March 10, 2015

Ms. Monet Vela
Office of Environmental Health Hazard Assessment
1001 I Street, 23rd Floor
Sacramento, California  95814

Re: Notice of Proposed Rulemaking Title 27, California Code of Regulations Amendment to Section 25705 Specific Regulatory Levels Posing No Significant Risk: Diisononyl Phthalate

Dear Ms. Vela,

On behalf of the High Phthalates Panel of the American Chemistry Council (ACC), we appreciate the opportunity to comment on OEHHA’s proposed No Significant Risk Level (NSRL) for diisononyl phthalate (DINP) under Proposition 65 in Title 27, California Code of Regulations, section 25705(b). According to the Initial Statement of Reasons, the proposed NSRL of 146 micrograms per day (µg/day) is based on carcinogenicity studies conducted in rodents, specifically the two DINP chronic bioassays conducted in male and female Fischer 344 (F344) rats reported by Moore (1998a) and Lington et al. (1997). The OEHHA derivation of an NSRL for DINP includes mononuclear cell leukemia (MNCL) data from these two chronic studies. The NSRL should not be based on these data because, as discussed below, MNCL observed in F344 rats is not relevant to humans and therefore is not an appropriate animal model of carcinogenesis. The researchers in Lington et al. came to the same conclusion with regard to the MNCL data in their study, finding that:

A review of the literature and our own data collectively suggest that the increase of MNCL in F344 rats is a common finding in aging F344 rats and that the increased incidence in rats treated with DINP is not relevant to humans.¹

A more appropriate animal model would be the rodent liver tumor data.² However, as detailed in previous comments submitted to OEHHA,³ incorporated by reference herein, because liver tumors observed in rodents are due to a mode of action involving the peroxisome proliferator-activated receptor-α (PPARα), at a minimum, OEHHA should account for differences in species sensitivity and apply a scaling factor of ten, as previously applied by OEHHA to di-(2-ethylhexyl) phthalate (DEHP), because of differences in receptor (PPARα) density. Using the most conservative data of male mouse liver incidence, and the approaches previously used by OEHHA, a scientifically-supported NSRL would be 2664 µg/day.

² No admission is made herein that such data indicates the mechanism of action is relevant to humans or was an appropriate basis for listing of DINP as “known to the State to cause cancer.”
I. **MNCL is a Strain-Specific Disease and Not a Relevant Predictive Model for Human Disease.**

MNCL was observed in two DINP chronic bioassays conducted in F344 rats (Lington et al., 1997; Moore, 1998a), but not in bioassays conducted in mice (Moore, 1998b) or the Sprague-Dawley rat (Monsanto, 1986). The 2001 Consumer Product Safety Commission (CPSC) Chronic Hazard Advisory Panel (CHAP) concluded that “the findings of mononuclear cell leukemia and renal tubular carcinoma in the rodent bioassay for DINP are of questionable relevance to humans.” Moreover, the European Chemicals Bureau (ECB) found little relevance to humans, citing International Agency for Research on Cancer (IARC) conclusions that MNCL had no known human counterpart:

> Regarding MNCL, a clear increased 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 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).

Other authoritative bodies, including the U.S. National Toxicology Program (NTP) and the National Industrial Chemicals Notification and Assessment Scheme (NICNAS) of the Australian Government Department of Health and Ageing, have questioned the relevance of MNCL data for human risk assessment.

At an NTP workshop in 2005, in addressing whether the currently used models, the F344/N rat and B6C3F1/N mouse, continued to be appropriate to identify substances that may pose a carcinogenic hazard for humans, the rat model breakout group recommended moving away from the F344 rat:

> The F344/N rat has been used in the NTP 2-year chronic toxicity and carcinogenicity bioassays for almost 30 years. The F344/N rat is known to have high background incidences of certain types of tumors including testicular interstitial cell tumors (Figure

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1a) and mononuclear cell leukemia (Figure 1b). From a statistical perspective, high background rates of such tumors in control animals will generally decrease the ability to detect an exposure-related effect. In addition, when a statistically significant tumor effect is found in test animals relative to concurrent controls, the effect may not be considered exposure-related if it falls within the range observed in historical controls (Haseman, et al., 1990). [Information on spontaneous frequencies of MNCL in male and female F344 rats is discussed in detail below.]

Figure 1b.

![Mononuclear Cell Leukemia](image)

In Figure 1b, the incidence of mononuclear cell leukemia in the F344/N rat and comparisons with other rats. Data presented at the NTP workshop (http://ntp.niehs.nih.gov see “Meetings & Workshop). (a): NTP F344/N, (b): NCTR F344, (c): Wistar Han CRL Data, (d): Wistar Han RCC Data, (e): Wistar proprietary data, and (f): Sprague Dawley proprietary data.

NICNAS released its Priority Existing Chemical Assessment Report (PEC No. 35) on DINP in 2012. In examining carcinogenicity, NICNAS states:

> Overall, the available data do not indicate a carcinogenic potential in humans for DINP. MCL [mononuclear cell leukaemia] was not found in other mammalian species and has no comparable type in humans.

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9 We have not included figure 1a as it is not relevant to this discussion.


The high spontaneous incidence of MNCL is confined to the F344 strain of rat, occurring in both sexes. As discussed in the comments submitted by Dr. Richard Irons (Attachments B and C), MNCL is a common cause of natural mortality in F344 rats over 18 months of age (Stromberg et al. (1985),12 Ward et al. (1990),13 Thomas et al. (2007)14). Haseman et al. (1998)15 reported an overall spontaneous incidence in NTP studies up to January 1997 of 50.5% (range 32–74%) in males and 28.1% (range 14–52%) in females. This compares with background incidence of less than 1% in other strains of commonly used laboratory rats. As discussed in the comments submitted by Dr. Robert R. Maronpot (Attachments D and E), the incidence in F344 rats is modulated by a variety of factors not clearly related to carcinogenicity. Corn oil gavage, for example, has been shown consistently to reduce the incidence of MNCL in male, but not female, controls. It is clear that the F344 rat is uniquely susceptible to developing MNCL, although the reason for this susceptibility is unknown.

