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American Chemistry Council Good Chemistry Makes It Possible

November 17, 2005

VIA EMAIL

Dr. C. W. Jameson
National Toxicology Program
Report on Carcinogens
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RE: 12th Report on Carcinogens
Nomination of DEHP for Delisting

Dear Dr. Jameson:

The American Chemistry Council Phthalate Esters Panel (Panel) submits these materials in response to the NTP request for comments on nominations to the 12th Report on Carcinogens (RoC). 70 Fed. Reg. 60548 (Oct. 18, 2005). The Panel's comments pertain to the request to delist di(2-ethylhexyl) phthalate (DEHP) from the RoC. The Panel consists of the major domestic producers and some users of phthalate esters, including DEHP.

These comments supplement comments submitted by the Panel on July 15 and 21, 2005. The information in these comments and in the Panel's previous submissions supports a conclusion that DEHP cannot be reasonably anticipated to cause cancer in humans. The Panel therefore supports the request to delist DEHP from the Report on Carcinogens.

If you have any questions, please call Marian K. Stanley, Senior Director and Manager of the Phthalate Esters Panel, at (703) 741-5623, email her at marian_stanley@americanchemistry.com, or write her at the address at the bottom of this letter.

Sincerely yours,

Courtney M. Price

BEFORE THE
NATIONAL TOXICOLOGY PROGRAM

SUPPLEMENTAL COMMENTS OF THE PHTHALATE ESTERS PANEL OF THE AMERICAN CHEMISTRY COUNCIL ON THE PROPOSAL TO REMOVE DI(2-ETHYLHEXYL) PHTHALATE FROM THE REPORT ON CARCINOGENS

National Toxicology Program (NTP); Report on Carcinogens; Status of Nominations to the 12th Report on Carcinogens (RoC): Request for Comments and Nominations of Scientific Experts 70 Federal Register 60548 (Oct. 18, 2005).

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EXECUTIVE SUMMARY

The American Chemistry Council Phthalate Esters Panel (Panel) submits these materials in response to the NTP request for comments on nominations to the 12th Report on Carcinogens (RoC). The Panel's comments pertain to the request to delist di(2-ethylhexyl) phthalate (DEHP) from the RoC. The Panel consists of the major domestic producers and a user of phthalate esters, including DEHP.

The Panel provided initial comments and data to NTP on July 15, 2005 and July 21, 2005. The comments provided here discuss information published since July of 2005, or not otherwise addressed in the original documents submitted by the Panel. As such, they supplement the original comments. These comments make the following points.

- Klaunig et al. (2003) have presented a mode of action (MOA) analysis for peroxisome proliferators in general, and for DEHP in particular. This approach is strongly recommended to identify the key events leading to carcinogenesis in animals exposed to DEHP, and whether those events are applicable to humans. Application of this approach demonstrates that rodent liver tumors caused by exposure to DEHP are not relevant to humans.
    - The majority of genetic toxicity studies for mutations (bacterial or mammalian) are negative. Thus, DEHP is classified as non-genotoxic by authorities such as the International Agency for Research on Cancer (IARC).
    - The lack of a primate chronic study is not an issue because using the MOA framework analysis allows for identification of key events from short-term studies that demonstrate that primates are not susceptible to tumors from exposure to DEHP.
    - New data for gene expression in primates following exposure to DEHP and other peroxisome proliferators indicates a lack of response for cell proliferation and apoptosis, key events in the carcinogenic process.
- Pancreatic acinar cell adenomas, Leydig cell adenomas, and MNCL are lesions have been observed only in rats, not mice or other species. These lesions are associated with peroxisome proliferators, and as such are not likely to be relevant for humans. Thus, they do not provide a basis for concluding that DEHP can be reasonably anticipated to cause cancer in humans.
- Comet assay results are insufficient to support an alternate mode of action for DEHP. Changes in comet assays have been reported and are apparently the consequence of the formation of abasic sites in DNA. However, these changes probably result from oxidative stress – a possible contributor to the peroxisome proliferation MOA, may or may not be toxicologically important, and may or may not play a role in liver tumor induction in rodents, but are not likely to be toxicologically relevant to humans.
- The information in these comments and in the Panel's previous submissions supports a conclusion that DEHP cannot be reasonably anticipated to cause cancer in humans. The Panel therefore supports the request to delist DEHP from the Report on Carcinogens.

