February 16, 2010

Sent via email (coshita@oehha.ca.gov) to:

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P.O. Box 4010
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RE: Diisononyl Phthalate (DINP) Carcinogenicity Hazard Assessment

Dear Ms. Oshita:

ExxonMobil Chemical Company is submitting this information in response to the California Office of Environmental Health Hazard Assessment (OEHHA) request for relevant information on diisononyl phthalate (DINP) to be considered by the OEHHA Science Advisory Board's Carcinogen Identification Committee (CIC). We request that OEHHA carefully review and consider this information as it prepares hazard identification materials on DINP.

DINP met OEHHA's screening criteria for consideration of Proposition 65 listing because of the observation of tumors in rats and mice treated with high doses of DINP. However, in contrast to most chemicals, there is a very robust data base for DINP demonstrating that those tumors in rodents are not relevant to a human cancer hazard assessment and that DINP is unlikely to cause cancer in humans. ExxonMobil therefore believes that DINP should not be listed as a human carcinogen under Proposition 65.

In addition to the materials being submitted at this time, additional information is anticipated in the near future that will be germane to development of hazard identification materials for DINP. ExxonMobil will submit information from the following when it becomes available:

- The American Chemistry Council Phthalate Esters Panel (Panel), of which ExxonMobil is a member, is holding a workshop on peroxisome proliferation. It will be attended by Panel member toxicologists and academic experts in the peroxisome proliferation mode of action as it relates to DINP and other phthalate esters. The goal of the workshop will be to address the significance of recent di(2-ethylhexyl) phthalate (DEHP) studies for understanding the peroxisome proliferation mode of action and the relevance to humans of phthalate-related rodent cancer. Originally, this workshop was to be held in December 2009, but that was prevented due to schedule conflicts for the experts; it appears it will be held in April.

DIISONONYL PHTHALATE (DINP)
Carcinogenicity Hazard Assessment

- A study sponsored by the European Council for Plasticizer and Intermediates has been conducted concerning the rate and extent of conversion of isotopically labelled DINP and DEHP into their primary and secondary metabolites in blood and urine following administration to human volunteers. A published report of the study originally was expected this Spring, but, due to need for additional analytical work, is now expected in Fall of 2010. These data will be directly relevant to pharmacokinetics, biomarkers and effects on biochemical and physiological processes in humans.

- The Hamner Institute currently is conducting mechanistic studies of DINP administered to pregnant dams. Preliminary results should be available late spring of this year. These studies will provide information on the dosage of DINP to the liver and related effects, and thus will contribute to understanding of the mechanism of rodent carcinogenesis.

As is evident from the submission materials, there is an extraordinary wealth of information pertaining to DINP, much of it technically complex. We note that OEHHA's usual practice for release of hazard identification materials for public comment is such that the CIC has about two weeks to review those comments prior to its meeting for consideration of listing. Because of the complexity of the database for DINP, ExxonMobil urges that there should be a longer period of time between the close of public comments and the CIC meeting on DINP so that the CIC members have adequate time to review and understand the various perspectives provided by those comments.

In addition, ExxonMobil toxicologists with specific expertise in DINP would be pleased to discuss the DINP data with OEHHA, by email, telephone, or face-to-face meeting. Please feel free to contact any of the following scientists with any questions about the DINP data:

Ammie Bachman, Ph.D., ammie.n.bachman@exxonmobil.com, (908) 730-2082
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In addition to the attached text discussion of the DINP database, ExxonMobil is separately submitting copies of key studies and reviews cited in the discussion. If OEHHA wishes copies of any cited materials not included in that submission or has any other questions or requests for information, please contact Angela Rollins at angela.rollins@exxonmobil.com or 281-870-6439.

We appreciate the opportunity to submit this information.

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Attachments

The following attachments are provided as separate pdfs.

A Statement of Ruth Angela Roberts, Ph.D.

B Statement of James Klaunig, Ph.D.

C Statement of Gary Williams, M.D. and Michael Iatropoulos, M.D., Ph.D.

D Statement of Richard Irons, Ph.D.

E Statement of James Swenberg, Ph.D.

DIISONONYL PHTHALATE (DINP)
Carcinogenicity Hazard Assessment

INTRODUCTION AND OVERVIEW

ExxonMobil Chemical Company is submitting this information in response to the California Office of Environmental Health Hazard Assessment (OEHHA) request for relevant information on diisononyl phthalate (DINP) to be considered by the OEHHA Science Advisory Board's Carcinogen Identification Committee (CIC).[1] OEHHA states that it will review and consider this information as it prepares hazard identification materials on DINP.

DINP met OEHHA's screening criteria for consideration of Proposition 65 listing because of the observation of tumors in rats and mice treated with high doses of DINP. However, in contrast to most chemicals, there is a very robust data base for DINP demonstrating that those tumors in rodents are not relevant to human cancer hazard assessment and that DINP is unlikely to cause cancer in humans. These conclusions are grounded in three basic aspects of the data:

1) DINP is not genotoxic, indicating that it does not interact directly with DNA;

2) The mechanisms leading to tumorigenesis in the rodents are irrelevant to humans; and

3) Primates treated with very high doses of DINP exhibit no effects indicative of the adverse effects leading to tumorigenesis in rodents, on the gross, cellular or biochemical level.

It is very important to keep the last point in mind. Primates are much more closely related to humans than are rodents. Because the tumors are observed in rodents, the bulk of this submission consists of detailed technical discussion of effects observed in studies of rodents or rodent tissue. In evaluating the mechanisms by which DINP causes cancer in rodents, OEHHA and the CIC should not lose sight of the primate data, which provide strong support for the mechanistic work demonstrating that the effects in rodents are not relevant to humans.

Prior to addressing the toxicological database, Section I of this submission discusses the identity of the chemical that is being evaluated. Commercial DINP is a complex substance that consists of more than simply phthalate molecules with nine-carbon arms. The cancer bioassays have been conducted using commercial DINP, and therefore OEHHA should associate any designation of DINP with its commercial CAS registry numbers – 68515-48-0 and 28553-12-0.

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[1] Announcement of Chemicals Selected by OEHHA for Consideration for Listing by the Carcinogen Identification Committee and Request for Relevant Information on the Carcinogenic Hazards of These Chemicals [10/15/09], http://www.oehha.org/prop65/CRNR_notices/state_listing/data_callin/sqe101509.html; Request for Relevant Information on Diisononyl Phthalate (DINP) to be Considered by the OEHHA Science Advisory Board's Carcinogen Identification Committee - Extension Of Public Comment Period [12/04/09], http://www.oehha.org/prop65/CRNR_notices/state_listing/data_callin/extDINP.html

Section II discusses the human and primate data relevant to assessing the carcinogenic potential of DINP. Primate studies and *in vitro* human and primate tests show no evidence of potential carcinogenicity, even under conditions that unquestionably would in rodents provoke responses that are part of the progression to cancer in those rodent species. Treatment for up to 90 days with doses as much as seven-fold greater than those that cause tumors in rodents showed no evidence of effects in the primates that are of the type associated with tumorigenesis in rodents.

Section III summarizes the *in vivo* and *in vitro* mutagenicity and genotoxicity tests on DINP. These uniformly demonstrate that DINP is not a genotoxic substance.

Section IV examines each type of cancer lesion seen in rodents – liver tumors, mononuclear cell leukemia (MNCL) and kidney tumors – and explains why they are not relevant for human hazard assessment.

Section IV.A addresses the liver tumors and shows that they are due to the peroxisome proliferation, or PPARα-agonism, mode of action that operates in rats and mice but not in humans. The section provides general background on peroxisome proliferation and then demonstrates that DINP meets both the International Life Sciences Institute (ILSI) and International Agency for Research on Cancer (IARC) criteria as being a peroxisome proliferator, such that the liver tumors observed in rodents are not relevant to humans. This is followed by a discussion of proposed alternative modes of action for tumorigenesis, including that proposed by Ito et al. (2007) and the constitutive androstane receptor (CAR) hypothesis, and demonstrates that the PPARα mode of action is the dominant and necessary mode of action driving liver tumorigenesis in rodent bioassays of DINP. The section then discusses the data demonstrating that the PPARα mode of action does not operate in humans. It also explains that, even if the PPARα mode of action theoretically could operate in humans, differences between rodent and human absorption make it virtually impossible for humans to achieve an internal dose that could produce tumors. Finally, Section IV.A summarizes the conclusions of a number of expert reviews that the liver tumors observed in rodents treated with DINP are not relevant for human cancer hazard assessment.

Section IV.B addresses MNCL, a lesion seen almost exclusively in F344 rats, in which it occurs spontaneously, and which has no clear analogue in humans. For this reason, authoritative bodies including IARC and the National Institutes of Health (NIH) have concluded that MNCL in rodents is irrelevant to evaluation of human cancer hazard.

Section IV.C shows that DINP toxicology meets the criteria of both the U.S. Environmental Protection Agency (USEPA) and IARC as producing kidney tumors by the $alpha_{2u}$-globulin mechanism. These agencies have determined that when those criteria are met, kidney tumors observed in rodents are not relevant for assessment of human cancer hazard. Therefore, as has been concluded by the reviews of several expert bodies, the kidney tumors observed in rodents exposed to DINP are not relevant to human cancer hazard assessment.

The conclusion from this large body of evidence is that DINP is very unlikely to cause cancer in humans and therefore should not be listed under Proposition 65 as known to the State of California to cause cancer.

We note that OEHHA's usual practice for release of hazard identification materials for public comment is such that the CIC has about two weeks to review those comments prior to its meeting for consideration of listing. Because of the complexity of the database for DINP, ExxonMobil urges that there should be a longer period of time between the close of public comments and the CIC meeting on DINP, so that the CIC members have adequate time to review and understand the various perspectives provided by those comments.

In addition, ExxonMobil toxicologists with specific expertise in DINP would be pleased to discuss the DINP data with OEHHA, by email, telephone, or face-to-face meeting, as it prepares the hazard identification materials. Please feel free to contact any of the following individuals with any questions:

Ammie Bachman, Ph.D., ammie.n.bachman@exxonmobil.com, (908) 730-2082
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I. DINP IDENTITY

Before addressing the toxicological data, it is important to discuss the identity of the chemical substance for which those data have been generated. In its data call-in notice for DINP and four other chemicals/chemical groups, OEHHA provided no CAS number for DINP. OEHHA and CIC consideration of DINP should be specific with reference to CAS Registry numbers (CASRNs) 68515-48-0 and 28553-12-0, which designate DINP as commercially produced and distributed.

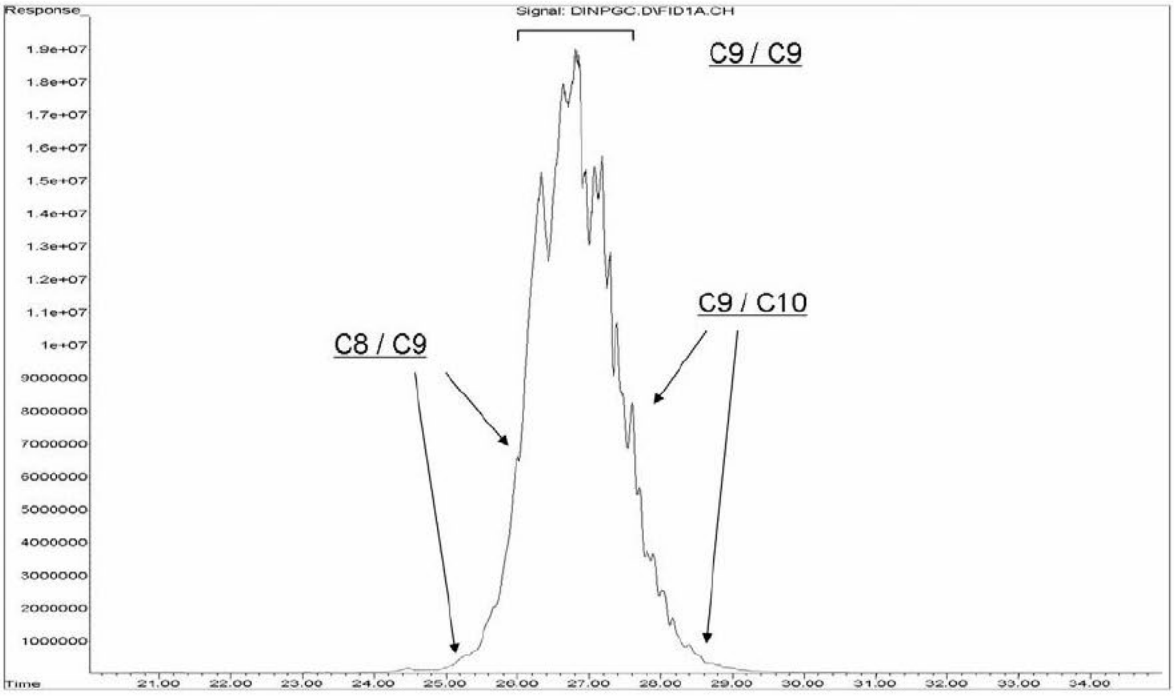
Unlike most lower molecular weight phthalates, DINP is not composed of a single molecule. Rather, it is produced by reaction of a phthalate moiety with alcohols. The majority of these alcohols have nine carbons (C9), but in various isomeric configurations. In addition, the alcohol fraction includes C8 and C10 alcohols. Since each phthalate has two hydrocarbon "arms", some molecules within commercial DINP have one arm that is C9, the other C8 or C10, and so forth. Thus, commercial DINP is not simply phthalate molecules with two C9 arms, but a complex substance consisting of C8-, C9- and C10-containing molecules. C9/C9 molecules predominant, but are not the only species.[2] See Figure 1.

[2] Although complex, the process to produce DINP is stable, and therefore the composition of the mixture is stable. The two commercial CASRNs describe mixtures that are commercially interchangeable (Babich et al., 2004; ECB, 2003a).

The primary cancer bioassays of DINP (Lington et al., 1997; Moore 1998a, b), have been bioassays of commercial DINP.[3] There are no bioassays of "pure" C9/C9 DINP. Thus, there is no basis to assign the rodent tumor results to C9/C9 versus any other types of molecule in the complex commercial substance. In fact, given the relatively high doses of DINP required to produce rodent tumors, it is plausible that the C9/C9 molecule is not the carcinogenic entity.

For these reasons, OEHHA should not list "DINP" with no associated CASRNs. Just as DIDP is listed on Proposition 65 under CASRNs 68515-49-1 and 26761-40-0, DINP should be considered as the entities CASRN 68515-48-0 and CASRN 28553-12-0.

Figure 1. Gas Chromatograph of DINP, CASRN 68515-48-0
Structural Components Confirmed by GC/MS Analyses



C8/C9 = the phthalate coester of C8 and C9 alcohols; 1,2-benzenedicarboxylic acid, isooctyl isononyl ester
C9/C9 = the phthalate diester of C9 alcohols; 1,2-benzenedicarboxylic acid, diisononyl ester
C9/C10 = the phthalate coester of C9 and C10 alcohols; 1,2 benzenedicarboxylic acid, isononyl isodecyl ester

[3] An additional bioassay was conducted using a form of DINP (CASRN 71549-78-5), that was never commercialized (Bio/Dynamics, 1986).

II. HUMAN AND PRIMATE DATA

As discussed in detail in Section IV, liver tumors, kidney tumors and mononuclear cell leukemia (MNCL) have been observed in rats and mice treated with high doses of DINP. For the reasons given below, all three of these lesions are not relevant for human hazard assessment. The greatest amount of concern has centered on the liver tumors, but the evidence clearly demonstrates that those tumors in rodents are due to peroxisome proliferation resulting from PPARα-agonism, which is not relevant to humans.

The bulk of the toxicological literature concerns studies designed to investigate the mechanism underlying rodent liver tumorigenesis. In reviewing this data, however, it is important to not lose sight of the unusually robust human and primate data for DINP. Those data provide a strong empirical basis for concluding that DINP is not likely to cause cancer in humans.

A. Epidemiology

There are no epidemiology studies on the carcinogenic potential of DINP. However, there are a number of clinical and population case-control studies of fibrate drugs. Fibrates are PPARα-agonists that are more potent than DINP (see Klaunig et al., 2003, Table 10). These studies are discussed below (Section IV.5.c) and show no evidence of a carcinogenic effect in humans from these PPARα agonists.

B. Human Cell Lines

Baker et al. (1996), Hasmall et al. (1999) and Kamendulis et al. (2002) have conducted studies of the effects of DINP in human cells in culture. These studies show a lack of the peroxisome proliferator response that is observed in rodents as a key event leading to development of liver tumors.

C. Primate Data

For DINP, there is an unusually large amount of data from *in vivo* studies in non-human primates as well as some *in vitro* data for humans and non-human primates. Primate studies and *in vitro* human and primate tests show no evidence of potential carcinogenicity, even under conditions that unquestionably would in rodents provoke responses that are part of the progression to cancer in those rodent species.

OEHHA and CIC should carefully consider this data, as primate data provides the best basis for determining whether chronic effects seen in rodents can reasonably be anticipated to occur in humans. Because monkeys are more closely related to humans than are rodents, primate studies provide a more relevant animal model for evaluating DINP than do rodent studies (e.g., Mazue and Richez, 1982). This is supported not only by the taxonomic, evolutionary and genetic evidence that places humans in the primate family, but also by toxicokinetic and mechanistic data.

1. *In Vivo* Primate Studies

Pugh et al. (2000) treated cynomolgus monkeys with DINP for 14 days at levels up to 500 milligrams per kilogram per day (mg/kg/day). Hall et al. (1999) treated marmosets with DINP up to the very high dose of 2500 mg/kg/day for 90 days; for a 70 kg human, this high dose would be about six ounces per day. In both of these primate studies, there was no evidence of those types of treatment-related effects which occur in rodents, even at the very high levels of treatment. More specifically, there were no treatment-related changes in weight or histopatholic changes in the liver, kidney and testes. There also were no treatment-related changes in serum chemistry measures, including lipids and cholesterol, or in measures of cellular function in the liver, including replicative DNA synthesis and peroxisomal enzymes.

The lack of adverse effects in the primate studies even at very high doses for up to 90 days is in contrast to the progression of pathology in rodents. For example, liver and kidney weights were increased in a 28-day study of rats (BIBRA, 1986). Liver weight increases were seen as early as 1 week after the beginning of treatment in the rat chronic bioassay (Moore, 1998a). Thus, the primate studies strongly indicate that primates are not adversely affected by DINP in the manner of rodents. In fact, the studies suggest that primates are refractory to any systemic toxicity from DINP. Thus, the primate studies – studies in species much more closely related to human than rodents – indicate that DINP is unlikely to be a human carcinogen.

2. *In Vitro* Primate Studies

Baker et al. (1996), Hasmall et al. (1999) and Kamendulis et al. (2002) found no evidence of peroxisome proliferation in human hepatocytes. Likewise, Benford et al. (1986) and Kamendulis et al. (2002) found no evidence of peroxisome proliferation in primate hepatocytes.

Thus, studies from several laboratories using hepatocytes from different individuals or different species of primates have demonstrated that a peroxisome proliferator response is not elicited by DINP in humans and other primates. These *in vitro* data further support a conclusion that it is unlikely that DINP is a human carcinogen.

III. GENOTOXICITY DATA

DINP has been evaluated in multiple *in vivo* and *in vitro* genotoxicity/mutagenicity assays and has been negative in all of them (Table 1). Even at very high doses of DINP, the tests have found neither DNA mutations nor chromosomal damage.

In vivo, a micronucleus test in mouse bone marrow found no evidence of chromosomal damage following administration of 2 g/kg/day (2000 mg/kg/day) of DINP for two consecutive days (McKee et al., 2000). In a rat bone marrow chromosome aberration test, DINP was negative at doses up to approximately 5 g/kg/day for five days, for a cumulative dose of up to 25 g/kg (Microbiological Associates, 1981).

In vitro, DINP has been tested in the *Salmonella* mutagenicity assay and found to be without activity in plate incorporation assays sponsored by the NIEHS (Zeiger et al., 1985) and in both plate incorporation and pre-incubation assays conducted by producing companies (McKee et al., 2000). DINP also tested negative in the mouse lymphoma test and the Balb/3T3 cell transformation assay (Barber et al., 2000), as well as the unscheduled DNA synthesis test in rat hepatocytes (Litton Bionetics, 1981). In an *in vitro* cytogenetics test in CHO cells, DINP was without activity even though the highest levels tested produced evidence of visible precipitation in the cell cultures (McKee et al., 2000).

These data strongly support a conclusion that DINP is not mutagenic or genotoxic.

Table 1.
Summary of Genetic Toxicology Information on DINP

Test System	Result	Reference
Salmonella (plate incorporation)	negative (+/- S9)	McKee et al., 2000
Salmonella (preincubation)	negative (+/- S9)	McKee et al., 2000; Zeiger et al., 1985
Mouse lymphoma	negative (+/- S9)	Barber et al., 2000
Cytogenetics (*in vitro*)	negative (+/- S9)	McKee et al., 2000
Unscheduled DNA synthesis (rat hepatocytes)	negative	Litton Bionetics, 1981
Mouse micronucleus test	negative	McKee et al., 2000
Cytogenetics (rat bone marrow)	negative	Microbiological Associates, 1981
Transformation assay (Balb/3T3)	negative	Barber et al., 2000

IV. RODENT BIOASSAYS

Three cancer bioassays have been conducted on commercial DINP, two in rats and one in mice.[4] Moore (1998a)[5] exposed F344 rats to dietary concentrations of 0, 500, 1500, 6000, or 12000 ppm (29, 88, 358, or 733 mg/kg/day for males and 36, 108, 442, or 885 mg/kg/day for females) DINP for two years. Similarly, Lington et al. (1997) administered dietary concentrations 0, 300, 3000, or 6000 ppm (mean daily intakes of 15, 152, and 307 mg/kg/day) of DINP for two years. Moore (1998b) administered 0, 500, 1500, 4000 or 8000 ppm (90, 275, 741, or 1560 mg/kg/day in males and 112, 335, 910, or 1,887 mg/kg/day in females) of DINP to B6C3F1/Crl BR mice for two years.

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[4] A dietary bioassay in Sprague-Dawley CD rats was conducted on a form of DINP that was never commercialized (Bio/Dynamics, 1986).

[5] In various reviews of DINP, the Moore studies alternatively are referred to as the Aristech studies (Aristech Chemical Company sponsored the studies) and as the Covance studies (Covance Laboratories conducted the studies).

In rodents, DINP at high doses produces liver tumors in rats and mice, MNCL in F344 rats but not in mice and kidney tumors only in male rats. However, there is a substantial body of research that provides compelling evidence that these tumors in rodents are not relevant for human health assessment. The overwhelming weight of the evidence is that DINP cannot reasonably be anticipated to cause cancer in humans. As discussed below, numerous independent scientists agree with this assessment, based on application of generally accepted scientific principles. The following sections consider each tumor type, in turn. In addition, because OEHHA included a reference to testicular dysgenesis syndrome (TDS) in its DINP summary, that hypothetical syndrome is also addressed.

A. Liver Tumors Observed In Rodents

Liver tumors have occurred in rats and mice exposed to high doses of DINP – 733-885 mg/kg/day in rats (Moore, 1998a) and 335-742 mg/kg/day in mice (Moore, 1998b).[6] DINP is in a class of chemicals known as "peroxisome proliferators" – chemicals that induce an increase in the size and number of a subcellular organelle known as a "peroxisome" in the liver cells of rodents. Many peroxisome proliferators are known to induce liver tumor formation in rodents. The peroxisome proliferation is mediated by the peroxisome proliferator-activated receptor α, or PPARα, and therefore the more current designation for chemicals causing peroxisome proliferation is PPARα-agonist.

Because many PPARα-agonists are important pharmaceutical agents (the fibrate class of hypolipidemic drugs), the toxicology of these chemicals has been extensively studied; a substantial amount of such work also has been performed with DINP and another phthalate compound, di(2-ethylhexyl) phthalate (DEHP). This has resulted in an extensive body of work that demonstrates that rodent liver tumors associated with peroxisome proliferation are not relevant for assessing potential human carcinogenicity. In fact, based on this evidence, the International Agency for Research on Cancer (IARC) and the International Life Sciences Institute (ILSI) have developed criteria for determining when tumors in rats and mice can be judged as not relevant to humans because they are due to peroxisome proliferation (IARC, 1995; Cattley et al., 1998; Klaunig et al., 2003).

The following first provides general background on peroxisome proliferation (subsection 1). It then demonstrates that DINP meets both the ILSI and IARC criteria as being a peroxisome proliferator, such that the liver tumors observed in rodents are not relevant to humans (subsections 2 and 3). This is followed by a discussion of proposed alternative pathways to tumorigenesis, independent of PPARα. This includes discussion of Ito et al. (2007), the CAR hypothesis and Yang et al. (2007) (subsection 4).

The unusually strong data base for DINP with respect to human cell lines and primate studies demonstrates that the PPARα mode of action does not operate in humans (subsection 5). Further, even if the PPARα mode of action theoretically could operate in humans, differences

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[6] No treatment-related preneoplastic or neoplastic liver lesions were observed in Lington et al. (1997).

between rodent and human absorption make it virtually impossible for humans to achieve an internal dose that could produce tumors (subsection 6). For these reasons, a number of expert reviews have concluded that the liver tumors observed in rodents treated with DINP are not relevant for human hazard assessment (subsection 7).

1. Background on Peroxisome Proliferation

It has been known for some years that certain substances – including some phthalate esters – produce a specific set of changes characterized as "peroxisomal proliferation" in livers of rats and mice following treatment at high levels. It also has been known for some years that chronic dietary administration of DEHP can produce liver tumors in rats and mice (Kluwe, 1982). A link between peroxisome proliferation and hepatocarcinogenesis in rats and mice, which was proposed 30 years ago (Reddy and Arzanoff, 1980), has engendered considerable research because humans do not appear susceptible to peroxisomal proliferation. For example, clinical studies of humans exposed for long periods to hypolipidemic drugs that are strong rodent peroxisome proliferators and are rodent hepatocarcinogens (reviewed in Ashby et al., 1994; Bentley et al., 1993) have shown no indication of any increase in cancer associated with those substances. As a result of this research, there is now a large body of evidence that demonstrates that the mode of action by which nongenotoxic peroxisome proliferators such as DINP lead to liver cancer in rodents is not relevant for humans (Ashby et al., 1994; Kluwe, 1994: Bentley et al., 1993; Lake, 1995a, b; Huber et al., 1996; Williams and Perrone, 1997; Cattley, et al., 1998; Klaunig et al., 2003). Rats and mice are uniquely susceptible to the morphological, biochemical and carcinogenic effects of peroxisome proliferators, while non-human primates and humans are completely non-responsive or refractory (e.g., Bentley et al., 1993. Elcombe et al., 1996; Hall et al., 1999; Huber et al. 1996; Kurata et al., 1998; Pugh et al., 2000).

The research was substantially advanced by the work of Issemann and Green (1990) who showed that peroxisome proliferator activity is mediated through a specific receptor (the peroxisome proliferator-activated receptor α, or PPARα) and by the demonstration that a mouse strain which lacks this receptor (PPARα-null mice) does not express peroxisomal proliferation or develop liver tumors following treatment for 11 months with a strong peroxisome proliferating agent (Peters et al., 1997).[7] These studies demonstrated an absolute requirement for activation of the PPARα receptor and expression of peroxisome proliferation in the development of rodent liver cancer.

There have been three particularly important reviews by independent scientific bodies of the evidence on peroxisome proliferation and its relationship to carcinogenic induction (IARC, 1995; Cattley et al., 1998; Klaunig et al., 2003). All three groups concluded that peroxisome proliferation-mediated rodent liver cancer has no practical significance to human health.

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[7] Peters, et al. (1997) compared the response of PPARα-deficient and normal PPARα mice following long-term administration of a potent peroxisome proliferating agent. The PPARα mice developed a 100% incidence of liver tumors following test material administration whereas the PPARα-deficient animals failed to develop tumors and did not exhibit liver cell proliferation of any type or peroxisome proliferation.

The first review was a 1994 working group of IARC which considered the relevance of peroxisome proliferation to humans as a generic mechanism (IARC, 1995). The IARC working group concluded that, when liver tumors in rats and mice were secondary to peroxisomal proliferation, this information could be used to modify the overall evaluation of the carcinogenicity data. One particular contribution by this group was to delineate the categories of evidence that could be used to establish whether rodent liver tumors are the consequence of a peroxisomal proliferation process.

The second review was by an international consensus workshop organized by the ILSI Health and Environmental Sciences Institute in December 1995, to consider specifically whether peroxisome proliferating compounds pose a liver cancer hazard to humans (Cattley et al., 1998). The symposium included approximately 100 scientists from government agencies, academia and industry, including leading researchers in the field from the United States and Europe. The final report of the workshop states, "The conclusion was reached that it is unlikely that peroxisome proliferators are carcinogenic to humans under anticipated conditions and levels of exposure, although their carcinogenic potential cannot be ruled out under extreme conditions of exposure." (Cattley et al., 1998, p. 57). One particular contribution of the ILSI working group was to delineate the criteria that could be used to define a substance as a peroxisome proliferator.

In 2001, the ILSI Risk Science Institute (ILSI RSI) formed a workgroup to review the information that had become available since 1995 on the relationship of peroxisome proliferation and liver tumors in rodents. The results of a series of meetings of that workgroup are presented in a paper titled "PPARα Agonist-Induced Rodent Tumors: Modes of Action and Human Relevance" (Klaunig et al., 2003). DINP is one of the examples of a peroxisome proliferator discussed in the document. The workgroup concluded:

> In summary, the weight of evidence overall currently suggests that the rodent [mode of action] for liver tumors is not likely to occur in humans, taking kinetic and dynamic factors into account. This conclusion is based upon evaluation of the existing body of evidence and would apply to the consequences of exposure to *known* examples of PPARα agonists.

(Klaunig et al., 2003, p. 693.) DINP is a known example of a PPARα agonist that was part of the basis for the workshop conclusions. Therefore, the conclusion of the ILSI RSI workgroup is that the liver tumors that occur in rodents treated with DINP are not likely to occur in humans.

Thus, there is consensus in the scientific community that peroxisome proliferators present, at most, a theoretical risk that could be expressed only under the most extreme conditions of exposure. The critical questions to evaluate the DINP data then become: (1) Is DINP a peroxisome proliferator; i.e., have the ILSI criteria been met? (2) Are the rodent liver tumors the consequence of a peroxisomal proliferation process, i.e., have the IARC criteria been met? (3) Is there any possibility of cancer, even under extreme circumstances? and, (4) If a theoretical possibility exists for human cancer, can the extreme exposure levels necessary be achieved? As shown below, the answers to these questions demonstrate that DINP cannot reasonably be anticipated to cause cancer in humans.

2. DINP Is a Peroxisome Proliferator Under the ILSI Criteria

As stated above, the 1995 ILSI workshop developed criteria for determining whether rodent liver tumors are the consequence of a peroxisomal proliferation process. Table 4 of Cattley et al. (1998) (reproduced here as Table 2) sets forth the minimum database to support characterization of a *non-genotoxic hepatocarcinogenic substance* as a peroxisome proliferator. DINP is a non-genotoxic substance as shown in Section II, above. DINP is a hepatocarcinogenic substance, as demonstrated by the observation of increased liver tumor incidence in rats and mice fed high doses of DINP (336 mg/kg/day in female mice; 700 to 900 mg/kg/day in male mice and in rats) (Moore 1998a; b). DINP also meets the criteria in Table 1, as shown in the text below.

Table 2.
Minimum database to support characterization of a nongenotoxic hepatocarcinogenic substance as a peroxisome proliferator (from Table 4, Cattley et al., 1998)

Key Element	Criteria	Measure
Gross hepatic morphology	Hepatomegaly	Increase in relative liver weight
Peroxisomes	Peroxisome proliferation	Increase in hepatocyte peroxisomes (V/V) by morphometry
Cell proliferation	Enhanced replicative DNA synthesis	Increase in hepatocellular BrdU nuclear labeling by light microscopy

(1) Hepatomegaly: DINP treatment causes significant increases in liver weight in rats and mice as documented in BIBRA (1986), Barber et al. (1987), Lington et al. (1997), Moore (1998 a; b), Valles et al. (2003) and Smith et al. (2000).

(2) Peroxisome Proliferation: That DINP produces peroxisomal proliferation in rats was first documented by Barber et al. (1987) and in the original study report (BIBRA, 1986). These reports also documented an increase in peroxisomal enzymes, also shown in Moore (1998a; b), Valles et al. (2003) and Smith et al. (2000). A study in mice demonstrated the dose-response relationship of DINP treatment to peroxisome proliferation, utilizing light microscopy, morphometric evaluation and peroxisomal enzyme induction (Kaufmann et al., 2002).

(3) Cell Proliferation: The induction of cell proliferation by DINP treatment in rat and mouse liver was first documented by Moore (1998a; b) and subsequently confirmed by Smith et al. (2000) and Valles et al. (2003). The enhanced cell proliferation was observed in the same hepatic compartment (perivenous, zone 3), where peroxisome proliferation starts initially, clearly indicating that the cell proliferation was the consequence of peroxisomal proliferation (Valles et al., 2003; Kaufmann et al., 2002).

Thus there are data from studies of DINP which satisfy the ILSI consensus criteria for peroxisomal proliferation. DINP produces liver tumors in rats and mice by a non-genotoxic

process. All of the hallmark criteria for peroxisomal proliferation, i.e., liver enlargement, peroxisome proliferation and cell proliferation, have been shown to occur in both rats and mice by at least three independent laboratories.

We note that, while DINP does meet the criteria from the 1995 ILSI workshop (Cattley et al., 1998), the subsequent ILSI RSI workgroup update found that "the demonstration of PPARα agonism was sufficient to abrogate the necessity for some of the more rigorous (and technically demanding) requirements determined by the previous working group" (Klaunig et al., 2003, p. 687). DINP is one example of a PPARα agonist used by the ILSI RSI workgroup to develop its conclusions (e.g., Klaunig et al., 2003, p. 667).

3. The DINP Liver Tumors Meet the IARC Criteria for Irrelevance to Humans

As stated above, IARC has reviewed the data on peroxisome proliferation and concluded that, when a tumor response in rats and mice is judged to be a consequence of peroxisome proliferation, the substance may be considered as not presenting a carcinogenic risk to man (IARC, 1995). IARC has in fact applied these criteria to determine that liver tumors in rodents treated with a phthalate are not relevant to humans. In February 2000, an IARC working group met to consider carcinogenicity data and other evidence of peroxisome proliferation for DEHP. Based on mechanistic data and other information, IARC concluded that the mechanism by which DEHP increases the incidence of hepatocellular tumors in rats and mice is not relevant to humans (IARC, 2000). Although DINP has not yet been evaluated by IARC, the available data are very similar to those for DEHP, so similar conclusions are anticipated.

The criteria established by IARC to make the determination that the tumors are not relevant to humans are (IARC, 1995 at 12-13):

> (a) Information is available to exclude mechanisms of carcinogenesis other than those related to peroxisome proliferation.
>
> (b) Peroxisome proliferation (increases in peroxisome volume density or fatty acid β-oxidation activity) and hepatocellular proliferation have been demonstrated under the conditions of the bioassay.
>
> (c) Such effects have not been found in adequately designed and conducted investigations of human groups and systems.

The data for DINP meet all of these criteria. With respect to the first criterion, alternative mechanisms of carcinogenicity, IARC relies substantially on the same types of information considered by ILSI, i.e., is there evidence that peroxisomal proliferation does occur in the species which develop cancer and can a role for a genotoxic process be ruled out.[8] (A genotoxic

[8] See, e.g., the IARC monograph discussion for DEHP (IARC, 2000, pp.116-121).

chemical is one that damages cellular DNA and may thereby trigger cancerous growth of the cell.) As described above, DINP does produce tumors in livers of rats and mice (Moore, 1998a; b) and there is clear evidence of peroxisomal proliferation in the livers of both species (Moore, 1998a; b; Smith et al., 2000; Valles et al., 2003; Kaufmann et al., 2002). That DINP is not genotoxic is shown in Section II, above. In addition, there is no evidence of any pathologic changes in the livers of these species unrelated to peroxisome proliferation which could provide an alternative explanation for tumor formation (Lington et al., 1997; Moore 1998a; b). Further, the electron microscopic evaluation in mice revealed, exclusively, findings related to peroxisome proliferation; no other degenerative findings on the subcellular level were observed in either sex (Kaufmann et al., 2002).

Ito et al. (2007) have proposed an alternative pathway for induction of liver tumors by another phthalate (DEHP) that is independent of PPARα activation. As discussed in Section IV.A.4.b, below, the limitations of the investigation using the mouse model employed by Ito et al. preclude this study as being sufficient to indicate there is a valid alternative mode of action of carcinogenesis other than that related to peroxisomal proliferation. Activation of CAR as a primary and independent pathway leading to tumors in mice has also been proposed for DEHP, see Section IV.A.4.b. However there is insufficient evidence to support CAR activation as a valid alternative mode of action underlying DEHP-induced liver tumorigenesis. Thus, the first IARC criterion is met.

The second criterion requires that peroxisome proliferation and hepatocellular proliferation be demonstrated under the conditions of the bioassay. As indicated above, increases in peroxisomal volume density, fatty acid β-oxidation and hepatocellular proliferation in livers of rats and mice treated with DINP have been documented (Barber et al., 1987; Moore, 1998a; b; Smith et al., 2000; Valles et al., 2003; Kaufmann et al., 2002). In the rat study (Moore, 1998a), the tumors appeared only at the highest dose (1.2% in the diet or approximately 733 mg/kg/day in male rats and 885 mg/kg/day in females). As also documented in the laboratory report describing that study (Moore, 1998a), DINP also caused significant increases in liver weight, peroxisomal enzyme induction and enhanced cell replication at that level. An independent study (Smith et al., 2000) confirmed these observations at the same levels in the same strain of rats. Thus the requirement that peroxisomal proliferation be demonstrated under the conditions of the bioassay has clearly been met in rats.

In the Moore mouse study, liver tumors were significantly increased in male mice given 4000 or 8000 ppm (approximately 740 and 1560 mg/kg/day) and in female mice given 1500, 4000 or 8000 ppm (approximately 336, 910 and 1888 mg/kg/day) in the diet for two years (Moore, 1998b). As defined by the study protocol, liver weights, peroxisomal enzyme induction and cell replication were examined in only the high dose group (8000 ppm) and the control, and all of these parameters were significantly elevated in the high dose group from that study (Moore, 1998b). An independent study also measured liver weight increase, peroxisomal enzyme induction and enhanced cell replication in the same strain of mice treated at 6000 ppm (Smith et al., 2000), and again all of these parameters were significantly elevated with respect to control. To evaluate peroxisome proliferation at the 1500 ppm and 4000 ppm levels, another study was conducted to determine the dose-response relationships for peroxisomal volume

density and peroxisomal enzyme induction in mice treated with DINP. The data indicated that both peroxisome volume density and peroxisomal induction were significantly elevated at the tumorigenic doses (Kaufmann et al., 2002). These new data provide direct evidence of peroxisomal proliferation under the conditions of the bioassay in the mouse as well as the rat. Taken together, these data demonstrate that, at every tumorigenic dose level in both rats and mice, there is a significant increase in peroxisome proliferation. Thus peroxisomal proliferation has been demonstrated under the conditions of the bioassay for DINP, meeting the second IARC criterion.

The third criterion requires evidence that peroxisome proliferation effects do not occur in "adequately designed and conducted investigations of human groups or systems." For this, IARC normally relies on data from studies in primates and/or human hepatocytes in culture. There have been two studies in non-human primates; in one of these DINP had no effects on the liver and showed no other evidence of peroxisome proliferation in marmosets following 90 days of treatment at levels up to 2500 mg/kg/day (Hall et al., 1999). In the other, DINP had no effects on the liver and showed no other evidence of peroxisome proliferation in cynomolgus monkeys following 14 days of treatment at levels up to 500 mg/kg/day (Pugh et al., 2000). Similarly, there was no evidence of peroxisome proliferation in either human hepatocytes (Baker et al., 1996; Hasmall et al., 1999; Kamendulis et al., 2002) or other primate hepatocytes tested under *in vitro* conditions (Benford et al., 1986; Kamendulis et al., 2002). Thus studies from several laboratories using hepatocytes from different individuals or different species of primates have demonstrated that a peroxisome proliferator response is not elicited by DINP in humans and other primates.

In summary, DINP meets all three IARC criteria for identifying a peroxisome proliferator for which liver tumors in rodents are not relevant to humans.

In 2000, IARC reviewed the evidence for DEHP in light of its criteria and determined that the classification of DEHP should be changed from Group 2B (probable human carcinogen) to Group 3 (not classifiable as to carcinogenicity). IARC summarized its determination for DEHP as follows:

> In making its overall evaluation of the possible carcinogenicity to humans of di(2-ethylhexyl) phthalate, the working group took into consideration that (a) di(2-ethylhexyl) phthalate produces liver tumours in rats and mice by a non-DNA-reactive mechanism involving peroxisome proliferation; (b) peroxisome proliferation and hepatocellular proliferation have been demonstrated under the conditions of the carcinogenicity studies of di(2-ethylhexyl) phthalate in mice and rats; and (c) peroxisome proliferation has not been documented in human hepatocyte cultures exposed to di(2-ethylhexyl) phthalate nor in the livers of exposed non-human primates. Therefore, the mechanism by which di(2-ethylhexyl) phthalate increases the incidence of hepatocellular tumours in rats and mice is not relevant to humans.

(IARC, 2000, p. 124.)

As shown above, the data for DINP completely parallel those for DEHP.

- DINP is not genotoxic (Barber et al., 2000; McKee et al., 2000; Zeiger et al., 1985). It produces peroxisome proliferation in rodent liver (Barber et al., 1987; Bird et al., 1986; Bio/Dynamics, Incorporated, 1982; Moore, 1998a;b; Smith et al., 2000; Kaufmann et al., 2002), but does not produce such effects in PPARα-deficient mice (Valles et al., 2003).

- Peroxisomal proliferation and hepatocellular proliferation have been demonstrated under the conditions of the carcinogenic studies of DINP (Moore, 1998a; b; Smith, et al., 2000; Kaufmann et al., 2002; Valles et al., 2003).

- Peroxisome proliferation has not been observed in cultured human hepatocytes treated with DINP or in hepatocytes from subhuman primates treated with DINP under both *in vivo* and *in vitro* conditions (Baker et al., 1996; Benford, et al., 1986; Hasmall, et al., 1999; Hall et al., 1999, Pugh et al., 2000; Kamendulis et al., 2002).

Therefore, for the same reasons IARC found that the liver tumors in rodents exposed to DEHP are not relevant to humans, the liver tumors observed in rats and mice exposed to high doses of DINP are not relevant for human hazard assessment.

4. Alternative Modes of Action for the Liver Tumors

Not only is there evidence that DINP induces peroxisomal proliferation in rats and mice, there is also direct evidence that induction of the peroxisomal functions is related to activation of the PPARα receptor. Clearly peroxisomal proliferation is the most plausible mode of action underlying the liver tumor response in rats and mice (Klaunig et al., 2003). Some have speculated on potential alternative pathways and targets whereby PPARα agonists could act via an independent and alternative mode of action leading to tumorigenesis. However, these data are insufficient to support other modes of action as sole drivers for the formation of liver tumors in rodents treated with DINP.

a. *DINP Data*

Since DINP is not genotoxic, the liver tumors could not have been initiated by a direct interaction with DNA. Therefore, the tumors must have been due to a secondary process related to cellular injury in the organ. There is no histologic evidence in the rodent studies for any liver changes other than those associated with peroxisomal proliferation. This was also confirmed by electron microscopy in mice, which revealed no other degenerative changes on the subcellular level (Kaufmann et al., 2002). In particular, there was no evidence of any other compensatory cell proliferation resulting from a toxic process other than enhanced replicative DNA synthesis, a PPARα-mediated process. DNA synthesis was statistically enhanced in the same hepatic compartment (perivenous, zone 3) where peroxisome proliferation was predominantly exhibited. There was evidence of inhibition of gap junctional intercellular communication (GJIC) (Smith et

al., 2000), but, as noted by IARC (1995), this is not inconsistent with a peroxisomal proliferation-mediated process. In fact, the ILSI RSI workgroup identified GJIC as a key event associated with the PPARα mode of action (Klaunig et al., 2003, p. 671). GJIC inhibition could act in concert with either enhanced cell replication or inhibition of apoptosis – which are the consequence of activation of the PPARα receptor – facilitating the expression of tumors in rodents following peroxisomal proliferator treatment (McKee, 2000).

b. *DEHP Data*

There are four non-exclusive hypotheses to explain the carcinogenic effects of peroxisome proliferators; (i) that oxidative stress related to induction of peroxisomal enzymes leads to malignant transformation, (ii) that enhanced replicative synthesis facilitates the expression of these (or spontaneously) transformed cells, (iii) that inhibition of apoptosis prevents transformed cells from being removed by normal homeostatic mechanisms and/or (iv) these in combination (Peters et al., 2000). The sufficiency of these processes to explain the carcinogenic response is consistent with current theoretical models.

The empirical evidence comes from a study in which a mouse strain lacking PPARα (i.e., PPARα-null mouse) did not have elevated levels of peroxisomal enzymes or enhanced cell replication and did not develop liver tumors following treatment with a potent peroxisome proliferating agent Wy-14,643 (Peters et al., 1997). Similarly, PPARα-null mice treated with high levels of DEHP (12,000 ppm) for six months developed *no* liver lesions, in comparison to significant liver lesions in wild-type mice (Ward et al., 1998).

On the basis of the strong body of evidence demonstrating that DEHP causes cancer in rodents via the PPARα mode of action and that that mode of action is unlikely to operate in humans, IARC and ILSI have determined that liver tumors in rodents treated with DEHP are not relevant for assessment of human cancer hazard from DEHP (IARC, 2000; Klaunig et al. (2003).[9] Despite these expert body determinations, some recent papers propose alternative mode of actions for induction of liver tumors by DEHP that are independent of PPARα activation. Because OEHHA's summary of DINP data includes references to these recent DEHP papers, we assume that OEHHA may be hypothesizing that the speculated alternative mode of actions might apply to DINP.

We stress that there is no evidence from DINP studies that would support a theory that DINP could operate via these alternative mode of actions. However, because DINP and DEHP both cause tumors in rodents by the same PPARα mode of action, we address these proposed alternatives below. We also address the work of Yang et al. (2007) regarding the sufficiency of the PPARα mode of action to explain the rodent tumors.

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[9] "The data lead to a conclusion that a carcinogenic response induced via the MOAs for liver tumorigenesis in the rodent is not likely to occur in humans following exposure to DEHP." Klaunig et al. (2003) at 704. "[T]he mechanism by which di(2-ethylhexyl) phthalate increases the incidence of hepatocellular tumours in rats and mice is not relevant to humans." IARC (2000) at 124.

Kupffer Cell Initiation

An early alternative proposal to the PPARα-mediated mode of action was that Kupffer cells initiated the proliferation response through production of tumor necrosis factor alpha (TNFα) by a process independent of PPARα (Rose et al., 1999). However, more recent data has shown that rodent liver hepatocytes respond to Kupffer cell-derived TNFα through mode of actions dependent on expression of PPARα in parenchymal cells (Peters et al., 2000), and the ILSI RSI workgroup identified Kupffer cell-mediated events as a key event associated with the PPARα mode of action (Klaunig et al., 2003, p. 671). Thus there is no plausible explanation for the rodent liver tumors except a PPARα-mediated process.

Oxidative Stress (Ito et al., 2007)

Ito et al. (2007) compared the effects of long-term dietary exposure of up to 0.05% DEHP on liver toxicity of wild type (+/+) and PPARα null (-/-) mice. They used a knockout PPARα -/- mouse strain, produced according to the method published by Lee et al. (1995), which is designed to cause both PPARα alleles in the mouse to be replaced by inactivated alleles, using the homologous recombination technique. Four biological endpoints were assessed after 24 months of treatment; the endpoints were referred to as: macroscopic liver findings, including tumors; microscopic liver findings; oxidative damage (8-hydroxy-2'-deoxyguanosine (8-OHdG) levels) and proto-oncogene expression levels (mRNA and/or protein). Ito et al. reported a statistically significant increase in the number of total liver tumors (i.e., hepatocellular carcinomas, hepatocellular adenomas and cholangiocarcinomas) from 2-8 (10-25.8%) between the wild type and knockout mice fed the top dose DEHP diet ($p<0.05$).

On the basis of their data, Ito et al. proposed an alternative mode of action for DEHP induced liver tumors independent of PPARα activation: DEHP-induced oxidative stress in mouse hepatocytes leading to inflammation and the activation of protooncogenes. However, several factors bring into question the utility of this paper for assessing DEHP (or by read-across, DINP) rodent carcinogenicity and the role (or lack of a role) of PPARα.

PPARα Null Mouse Model

Ito et al. reported the use of a PPARα null mouse strain produced according to a method published by Lee et al. (1995). The Lee et al. knockout mouse had both PPARα alleles replaced using the homologous recombination technique. It should be noted that in a knockout model, the possibility that other genes overlap with the PPARα function cannot be eliminated. Lee et al. were able to demonstrate that their PPARα mice no longer had detectable levels of the PPARα gene, mRNA or protein via the Southern, Northern and Western blotting techniques, respectively. Ito et al. (2007) provide no such data, other than a mention that PPAR mRNA was only measured in wild-type animals. Without supporting data, the authors cannot demonstrate that their animals were truly PPARα knockouts.

Survival Rates

Ito et al. reported percent survival for the control (i.e., 0% DEHP) wild type mice and the PPARα null mice through 23 months to be 96%. This survival rate is significantly higher than that earlier reported by Howroyd et al. (2004), where the percent survival for 22 wild-type and 12 null mice at 23 months were ~60% and 35%, respectively. As Ito et al. and Howroyd et al. both cite the same laboratory and background for their mice, the mice used by the two research groups apparently are from the same stock colony. The survival rates reported by Howroyd et al. are much more in line with typical survival rates of transgenic mouse strains. Therefore, the unusually high survival rates reported by Ito et al. raise serious questions about their data. Ito et al. did not address in their paper why the survival rate in their study was so different from that of the earlier Howroyd et al. study.

Liver Weight

Ito et al. reported no significant effect on bodyweight or liver weight in either the wild or the null mice. However, the data suggest a trend towards an increase in liver weight for the PPARα -/- (null) animals, especially the 0.05% DEHP exposed group (+/+ mean = 1.27g ±0.18; -/- mean = 1.78g±0.84). A trend of increased liver weight was not observed in the wild mice. In addition, the data indicate that peroxisome proliferation was not occurring in the wild-type mice at the doses tested. These results are the opposite of what would be expected for a PPARα agonist hepatocarcinogen (Klaunig et al., 2003). If the PPARα mode of action was induced in the wild type mice with 0.01 or 0.05% DEHP, then increases in liver weight and in size and number of hepatocyte peroxisomes would have been observed (e.g., David et al., 1999; Klaunig et al., 2003 Table 5), whereas no increase in liver weight would be anticipated for the null mice. Therefore, the DEHP treated wild-type mice were not an adequate control comparison to the DEHP treated null mice in this study.

8-hydroxy-2'-deoxyguanosine (8-OHdG) Levels

As indicated above, 8-OHdG is a marker of oxidative damage to DNA. Ito et al. reported that DEHP treatment dose-dependently increased 8-OHdG levels in the livers of both PPARα null mice and wild-type mice; however, the degree of increase was greater in the null mice. This could be a reflection of the fact that levels of 8-OHdG were significantly higher in the PPARα null control (i.e., 0% DEHP) mice than the wild-type control mice, which would indicate that the PPARα null mice suffered from an increased hepatic oxidative stress, as compared with wild-type mice, *with or without* DEHP treatment.

Although previous research has suggested that oxidative DNA damage is a PPARα dependent event (Rusyn et al., 2004), the Ito data suggest that mice fundamentally have increased oxidative damage even in the absence of PPARα. As oxidative stress increases and 8-OHdG accumulates, DNA repair is induced as a compensatory mechanism in the wild-type animal. Chronic treatment with peroxisome proliferators, including DEHP, has induced increased repair in both rat and mouse liver (Rusyn et al., 2000). Ito et al. demonstrated that the mRNA levels of an 8-OHdG repair enzyme, 8-oxoguanine DNA-glycosylase 1, were unchanged in both wild-type and null mice suggesting that repair was not induced in either the wild-type or

null mice. This could explain why DEHP-induced increased oxidative stress was observed with both genotypes.

Tumors

Ito et al. reported that a statistically significant increase in the number of liver tumors (i.e., hepatocellular carcinomas, hepatocellular adenomas and cholangiocarcinomas) from 2-8 (10-25.8%) was seen between the wild type and null mice fed the top dose DEHP diet ($p<0.05$). This was mostly due to a jump from 2 to 6 in hepatocellular adenoma (i.e., benign tumors) incidence between these two groups. Statistical significance was reached *only* when the total numbers of tumors were combined. Ito et al. discuss the low number of tumors and report them to be a reflection on the relatively low doses of DEHP used in the study. Again, these same doses of DEHP did not induce peroxisome proliferation or any indications of hepatomegaly in the wild-type animals.

The utility of these data is limited in that a number of reports have indicated that aged (e.g., 24 month) PPARα-null mice are more vulnerable to tumorigenesis than wild-type mice, due to fundamental mechanistic differences in the two types of mice (Mandard et al., 2004; Kostadinova et al., 2005; Balkwill and Couseens, 2005; Pikarsky et al., 2004; Takashima et al., 2008).

Howroyd et al. (2004) compared age-dependent lesions in the liver, kidney and heart in PPARα-null mice with those observed in wild-type SV129 mice, *in the absence of any chemical treatment*. (SV129 is also the strain used by Ito et al.) Various non-neoplastic spontaneous aging lesions occurred at higher incidence, shorter latency, or increased severity in PPARα-null mice compared with wild-type mice. In addition, a greater number of hepatocellular carcinomas and multiple hepatocellular adenomas were seen in PPARα-null mice compared with wild-type. Thus, as spontaneous tumors are known to occur in the PPARα-null mice at 24 months, the Ito et al. data indicate the possibility that DEHP merely promoted the formation of the spontaneous liver tumors in the aged null mice. As suggested by Howroyd et al. (2004), PPARα may, in fact, delay the development of some spontaneous lesions associated with aging in the liver of SV129 mice.

Takashima et al. (2008) examined gene expression profiles of hepatocellular adenoma tissues as well as control livers of wild-type and PPARα null mice. The genes identified and hypothesized to contribute to spontaneous tumorigenesis (i.e., Gadd45a and caspase 3-dependent apoptosis genes) in the null mice were unique to the null mice. These data indicate that the underlying biology between the wild-type and knock out mice differs. These fundamental differences complicate the interpretation and explanation of liver tumor formation in the null mice.

On the basis of the null-mouse data, Guyton et al. (2009) have suggested that a mode of action independent from PPARα may contribute to tumorigenesis. However, Guyton et al. attempted to compare chemically induced tumor incidences across strains of mice (e.g., SV129

vs. B6C3F1). Such comparison may be confounded by the strain-specific susceptibility to spontaneous tumorigenesis (Krupke et al., 2008).[10]

Importantly, with respect to DINP, literature searches reveal no reports that DINP induces production of reactive oxygen species in livers of rodents, humans or non-human primates, or in cultured liver cells from these species.

For all the above reasons, the Ito et al. (2007) data are not sufficient to indicate that, for DINP, there is a valid alternative mode of action resulting in liver tumors in rodents other than that related to peroxisomal proliferation.

Other Nuclear Receptors, including CAR

Another suggestion is that peroxisome proliferator agonists induce effects in the liver through nuclear receptors other than PPARα. Under this hypothesis, such activation of other receptors potentially represents a secondary mode of action contributing to liver tumorigenesis.

Gonzalez et al. (1998) concluded that all peroxisome proliferators are likely to cause tumors through activation of PPARα, and not via other nuclear receptors, including PPARβ or PPARγ. The activity of PPARα is not the same in humans as in rodents. There is only one function related to PPARα activation in rodents which is also expressed in humans – fatty acid metabolism – and that proceeds by different pathways in these species. As reviewed by Vameq and Latruffe (1999), PPARγ is involved in adipocyte differentiation, formation of foam cells and interference with tumor growth. Thus, activation of PPARγ seems more likely to be involved in tumor protection than tumor induction. Further, in contrast to PPARα, the activity of this receptor seems to be conserved across species. PPARβ may be involved in adipocyte differentiation but is not well understood.

More recently, activation of the constitutive activated/androstane receptor (CAR) and/or pregnane X receptor (PXR) have been suggested as alternative pathways. CAR and PXR regulate an overlapping set of xenobiotic metabolizing enzymes (XMEs), including members of the cytochrome P450 (Cyp) 2b and 3a families and genes associated with growth regulation in the rat and mouse liver (Nelson et al., 2006).

Of particular interest is CAR, an orphan nuclear receptor which regulates the expression of XMEs and transport proteins in response to exposure to xenobiotics. CAR received its name because of its high constitutive activity, and, when it was originally cloned, it was thought to be a permanent resident of the nuclear compartment; an observation made in a cell line (Baes et al., 1994). However, further work in primary hepatocytes indicates that, in its inactive state, CAR is localized to the cytosol and only translocates to the nucleus in response to an inducer (Kawamoto et al., 1999). While some xenobiotics are able to bind to CAR, facilitating activation, ligand binding to CAR is not a requirement. In fact, it is hypothesized that the majority of CAR

[10] Mouse Tumor Biology Database (MTB), Mouse Genome Informatics, The Jackson Laboratory, Bar Harbor, Maine, http://www.informatics.jax.org/.

activators work through indirect mechanisms (Baldwin and Roling, 2009). For example, phenobarbital (PB) activates CAR through an AMP kinase phosphorylation cascade (Rencurel et al, 2005; Shindo et al, 2007). Once in the nucleus, CAR hetero-dimerizes with retinoid X receptor alpha (RXRα), the most abundant of the three RXR receptors, prior to binding DNA and inducing gene expression.

Transactivation assays have shown that the phthalate DEHP and its primary metabolite monoethylhexyl phthalate (MEHP) can activate mouse CAR and PXR. In the presence of an inverse agonist to increase assay sensitivity, MEHP was demonstrated to activate mouse CAR approximately 2-fold above control in an *in vitro* luciferase reporter assay (Baldwin and Roling, 2009). MEHP was also shown to activate mouse PXR (Hurst and Waxman, 2004). In a transactivation assay designed to measure mRNA expression of the CAR target gene Cyp2b10, MEHP did not induce any change in gene expression, while DEHP up-regulated Cyp2b10 approximately 2-fold (Eveillard et al, 2009). Upon oral administration, DEHP is rapidly metabolized to MEHP (IARC, 2000, at 74-75); therefore, the mouse liver is likely exposed predominantly to MEHP. The significance of the *in vitro* DEHP-induced activation becomes questionable due to the influence of this rapid metabolism *in vivo*.

The PPARα independent biological events underlying the observed DEHP-induced mouse liver tumors in PPARα-null mice (Ito et al., 2007) may involve activation of CAR. The contribution of CAR-regulated gene expression changes in wild-type and PPARα null mice was recently investigated (Ren et al., 2010; Rosen et al., 2008). Wild-type and PPARα null mice were given a daily gavage dose of 200 mg/kg or 1150 mg/kg DEHP for 4 days. A dose of 200 mg/kg is comparable to the 0.05% dose of DEHP used by Ito et al. (2007). A comparison of DEHP-treated wild-type and PPARα-null mice revealed that PPARα is required for approximately 94% of all transcriptional changes in wild-type mice (Ren et al., 2010). The remaining 6% of transcriptional changes are dominated by genes involved in xenobiotic metabolism, which have the potential to be under the regulation of CAR. Transcription profiling of the 6% remaining genes in DEHP treated mice indicates that a number of xenobiotic metabolizing enzymes that are known CAR target genes are induced to a greater extent by DEHP in PPARα-null mice than in wild type mice (Ren et al., 2010). This research indicates that, only in the absence of PPARα (i.e., PPARα-null mice), chronic activation of CAR becomes the predominant mode of action contributing to the low level of liver tumors induced. Importantly, in wild-type mice, DEHP transcriptional responses are shown to be overwhelmingly dependent on PPARα. This is consistent with data showing that PPARα is expressed in higher levels in liver compared to CAR; therefore, PPARα gene-expression may be favored (Ren et al., 2010).

These data suggest that DEHP/MEHP activates CAR; however, the data do not elucidate whether this occurs via direct ligand binding or through some indirect pathway activation. Questions also remain as to the dominance of this pathway in a wild-type animal. These early studies indicate that CAR activation is a minor pathway affected by MEHP and this activation would, in essence, be "swamped out" by the activation of PPARα and its ensuing effects. The minor contribution of DEHP-induced CAR activation to liver tumorigenesis in the wild-type mouse is not sufficient to drive tumorigenesis independent of PPARα.

To date, there has only been one study which investigated the ability of DEHP and MEHP to activate human CAR (DeKeyser et al., 2009). In human livers, the CAR gene expresses a number of differentially spliced mRNA transcripts, (Savkur et al., 2003; Arnold et al., 2004; Jinno et al., 2004; Lamba et al., 2004). The CAR2 splice variant, which lacks constitutive activity, is expressed at approximately 30% of the reference transcript level in human hepatocytes (Xu et al., 2004; DeKeyser et al., 2009). The CAR2 transcript cannot be generated in marmoset, mouse and rat, indicating that CAR2 may be unique to humans (Kent et al., 2002; DeKeyser et al., 2009). DEHP has been shown to activate CAR2 *in vitro* in a transactivation study in which CAR2 was added to a kidney epithelial cell line derived from the African green monkey (i.e., COS-1). However, when MEHP was tested in the same assay, only weak activity was demonstrated even at a concentration of 10uM. From this, DeKeyser et al. (2009) concluded that DEHP, not MEHP, is a potent agonist of CAR2. However, this conclusion is inconsistent with the prevailing hypothesis that MEHP is the active metabolite in animals and humans due to the high rate of metabolism of the parent compound (see, e.g., ECB, 2008; Rhodes et al., 1996; Tomita et al., 1982). Thus, these data suggest that activation of CAR2 is not a plausible mode of action whereby DEHP could cause cancer in humans (or even mice).

Although CAR2 was not seen to be conserved across species (e.g., rat, mouse and marmoset), CAR1, the predominant nuclear hormone receptor in rodents and humans, is conserved. In a mammalian two-hybrid system set up to test human CAR1 affinity, DEHP was only a weak competitor of the inverse agonist androstanol at 10uM; the same result was obtained when mouse CAR was tested (DeKeyser et al., 2009). Unfortunately, MEHP was not tested for affinity to either human or mouse CAR1. These data suggest that if DEHP and/or MEHP does not readily bind the highly conserved CAR1, and CAR1 is the only active CAR in rodents, then DEHP/MEHP is likely an indirect activator of CAR in rodents. Further evidence is needed to understand whether CAR activation occurs simultaneously with activation of PPAR and contributes as a secondary pathway to upregulation of *cyp* genes or whether CAR activation is compensatory under conditions where there is a breakdown in the PPAR signaling cascade.

The same study also showed, in primary human hepatocytes, 50 uM DEHP up-regulated the two predominant genes regulated by CAR, CYP2B6 and CYP3A4 (DeKeyser et al, 2009). This up-regulation is likely a net effect of DEHP-induced activation of PPARα, PXR and possibly CAR. It is not direct evidence for CAR activation in human primary hepatocytes.

There are currently no *in vivo* or *in vitro* human data regarding DINP binding to or indirect activation of CAR-regulated genes.

In summary, the available data on phthalate-induced activation of CAR and the formation of rodent liver tumors indicates that (1) only in the absence of PPARα (i.e., PPARα-null mice); does chronic activation of CAR contribute significantly to the low level of liver tumors observed (2) DEHP and/or MEHP activates CAR in rodents, but this is a minor pathway and would, in essence, be “swamped out” by the activation of PPARα and its ensuing effects in wild-type animals; and (3) CAR is not conserved across species and therefore effects of CAR activation in rodents may not be relevant to humans.

Sufficiency of PPARα to Cause Liver Tumors (Yang et al., 2007)

Yang et al. (2007) have conducted research that does not provide an alternative mode of action for the rodent livers tumors, but from which they speculate that the PPARα mode of action is not sufficient to explain the tumorigenesis. However, the results of this study must be interpreted with caution. Questions about the study must be addressed before it can be considered a serious challenge to the prior conclusions of expert body reviews on rodent liver tumor formation from treatment with PPARα agonists.

Yang et al. created a transgenic mouse model, termed LAP-V16 PPARα, which displays a constitutively active PPARα restricted to hepatocytes.[11] Because the animals are not in a PPARα-null background, they also express endogenous PPARα in multiple tissues; including the liver.

For the most part, the LAP-V16 PPARα mice exhibited molecular and cellular responses similar to that of wild-type mice fed the potent PPARα agonist WY-14,643.[12] The major difference between the LAP-V16 PPARα transgenic animals and the wild-type mice was the absence of liver tumors in aged, 1 year, LAP-V16 PPARα mice. Unlike the wild type mice in the chronic feeding study with WY-14,643, which exhibited a hepatic tumor incidence rate of 100%, the LAP-V16 PPARα transgenic animals did not exhibit any grossly visible hepatic lesions. On the other hand, the induction of hepatocyte proliferation was similar between the LAP-V16 PPARα mice and the wild-type mice fed WY-14,643. Therefore, the results indicate that constitutive activation of PPARα in mouse hepatocytes induces hepatocyte proliferation, but is not sufficient to induce liver tumors.

As stated, the results of this study must be interpreted with caution. The authors of the study created the transgenic mice by introducing the LAP-V16 PPARα transgene in a wild-type (129/Sv) background. In LAP-V16 PPARα animals, the viral coactivator V16 functions by

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[11] The potent viral transcriptional activator VP16 was fused to the mouse PPARα cDNA construct to create a transcription factor that constitutively activates PPARα responsive genes in the absence of ligands (Yang et al., 2006). Transgenic mice were produced whereby inducible expression of the VP16 PPARα transgene was targeted to hepatocytes using the tetracycline regulatory system under the control of the liver enriched activator protein promoter (LAP).

[12] Hepatomegaly was observed in the LAP-V16 PPARα mice, but at a much lower extent when compared to wild-type mice treated with WY-14,643 for 2-weeks. Histological examination revealed that the wild-type mice treated with WY-14,643 had hepatocyte hypertrophy while the LAP-V16 PPARα did not. The LAP-V16 PPARα mice exhibited similar expression levels of a few PPARα target genes involved with peroxisomal, mitochondrial and microsomal fatty acid catabolism as compared to wild-type mice treated with WY-14,643. Furthermore, the induction of peroxisome proliferation as measured by the marker protein peroxisomal membrane protein 70, the reduction of serum lipids and the quantity of hepatocyte proliferation was equivalent between the LAP-V16 PPARα mice and the wild-type mice treated with WY-14,643. Non-parenchymal cell (NPC) proliferation was not observed in LAP-V16 PPARα mice in contrast to the dramatic proliferation of these cells in WY-14,643 treated wild-type mice. NPCs include Kupffer cells, hepatic stellate cells and sinusoid endothelial cells.

recruiting coactivator complexes, including histone acetyltransferases, to the vicinity of PPARα dependent genes (Herrera and Triezenberg, 2004). The over-expression of PPARα results in an increased likelihood that the protein, *based on sheer quantity*, will be near an inducible gene leading to changes in gene transcription. Thus, while this model is useful for deciphering molecular and cellular events, the results are not necessarily an accurate representation of the effects resultant from ligand activation of PPARα.

Perhaps the biggest drawback for the study is that no attempt to distinguish global changes in gene expression between ligand-exposed wild-type mice and the constitutively active transgenic animals is made. Without this key piece of information, it is not possible to have confidence in the transgenic model as a surrogate for ligand activation of PPARα. Furthermore, this information would shed light on the observed differences between the transgenic animals and the wild-types. Another issue not addressed in the study is what influence, if any, the endogenous (normal) PPARα had in the transgenic mice, since they were not generated in a PPARα -/- null background.

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Thus, while there have been some recent papers that suggest alternative mode of actions, significant questions about the studies detract from their plausibility. There is no mode of action other than a PPARα process that provides a plausible mode of action for the liver tumors observed in DINP-treated rodents. Even if the speculated alternative processes do occur, the weight of the evidence is that the PPARα process is predominant and necessary for rodent hepatic tumor formation.

5. The PPARα Mode of Action Does Not Operate in Humans

Having established that the mode of action by which DINP causes liver tumors is PPARα-mediated, one could ask whether there is a theoretical possibility that tumors could arise in humans as a consequence of a peroxisome proliferation-mediated response. The evidence indicates that the answer to this question is no. There are notable species differences with respect to peroxisome proliferation and the induction of liver tumors from PPARα agonists. Rodents such as rats and mice readily exhibit peroxisomal proliferation and tumor formation while guinea pigs, non-human primates and humans are significantly less responsive to peroxisomal proliferation.

a. *PPARα Expression and Activation in Rodents and Humans*

The demonstration that activation of PPARα was an absolute requirement in the induction of lesions leading to tumor formation (Ward et al., 1998) established a basis for species differences; levels of PPARα in humans are substantially lower than they are in rodents. Palmer et al. (1998) have shown that humans have less than one-tenth the level of PPARα expression observed in mice. These reduced levels appear to be the result of lower transcription rates, inefficient pre-messenger RNA splicing, or both (Palmer et al., 1998; Tugwood et al., 1996).

In addition to the reduced levels of PPARα in humans, there is strong evidence that there are additional factors which prevent the expression in humans of the PPARα-mediated functions which play a role in rodent cancer. Woodyatt et al. (1999) showed that, although human PPARα could bind peroxisome proliferating agents (PP-agents) and that this complex could drive transcription of the acetyl co-enzyme A (ACO) in mouse cells, it could not drive transcription of this gene in human cells. In fact, the activity of the PPARα/PP-agent complex may be a basis for species differences in metabolism of fatty acids: in rodents fatty acid metabolism involves activation of PPARα by a PP-agent and transcription of the ACO complex, whereas in humans the PPARα/PP-agent complex binds to a different response element and transcribes the apoA1 and apoCIII regions (summarized in Roberts, 1999).[13] Vanden Huevel (1999) noted that there was interindividual variability in human PPAR sequences and wondered whether that could lead to individuals at increased risk. However, the identified human PPARα variants have been either inactive (Woodyatt et al., 1999) or dominant negative suppressors (Gervois et al., 1999). Thus, the interindividual variability which has been identified has tended to reduce effective PPARα levels in humans rather than to increase them. Further, Lawrence et al. (2001) tested this hypothesis directly with human cell lines (HepG2 cells) that "over-expressed" human PPARα. They found that the PPARα-related functions were not increased by PPARα agonists, demonstrating that, although PPARα is present in human cells, higher PPARα levels, if present, could not lead to greater risk.

Thus, since the 1990's, the data have indicated that there are both quantitative and qualitative differences between rodents and humans. The data shows that the levels of PPARα in humans are at least an order of magnitude below those found in rodents. Further, although some fraction of human PPARα can bind agonists and is active when tested with rodent receptors, the evidence suggests that it does not lead to transcription of similar functions in humans. Specifically, MEHP has been shown to be a less avid agonist for the human PPARα receptor than for the mouse and rat receptor (Bility et al., 2004).

There also was been inferential evidence from the late 1990's that the PPARα-related functions related to rodent liver carcinogenicity are not expressed in humans. A review article by Gonzalez et al. (1998) noted that the mechanisms of rodent liver carcinogenicity associated with peroxisome proliferation included oxidative stress (which the authors associated with

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[13] In rodents, lipid metabolism is mediated by peroxisomal enzymes, specifically acetyl CoA oxidase (ACO), whereas human lipid metabolism is mediated through alterations in gene expression of the major high density apoliproteins, apoAI, apoAII and apoCIII as well as lipoprotein lipase (LPL) (reviewed in Vamecq and Latruffe, 1999). Roberts and coworkers (Lambe et al, 1999; Woodyatt et al., 1999) have shown that the human peroxisome proliferation response element (PPRE) differs in sequence from that of the rat. They have shown further that whereas both human and mouse PPARα can drive transcription of mouse ACO, neither can drive transcription of the human ACO gene sequence (Woodyatt et al., 1999). Conversely, there are also differences between humans and rats in the sequence of the ApoA1 gene promoter; the human gene is activated by hypolipidemic agents whereas the rat gene sequence is not (Vu-Dac et al., 1998). Thus, the lack of expression of residual peroxisomal function in primates and cultured human cells seems to be a consequence of differences between humans and rats at the transcriptional level in control of lipid metabolism.

expression of peroxisomal enzyme induction) and enhanced cell proliferation. They also believed there to be a role for apoptosis (programmed cell death, inhibited by peroxisome proliferators) and tissue necrosis factor α (TNF-α), a hepatocyte growth factor secreted by Kupffer cells. They reported that humans differed from rodents in expression of PPARα-related functions in a number of ways (Table 3).

Since the publication of that table, the two question marks in the human column have been answered. Apoptosis in human hepatocytes has been shown to be unaffected by DINP (Hasmall et al., 1999), and PPARα activation seems to have no role in inflammatory processes in humans (Vameq and Latruffe, 1999). In addition, the positive hypolipidemic effects in humans have been shown to occur by a process which is different from that which is active in rats and mice (Vameq and Latruffe, 1999).

Table 3.
Comparison of Human and Rodent Expression of PPARα-Related Functions
(from Table 2 in Gonzalez et al., 1999)

Response to peroxisome proliferators	Mice and Rats	PPARα-Null Mice	Humans
PPARα expression	+	-	+/10 (10 fold less than mice)
increase in peroxisomes	+	-	-
enzyme induction	+	-	-
cell proliferation	+	-	-
apoptosis inhibition	+	-	? [see text]
hypolipidemic effects	+	-	+
anti-inflammatory effects	+	-	? [see text]
increased risk of cancer	+	-	-

b. *PPARα Function and Expression in Humanized Mouse Models*

To decipher the molecular differences between the human and mouse PPARα, several mouse models that only express human PPARα have been created (Cheung et al, 2004; Morimura et al, 2006; Yang et al, 2008). These models indicate that the observed species differences could potentially be attributable to differential changes in gene expression and further emphasize the difference between humans and rodents in the response to peroxisome proliferators. Unlike wild-type mice, liver-specific humanized PPARα mice do not develop liver cancer after chronic treatment with the PPARα agonist WY-14,643 (Morimura et al, 2006). Simply stated, the molecular message relayed from the mouse PPARα is different than the message from the human PPARα. Furthermore, these transgenic models also demonstrate that the effects of PPARα agonists on lipid metabolism are distinct from the effects on hepatomegaly and liver carcinogenesis, thereby suggesting a mode of action by which humans can be resistant

to the development of liver cancer but yet still exhibit decreased triglycerides; from fibrate pharmacotherapy for example.

The first humanized PPARα (hPPARα) mouse model was reported by Cheung et al. (2004); subsequently used by Morimura et al. (2006). This transgenic model specifically expresses the human receptor in the liver of PPARα null mice. To generate this model, hPPARα was placed under the control of the Tet-Off system of doxycycline control with the liver-specific LAP1 promoter; leading to constitutive expression of hPPARα in the absence of doxycycline only in the liver and not in any other tissues.

The expression level of hPPARα in this model was comparable to the wild-type mouse PPARα (mPPARα). Treatment of the hPPARα transgenic mice with either WY-14,643 or fenofibrate, two well known PPARα agonists, resulted in the induction of peroxisomal lipid-metabolizing enzymes; demonstrating that the hPPARα is functionally active. Wild-type mice treated with the PPARα agonists showed a marked hepatomegaly that was due to enhanced cell proliferation as well as cell hypertrophy resulting from an increase in the number and size of peroxisomes. In contrast, the hPPARα transgenic mice did not exhibit any hepatocellular proliferation nor did they have an induction of the cell cycle genes typically associated with proliferation. More importantly, the hPPARα transgenic mice were found to be resistant to WY-14,643 induced hepatocarcinogenesis after 11 months of treatment; which is in direct contrast to the 100% incidence rate observed in the wild-type mice which had both hepatocellular adenomas and carcinomas (Morimura et al, 2006).

A second humanized transgenic mouse model was created by Yang et al. (2008) in which the human PPARα gene isolated from a PAC genome library, with 5' and 3' flanking sequences spanning approximately 100 kilobases (kb) upstream of exon 1 and 28 kb downstream of exon 8, was introduced into transgenic mouse founders that were further bred with PPARα-null mice, resulting in a mouse model that only expressed the hPPARα gene.

Initial experiments showed that the expression patterns and relative expression amount of hPPARα in the transgenic animals were identical to wild-type mice; hPPARα was expressed in organs or tissues with high fatty acid catabolism as expected. Responsiveness of the hPPARα transgenic model was also similar to wild-type animals in that hPPARα responsive gene and protein levels were up-regulated by overnight fasting. Furthermore, following two weeks of fenofibrate treatment, a robust induction of genes encoding enzymes for peroxisomal, mitochondrial, microsomal and cytosolic fatty acid metabolism were found in the liver, kidney and heart was observed in both wild-type and hPPARα transgenic mice, similar to the effects observed in PPARα-null mice transfected with an adenovirus containing either the human or mouse PPARα (Yu et al., 2001).

Hepatomegaly was observed in hPPARα transgenic mice following 2 weeks of exposure to WY-14,643. However, the extent of hepatomegaly was markedly lower than in wild-type mice. Peroxisome proliferation was also noted in both the transgenic mice and wild-type mice. A significant difference in hepatic gene expression was noted between the wild-type and transgenic mice; more genes were induced by WY-14,643 in wild-type mice as compared to the transgenic mice. Additionally, the expression of pri-let-7C and mature let-7C, a microRNA

transcript critical for cell growth and shown to target c-*myc*, a known oncogene, was not inhibited by WY-14,643 in the transgenic mice as compared to the decrease observed in the wild-type mice.

In conclusion, the recent work with humanized mouse models provides insight into the notable species differences with respect to peroxisome proliferation and the induction of liver tumors from PPARα agonists. The use of humanized PPARα transgenic mouse models suggests that the differences could potentially be attributable to differential changes in gene expression. Further emphasizing the difference between humans and rodents in the response to peroxisome proliferators is that humanized PPARα mice do not develop liver cancer after treatment with the PPARα agonist WY-14,643 in contrast to the observations in wild-type mice. These data provide further support for the conclusion that the PPARα mode of action for liver tumorigenesis in rodents is not relevant to humans.

c. *Empirical Data*

From the foregoing, the most plausible interpretation consistent with the data is that the PPARα-mediated functions associated with carcinogenic induction in mice and rats are not expressed in humans. A large body of empirical evidence which is consistent with that view supports this assertion.

In primate *in vivo* studies, high doses of DINP do not produce liver changes of any kind. Hall et al. (1999) administered of 2500 mg/kg/day DINP to marmosets for 13 weeks and reported no pathological changes in liver, kidneys or testes. In addition, the Hall et al. study showed that DINP treatment did not induce peroxisomal proliferation and had no effects on levels of peroxisomal enzymes in marmosets, at levels well above those associated with effects in rats and mice. These results were confirmed by Pugh et al. (2000), who performed a 14-day study of cynomolgus monkeys in which no liver effects – including no change in hepatic peroxisome β-oxidation, DNA synthesis, or GJIC – were observed from high doses of DEHP and DINP (500 mg/kg/day).

Similarly, under *in vitro* conditions, DINP increased replicative DNA synthesis and suppressed apoptosis in rodent hepatocytes but not in human cells (Hasmall et al., 1999). MINP, the monoester metabolite of DINP, had no effects on peroxisomal enzyme levels in either human or primate hepatocytes in culture (Benford et al., 1986; Kamendulis et al., 2002), nor on GJIC (which is associated with peroxisome proliferation) in human hepatocytes and a human liver cell line (Baker et al., 1996).

d. *Clinical and Epidemiological Data*

Support for the conclusion that the PPARα mode of action does not operate in humans is provided by studies of human beings treated with members of the fibrate family of drugs. Fibrates are therapeutic agents which were developed to treat hyperlipidemia and are PPARα agonists. Members of this family of pharmaceutical agents have varying degrees of affinity for PPARα. Some human data on PPARα agonist effects are available from several clinical trials and population case-control studies pertaining to fibrate pharmacotherapy (Benzafibrate

Infarction Prevention Study Group, 1992, 2000; Canner et al., 1986; Committee of Principal Investigators, 1978, 1980, 1984; Coronary Drug Research Group, 1975, 1977; De Faire et al., 1995; Diabetes Atherosclerosis Intervention Study Investigators, 2001; Freeman et al., 2006; Frick et al., 1987, 1997; Huttunen et al., 1994; Keech et al., 2005, 2006; Meade, 2001; Rubins et al., 1993, 1999; Tenkanen et al., 2006). These studies examined a range of human responses to PPARα agonistic effects including atherosclerosis, cardiovascular disease, serum biomarkers of fatty acid metabolism, acute toxicity and organ-specific chronic toxicity including cancer.

In the Helsinki Heart Study, a total of 4081 men aged 40–55 with elevated serum cholesterol were treated with either gemfibrozil or placebo for a 5-year period (Frick et al., 1987; Huttunen et al., 1994). Despite a significant lowering of serum lipids which prevented coronary heart disease in the gemfibrozil-treated group, no differences in total death rate or liver cancer incidence were observed between treatment groups. However, liver cancer incidence was not reported as a single endpoint; the incidence was either reported as total deaths from cancer, or deaths from liver, gallbladder and intestinal cancers grouped together. No statistically significant differences were found for any class of cancers examined following this five year exposure and follow-up period (Frick et al., 1987). Importantly, the incidence of cancer mortality in this study, for placebo and fibrate-treated patients, was less than 2% for each group, compared to greater than 50% in PPARα agonist-treated rodents (Ashby et al., 1994; Bentley et al., 1993; Lake, 1995a, 1995b; Reddy and Lalwani, 1983).

The other randomized clinical trial was conducted over a total of thirteen years by the World Health Organization (WHO) to determine whether clofibrate would lower the incidence of ischemic heart disease (IHD) in men. It was carried out in 15,745 men with a treatment group and two control groups (one high and one low cholesterol level) of about 5000 men each (Committee of Principal Investigators, 1978). The average treatment period was 5.3 years and follow-up reports were provided 4.3 and 7.9 years after this period. Clofibrate was reported to cause a statistically significantly higher age-adjusted total mortality as compared with the high cholesterol placebo-treated control groups in this study, due to a 25% increase in non-cardiovascular causes from diseases of the liver, gall bladder, pancreas and intestines, including malignant neoplasms of these sites (Committee of Principal Investigators, 1980). However, in the final follow-up study (5.3 years in the treatment phase with 7.9 years follow-up for a total of 13.2 years), neither the number nor rate of cancer deaths in the clofibrate-treated group was statistically different from the control groups (Committee of Principal Investigators, 1984). The reason for the difference in mortality at the earlier time point is uncertain.

Similar to the Helsinki Heart Study, no specific data on the incidence of liver cancer was provided for the WHO study. It should be noted that in this final follow-up study there was an excess of only 12% deaths from all causes other than IHD, compared with 25% in the earlier studies of that cohort. Furthermore, the proportional differences between the treated group and the control groups in the final follow-up study was diminished for malignant disease but increased for nonmalignant diseases. The results indicate that the excess in deaths from diseases other than IHD was largely confined to the clofibrate treatment period (average 5.3 years). However, 7.9 years post-treatment, there were 27 deaths associated with liver, gallbladder, or intestinal cancers in the clofibrate treated group, compared to 18 and 11 deaths associated with

the same endpoints in the high cholesterol and low cholesterol control groups, respectively (out of about 5000 persons per group). Similar to the Helsinki Heart Study, this incidence is less than 1% for both control and treatment groups.

Finally, a limited epidemiological study showed no evidence of increased cancer risk as a result of fibrate therapy (Law et al., 1994). (As with the clinical trials, cancer incidence is not reported specifically for liver cancer.) If PPARα acts in humans in a similar manner as in rodents, it would be expected that there would at least been an effect such as hepatomegaly observed in these clinical and epidemiological studies, but it was not.

Thus, these studies are consistent with the other strong evidence that the PPARα mode of action which causes cancer in rodents is not relevant for assessing human cancer hazard from PPARα agonists. Despite some limitations in data analysis (i.e., not reporting single-endpoint organ data), these studies suggest that chronic administration with PPARα agonists does not increase cancer risk in humans.

These studies add to the weight of evidence given by the human and primate data discussed above that DINP is unlikely to cause liver tumors in humans.

6. Even if DINP Could Cause Peroxisome Proliferation in Humans, Human Internal Dose Levels Cannot Reach Carcinogenic Levels

The foregoing makes clear that the liver tumors observed in rodents treated with DINP simply are not relevant to humans. However, even assuming it were possible for DINP to cause some peroxisome proliferator response in humans, there is no conceivable scenario under which humans could be exposed to sufficient amounts of DINP to cause liver tumors. Because of differences between primate and rodent absorption of DINP, internal doses equivalent to those required to produce tumors in rodents simply cannot be achieved in humans.

The ILSI RSI workgroup concluded that, for PPARα agonists in general, taking into account kinetic and dynamic factors, the animal mode of action is not plausible in humans (Klaunig et al., 2003, pp. 691-693). This is specifically demonstrated by phthalate data on differences in absorptive capacity between rodents and primates, which demonstrate that the relatively high internal doses associated with effects in rodents cannot be achieved in humans.

The rodent data indicate that approximately 50% of orally administered DINP is absorbed as the corresponding monoester at dose levels up to 500 mg/kg per day (mg/kg/day) (Lington et al., 1985; El-Hawari, et al., 1985; 1983). Data from studies of absorption of DEHP in rodents indicate that this relationship is preserved at even higher treatment levels (Rhodes et al., 1986). Primates, however, respond very differently. Data from studies with DEHP in both the marmoset (Rhodes et al., 1986) and cynomolgus monkeys (Astill, 1989) show that, at very high dose levels, absorption in the primates is limited and that internal doses do not exceed those measured in rats exposed to 150-200 mg/kg/day (which are non-tumorigenic doses).

Comparative dosimetry studies (Pugh et al., 2000) indicate that DINP is even more poorly absorbed by primates than DEHP. Studies with volunteers also indicate that humans

absorb a much lower fraction of the dose than rodents for doses up to 500 ug/kg (Anderson et al., 2001).[14] These data emphasize that consideration of the likely internal dose, based on toxicokinetic considerations, is crucial to an evaluation of the potential for toxicological effects in humans from DINP exposures. In other words, absorption of phthalates in rodents and humans may be similar at very low doses (<100 ug/kg), but at the higher doses, seen to produce tumorigenic responses in rodents, humans absorb much less. The data indicate that effects produced in rodents by DINP will not occur in humans, because the high internal doses required to produce these effects in rodents cannot be achieved in humans due to decreased absorption with increasing dose.

The lowest DINP dose that has been associated with tumor induction is 336 mg/kg/day in female mice with effects in other species and sexes occurring at levels ranging from approximately 700 to 900 mg/kg/day (Moore, 1998a; b). As stated above, the maximum level absorbed by primates corresponds to a rodent level of 150-200 mg/kg, well below the dose required to induce tumors in the more sensitive rodents. Thus, the evidence indicates that, regardless of the level of exposure, humans could never absorb enough DINP to achieve the internal doses associated with liver tumors in rodents. That the doses which can be achieved in humans would not pose any concern is indicated by the fact that 2,500 mg/kg/day for 13 weeks produced no liver effects whatsoever in marmosets (Hall et al., 1999).

In summary, there is strong evidence that the PPARα mode of action which is responsible for liver tumors in DINP-treated rodents is not operable in humans. However, even if PPARα in humans did respond to DINP in a manner similar to rodent PPARα, it simply is not possible for humans to achieve sufficient doses of DINP to result in liver tumors.

7. Expert Body Reviews Have Concluded that the Rodent Liver Tumors in DINP Studies Are Not Relevant to Humans

The CPSC CHAP concluded "that DINP causes liver cancer in rodents by a PPARα-mediated mechanism that is pronounced in rodents and believed not readily induced in humans, especially at doses resulting from current use of consumer products" (CPSC, 2001, p. 122). Subsequently, the CPSC staff, using the CHAP report and the report of the ILSI workshop, "concluded that DINP, which is a peroxisome proliferator, is not likely to present a cancer risk in humans" (CPSC, 2003).

The ILSI RSI workgroup concluded:

> In summary, the weight of evidence overall currently suggests that the rodent [mode of action] for liver tumors is not likely to occur in humans, taking kinetic and dynamic factors into account. This

[14] Koch et al. (2005) reported surprisingly high absorption of DEHP in a single human volunteer; however, it appears the experimental conditions may have allowed for additional DEHP exposure form the environment during the sampling period. To our knowledge, this anomalous result is not corroborated by any other publication. The results of the ECPI study (discussed in the cover letter to this submission) will provide further data, specifically for DINP as well as DEHP.

> conclusion is based upon evaluation of the existing body of evidence and would apply to the consequences of exposure to *known* examples of PPAR*α* agonists.

