From:<laura.n.winks@exxonmobil.com>
To: <coshita@oehha.ca.gov>
Date: 5/5/2009 2:51 PM
Subject: ExxonMobil Chemical submission to OEHHA: Prioritization Comments Part II
Attachments: ExxonMobil DINP submission Part II.pdf; AttARoberts.PDF; AttBKlaunig.PDF; AttCWilliams.PDF; AttDIrons.PDF; AttESwenberg.PDF

Dear Ms. Oshita:

This is the second of three emails containing comments from ExxonMobil Chemical regarding the prioritization of chemicals to be considered at the May 29 meeting of the Carcinogen Identification Committee. These comments pertain specifically to diisononyl phthalate (DINP).

The content of the three emails is as follows:

1st email: The email has attached Part I of ExxonMobil's comments, which is a summary of the pertinent data.

2nd email: This second email has attached Part II of our comments, which provides greater detail on the scientific data, and the following attachments:

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.

3rd email: The third email has attached a copy of Klaunig et al. (2003), which is a review of the peroxisome proliferation mechanism, based on data for DINP and other chemicals. Other expert body reviews of the DINP data (CPSC CHAP, EU Risk Assessment, NTP CERHR) have been provided to the CIC by OEHHA and/or are readily available on the Internet. Because Klaunig et al. (2003) is not readily available on the Internet and is not cited by OEHHA, we are providing a copy. It is 6 MB -- if not received please let us know and we will break it down.

ExxonMobil would be pleased upon request to provide a copy of any other study cited in our comments.

If the CIC or OEHHA have any questions or need additional information, please contact me or any of the following scientists:

Ammie Bachman, Ph.D., ammie.n.bachman@exxonmobil.com, (908) 730-2082
Bob Barter, Ph.D., robert.a.barter@exxonmobil.com, (908) 730-2153
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Thank you.
(See attached file: ExxonMobil DINP submission Part II.pdf)

(See attached file: AttARoberts.PDF)(See attached file: AttBKlaunig.PDF)(See attached file: AttCWilliams.PDF)(See attached file: AttDIrons.PDF)(See attached file: AttESwenberg.PDF)

Regards,

Laura N. Winks
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PART TWO
IN-DEPTH DISCUSSION OF PERTINENT DINP DATA

ExxonMobil Chemical is submitting these comments in response to notification by the California Office of Environmental Health Hazard Assessment (OEHHA) of 38 chemicals for consultation by the Carcinogen Identification Committee (CIC) at its meeting on May 29, 2009. The purpose of these comments is to provide the CIC with relevant scientific information to support the CIC's evaluation of diisononyl phthalate (DINP), and specifically the CIC's recommendation to OEHHA concerning the level of priority that should be assigned to DINP for future consideration as a candidate chemical for listing under Proposition 65. Part One of the comments presented a summary of the strong body of evidence that neoplasms observed in rodent studies of DINP are caused by mechanisms that are not relevant for humans. This Part Two of the comments provides greater detail on that body of evidence.[1]

Section I discusses the human data available for DINP. There are no epidemiology studies, but there are other data that demonstrate DINP does not affect humans in the same manner as rodents.

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

Unlike most other substances, DINP has been studied in primates (including human cell cultures) as well as rodents. Section III summarizes the primate data which show no toxic effects even from very large doses of DINP.

Section IV discusses the conclusions of several expert body reviews of DINP in the past decade, which have found that the tumors observed in rodent studies are not relevant for human risk assessment.

Section V then 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 risk 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 a priority for evaluation and consideration of listing under Proposition 65.

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[1] ExxonMobil recognizes that this Part Two addresses a large body of information. Questions or requests for additional information (including copies of studies cited herein) may be directed to any of the following individuals:
Ammie Bachman, Ph.D., ammie.n.bachman@exxonmobil.com, (908) 730-2082
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I. AVAILABLE HUMAN DATA SUPPORT THE CONCLUSION THE DINP IS NOT LIKELY TO CAUSE CANCER IN HUMANS

There are no epidemiology studies on the carcinogenic potential of DINP. Nevertheless, there are some other types of human data which support a conclusion that tumors observed in rodent studies of DINP are unlikely to occur in humans. As discussed further in Section V.A.5 below, studies in human cell cultures (Baker *et al.*, 1996; Hasmall *et al.*, 1999; Kamendulis *et al.*, 2002) show a lack of the peroxisome proliferator response observed in rodents as a key event leading to development of liver tumors. In addition, because of differences in human and primate absorption of phthalates, internal doses equivalent to those required to produce tumors in rodents cannot be achieved in humans. Further, data on human exposure to DINP show that exposures are orders of magnitude below levels required to produce tumors in rodents (see Section V.A.6).

II. DINP IS NOT GENOTOXIC

A genotoxic chemical can damage cellular DNA and thereby trigger cancerous growth. DINP is not such a substance. As shown in Table 1, DINP has been evaluated in multiple *in vivo* and *in vitro* genotoxicity/mutagenicity assays and has been negative in all of them. 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 g/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 5.0 grams/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).

Thus, the conclusion that DINP is not mutagenic or genotoxic is supported by a strong database.

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

III. STUDIES IN PRIMATES SHOW NO EVIDENCE OF A POTENTIAL CANCER RESPONSE

Often, toxicological data on a chemical is limited to studies in rats and mice. For DINP, however, there is also an unusually large amount of data from studies in non-human primates – species that are much more closely related to humans than are rodents – as well as some *in vitro* data for humans. This 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.

There have been two *in vivo* studies of DINP in non-human primates. In one, cynomolgus monkeys were treated with DINP for 14 days at levels up to 500 mg/kg/day (Pugh *et al.*, 2000). In the other, marmosets were treated with levels up to the very high dose of 2500 mg/kg/day for 90 days (Hall *et al.*, 1999). (For a 70 kg human, this dose would be about six ounces per day.) In both of these primate studies, there was <u>no evidence</u> of treatment-related effects, including no changes in liver or kidney weights and no treatment-related changes in histopathology, even at the very high levels of treatment.

These studies were subchronic, versus the chronic bioassays in rodents. However, the lack of adverse effects in the primate studies even at very high doses 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.

As discussed in Section V.A below, liver tumors in rodents treated with DINP are due to the peroxisome proliferation mechanism. 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. Further, as discussed in Section V.A.6, toxicokinetic data in primates and human volunteers show that the high internal doses associated with adverse effects in rodents cannot be achieved in humans.

Thus, 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 species.

IV. EXPERT BODY REVIEWS HAVE DETERMINED THAT DINP IS UNLIKELY TO POSE A HUMAN CANCER RISK

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).[2] However, 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). 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 conclusion that the rodent mode of action for liver tumors from such compounds is not relevant to humans.

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[2] 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, 2009).

- 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 labelled for human health effects, including no cancer designation (EC, 2000).

These consensus opinions support the conclusion that DINP is highly unlikely to cause adverse human health effects.

ExxonMobil notes that, while the U.S. Environmental Protection Agency (EPA) has reviewed the data for DINP, it *has not* made a final determination regarding the carcinogenicity of DINP. EPA 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 EPA proposed to list DINP in part based on the animal cancer data (Fed. Reg., 2000). However, after receipt of comments, EPA 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). EPA accepted further comments and to date has not issued a final decision.

V. CANCER LESIONS OBSERVED IN RODENT BIOASSAYS OF DINP ARE NOT RELEVANT TO HUMANS

In rodents, DINP at high doses produces liver tumors in rats and mice, kidney tumors in male rats, and mononuclear cell leukemia (MNCL) in 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. Numerous independent scientists agree with this assessment, based on application of generally accepted scientific principles.