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INTRODUCTION

The American Chemistry Council Phthalate Esters Panel (Panel) submits these materials in response to the NTP request for comments on nominations to the 12th Report on Carcinogens (RoC). 70 Fed. Reg. 60548 (Oct. 18, 2005). The Panel's comments pertain to the request to delist di(2-ethylhexyl) phthalate (DEHP) from the RoC. The Panel consists of the major domestic producers and a user of phthalate esters, including DEHP.[1]

The Panel provided initial comments and data to NTP on July 15, 2005 and July 21, 2005. The comments provided here discuss information published since July of 2005, or not otherwise addressed in the original documents submitted by the Panel. As such, they supplement the original comments.

Part I of these comments describes the mode of action (MOA) analysis for peroxisome proliferators in general, and for DEHP in particular, developed by Klaunig et al. (2003). This approach, which identifies the key events leading to carcinogenesis in animals exposed to DEHP, provides the best approach for evaluation DEHP carcinogenicity. Application of this approach demonstrates that rodent liver tumors caused by exposure to DEHP are not relevant to humans. Supporting that conclusion are the following points:

- The majority of genetic toxicity studies for mutations (bacterial or mammalian) are negative. Thus, DEHP is classified as non-genotoxic by authorities such as the International Agency for Research on Cancer (IARC).

- The lack of a primate chronic study is not an issue because using the MOA framework analysis allows for identification of key events from short-term studies that demonstrate that primates are not susceptible to tumors from exposure to DEHP.

- New data for gene expression in primates following exposure to DEHP and other peroxisome proliferators indicates a lack of response for cell proliferation and apoptosis, key events in the carcinogenic process.

Part II explains that pancreatic acinar cell adenomas, Leydig cell adenomas, and MNCL observed in studies of DEHP do not provide a basis for concluding that DEHP can be reasonably anticipated to cause cancer in humans. These lesions have been observed only in rats, not in mice or other species. These lesions are associated with peroxisome proliferators, and as such are not likely to be relevant for humans.

Part III explains that comet assay results are insufficient to support an alternate mode of action for DEHP. Changes in comet assays have been reported and are apparently the consequence of the formation of abasic sites in DNA. However, these changes probably result from oxidative stress – a possible contributor to the peroxisome proliferation MOA, may or may not be toxicologically important, and may or may not play a role in liver tumor induction in rodents, but are not likely to be toxicologically relevant to humans.

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[1] The Panel members are BASF Corporation, Eastman Chemical Company, ExxonMobil Chemical Company, Ferro Corporation, and Teknor Apex, Inc.

The information in these comments and in the Panel's previous submissions supports a conclusion that DEHP cannot be reasonably anticipated to cause cancer in humans. The Panel therefore supports the request to delist DEHP from the Report on Carcinogens.

I. A MODE OF ACTION FRAMEWORK ANALYSIS DEMONSTRATES THAT DEHP CANNOT BE REASONABLY ANTICIPATED TO CAUSE LIVER TUMORS IN HUMANS

As discussed in the Panel's July 15, 2005 comments, the International Life Sciences Institute (ILSI) Risk Science Institute formed a workgroup in 2001 to review information on the mechanisms by which peroxisome proliferating chemicals produce carcinogenic responses in rats and mice. The report of the workgroup was published in late 2003 (Klaunig et al., 2003). For peroxisome proliferators in general, 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" (Klaunig et al., 2003, p. 693). DEHP was included as a case study by the group, with the following outcome: "The data lead to a conclusion that a carcinogenic response induced via the [modes of action] for liver tumorigenesis in the rodent is not likely to occur in humans following exposure to DEHP" (Klaunig et al., 2003, p. 704). The following explains the why the mode of action (MOA) framework used by Klaunig et al. provides the best approach for evaluating DEHP data and how the data demonstrate that liver tumors in rodents are not relevant to humans.

A. A Mode of Action Framework Provides the Best Approach for Evaluation of the Relevance to Humans of Liver Tumors in Rodents Exposed to DEHP

DEHP has been characterized as a non-genotoxic carcinogen due to its lack of mutation or other direct action on genetic material associated with the production of cancer (IARC, 2000). While this statement is an oversimplification of the data available on genotoxicity studies conducted with DEHP, and selective assays have produced positive results under some conditions, the weight of the evidence indicates that a genetic mechanism for tumorigenesis is not relevant for DEHP (IARC, 2000). Instead, the hepatocellular tumorigenic process for DEHP has long been associated with biochemical (and cell proliferative) changes known as peroxisome proliferation.