(Klaunig et al., 2003, p. 693.) DINP is a known example of a PPARα agonist that was part of the basis for the workshop conclusions. Therefore, the conclusion of the ILSI workshop is that the liver tumors that occur in rodents treated with DINP are not likely to occur in humans.

The EU in its risk assessment of DINP stated:

> The current literature suggests that only rats and mice are responsive to the carcinogenic effects of peroxisome proliferator, while dogs, non-human primates and humans are essentially non-responsive or refractory. In this way, it should be noted that in monkey, following oral administration of DINP for 14 days or 13 weeks there was no evidence of peroxisome proliferation. This indicates that monkeys and subsequently probably humans are far less sensitive than rodents to peroxisome proliferation and its relative liver effects. It should be noted that recently IARC gave a ruling on the carcinogenicity of DEHP and concluded that the mechanism (peroxisome proliferation and PPARα activation) by which DEHP increased the incidence of liver tumours in rodents was not relevant to humans. (ECB, 2003a, p. 243)

In its formal review of risks and an assessment of classification, the EU did not identify carcinogenicity as a critical endpoint (ECB, 2003a, 2003b) and did not classify DINP as a carcinogen (EC, 2000). In the risk assessment summary document, the EU stated that, on the basis of the peroxisome proliferation evidence, "there is no concern for a potential carcinogenic effect in humans." (ECB, 2003b, p. 14)

When USEPA originally proposed to list DINP under EPCRA Section 313 (Fed. Reg. 2000), the American Chemistry Council requested that several prominent researchers provide opinions on the potential human carcinogenicity. Those opinions were provided in comments submitted to USEPA in 2001; copies as provided with these comments, as follows:

- Attachment A is a statement by Ruth Roberts, Ph.D., currently Senior Director of Toxicology at Astra Zeneca in the United Kingdom. She holds a Doctorate in Medical Oncology and completed a Postdoctoral Fellowship in molecular oncology. Dr. Roberts has performed some of the foremost research on the mechanism by which peroxisome proliferators cause cancers in rodents and whether that mechanism operates in humans. Dr. Roberts concludes: "weight of the evidence supports the position that the rodent liver tumors caused by peroxisome proliferators such as DINP are not relevant to man since we differ from rodents at the molecular level in our response to peroxisome proliferators."

- Attachment B is a statement by James Klaunig, Ph.D. Dr. Klaunig is Professor and Director of Toxicology at the Indiana University School of Medicine and is Director of the State Department of Toxicology for the State of Indiana. He holds his Doctorate in Experimental Toxicology/Pathology and has done Postdoctoral work in pathology. He serves and has served on numerous review committees for government agencies, including USEPA, NTP and NIH. Dr. Klaunig has conducted significant research on peroxisome proliferation mechanisms and participated in the ILSI RSI workshop on peroxisome proliferators. He concludes that the data "provide mechanistic evidence that rodent liver tumor induction by DINP is by a peroxisomal proliferation process which does not occur in humans or other primates." [Note that the "unpublished data" provided with Dr. Klaunig's statement has now been published (Kamendulis et al., 2002).]

- Attachment C is a cancer risk assessment for DINP by Gary Williams, M.D., and Michael Iatropoulos, M.D., Ph.D. Dr. Williams is Professor of Pathology, Director of Environmental Pathology and Toxicology, and Head of the Program on Medicine, Food and Chemical Safety, at the New York Medical College. Dr. Williams is a recognized expert in chemical carcinogenesis; Dr. Iatropoulos is a Research Professor of Pathology at New York Medical College and is also an expert in chemical carcinogenesis. Drs. Williams and Iatropoulos reviewed the data for DINP with respect to liver and kidney tumors and MNCL. They concluded, "the increases in all three spontaneously occurring tumors seen with DINP occurred through processes not relevant to humans and at exposures vastly beyond that which would take place with product use."

In summary, numerous independent scientists have evaluated the potential for peroxisome proliferators in general or DEHP and DINP in particular to cause cancer in humans. The overwhelming scientific consensus is that DINP cannot reasonably be anticipated to cause cancer in humans.

B. Mononuclear Cell Leukemia Observed in Fisher 344 Rats

Mononuclear call leukemia (MNCL) was observed in the two DINP bioassays conducted in Fisher 344 rats, but not in the bioassay conducted in mice (Lington et al., 1997; Moore, 1998a; b). MNCL is a lesion that occurs almost exclusively in the F-344 rat and that occurs spontaneously in that species. MNCL is discounted by authoritative agencies such as the National Toxicology Program (NTP) and the International Agency for Research on Cancer (IARC). As described below and in the attached opinion from Dr. Richard Irons (Attachment D), a preeminent researcher of leukemogenesis, the use of MNCL as a basis for human health hazard assessment is not scientifically supportable. In fact, Dr. Irons notes that a proposal he made to the National Institutes of Health (NIH) had been rejected because of the "obvious lack of significance of MNCL to human disease."

1. MNCL in Fischer Rats Is Generally Disregarded for Human Hazard Assessment

MNCL is a spontaneous tumor which occurs frequently in the F-344 rat and is the most common cause of spontaneous death in that strain and species (e.g., Haseman et al., 1998). NTP historical control data show that MNCL occurs in 14 to 74 percent of control animals (Haseman et al., 1998). Background incidence is seen to be highly variable and has more than doubled during the two decades since the Haseman et al. report in 1985. (Thomas et al., 2007). MNCL is found at much lower incidence in other rat strains (Iatropoulos, 1983) and has not been reported in mice (e.g., Harleman et al., 1994). There may also be differences within strains – the incidence of MNCL seems much lower in Japanese F-344 rats than in the F-344 strain historically used by the NTP (Whysner et al., 1995).

The results of DINP chronic studies are consistent with these findings. MNCL was found in two studies in the F-344 rat (Lington et al., 1997; Moore, 1998a) but not in the B6C3F1 mouse (Moore, 1998b) or, for a non-commercial DINP, the Sprague-Dawley rat (Bio/dynamics, 1986).

When assessing the significance of changes in MNCL incidence, points to consider include: (1) that the factors contributing to a high, variable, spontaneous incidence of MNCL in the F-344 rat are unknown; (2) that there are a number of factors which contribute to variability in MNCL frequency for unknown reasons – including the use of corn oil as a vehicle (Haseman et al., 1985), single vs. group housing (Haseman et al., 1998), splenic toxicity, lifespan, body weight and dietary fat (but not dietary restriction) (Elwell et al., 1996); and (3) that treatment with genotoxic agents that might logically be expected to increase the incidence of cancer in general has either no effect or actually reduce MNCL incidence (Waalkes et al., 1991; Lijinsky et al., 1993; Elwell et al., 1996).

Many authoritative sources have questioned the relevance of MNCL data for human cancer hazard assessment purposes. For example, the NTP, in its review of the carcinogenesis data for diallyl phthalate wrote:

> The relatively high and variable spontaneous incidence of mononuclear cell leukemia in aged F-344 rats confounds the interpretation of this tumor type in dosed animals as evidence of a carcinogenic response. That is, statistical evidence of an increased occurrence of mononuclear cell leukemia in dosed animals as an indication of carcinogenicity may appropriately be regarded with less confidence than would similar incidence data for other tumor types in the F-344 rat. (NTP, 1984).

More recently, the NTP has decided to stop use of the F344 strain, in part because of the high spontaneous incidence of MNCL is that strain (King-Herbert and Thayer, 2006; NTP BSC, 2007).

In a review of tetrachloroethylene, the United Kingdom Health and Safety Executive (HSE) noted that MNCL was a common neoplasm that occurred at high and variable frequency

in the F-344 rat. They did not consider an excess of MNCL as evidence for a carcinogenic response even though the frequency exceeded the historical averages of both the NTP and the testing laboratory (HSE, 1987). As noted above, NIH rejected a proposal by Dr. Irons because of the "obvious lack of significance of MNCL to human disease."

The National Research Council has stated, "It is unclear whether [MNCL] is a relevant predictor of human leukemias or other adverse health effects" (NRC, 2010, p. 56). With respect to tetrachloroethylene, NRC states that "the high backgrounds [of MNCL in F344 rats] make it difficult to interpret the biological significance of the increase in the incidence of [MNCL] observed in the treatment groups" (NRC, 2010, p. 54).

In his opinion (Attachment D), Dr. Richard Irons, a pre-eminent researcher in the field of leukemogenesis, states, "In my view, MNCL in the F344 rat is not a useful model for the direct study of human disease and is certainly not an appropriate endpoint for predicting or extrapolating carcinogenic risk in humans," and "there is no biologic rationale for concluding that F-344 MNCL is a relevant surrogate for a comparable disease entity or, independently, any disease that has been associated with chemical exposure in humans."

A recent review of MNCL (Thomas et al., 2007) suggests that a weight of evidence approach be taken when statistically identified increases in MNCL occur with exposure. The authors propose similarities between F344 MNCL and human NK-LGL leukemia based on functional, clinical and morphological characteristics, but emphasize that the mechanisms of leukemogenesis may be very different. NRC (2010, p. 56) points out that Thomas et al. note that "in contrast with F344 rats, human NK-LGL leukemia is rare, occurs primarily in the young and may be associated with Epstein Barr virus (EBV) although no such virus-leukemia is known to contribute to the etiology of rat LGLL/[MNCL]" (NRC, 2010, pp. 56-57).

Without further research to clarify the leukemic cell of origin and define candidate molecular targets, the case for potential human relevance of MNCL remains weak, particularly in light of the high, variable spontaneous incidence of MNCL in the Fischer 344 rat – the only species in which MNCL was seen in conjunction with DINP administration.

2. Expert Body Reviews Have Concluded that the MNCL in DINP Studies Is Not Relevant to Humans

The CPSC CHAP concluded:

> The findings of mononuclear cell leukemia and renal tubular carcinoma in the rodent bioassay for DINP are of questionable relevance to humans. (CPSC, 2001, p. 122).

The EU Risk Assessment states:

> Regarding MNCL, a clear increase incidence is observed in the two studies conducted with Fisher rats (outside the historical range of spontaneous leukemia), along with shortening of the onset of

> MNCL. However, MNCL is a common neoplasm in the Fischer 344 rats and the increased incidence after chronic exposure to some substances is likely a strain specific effect with little relevance for humans. Of interest, the IARC categorised MNCL as "an unclassified leukemia with no known human counterpart" and substances which increase MNCL frequency as "not classifiable as to carcinogenicity in humans" (IARC, 1990). (ECB, 2003a, p. 225).

As noted above, the NIH rejected a proposal by Dr. Irons because of the "obvious lack of significance of MNCL to human disease" Dr. Irons reviewed the Lington and Moore data and concluded that "specifically with respect to bioassays of di-isononyl phthalate, the dose-dependent nature of treatment-related MNCL is not impressive, suggesting that the observed increases represent a non-specific high dose effect that cannot be meaningfully attributed to a carcinogenic event." (Attachment D).

Thus, the opinion of several authoritative bodies is that MNCL is not relevant for human health assessment. In addition, the CPSC CHAP, the EU and Dr. Irons have specifically found that MNCL in the DINP bioassays is not relevant for human health assessment.

C. Kidney Tumors in Male Rats

Kidney tumors have been observed in male rats exposed to high doses of DINP (733-885 mg/kg/day) for two years (Moore, 1998a), but not in female rats and not in mice of either gender (Moore, 1998a; b). Male rats are known to be susceptible to formation of kidney tumors through a mechanism involving alpha$_{2u}$-globulin accumulation. Because humans do not produce alpha$_{2u}$-globulin, such male rat kidney tumors are not relevant for human health assessment (USEPA, 1991; Swenberg and Lehman-McKeeman, 1998). The kidney tumors observed in the DINP study were malignant tubule cell carcinomas, found in male rats given high dietary doses but not in female rats or in mice of either sex. See Table 4. The tumors found were of a type associated with an alpha$_{2u}$-globulin process and also demonstrated the sex- and species-specific responses expected for an alpha$_{2u}$-globulin process.

In the DINP study in rats, there was evidence in the male rats of microscopic changes characteristic of alpha$_{2u}$-globulin induction (Moore, 1998a). Subsequent studies have demonstrated that all the criteria established by the USEPA and by IARC to verify that a carcinogenic response is the consequence of the alpha$_{2u}$-globulin mechanism are met for DINP (Caldwell et al., 1999; Schoonhoven et al., 2001). Attachment E is a letter from Dr. James Swenberg who is an expert in the alpha$_{2u}$-globulin mechanism (he is a co-author of the IARC scientific publication on the alpha$_{2u}$-globulin mechanism) and who has conducted some of the research on DINP. As stated by Dr. Swenberg, the data "clearly demonstrate that DINP causes [alpha$_{2u}$-globulin nephropathy]" and that "the data on kidney tumors is not relevant for human risk assessment."

Table 4.
Incidence of malignant tubule cell carcinomas in rats and mice following chronic dietary administration of DINP – number of rats per dose group (mg/kg/day)

	control	~30	~90	~400	~800	recovery *
male rats	0	0	0	0	2	4
female rats	0	0	0	0	0	0
	control	~100	~300	~800	~1600	recovery *
male mice	0	0	0	0	0	0
female mice	0	0	0	0	0	0

* Animals in the recovery group were given the high dose for 18 months and then held without treatment until terminal sacrifice (24 months).

The following discusses in greater detail how the DINP data meet the USEPA and IARC alpha$_{2u}$-globulin mechanism criteria (subsections 1 and 2). It then discusses the fact that expert reviews have determined on that basis that the kidney tumors observed in male rats treated with DINP are not relevant to humans (subsection 3).

1. The DINP Data Meet USEPA's Criteria for an Alpha$_{2u}$-Globulin Mechanism

In 1991 the USEPA reviewed the evidence for alpha$_{2u}$-globulin accumulation as a potential mechanism of renal cancer and its relevance to humans (USEPA, 1991). This review culminated in a two part USEPA science policy statement (USEPA, 1991, p. 85):

> (1) Male rat kidney tumors arising as a result of a process involving [alpha$_{2u}$-globulin] accumulation do not contribute to the qualitative weight-of-evidence that a chemical poses a human carcinogenic hazard. Such tumors are not included in dose-response extrapolations for the estimation of human carcinogenic risk.
>
> (2) If a chemical induces [alpha$_{2u}$-globulin] accumulation in male rats, the associated nephropathy is not used as an endpoint for determining non-carcinogenic hazard. Estimates of non-carcinogenic risk are based on other endpoints.

USEPA also provided guidance for determining whether the alpha$_{2u}$-globulin process could be a factor in renal effects. Each of three factors, set forth in Section XVII-A of USEPA (1991, pp. 86-87) must be met. As the following shows, all three factors are met for DINP.

> "(1) Increased number and size of hyaline droplets in renal proximal tubule cells of treated male rats

> The abnormal accumulation of hyaline droplets in the P2 segment of the renal tubule is necessary to attribute the renal tubule tumors to the [alpha$_{2u}$-globulin] sequence of events. This finding helps differentiate the [alpha$_{2u}$-globulin] inducers from chemicals that produce renal tubule tumors through other means." (USEPA, 1991, p. 86)

As shown in Caldwell et al. (1999), hyaline droplets were evaluated by immunohistochemical staining (a process specific for α_{2u}-g) in male and female rats. Droplets were present in male rat kidneys, and both droplet size and area involved were significantly increased with dose. Droplets were not present in kidneys from female rats. The accumulation of α_{2u}-g in male rat kidneys with increasing dose was independently confirmed by a second laboratory (Schoonhoven et al., 2001). These data demonstrate the abnormal accumulation of hyaline droplets in the renal proximal tubules of treated rats and show also that this does not occur in female rats, thus demonstrating the sex specificity of this finding.

> "(2) Accumulating protein in the hyaline droplets is [alpha$_{2u}$-globulin]
>
> Hyaline droplet accumulation is a nonspecific response to protein overload in the renal tubule and may not be due to [alpha$_{2u}$-globulin]. Therefore, it is necessary to demonstrate that [alpha$_{2u}$-globulin] accounts for the hyaline droplet accumulation found in the male rat." (USEPA, 1991, p. 86)

As shown above, the evaluation of hyaline droplets utilized immunohistochemistry to detect the highly specific binding of a monoclonal antibody to alpha$_{2u}$-globulin. As documented by both Caldwell et al. (1999) and Schoonhoven et al. (2001), the accumulating protein in the hyaline droplets is alpha$_{2u}$-globulin. As stated above, the absence of alpha$_{2u}$-globulin in kidneys from female rats was also demonstrated, confirming the sex specificity of the observation.

> "(3) Additional aspects of the pathological sequence of lesions associated with [alpha$_{2u}$-globulin] nephropathy are present.
>
> Typical lesions include single cell necrosis, exfoliation of epithelial cells into the proximal tubular lumen, formation of granular casts, linear mineralization of papillary tubules, and tubule hyperplasia. If the response is mild, all of these lesions may not be observed; however, some elements consistent with the pathological sequence must be demonstrated to be present." (USEPA, 1991, pp. 86-87)

As documented in Caldwell et al. (1999), tubular regeneration and tubular epithelial hyperplasia were present in male rat kidneys, predominantly in the P2 segment of the proximal tubule of the renal cortex, and increased in a dose-responsive manner. In contrast, tubular regeneration was present in only one of the high dose female rats. Mineralization was

documented in the pathology reports of the chronic studies (Moore, 1998a; b). This also showed a strong dose response relationship in the male rat kidneys. Mineralization was present in kidneys of some female rats, but did not increase with dose, and was not present in kidneys of mice (Table 5). Lington et al. (1997) reported a statistically significant increase in renal epithelial cells in the urine. This is the consequence of exfoliation of epithelial cells into the proximal tubular lumen. Single cell necrosis and formation of granular casts were not reported, but as DINP is clearly a weak inducer of α_{2u}-g, all of the histological changes are not to be expected, and the absence of some, as noted by the USEPA, is not inconsistent with an [alpha$_{2u}$-globulin] mediated response.

Table 5.
Incidence of kidney mineralization following dietary administration of DINP.
No. affected rats/total no. rats in each dose group (mg/kg/day)

	control	~30	~90	~400	~800	recovery *
male rats	16/60	14/50	11/50	59/60	57/60	50/50
female rats	11/60	9/50	4/50	14/50	16/60	10/50
	control	~100	~300	~800	~1600	recovery *
male mice	NP	NP	NP	NP	NP	NP
female mice	NP	NP	NP	NP	NP	NP

*Animals in the recovery groups were treated with the high dose for 18 months and then held without further treatment until terminal sacrifice (24 months).
NP – not present.

In a dietary study of DINP in Sprague-Dawley rats at levels of 0.3 and 1.0% for 13 weeks, tubular regeneration, nephritis, tubular casts and nephrosis were observed primarily in male rats and increasing with dose (Bird et al., 1986; Bio/Dynamics, 1982). These lesions are consistent with [alpha$_{2u}$-globulin] pathology and provide further evidence that the α_{2u}-g process was operative in causing the kidney tumors in male rats treated with DINP. Additionally, the appearance and extent of these lesions at 13 weeks further differentiate them from those associated with chronic progressive nephropathy, providing further evidence they are the consequence of an [alpha$_{2u}$-globulin] mediated process.

Thus, all three of USEPA's obligatory criteria are met for DINP. When this is the case, then USEPA's guidance states that additional information is reviewed (USEPA, 1991, Section XVII-B, pp. 87-88). Data are available for several of the categories of USEPA describes,[15] as follows:

[15] Data for all categories of additional information listed by USEPA are not required. As USEPA states: "the information may not always be available; nor should this list be considered exhaustive." (USEPA, 1991, p. 87).

(a) Additional biochemical information (including reversible binding of the chemical to alpha$_{2u}$-globulin): As documented by Schoonhoven et al. (2001), reversible binding of DINP metabolites to alpha$_{2u}$-globulin has been demonstrated.

(b) Sustained cell division in the proximal tubule of the male rat: This was documented by Caldwell et al. (1999) through the use of immunochemical techniques -- specifically, the use of the proliferating-cell nuclear antigen (PCNA) -- and was subsequently confirmed by Schoonhoven et al. (2001) through the use of an alternative technique -- BrdU labelling.

(c) Genotoxicity (i.e., information on potential genotoxicity in a standard battery of short-term tests relevant to the evaluation of potential carcinogenicity provides a possible means for distinguishing between genotoxic and non-genotoxic processes): As described in Section II, DINP is not genotoxic as evidenced by negative results in a number of short term tests including Salmonella, mouse lymphoma and micronucleus tests (Barber et al., 2000; McKee et al., 2000; Zeiger et al., 1985).

(d) Animal bioassay data in other sex-species combinations: As described above, DINP produces tubule cell carcinomas in male rats but not in female rats or in mice of either sex. This is consistent with the expected pattern of response for an alpha$_{2u}$-globulin mechanism. It also provides indirect evidence that, if there are other toxic processes associated with DINP treatment, they do not contribute to kidney cancer as no kidney tumors were found except in male rats and under conditions in which alpha$_{2u}$-globulin was increased.

USEPA's guidance summarizes the evaluation of the three "must have" factors, plus additional evidence, as follows:

> Confidence in determining which of the three categories [i.e., compounds producing renal tumors in male rats attributable solely to chemically induced alpha$_{2u}$-globulin accumulation; compounds producing renal tubule tumors that are not linked to alpha$_{2u}$-globulin accumulation; compounds producing some renal tubule tumors in male rats attributable to the alpha$_{2u}$-globulin process and some attributable to other carcinogenic processes] applies depends on the comprehensiveness and consistency of the available data. If all the data (two species, two sex combination bioassay, all elements in XVII-A [the 3 specific findings described above], and additional information such as that described in XVII-B [including points a-d above]) are consistent with a role for chemically induced [alpha$_{2u}$-globulin], there is a high degree of confidence that the [alpha$_{2u}$-globulin] syndrome alone accounts for the renal tubule tumors. (USEPA. 1991, p.88)

Application of this reasoning to the DINP data shows a high degree of confidence that the alpha$_{2u}$-globulin syndrome alone accounts for the renal tubule tumors observed in male rats treated with DINP. As documented above, there is a two-species, two-sex bioassay that provides data consistent with the alpha$_{2u}$-globulin process, i.e., malignant tubule cell tumors in kidneys of male rats but not female rats or mice (Moore, 1998a and b). The three required criteria (Section XVII-A) are met: there is evidence of hyaline droplet accumulation, a demonstration that the accumulating protein in the hyaline droplets is alpha$_{2u}$-globulin and histopathological evidence consistent with an alpha$_{2u}$-globulin process. There is also additional information as described in section XVII-B that is consistent with a role for chemically-induced alpha$_{2u}$-globulin. No data for DINP are inconsistent with an alpha$_{2u}$-globulin process. Thus, under USEPA's guidance, an alpha$_{2u}$-globulin mediated process is the most plausible mechanism for kidney tumor induction, the male rat kidney tumors should be attributed to an alpha$_{2u}$-globulin process and neither those tumors nor any associated renal toxicity should be used for human health hazard identification.

2. The DINP Data Meet the IARC Criteria for an Alpha$_{2u}$-Globulin Mechanism

A review of the significance of alpha$_{2u}$-globulin induction to human health was conducted in 1997 by the International Agency for Research on Cancer (IARC) (Swenberg and Lehman-McKeeman, 1998). An expert panel reviewed the evidence for alpha$_{2u}$-globulin as a mechanism for renal-cell neoplasms and concluded that this mechanism was operative only in male rats and had no clinical significance for humans. The panel further determined that kidney tumors in male rats which are the consequence of an alpha$_{2u}$-globulin-mediated process should not be used in an assessment of human carcinogenic hazard. Finally, the IARC panel defined a set of criteria, similar to those established by the USEPA, which could be used to determine whether a substance acts via an alpha$_{2u}$-process (Swenberg and Lehman-McKeeman, 1998).

The IARC criteria, and how the DINP compare to those criteria, are as follows:

(a) Lack of genotoxic activity (agent and/or metabolite) based on an overall evaluation of in-vitro and in-vivo data. As described in Section II of these comments, DINP has been tested in a number of in vivo and intro tests for genotoxic activity and all have produced negative results (Barber et al., 2000; McKee et al., 2000; Zeiger et al., 1985).

(b) Male rat specificity for nephropathy and renal tumorigenicity. As shown in Table 4 (above), the renal tumors were in male rats; there were none in female rats or in mice of either sex. The male rat specificity for an alpha$_{2u}$-globulin nephropathy is documented in Caldwell et al. (1999). Thus the male rat specificity for both nephropathy and renal tumorigenicity has been documented.

(c) Induction of the characteristic sequence of histopathological changes in shorter-term studies of which protein droplet accumulation is obligatory. As described above, protein droplet accumulation is documented in Caldwell et al. (1999) along with evidence that the protein which is being accumulated is alpha$_{2u}$-globulin. Other aspects of characteristic pathology – including tubular regeneration and tubular

hypertrophy in male but not female rat kidney – are also documented in Caldwell et al. (1999). Evidence of mineralization of renal tubules is documented in Moore (1998a).

(d) Identification of the protein accumulating in tubular cells as alpha$_{2u}$-globulin. This was documented by Caldwell et al. (1999) and confirmed by Schoonhoven et al. (2001).

(e) Reversible binding of the chemical or metabolite to alpha$_{2u}$-globulin. This is documented in Schoonhoven et al. (2001). See also Attachment E.

(f) Induction of sustained increased cell proliferation in the renal cortex . This was documented in Caldwell et al. (1999) and confirmed by Schoonhoven et al. (2001) by a different technique.

(g) Similarities in dose-response relationship of the tumor outcome with the histopathological end-points (protein droplets, alpha$_{2u}$-globulin accumulation, cell proliferation). Kidney tumors were found only after dietary administration of DINP at a level of 1.2% (733 mg/kg/day in the male rats). As documented in Caldwell et al. (1999), protein droplets and alpha$_{2u}$-globulin accumulation were significantly elevated in comparison to control values at 0.6% (307 mg/kg/day) but not at lower levels (307 mg/kg/day was the highest dose used in the Caldwell et al. study). As shown by Caldwell et al. (1999), cell proliferation was elevated at 0.6% in the diet, but was not significantly different from controls. Schoonhoven et al. (2001) reported a doubling in cell proliferation in animals given 900 mg/kg. Thus it is evident that significant effects in the critical parameters are found at doses approximating the tumorigenic levels.

Thus, DINP meets all of the IARC criteria, showing that the male rat kidney tumors associated with DINP treatment are the result of an alpha$_{2u}$-globulin-mediated process and are not relevant to humans.

3. Expert Body Reviews Have Concluded that DINP Data Meet the Criteria for an Alpha$_{2u}$-Globulin Mechanism

Reviewing bodies have agreed the DINP data meet the criteria for an alpha$_{2u}$-globulin-mediated process and have therefore found that kidney tumors seen in male rats treated with DINP are not relevant for human cancer hazard assessment.

The CPSC CHAP report states:

> Male rat specificity in tumor response, lack of genotoxicity, histopathology findings of cytotoxicity and regeneration, α2μ-globulin accumulation**,** and demonstrated cell proliferation strongly support the criteria for demonstrating α2μ-globulin mechanism (IARC, 1998). Therefore, the renal tumors in male rats

> at the high dose of DINP are assumed to be rat specific and are not used to predict human cancer risk. (CPSC, 2001, p. 91)

The EU risk assessment states: "Pertaining to kidney tumours, the species and sex-specific alpha 2u globulin mechanism likely responsible for kidney tumours seen in male rats is not regarded as relevant to humans." (ECB, 2003a, p. 223; ECB, 2003b, p. 14)

D. Testicular Dysgenesis Syndrome

On March 5, 2009, OEHHA announced that the Carcinogen Identification Committee (CIC) would provide advice to OEHHA regarding prioritization of 38 chemical for preparation of hazard identification materials.[16] With that notice, OEHHA made available its summary of scientific information on DINP.[17] Under the header "Mechanisms", that summary lists "Testicular dysgenesis syndrome" (TDS), citing to Borch et al. (2004). However, there are no reported studies that have linked exposure to DINP with TDS or testicular cancer in humans. The data base for DINP does not otherwise provide a basis for associating DINP with the hypothetical TDS mechanism or with testicular cancer.

Skakkebaek et al. (2001) first coined the term testicular dysgenesis syndrome (TDS), hypothesizing that abnormal spermatogenesis, cryptorchidism (undescended testicles), penile malformations such as hypospadias and incidences of testicular cancer observed in humans had a common etiology. The hypothesis states that these clinical problems may result from an irreversible developmental disorder occurring early in fetal life consequential to either a genetic predisposition and/or environmental insult(s). Currently, no biological mechanism is defined for TDS, but it is theorized that abnormal spermatogenesis and testicular cancer may be the result of disturbed Sertoli cell function, while hypospadias and cryptorchidism may result from decreased Leydig cell function (Wohlfahrt-Veje et al., 2009).

Several rigorous scientific reviews of DINP, including those of the National Toxicology Program (NTP) Center for the Evaluation of Risks to Human Reproduction (CERHR) (NTP CERHR, 2000), the Consumer Product Safety Commission (CPSC) Chronic Hazard Advisory Panel (CHAP) for DINP (CPSC, 2001) and the European Union Risk Assessment for DINP (ECB, 2003a, b), clearly indicate that exposure to DINP does not induce the symptoms of TDS in humans or laboratory animals. In all of the reproductive toxicity, developmental toxicity and chronic carcinogenicity studies performed with commercial DINP, no symptoms of TDS, including hypospadias, cryptorchidism, or testicular cancer have been reported. These studies are briefly summarized below.

In the chronic 2-year carcinogenicity studies in F344 rats, reported by Moore (1998a) (daily exposure to 0, 500, 1500, 6000, or 12000 ppm) and Lington et al. (1997) (daily exposure

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[16] Prioritization: Chemicals for Consultation by the Carcinogen Identification Committee, 3/5/09, www.oehha.org/prop65/CRNR_notices/state_listing/prioritization_notices/prior030509.html.

[17] Diisononyl Phthalate (DINP), http://www.oehha.org/prop65/CRNR_notices/state_listing/prioritization_notices/pdf/DINP.pdf

to 0, 300, 3000, or 6000 ppm), benign testicular interstitial cell tumors were found in nearly all animals – both controls and those treated chronically with DINP. F344 rats normally display a high incidence of testicular tumors, and the incidences of treated animals in these studies were within the historical control range. Therefore, these studies do not indicate that commercial DINP causes testicular cancer.

A 2-year carcinogenicity study was conducted in Sprague Dawley rats treated with a form of DINP (Santicizer 900, CASRN 71549-78-5) that was never commercially produced (Bio/dynamics, 1986). The rats were exposed daily to 0, 500, 5000, or 10000 ppm of the test substance. The high dose males (the only exposure group examined histopathologically) had an increased incidence of testicular interstitial cell hyperplasia. A slightly higher incidence of interstitial cell tumors (1%) was also observed in the testes of the high dose males in comparison to their concurrent control animals (0%). However, the incidence was lower than that of the historical controls (9.8%). Therefore these findings have questionable toxicological significance. Further, this study has low reliability as a basis for assessing commercial DINP, as it was performed with a DINP mixture that was not well defined and never commercially produced.

In a two generation reproduction and developmental toxicity study, P1 males and females received test material (0.2, 0.4, or 0.8% in the diet) daily for at least ten weeks prior to mating and during the mating period (Waterman et al., 2000). Additionally, P1 female animals received test material during the gestation and postpartum periods, until weaning of the F1 offspring on post natal day (PND) 21. P2(F1) males were dosed from PND 21 for at least 10 weeks prior to mating and through the mating period for F2 litters, until sacrificed following delivery of their last litter sired. P2(F1) females were dosed from PND 21 for at least 10 weeks prior to mating, during mating, gestation, lactation and until they were sacrificed following weaning of the F2 animals on PND 21. There were no statistically significant differences in male mating, male fertility, female fertility, female fecundity or female gestational indices in P1 generation. A slight decrease, not statistically significant, of male mating, male fertility, female fertility and female fecundity indices was observed in P2 generation. Mean days of gestation of the P1/P2 treated and control animals were essentially equivalent. There were no adverse testicular effects reported for either the P1 or P2 generation, and there were no signs of TDS-related effects.

In a one-generation reproductive and developmental toxicity study (Exxon Biomedical Sciences, 1996), rats were administered 0.5, 1, or 1.5% DINP from 10 weeks prior to mating, through gestation and ending on PND 21. Pertaining to P1 male organ toxicity, there was a statistically significant increase in the mean absolute and relative right testis weight, left testis and right epididymis weights and the mean relative left epididymis and seminal vesicle weights in the high-dose males compared with controls. It was not determined if any structural changes occurred in reproductive organs at any dose level; microscopic evaluation was not performed on any organs in both sexes. Thus significance of organ weight changes could not be assessed because of the limitation of the study. However, there were no statistically significant differences in male mating, male fertility, female fertility, female fecundity, or female gestational indices between treated and control animals. There were no instances of testicular tumors.

It has also been proposed that suppression of fetal androgen production and/or increased estrogen exposure might underlie the occurrence of TDS with respect to certain phthalates

(Sharpe, 2003). However, the data for DINP are inconsistent with respect to anti-androgenic effects in young male rats. Two studies, which used an unrealistically high dose of DINP – 750 mg/kg/day, administered by gavage, resulted in a questionably significant increase in malformation of the male reproductive tract (Gray et al., 2000) or decreased testosterone in male rats (Borch et al., 2004). In contrast, no anti-androgenic effects were observed in male offspring of pregnant rats exposed to higher levels of DINP in the diet (Masutomi et al., 2003) or in any of the previously mentioned reproductive toxicity, developmental toxicity and chronic carcinogenicity studies performed with commercial DINP; summarized below.

The study conducted by Gray et al. (2000) shows a low incidence of effects without any dose response and with effects of unclear significance. As infants, male rats were exposed to a single 750 mg/kg dose of DINP between gestation day 14 and post natal day 3. The authors reported males displaying retained areolas (22% reported as statistically significant). No other single endpoint (nipple retention, epididymal agenesis, fluid filled testes and testes weight) on its own was significantly different from control values. However, the authors pooled all observed effects to produce the 7.7% adverse incidence reported in the study. Only by pooling different effects was statistical significance demonstrated. This type of data manipulation is not routinely performed in toxicological safety evaluations, nor is it considered good statistical practice. It should also be noted that Gray et al. (2000) did not observe any effects on anogenital distance or on reduction of testosterone levels in the blood with DINP treated animals. Based on the above points it is unclear whether adverse effects have been found for DINP in this study or not. Importantly, there were no instances of hypospadias or cryptorchidism reported in the study.

Likewise, the paper by Borch et al. (2004) does not present data demonstrating DINP induces TDS and should not be considered as evidence for a mechanism of toxicity. In this report, 32 pregnant female rats were exposed to either 300 mg/kg-bw DEHP or 750 mg/kg-bw DINP, alone or in combination, from gestation day 7 to gestation day 21. The dams were sacrificed on gestation day 21 and the pups were harvested for analysis of testicular testosterone production, testicular testosterone content, plasma testosterone levels and plasma luteinizing hormone (LH) levels. The results indicate that testicular testosterone production and testicular testosterone content were significantly decreased in the DINP exposed pups while plasma testosterone and plasma LH levels were unaltered. However, no mechanism of toxicity can be determined from this paper since it is limited by several confounding factors. First, the dose was administered via a single oral gavage exposure each day of testing. This method of administration can result in the overwhelming of normal detoxifying processes which can lead to overt toxicity. Second, there were no adverse phenotypic effects such as testicular malformations reported in the study. Therefore it is unclear if the decrease in testosterone content is in fact a toxicologically significant response. Third, while DEHP and DINP individually appeared to induce a decrease in testosterone content, there was no indication of a modulating effect of DINP on DEHP when co-administered. Finally, the authors sampled testosterone levels on gestation day 21, a time point after the developmental surge of testosterone that occurs during gestation day 16-18 in the rat. After gestation day 18, plasma testosterone levels are naturally declining in the fetal rat.

As stated by the CERHR expert panel (NTP CERHR, 2000):

Reproductive performance and histological effects on gonads and accessory sex organs were assessed in one- and two-generation dietary studies. Parental doses of up to 0.8% in feed (665–779 [M] and 696–802[F] mg/kg bw/day) did not affect fertility or sex organ histology in either the F0 or F1 male or female pups. A 13-week gavage study in adult marmosets resulted in no evidence of microscopic testicular changes at doses that did adversely affect body weight gain (2,500 mg/kg bw/day). Testicular lesions were not observed in prepubertal cynomolgus monkeys that were gavaged for 2 weeks with 500 mg/kg bw/day, reportedly the maximum dose that can be absorbed by the monkeys. Chronic 2-year studies in rats and mice gave no gross or histologic evidence of effects on testes or ovaries at doses that did cause liver and kidney effects and other clinical signs of toxicity. Thus, the data are sufficient to conclude that neither the reproductive organs nor fertility are affected by extended oral exposure to DINP.

In summary, there is no reliable evidence that commercial DINP induces testicular cancer in laboratory animals or humans, nor that the hypothetical TDS mechanism applies to DINP.

V. EXPERT BODY REVIEWS OF DINP HUMAN CANCER POTENTIAL

DINP has not been listed as a carcinogen, nor even considered for listing, by the International Agency for Research on Cancer (IARC) or the National Toxicology Program (NTP).[18] That being said, DINP has been the subject of other rigorous scientific reviews, which have concluded DINP is unlikely to pose a cancer risk to humans.

- A Chronic Hazard Advisory Panel (CHAP) of the Consumer Product Safety Commission (CPSC), consisting of seven independent experts, held three public meetings in the year 2000 to evaluate the toxicological data for DINP. The CHAP's report was published in 2001 (CPSC, 2001; see also Babich et al., 2004). The CHAP concluded that: the criteria for the $alpha_{2u}$-globulin mechanism were met and therefore the kidney tumors observed in male rats are rat-specific; the MNCL observed in Fisher 344 rats treated with DINP is of questionable significance due to its high and variable background and possible strain specificity; and the liver tumors in rodents are not relevant for human risk assessment because, even if DINP could activate the PPARα mechanism in humans, the dose that would be required to do so is far in excess of any reasonably anticipated human exposures (CPSC, 2001).

- In 2003 a workgroup of the International Life Sciences Institute (ILSI) Risk Science Institute reviewed the relationship of peroxisome proliferation and liver tumors in rodents, publishing its results as Klaunig et al. (2003). This effort was to update the 1995 ILSI workshop on peroxisome proliferation and rodent tumors, reported by Cattley et al. (1998). DINP was one of the peroxisome proliferators used to develop the workgroup's

[18] The Natural Resources Defense Council has nominated DINP for consideration by IARC (IARC, 2008), but to date IARC has not scheduled DINP for consideration (IARC, 2010).

conclusion that the rodent mode of action for liver tumors from such compounds is not relevant to humans.

- The European Union (EU) has conducted a very thorough risk assessment of DINP, with input from governmental scientists throughout Europe (ECB 2003a; 2003b). The EU risk assessment concluded that the liver tumors observed in rodents are due to a peroxisome proliferation process that is not relevant to humans, the kidney tumors in male rats were due to an $alpha_{2u}$-globulin process that is not relevant to humans and the MNCL was a strain-specific effect not relevant to humans (ECB, 2003a, Section 4.1.2.8). On the basis of its review, the EU has concluded that there is no basis to expect human risk of cancer, reproductive or developmental, or any other health effect from exposure to DINP. Accordingly, the EU also has determined that DINP should not be classified or labeled for human health effects, including no cancer designation (EC, 2000).

These consensus opinions support the conclusion that DINP is highly unlikely to cause cancer in humans.

ExxonMobil notes that, while the U.S. Environmental Protection Agency (USEPA) has reviewed the data for DINP, it *has not* made a final determination regarding the carcinogenicity of DINP. USEPA undertook its review in response to a petition to list DINP under Section 313 of the Emergency Planning and Community Right-to-Know Act (EPCRA). OEHHA has provided to the CIC a 2000 Federal Register notice in which USEPA proposed to list DINP in part based on the animal cancer data (Fed. Reg., 2000). However, after receipt of comments, USEPA published a revised notice on June 14, 2005, in which it reserved judgment on the potential for DINP to cause cancer in humans (Fed. Reg., 2005). USEPA accepted further comments and to date has not issued a final decision.

CONCLUSION

For the reasons presented, ExxonMobil believes the data support the conclusion that the cancer findings in rodent bioassays of DINP are not relevant to humans. Further, primate data indicates that primates are refractory to DINP. Therefore, ExxonMobil respectfully submits that DINP should not be listed under Proposition 65 as a carcinogen.

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Attachments

The following attachments are provided as separate pdfs.

A Statement of Ruth Angela Roberts, Ph.D.

B Statement of James Klaunig, Ph.D.

C Statement of Gary Williams, M.D. and Michael Iatropoulos, M.D., Ph.D.

D Statement of Richard Irons, Ph.D.

E Statement of James Swenberg, Ph.D.

Attachment A

Diisononyl Phthalate (DINP): relevance of rodent cancer data to humans

Ruth A Roberts, PhD[1]

1. I am Head of Cell Biology Research and Cancer Project Manager at Syngenta Central Toxicology Laboratory in the UK. I received a PhD in 1987 from the Paterson Institute for Cancer Research, UK followed by post-doctoral training in Molecular Oncology at the Imperial Cancer Research Fund, London, UK. My work focuses on understanding the mechanisms through which peroxisome proliferators contribute to the development of cancer in rodents and whether the mechanism of carcinogenesis for these chemicals will operate in humans. I serve on the UK Government Committee on Carcinogenicity and have published more than 50 scientific articles, many of which address the mechanism of peroxisome proliferator-induced rodent carcinogenesis, including studies of DEHP and DINP.

2. I would like to comment on the EPAs recent preliminary decision to add diisononyl phthalate (DINP) to its list of substances which need to be reported under the Emergency Planning and Community Right to Know Act. A reason given is that DINP could be reasonably anticipated to be carcinogenic in humans.

3. **I disagree with this position since I believe that humans differ from rodents in their response to peroxisome proliferators and weight of evidence supports the position that peroxisome proliferators do not pose a carcinogenic risk to humans.**

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The foregoing opinion is given in my personal capacity as an expert in cancer toxicology and not in my capacity as an employee of Syngenta Central Toxicology Laboratory and does not necessarily reflect the opinion of Syngenta Central Toxicology Laboratory.

4. **The evidence consists of four key points:**

   a) peroxisome proliferators cause peroxisome proliferation, cell proliferation and suppression of apoptosis in rat and mouse hepatocytes

   b) this represents a mode of action (MOA) for the rodent liver tumors

   c) human hepatocytes are resistant to the effects of peroxisome proliferators seen in rodents hepatocytes so it is reasonable to assume that the downstream tumorigenic response seen in rodents will not occur in humans

   d) specifically, DINP is unable to induce peroxisome proliferation, cell proliferation or to suppress apoptosis in donor human hepatocytes in vitro (1).

5. There is no evidence and only speculation to contest points a-d.

6. Scientific consensus supports a relationship between the growth perturbation caused by peroxisome proliferators and liver tumors. This is explained by considering the multistage process by which a normal cell progresses through to malignancy. A cell sustains DNA damage either spontaneously or via exposure to genotoxic chemicals. This damage can be repaired or can be fixed into the genome by missense/mismatch mutation during DNA synthesis. If the damage is fixed into a gene key for cell growth control, the cell is referred to as initiated. The growth of this initiated cell can then be promoted by further rounds of cell replication to form a focus which may ultimately progress to a tumor. Conversely to cell proliferation, apoptosis or programmed cell death can remove unwanted or potentially damaged cells from the cell pool, protecting from cancer and reducing the pool size of initiated cells (2). Thus, proliferation and apoptosis regulate the fixation and ultimately the expression of DNA damage as a tumor (3). It follows, therefore,

that a chemical that can stimulate proliferation and suppress apoptosis is likely to cause cancer.

7. DINP belongs to the peroxisome proliferator class of rodent hepatocarcinogens. In rodent liver both *in vivo* and *in vitro*, PPs cause hepatocyte DNA synthesis and cell proliferation and suppress the apoptosis that may normally serve to remove damaged cells (reviewed in 4). As expected, DINP induces cell proliferation and suppresses apoptosis in rat hepatocytes *in vitro* as well as causing the increase in β-oxidation associated with peroxisome proliferation (1). This growth perturbation provides a mode of action for peroxisome proliferator-induced rodent hepatocarcinogenesis.

8. The effects of PPs in the rodent are mediated by the peroxisome proliferator activated receptor α (PPARα) since transgenic mice where the PPARα gene is deleted are refractory to PP-induced liver enlargement, proliferation and carcinogenesis (5-7). *In vitro*, PPs can no longer induce DNA synthesis nor suppress apoptosis in hepatocytes isolated from PPARα null mice (8,9).

9. Despite the lack of human hepatocyte response to the adverse effects of peroxisome proliferators, humans do show a therapeutic response to the fibrate peroxisome proliferators via an alteration in lipid metabolism (10). Such marked species differences in response to peroxisome proliferators probably arise from both quantitative and qualitative differences in PPARα, the receptor that mediates the response to peroxisome proliferators.

10. PPARα binds to the promoters regions of peroxisome proliferator responsive genes at a specific DNA sequence known as a peroxisome proliferator response element (PPRE). Analysis of human liver samples provides evidence to support the quantitative hypothesis since there is much less PPARα compared with rat or mouse liver (11). In human liver, levels of PPARα may be sufficient to mediate hypolipidaemia but insufficient to activate

the full gene battery associated with the rodent response (12). These lower levels of full length PPARα could be explained by splicing errors since a truncated, inactive form of PPARα, hPPARα8/14 results from skipping at exon 6 has been cloned from human liver (13) and is present in all the human livers examined to date (4,14).

11. In addition to quantitative differences in PPARα, species differences could be attributed to qualitative differences in the PPARα-mediated response. The rat ACO gene is a marker for rodent peroxisome proliferation and is switched on by PPARα (15). As expected, the rat ACO gene contains a PPRE that acts as a binding site for PPARα, allowing the rat ACO gene to be switched on by peroxisome proliferators (15). However, the promoter for human ACO differs in sequence from the rat equivalent and we find that this human sequence cannot be switched on by peroxisome proliferators (16,17). Thus, even in the presence of sufficient PPARα, the human equivalent of rodent genes associated with peroxisome proliferation may remain inactive.

12. These data on quantitative and qualitative differences in PPARα-mediated responses contribute to our understanding of how chemicals may cause tumours in rodents and how this response may differ in humans.

13. In summary, peroxisome proliferators such as DINP alter the rates of proliferation and apoptosis, providing a MOA for peroxisome proliferator-induced carcinogenesis. Human hepatocytes are resistant to peroxisome proliferator-induced growth perturbation providing evidence that the rodent MOA does not operate in humans. These marked species differences in response to PPs are probably due both to quantity of PPARα and the quality of the PPARα-mediated response. Thus, weight of evidence supports the position that the rodent liver tumors caused by peroxisome proliferators such as DINP are not relevant to man since we differ from rodents at the molecular level in our response to peroxisome proliferators.

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Attachment B

INDIANA UNIVERSITY

SCHOOL OF MEDICINE

January 26,2001

Ms. Marian Stanley
American Chemistry Council
1300 Wilson Blvd.
Arlington, VA 22209

703-741-5623

Re: Comments on Proposed Rule "Addition of Di-isononyl Phthalate Category; Community Right-to-Know Toxic Chemical Reporting

Dear Ms. Stanley:

I am writing in address to the EPA proposal to add "Di-isononyl phthalate category" to the list of toxic chemicals subject to the reporting requirements under EPCRA section 313 and section 6607 of the Pollution Prevention Act (PPA). Specifically, I would like to comment on the EPA review of the available rodent liver tumor data and the subsequent determination by the EPA that DINP can be reasonably anticipated to cause cancer in humans. Based on, among other things, data produced in my laboratory, it is clear that DINP produces peroxisomal proliferation in rat and mouse liver. It appears that peroxisome proliferation may be a necessary step in liver tumor development in these two species by DINP and other members of the class of peroxisome proliferators. In contrast to that seen in rodents, there is no evidence of peroxisomal proliferation in primate liver or in primate or human liver cells in culture. Therefore the apparent mechanism by which DINP increases the incidence of hepatocellular tumors in rats and mice is not relevant to humans. The International Agency for Research in Cancer recently reached a similar conclusion in its review of another member of the peroxisome proliferating group of compounds, di-2 (ethylhexyl) phthalate (DEHP) (IARC, 2000).

Extensive evidence exists that chemicals in the class of peroxisome proliferators are hepatocarcinogenic in rats and mice (e.g., Ashby et al., 1994; Cattley et al., 1998; IARC, 1995). The carcinogenicity of peroxisome proliferators appears to be dependent on the presence of the peroxisome proliferator-activated receptor alpha (PPARa). Recent work has demonstrated that wild type mice develop liver changes consistent with peroxisomal proliferation and ultimately liver tumors whereas there are no effects in livers of mice that lack PPARα (Peters et al., 1997; Ward et al., 1998). DINP is a peroxisome proliferator in rats and mice (Ashby et al., 1994; Barber et al., 1987; Butala et al., 1996; 1997; Lington et al., 1997; Smith et al., 2000; Valles et al., 2000). From these data it appears that the rodent tumors induced by DINP are associated with the peroxisomal proliferation process.

My laboratory for over 20 years has been involved in understanding the mechanisms of hepatic chemical carcinogenesis. A specific interest of my laboratory has been to investigate the cellular mechanisms by which nongenotoxic agents such as peroxisome proliferators induce neoplastic development in the rodent liver. We have examined several important endpoints that appear to be involved in the nongenotoxic carcinogenic process, including, induction of cell proliferation, modulation of gene expression, inhibition of cell to cell communication via gap junctions and induction of peroxisome proliferation. All of these effects have been shown by laboratory investigations to be important in the process of nongenotoxic carcinogenesis.

DEPARMENT OF PHARMACOLOGY AND TOXICOLOGY

DIVISION OF TOXICOLOGY

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We have recently examined the effect of phthalate esters (specifically DEHP and DINP) on these important effects in rodent and primate systems (both in vivo and in vitro). The in vivo studies demonstrated a correlation in the rodents to the induction of peroxisome proliferation, increased hepatic cell proliferation, inhibition of gap junctional intercellular communication and phthalate ester induced hepatic tumorigenesis. In vitro studies employing primary cultured hepatocytes from rodents and primates showed that DINP and DEHP similarly modified these parameters in rodent hepatocytes in a dose dependent manner but had no effect on these parameters in primate hepatocytes. These data provide evidence that the mechanisms involved in phthalate ester induction of rodent liver tumors would not occur in humans. Therefore these compounds (specifically DINP) would not induce neoplasia in humans and thus the phthalate ester rodent tumorigenicity data should not be used as a basis for human health risk assessment.

It is useful to review the views of the International Agency for Research in Cancer on this subject. Specifically the International Agency for Research in Cancer determined in 1995 that it could be concluded that liver tumors in rats and mice were a secondary to peroxisomal proliferation if:

(a) other mechanisms of carcinogenesis could be excluded,
(b) peroxisome proliferation could be demonstrated under the conditions of the bioassay, and
(c) such effects were not found in adequately designed and conducted investigations of human groups and systems.

In its review of DEHP earlier this year, the International Agency for Research in Cancer determined that this final criterion could be met through studies of human hepatocytes under in vitro conditions.

In recently published studies from my laboratory, DEHP (20,000 mg/kg diet) was given to F344 rats for one or two weeks. DEHP containing diet was then removed for one or two weeks. Relative liver weights, peroxisomal enzyme activity, and rates of DNA synthesis were all significantly elevated and inhibition of gap junctional intercellular communication (GJIC) was observed during the DEHP treatment. When DEHP was removed from the diet, all of these parameters returned to levels at or near control values (Isenberg et al., 2000).

A similar experiment with DINP in my laboratory demonstrated that 12000 mg/kg diet DINP (the tumorigenic dose in chronically treated F344 rats) also significantly elevated liver weights, peroxisomal enzyme activity and DNA synthesis and inhibited GJIC. These parameters were also elevated in B6C3F1 mice given DINP at dietary levels of 6000 mg/kg diet (Smith et al., 2000).

To directly assess the effects of DINP (and DEHP) in non-human primates, adult male cynomolgus monkeys were given 500 mg/kg DINP daily by intragastric administration. After 14 days of treatment, animals were killed and examined for pathologic changes, GJIC, and peroxisomal proliferation. No effects on liver, kidney or testes weights were observed. Similarly no pathological changes and no evidence of peroxisomal proliferation or GJIC

inhibition were observed (Pugh et al., 2000). These findings agree with other reported studies with DEHP (Kurata et al., 1997) and DINP (Hall et al., 1999) in marmosets at levels up to 2500 mg/kg/day treatment for 90 days.

To further assess whether these species differences could be demonstrated under in vitro conditions, studies of peroxisomal proliferation and inhibition of GJIC were carried out using hepatoctytes from rats, mice, cynomolgus monkeys and humans. Human liver cells, and primary human hepatocytes were also examined. MEHP, the principal metabolite of DEHP, elevated peroxisomal enzyme activity in rodent but not primate cells and similarly inhibited GJIC in rat and mouse hepatocytes but had no effects on these parameters in monkey or human hepatocytes or in human liver cells (Baker et al., 1995; Klaunig, unpublished data, attached).

The results with MINP, the principal metabolite of DINP, were similar. Peroxisomal enzyme levels were elevated in rat and mouse but unaffected in humans. There was also clear evidence of inhibition of GJIC in rat and mouse hepatocytes but monkey and human hepatocytes were unaffected (Klaunig, unpublished data, attached).

In summary, DINP increased the frequency of liver tumors in rats and mice at high doses. It also induced peroxisomal proliferation and inhibited GJIC in the same species. DINP had no effects on these parameters in primate liver at levels that were clear effect levels in rodents. Similar results were found in in vitro studies; peroxisomal enzymes were elevated and GJIC inhibited in rat and mouse hepatocytes but there were no effects in monkey or human hepatocytes.

In summary, these data provide mechanistic evidence that rodent liver tumor induction by DINP is associated with a peroxisomal proliferation process which does not occur in humans or other primates.

Sincerely,

[redacted]

James E Klaunig, Ph.D.
Professor and Director of Toxicology

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Species Specificity of DINP- induced Peroxisomal Proliferation and Inhibition of Gap Junctional Intercellular Communication

James E. Klaunig, Ph.D
Professor and Director of Toxicology
Indiana University School of Medicine
Indianapolis, Indiana

Species Specificity of DINP- induced Peroxisomal Proliferation and Inhibition of Gap Junctional Intercellular Communication

- IARC (1995)
  - questioned the relevance to humans of rodent liver tumors resulting from peroxisomal proliferation processes
- IARC (2000)
  - provided the criteria to determine whether liver tumors were the consequence of peroxisomal proliferation in its evaluation of the tumor-response data for DEHP
  - One criteria was a demonstration that these effects are rodent specific and not expressed in primates

- Studies in my laboratory over the past 20 years have examined the comparative species specificity of chemical carcinogenesis. Recent studies have included studies with peroxisome proliferators including DEHP and DINP.

Species Specificity of DINP- induced Peroxisomal Proliferation and Inhibition of Gap Junctional Intercellular Communication

Nongenotoxic Carcinogenesis

involves:

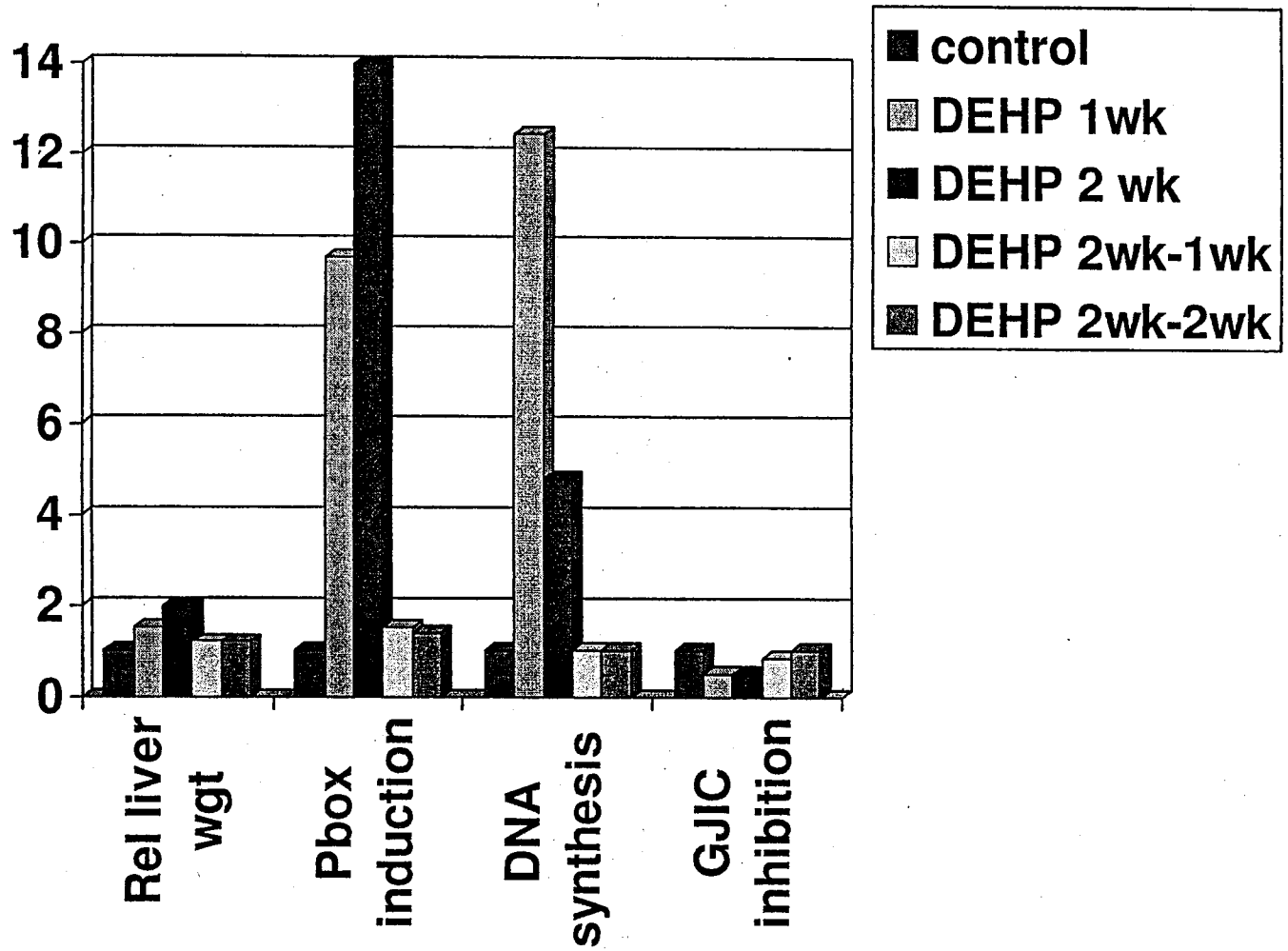
- modulation of cell growth
  - cell proliferation
  - cell death (apoptosis)
- changes in cell to cell communication (gap junctional communciation)
- modification of gene expression
- induction of peroxisomal proliferation

EFFECTS OF DEHP (20,000 PPM) ON F344 RATS[1]

	Relative Liver Weight	PBOX Induction	DNA Synthesis	GJIC Inhibition
+ DEHP (1 week)	1.5x*	9.7x*	12.5x*	0.57x*
+ DEHP (2 weeks)	2.0x*	14x*	4.8x*	0.57x*
+ DEHP (2 weeks) – DEHP (weeks)	1.2x*	1.5x	1.0x	0.85x*
+ DEHP (2 weeks) – DEHP (2 weeks)	1.2x*	1.4x*	1.0x	1.0x

[1] Isenberg et al., (2000) * - $P \leq 0.05$

EFFECTS OF DEHP (20,000 PPM) ON F344 RAT HEPATIC ENDPOINTS

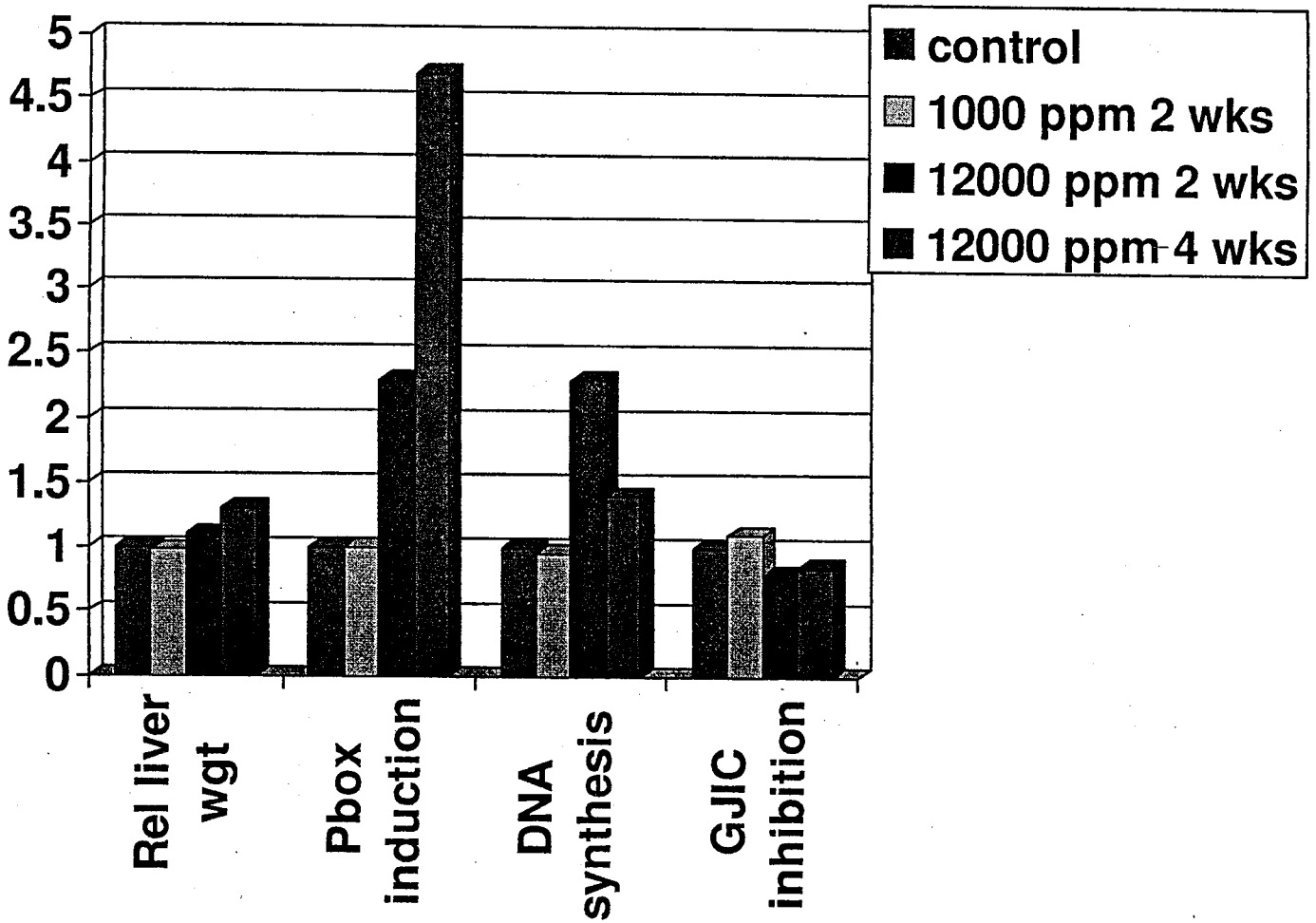


EFFECTS OF DINP ON F344 RAT LIVER[1]

	Relative Liver Weight	PBOX Induction	DNA Synthesis	GJIC Inhibition
1000 ppm (2 Weeks)	0.98x	1.0x	0.96x	1.1x
12,000 ppm (2 Weeks)	1.1x	2.3x*	2.3x*	0.79x*
12,000 ppm (4 Weeks)	1.3x*	4.7x*	1.4x	0.85x

[1] Smith et al., 2000 * - $P \leq 0.05$

EFFECTS OF DINP ON F344 RAT HEPATIC ENDPOINTS



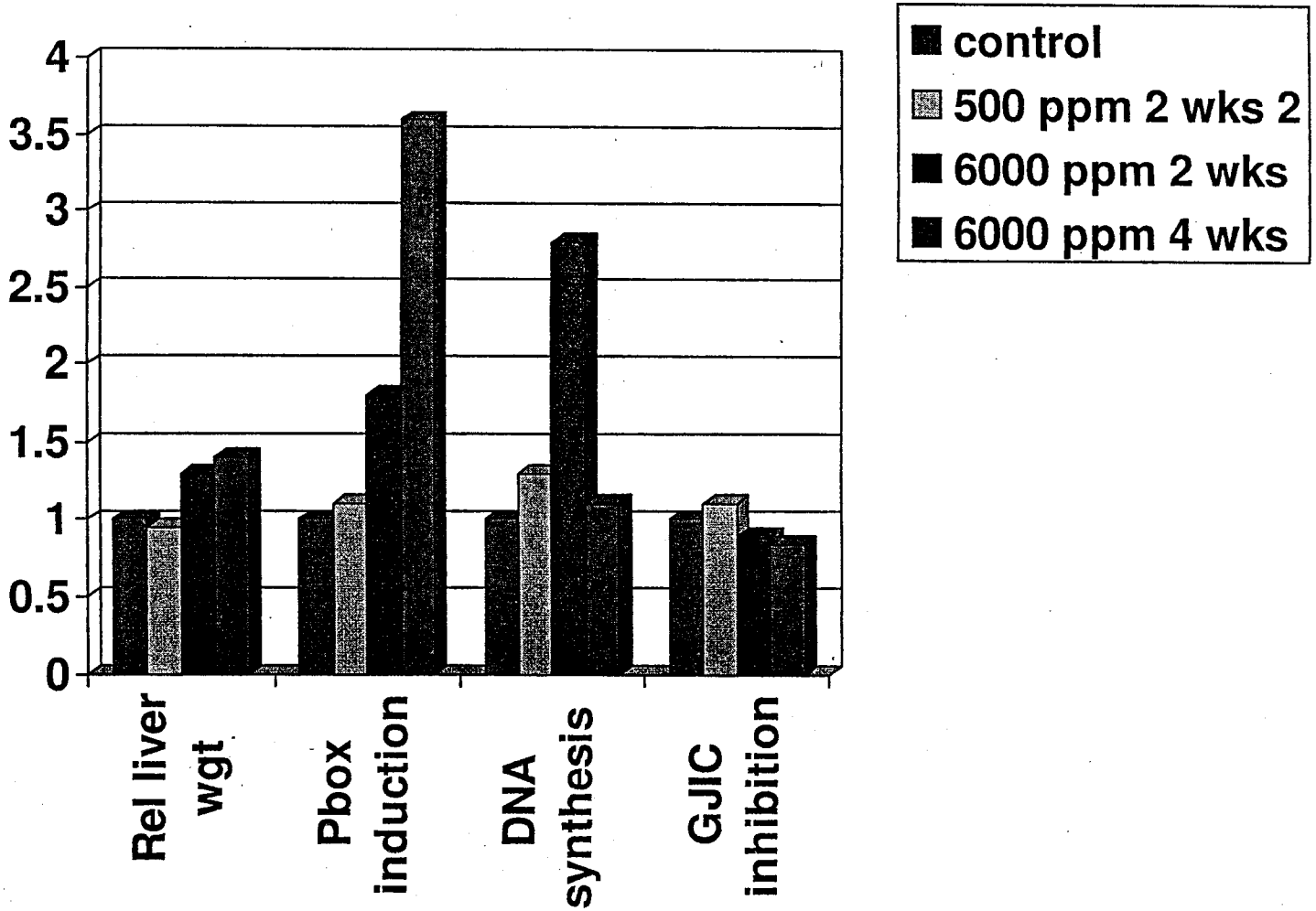
EFFECTS OF DINP ON B6C3F1 MOUSE LIVER[1]

	Relative Liver Weight	PBOX Induction	DNA Synthesis	GJIC Inhibition
500 ppm (2 Weeks)	0.95x	1.1x	1.3x	1.1x
6000 ppm (2 Weeks)	1.3x*	1.8x*	2.8x*	0.89x
6000 ppm (4 Weeks)	1.4x*	3.6x*	1.1x	0.84x*

[1] Smith et al., 2000

* - $P \leq 0.05$

EFFECTS OF DINP ON B6C3F1 MOUSE HEPATIC ENDPOINTS



EFFECTS OF DINP AND DEHP ON PRIMATES[1]

- Adult male cynomolgus monkeys
- DINP (500 mg/kg) or DEHP (500 mg/kg) daily for 14 days
- No effects on liver, kidney or testes weights
- No pathological changes in liver, kidneys or testes
- No differences in DNA synthesis, PBOX induction or GJIC inhibition

  Similar results in 90 day studies in marmosets for both DEHP (Kurata et al., 1997) and DINP (Hall et al., 1999)

[1] Pugh et al., (2000)

EFFECTS OF MEHP ON GJIC[1] (IN VITRO STUDIES)

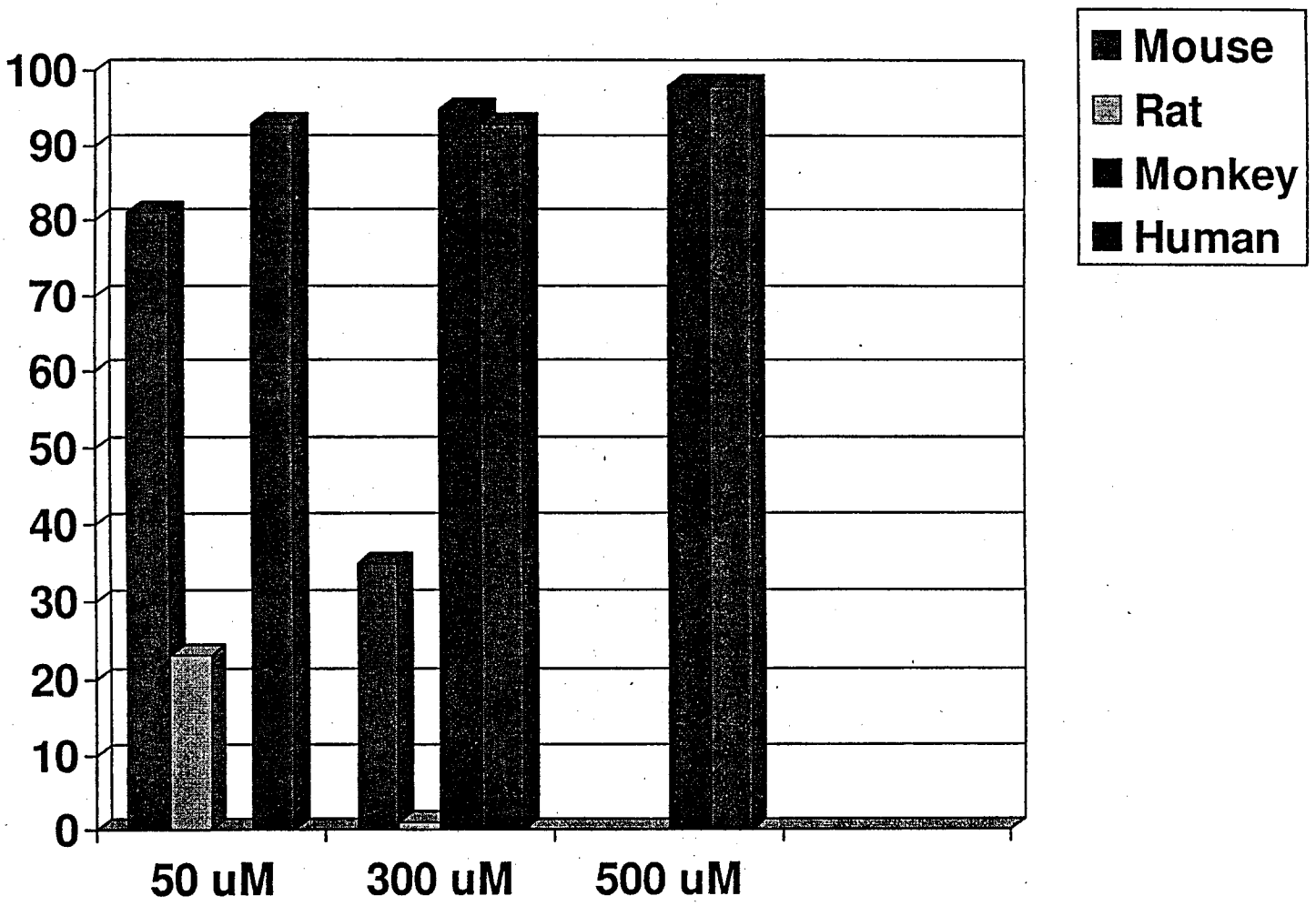
	Mouse	Rat	Monkey	Human
50 uM	81%*	23%*	Not Tested	93%
300 uM	35%*	1%*	95%	93%/93%[2]
500 uM	Not Tested	Not Tested	98%	98%

[1] Kamendulis et al. (in preparation)

[2] Data given or primary human hepatocytes / established cell line

* - $P \leq 0.05$

EFFECTS OF MEHP ON GJIC
(IN VITRO STUDIES)



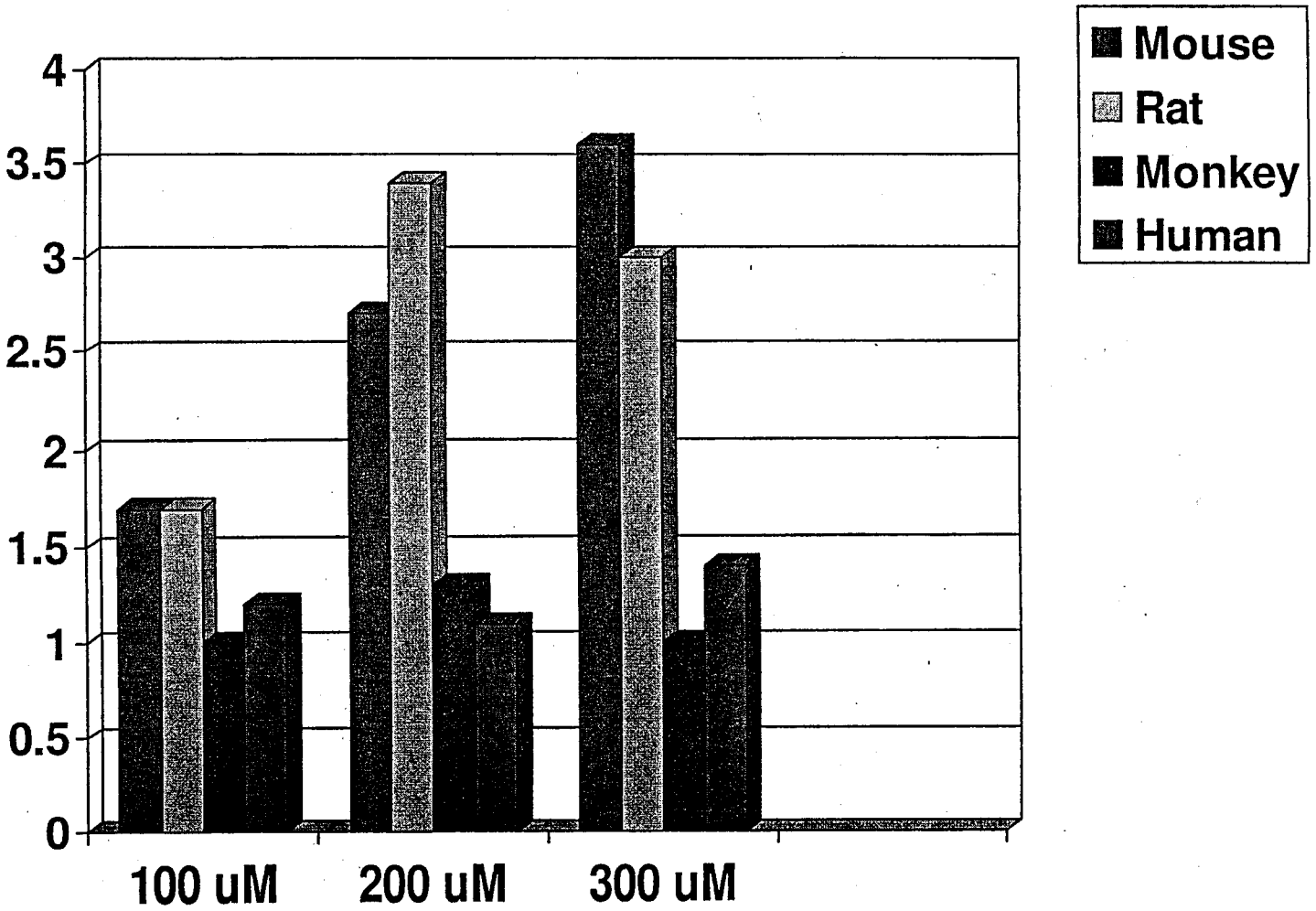
EFFECTS OF MEHP ON PBOX INDUCTION[1] (IN VITRO STUDIES)

	Mouse	Rat	Monkey	Human
100 uM	1.7x	1.7x	1.0x	1.2x
200 uM	2.7x	3.4x	1.3x	1.1x
300 uM	3.6x*	3.0x*	1.0x	1.5x

[1] Kamendulis et al. (in preparation)

* - $P \leq 0.05$

EFFECTS OF MEHP ON HEPATOCYTE PBOX INDUCTION (IN VITRO STUDIES)



EFFECTS OF MINP ON GJIC INHIBITION[1] (IN VITRO STUDIES)

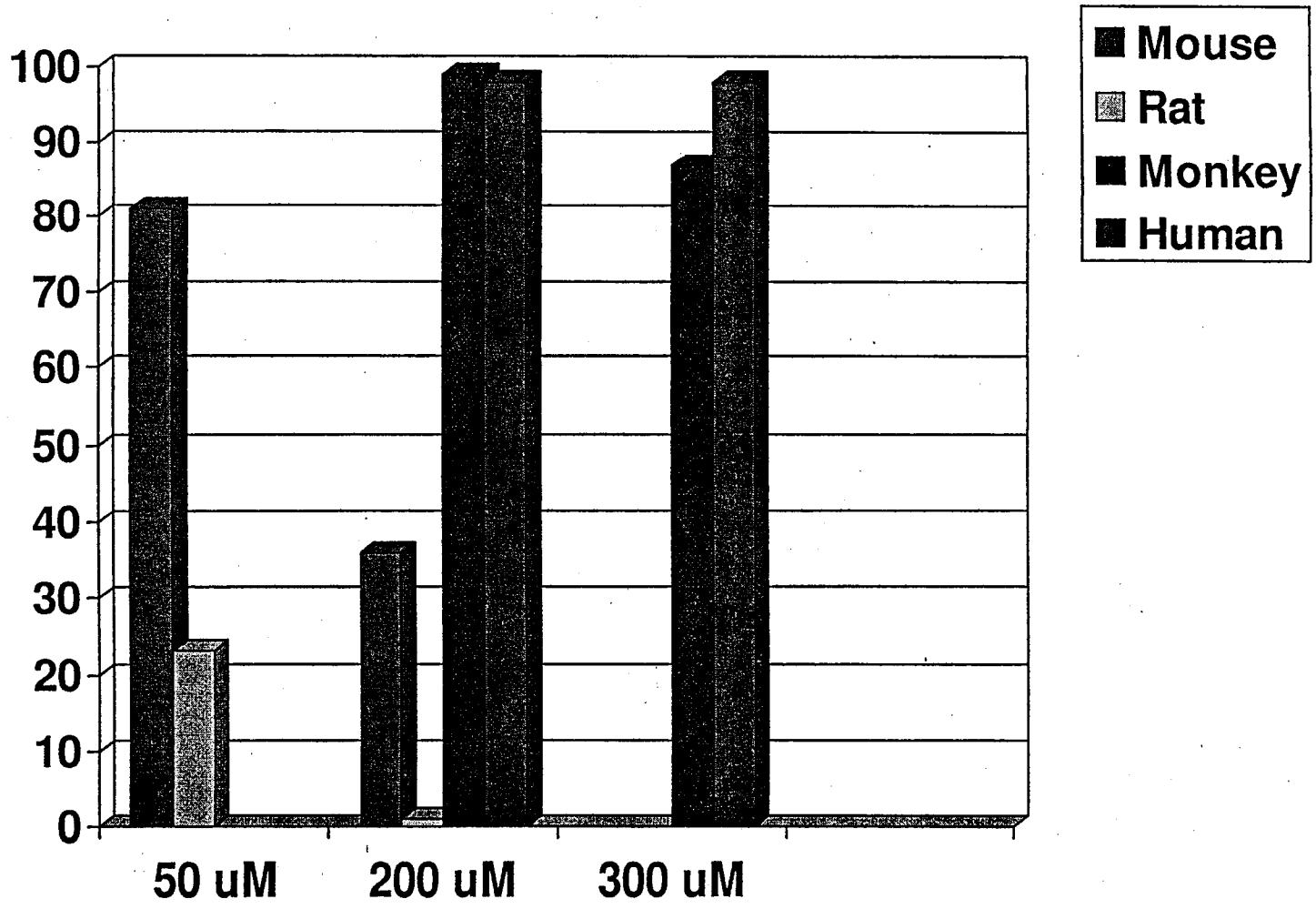
	Mouse	Rat	Monkey	Human
50 uM	81%*	23%*	Not Tested	Not Tested
200 uM	36%*	1%*	99%	93%/98%[2]
300 uM	Not Tested	Not Tested	87%	Not Tested/98 %

[1] Kamendulis et al. (in preparation)

[2] Results given as primary human hepatocytes / established cell line

* - $P \leq 0.05$

EFFECTS OF MINP ON HEPATIC GJIC (IN VITRO STUDIES)



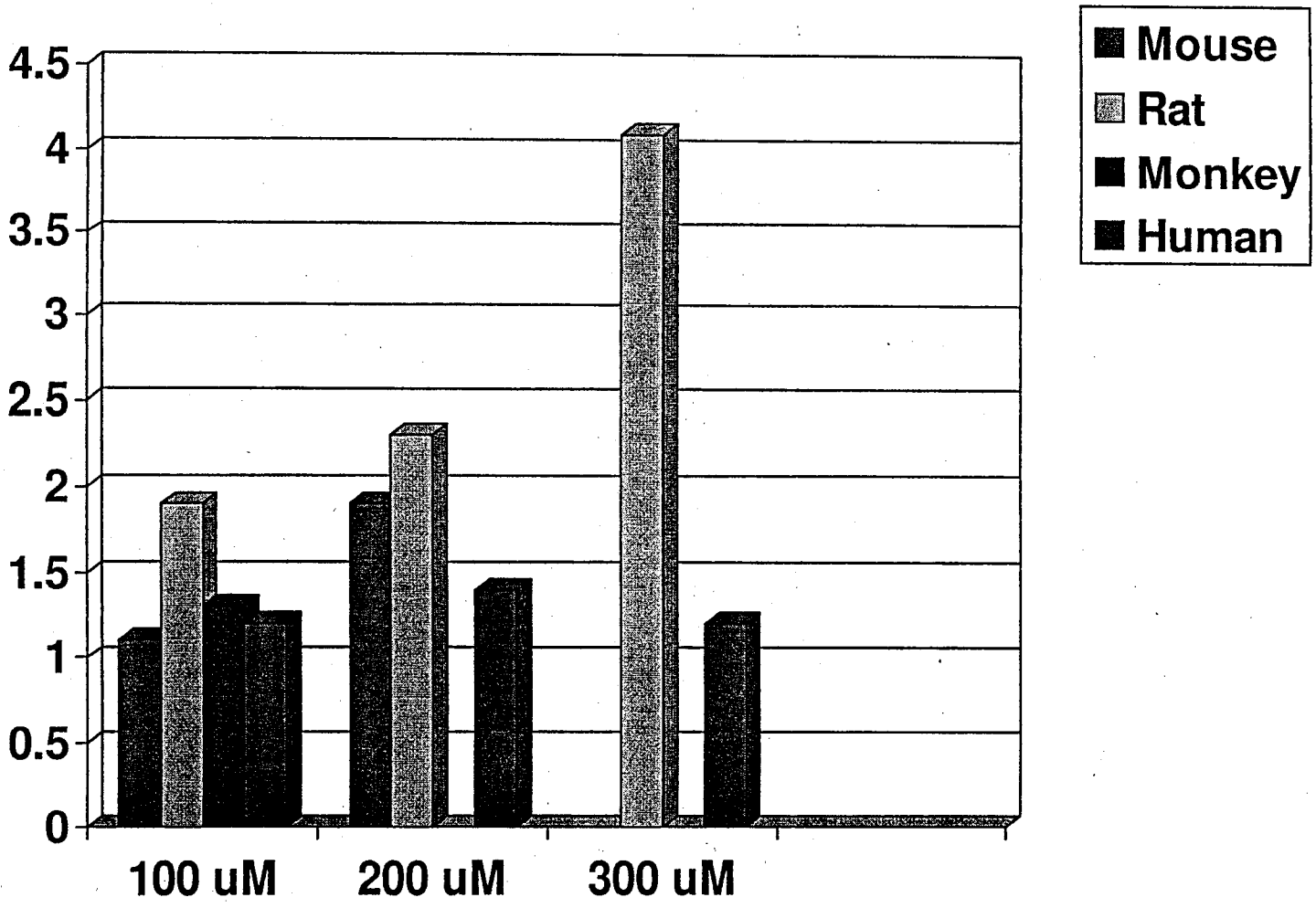
EFFECTS OF MINP ON PBOX INDUCTION[1] (IN VITRO STUDIES)

	Mouse	Rat	Monkey	Human
100 uM	1.1x	1.9x	1.3x	1.2x
200 uM	1.9x	2.3x	Toxic	1.4x
300 uM	Toxic	4.1x*	Toxic	1.2x

[1] Kamendulis et al. (in preparation)

* - $P \leq 0.05$

EFFECTS OF MINP ON HEPATOCYTE PBOX INDUCTION (IN VITRO STUDIES)



SPECIES SPECIFICITY OF LIVER EFFECTS OF DINP

- DINP Induces Liver Tumors at High Doses in Rats and Mice
- DINP Induces Peroxisomal Proliferation and Inhibits GJIC in Rat and Mouse Liver
- DINP Has No Effects in Primate Liver at Rat and Mouse Effect Levels
- DINP Induces Peroxisomal Proliferation and Inhibits GJIC in Rat and Mouse Hepatocytes in vitro but not in Monkey or Human Hepatocytes
- These Data Provide Mechanistic Evidence that Rodent Liver Tumor Induction by DINP would not Occur in Humans or Other Primates

Attachment C

Cancer Risk Assessment
Di(isononyl) phthalate

Prepared for the
American Chemistry Council

by

Gary M. Williams, M.D.
Professor of Pathology
Director, Environmental Pathology and Toxicology

and

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New York Medical College
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June 13, 2000

STRUCTURE

Di(isononyl)phthalate (DINP) (Fig. 1) is a complex substance containing isomeric mixture of diesters of ortho-phthalic acid and primary aliphatic branched chain alcohols, predominantly of the C_9 chain length. Currently, two principal types of DINP are available in commerce. The main difference is related to the isomeric distribution. The processes of production are stable for both, with their isomeric distributions consistent over time. DINP-1 is CAS RN 68515-48-0 and DINP-2 is CAS RN 28553-12-0. For both, the principal constituents of the alcohol feed stocks are dimethyl heptanols and methyl octanols. The alkyl side chains are primarily comprised of 7-9 carbon chains with 0-2 methyl groups.

No potentially DNA-reactive structure is present in DINP, MINP and other components of the mixture, not is there any evidence that any are created by metabolic processes.

GENOTOXICITY TESTING

DINP has been extensively tested for genotoxicity by a variety of endpoints (Table 1).

DINP was tested in the Salmonella/histidine locus mutagenicity assay using the preincubation procedure (Zeiger et al, 1985) (Table 1). In tester strains TA 100, TA 1535, TA 1537, and TA 98 at up to 10,000 µg/plate, no increase in revertants occurred without exogenous activation or in the presence of either Aroclor 1254-induced liver 9000Xg supernatant (S-9 fraction) from male Sprague-Dawley rats or Syrian hamsters.

In another study, DINP was tested in the Salmonella/histidine locus mutagenicity assay (McKee et al, 2000). In a plate incorporation test, DINP at up to 5000 µg/plate either without an activation system or with a metabolic activating mix (S-9) prepared from Aroclor-induced rat liver, produced no increase in revertants in strains TA 98, TA 100, TA 1535, TA 1532 and TA 1538, whereas positive controls were active. A preincubation assay using strains TA 98, TA 100, TA 1535 and TA 1537 was also negative both without and with an activation system, while positive controls were active.

DINP was tested in the L51784 mouse lymphoma cell/thymidine kinase locus mutagenicity assay (Barber et al, 2000). DINP at up to 8 µl/ml without exogenous activation

and at up to 0.600 µl/ml in the presence of a rat liver S-9 activation system produced no increase in mutant frequency, while the positive controls ethylmethane sulfonate and 3-methylcholanthrene were active.

DINP was tested in Chinese hamster ovary (CHO) cells for chromosome aberrations (McKee et al, 2000). Cells were exposed in one test for 3 hours either without exogenous metabolic activation or in the presence of an Aroclor-induced rat liver S-9 preparation. In a second test, the exposure was 3 hours with the activation system and 20 hours without. At up to 160 µg/ml, DINP produced no increase in aberrant cells, while the positive controls were active.

DINP was tested in the Balb/3T3 cell transformation assay (Barber et al, 2000). Cells were incubated with concentrations of up to 3.750 µl/ml for 3 days. No increase in transformed foci was found, in contrast to the positive controls 3-methycholanthrene and methyl-N-nitro-N´-nitrosoguanidine.

DINP was tested in groups of 5 male and female CD-1 mice for bone marrow micronucleus induction (McKee et al, 2000). DINP was given by gavage and 3 days later femoral bone marrow was harvested for analysis. Doses of up to 5000 mg/kg produced no increase in micronucleated polychromatophilic erythrocytes in bone marrow, while the positive control cyclophosphamide was active.

In summary, DINP was negative in all systems studied.

ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION (ADME)

The ADME of ^{14}C-DINP were investigated in male and female F344 rats exposed to a single oral dose of 50 or 500 mg/kg. In addition, in males, 5 daily oral doses of 50, 150, and 500 mg/kg bw were administered (El-hawari et al, 1983). A single oral dose of 50 mg/kg was readily absorbed and distributed mainly to the liver and then systemically to the kidneys and other tissues. Thus ^{14}C-DINP is absorbed via the portal system initially. A single oral dose of 500 mg/kg, or all 3 five daily doses , showed saturation of absorption. The highest radioactivity was in the liver, followed by kidneys, then blood. Other tissues including fat had low levels of radioactivity. At all exposures, radioactivity rapidly disappeared from the liver, kidneys or blood, reaching low levels at 72 hours. DINP was de-esterified to MINP, which was

further metabolized by side-chain oxidation of the ester or by hydrolysis to polar phthalic acid (PA). High dose levels seem to favor formation of oxidation products of MINP over hydrolysis to PA. DINP metabolites were readily excreted in urine (60-80%) and to a lesser extent in the feces (20-40%). Non absorbed DINP, or bile-excreted DINP and its liver metabolites, were metabolized in the intestine of male rats to MINP and its oxidation products (80%), whereas in female rats the diesters (DINP) and monoesters (MINP) were excreted (80%).

In male B6C3F1 mice and F344 rats fed DINP at 500 and 6000 ppm and 1000 and 12,000 ppm in the diet, respectively, for up to 4 weeks, analysis of DINP and metabolites revealed that the levels of the compound and its metabolites were not dose proportional in either liver or serum (Smith et al, 2000), indicating saturation of absorption. Moreover, in the serum of rats and mice at 2 and 4 weeks, only MINP and PA were present but not DINP, whereas in the liver, DINP was present. The concentrations of MINP-1 in liver and serum were greater after 4 weeks than at 2 weeks of exposure, indicating that steady state conditions had not been achieved.

In summary, DINP is highly absorbed, readily metabolized and excreted.

SUBCHRONIC TOXICITY

Groups of 5 F344 male and female rats fed DINP diet at 600, 1200 and 2500 ppm for 3 weeks (BIBRA, 1985; Barber et al, 1987). The high dose produced about a 12-fold increase in activity of the peroxisome marker cyanide-insensitive palmitoyl-CoA oxidase or fatty acid oxidase (FAO). DINP was ranked as a slightly less potent peroxisome proliferator than DEHP (Barber et al, 1987).

A 13- week dietary study was conducted in male and female F344 rats at doses of 0, 1000, 3000, 6000, 10,000 and 20,000 ppm DINP in the diet (Bird et al, 1986). These represent approximately 50, 150, 320, 530 and 1260 mg/kg bw/day, respectively. Decreases in body weight gain were present in the high dose group of both genders. Liver weight increase, hepatocellular hypertrophy at doses 3000 ppm and above were present in both males and females. Moreover, nephrosis was observed in male rats in a dose-related fashion. Increased

numbers of peroxisomes in liver cells were confirmed ultrastructurally in the two highest doses in both genders, being more prominent in males.

Groups of 10 F344 male and female rats were fed DINP diet at 0, 2500, 5000, 10,000 and 20,000 ppm for 13 weeks (Myers, 1991). Based on the average consumed dose, DINP doses were 146, 292, 584 and 1168 mg/kg bw/day in males, and 182, 364, 728 and 1456 mg/kg bw/day in females. No mortality occurred. There was a decrease in body weight gain in high dose males and females. Anemia was present at 5000 ppm and above in both genders. Increases in serum albumin and decreases in serum globulin were present in both mid-high and high dose groups of both genders. Liver weight increases were present in all groups except the low dose groups. Hepatocellular hypertrophy was present in both genders of the high dose group. Increased renal weight and BUN were present in high dose males and females. Increases in regenerative basophilic renal tubule cells were present in males of all groups except in the low dose group.

A 13-week dietary study was conducted in male and female B6C3F1 mice (10/group/gender) at doses of 0, 1500, 4000, 10,000 and 20,000 ppm DINP in the diet (Wolfe, 1992). These doses represent approximately 365, 972, 2600 and 5770 mg/kg bw/day, respectively. In addition, 15 mice /group/gender received WY14643 as a positive control group for 3, 30 and 90 to study hepatocellular proliferation and peroxisomal proliferation. There was no exposure-related effect on survival. Body weight gain decreases were present from week 6 until the end of the study in the two top dose groups of both genders. Significant organ weight changes included the uterus (decrease), spleen (decrease), kidney (decrease in males and increase in females) and testes (decrease) in high dose animals. Liver weights were increased in groups 3, 4 and 5 of both sexes. AST and ALT were increased in high dose males, along with an increase in urine volume in groups 4 and 5 males. Hepatocellular enlargement and tubular nephrosis, and lymphoid depletion in the spleen and thymus were present in the high dose groups of both genders, along with uterine hyperplasia, absence of corpora lutea and immature sperm in epididymides of group 5 animals. The labeling index for liver cells was not increased, but there was increased FAO activity in hepatocytes. The positive control

WY14643 was active in producing increases in both hepatocellular proliferation and FAO activity.

Groups of five male B6C3F1 mice and F344 rats were fed DINP at 500 and 6000 ppm diet and 1000 and 12,000 ppm diet, respectively, for up to 4 weeks (Smith et al, 2000). In mice, DINP produced liver weight increases at the high dose at 2 and 4 weeks. In rats, the high dose produced an increase at 4 weeks. FAO was increased in mice at the high dose by about 4-fold at two weeks and by 5-fold at 4 weeks. In rats, the increase in the high dose group was about 3-fold at 2 weeks, and 5-fold at 4 weeks. The BrdU labeling index was increased in high dose mice and rats by about 2-fold only at 2 weeks. Analysis of DINP and metabolites revealed that at both 2 and 4 weeks in mice, DINP and MINP were detectable in liver while only MINP was detectable in serum. The concentrations in liver were related to dose and tended to increase from 2 to 4 weeks. Likewise in rats, DINP and MINP were detectable in liver while only MINP was detectable in serum. The concentration of MINP was related to dose and increased from 2 to 4 weeks.

Groups of 4 male and female marmosets approximately 16-25 months of age were administered DINP at up to 2500 mg/kg/day by gavage for 13 weeks (Hall et al, 1999). There were no gross or histologic changes in liver, kidneys or testes. No increase in the FAO activity or cytochrome P450 concentration were found, in contrast to a 2-fold increase in FAO activity by the positive control clofibrate.

Groups of 4 young adult (about 2 years old) male cynomolgus monkeys were administered DINP at 500 mg/kg/day by nasogastric feeding tube for 14 days (Pugh et al, 2000). There was no effect on liver weight, liver FAO activity, or liver cell proliferation similar to clofibrate at 250 mg/kg/day. Also, in fresh liver slices, no alteration of gap junctional intercellular communication was found using *in situ* dye transfer measurement.

In summary, DINP produces peroxisome proliferation and increased hepatocellular proliferation in rodents at high doses, but no effects were observed in primates at up to 2500 mg/kg/day.

CHRONIC TOXICITY AND CARCINOGENICITY TESTING IN RATS

Lington et al (1997) Study

In a study reported by Lington and coworkers (1997), groups of 110 F344 rats/gender were fed DINP at dietary levels of 0, 300, 3000, and 6000 ppm for periods up to 2 years. These represent 15, 152 and 307 mg/kg bw/day in males and 18, 184 and 375 mg/kg bw/day in females, respectively. Interim sacrifices of 10 rats/gender/dose were made at 6, 12, and 18 months. At termination (24 months), survival was in excess of 60% for all groups. At the mid and high doses, body weight gain and food consumption were reduced. There was a dose-related increase in liver and kidney weights, and anemia (only high dose in both genders), increased BUN, creatinine, AST, ALT (high dose in both genders) alkaline phosphatase (high dose males).

Mononuclear cell leukemia was increased in mid and high doses in both genders (Table 2). However, the in-study control group values were low compared to historical control data (Thurman et al, 1994; NTP, 1997; Haseman et al, 1998; Caldwell, 1999). No clear exposure-related nonneoplastic or neoplastic lesions were found.

A clear no-observed-effect level was demonstrated for all biological end points at a dietary level of 300 ppm or 17 mg/kg/day of DINP.

Moore (1998b) and Butala et al (1996) Study

In a study reported by Moore (1998b) and Butala and coworkers (1996), DINP was administered daily to F344 rats in the diet for 104 weeks at dietary concentrations of 0, 500, 1500, 6000, and 12000 ppm for groups 1, 2, 3, 4, and 5, respectively. Group 6 received 12000 ppm for 78 weeks, followed by 26 weeks of recovery. Based on the average consumed dose, DINP doses were 29.2, 88.3, 358.7, and 733.2 mg/kg/body weight/day in males, and 36.4, 108.6, 442.2, and 885.4 mg/kg bw/day in females for groups 2, 3, 4, and 5, respectively. For group 6, the concentration of DINP was 637.3 mg/kg bw/day in males, and 773.6 mg/kg bw/day in females. Consequently, in each DINP dose group, the daily dose consumed by females was 21-25% greater than that consumed by males.

Parameters evaluated were mortality, clinical observations, body weight, food consumption, and compound consumption data, clinical pathology parameters, organ weights,

and necropsy and histopathological findings. Ancillary analyses were also conducted at weeks 1, 2, 13, 79, and 104 to evaluate hematology, clinical chemistry, proliferation and peroxisome proliferation in the livers. As a positive control (Group 7) in only male rats, WY14643 was administered at 1000 ppm in the diet for at least 13 weeks.

The no-observable-adverse-effect level for systemic toxicity was 1500 ppm, or 88.3 mg/kg/day in males, and 108.6 mg/kg/day in females. The systemic toxicity included reduction in body weight gain, 10% in males and 15% in females, anemia in both males and females, 15% increase in BUN in both genders, hypoactivity and increase in AST, ALT and liver weight in both genders. FAO activity, an indicator of peroxisome proliferation, was increased in high dose males and females. The rate of hepatocellular proliferation measured by BrdU in high dose animals was not increased throughout the entire study in both genders, except during the initial evaluation after the first week. Microscopically, there was evidence of compensatory extramedullary hematopoiesis, hepatocellular hypertrophy and increased mineralization of the renal papillae. The incidence of most of these findings decreased during the exposure-free interval (group 6), indicating a reversibility trend.

Group 7 given WY14643 for up to 13 weeks exhibited increased cell proliferation and liver weight, demonstrating the responsiveness of the rats to a peroxisome proliferator.

Exposure related neoplastic findings were limited mainly to hepatocellular neoplasms, which were increased in high dose animals of both genders (Table 3). In the recovery groups, the incidence of these hepatocellular neoplasms was reduced compared to the high dose incidence. In both genders, the incidence of the in-study control values were similar to the historical control values for this strain (NTP, 1997).

In males of groups 5 (high dose) and 6 (high dose with recovery), 2.5% and 8% incidences of renal tubule cell carcinomas were present (Table 3). Group 6 value was significant compared to in-study (none) and historical control values (0.9%) (NTP, 1997). Caldwell and coworkers (1999) provided evidence for the occurrence of α2μ–globulin in the kidneys of male rats exposed to high doses of DINP (see below).

Finally, increases in the incidence of mononuclear cell leukemia in some groups were present in a non-dose related pattern in both genders (Table 3). The in-study control group incidence of this neoplasm was quite low compared to the historical control values for this strain, i.e. 62% for the males and 42% for the females. If the historical control values were to be applied, then none of the DINP-exposed group incidences would be outside those values. Thus, the increases do not appear to be compound related.

CHRONIC TOXICITY AND CARCINOGENICITY TESTING IN MICE

In a study reported by Moore (1998a) and Butala and coworkers (1997), DINP was administered daily to B6C3F1 mice in the diet for 104 weeks at dietary concentrations of 0, 500, 1500, 4000, and 8000 ppm for groups 1, 2, 3, 4, and 5, respectively. Group 6 received 8000 ppm for 78 weeks, followed by 26 weeks of recovery. Based on the average consumed dose, DINP doses were 90.3, 275.6, 741.8, and 1560 mg/kg bw/day in males and 112, 335.6, 910.3, and 1887.6 mg/kg bw/day in females for groups 2, 3, 4, and 5, respectively. For group 6, the concentration of DINP was 1377 mg/kg bw/day in males and 1581 mg/kg bw/day in females. Consequently, in each DINP dose group, the daily dose consumed by females was 15-24% greater than that consumed by males.

Parameters evaluated were mortality; clinical observations; body weight, food consumption, and compound consumption data; and necropsy and histopathological findings. Ancillary analyses were also conducted at weeks 78 and 104 to evaluate hematology, clinical chemistry, cell proliferation, and peroxisome proliferation in the livers.

The no-observable-adverse-effect level for systemic toxicity was 1500 ppm, or 275.6 mg/kg/day in males, and 500 ppm, or 112 mg/kg/day in females. The systemic toxicity included reduction in body weight gain, 40% in males and 20% in females, for the high dose groups. Even at the mid-high dose the reduction was 22% in males and 18% in females. In addition, leukopenia and an increase in liver weights were present in high dose males and females. FAO oxidase activity, an indicator of peroxisome proliferation, was evaluated in high dose animals and was found to be increased in both males and females. On the other hand, the rate of hepatocellular proliferation measured in BrdU was not increased in either gender at

either 78 or 104 weeks. Microscopically, there was evidence of hepatocellular hypertrophy. The incidence of most of these findings decreased during the exposure-free interval (group 6), indicating a reversibility trend.

Exposure related neoplastic findings were limited to hepatocellular neoplasms, which were increased in the two high doses of both genders and in the mid-low dose of the females (Table 4). In the recovery group, the incidence of hepatocellular neoplasms was reduced, especially in males, compared to the incidence at the end of exposure. In females, the in-study control incidence value was very low compared to the historical control values (NTP, 1997). Had these control values been higher, then only the high dose incidence value would be significantly increased.

In summary, increases were found in liver tumors in mice and rats and in kidney tumors in male rats. Non-dose related increases in mononuclear cell leukemia in rats are not considered to be treatment related.

MECHANISTIC STUDIES

The response of cultured hepatocytes from rats and marmosets to DINP and MINP was studied (Benford et al, 1986). DINP produced a small increase in FAO activity in rat hepatocytes, while MINP produced a large increase. In marmoset hepatocytes only minimal changes were produced by DINP and MINP. Likewise for laurate hydroxylation activity in rat hepatocytes, MINP produced a larger effect (about a 13-fold increase) than DINP. In marmoset hepatocytes increases of about 3-fold were produced by both DINP and MINP.

To elucidate the pathogenesis of renal changes and kidney tumors in a chronic study with DINP reported by Butala and coworkers (1996), a retrospective immunohistochemical analysis of α 2 μ-globulin (α 2 UG), the protein associated with nephropathy and kidney tumors, cell proliferation (PCNA) and renal histopathology was conducted immunohistochemically with renal cells quantified by image analysis (Caldwell et al, 1999). The doses of DINP examined were 0.03, 0.3 and 0.6% in males and 0.6% in females. In male rats the mean % area positive for α 2 UG was increased in the 0.6% group, but not at other doses. The findings were

considered to provide evidence for an α 2 UG mechanism of tumorigenesis, which is considered to be not relevant to humans (USEPA, 1991; Rice et al, 1999).

The response of human hepatocytes in culture to DINP was compared to that of rat hepatocytes by Hasmall et al (1999). In rat hepatocytes DINP produced an induction of FAO activity, an increase in DNA synthesis and suppression of apoptosis. In contrast in human hepatocytes, at the same concentrations, there was no effect on these parameters.

To determine whether DINP effects in liver are mediated by the peroxisome proliferator activated receptor (PPAR), male and female 129/SV PPARα (+/+) and 129/SV PPARα-null (-/-) (C 57 BL 16 derived) mice and B6C3F mice (Valles et al, 2000). Mice were fed control diet or diets containing 8000 ppm DINP for 1 or 3 weeks. Liver weights were increased at 1 week in male and female PPARα (+/+) and B6C3F1 mice, but not in PPARα (-/-) mice. FAO and Cyp 4a proteins were increased in livers of female PPARα (+/+) mice, but not PPARα (-/-) at 3 weeks.

In summary, DINP is a peroxisome proliferator in rodents, but not primates in relationship to expression of PPARα .

CONCLUSIONS

1. An extensive data base documents that DINP is non genotoxic.
2. DINP is hepatocarcinogenic in mouse liver at 4000 ppm in males and 1500 ppm in females, and in rat liver at 12,000 ppm in both males and females. DINP produced increases in kidney tumors at 12,000 ppm in male rats.
3. DINP is a weak peroxisome proliferator in mouse and rat liver, but not primate liver, in relationship to expression of PPARα.
4. DINP produces α2UG accumulation in male rat kidney.

RISK ASSESSMENT

DINP is nongenotoxic like other phthalates (Zeiger et al, 1985; Doull et al, 1999; Barber et al., 2000) and peroxisome proliferators (Budroe and Williams, 1993). Based on these findings and its structure, it can be concluded that DINP, either itself or its metabolite MINP, is devoid of DNA reactivity. Accordingly, the tumor increases must stem from another mode of action.

The liver tumors can be ascribed to the epigenetic effects of DINP. Clearly, several effects, notably peroxisome proliferation and hepatomegaly, are a consequence of activation of the PPARα receptor. This receptor binds to DNA as a heterodimer with the retinoid X receptor to act as transcription factor for a number of genes including those for peroxisomal and microsomal FAO enzymes (Lee et al, 1995). PPARα is also required for induction of cell replication by peroxisome proliferators (Peters et al, 1997). These responses have both been postulated to be involved in the hepatocarcinogenicity of peroxisome proliferators (Rao and Reddy, 1996). The increased peroxisomal fatty acid β-oxidation activity generates H_2O_2 which can lead to oxidative stress. The increased cell proliferation can render cells more susceptible to transformation, including by oxidative DNA damage. Regardless of the precise mechanisms, species differences in PPARα expression determine susceptibility to hepatocarcinogenicity by peroxisome proliferators (Williams and Perrone, 1996). In particular, humans have low expression of PPARα and do not respond to peroxisome proliferators (Palmer et al, 1998; Holden and Tugwood, 1999; Maloney and Waxman, 1999). In fact, human hepatocytes in culture respond to peroxisome proliferators with reduced DNA synthesis and increased apoptosis (Perrone et al, 1998), as was demonstrated for DINP. These responses would not lead to a tumorigenic effect (Williams, 1997). Thus, the hepatic effects of DINP in rodent bioassays are not applicable to human hazard identification. This position has also been adopted by the IARC (1995).

No effect levels (NEL) for hepatocellular neoplasia were found at 359 mg/kg bw/day for male and at 442 mg/kg bw/day for female rats, and at 276 mg/kg bw/day for male and 112 mg/kg bw/day for female mice (Butala et al, 1996, 1997; Moore, 1998a, 1998b). DINP is a weak peroxisome proliferator and these doses are below the levels required for peroxisome proliferation. Thus, DINP exhibits a threshold for hepatocarcinogenicity at doses required to elicit the cellular effects that are the basis for its carcinogenicity. Accordingly, a linear extrapolation approach to human risk assessment is not scientifically justified.

The male rats in the high dose group of the study of Butala et al (1996) and Moore (1998b), using 12,000 ppm, developed a small incidence of kidney tubular cell tumors (Table 3). In

short term studies, DINP produced nephrosis. In male rats from the carcinogenicity study, amounts of α2UG were increased (Caldwell et al, 1999). Increases in renal cell neoplasms in male rats mediated by the α2μ–globulin nephropathy are not considered to be a cancer hazard to humans (USEPA, 1991; Rice et al, 1999), because humans lack this urinary protein.

Rats in both studies exhibited questionable increases in mononuclear cell leukemia. This tumor is an F344 rat strain-specific common tumor with no established biological relevance for humans (Thurman et al, 1994; NTP, 1997; Haseman et al, 1998; Caldwell, 1999). No agent producing an increase in mononuclear cell leukemia in rats has been associated with human leukemia/lymphoma.

In conclusion, the increases in all three spontaneously occurring tumors seen with DINP treatment occurred through processes not relevant to humans and at exposures vastly beyond that which would take place with product use. A similar assessment was made by Wilkinson and Lamb (1999). Also, an expert group arrived at the same conclusion for di(2-ethylhexyl)phthalate, a more potent peroxisome proliferator (Doull et al, 1999).

Table 1. Genotoxicity Studies with Di (isononyl) phthalate

Test system	Result	Reference
Salmonella typhimurium assay (plate incorporation procedure)	negative (+/- S9)[a]	McKee et al, 2000 (unpublished manuscript)
Salmonella typhimurium assay (preincubation procedure)	negative (+/- S9)[b]	Zeiger et al, 1985; McKee et al, 2000 (unpublished manuscript)
In vitro L51784 mouse lymphoma assay	negative (+/- S9)[ab]	Barber et al, 2000
In vitro cytogenetics in Chinese hamster ovary assay	negative (+/- S9)[a]	McKee et al, 2000 (unpublished manuscript)
In vitro Balb/3T3 cell transformation assay	negative[ab]	Barber et al, 2000
In vivo mouse (CD-1) micronucleus assay	negative[a]	McKee et al, 2000 (unpublished manuscript)

[a] CAS RN 68515-48-0
[b] CAS RN 28553-12-0

Table 2. Total Percent Incidence of Mononuclear Cell Leukemia in DINP-Exposed F344 Rats in the Study of Lington et al (1997).

Gender: Males

Group Number	1	2	3	4
Mononuclear cell leukemia	33	28	48*	51*

Gender: Females

Group Number	1	2	3	4
Mononuclear cell leukemia	22	20	30*	43*

Group 1 control, group 2 300 ppm, group 3 3000 ppm, and group 5 6000 ppm; *, significantly increased at $p < 0.05$.

Table 3. Total Percent Incidence of Significant Neoplasms in DINP-Exposed F344 Rats in the Study of Butala et al (1996) and Moore (1998b)

Gender: Males

Group Number[a]	1	2	3	4	5	6 (recovery)
Number examined	80	50	50	65	80	50
Hepatocellular adenoma/ carcinoma	6.3[b]	8.0	4.0	10.8	21.3*	14.0*
Mononuclear cell leukemia	27.5[c]	46.0*	42.0	49.2*	37.5	62.0*
Renal tubule cell carcinoma	0	0	0	0	2.5	8*

Gender: Females

Group Number[a]	1	2	3	4	5	6 (recovery)
Number examined	80	50	50	65	80	50
Hepatocellular adenoma/ carcinoma	1.3[b]	2.0	0	3.1	10.0*	4.0*
Mononuclear cell leukemia	21.3[c]	32.0	18.0	44.6*	37.5	48.0*
Renal tubule cell carcinoma	0	0	0	0	0	0

a, group 1 control, group 2 500 ppm, group 3 1500 ppm, group 4 6000 ppm, group 5 12,000 ppm and group 6 12,000 ppm for 78 weeks with recovery; b, the historical control data base is 6% for males and 1% for females; c, the historical control data base is 62% for males and 42% for females, Thurman et al, 1994, NTP, 1997, Haseman et al, 1998; *, significantly increased at $p < 0.05$;.

Table 4. Total Percent Incidence of Significant Neoplasms in DINP-Exposed B6C3F1 Mice in the Study of Butala et al, 1997 and Moore, 1998a

Gender: Males

Group Number[a]	1	2	3	4	5	6 (recovery)
Number examined	70	60	60	60	70	50
Hepatocellular adenoma/ carcinoma	22.9[b]	21.7	30.0	46.7*	44.3*	38

Gender: Females

Group Number[a]	1	2	3	4	5	6 (recovery)
Number examined	70	60	60	60	70	50
Hepatocellular adenoma/ carcinoma	4.3[b]	8.2	16.7*	18.3*	45.7*	36.0*

a, group 1 control, group 2 500 ppm, group 3 1500 ppm, group 5 4000 ppm, group 5 8000 ppm and group 6 8000 ppm for 78 weeks with recovery; b, the historical control data base is 29% for males and 30% for females (NTP, 1997);
*, significantly increased at $p < 0.05$.

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Attachment D

University of Colorado Health Sciences Center

School of Pharmacy
Molecular Toxicology and Environmental Health Sciences Program

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September 4, 2000

Dr. Marian K. Stanley
American Chemistry Council
1300 Wilson Boulevard
Arlington, VA 22209

Dear Dr. Stanley:

You have asked me to outline my views on the human health signficance of F-344 mononuclear cell leukemia (MNCL). As you may be aware I have spent the better part of my career studying mechanisms of human leukemogenesis including 10 years that were devoted to identifying and characterizing animal models that might be relevant for the study of human leukemogenesis. Some of that effort focused on MNCL. In my view, MNCL in the F344 rat is not a useful model for the direct study of human disease and is certainly not an appropriate endpoint for predicting or extrapolating carcinogenic risk in humans. The principal reasons for my opinion are that MNCL occurs spontaneously in a large majority of F-344 rats surviving through maturity, and that a strong case can be made that non-carcinogenic stimuli can influence the onset of the disease. Therefore, with respect to MNCL, any attempt to distinguish between a *de novo* carcinogenic event and the exacerbation of a spontaneously occurring lesion is problematic.

MNCL is the leading spontaneous cause of death in F-344 rats (Maloney et al, 1970; Davey et al, 1970). The spontaneous incidence of MNCL varies widely with a nominal unadjusted incidence exceeding 60% in untreated male controls in some bioassays. The spontaneous incidence in female F-344 rats is somewhat lower, although there is considerable overlap. For example, the unadjusted lifetime incidence of spontaneous MNCL in untreated control male and female F-344 rats was 62%/28%; 58%/48%; 38%/28%; 34%/28%, respectively, for four different studies picked at random (NTP Technical Report Series: 377;398;403;419). The genetic predisposition of the F-344 to the development of this disease has been elegantly confirmed in cross and back-cross experiments (Lipman et al, 1996). The onset of MNCL spontaneously occurring in untreated F-344 rats is abrupt with almost no cases appearing before 15 months of age

and over 90% of cases presenting between 18-24 months of age. This observation has led some authorities to suggest that bioassays in the F-344 rat should be terminated at 18 months to avoid obscuring genuine tumorigenic responses.

Dietary restriction has been shown to influence the onset but not the progression of MNCL (Higami et al. 1996) as has chronic exposure to a variety of chemicals. Some carcinogenic agents actually appear to *decrease* the lifetime incidence of MNCL in F-344 rats. Cadmium, for example, which is carcinogenic in its own right, produces a 66% incidence of sarcomas, both histiocytic and osteogenic as well as fibrosarcoma, but also results in a four fold *reduction* in MNCL that cannot be explained in terms of competing causes of mortality (Waalkes et al, 1991).

I have reviewed the results of two chronic studies conducted on di-isononyl phthalate, the Lington study (1997) and a bioassay conducted by Aristech. In the Lington study, predictably no significant incidence MNCL cases were reported prior to the 18 month sacrifice. Between 18 and 24 months there was a sharp increase in the spontaneous incidence of MNCL in control animals, with 60% and 44% of unscheduled deaths in males and females, respectively, due to the disease. Although the incidence of MNCL in high dose animals dying during this period was arguably higher (79% and 81%), the incidence in low dose animals was actually lower than controls. In the Aristech study, no significant incidence of MNCL was observed prior to the 18 month sacrifice. At terminal sacrifice the spontaneous incidence of MNCL in control animals was 34% and 26%, respectively, for males and females, and the cumulative incidence of MNCL in high dose animals was 46% for both males and females. Again, there was no significant dose-response and values reported for the high dose group are well within the historical range for the incidence of MNCL in untreated rats.

Parenthetically, I believe that a great deal of circumstantial evidence can be brought to bear to support the hypothesis that any non-specific stress leading to chronic adrenal corticoid stimulation or, alternatively, mild immunosuppression, will exacerbate the onset of the disease. It may be informative that a proposal I once submitted to test this hypothesis was denied funding by NIH because of the "obvious lack of significance of MNCL to human disease."

It is my opinion that MNCL in the F-344 rat is a genetically programmed disease, the pathogenesis of which has no obvious biologically relevant counterpart in humans. Acute myelogenous leukemia (AML) is widely recognized by medical, scientific and regulatory authorities to be the predominant, if not only human leukemia associated with previous exposure to alkylating chemotherapeutic agents and occupational exposure (See Irons & Stillman, 1996; Irons, 1997 for reviews). For example, the 1997 Toxicological Profile for Benzene published by the Agency for Toxic Substances and Disease Registry (ATSDR) of the U.S. Department of Health and Human Services states:

> "Long term exposure to relatively high levels of benzene in air can cause cancer of the blood forming organs. This condition is called leukemia. Exposure to

benzene has been associated with the development of a particular type of leukemia called acute myeloid leukemia (AML)" (ATSDR).

Fischer rat MNCL is a not related to AML, either with respect to cell of origin, pathogenesis or molecular mechanism. MNCL originates in a non-myeloid/non-lymphoid cell called an NK (natural killer) cell (Reynolds et al, 1984). Human AML arises in a myeloid progenitor cell which is representative of an entirely separate cell lineage. Human AML developing secondary to drug or chemical exposure is associated with early clastogenic events involving specific clonal chromosome abnormalities (involving predominantly chromosomes 5 and 7) that reflect large deletions of DNA. Because of the unique genetic mapping of hematopoietic genes, these events are necessarily species- specific (Irons, 1997). Alternatively, the closest morphologic counterpart to MNCL in humans , "large granular cell leukemia" (LGL) is a cell that originates in a cytotoxic T lymphocyte and has never been associated with drug or chemical exposure. A human leukemia variant of NK cell origin does occur, is rare, presents primarily in children or young adults, and has never been associated with drug or chemical exposure (Jandl, 1996). Consequently, there is no biologic rationale for concluding that F-344 MNCL is a relevant surrogate for a comparable disease entity or, independently, any disease that has been associated with chemical exposure in humans.

Although a variety of agents have been shown to exacerbate the onset of F-344 MNCL when administered in high doses, the relevance of this finding to human carcinogenesis has not been established. Although MNCL is the most frequent spontaneous cause of death in F-344 rats, the incidence in untreated animals varies widely, often rendering interpretation of treatment-related cases an impossibility on quantitative grounds alone. Specifically with respect to bioassays of di-isononyl phthalate, the dose-dependent nature of treatment-related MNCL is not impressive, suggesting that the observed increases represent a non-specific high dose effect that cannot be meaningfully attributed to a carcinogenic event.

If you have any further questions, please do not hesitate to contact me.

Sincerely,

[redacted]

Richard D. Irons, PhD, D.A.B.T.
Professor and Director

References:

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Attachment E

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January 25, 2001

Ms. Marian K. Stanley
American Chemistry Council
1300 Wilson Boulevard
Arlington, VA. 22209

Reference: Docket Control Number: OEI-100004

Dear Ms. Stanley:

I am writing to address the proposed listing of DINP under section 313 of the Community Planning and Right-to-know Act on the basis of potential carcinogenicity. My laboratory has been conducting research on the mechanisms by which DINP causes cancer in rodents for the past two years. These studies have focused on two major issues that are also the issues cited in the Federal Register (Vol 65, 172, 53618-53689, 2000) as major reasons for the above listing. The research covers two areas, rodent liver tumors, part of which is now in press in Carcinogenesis (Rusyn et al, 2000), and alpha$_{2u}$-globulin nephropathy (α_{2u}-G) which is being presented at the 2001 Society of Toxicology meeting (Schoonhoven et al., 2001).

Our research on α_{2u}-G has extended the previous work in this area, filling in data gaps to meet the EPA criteria for α_{2u}-G. Specifically we have demonstrated that [^{14}C]-DINP binds reversibly to male rat kidney cytosol, but not to female rat kidney cytosol. We also did extensive research on cell proliferation. Using BrdU to label proliferating cells, we demonstrated that male, but not female F344 rats had a doubling in the number of replicating cells compared with controls. These data clearly demonstrate that DINP causes α_{2u}-G. The argument that some nephrotoxicity was demonstrated in female rats and that there was an increase in chronic progressive nephropathy in mice precludes using the α_{2u}-G mechanism is not valid, as these groups did not develop renal cancer. Only the male rats developed an increase in renal tumors after two years. It is not scientifically justified to use an argument that other mechanisms were responsible, when these other mechanisms did not cause cancer. Thus, the data on rat kidney tumors is not relevant for human risk assessment.

We have also been conducting research on peroxisome proliferation under a grant from NIEHS. The main focus of this grant is to determine the role of the PPARα receptor in the induction of oxidative stress. We have shown that rodents exposed to peroxisome proliferators show dose-, time-, and potency-related increases in expression of base excision repair enzymes associated with oxidative DNA damage. This response requires a functional PPARα, as knockout mice do not show any evidence for induction of DNA repair. These data strongly suggest that peroxisome proliferator mediated

oxidative damage only occurs in species with a functional PPARα receptor and response element. Thus, all three of the mechanisms involved in the induction of liver cancer by these agents (increased cell proliferation, decreased apoptosis and oxidative stress) require a functional PPARα receptor and response element. Since humans do not have a functional PPARα receptor and response element, these mechanisms are not relevant for human risk assessment.

In summary, a vast literature and the above new studies do not support the extrapolation of rodent carcinogenicity on DINP to human risk of cancer. Both the kidney and liver tumors occur by mechanisms that are not relevant for humans.

Rusyn, I. et al. (2000). Expression of base excision repair enzymes in rat and mouse liver is induced by peroxisome proliferators and is dependent upon carcinogenic potency. *Carcinogenesis*: 21:2141-2145.

Schoonhoven, R. et al. (2001). Di(isononyl)phthalate binds reversibly to α_{2u}-globulin and induces cell proliferation in male rat kidneys. To be presented at the 2001 Society of Toxicology meeting. (Abstract attached).

Sincerely,

[redacted]

James A. Swenberg

DI(ISONONYL) PHTHALATE BINDS REVERSIBLY TO α_{2u}-GLOBULIN AND INDUCES CELL PROLIFERATION IN MALE RAT KIDNEYS

R Schoonhoven, E Bodes* and J A Swenberg. Curriculum in Toxicology, University of North Carolina, Chapel Hill, NC, USA.

Di(isononyl) phthalate (DINP) is a commonly used plasticizer that has preliminary data suggesting that it induces male rat specific α_{2u}-globulin (α_{2u}G) nephropathy. We have conducted a series of studies to further investigate this issue. In the first study, reversible binding between DINP and α_{2u}G was evaluated. Male and female Fischer 344 (F344) rats were gavaged with a single dose (900mg/kg) of radiolabeled [^{14}C]-DINP. Kidney cytosol samples were pooled by gender, and dialyzed against buffer with or without 0.1 % SDS. Cytosol dialyzed against buffer with SDS, but not without SDS, lost radioactivity to background levels, demonstrating reversible binding of DINP to α_{2u}G. A second study was done using immunohistochemistry (IHC) to quantify dose dependent accumulation of α_{2u}G and changes in cell proliferation in kidneys of rats exposed to DINP. Male F344 rats were gavaged (90, 300 or 900 mg/kg) daily for 5 days. A negative and positive control group received corn oil or d-limonene, respectively. Animals received BrdU in drinking water for 72 hours prior to sacrifice. IHC showed an increase in the amount of α_{2u}G staining in the treated over control groups. BrdU cell proliferation was measured to establish a labeling index (LI). When random fields of 2,000 cells were counted, large standard errors (SE$\geq$0.5) were present. This was due to localized lesions within the cortex which were counted or missed due to selection. Using counts of 10,000 and 20,000 cells, most of the cells in the cortex were counted with a SE<0.2 in all groups. There was a doubling in cell proliferation of DINP treated over control groups. Collectively, these data demonstrate that DINP induces α_{2u}G nephropathy. (Supported in part by the American Chemical Council.)

Carcinogenesis vol.21 no.12 pp.2141–2145, 2000

ACCELERATED PAPER

Expression of base excision repair enzymes in rat and mouse liver is induced by peroxisome proliferators and is dependent upon carcinogenic potency

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Elevated and sustained cell replication, together with a decrease in apoptosis, is considered to be the main mechanism of hepatic tumor promotion due to peroxisome proliferators. In contrast, the role of oxidative stress and DNA damage in the carcinogenic mechanism is less well understood. In view of possible induction of DNA damage by peroxisome proliferators, DNA repair mechanisms may be an important factor to consider in the mechanism of action of these compounds. Here, the ability of peroxisome proliferators to induce expression of base excision repair enzymes was examined. WY-14,643, a potent carcinogen, increased expression of several base excision DNA repair enzymes in a dose- and time-dependent manner. Importantly, expression of enzymes that do not repair oxidative DNA damage was not changed. Moreover, less potent members of the peroxisome proliferator group had much weaker or no effects on expression of DNA repair enzymes when compared with WY-14,643. Collectively, these data suggest that DNA base excision repair may be an important factor in peroxisome proliferator-induced carcinogenesis and that induction of DNA repair might provide further evidence supporting a role of oxidative DNA damage by peroxisome proliferators.

Introduction

Peroxisome proliferators are a diverse group of chemicals and therapeutic agents. In rodents these compounds cause hepatomegaly, proliferation of peroxisomes in hepatic parenchymal cells and marked increases in the activity of enzymes required for peroxisomal β-oxidation of fatty acids (1). These changes persist at steady-state levels as long as peroxisome proliferators are administered. Long-term exposure results in the development of liver tumors in rodents (2). Rodents are much more sensitive to the effects of peroxisome proliferators than dogs, non-human primates or humans. This difference in sensitivity appears to be due to differences in the PPARα receptor and its response elements (reviewed in ref. 3). Most reviews of peroxisome proliferators conclude that humans are at little or no risk for cancer from exposure to these agents (4).

The major mechanisms that have been proposed for peroxisome proliferator-induced hepatocarcinogenesis are: (i) increased cell proliferation leading to promotion of spontaneously initiated lesions; (ii) oxidative stress, resulting from disproportionate increases in the levels of oxidants. However, recent observations that oxidant-dependent activation of the transcription factor NF-κB plays a critical role in increased hepatocyte proliferation caused by WY-14,643 (5) and ciprofibrate (6) suggest that these mechanisms may not be mutually exclusive and oxidants may be involved in signaling increased cell proliferation.

It has been suggested that reactive oxygen species play a role in the initiation and promotion steps of carcinogenesis induced by peroxisome proliferators. Recently, direct evidence of rapid peroxisome proliferator-induced generation of hydroxyl radicals *in vivo* has been presented (7). Indeed, production of reactive oxygen species may lead to DNA damage via hydroxyl radicals and products of lipid peroxidation. Oxidative stress is hypothesized to be a common pathway for many non-genotoxic chemical carcinogens (8). However, the role of oxidative stress has been questioned. Indeed, when compared with direct DNA damaging agents, the magnitude of response following carcinogenic exposures to chemicals thought to work through oxidants has been small. Several attempts to assess oxidative DNA damage by peroxisome proliferators using direct measurement of adducts produced equivocal results (9,10). Moreover, the artifactual formation of oxidized base damage due to artifactual auto-oxidation reactions in assays requiring extraction of DNA has plagued this experimental approach (11).

On the other hand, it is known that DNA repair enzymes are induced both *in vivo* and *in vitro* by oxidative stress (12). Several DNA repair genes involved in oxidative damage have been identified and it is believed that the predominant pathway used for removal of oxidized bases is the base excision repair (BER) pathway. Several proteins are involved in this multistep repair process (12). For instance, the primary pathway for removal of 8-hydroxydeoxyguanosine (8-OH-dG) appears to be OGG1, a glycosylase/lyase, which excises this adduct from DNA and cleaves 3′ to AP (abasic) sites, leaving a 3′-cleaved AP site (13). The DNA is then cleaved 5′ to the AP site by AP endonuclease (APE), the gap is filled by polymerase β (Pol β) and the newly synthesized DNA is sealed by ligase (14). While BER is considered the main pathway for oxidative DNA damage, nucleotide excision repair and long patch repair have also been shown to remove oxidative damage from DNA (15). Importantly, expression of enzymes that participate in these processes may be induced following increased production of reactive oxygen species (16) or chemical exposure (17).

No previous reports, however, have described changes in DNA repair enzymes associated with peroxisome proliferator-induced carcinogenesis. Since BER is a major mechanism for removal of oxidative lesions from DNA (18), we investigated the expression of several DNA glycosylases, APE, DNA

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polymerases and ligases in livers of rats (Fisher 344) and mice (C57Bl/6) treated with peroxisome proliferators. To assemble a comprehensive set of liver tissues, a combination of samples from several different studies performed at the University of North Carolina at Chapel Hill, Chemical Industry Institute of Toxicology, or by the National Toxicology Program has been used.

WY-14,643 causes a dose-dependent increase in expression of DNA BER enzymes in rodent liver

Mammalian *N*-methylpurine-DNA glycosylase (MPG) has broad substrate specificity and primarily is capable of hydrolysis of 3-methyladenine. It has also been shown to hydrolyze 1,N^6-ethenoadenine *in vitro* and weak activity towards 8-OH-dG has been reported (19). A quantitative reverse transcriptase PCR assay (19) was used to test the hypothesis that peroxisome proliferators induce expression of MPG in rat liver. The use of *in vitro* synthesized reference RNA allowed quantitation of the PCR products (MPG and standard) after gel electrophoresis and autoradiography (Figure 1A). The effect of a 21 day treatment with WY-14,643 (1000 p.p.m.) on MPG mRNA in whole rat liver is shown in Figure 1B. In livers of animals fed a regular chow diet, expression of MPG mRNA was low (89 ± 16 fg/μg total RNA), however, it was elevated 2-fold after treatment with WY-14,643 (164 ± 21 fg/μg total RNA). Moreover, a dose-dependent increase in expression of MPG was observed in rats treated with WY-14,643 for 90 days (Figure 1C). The amount of MPG mRNA was 2- or 6.5-fold higher in livers of rats treated with 100 or 500 p.p.m. WY-14,643, respectively, than in control animals. Collectively, these results support the hypothesis that potent peroxisome proliferators (i.e. WY-14,643) may inflict DNA damage and that levels of DNA repair gene expression could be used to establish a gene response profile after exposure to these chemicals.

Next, we used a recently developed multi-probe RNase protection assay for BER enzymes. This approach distinguishes the presence of multiple expressed DNA repair genes simultaneously from a single sample, which allows comparative analysis of different mRNA products both within and between samples. This is a highly sensitive and specific approach for detection and quantitation of gene expression at the mRNA level. It should be noted, however, that the RNase protection assay template sets (a kind gift of BD PharMingen, San Diego, CA) used here for rat and mouse tissues were under development and differed slightly in the composition of DNA repair genes evaluated. Total mRNA was isolated from livers of rats fed control or WY-14,643 (1000 p.p.m.)-containing diet for up to 22 weeks. A time-dependent 3- to 12-fold increase in mRNA for OGG1, APE, MPG and Pol β was observed (Figure 2). Importantly, expression of several enzymes that are not related to oxidative DNA damage (e.g. O^6-methylguanine-DNA methyltransferase and polymerase δ) was not changed. It should be noted that our findings of an increased expression of mRNA for BER proteins were corroborated recently when it was reported that Pol β and APE protein levels were increased markedly after treatment with WY-14,643 for 6 days (20).

The single nucleotide BER pathway is a favorable mechanism for removal of oxidized bases and is dependent upon the interaction between DNA Pol β and DNA ligase I (21). Indeed, only ligase I, but not ligase III or other enzymes of the 'long patch' repair pathway, was induced in mouse liver after dietary treatment with WY-14,643 (500 p.p.m.) for 7 days (Figure 3). Similar to what was observed in rats, WY-14,643 (500 p.p.m.) caused an ~3-fold increase in mRNA for OGG1, TDG, APE, MPG and Nth1 in mouse liver (data not shown). These findings

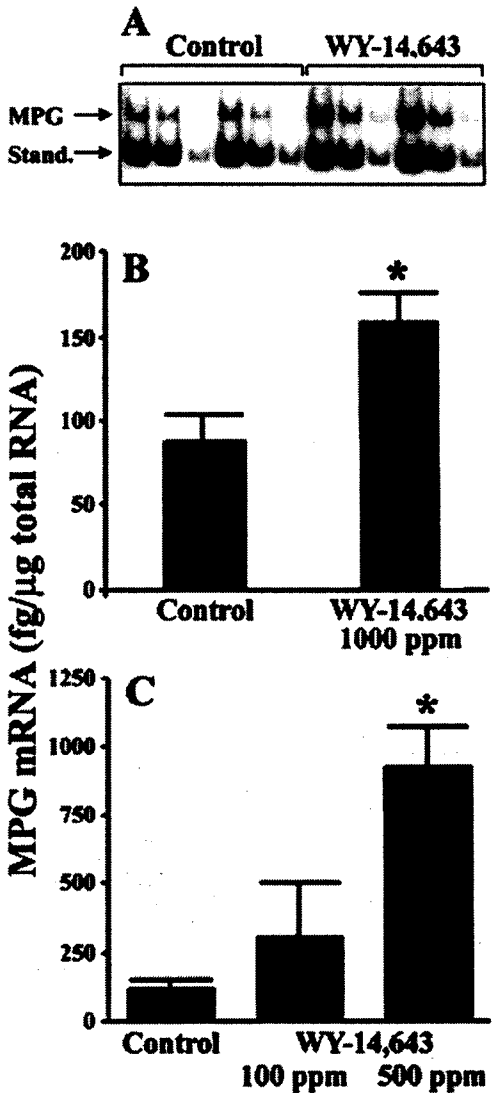

Fig. 1. MPG mRNA is induced in rat liver by dietary feeding of WY-14,643. (**A**) An autoradiogram of PCR products of native mRNA and synthetic MPG RNA (a kind gift of Dr S. Mitra, University of Texas, Galveston, TX) after *Eco*RI digestion is shown. Fisher 344 rats were fed either control diet (NIH-07) or diet containing WY-14,643 (1000 p.p.m. for 21 days). Total cellular RNA was isolated using a QuickPrep extraction kit (Amersham Pharmacia Biotech) and RT–PCR was performed as described in Roy *et al.* (19). Three 1:2 serial dilutions of each sample mixture containing 2 μg total cellular RNA and 540 fg synthetic RNA standard were made, amplified by PCR and resolved on 6% polyacrylamide gels. The 471 bp product of cellular MPG amplification and 366 bp product of reference RNA amplification after enzymatic cleavage of the 105 bp non-radioactive fragment are indicated. Representative autoradiogram. (**B**) Data shown are results of densitometry analysis of images from the experiment detailed in (A). (**C**) As above but rats were given 0 (Control), 100 or 500 p.p.m. WY-14,643 for 90 days. Data reported as means ± SEM from four separate experiments. Asterisks (*) denote statistical differences ($P < 0.05$) from the control group by one way ANOVA and Student–Newman–Keuls *post hoc* test.

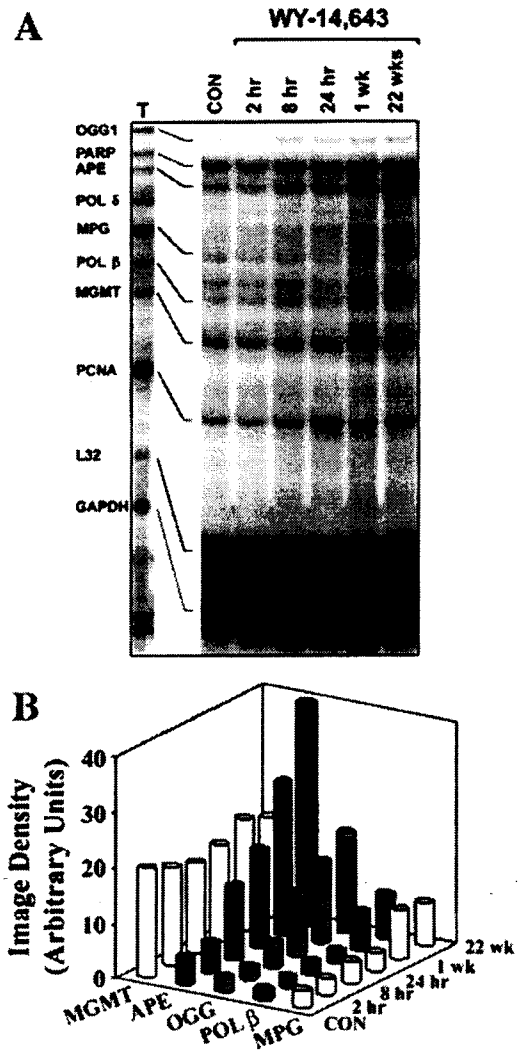

Fig. 2. WY-14,643 causes a time-dependent increase in expression of base excision DNA repair enzymes in rat liver. (**A**) Total RNA was isolated from livers of rats treated with WY-14,643 (1000 p.p.m.) for up to 22 weeks using a QuickPrep extraction kit (Amersham Pharmacia Biotech) and dissolved in RNase-free water. Expression of base excision DNA repair enzymes was analyzed with an RNase protection assay using rodent multi-nucleotide RNA probe template sets (a generous gift of BD PharMingen). Riboprobes were synthesized in the presence of [^{32}P]dUTP to yield labeled antisense RNA probes. The RNase protection assay was performed on 40 μg of individual RNA samples using a RiboQuant multi-probe RNase protection assay kit (BD PharMingen). Protected fragments were separated on QuickPoint nucleic acid separation gels (Novex, San Diego, CA), dried and exposed to X-ray film. Representative autoradiogram from three separate experiments. (**B**) Data shown are results of densitometry analysis of images from the experiment detailed in (A). The intensity of protected bands was quantified using an image analyzer and normalized to the intensity of housekeeping genes L32 and GAPDH.

are important because they show that WY-14,643 induces expression of the whole pathway responsible for the repair of oxidative DNA damage, DNA glycosylases, APE, Pol β and ligase I.

Potent carcinogens are more potent inducers of BER enzymes gene expression

WY-14,643 is one of the most potent carcinogens among the peroxisome proliferators. To test whether less potent

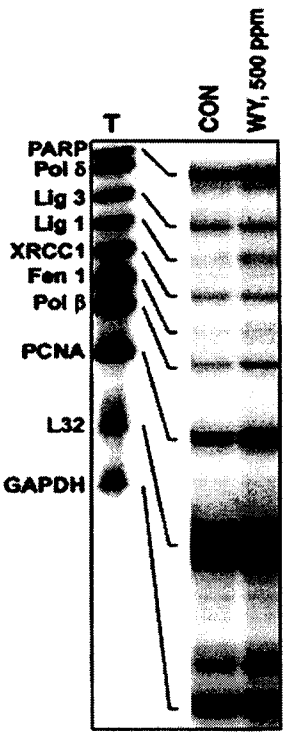

Fig. 3. Expression of ligase I is induced in mouse liver by WY-14,643. Mice (C57Bl/6) were fed a diet containing the potent carcinogen WY-14,643 (WY, 500 p.p.m.) for 7 days. Total RNA was isolated from liver samples and used in the RNase protection assay as detailed in Figure 2. Representative autoradiograms from three separate experiments.

carcinogens from this group of compounds have similar effects on DNA repair enzymes, rats were fed WY-14,643 or the weaker carcinogens di(2-ethyhexyl)phthalate (DEHP) and clofibric acid for 22 weeks. Both DEHP (12000 p.p.m.) and clofibric acid (5000 p.p.m.) increased expression of mRNA for OGG1, APE, MPG and Pol β by ~2- to 3-fold, however, these effects fell short of the profound changes caused by WY-14,643 (1000 p.p.m.) (Figure 4). It should be noted that since WY-14,643, DEHP and clofibric acid cause a similar initial increase in cell proliferation, the differences in expression of DNA repair enzymes observed here are not due to a rapid growth of liver mass. Furthermore, when WY-14,643 (1000 p.p.m.) and DEHP (12000 p.p.m.) were administered to rats for 7 days, induction of OGG1 APE and Nth1 was observed only in WY-14,643-treated animals (data not shown). Similar effects were observed with gemfibrozil, another potent rodent carcinogen, but not with the weak carcinogen dibutyl phthalate (Figure 5). Specifically, both WY-14,643 (500 p.p.m.) and gemfibrozil (16000 p.p.m.) administered to rats for 90 days increased expression of DNA BER enzymes, while dibutyl phthalate (10000 p.p.m.) had no effect.

It has been hypothesized that the hepatocarcinogenicity of peroxisome proliferators is due to oxidative stress. At least two possible sources of oxidants following administration of peroxisome proliferators have been proposed: (i) peroxisomal acyl CoA oxidase in parenchymal cells; (ii) NADPH oxidase in Kupffer cells (reviewed in ref. 22). The data presented here are important for mechanistically based risk assessment of peroxisome proliferators for several reasons. First, further evidence supporting the role of oxidative DNA damage in the

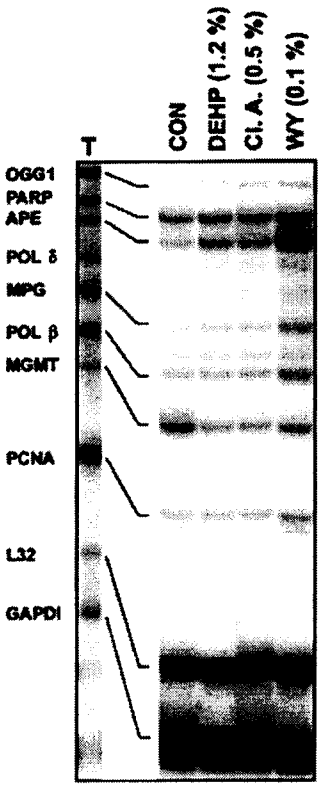

Fig. 4. Chronic treatment with peroxisome proliferators that are potent carcinogens induces increases in DNA BER enzymes expression. Total RNA was isolated from livers of rats treated with DEHP (1.2% w/w), clofibric acid (Cl. A., 0.5% w/w) or WY-14,643 (WY, 0.1% w/w) for 22 weeks and used in the RNase protection assay as detailed in Figure 2. Representative autoradiogram from three separate experiments.

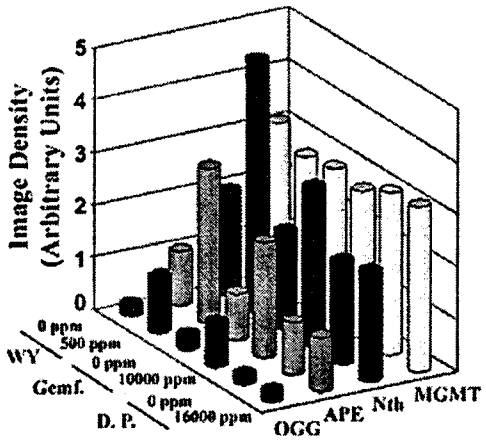

Fig. 5. Sub-chronic treatment with the potent carcinogens WY-14,643 and gemfibrozil, but not with the weak carcinogen dibutyl phthalate, increases expression of DNA excision repair enzymes. Total RNA was isolated from livers of rats treated with WY-14,643 (WY, 500 p.p.m.), dibutyl phthalate (D.P., 10000 p.p.m.) or gemfibrozil (Gemf., 16000 p.p.m.) for 90 days and used in the RNase protection assay as detailed in Figure 2. Data shown are results of densitometry analysis and the intensity of protected bands was quantified using an image analyzer and normalized to the intensity of housekeeping genes L32 and GAPDH.

mechanism of action of peroxisome proliferators is provided. Specifically, we suggest that DNA BER may be an important factor in peroxisome proliferator-induced carcinogenesis and that induction of DNA repair reflects an increase in oxidized bases following treatment with these compounds. The small and inconsistent increases in oxidative DNA damage observed in previous studies may be due to an inability to observe real increases due to the artifactual formation of oxidative DNA damage during DNA isolation. Most of the studies have reported 8-OH-dG values in control rat livers of ~2 per 10^{-5} bp. These values can be dramatically reduced through the use of free radical trapping reagents and antioxidants (23, 24). Reductions in artifactual 8-OH-dG are likely to increase the level of detectable oxidative DNA damage resulting from exposure to peroxisome proliferators.

Second, overexpression of DNA repair enzymes *per se* may play an important role in the mechanism of action of peroxisome proliferators. For instance, Pol β is normally expressed at low levels and has high infidelity in replicating DNA (25). Recently it was shown that overexpression of Pol β may result in increased spontaneous mutagenesis (26). Moreover, it was hypothesized that an excess of Pol β may disrupt functions of other DNA polymerases by introducing illegitimate deoxyribonucleotides or mutagenic base analogs like those produced by oxidative stress (27).

In addition, APE (Ref-1) is a multifunctional protein that stimulates DNA binding by a number of transcription factors, such as NF-κB and AP-1 (28). Interestingly, peroxisome proliferators increase the activity of NF-κB in rodent liver and it was hypothesized that NF-κB activation plays a role in increased cell proliferation induced by these compounds (reviewed in ref. 22). Therefore, the marked increase in APE expression observed here might be important for the promotional activity of peroxisome proliferators. On the other hand, APE is known to regulate transactivation of p53 (29). This transactivation only requires the C-terminus and has been suggested to delay the G_1/S transition and enhance BER (29). Given that p53 has been shown to enhance BER (30), it is possible that up-regulation of APE expression by peroxisome proliferators leads to enhanced p53-dependent BER. The significance of APE transactivation of the pro-apoptotic functions of p53 is unclear, since peroxisome proliferators are known to decrease apoptosis in liver (31).

An additional concern is that DNA repair enzymes could be up-regulated unevenly, so that a state of imbalanced DNA repair may exist. Indeed, imbalanced DNA repair may lead to formation of both mutagenic and clastogenic lesions. In the case of oxidative DNA damage, if glycosylase and APE are overabundant (relative to DNA polymerase or DNA ligase) then DNA strand breaks might accumulate that could consequently influence cell viability and induce chromosomal damage (32). Whether peroxisome proliferators cause imbalanced repair has yet to be determined.

Finally, since current risk assessment of peroxisome proliferators is based on important differences between humans and rodents in expression of PPARα (reviewed in ref. 3), the approach of analyzing expression of DNA repair enzymes can be used in further studies to assess whether or not BER enzymes, as a biomarker of oxidative stress, are induced by WY-14,643 in PPARα knockout mice.

In summary, the results of this study provide new information that supports a role of oxidative stress as a mechanism of carcinogenesis for peroxisome proliferators. It demonstrates a

clear induction of DNA repair pathways associated with oxidative DNA damage that is related to the dose and length of exposure, as well as the potency of the peroxisome proliferator for inducing hepatic carcinogenesis.

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