A. Liver Tumors Observed In Rodents Are Due to Peroxisome Proliferation

Liver tumors have occurred in rats and mice exposed to high doses of DINP (Moore 1998a and 1998b).[3] 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 cause liver tumors in rodents.

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[3] 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).

Because many peroxisome proliferators 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 the related 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 secondary to peroxisome proliferation (IARC, 1995; Cattley *et al.*, 1998; Klaunig *et al.*, 2003).

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 20 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 mechanism by which nongenotoxic peroxisome proliferators such as DINP and DEHP cause liver carcinogenicity in rodents is not relevant for humans (Ashby *et al.*, 1994; Kluwe, 1994: Bentley *et al.*, 1993; Lake, 1995; 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 12 months with a strong peroxisome proliferating agent (Peters *et al.*, 1997).[4] These studies demonstrated an absolute requirement for activation

[4] 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.

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.

The first review was a 1994 working group of IARC to consider the relevance to humans of peroxisome proliferation 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 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 "PPAR*α* 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 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 RSI workgroup is that the liver tumors that occur in rodents treated with DINP are not likely to occur in humans.

Thus, there was a consensus in the scientific community that peroxisome proliferators presented, at the 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) Were 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 1) 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 1.
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 weights 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 of examples 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 secondary to 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.[5] (A genotoxic 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 mechanism for induction of liver tumors by another phthalate (DEHP) that is independent of PPARα activation. As discussed in Section V.A.4.a, below, the utility of the mouse model employed by Ito *et al.* is limited, such that the data of Ito *et al.* are not sufficient to indicate there is a valid alternative mechanism of carcinogenesis other than that related to peroxisomal proliferation. 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

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

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.

- Like 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 risk assessment.

4. A PPARα-Dependent Mechanism Is the Only Plausible Mechanism 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 mechanism for the liver tumor response in rats and mice (Klaunig *et al.*, 2003). With respect to the question of whether there are any other processes that provide a plausible explanation for the tumors in rodents, there are three sub-questions: (a) Is there evidence for processes in rodents other than those associated with peroxisome proliferation which could explain the liver tumor response? (b) Is there plausible evidence that the consensus view of this carcinogenic process, *i.e.*, that it involves a PPARα-mediated process, is inaccurate? and (c) Is there plausible evidence for an alternative process which could be operative in humans?

a. *Is there evidence for processes in rodents other than those associated with peroxisome proliferation which could explain the liver tumor response?*

Other mechanisms for carcinogenicity in rodents are not supported by the data. Since DINP is not genotoxic, the liver tumors could not have been initiated by a direct interaction between chemical and 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).

Ito *et al*. (2007) have proposed an alternative mechanism for induction of liver tumors by another phthalate (DEHP) that is independent of PPARα activation. The report compares the effects of long-term dietary exposure of up to 0.05% DEHP on liver toxicity of wild type and PPARα null (-/-) mice. The results presented must be carefully considered in light of the utility of the PPARα null mouse model used and additional reports indicating an inherent susceptibility of these mice to tumorigenesis.

Ito *et al.* reported the use of a PPARα -/- mouse strain produced according to a method published by Lee *et al.* (1995). These knockout mice had both PPARα alleles replaced using the homologous recombination technique. Four biological endpoints were assessed after 24 months of treatment; the endpoints were referred to as: macroscopic liver findings; microscopic liver findings; oxidative damage (8-OHdG levels) and proto-oncogene expression levels (mRNA and/ or protein). A statistically significant increase in the number of liver tumors (*i.e.,* hepatocellular carcinomas, hepatocellular adenomas, and chologiocellular carcinomas) from 2-8 (10-25.8%) was seen between the wild type and knockout mice fed the top dose DEHP diet ($p<0.05$). This was mostly due to a jump from 2 to 6 in hepatocellular adenoma incidence between these two groups. It should be noted that 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.

There was no significant effect reported on bodyweight or liver weight, though the data suggested a trend towards an increase in liver weight for the PPARα -/- animals, especially the 0.05% DEHP exposed group (+/+ mean = 1.27g ±0.18; -/- mean =1.78g±0.84). The reported 8-

OHdG data suggest -/- mice suffered from an increased hepatic oxidative stress with DEHP as compared to the +/+ mice, though this was not supported by unchanged mRNA levels for 8-oxoguanine DNA-glycosylase 1, an 8-OHdG repair enzyme. As +/+ mice showed lower 8-OHdG levels than -/- mice at all DEHP exposure levels *and* in the controls (0% DEHP), PPARα may prevent the oxidation of DNA dG. Ito *et al.* did not address the plausibility of any biological relevance between raised 8-OHdG levels and increased incidence of liver tumors in -/- mice exposed to DEHP.

On the basis of their data, Ito *et al*., 2007 proposed an alternative mechanism for DEHP induced liver tumors, which is independent of PPARα activation in that inflammation and protooncogenes altered by 0.05% DEHP-derived oxidative stresses may be involved in the tumorigenesis found in the PPARα-null mice, but not in wild-type mice. However, the utility of these data is limited in that a number of reports have indicated that aged PPARα null mice are more vulnerable to tumorigenesis due to fundamental mechanistic differences (Mandard *et al*., 2004; Kostadinova *et al*., 2005; Balkwill and Couseens, 2005; Pikarsky *et al*., 2004; Takashima *et al*., 2008). Most recently, gene expression profiles of hepatocellular adenoma tissues as well as control livers of wild-type and PPARα null mice were examined (Takashima et al., 2008). The genes identified to contribute to tumorigenesis (i.e. Gadd45a and caspase 3-dependent apoptosis genes) in the null mice were unique to the null mice.

As spontaneous tumors are known to occur in the PPARα null mice at 24 months, Ito *et al.* indicate the possibility that DEHP merely promoted the formation of the spontaneous liver tumors in the aged null mice; a mechanism that is unique to the null mice and would not exist in the wild type mice. 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. Therefore, the Ito *et al*. (2007) data are not sufficient to indicate there is a valid alternative mechanism of carcinogenesis other than that related to peroxisomal proliferation.

b. *Is there plausible evidence that the consensus view of this carcinogenic process, i.e., that it involves a PPARα-mediated process, is inaccurate?*

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 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 (Peters *et al.*, 1997). An alternative proposal 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 mechanisms 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.

c. *Is there plausible evidence for an alternative process which could be operative in humans?*

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. As described in more detail below, 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. There is no plausible evidence that differences between humans and rodents could lead to an increased risk of cancer to humans. Again, this is demonstrated empirically. In studies of primate and human hepatocytes in culture, DINP does not produce peroxisome-proliferation related effects (Benford *et al.*, 1986; Baker *et al.*, 1996; Hasmall *et al.*, 1999; Kamendulis *et al.*, 2002). In primate *in vivo* studies, high doses of DINP do not produce liver changes of any kind. Pugh *et al.* (2000) performed a 14-day study of cynomolgus monkeys in which no liver effects – including no change in hepatic peroxisome β-oxidation – were observed from high doses of DEHP and DINP (500 mg/kg/day). Hall *et al.* (1999) reported that administration of 2500 mg/kg/day DINP to marmosets for 13 weeks produced no pathological changes in liver, kidneys or testes, and no evidence of peroxisomal proliferation.

* * * * *

In summary, for the reasons given above, there is no mechanism other than a PPARα process that provides a plausible mechanism for the liver tumors observed in DINP-treated rodents.

5. <u>The PPARα Mechanism Does Not Operate in Humans</u>

Having established that the mechanism 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.

The demonstration that activation of PPARα was an absolute requirement in the induction of liver carcinogenesis (Peters *et al.*, 1997) 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).[6] Vanden Huevel (1999) noted that there was interindividual variability in human PPAR sequences and wondered whether than 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, all of the available data indicate 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.

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[6] 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.

There is also inferential evidence 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 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).

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	+	-	-

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).

Thus 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. Hall *et al.* (1999) showed that DINP treatment could 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) through studies with cynomolgus monkeys. Similarly under *in vitro* conditions, DINP increased replicative DNA synthesis and suppressed apoptosis in rodent hepatocytes but had no effects in human cells (Hasmall *et al.*, 1999), and MINP, the monoester corresponding to DINP, had no effects on peroxisomal enzyme levels in either human or primate hepatocytes in culture (Benford *et al.*, 1986; Kamendulis *et al.*, 2002).

6. Even if DINP did 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, for two reasons. First, 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. Second, even if sufficiently high internal doses could somehow be achieved, actual exposures to such doses of DINP would not occur under any plausible scenario.

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 milligrams per kilogram 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, even at very high treatment 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. 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 (Anderson *et al.*, 2001). 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. The data indicate that effects produced in rodents by DINP will not occur in humans, at least in part because the high internal doses required to produce these effects in rodents cannot be achieved in humans.

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 *et al.*, 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).

Even if human doses were not limited by absorptive capacity, however, exposure to the amount of DINP that would be required to cause liver tumors simply would not occur under any plausible exposure scenario. As stated above, the lowest level at which DINP produced a carcinogenic response was 336 mg/kg/day (female mice). If one were to assume that humans and mice were equally sensitive, a 70 kg human would have to ingest an average of 23,500 mg/day over an entire lifetime to achieve a dose to that which caused liver tumors in mice.

However, humans and rodents are not equally sensitive. As described above, the levels of PPARα in humans are at least an order of magnitude below those found in rodents (Tugwood *et al.*, 1996; Palmer *et al.*, 1998). Thus, humans should be at least an order of magnitude less sensitive than animals. That brings the required dose to approximately 235,000 mg/day for a 70 kg adult (or approximately half a pound per day of undiluted material). (In fact, the required dose would be yet higher, because, as discussed above, human PPARα does not respond to DINP in the same manner as rodent PPARα.) Such high doses simply cannot be reasonably foreseen for humans.

Since 1999, the CDC has been analyzing samples of urine from the U.S. population for phthalate metabolites. CDC has reported its biomonitoring findings in reports issued in 2001, 2003 and 2005. The 2003 report includes the data from the 2001 report, and provides results for samples from 2541 persons (CDC, 2003). The 2005 report provides data for an additional 2772 persons (CDC, 2005). The DINP exposure corresponding to the urinary metabolite concentration can be calculated using the method of David (2000).

For the results reported in 2003, no DINP metabolite was detected at the 50th or 75th percentile levels. At the 95th percentile, the creatinine-corrected value for the total population was 4.29 μg/g, which corresponds to a DINP exposure of 0.88 μg/kg/day. The 95th percentile creatinine-corrected value for children, age 6-11, was 6.00, corresponding to a DINP exposure of 0.67 μg/kg/day.[7]

For the results reported in 2005, no DINP metabolite was detected in the overall population even at the 95th percentile, nor in subgroups divided by age. It was detected at the 95th percentile for Mexican Americans and Non-Hispanic Blacks, although the levels reported were lower than those reported in 2003. The higher of the two 95th percentile levels was for Mexican Americans – 2.31 μg/g. That converts to an exposure of 0.67 μg/kg/day.

Conservatively assuming a 70 kg person were exposed to the 2003 95th percentile level of exposure every day of their life, that person's exposure would be approximately 62 μg/day – a level that is approximately <u>3,800,000 times</u> less than 235,000 mg/day, the minimum shown above that would be required to cause tumors (assuming human PPARα could respond to DINP in an equivalent manner to rodent PPARα).

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[7] Restrictions on use of DINP in toys and child care products became effective in 2009 under both California and Federal law. Thus, future exposures to DINP will be even less, particularly for children.

Thus, it is not plausible that exposures of Californians to DINP would contribute to cancer incidence.

* * * * *

In summary, there is strong evidence that the PPARα mechanism which is responsible for liver tumors in DINP-treated rodents is not operable in humans. 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. This is because actual exposure to DINP, even under extreme scenarios, is far below the doses that would be required and because, even if humans were to receive high doses of DINP, they would not absorb sufficient DINP to result in internal doses high enough to lead to liver tumors. Thus, DINP cannot be reasonably anticipated to cause liver cancer in humans.

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, based on the CHAP and on the ILSI workshop, have "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 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)

The EU did not identify carcinogenicity as a critical endpoint (ECB, 2003a, 2003b) and has not classified 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 EPA 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 EPA in 2001; copies as provided with these comments, as follows:

- Attachment A is a statement by Ruth Roberts, Ph.D., Head of Cell Biology Research and Cancer Project Manager at Syngenta Central Toxicology Laboratory 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 EPA, 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 Is Not Relevant to Humans

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 risk 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 Risk 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 those 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 *et al.*, 1998a) but not in the B6C3F1 mouse (Moore *et al.*, 1998b) or 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 have 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 risk 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).

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."

EPA has not generally regarded MNCL in Fisher 344 rats to be indicative of any human cancer concern. For example, EPA reviewed the Lington DINP study, including the MNCL data, and concluded that "no evidence of carcinogenicity has been found in these studies" (Hirzy, 1989). Additionally, in its review of butylbenzyl phthalate, EPA stated that the available evidence, including increased MNCL in F-344 rats, "does not indicate that BBP causes or can reasonably be anticipated to cause cancer in humans" (EPA, 1987).

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." 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."

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. Without further research to clarify the leukemic cell of origin, and define candidate molecular targets, the case for potential human relevance remains weak, particularly in light of the high, variable spontaneous incidence of MNCL in the Fischer 344 rat – the 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).

Thus, the opinion of many authoritative bodies and the current literature continue to support the position 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. The Mechanism for Formation of Kidney Tumors in Male Rats Exposed to DINP Is Not Relevant to Humans

Kidney tumors have been observed in male rats exposed to high doses of DINP 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 (EPA, 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 EPA 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).

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

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

EPA 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 EPA (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." (EPA, 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." (EPA, 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." (EPA, 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 EPA, 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 EPA's obligatory criteria are met for DINP. When this is the case, then EPA's guidance states that additional information is reviewed (EPA, 1991, Section XVII-B, pp. 87-88). Data are available for several of the categories of EPA describes,[8] as follows:

(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.

EPA'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

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

> 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. (EPA. 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 EPA'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 EPA, 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 risk 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. Exposure to DINP Does Not Result in Testicular Dysgenesis Syndrome

In the compilation of studies on DINP, under the heading "Mechanisms", OEHHA lists "Testicular dysgenesis syndrome" (TDS) and references the paper published by Borch *et al*. (2004). However, based on all currently available data, DINP does not induce TDS and TDS should not be considered as a mechanism of toxicity for DINP.

The TDS term was first coined in 2001 when it was hypothesized that cases of abnormal spermatogenesis, cryptorchidism (undescended testicles), penile malformations such as hypospadias, and incidences of testicular cancer observed in humans may all have a common etiology (Skakkebaek *et al*., 2001). The hypothesis states that these clinical problems may result from an irreversible developmental disorder 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).

There have been 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). In all of the summaries of reproductive, developmental, and chronic carcinogenicity studies, DINP has not been identified as producing any adverse TDS-like effects.