Thomas et al. (2007) discuss the character of the disease and cell type of the F344 MNCL and relate these to human malignancies. It is probable that most, if not all, F344 MNCL is derived from natural killer cells. In the F344 rat, MNCL is an aggressive, often fatal disease in older animals. The closest analogue in humans is a natural killer cell-derived malignancy (Aggressive NK-cell Leukemia) (ANKL) that is extremely aggressive but occurs in younger adults. The human disease is rare, believed to have an Epstein-Barr virus mediated etiology, and has not been associated with exposure to chemicals. As Dr. Irons discusses in his technical comments, only human Aggressive NK-cell Leukemia shares clinical features and a presumed cell of origin with F344 MNCL. As he notes, human ANKL is one of the rarest hematologic diseases known and only 98 cases have ever been reported worldwide. Additionally, Epstein-Barr virus, which is unique to humans, has been found in 100% of ANKL cases. Thus, as Dr. Irons notes, as MNCL in the F344 rat has a completely different etiology from human ANKL, the F344 MNCL is a strain-specific disease and not a relevant predictive model for human disease.

II. OEHHA Should Base an NSRL on the Male Mouse Liver Data

The rodent liver tumor response provides the most consistent response across sex, species and strains in the life time bioassays. To determine concordance of response across the bioassays, ACC combined liver tumor response in male F344 rats from the Lington et al., and Moore studies, and assessed cancer slope model fit. The combination of the data provided a good fit to model, indicating the treatment related nature of the response. Given the consistency of response and the ability to model the combined data sets, ACC calculated an NSRL for DINP of 2664 µg/day using rodent liver tumor data.

A. Derivation of animal cancer potency factors (q1*)

ACC determined the 95% lower-bound confidence limit on the benchmark dose (BMDL) for the liver data using the multi-stage cancer model in the most current version (v 2.4) of USEPA’s Benchmark Dose Software. The model was run using a polynomial term between 1 and 2; the BMDL (mg/kg-day) was selected from among the models that adequately fit the data (P-value > 0.1) by choosing the model (and associated BMDL) with the lowest Akaike’s Information Criterion (AIC) score.

Table 1. Rodent liver tumor potency estimates from primary cancer studies of DINP in rodents

<table>
<thead>
<tr>
<th>Study</th>
<th>Sex</th>
<th>Dose Concentration (mg/kg feed)</th>
<th>Calculated Intake (mg/kg day)</th>
<th>Combined liver tumors</th>
<th>q1* (mg/kg/day)1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rat Studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lington et al 1997</td>
<td>Male</td>
<td>0, 300, 3000, 6000</td>
<td>0, 15, 152, 307</td>
<td>3/81, 1/80, 1/80,</td>
<td>0.000272411</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4/80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0, 300, 3000, 6000</td>
<td>0, 18, 184, 375</td>
<td>1/81, 2/81, 0/80,</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2/80</td>
<td></td>
</tr>
<tr>
<td>Moore 1998a</td>
<td>Male</td>
<td>0, 500, 1500, 6000, 12000</td>
<td>0, 29.2, 88.3, 358.7, 733.2</td>
<td>5/65, 4/50, 2/50, 7/65, 17/65</td>
<td>0.000320818</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0, 500, 1500, 6000, 12000</td>
<td>0, 36.4, 108.6, 442.2, 885.4</td>
<td>1/65, 1/50, 0/50, 2/65, 8/65</td>
<td>0.000152717</td>
</tr>
<tr>
<td>Monsanto 1986</td>
<td>Male</td>
<td>0, 500, 5000, 10000</td>
<td>0, 27, 271, 553</td>
<td>4/70, 7/69, 12/69, 9/70</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0, 500, 5000, 10000</td>
<td>0, 33, 331, 672</td>
<td>1/70, 1/70, 10/70, 9/70</td>
<td>0.000255301</td>
</tr>
<tr>
<td><strong>Mouse Study</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moore 1998b</td>
<td>Male</td>
<td>0, 500, 1500, 4000, 8000</td>
<td>0, 90.3, 276, 742, 1560</td>
<td>16/70, 13/67, 18/66, 28/65, 31/70</td>
<td>0.00037808</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0, 500, 1500, 4000, 8000</td>
<td>0, 112, 335.6, 910, 1888</td>
<td>3/70, 5/68, 10/68, 11/67, 33/70</td>
<td>0.000328649</td>
</tr>
</tbody>
</table>

The most conservative cancer potency estimate (q1 = 0.00037808) was generated from the male mouse data from Moore (1998b). This identified the Moore mouse study as the most sensitive and appropriate for use in the NSRL derivation.

B. Derivation of human cancer potency factors

Next, ACC converted the animal data into a human potency estimate to derive an NRSL, in accordance with Section 25703. This conversion should not be taken as any indication that the mechanism of carcinogenicity occurring in the rodent bioassays is relevant to humans. The cancer potency estimates described above are animal potency estimates. These values were converted to human cancer potency estimates using the following allometric scaling equation (CCR Title 27, Sections 25701-25703):

\[
\text{human cancer potency} = q1^* \times \left(\frac{bW_{human}}{bW_{animal}}\right)^{1/4}
\]

Given the lower bodyweight for mice compared to rats, the mouse to human scaling factor will be larger than the rat to human scaling factor. The combination of the most conservative tumor potency estimate, derived from the mouse data, and the use of the mouse bodyweight in the allometric scaling equations yields the most conservative human cancer potency estimate.
Default human bodyweights for human males and females are set by the State of California for NRSL development as 70 kg and 58 kg, respectively. The mouse bodyweights were estimated using the study specific bodyweight data. For the Moore mouse study, male bodyweights were between 0.03 and 0.038 kg for the duration of the study.

III. OEHHA Should Apply the 10-fold Scaling Factor to the NSRL Derivation to Account for Differences in Species Sensitivity.

For the NSRL for DEHP OEHHA adopted a reduced potency estimate for agents which produce liver tumors in rodents through the PPAR-α mode of action.\textsuperscript{16} DINP has been shown to induce liver tumors in rodents through this mechanism (Valles \textit{et al.}, 2003).\textsuperscript{17} It has not been shown, however, that these rodent tumors are relevant to humans.

For DEHP, OEHHA applied a reduction in potency by a factor of ten. A tenfold reduction in potency is a conservative estimate of potency difference and OEHHA should apply that same scaling factor to its proposed NSRL for DINP. Thus the equation for the calculation of human cancer potency is: animal cancer potency (q\textsubscript{1}) multiplied by the allometric scaling factor multiplied by the sensitivity scaling factor (1/10\textsuperscript{th}) resulting in the following equation.

\[
\text{human cancer potency} = q_1 \times \left(\frac{bw_{human}}{bw_{mouse}}\right)^{1/4} \times \frac{1}{10}
\]

IV. Conclusion

OEHHA should revise its proposed NSRL for DINP since the scientific basis of the proposed NSRL is unsupportable. MNCL as observed in F344 rats is not an appropriate animal model of carcinogenicity in humans.