DEHP was listed in 1987 by the NTP as reasonably anticipated to be a human carcinogen based on a finding of liver tumors in rats and mice exposed to high doses in the diet (NTP, 1983). In evaluating the relevance to humans of liver tumors observed in the bioassay of DEHP, several approaches can be taken: one is to determine relevance based on extensive mechanistic information, another is to evaluate the carcinogenicity in an animal model closely resembling humans such as a non-human primate, another is use epidemiology data, and another is to use a mode of action framework analysis.

Mechanistic Information. Since the results of the bioassay were first reported, there have been numerous studies on the mechanism of carcinogenesis for DEHP and other peroxisome proliferators. These studies have identified many of the gene expression, cellular, and biochemical events that lead to liver tumors. They have shown that peroxisome proliferation is triggered by a nuclear receptor PPARα; that increase in cell proliferation and inhibition

apoptosis work in concert to maintain and promote damaged cells that would ordinarily have died; that biochemical processes involved in fatty acid metabolism are separate from other processes involved in cell turnover, and that Kupffer cells play a role in cell proliferation. There are gaps, however, in the understanding of the process, so that relying solely on traditional mechanistic data may be inadequate to evaluate the relevance of rodent liver tumors to humans.

Non-Human Primate Model. Evaluating the relevance of tumors in rodents by comparing the results to other species, such as non-human primates, requires either an assumption or understanding about the relevance of that animal model to humans compared with the rodent model. To date, no primate study of sufficient duration has been conducted with any peroxisome proliferator to ascertain that tumors would not develop following lifetime exposure. On the other hand, studies have been conducted for sufficient duration to demonstrate that not all the cellular and biochemical events seen in rodents occur in non-human primates. Of the key events identified in rodents, there is evidence that the biochemical processes associated peroxisomal enzymes for fatty acid metabolism are not increased in human or primate liver *in vitro* or primate liver *in vivo* (IARC, 2000; Klaunig et al., 2003). Furthermore, cell cycle events such as increased cell proliferation or inhibition of apoptosis have not been observed in human or primate liver, and inhibition of gap junction intracellular communication (GJIC) has not been observed (IARC, 2000; Klaunig et al., 2003). While these data have stood for some time, there have been questions about the sensitivity of measurements and whether these events could be accurately measured *ex vivo*. However, recent data from *in vivo* studies of primates treated with ciprofibrate, a more potent peroxisome proliferator than DEHP, demonstrate that gene expression for these cellular events are not upregulated as they are in rodents (Cariello et al., 2005). Therefore, the data showing no increase in cell proliferation or inhibition of apoptosis following exposure of human cells to DEHP are supported by evidence that the genes for these events are not turned on, rather than any peculiarities of the test system.

Epidemiology. Epidemiological data populations exposed to environmental or workplace levels of DEHP are not available. Mortality studies of populations exposed to DEHP via medical devices are inconclusive because of the short period of time of exposure and the fact that these populations are medically compromised. However, epidemiological studies for therapeutic peroxisome proliferators such as hypolipidemic drugs have failed to suggest even minimal association of cancer mortality with exposure.

Mode of Action Framework. What has been used to evaluate the relevance of rodent liver tumors to humans is a mode of action (MOA) framework analysis (Klaunig et al., 2003) that uses the Bradford-Hill criteria for causality (Hill, 1965). In this process, the key events leading to tumorigenesis are identified and supported by evidence in rodents. Reversibility, temporal response, and dose-response information are used to establish biological plausibility. These key events are then evaluated for their occurrence in humans (or non-human primates). If any key event does not occur in humans, then the tumors are considered to be not biologically plausible in humans. The Panel believes that this approach is the most scientifically defensible and rational for evaluating the relevance of rodent liver tumors to humans. Since Klaunig et al. (2003) already provided the state of the science up to 2002, the NTP can build on that beginning by applying data generated since 2002. In doing so, it is assumed that all peroxisome proliferators act via a common mode of PPARα activation, and that compounds

differ only in their potency. Thus, the lack of a key event for one peroxisome proliferator likely is applicable to all peroxisome proliferators.

The Panel submits, then, the following MOA Framework analysis from Klaunig et al., with additional data developed since 2002, for consideration by NTP in determining the relevance to humans of rodent liver tumors observed in DEHP studies.

B. A Mode of Action Framework Analysis for DEHP Shows that Key Events Leading to Liver Tumors in Rodents Are Not Operative in Humans or Other Primates

Although the NTP may choose to develop its own MOA list of key events, Klaunig et al. (2003) provides a MOA for DEHP, and peroxisome proliferators in general, which can be used as a starting point (Table 1). Given that this MOA has undergone peer-review through a workshop of invited experts, and through publication, it is an accepted list of key events for liver tumorigenesis in rodents. Following this publication, another study has been reported that provides evidence of gene expression for cell proliferative triggers. Currie et al. (2005) reported on gene up- and down-regulation in rodent liver following short-term exposure to DEHP. $B_6C_3F_1$ mice were treated with 1150 mg/kg DEHP by oral gavage for up to 3 days. Gene expression was determined at 1, 2, 4, 8, 24, 48, and 72 hours after the first dose. Genes involved in cell growth and inhibition of apoptosis (NF-κB) are up-regulated following treatment. Furthermore, genes associated with epigenetic events are up-regulated, particularly those involved in one-carbon metabolism, which may help explain some of the spurious positive genetic toxicity results with this non-genotoxic carcinogen.

In addition to a MOA table for the key events in rodent tumorigenesis, Klaunig et al. provide evidence for the occurrence of these key events in humans or non-human primates (Table 2). While there are few key events in the mode of action that have been observed in human cells *in vitro* or non-human primates *in vitro* or *in vivo*, questions about the relevance of the MOA to humans still exist because human liver (and other tissues) contain PPARα, and because therapeutic PPARα-agonists have elicited some of the key events, especially ones associated with fatty acid metabolism. Thus, although the evidence for the effects of DEHP on human or non-human primate tissue indicates that the key events are not operative, the data for some strong PPARα agonists suggests that at least some events are operative. However, recent data for such potent PPARα agonists indicates that not all the tumorigenic key events in rodents are operative for humans, and therefore that formation of liver tumors in humans is not plausible.

While alteration in fatty acid metabolism is an effect in humans, other metabolic enzymes and cellular processes associated with peroxisome proliferation are not. Colton et al. (2004) demonstrated that primate liver did not show the same increase in peroxisome number and size that defines 'peroxisome proliferation'. Using quantum dots to visualize peroxisomes, Colton et al. found only an increase in numbers of peroxisomes, not in volume following treatment of cynomolgus monkeys with up to 400 mg/kg ciprofibrate, compared with an increase in both in rats. Furthermore, Vanden Heuvel et al. (2003) showed that gene expression in human hepatoma cells was much different than for rat hepatoma cells exposed to the potent PPARα agonist, WY 14,643; in fact, far fewer genes were affected in human cells than in rat cells. These differences include genes for metabolic enzymes and signaling factors. Investigators have

Table 1. Framework Analysis of Key Events in Rodents

In **bold** are new data (since 2002) in support of key events.

Event	Evidence in animals	Reference
Hydrolysis to monoester essential for bioactivation	*In vitro* for downstream events in primary rodent hepatocytes; in vitro for PPAR activation	Gray et al. (1983), Maloney and Waxman (1999), Mitchell et al. (1985a)
Activation of PPAR	Concentration-related activation in vitro; no downstream events in PPAR-null mice in vivo	Hasmall et al. (2000), Issemann and Green (1990), Maloney and Waxman (1999) in vitro; Ward et al. (1998) in vivo
PPAR-dependent regulation of genes encoding for peroxisomal enzymes	In vivo increases in mRNA for CYP in WT versus PPAR-null mice	Reddy et al. (1986), Ward et al. (1998)
PPAR-dependent regulation of genes encoding for cell cycle growth and apoptosis	~~No data~~ **Increase in gene expression for cell proliferation and inhibition of apoptosis in vivo**	**Currie et al. (2005)**
PPAR-dependent expression of genes encoding for fatty acid metabolism	Increase in gene expression for fatty acid metabolism enzymes	Reddy et al. (1986)
Peroxisome proliferation	Dose-related increases in CYP and peroxisomal enzymes	Barber et al. (1987), David et al. (1999), Dirven et al. (1990, 1992), Huber et al. (1996), Moody and Reddy (1978), Mitchell et al. (1985b), Reddy et al. (1986)
Perturbation of cell proliferation and/or apoptosis	Bursts of cell proliferation in vivo Prolonged cell replication Inhibition of apoptosis	Conway et al. (1989), David et al. (1999), James et al. (1998), Smith-Oliver and Butterworth (1987) Marsman et al. (1988), Mitchell et al. (1985), Ward et al. (1998) Hasmall et al. (1999, 2000), James et al. (1998)
Inhibition of GJIC	GJIC inhibited	Isenberg et al. (2000, 2001), Kamendulis et al. (2002)
Oxidative stress	Conflicting data in vivo; increased H_2O_2 levels in vitro	Cattley and Glover (1993), Takagi et al. (1990), Tomaszewski et al. (1986)
Kupffer cell-mediated events	Kupffer cell-mediated cell proliferation activation in vitro	Rose et al. (1999)
Selective clonal expansion	DEHP promotes initiated cells in vivo	Osterle and Deml (1988), Ward et al. (1983, 1986)

Table 2. Key Events Comparison between Rodents and Humans (or Other Primates)

In **bold** are new data in support of key events.

Event	Evidence in rodents	Evidence in primates
Hydrolysis to monoester essential for bioactivation	In vitro for downstream events in primary rodent hepatocytes; in vitro for PPAR activation	Hydrolysis and absorption can occur
Activation of PPAR	Concentration-related activation in vitro; no downstream events in PPAR-null mice in vivo	hPPAR can be activated by MEHP
PPAR-dependent regulation of genes encoding for peroxisomal enzymes	In vivo increases in mRNA for CYP in WT versus PPAR-null mice	No evidence of increased gene expression/transcription
PPAR-dependent regulation of genes encoding for cell cycle growth and apoptosis	**In vivo increase in gene expression for NF-κB (Currie et al., 2005)**	~~No data~~ **No increase in gene expression for cell proliferation or inhibition of apoptosis (Hoivik et al., 2004; Cariello et al., 2005) using ciprofibrate**
PPAR-dependent expression of genes encoding for fatty acid metabolism	Increase in gene expression for fatty acid metabolism enzymes	No evidence of increased transcription
Peroxisome proliferation	Dose-related increases in CYP and peroxisomal enzymes	No peroxisomal enzyme activity in human cells
Perturbation of cell proliferation and/or apoptosis	Bursts of cell proliferation in vivo; Prolonged cell replication; Inhibition of apoptosis	No evidence of cell replication or inhibition of apoptosis
Inhibition of GJIC	GJIC inhibited	No evidence of inhibition of GJIC in primates in vivo or human or primate hepatocytes in vitro
Oxidative stress	Conflicting data in vivo; increased H_2O_2 levels in vitro	No data for DEHP, but other PPs show no effect (O'Brien *et al*., 2005)
Kupffer cell-mediated events	Kupffer cell-mediated cell proliferation activation in vitro	No data
Selective clonal expansion	DEHP promotes initiated cells in vivo	No data

long thought that the difference in response was related to the response element, PPRE (Hasmall et al., 2000); however, data from Cheung et al. (2004) suggest that the receptor itself may play a role. Cheung et al. developed a transgenic mouse with hPPARα cDNA. This animal model was treated with Wy 14,643 or fenofibrate for up to 8 weeks. Genes and the resulting enzymes for fatty acid metabolism were upregulated in the humanized animals. Cell proliferation (as measured by BrdU incorporation) was not observed in the humanized animal model. Furthermore, in a recent study by Hoivik et al. (2004), cynomolgus monkeys treated for two weeks with ciprofibrate at doses up to 9 times the therapeutic level (*i.e.,* 400 mg/kg-day) exhibited a dose-related 2- to 3-fold increase in relative liver weight as well as peroxisome and mitochondrial number. However, the authors also noted that there was no evidence of cell proliferation based on the number of mitotic figures and immunohistochemical staining for the Ki-67 antigen. Consistent with these light microscopic findings, there was no treatment-related effect on hepatic mRNA levels for proteins involved in cell division or apoptosis or on most proteins known to respond to oxidative stress. As expected from the known pharmacology of PPARα agonists, a mild induction of mRNA levels of beta-oxidation and detoxification enzymes was observed.

Using the same test system, Cariello and coworkers (2005) recently evaluated the hepatic transcriptional profile in cynomolgus monkeys exposed to ciprofibrate at doses up to 400 mg/kg-day for either 4 or 15 days using Affymetrix human GeneChips®, an effective detection system for rhesus monkey RNA (Chrismar et al., 2002). As expected, genes associated with fatty acid metabolism and mitochondrial oxidative phosphorylation were upregulated, albeit at levels about 10-fold less than that observed in rodents. Additional analyses emphasized transcriptional responses for the following processes:

- Apoptosis. In cynomolgus monkeys, genes associated with the suppression of apoptosis in the liver were either downregulated (NFκB) or unchanged (TNFα), while a gene associated with the enhancement of apoptosis (TGFβ1) was unchanged. In a supervised analysis of 90 probsets, 9 exhibited disregulation with the principle effect being the upregulation of pro-apoptosis genes. The authors concluded that PPARα-receptor activation by ciprofibrate results in a pro-apoptotic signal; a conclusion consistent with the subcapsular single-cell necrosis observed in the liver microscopically. PPARα-receptor activation in rodents results in an anti-apoptotic response.

- Cell Proliferation. In cynomolgus monkeys, genes associated with the cell growth (c-MYC and JUN) were downregulated; the opposite is observed in rodents. In a supervised analysis of 99 probsets, 11 exhibited disregulation with the principle effect being the downregulation of pro-proliferative genes or the up-regulation of a pro-apoptotic anti-proliferative gene. The authors concluded that PPARα-receptor activation by ciprofibrate results in an anti-proliferative and pro-apoptotic effect.

- Oxidative Stress. In a supervised analysis of 99 probsets related to oxidative stress, 13 exhibited disregulation. The authors concluded that PPARα-receptor activation in the cynomolgus monkey does not result in significant oxidative stress because (a) the expression of several genes that are increased after oxidative stress in a variety of tissues (catalase, glutathione peroxidase) is decreased, (b) upregulation and downregulation of

the same gene occurred under different exposure conditions, and (c) there was a lack of dose-response.

- DNA Repair. In a supervised analysis of 148 probsets, 10 exhibited disregulation. Three DNA repair related genes were downregulated while seven were upregulated. The authors concluded that PPARα-receptor activation by ciprofibrate in the cynomolgus monkey did not show a clear indication of DNA repair, as many of the disregulated genes were not altered in a dose-dependent manner.

Thus, while some key events are operative in humans, primarily those involved in fatty acid metabolism, the key events for cell proliferation and inhibition of apoptosis leading to tumor formation are absent in human or primate liver even for potent PPARα agonists. Given the absence of a key event in humans, the conclusion is that DEHP, or any PPARα agonist, is incapable of producing tumors through this mode of action, and liver tumors observed in rodents are not relevant to humans.

II. OTHER PEROXISOME PROLIFERATOR-ASSOCIATED LESIONS DO NOT PROVIDE A BASIS FOR CONCLUDING THAT DEHP CAN BE REASONABLY ANTICIPATED TO CAUSE CANCER IN HUMANS

In addition to liver tumors, mononuclear cell leukemia, pancreatic acinar cell adenomas, and Leydig cell adenomas are associated with PPARα agonists (Klaunig et al., 2003). All these lesions have been observed in animals treated with DEHP and other peroxisome proliferators; all these lesions are found exclusively in rats (F-344 and Sprague-Dawley, depending on the lesion); and all these lesions have been observed at relatively high doses at which peroxisome proliferation is elevated. While the MOA for each lesion has not been established, the close association with PPARα agonists strongly indicates that these lesions have questionable significance for humans. Therefore, these lesions do not provide a basis for concluding that DEHP can reasonably be anticipated to cause cancer in humans.

A. Mononuclear Cell Leukemia Is Not Relevant to Humans

Mononuclear call leukemia (MNCL) has been observed in bioassays of DEHP conducted in Fisher 344 rats, but not in bioassays conducted in mice (NTP, 1982; David, 2000a; b). MNCL is a lesion that occurs almost exclusively in the F-344 rat, and that occurs spontaneously in that species. The International Agency for Research on Cancer (IARC) has categorized 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).

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). 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 DEHP chronic

studies are consistent with these findings. MNCL was found in two studies in the F-344 rat (NTP, 1982; David et al., 2000a) but not in the B6C3F1 mouse (David et al., 2000b) or the Sprague-Dawley rat (Ganning et al., 1991; Voss et al., 2005).

When assessing the significance of changes in MNCL incidence, the following are points to consider. (1) The factors contributing to a high spontaneous incidence of MNCL in the F-344 rat are unknown. (2) 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, 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). (3) 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 (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, 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. It 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).

In its review of butylbenzyl phthalate, U.S. Environmental Protection Agency 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). The Consumer Product Safety Commission (CPSC) Chronic Hazard Advisory Panel (CHAP) on diisononyl phthalate specifically reviewed the MNCL data for DINP and concluded that it is not relevant for assessing human health risk (CHAP 2001).

Thus, the opinion of many authoritative bodies is that MNCL is not relevant for human health assessment.

B. Pancreatic Acinar Cell Adenomas in DEHP Studies Are Not Relevant for Humans

Pancreatic acinar cell adenomas (PACA) are also associated with exposure to DEHP and other peroxisome proliferators (Klaunig et al., 2003). Although suggested by some to be a new lesion not reported previously for DEHP-exposed animals, the NTP observed non-significant, elevated incidences of this lesion in its own study of DEHP (NTP, 1982). Like other PPARα-triggered events, the incidence of PACA is elevated only in animals that have elevated levels of peroxisome proliferation. Not all the key events for this MOA are clearly defined (Klaunig et al., 2003), but the overall sequence of events is thought to center on cholestasis and increases in cholecystokinin (CCK) that lead to hyperplasia, cell proliferation, and carcinogenesis.

PPARα agonists have been shown to down-regulate genes for bile acid biosynthetic enzymes; this decrease leads to an increase in CCK and cholestasis which triggers acinar cell proliferation. Regulation of bile acid biosynthesis has been demonstrated with several peroxisome proliferators (WY 14,643, fibrates), but has not been studied substantially with DEHP. What has been demonstrated is a lack of response in primates. Kurata *et al*. (1998) reported that CCK levels were unchanged from controls in marmosets treated with up to 2500 mg/kg/d. Furthermore, no histopathological change was observed in the pancreas of these animals. Thus, primates (and presumably humans) are unresponsive to a key event that is likely causal in the tumorigenic process for PACA.

C. Leydig Cell Adenomas in Rodents Are Not Relevant for Humans

It has been suggested that another PPARα-related lesion is Leydig cell adenoma (Klaunig et al., 2003). A recent study by Voss et al. (2005) reported an increase in these lesions in Sprague-Dawley rats treated with 300 mg/kg DEHP for their lifetime. This report contrasts with a previous study reported by Ganning et al. (1991), also using Sprague-Dawley rats treated with 20000 ppm DEHP (~ ≥ 1000 mg/kg/d), in which liver and Leydig cell tumors were not observed. However, the duration of treatment differed in the two studies. The Voss et al. study was of unusual design in that the authors started with 730 male Sprague-Dawley rats divided into 4 groups: control (n = 390), 30 mg/kg/day (n=180), 95 (n=100) and 300 (n=60) mg/kg/day, each treated until death. This made it a 159 week study as compared to a normal 104 week (2 year) study. Since the Leydig cell tumors are an age-related lesion, the increased study length contributed to the tumor frequency. The first Leydig cell tumors were found only after 700 days on test, so most of the animals that developed Leydig cell tumors only did so after the end of a conventional cancer study. The significance of a elevated incidences of a spontaneous tumor is unclear, especially when incidences are altered very late in the life-span of the animal. Furthermore, the incidence in the 300 mg/kg/day dose group was significantly higher than for the other groups, a dose level at which peroxisome proliferation would be elevated (David et al., 1999; Ganning et al., 1991).

The association of this tumor type with PPARα agonists is strong. Of 15 peroxisome proliferators tested, 9 had Leydig cell adenomas observed in male Sprague-Dawley rats. The MOA proposed by Klaunig et al. (2003) is not complete, but the association of this lesion with the biochemical events of peroxisome proliferation makes this MOA plausible. As with other PPARα lesions, there is no evidence that events observed in rodents occur in primates treated with DEHP. In general, testicular lesions or Leydig cell hyperplasia have not been observed in primates following DEHP exposure (Rhodes et al., 1986; Kurata et al., 1998; Pugh et al., 2000; unpublished report from Mitsubishi Chemical Safety Institute, 2004 as reported by CERHR, 2005).

III. COMET ASSAY RESULTS ARE INSUFFICIENT TO ESTABLISH AN ALTERNATE MOA

Recent data from studies using the comet assay have suggested that DNA damage may result from exposure of both rodent and human cells to PPARα agonists including mono(2-ethylhexyl)phthalate (MEHP), a primary and active metabolite of DEHP (Anderson et al., 1999; Deutsch et al., 2001; Kleinsasser et al., 2004). On this basis, some have questioned whether

there is an alternate MOA to that described above. However, these data can be interpreted to support the peroxisome proliferation MOA. It is believed that the carcinogenic effects of PPARα agonists are due to one or more of three non-exclusive processes: increased cell proliferation, inhibition of apoptosis, and oxidative stress. It has been reported that peroxisome proliferation (one of the consequences of PPARα activation) results in the generation of hydroxyl radicals, and these in turn can produce DNA damage, usually abasic sites (Rusyn et al., 1999). The comet assay data provide support for the oxidative stress mode of action associated with peroxisome proliferation.

An increase in the number of abasic sites should be readily detectable through the use of the comet assay in which, if alkaline conditions are used (as was the case in the 4 papers referenced above), the abasic sites are converted to single strand breaks which, after the DNA unwinds to form single strands, result in the production of DNA fragments. Thus there is some evidence that DEHP (and/or its principal metabolite MEHP) may produce DNA damage through an indirect mechanism. However, it is less certain that this is a contributory mechanism to rodent liver tumors, for the following reasons:

- First, these lesions are readily repaired. In fact, as shown by Rusyn et al. (2000), the levels of enzymes which repair these lesions are also increased by treatment.

- Second, although the studies by Anderson et al. (1999) and Kleinsasser et al. (2004) did utilize human cells, it is not clear to what extent the *in vitro* conditions mimicked the *in vivo* conditions (note that the study by Deutsch et al. (2001) did not examine DEHP or any other phthalates). Certainly the treatment levels (mM concentrations) were extremely high, and physiological protective mechanisms either may have not been expressed under the *in vitro* conditions or may have been overwhelmed by the high concentrations tested. Indeed, isolated single epithelial cells were much more sensitive to the effects of MEHP than were epithelial cells exposed in isolated tissue samples (mini-organ cultures) (Kleinsasser et al., 2004). As noted by the authors, these mini-organ culture experiments were conducted expressly to circumvent some of the interpretational difficulties associated with single cell in vitro systems.

- Additionally, as discussed below, changes in comet assays cannot be interpreted as genotoxic changes without other corroborative evidence.

- Further, even if these changes are relevant to the production of liver tumors in rodents, the human relevance is unclear. For example, it has been shown that the genes which produce oxidative stress in rodents are not activated by PPARα agonists in primates (Cariello et al., 2005). Thus, this may simply be one more manifestation of the profound species differences in responses to PPARα agonists.

The version of the comet assay described above, while maximizing sensitivity, detects several kinds of DNA damage in single cells (Singh, 1988; Olive et al., 1991; Fairbairn et al., 1995). This version incorporates alkaline denaturation for unwinding DNA double helixes, thereby allowing free ends resulting from DNA single strand breaks (SSB) to migrate in a gel during electrophoresis. The alkaline version of the comet assay detects SSB in addition to double-strand breaks (DSB) in DNA, but these may not be important genotoxic endpoints as they

are usually rapidly and correctly repaired without leading to lethal or mutagenic effects (Collins et al., 1997). In addition, the alkaline comet assay detects other kinds of disturbances in DNA such as alkali labile sites and even DNA repair in progress. Because of the variety of DNA perturbations detected in this version of the assay, with most being in the form of SSB and alkali labile sites, their toxicological significance is uncertain, requiring correlation with other measures of genotoxicity for interpretation. The very multiplicity of changes that can give alterations in alkaline comets makes the assay useful for mechanistic studies (Fairbairn et al., 1995). However, the value of the comet assay results is questionable for developing an alternate MOA. The European Union Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers (SCCNFP) has stated that comet formation is due to primary DNA lesions, and that, for the interpretation of test results, one should elucidate whether the primary DNA damage is converted into biologically relevant chromosome or gene mutations (SCCNFP, 2004).

In summary, changes in comet assays have been reported and are apparently the consequence of the formation of abasic sites in DNA. However, these changes probably result from oxidative stress, may or may not be toxicologically important, and may or may not play a role in liver tumor induction in rodents, but are not likely to be toxicologically relevant to humans. The conclusion from the above is that the comet assay results provide support for the current peroxisome proliferation MOA, more than for an alternate MOA.

CONCLUSION

The information in these comments and in the Panel's previous submissions supports a conclusion that DEHP cannot be reasonably anticipated to cause cancer in humans. The Panel therefore supports the request to delist DEHP from the Report on Carcinogens.

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