In chronic 2-year carcinogenicity reports published by Moore *et al.* (1998a) (daily exposure to 0, 500, 1500, 6000, or 12000 ppm) and Lington *et al.* (1997) (daily exposure to 0, 300, 3000, or 6000 ppm), benign testicular interstitial cell tumors were found in nearly all control and F344 rats treated chronically with DINP. However, this finding is not considered relevant since the incidences of the tumors were found to be within the historical control range and F344 rats normally display a high incidence of testicular tumors. Similarly, a 2-year carcinogenicity study involving Sprague Dawley rats exposed daily to 0, 500, 5000, or 10000 ppm to a DINP

mixture that was never commercially produced, both testicular (interstitial) cell hyperplasia and tumors were elevated above concurrent controls in the only exposure group examined, the high dose exposure group (Biodynamics, 1986). The incidence of the testicular tumors in the high dose animals were significantly above those in the controls, but within the range of historical control values and are therefore not considered reliable for interpretation.

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 post natal day (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.

In a two generation study (Waterman *et al*., 2000), P1 males and females received test material daily for at least ten weeks prior to mating and during the mating period. Additionally, P1 female animals received test material during the gestation and postpartum periods, until weaning of the F1 offspring on Post Partum Day (PPD) 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 PPD 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.

Sharpe (2003) proposed that suppression of fetal androgen production and/or increased estrogen exposure might underlie the occurrence of TDS with respect to certain phthalates. However, the data for DINP are inconsistent with respect to anti-androgenic effects in young male rats. Two studies, which used an unrealistically high doses of DINP 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).

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 that some males displayed 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 to 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 see 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.

Likewise, the paper by Borch *et al*. (2004) does not present data demonstrating that 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 days 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 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 alone 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.

In conclusion, there is no evidence that DINP induces TDS in laboratory animals. 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.

CONCLUSION

The foregoing information is presented to assist the CIC in developing a recommendation concerning the level of priority that should be assigned to DINP for possible evaluation for listing under Proposition 65. 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. Therefore, ExxonMobil respectfully submits that DINP should not be listed under Proposition 65 as a carcinogen. The ranking of DINP for development of hazard identification materials should be "no priority" or, at most, "low priority."

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NOTE: ExxonMobil would be pleased upon request to provide a copy of any of these studies.

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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.

Also provided as a separate pdf is the full text of Klaunig *et al.,* 2003.

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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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.

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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,

James E Klaunig, Ph.D.
Professor and Director 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

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

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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:

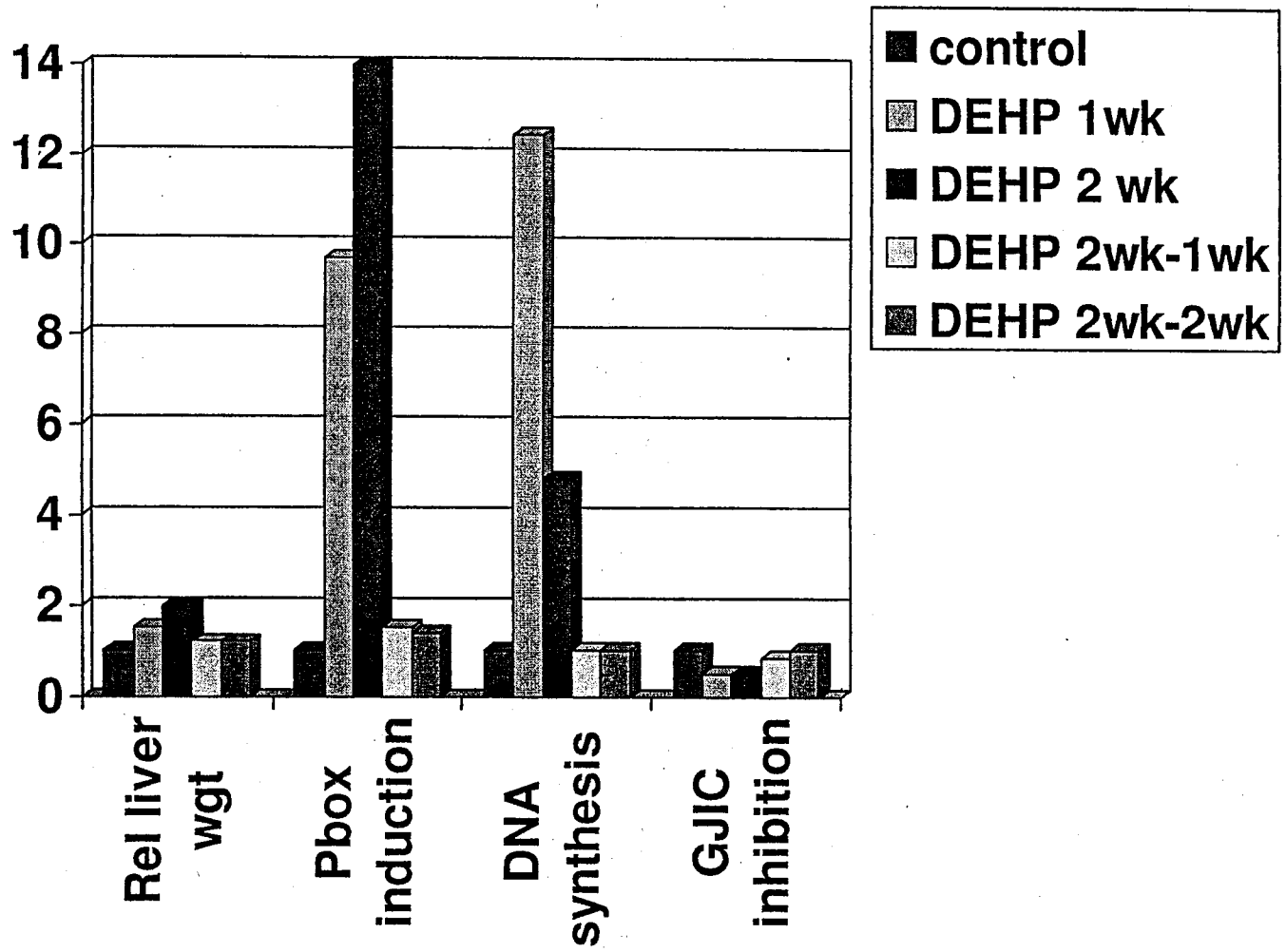
- modulation of cell growth
  - cell proliferation
  - cell death (apoptosis)
- changes in cell to cell communication (gap junctional communciation)
- modification of gene expression
- induction of peroxisomal proliferation