A more appropriate animal model would be the rodent liver tumor data. Using the most conservative data of male mouse liver incidence, and accounting for differences in species sensitivity by applying a scaling factor of ten, as applied to the NSRL for DEHP, a scientifically-supported NSRL would be 2664 µg/day.

\textsuperscript{17} Valles \textit{et al.} (2003). Role of the peroxisome proliferator activated receptor alpha in responses to diisononyl phthalate. Toxicology 191:211-225.
Please contact me at eileen_conneely@americanchemistry.com or at 202-249-6711 if you require further information.

Sincerely,

Eileen Conneely

Eileen Conneely, J.D., M.P.H.
Director, Chemical Products and Technology Division
American Chemistry Council

Attachments:

A  ACC Phthalate Esters Panel Submission of Information on Diisononyl Phthalate (Feb. 16, 2010)
B  Comment of Dr. Richard D. Irons Regarding OEHHA Proposed NSRL (Feb. 12, 2015)
C  Presentation of Dr. Richard D. Irons at Public Hearing (Feb. 25, 2015)
D  Comment of Dr. Robert R. Maranpot Regarding OEHHA Proposed NSRL (Mar. 2, 2015)
E  Presentation of Dr. Robert R. Maranpot at Public Hearing (Feb. 25, 2015)
F  Presentation of Dr. Richard McKee at Public Hearing (Feb. 25, 2015)
BEFORE THE
OFFICE OF ENVIRONMENTAL HEALTH HAZARD ASSESSMENT
CALIFORNIA ENVIRONMENTAL PROTECTION AGENCY

Announcement of Chemicals Selected by OEHHA
for Consideration for Listing by the Carcinogen Identification Committee
and Request for Relevant Information
on the Carcinogenic Hazards of These Chemicals
(October 15, 2009)

Submission of Information on Diisononyl Phthalate (DINP)
Phthalate Esters Panel
American Chemistry Council
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Arlington, Virginia 22209

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steve_risotto@americanchemistry.com

OF COUNSEL
David B. Fischer
Assistant General Counsel

February 16, 2010
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I. INTRODUCTION

Based on “evolving scientific knowledge” there is insufficient evidence to support a listing of DINP as a carcinogen under Proposition 65. DINP meets none of the criteria identified in Section 25249.8 of the Health and Safety Code as a basis for listing. It has not been identified by an authoritative body to cause cancer, no state or federal government has required it to be identified as causing cancer, and as discussed below it cannot be “clearly shown through scientifically valid testing according to generally accepted principles to cause cancer.”

As indicated by OEHHA, no cancer epidemiology studies exist for DINP. Instead OEHHA references as evidence of DINP’s potential carcinogenicity data from genotoxicity studies, animal bioassays, effects on testosterone synthesis in rodents, structure-activity considerations, and mechanistic studies related to tumor induction and Testicular Dysgenesis Syndrome. As set forth in more detail below, the available evidence indicate that –

- DINP is not mutagenic or genotoxic;
- There is a strong and compelling evidence that DINP-induced lesions in rodents are not relevant to humans – a finding that has been confirmed by several expert reviewing bodies;
- Suggestions of an alternative mechanisms for tumorigenesis, based on results with DEHP in genetically-altered mice, are not supported by the available evidence;
- Exposure to DINP does not result in Testicular Dysgenesis Syndrome; and
- DINP does not affect testosterone synthesis in primates.

As a result, DINP therefore does not warrant listing under Proposition 65 as “known to the state to cause cancer” because it has not been clearly shown to cause cancer.

II. DINP IS NOT MUTAGENIC OR GENOTOXIC

The conclusion that DINP is not mutagenic or genotoxic is supported by a robust database. DINP was not mutagenic in an Ames assay using 5 strains of *Salmonella typhimurium* and did not induce an increase in chromosomal aberrations in Chinese hamster ovary cells (Zeiger et al., 1985; McKee et al., 2000). Additionally, it did not cause unscheduled DNA synthesis in rat hepatocytes (Litton Bionetics, 1981). In vivo, DINP was inactive in a micronucleus test in mouse bone marrow (McKee et al., 2000) and did not induce an increase in chromosomal aberrations in rat bone marrow cells (Microbiological

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DINP was inactive in a mouse lymphoma test and in a cell transformation assay in Balb 3T3 cells (Barber et al., 2000).\(^5\)

The available evidence led the European Chemicals Branch (ECB) to conclude in its 2003 risk assessment for DINP –

DINP is not mutagenic \textit{in vitro} in bacterial mutation assays or mammalian gene mutation assay (with and without metabolic activation) and is not clastogenic in one cytogenetic assay \textit{in vitro} on CHO cells and in one \textit{in vivo} assay on bone marrow cell of Fisher 344 rats. This suggests that DINP is not genotoxic \textit{in vivo} or \textit{in vitro}.\(^7\)

The reference cited by OEHHA (Babich et al, 2004), moreover, concludes that “[a]s with other peroxisome proliferators, DINP exhibits little or no evidence of genotoxicity.”

### III. \textbf{ANIMAL CANCERS IN RODENTS EXPOSED TO DINP ARE NOT RELEVANT TO HUMANS}

DINP at high doses produces liver tumors in rats and mice, kidney tumors in male rats, and mononuclear cell leukemia (MNCL) in rats (Lington et al., 1997; Moore, 1998a,b; Bio/dynamics, 1986). There is a substantial body of research providing compelling evidence that these tumors in rodents are not relevant for human health assessment. As a consequence, numerous expert reviews have declined to consider DINP as a known, probable, or possible human carcinogen.

#### A. Kidney Tumors Observed in Male Rats Are Due to Alpha-2u-Globulin Nephropathy and Are Not Relevant to Humans

The kidney tumors observed in bioassays in male rats were the result of induction of alpha-2u-globulin. Male rats are known to be susceptible to formation of kidney tumors through a mechanism involving alpha2u-globulin accumulation. Because humans do not produce alpha2u-globulin, such male rat kidney tumors are not relevant for human health assessment. (USEPA, 1991; Swenberg and Lehman-McKeeman, 1998).\(^8,9\)


As acknowledged in the US Environmental Protection Agency’s (USEPA) revised hazard assessment on DINP, the Consumer Product Safety Commission’s (CPSC) Chronic Hazard Advisory Panel (CHAP, 2001), and the ECB’s risk assessment for DINP (ECB, 2003), the data for DINP meet the criteria for the existence of alpha-2u-globulin nephropathy, a male rat-specific mechanism not relevant to humans. In particular, USEPA states –

The data obtained in [DINP] studies were evaluated against published criteria for evaluating male-specific nephropathy and its relevance to human. The results of this evaluation indicate that: (1) all three EPA criteria for existence of the alpha-2u-globulin mode of action have been met; (2) six of the seven International Agency for Research on Cancer (IARC) criteria for existence of the alpha-2u-globulin process have been met; and (3) EPA has not found other information or data to suggest that another mechanism is likely to be involved. Based on this evaluation, the Agency believes that DINP-induced kidney tumors are associated with a male rat-specific mechanism involving alpha-2u-globulin accumulation in the kidney and that this mechanism is not appropriate for estimating hazard in humans.12

The seventh IARC criterion that USEPA asserts has not been met is evidence that DINP binds to alpha-2u-globulin. Binding of DINP to alpha-2u-globulin, however, was subsequently observed by Schoonhoven et al. (2001). Thus all criteria of both the USEPA and IARC criteria for the alpha-2u-globulin mechanism are met by the DINP data. For chemicals that meet the alpha-2u-globulin, USEPA noted that –

Male rat renal tubule 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.15

B. Mononuclear Cell Leukemia Observed in Fisher F344 Rats Is Not Relevant to Humans

Mononuclear cell leukemia (MNCL) was observed in the two DINP chronic studies conducted in Fisher 344 rats (Lington et al., 1997; Moore et al., 1998a), but not in the bioassay conducted in mice

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11 ECB 2003, at 225.

12 USEPA, 2005, at 68-69 (citations and footnotes deleted, emphasis added).

13 Id. at 69, note 4.


(Moore et al., 1998b). Reviewing this data, the CPSC CHAP concluded that “[t]he findings of mononuclear cell leukemia and renal tubular carcinoma in the rodent bioassay for DINP are of questionable relevance to humans.” Moreover, the ECB Risk Assessment states –

Regarding MNCL, a clear increased 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 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).

Other authoritative bodies, including USEPA, the National Toxicology Program, the National Institutes of Health, and a recently published review (Thomas et al., 2007) have questioned the relevance of MNCL data for human risk assessment purposes and have suggested the application of a weight-of-evidence approach when statistically identified increases in MNCL are observed.

The high spontaneous incidence of MNCL is confined to the F344 strain of rat. It occurs in both sexes in the F344 rats. The incidence has been rising over the course of the NTP bioassay program, but is highly variable from laboratory to laboratory and study to study. Haseman et al. (1998) reported an overall spontaneous incidence in NTP studies up to January 1997 of 50.5% (range 32–74%) in males and 28.1% (range 14–52%) in females. This compares with background incidence of less than 1% in other strains of commonly used laboratory rats. The incidence in F344 rats is modulated by a variety of factors not clearly related to carcinogenicity. Corn oil gavage, for example, has been shown consistently to reduce the incidence of MNCL in male, but not female, controls. It is clear that the F344 rat is uniquely susceptible to developing MNCL, although the reason for this susceptibility is unknown.

Thomas et al. discuss the character of the disease and cell type of the F344 MNCL and relate these to human malignancies. It is probable that most if not all F344 MNCL is derived from a natural killer cell subset of large granular cell lymphocytes (LGL). In the F344 rat, MNCL is an aggressive, often fatal disease in older animals. The closest analogue in humans is a natural killer cell, LGL-derived malignancy that is extremely aggressive but occurs in younger adults. The human disease is rare and is believed to involve a viral mechanism; it has not been associated with exposure to chemicals. Thus,

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16 CHAP, 2001, at 122.
17 ECB, 2003, at 225.
although some have suggested that there may be a possible human analogue to MNCL, the high susceptibility is clearly confined to the F344 rat strain.

C. Liver Tumors Observed in Rodents Are Due to Peroxisome Proliferation

There is a robust body of data indicating that the liver tumors observed in DINP rodent studies are due to a mode of action involving the peroxisome proliferator-activated receptor-α (or PPARα). The progression of liver effects caused by peroxisome proliferating compounds (PPCs), leading up to and including tumors, has been well characterized, and there is a strong scientific consensus that such effects are not relevant for human risk assessment (e.g., Cattley et al., 1998; Klaunig et al., 2003).20,21 All liver effects observed in the DINP rodent studies, with the possible exception of spongiosis hepatitis (a spontaneous lesion in rats), were consistent with the PPARα mode of action. In contrast, no such effects were seen in the primate studies, even at doses of 2500 mg/kg/day (Hall et al., 1999; Pugh et al., 2000).22,23

Primate data provide the best basis for determining whether chronic effects seen in rodents can reasonably be anticipated to occur in humans. 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 a colossal 2,500 mg/kg/day for 90 days (Hall et al., 1999). In both of these primate studies, there was no evidence of treatment-related effects, including liver or kidney weights or treatment-related changes in histopathology, even at the very high levels of treatment. There was no evidence of peroxisome proliferation in non-human primate hepatocytes tested under in vitro conditions (Benford et al., 1986; Kamendulis et al., 2002)24,25 or in human hepatocytes (Baker et al., 1996; Hasmall et al., 1999; Kamendulis et al., 2002).26,27 These findings suggest a “marked species difference” in peroxisomal proliferation resulting from phthalate exposure.28

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28 ECB, 2003, at 223.
The potential human response to PPCs also has been examined in liver biopsies from patients treated with hypolipidemic drugs (i.e., fibrates) which are potent PPCs. Morphohometric measurements in liver biopsies did not reveal evidence for peroxisome proliferation. As noted by IARC, the potential carcinogenic risk of hypolipidemic therapy with fibrates has been evaluated in two limited clinical trials with no evidence for carcinogenesis. (IARC, 1996)\(^9\)

Thus, studies from several laboratories using hepatocytes from different individuals or different species of non-human primates have demonstrated that a peroxisome proliferator response is not elicited by DINP in these species. And of particular relevance to the CIC’s review of DINP, these studies show no evidence of potential carcinogenicity, even under conditions that unquestionably would elicit tumorigenesis in rodents, including the progression to cancer.

D. Numerous scientific reviews provide further support for the position that the rodent liver tumors have no relevance to humans

A review of peroxisome proliferation by IARC concluded that rats and mice had a much higher propensity for peroxisomal proliferation than other species including humans (IARC, 1995).\(^10\) More specific to phthalates, IARC revised its classification of DEHP in 2000 from “possibly carcinogenic to humans” (Group 2B) to “not classifiable as to its carcinogenicity to humans” (Group 3)\(^31\) based in large part on its consideration of the relevance of the rodent liver tumors. In summarizing its conclusion, IARC explained –

In making its overall evaluation of the carcinogenicity to humans of [DEHP], the Working Group took into consideration that (a) [DEHP] 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 [DEHP] in rats and mice; and (c) peroxisome proliferation has not been documented in human hepatocyte cultures exposed to [DEHP] nor in the liver of exposed non-human primates. Therefore, the mechanism by which [DEHP] increases the incidence of hepatocellular tumours in rats and mice is not relevant to humans.\(^32\)

Although DINP has not been evaluated by IARC, the available data are very similar to those for DEHP, so similar conclusions would be anticipated.

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\(^32\) Id, at 124.
In 2003, a workgroup of the ILSI Risk Science Institute reviewed the relationship of peroxisome proliferation and liver tumors in rodents. The results of that workshop are presented in a paper titled “PPARα Agonist-Induced Rodent Tumors: Modes of Action and Human Relevance” (Klaunig et al., 2003). DINP is one of the examples of a peroxisome proliferator discussed in the document. The workshop concluded –

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

DINP is a known example of a PPARα agonist that was part of the basis for the workshop conclusions.

Other reviews support the ILSI and IARC conclusions. For example, CPSC’s 2001 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.”  

Subsequently the CPSC staff, based on the CHAP and on the ILSI workshop, “concluded that DINP, which is a peroxisome proliferator, is not likely to present a cancer risk in humans” (CPSC, 2003).  

Similarly, the European Union (EU) in its risk assessment of DINP concludes –

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.  

The EU did not identify carcinogenicity as a critical endpoint (ECB, 2003) and has not classified DINP as a carcinogen (EC, 2000).

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33 Klaunig et al., 2003, at 693 (emphasis added).
34 CHAP, 2001, at 122.
36 ECB, 2003, at 243 (emphasis added).
In an earlier summary of available scientific information on DINP, OEHHA referenced the USEPA’s 2000 proposal to add DINP to the list of toxic chemicals subject to the reporting requirements under EPCRA section 313. In its original proposal to list DINP, USEPA stated that DINP can reasonably be anticipated to cause carcinogenicity, based on liver tumors, kidney tumors and mononuclear cell leukemia MNCL observed in rodent studies. In 2005, however, USEPA revised its hazard assessment in response to public comments and subjected the revised assessment to peer review by both USEPA experts and external peer reviewers. Importantly, in this revised assessment, USEPA reconsidered the evidence of DINP carcinogenicity and stated that “at this time, USEPA reserves judgment on whether DINP can reasonably be anticipated to cause cancer in humans.”

IV. RECENTLY PROPOSED PPARα-INDEPENDENT MECHANISMS OF TUMORIGENESIS ARE NOT SUPPORTED BY THE AVAILABLE INFORMATION

The conclusion that activation of the PPARα receptor is the key event in the induction of liver tumors in the laboratory animals is supported by the absence of tumors in nine PPAR-α–null (knock-out, or KO) mice exposed to a strong peroxisome proliferator (Wy-14,643) after 11 months, whereas each of the six similarly exposed wild-type mice had multiple hepatocellular neoplasms (Peters et al., 1997). Subsequent work further suggested that DEHP failed to induce peroxisomal enzymes and peroxisome proliferation in KO mice after 24 weeks of exposure (Ward et al. 1998).

Recent studies conducted in Japan using KO mice by Ito et al. (2007), however, have led some to suggest that liver tumors might occur in wild-type mice via a PPARα-independent mechanism and that the observed liver tumors may therefore have relevance to humans (Ito et al., 2007; Guyton et al., 2009). The Japanese researchers observed that chronic exposure to DEHP resulted in a low level of liver tumors in the KO mice. No such tumors were observed in wild-type mice with an intact PPAR receptor, however, and the relevance of these tumors in KO mice to humans and the PPARα mode of action is unclear. The unusually high survival rates reported by Ito et al. also raise serious questions about their data. Ito et al. did not address in their paper why the survival rate in their study for both the wild-type and KO mice was so different from that reported in Howroyd et al. (2004). In addition, data confirming that Ito et al. had in fact knocked out the PPARα alleles was not included in the published report, key information for validating their model and their results.

Ito et al. proposed an alternative mechanism for rodent liver tumors that is independent of PPARα activation. Their hypothesis suggests increased production of reactive oxygen species as a result of increased oxidative stress in mouse hepatocytes due to DEHP exposure. However, as suggested previously (Kostadinova et al., 2005; Balkwill and Cousens, 2005)\(^ {42,43} \), the underlying increased susceptibility of the KO mice to tumorigenesis in the absence of chemical treatment may be due to fundamental mechanistic differences limiting the applicability of the model for testing the proposed hypothesis. Spontaneous tumors are known to occur in the KO mice at 24 months (Takashima et al., 2008).\(^ {44} \) The utility of this mouse model to assess alternative mechanisms of tumorigenesis, therefore, is problematic as is its relevance to humans. Importantly, there are no known reports on the ability of DINP to induce production of reactive oxygen species in livers of rodents, humans or non-humans primates, or in cultured liver cells from these species.

Subsequent analysis of gene expression in wild-type and KO mice by Ren et al. (2010)\(^ {45} \) indicate that transcriptional responses to DEHP and other peroxisome proliferating chemicals are overwhelmingly dependent on PPARα. Ren et al. point out that a number of their findings argue against the view that the induction of tumors in the KO mice supports a mode of action other than PPARα activation in wild-type strains—

First, the transcript profile comparison in the present study showed that the vast majority of genes altered by DEHP in wild-type mice were not similarly altered in PPARα-null mice. Thus, the DEHP-induced tumors in wild-type mice could only be PPARα independent if the ~6% of the PPARα-independent gene changes were responsible for the tumors, an unlikely scenario given the magnitude of the PPARα-dependent effects. Second, wild-type and PPARα-null mice exhibited differences in DEHP-induced carcinogenesis. DEHP did not induce equivalent levels of tumors in the wild-type and PPARα-null mice; there were no statistically significant increases in liver tumors in the wild-type mice under these exposure conditions (200 ppm). DEHP increased the expression of growth control genes in PPARα-null mice but not in wild-type mice at equivalent doses (Ito et al., 2007). Transcript profiling and RTPCR showed highly dissimilar changes in gene expression in the liver tumors from the wild-type and PPARα-null mice, indicating different molecular mechanisms of their origins (Takashima et al., 2008). Lastly, in the absence of PPARα, DEHP altered a unique set of genes not similarly altered in wild-type mice. Some of these genes are known targets of [constitutive-activated receptor or CAR]. Taken together, these data indicate that although DEHP can induce marginal increases in liver tumors in PPARα-null mice, the mode of action is different from that in wild-type mice. The transcriptional responses induced by DEHP in

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\(^ {44} \) Takashima K et al. Different mechanisms of DEHP-induced hepatocellular adenoma tumorigenesis in wild-type and PPARα-null mice. *J Occup Health* 50: 169-180 (2008).

wild-type and nullizygous mouse strains indicate that CAR is important in the induction of tumors in PPARα-null mice.46

Ren et al. point to evidence that PPARα and CAR have antagonist properties and note that “[t]he expression of the CAR gene itself was increased by DEHP in PPARα-null but not in wild-type mice.” (emphasis added) CAR activation is a minor pathway in wild-type mice and this activation, likely by MEHP, would, in essence, be “swamped out” by the activation of PPARα and its ensuing effects. The minor contribution of DEHP-induced CAR activation to liver tumorigenesis in the wild-type mouse is not sufficient to drive tumorigenesis independent of PPARα.

To date, there has only been one study which investigated the ability of DEHP and MEHP to activate human CAR (DeKeyser et al., 2009).47 In human livers, the CAR gene expresses a number of differentially spliced mRNA transcripts, (Savkur et al., 2003; Arnold et al., 2004; Jinno et al., 2004; Lamba et al., 2004).48,49,50,51 The CAR2 splice variant, which lacks constitutive activity, is expressed at approximately 30% of the reference transcript level in human hepatocytes (Xu et al., 2004; DeKeyser et al., 2009).52 The CAR2 transcript cannot be generated in marmoset, mouse or rat, indicating that CAR2 may be unique to humans (Kent et al., 2002; DeKeyser et al., 2009).53 DEHP has been shown to activate CAR2 in vitro in a transactivation study in which CAR2 was added to a kidney epithelial cell line derived from the African green monkey (i.e., COS-1). However, when MEHP was tested in the same assay, only weak activity was demonstrated even at a concentration of 10uM. From this, DeKeyser et al. (2009) concluded that DEHP, not MEHP, is a potent agonist of CAR2. However, this conclusion is inconsistent with the prevailing hypothesis that MEHP is the active metabolite in animals and humans due to the high rate of metabolism of the parent compound (see, e.g., ECB, 2008; Rhodes et al., 1996; Tomita et al., 1982).54,55,56 Thus, these data suggest that activation of CAR2 is not a plausible mode of action whereby DEHP could cause cancer in humans (or even mice).

46 Id. at 55-56.
V. EXPOSURE TO DINITRILE DOES NOT RESULT IN TESTICULAR DYSGENESIS SYNDROME

The term Testicular Dysgenesis Syndrome (TDS) was introduced 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 all have a common etiology (Skakkebaek et al., 2001). In particular, it has been proposed that suppression of fetal androgen production and/or increased estrogen exposure is the underlying basis of this syndrome (Sharpe, 2003). The available data, however, are inconsistent in demonstrating that DINITRILE produces antiandrogenic effects in young male rats. Two studies, which used an unrealistically high dose of DINITRILE 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 antiandrogenic effects were observed in male offspring of pregnant rats exposed to higher levels of DINITRILE in the diet (Masutomi et al., 2003). Additionally, more definitive rat studies (i.e., 2-generation reproduction and developmental studies) indicate that DINITRILE does not induce cryptorchidism, hypospadias or low sperm counts and therefore do not support attributing TDS to DINITRILE exposure (Waterman et al., 2000).

VI. DINITRILE DOES NOT AFFECT TESTOSTERONE SYNTHESIS IN PRIMATES

The available cancer studies do not support an association between DINITRILE exposure and testicular cancer in laboratory animals. Linton et al. 1997 reported no significant excess of bilateral interstitial cell tumors in Fischer 344 rats exposed to up to 307 mg/kg/day. Although testicular tumor incidence in high dose animals was significantly elevated in the cancer study conducted by Bio/dynamics (1986), the incidence fell within the range of historical control values. In a two-generation reproductive study, however, testicular histology was unaffected in both generations at doses up to 779 mg/kg bw/day (Waterman, 2000). The 13-week gavage study in adult marmosets conducted by Hall et al. (1999) resulted in no evidence of microscopic testicular changes at doses that did adversely affect body

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weight gain (2500 mg/kg bw/day). Testicular lesions were not observed by Pugh et al. (2000) in prepubertal Cynomolagus monkeys that were gavaged for 2 weeks with 500 mg/kg bw/day, reportedly the maximum dose that can be absorbed by the monkeys.

In reviewing the results of Borch et al. (2004), an expert panel convened by the National Toxicology Program’s Center for Evaluation of Risks to Human Reproduction noted that “[e]x vivo testosterone production is of questionable relevance to human risk, especially when no in vivo plasma testosterone or LH changes were noted.”62 This caution is underscored by the results of a 13-week study in marmoset monkeys which observed no treatment-related changes in the testosterone concentrations assessed, as well as no changes in testis weight or testis microscopic examination (Hall et al., 1999). A more recent, small study of fetal exposure of marmosets to benzyl butyl phthalate (BBP) found no measurable effects on testis development/function, testicular dysgenesis, and observed no effects by adulthood (McKinnell et al., 2009).63

VII. **HUMAN EXPOSURES TO DINP ARE FAR BELOW LEVELS THAT COULD CAUSE CANCER, EVEN IF THE ANIMAL DATA ARE ASSUMED RELEVANT FOR HUMAN RISK ASSESSMENT**

Since 1999, the Centers for Disease Control and Prevention (CDC) have been analyzing samples of urine from the U.S. population for phthalate metabolites, including mono-isononyl phthalate (MINP). CDC has reported its biomonitoring findings, representative of the U.S. population, in reports issued in 2001, 2003, 2005, and 2009. The 2009 report includes the information from the previous reports and provides data for an additional 2600 persons (CDC, 2009).64

For the results reported in 2009, MINP was not detected at the 50th or 75th percentile levels. At the 95th percentile, the creatinine-corrected value for the total population was 2.92 micrograms per gram creatinine (µg/g), which corresponds to an estimated DINP exposure of 1.3 µg/kg/day. The 95th percentile creatinine-corrected value for children, age 6-11, was 3.27 µg/g, corresponding to an estimated DINP exposure of 0.82 µg/kg/day. In all of the reported groups (by age, by gender, and by race/ethnicity), the 95th percentile exposure levels for DINP appear to have declined slightly from the 1999-2000 CDC survey.

An analysis of DINP metabolites by Silva et al. (2006) suggest that the oxidative metabolites may be better biomarkers of DINP and that use of MINP as the sole biomarker may underestimate the

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prevalence of human exposure to DINP. While their investigation of samples from 129 adults indicate that DINP exposure may be widespread, the (uncorrected) metabolite levels reported suggest that mean exposures are below 3 μg/kg/day and exposures at the 95th percentile are around 15 μg/kg/day or less. Wittassek et al. (2007) also measured oxidative metabolites in the urine of 240 students sampled during 1988-1993 and an additional 119 sampled between 2001 and 2003 and calculated exposure levels based on the measured levels of these oxidative metabolites. The 50th percentile levels they reported were 0.22 and 0.37 μg/kg/day respectively with 95th percentile values of 1.6 and 1.5 μg/kg/day – consistent with the estimates based on MINP.

The lowest DINP dose that has been associated with tumor induction is 336 mg/kg/day in female rats, with effects in other species and sexes occurring at levels ranging from approximately 700 to 900 mg/kg/day (Moore et al., 1998a,b). This is more than 20,000 times greater than the highest 95th percentile exposure estimated for this non-genotoxic substance, assuming that humans were as sensitive to the effects of DINP exposure as mice. The scientific evidence, however, is that humans are far less sensitive than mice. Thus, it is not plausible that exposures of Californians to DINP would contribute to cancer incidence, even if the animal data are assumed relevant to human risk assessment.

VIII. CONCLUSION

Based on the available data for DINP, it can be concluded that the chemical is not genotoxic, cancer findings in rodent bioassays are not relevant to humans, proposed alternative mechanisms for liver tumorigenesis are not supported by the available information, and a role for DINP in TDS is not well substantiated. Moreover, it is not plausible that exposures of Californians to DINP would contribute to cancer incidence, even if the animal data are assumed relevant to human risk assessment.

As a result, DINP therefore does not warrant listing under Proposition 65 as “known to the state to cause cancer” because it has not been clearly shown to cause cancer as required by California law.

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65 Silva MJ et al. Oxidative metabolites of diisononyl phthalate as biomarkers for human exposure assessment. Environ Health Persp 114(8): 1158-1161 (2006). The metabolites measured were mono(hydroxylisononyl) phthalate (MHINP), mono(carboxylisononyl) phthalate (MCINP), and mono(oxoisononyl) phthalate (MOINP).

66 The metabolite levels reported by Silva et al. were not corrected for creatinine. CDC information for MINP were used to make the creatinine correction so that DINP exposure could be estimated. Molar conversion factors for MHINP (0.202), MCINP (0.106), and MOINP (0.016) were based on information from Fromme et al., 2007 (Environ Int 33: 1012-1020).

California State Office of Environmental Hazard Assessment proposal to establish a specific regulatory level posing no significant risk for Diisononyl Phthalate by amending Title 27, California Code of Regulations, section 25705.

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February 12, 2015

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The Fischer344 rat mononuclear cell leukemia (F344-MNCL) is a strain-specific disease and not an appropriate animal model of carcinogenesis.

F344-MNCL is a spontaneous, commonly occurring acute leukemia that is a prominent cause of natural mortality in F344 rats over 18 months of age (Stromberg 1985, Ward et al 1990, Thomas et al 2007). Reported incidences for F344-MNCL in untreated rats vary between 32%-74% for males and 14%-52% for females and are highly variable from one study/laboratory to another (Haseman et al 1998, 2003, Thomas 2007). Clinically, F344-MNCL is an acute rapidly fulminating leukemia characterized at onset by multi-organ involvement primarily in the spleen, liver and lung, and in relatively late stages of the disease, bone marrow (Stromberg & Vogtsberger 1983). MNCL is characterized by a sudden onset, rapid progression and is uniformly fatal.

The precise origin of the leukemia-initiating cell in F344-MNCL has not been fully elucidated. The first descriptions of MNCL in the 1960’s and 1970’s were limited to morphologic evidence and referred to as “mononuclear cell leukemia.” Subsequently, immunophenotypic, molecular and functional evidence has accumulated to support the conclusion that MNCL is an acute natural killer cell (NK) leukemia (Thomas et al 2007). The cause of F344-MNCL remains unknown, and there is no definitive evidence of an endogenous- or infectious- viral etiology (Stromberg 1990). However, the incidence of F344-MNCL in untreated rats is age-dependent with onset increasing between 15 and 24 months (Caldwell et al 1999). The high cumulative incidence of MNCL in untreated F344 rats strongly favors an underlying age-dependent genetic origin for the disease (Thomas et al 2007). However, there are numerous factors that influence its onset and incidence. These include: gender, nutritional, caloric, hormonal, immunological, environmental, physical (i.e. ionizing radiation) and chemical influences in addition to the presence of concurrent disease. The predictability of many of these influences remains obscure and counter-intuitive. For example, one of the most commonly encountered influences in bioassays conducted using the F344 rat is a decrease in the background incidence of MNCL in animals when oils have been used as a vehicle in gavage studies (Haseman et al 1985, NTP 1994).

The absence of any clearly defined mode of action for causation of F344 MNCL, together with myriad competing influences on onset and incidence, renders it impossible to reliably assess potential contributions to variance for the disease. Consequently, the evaluation of treatment-related effects in the incidence of MNCL is primarily dependent on the underlying assumptions inherent in the statistical methodologies used, and conflicting results are often obtained by simply using lifetable
vs incidental tumor/logistic regression analyses (Thomas et al 2007). Further, there is often a lack of concordance between dose-related incidence of neoplasms in other test species and the threshold-dependent-incidence of F344 MNCL in rats exposed to relatively high doses of many chemicals, including alkyl phthalates (Caldwell 1999).

The pathogenesis of F344 mononuclear cell leukemia is not a relevant predictor of human disease.

Historically, F344 MNCL was proposed to be a relevant model for possibly two human lymphoproliferative disorders, T-cell LGL and aggressive NK-cell leukemia (ANKL), based solely on morphology (see Thomas et al 2007). However, only human ANKL shares clinical features and a presumed cell of origin with F344 MNCL (Jaffe et al 2001, Ryder et al 2007, Chan et al 2008). Unlike F344 MNCL, Human T-cell LGL is a T-cell neoplasm characterized by a typically indolent (i.e., chronic) course. The normal counterpart for T-cell LGL is a CD8-positive T lymphocyte- not an NK cell (Chan et al, 2008a). Similar to F344 MNCL, human ANKL is an acute leukemia of NK cells that almost always exhibits disseminated multi-organ involvement at diagnosis and exhibits an aggressive and uniformly fatal course (Ryder et al 2007).

In contrast to MNCL in the F344 rat, human ANKL is one of the rarest hematologic diseases known: only 98 cases of ANKL have been reported to date worldwide, and series involving Caucasians have been limited to 1, and in a single instance, 2 patients (see Ryder et al 2007). We published 9 cases of ANKL diagnosed in our laboratory in Shanghai over a 2 year period, representing the third largest series reported worldwide and the largest series from mainland China (Ryder et al 2007). Similar to the aggressive clinical course observed for F344 MNCL, survival in our ANKL patients was measured in weeks. Nevertheless, human ANKL possesses a distinct viral etiology not found in F344 MNCL. Epstein-Barr virus (EBV) infection, which is unique to humans, is almost invariably found in ANKL and is observed in 100% of our ANKL cases. Taken together, the unique viral etiology and extremely rare occurrence of ANKL in humans provides additional evidence to indicate that F344 MNCL is not a relevant predictive model for human disease.
References


Comments regarding proposal to establish a specific regulatory level posing no significant risk for diisononyl phthalate

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*On behalf of the American Chemistry Council
Fischer 344 mononuclear cell leukemia (MNCL)

<table>
<thead>
<tr>
<th>Cell of Origin</th>
<th>Clinical features/tissue involvement</th>
<th>Etiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature NK cell</td>
<td>Acute leukemia / spleen, liver, lung, bone marrow</td>
<td>Genetic strain-specific /age-dependent</td>
</tr>
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</table>

- MNCL is the principal cause of death in untreated F344 rats
  - 14-52% of females
  - 32-74% of males
- Onset of MNCL influenced by: age, gender, nutrition, immunology, stress, presence of other diseases, etc.
- No evidence of a viral etiology
- No evidence to support an isolated finding of F344 MNCL following lifetime exposure to high doses of non-genotoxic compounds (e.g. alkyl-phthalates) is relevant for humans.
Human Aggressive NK cell leukemia (ANKL)

<table>
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<th>Cell of Origin</th>
<th>Clinical features/tissue involvement</th>
<th>Etiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature NK cell</td>
<td>Aggressive disease / spleen, liver, bone marrow</td>
<td>Epstein-Barr virus (EBV) (100%)</td>
</tr>
</tbody>
</table>

- One of the rarest hematopoietic neoplasms known.
- Tumor cells express clonal EBV.
- Most cases occur in teenagers and young adults
Aggressive NK cell leukemia (ANKL)

- First described in 1990
- ~100 cases have been reported worldwide


F344 MNCL shares common cell of origin, immunophenotype, as well as certain molecular and clinical features with ANKL in humans. However, human ANKL is an extremely rare disease involving clonal Epstein-Barr virus (EBV) while F344 MNCL is a strain-specific genetically-dependent disease with no evidence of a viral etiology.

There is no biological basis to support F344 MNCL as a relevant predictive model for human disease associated with exposure to non-genotoxic agents e.g. alkyl-phthalates.
FISCHER 344 (F344) RAT PREDILECTION FOR MONONUCLEAR CELL LEUKEMIA (MNCL)

- MNCL CAUSATIVE MECHANISMS UNKNOWN
- F344 HAVE AN AGE-RELATED SUSCEPTIBILITY WITH A HIGH INCIDENCE
- LOW INCIDENCE IN OTHER RAT STRAINS/STOCKS
- F344 MNCL IS NOT DRIVEN BY A DOMINANT GENETIC FACTOR
- INCIDENCE INFLUENCED BY A WIDE VARIETY OF MODIFYING FACTORS

It is well established that there is a wide variability of spontaneously occurring MNCL in F344 rats. Maximum background incidence in male F344 rats ranges from 10 to 74% and in females from 6 to 52% (Thomas et al., 2007). The background incidence more than doubled in the two decades from an initial incidence report in 1985 (Haseman et al., 1985). MNCL is an age-related disease and a major concern cause of early mortality in F344 rat carcinogenicity bioassays. The causative mechanism remains unknown. MNCL is less frequent in other commonly used rat stocks with spontaneous incidences of 0.5 to 0.6% in Sprague Dawley rats (Frith 1988; Brix et al., 2005). There are no published accounts documenting a treatment-related increase in MNCL in any non-F344 rat strain or stock. Based on comparison of F344 and Brown Norway rats in a diet restriction study, F344 MNCL is not driven by a dominant genetic factor and most probably is influenced by multiple genes in addition to being influenced by a wide variety of non-genetic modifying factors (Lipman et al., 1999). The F344 rat is isogenic and, therefore, has uniform susceptibility to MNCL. Thus, the high degree of variability in MNCL response favors a significant contribution from environmental and other unknown non-genetic factors.
Factors Affecting Variability of Control F344 MNCL Incidences

- **Nutritional factors**
  - Diet restriction
  - Diet – NIH-07 vs NTP-2000
  - Oil gavage studies

- **Environmental factors**
  - