Balb/3t3 cell *in vitro* transformation assay for eight phthalate esters. *J Appl Toxicol* **20**:69-80.

Becker K, Goen T, Seiwert M, Conrad A, Pick-Fuss H, Muller J, Wittassek M, Schulz C, Kolossa-Gehring M (2009). GerES IV: Phthalate metabolites and bisphenol A in urine of German children. *Int J Hyg Envir Heal* **212**:685-692.

Bennasroune A, Rojas L, Foucaud L, Goulaouic S, Laval-Gilly P, Fickova M, Couleau N, Durandet C, Henry S, Falla J (2012). Effects of 4-nonylphenol and/or diisononylphthalate on THP-1 cells: impact of endocrine disruptors on human immune system parameters. *Int J Immunopathol Pharmacol* **25**:365-376.

Berger J, Moller DE (2002). The mechanisms of action of PPARs. *Annu Rev Med* **53**:409-435.

Bility MT, Thompson JT, McKee RH, David RM, Butala JH, Vanden Heuvel JP, Peters JM (2004). Activation of mouse and human peroxisome proliferator-activated receptors (PPARs) by phthalate monoesters. *Toxicol Sci* **82**:170-182.

Bio\dynamics, Inc. (1986). A chronic toxicity carcinogenicity feeding study in rats with Santizer 900. Submitted to Monsonato Company by Bio\dynamics, Inc. Project No. 81-2572. June 20, 1986 *[as reviewed by CPSC, 2001]*.

Boberg J, Christiansen S, Axelstad M, Kledal TS, Vinggaard AM, Dalgaard M, Nellemann C, Hass U (2011). Reproductive and behavioral effects of diisononyl phthalate (DINP) in perinatally exposed rats. *Reprod Toxicol* **31**:200-209.

Borch J, Ladefoged O, Hass U, Vinggaard AM (2004). Steroidogenesis in fetal male rats is reduced by DEHP and DINP, but endocrine effects of DEHP are not modulated by DEHA in fetal, prepubertal and adult male rats. *Reprod Toxicol* **18**:53-61.

Bookout AL, Jeong Y, Downes M, Yu RT, Evans RM, Mangelsdorf DJ (2006). Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. *Cell* **126**:789-799.

Calafat AM, Wong LY, Silva MJ, Samandar E, Preau JL, Jia LT, Needham LL (2011). Selecting adequate exposure biomarkers of diisononyl and diisodecyl phthalates: Data from the 2005-2006 National Health and Nutrition Examination Survey. *Environ Health Persp* **119**:50-55.

Caldwell JC (2012). DEHP: Genotoxicity and potential carcinogenic mechanism- A review. *Mut Res* **751**:82-157.

Caldwell DJ, Eldridge SR, Lington AW, McKee RH (1999). Retrospective evaluation of alpha 2u-globulin accumulation in male rat kidneys following high doses of diisononyl phthalate. *Toxicol Sci* **51**:153-160.

Chandra M, Riley MG, Johnson DE (1992). Spontaneous neoplasm in aged Sprague-Dawley rats. *Arch Toxicol* **66**:496-502.

Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ (2001). Nuclear receptors and lipid physiology: opening the X-files. *Science* **294**:1866-1870.

Chopra B, Hinley J, Oleksiewicz MB, Southgate J (2008). Trans-species comparison of PPAR and RXR expression by rat and human urothelial tissues. *Toxicol Pathol* **36**:485-495.

CPSC (2001). Consumer Product Safety Commission. Report to the U.S. Consumer Product Safety Commission by the Chronic Hazard Advisory Panel on diisononyl phthalate (DINP).

CPSC (2010). Consumer Product Safety Commission. Toxicity review of diisononyl phthalate (DINP).

Dekeyser JG, Stagliano MC, Auerback SS, Prabhu AD, Jones CJ (2009). Di(2-ethylhexyl) phthalate is a highly potent agonist for the human constitutive androstane receptor splice variant CAR2. *Mol Pharmacol* **75**:1005-1013.

DeKeyser JG, Laurenzana EM, Peterson EC, Chen T, Omiecinski CJ (2011). Selective phthalate activation of naturally occurring human constitutive androstane receptor splice variants and the pregnane X receptor. *Toxicol Sci* **120**:381-391.

Diehl AM (2000). Cytokine regulation of liver injury and repair. *Immunol Rev* **174**:160-171

Dinse GE, Peddada SD, Harris SF, Elmore SA (2010). Comparison of NTP historical control tumor incidence rates in female Harlan Sprague Dawley and Fischer 344/N rats. *Toxicol Pathol* **38**:765-775.

ECHA (2010). European Chemicals Agency. Review of new available information for Di-'isononyl' phthalate (DINP) CAS No. 28553-12-0 AND 68515-48-0. Document developed in the context of evaluation of new scientific evidence concerning the restrictions contained in Annex XVII to regulation (EC) No. 1907/2006 (REACH).

EC JRC (2003). European Commission Joint Research Centre. European Union Risk Assessment Report. 1,2-benzenedicarboxylic acid, di-C8-10-branched alkyl esters, C9 rich and di-"isononyl" phthalate (DINP). Luxembourg: Office for Official Publications of the European Communities.

Erkekoglu P, Giray B, Durmaz E, Ozmert E, Kizilgun M, Derman O, Yurdakok K (2010). Evaluation of the correlation between plasma amylase and lipase levels and phthalate exposure in pubertal gynecomastia patients. *Turk Pediatri Arsivi (Turkish Archives of Pediatrics)* **45**:366-370.

Erkekoglu P, Rachidi W, Yuzugullu OG, Giray B, Ozturk M, Favier A, Hincal F (2011). Induction of ROS, p53, p21 in DEHP- and MEHP-exposed LNCaP cells-protection by selenium compounds. *Food Chem Toxicol* **49**:1565-1571.

Gray LE, Ostby J, Furr J, Price M, Veeramachaneni DNR, Parks L (2000). Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. *Toxicol Sci* **58**:350-365.

Guyton KZ, Chiu WA, Bateson TF, Jinot J, Scott CS, Brown RC, Caldwell JC (2009). A reexamination of the PPAR-alpha activation mode of action as a basis for assessing human cancer risks of environmental contaminants. *Environ Health Perspect* **117**:1664-1672.

Hannas BR, Furr J, Lambright CS, Wilson VS, Foster PM, Gray LE Jr. (2011). Dipentyl phthalate dosing during sexual differentiation disrupts fetal testis function and postnatal development of the male Sprague-Dawley rat with greater relative potency than other phthalates. *Toxicol Sci* **120**:184-193.

Hannas BR, Lambright CS, Furr J, Evans N, Foster PM, Gray EL, Wilson VS (2012). Genomic biomarkers of phthalate-induced male reproductive developmental toxicity: a targeted RT-PCR array approach for defining relative potency. *Toxicol Sci* **125**:544-557.

Harris CA, Henttu P, Parker MG, Sumpter JP (1997). The estrogenic activity of phthalate esters *in vitro*. *Environ Health Perspect* **105**:802-811.

Haseman JK, Hailey JR, Morris RW (1998). Spontaneous neoplasm incidences in Fischer 344 rats and B6C3F1 mice in two-year carcinogenicity studies: a National Toxicology Program update. *Toxicol Pathol* **26**:428-441.

Hester S, Moore T, Padgett WT, Murphy L, Wood CE, Nesnow S (2012). The hepatocarcinogenic conazoles: cyproconazole, epoxiconazole, and propiconazole induce a common set of toxicological and transcriptional responses. *Toxicol Sci* **127**:54-65.

Hines CJ, Hopf NBN, Deddens JA, Silva MJ, Calafat AM (2011). Estimated daily intake of phthalates in occupationally-exposed groups. *J Exp Sci Environ Epi* **21**:133-141.
IARC (1992). International Agency for Research on Cancer. Part 1. The Rat, urinary system In: *International Classification of Rodent Tumors.* IARC Scientific Publication No. 122. U. Mohr (Ed.). Lyon, France: World Health Organization.

IARC (2000). International Agency for Research on Cancer. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Volume 77. Some Industrial Chemicals. Di(2-ethylhexyl) Phthalate. World Health Organization. Lyon, France.

IARC (2013). International Agency for Research on Cancer. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Volume 101. Some Chemicals Present in Industrial and Consumer Products, Food and Drinking-water, Di(2-ethylhexyl) Phthalate. World Health Organization. Lyon, France.

Imaoka S, Yamada T, Hiroi T, Hayashi K, Sakaki T, Yabusaki Y, Funae Y (1996). Multiple forms of human P450 expressed in Saccharomyces cerevisiae. *Biochem Pharmacol* **51**:1041-1050.

Issemann I, Green S (1990). Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferation. *Nature* **347**:645-650.

Ishmael J, Dugard P (2006). A review of perchloroethylene and rat mononuclear cell leukemia. *Reg Toxicol Pharmacol* **45**:178-184.

Ito Y, Yamanoshita O, Asaeda N, Tagawa Y, Lee CH, Aoyama T, Ichihara G, Furuhashi K, Kamijima M, Gonzalez FJ, Nakajima T (2007). Di(2-ethylhexyl)phthalate induces hepatic tumorigenesis through a peroxisome proliferator-activated receptor alpha-independent pathway. *J Occup Health* **49**:172-182.

Ito Y, Nakamura T, Yanagiba Y, Ramdhan DH, Yamagishi N, Naito H, Kamijima M, Gonzalez FJ, Nakajima T (2012) Plasticizers May Activate Human Hepatic Peroxisome Proliferator-Activated Receptor α Less Than That of a Mouse but May Activate Constitutive Androstane Receptor in Liver. *PPAR Res* 2012. pii: 201284, 2012.

Jia Y, Qi C, Kashireddi P, Surapureddi S, Zhu YJ, Rao MS, Le Roith D, Chambon P, Gonzalez FJ, Reddy JK (2004). Transcription coactivator PBP, the peroxisome proliferator-activated receptor (PPAR)-binding protein, is required for PPAR alpha-regulated gene expression in liver. *J Biol Chem* **279**:24427-24434.

Kaufmann W, Deckardt K, McKee RH, Butala JH, Bahnemann R (2002). Tumor induction in mouse liver: Di-isononyl phthalate acts via peroxisome proliferation. *Regul Toxicol and Pharmacol* **36**:175-183.

Klaunig JE, Babich MA, Baetcke KP, Cook JC, Corton JC, David RM, DeLuca JG, Lai DY, McKee RH, Peters JM, Roberts RA, Fenner-Crisp PA (2003). PPAR α agonist-induced rodent tumors: Modes of action and human relevance. *Crit Rev Toxicol* **33**:655-780.

Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM, Lehmann JM (1997). Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc Natl Acad Sci USA* **94**:4318-4323.

Koch HM, Angerer J (2007). Di-iso-nonylphthalate (DINP) metabolites in human urine after a single oral dose of deuterium-labelled DINP. *Int J Hyg Environ Heal* **210**:9-19.

Koch HM, Wittassek M, Bruning T, Angerer J, Heudorf U (2011a). Exposure to phthalates in 5-6 years old primary school starters in Germany - A human biomonitoring study and a cumulative risk assessment. *Int J Hyg Environ Health* **55**:7-31.

Koch HM, Haller A, Weiss T, Kafferlein HU, Stork J, Bruning T (2011b). Phthalate exposure during cold plastisol application - a human biomonitoring study. *Toxicol Lett* **213**:100-106.

Lee HC, Yamanouchi K, Nishihara M (2006). Effects of perinatal exposure to phthalate/adipate esters on hypothalamic gene expression and sexual behavior in rats. *J Reprod Dev* **52**:343-352.

Lee BM, Koo HJ (2007). Hershberger assay for antiandrogenic effects of phthalates. Part A - Current Issues. *Journal of Toxicology and Environmental Health,Part A:-Current Issues* **70**:1365-1370.

Lee MH, Park J, Chung SW, Kang BY, Kim SH, Kim TS (2004). Enhancement of interleukin-4 production in activated CD4+ T cells by diphthalate plasticizers via increased NF-AT binding activity. *Int Arch Allergy Immunol* **134**:213-222.

Lin S, Ku HY, Su PH, Chen JW, Huang PC, Angerer J, Wang SL (2011). Phthalate exposure in pregnant women and their children in central Taiwan. *Chemosphere* **82**:947-955.

Lington AW, Bird MG, Plutnick RT, Stubblefield WA, Scala RA (1997). Chronic toxicity and carcinogenic evaluation of diisononyl phthalate in rats. *Funda Appl Toxicol* **36**:79-89.

Mamtani R, Haynes K, Bilker WB, Vaughn DJ, Strom BL, Glanz K, Lewis JD (2012). Association between longer therapy with thiazolidinediones and risk of bladder cancer: a cohort study. *J Natl Cancer Inst* **104**:1411-1421.

Maronpot RR, Montgomery CA, Boorman GA Jr, McConnell EE (1986). National toxicology program nomenclature of hepatoproliferative lesions of rats. *Toxicol Pathol* **14**:263-273.

Masutomi N, Shibutani M, Takagi H, Uneyama C, Takahashi N, Hirose M (2003). Impact of dietary exposure to methoxychlor, genistein, or diisononyl phthalate during the perinatal period on the development of the rat endocrine/reproductive systems in later life. *Toxicology* **192**:149-170.

Masutomi N, Shibutani M, Takagi H, Uneyama C, Lee KY, Hirose M (2004). Alteration of pituitary hormone-immunoreactive cell population in rat offspring after maternal dietary exposure to endocrine active chemicals. *Arch Toxicol* **78**:232-240.

McKee RH, Przygoda RT, Chirdon MA, Engelhardt G, Stanley M (2000). Di(isononyl) phthalate (DINP) and di(isodecyl) phthalate (DIDP) are not mutagenic. *J Appl Toxicol* **20**:491-497.

Montgomery CA Jr, Seely JC (1990). Kidney. In: *Pathology of the Fischer Rat*. G Boorman, S Eustis, M Elwel, CA Montgomery, Jr. and WF MacKenzie (Eds.). San Diego: Academic Press, Inc., pp.127-152.

Moore MR (1998a). Oncogenicity study in rats with di(isononyl)phthalate including ancillary hepatocellular proliferation and biochemical analysis. Covance laboratories, Inc., Vienna, VA. Study No. 2598-104.

Moore MR (1998b,). Oncogenicity study in mice with di(isononyl)phthalate including ancillary hepatocellular proliferation and biochemical analysis. Covance Laboratories Inc., Vienna VA. Study No. 2598-105.

NRC (2008). National Research Council. *Phthalates and Cumulative Risk Assessment: The Tasks Ahead.* Washington D.C.: National Academic Press.

NTP (2003). National Toxicology Program. NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Di-isononyl Phthalate (DINP). NIH Pub. 03-4484. U.S. Department of Health and Human Services, National Toxicology Program, Center for the Evaluation of Risks to Human Reproduction.

NTP (2013). National Toxicology Program. NTP Historical Control Information for the NIH-07 Diet. Oral, Feed. Fischer F344 Rats: Pathology Tables. At: http://ntp-server.niehs.nih.gov/ntp/research/database_searches/historical_controls/path/r_orlfd.txt

Office of Environmental Health Hazard Assessment (OEHHA) (2002). No Significant Risk Level (NSRL) for the Proposition 65 Carcinogen Di(2-Ethylhexyl)Phthalate.

Office of Environmental Health Hazard Assessment (OEHHA) (2013). Evidence on the Carcinogenicity of Butyl Benzyl Phthalate. October, 2013.

Oleksiewicz MB, Southgate J, Iverson L, Egerod FL (2008). Rat urinary bladder carcinogenicity by dual acting PPARα+γ agonist. *PPAR Res* 2008. pii: 103167, 2008.

Ostby JS, Hotchkiss AK, Furr JR, Gray LE (2001). Investigation of the ability of diisononyl phthalate (DINP) to alter androgen-dependent tissue development in Sprague-Dawley rats. *The Toxicologist* #**1070** *[abstract]*.

Palmer CN, Hsu MH, Griffin KJ, Raucy EF, Johnson EF (1998). Peroxisome proliferator activated receptor-alpha expression in human liver. *Mol Pharmacol* **53**:14-22.

Peters JM, Cattley RC, Gonzalez FJ (1997). Role of PPAR alpha in the mechanism of action of the nongenotoxic carcinogen and peroxisome proliferator Wy-14,643. *Carcinogenesis* **18**:2029-2033.
Pyper SR, Viswakarma N, Yu S, Reddy JK (2010). PPARalpha: energy combustion, hypolipidemia, inflammation and cancer. *Nucl Recept Signal* **8**:002.

Rakhshandehroo M, Hooiveld G, Muller M, Kersten S (2009). Comparative analysis of gene regulation by the transcription factor PPARalpha between mouse and human. PLoS One **4**(8):e6796.

Rakhshandehroo M, Knoch B, Muller M, Kersten S (2010). Peroxisome proliferator-activated receptor alpha target genes. *PPAR Res* 2010. pii: 612089, 2010.

Rao MS, Reddy JK (1987). Peroxisome proliferation and hepatocarcinogenesis. *Carcinogenesis* **8**:631-636.

Reddy JK, Azarnoff DL, Hignite CE (1980). Hypolipidaemic hepatic peroxisome proliferators form a novel class of chemical carcinogens. *Nature* **283**:397-398.

Reddy JK, Warren JR, Reddy MK, Lalwani ND (1982). Hepatic and renal effects of peroxisome proliferators: Biological implication. *Ann NY Acad Sci* **368**:81-110.

Reynolds CW, Timonen T, Herberman RB (1981). Natural killer (NK) cell activity in the rat. I. Isolation and characterization of the effector cells. *J Immunol* **127**:282-287.

Rusyn I, Rose ML, Bojes HK, Thurman RG (2000). Novel role of oxidants in the molecular mechanism of action of peroxisome proliferators. *Antioxid Redox Signal* **2**:607-621.

Rusyn I, Corton JC (2012). Mechanistic consideration for human relevance of cancer hazard of di(2-ethylhexyl) phthalate. *Mut Res* **750**:141-158.

Saravanabhavan G, Murray J (2012). Human biological monitoring of diisononyl phthalate and diisodecyl phthalate: a review. *J Environ Public Health* **2012**: article ID 810501.

Scott HM, Mason JI, Sharpe RM (2009). Steroidogenesis in the fetal testis and its susceptibility to disruption by exogenous compounds. *Endocrine Reviews* **30**:883-925.

Sharma R, Lake BG, Gibson GG (1988). Co-induction of microsomal cytochrome P-452 and the peroxisomal fatty acid beta-oxidation pathway in the rat by clofibrate and di-(2-ethylhexyl)phthalate. Dose-response studies. *Biochem Pharmacol* **37**:1203-1206.

Sharpe RM, Skakkebaek NE (2008). Testicular dysgenesis syndrome: Mechanistic insight and potential new downstream effects. *Fertil Steril* **89**(2):33-38.

Shaw D, Lee R, Roberts RA (2002). Species differences in response to the phthalate plasticizer monoisononylphthalate (MINP) *in vitro*: a comparison of rat and human hepatocytes. *Arch Toxicol* **76**:344-350.
Silva MJ, Kato K, Wolf C, Samandar E, Silva SS, Gray EL, Needham LL, Calafat AM (2006a). Urinary biomarkers of di-isononyl phthalate in rats. *Toxicology* **223**:101-112.

Silva MJ, Reidy JA, Preau JL, Needham LL & Calafat AM (2006b). Oxidative metabolites of diisononyl phthalate as biomarkers for human exposure assessment. *Environ Health Perspect* **114**(8):1158-1161.

Skakkebaek NE, Rajpert-De Meyts E, Main KM (2001). Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Hum Reprod* **16**:972-978.

Smith JH, Isenberg JS, Pugh G, Jr., Kamendulis LM, Ackley D, Lington AW, Klaunig JE (2000). Comparative in vivo hepatic effects of di-isononyl phthalate (DINP) and related C7-C11 dialkyl phthalates on gap junctional intercellular communication (GJIC), peroxisomal beta-oxidation (PBOX), and DNA synthesis in rat and mouse liver. *Toxicol Sci* **54**:312-321.

Stromberg PC (1985). Large granular lymphocyte leukemia in F344 rats. *Toxicol Pathol* **14**:263-273.

Swenberg, JA, Lehman-McKeeman LD. (1999). α2-Urinary globulin-associated nephropathy as a mechanism of renal tubule cell carcinogenesis in male rats. In: *Species Differences in Thyroid, Kidney and Urinary Bladder Carcinogenesis.* CC Capen, E Dybing, JM Rice, JD Wilbourn (Eds.). Lyon, France: IARC Scientific Publications. pp. 95-118.

Thomas J, Haseman JK, Goodman JI, Ward JM, Loughran TP Jr., Spencer PJ (2007). A review of large granular lymphocytic leukemia in Fischer 344 rats as an initial step toward evaluating the implication of the endpoint to human cancer risk assessment. *Toxicol Sci* **99**:3-19.

Thoolen B, Maronpot RR, Harada T, Nyska A, Rousseaux C, Nolte T, Malarkey DE, Kaufmann W, Kuttler K, Deschl U, Nakae D, Gregson R, Vinlove MP, Brix AE, Singh B, Belpoggi F, Ward JM (2010). Proliferative and nonproliferative lesions of the rat and mouse hepatobiliary system. *Toxicol Pathol* **38**:5S-81S

Trosko JE, Chang CC, Madhukar BV (1990). Modulation of intercellular communication during radiation and chemical carcinogenesis. *Radiat Res* **123**:241-251.

Tseng CH, Tseng FH (2012). Peroxisome proliferator-activated receptor agonists and bladder cancer: lessons from animal studies. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* **30**:368-402.

Turpeinen M, Raunio H, Pelkonen O (2006). The functional role of CYP2B6 in human drug metabolism: substrates and inhibitors *in vitro*, *in vivo* and *in silico*. *Curr Drug Metab* **7**:705-714.

U.S. EPA (1993). IRIS Toxicological Review and Summary Documents for Di(2-ethylhexyl)phthalate (DEHP) (CASRN 117-81-7). U.S. Environmental Protection Agency, Washington, DC.

U.S. EPA (1993). IRIS Toxicological Review and Summary Documents for Butyl benzyl phthalate (CASRN 85-68-7). U.S. Environmental Protection Agency, Washington, DC.

U.S. EPA (2005a). Revised Technical Review of Diisononyl Phthalate. Office of Environmental Information, Environmental Analysis Division, Analytical Support Branch. March 2005.

U.S. EPA (2005b). Addition of Diisononyl Phthalate Category; Community Right-to-Know Toxic Chemical Release Reporting; Notice of Data Availability. 40 CFR Part 372 [TRI–2005–0004; FRL–7532–4] RIN 2025–AA17.

U.S. EPA (2009). Phthalates Action Plan. U.S. Environmental Protection Agency, Washington, D.C. December 2009.

U.S. EPA (2012a). Phthalates Action Plan (Revised 03/14/2012). U.S. Environmental Protection Agency, Washington, D.C. http://www.epa.gov/oppt/existingchemicals/pubs/actionplans/phthalates_actionplan_revised_2012-03-14.pdf

U.S. EPA (2012b). Toxicological Review of Tetrachloroethylene (Perchloroethylene) (CAS No. 127-18-4) In Support of Summary Information on the Integrated Risk Information System (IRIS). EPA/635/R-08/011F. February 2012. U.S. Environmental Protection Agency, Washington, DC.

Valles EG, Laughter AR, Dunn CS, Cannelle S, Swanson CL, Cattley RC, Corton JC (2003). Role of the peroxisome proliferator-activated receptor alpha in responses to diisononyl phthalate. *Toxicology* **191**:211-225.

Ward JM, Peters JM, Perella CM, Gonzalez FJ (1998). Receptor and nonreceptor-mediated organ-specific toxicity of di(2-ethylhexyl)phthalate (DEHP) in peroxisome proliferator-activated receptor alpha-null mice. *Toxicol Pathol* **26**:240-246.

Ward JM, Rehm S, Reynlds CW (1990). Tumors of the hematopoietic system. In: *Pathology of Tumors in Laboratory Animals, Volume 1 - Tumors of the rat*. V Turusov and U Mohr (Eds.). Lyon, France: IARC Scientific Publications. pp. 625-657. [as cited in Thomas *et al*. 2007].

Yang Q, Ito S, Gonzalez FJ (2007). Hepatocyte-restricted constitutive activation of PPARα induces hepatoproliferation but not hepatocarcinogenesis. *Carcinogenesis* **28**:1171-1177.

Yeldandi AV, Rao MS, Reddy JK (2000). Hydrogen peroxide generation in peroxisome proliferator-induced oncogenesis. *Mutat Res* **448**:159-177

Zeman FA, Boudt C, Tack K, Barneaud AF, Brochot C, Pery ARR, Oleko A, Vandentorren S (2013). Exposure assessment of phthalate in French pregnant women: Results of the ELFE pilot study. *Int J Hyg Environ Heal* **16**:271-279.

Zhou S, Yung Chan S, Cher Goh B, Chan E, Duan W, Huang M, McLeod HL (2005). Mechanism-based inhibition of cytochrome P450 3A4 by therapeutic drugs. *Clin Pharmacokinet* **44**:279-304.