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

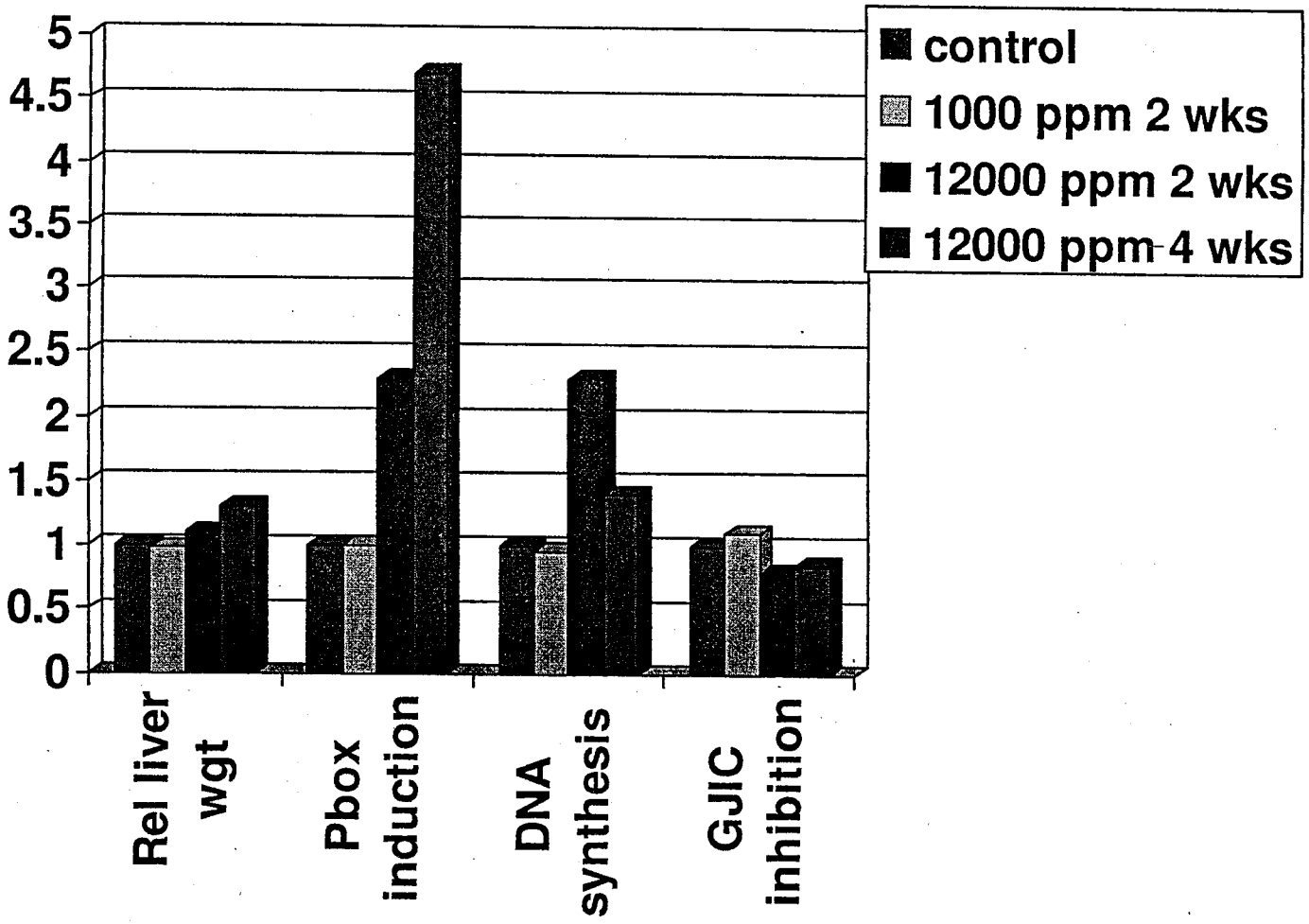


EFFECTS OF DINP ON F344 RAT LIVER[1]

	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



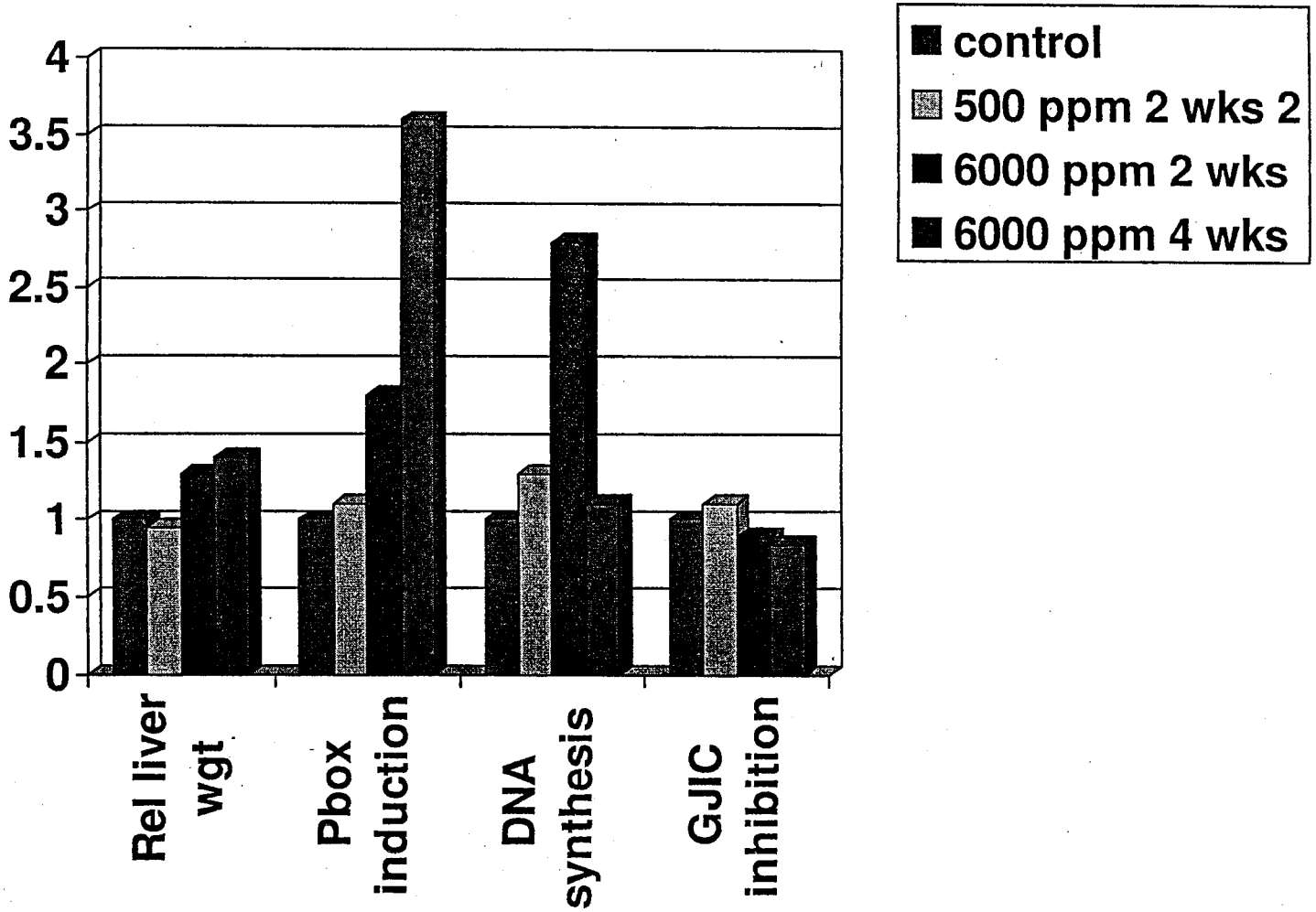
EFFECTS OF DINP ON B6C3F1 MOUSE LIVER[1]

	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)

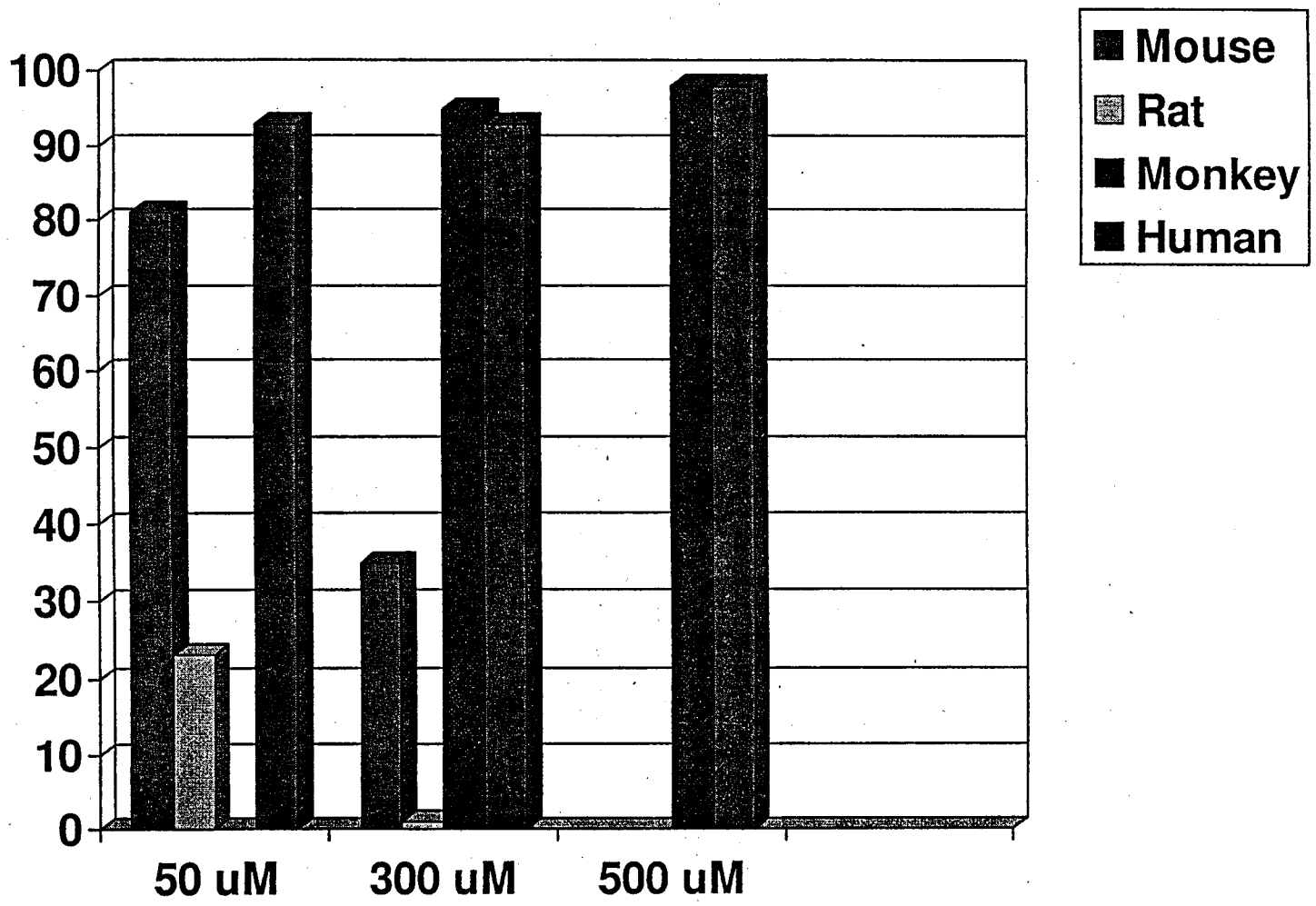
[1] Pugh et al., (2000)

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)



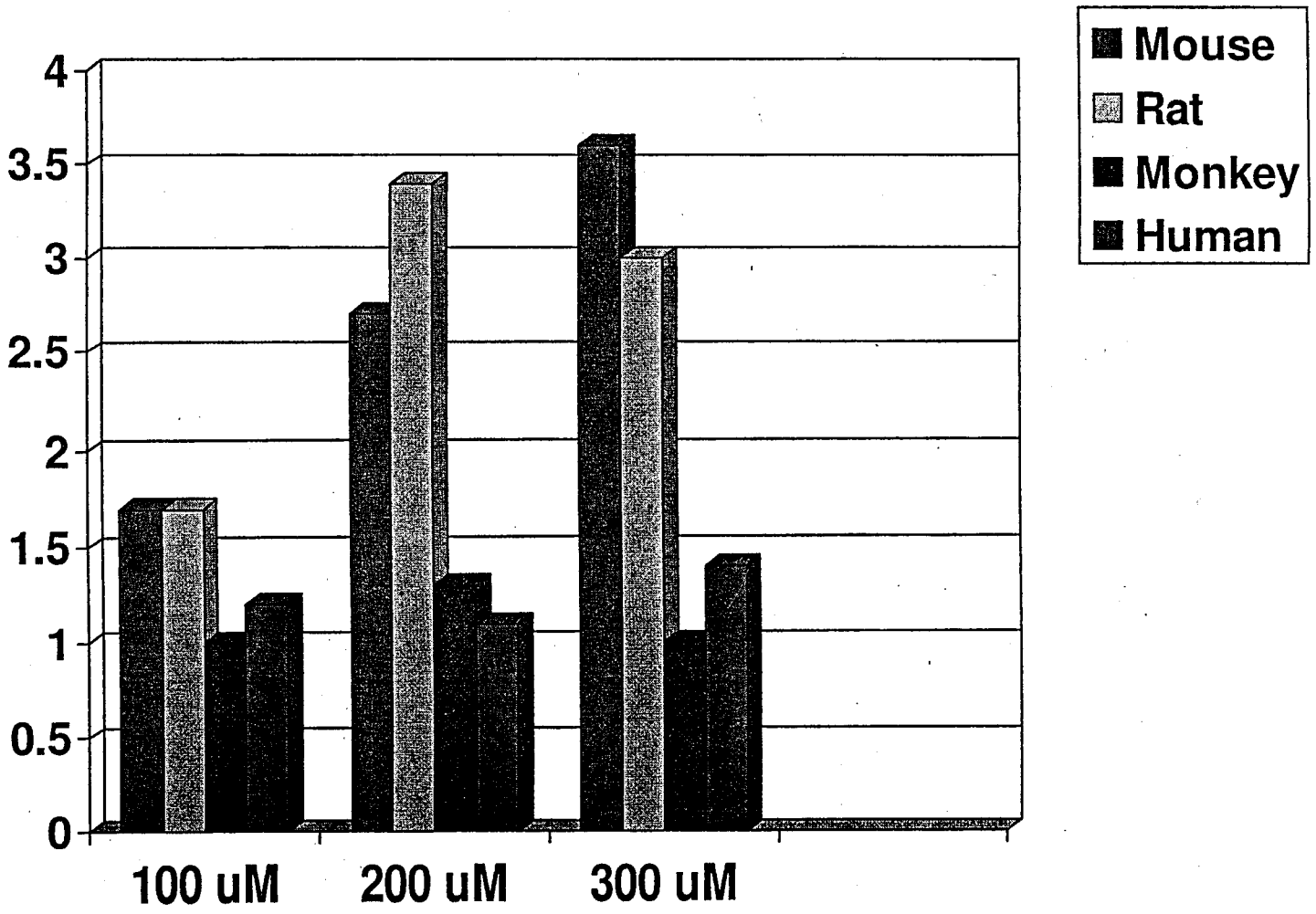
EFFECTS OF MEHP ON PBOX INDUCTION[1] (IN VITRO STUDIES)

	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)

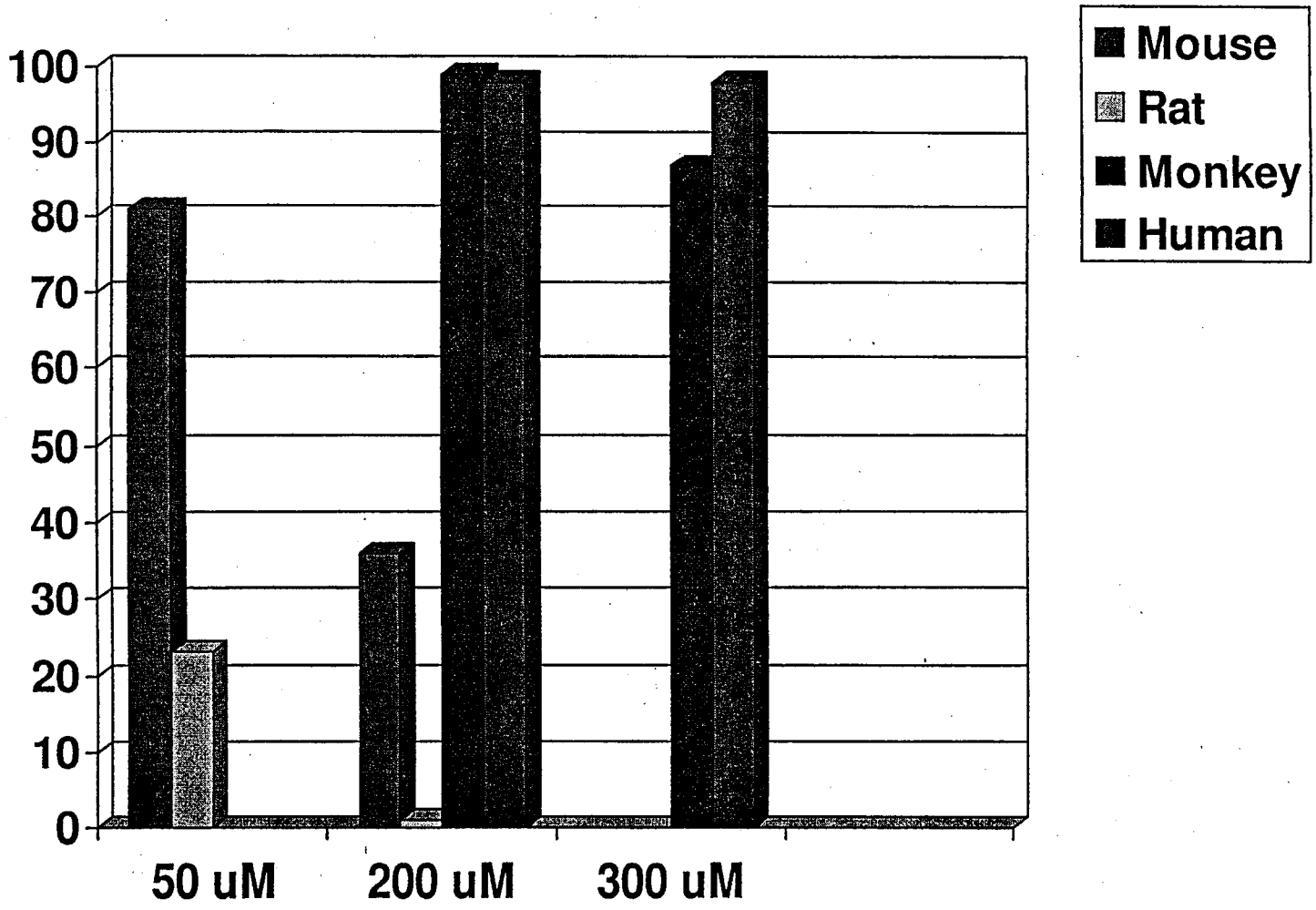


EFFECTS OF MINP ON GJIC INHIBITION[1] (IN VITRO STUDIES)

	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)

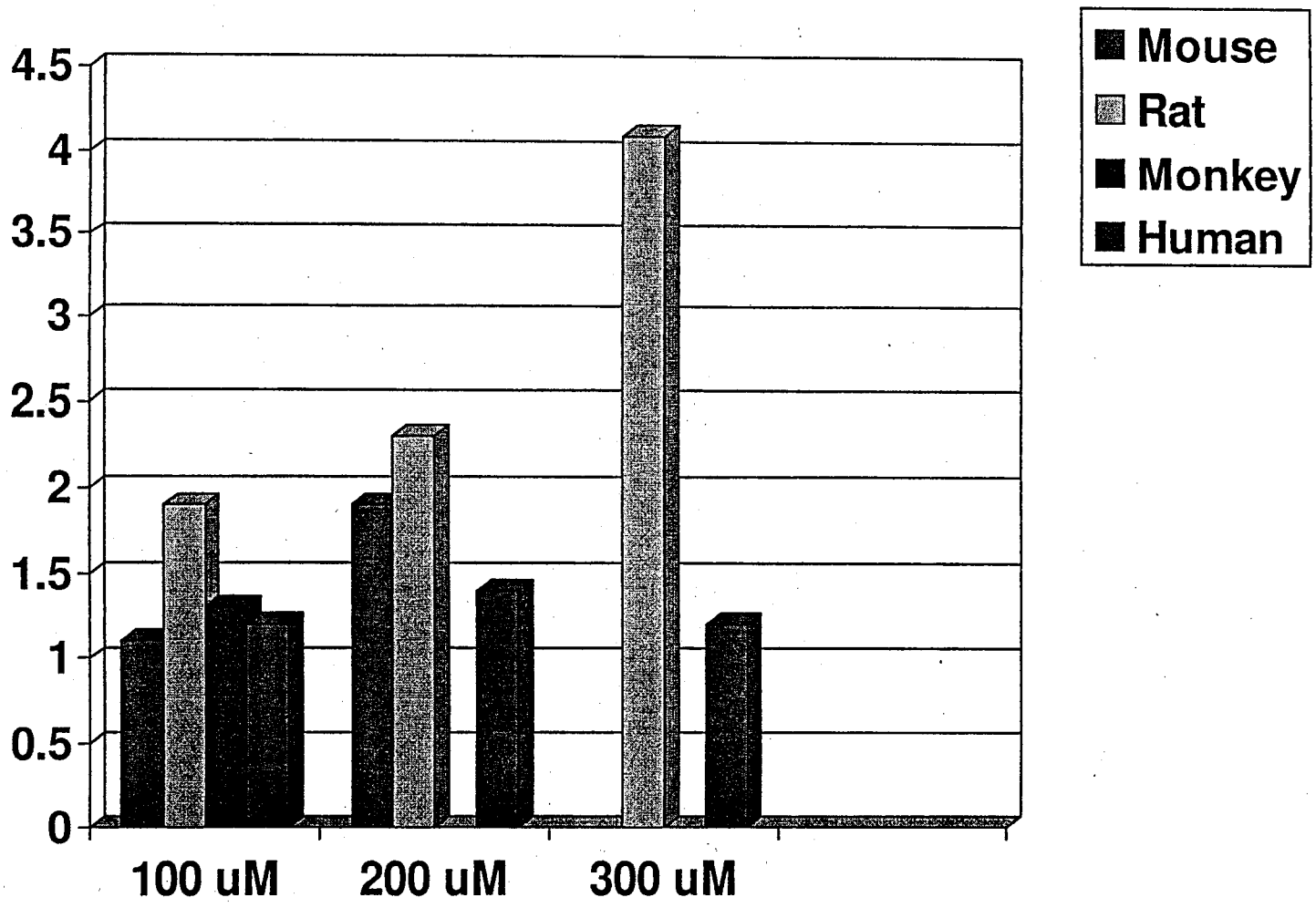


EFFECTS OF MINP ON PBOX INDUCTION[1] (IN VITRO STUDIES)

	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

Cancer Risk Assessment
Di(isononyl) phthalate

Prepared for the
American Chemistry Council

by

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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 $\alpha 2\mu$–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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September 4, 2000

Dr. Marian K. Stanley
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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,

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

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January 25, 2001

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

James A. Swenberg

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

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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≥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

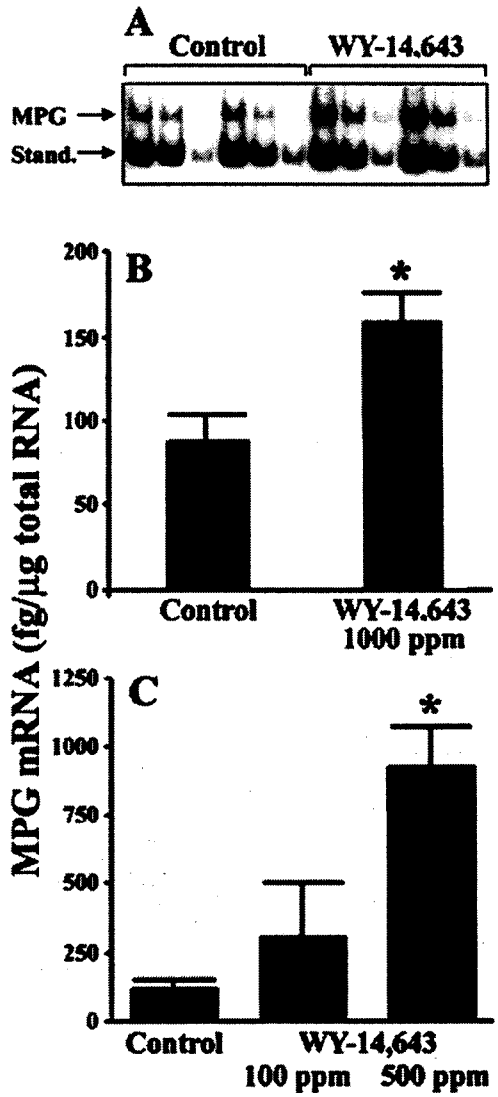

Fig. 1. MPG mRNA is induced in rat liver by dietary feeding of WY-14,643. (**A**) An autoradiogram of PCR products of native mRNA and synthetic MPG RNA (a kind gift of Dr S. Mitra, University of Texas, Galveston, TX) after *Eco*RI digestion is shown. Fisher 344 rats were fed either control diet (NIH-07) 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.

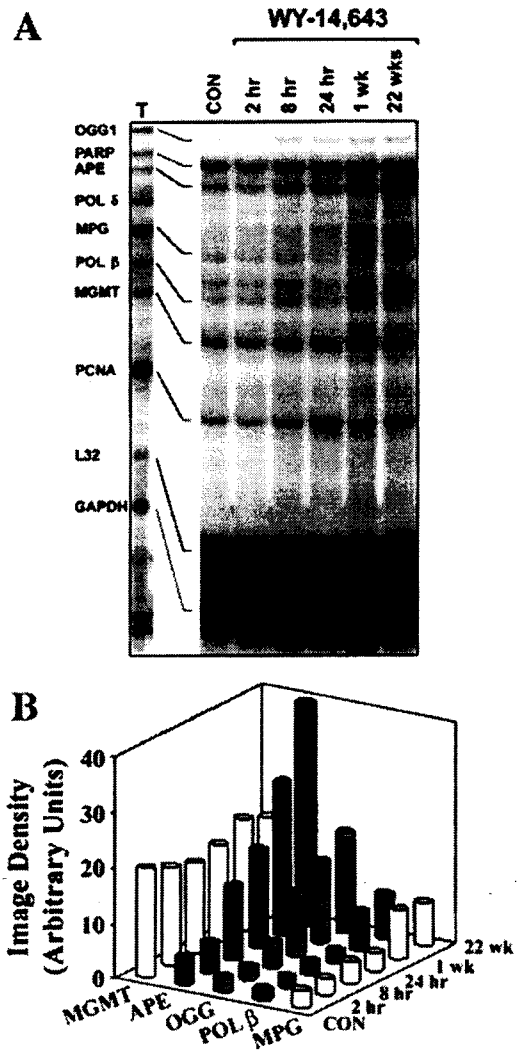

Fig. 2. WY-14,643 causes a time-dependent increase in expression of base excision DNA repair enzymes in rat liver. (**A**) Total RNA was isolated from livers of rats treated with WY-14,643 (1000 p.p.m.) for up to 22 weeks using a QuickPrep extraction kit (Amersham Pharmacia Biotech) and dissolved in 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

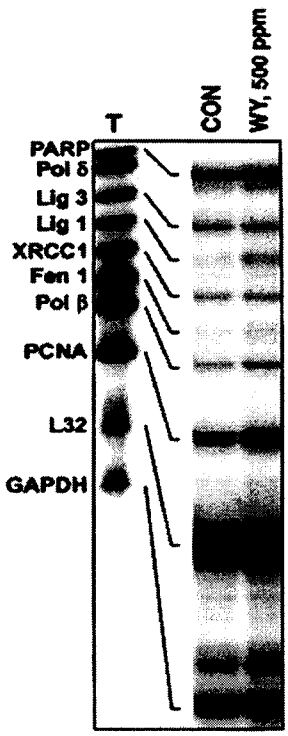

Fig. 3. Expression of ligase I is induced in mouse liver by WY-14,643. Mice (C57Bl/6) were fed a diet containing the potent carcinogen WY-14,643 (WY, 500 p.p.m.) for 7 days. Total RNA was isolated from liver samples and used in the RNase protection assay as detailed in Figure 2. Representative autoradiograms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

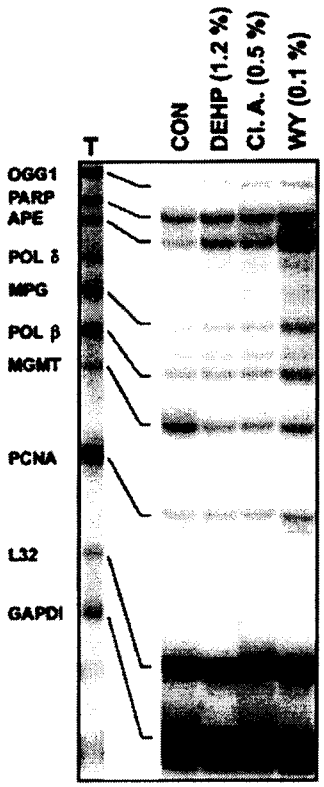

Fig. 4. Chronic treatment with peroxisome proliferators that are potent carcinogens induces increases in DNA BER enzymes expression. Total RNA was isolated from livers of rats treated with DEHP (1.2% w/w), clofibric acid (Cl. A., 0.5% w/w) or WY-14,643 (WY, 0.1% w/w) for 22 weeks and used in the RNase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

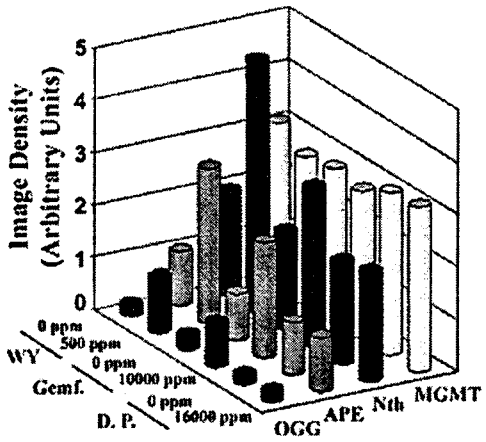

Fig. 5. Sub-chronic treatment with the potent carcinogens WY-14,643 and gemfibrozil, but not with the weak carcinogen dibutyl phthalate, increases expression of DNA excision repair enzymes. Total RNA was isolated from livers of rats treated with WY-14,643 (WY, 500 p.p.m.), dibutyl phthalate (D.P., 10000 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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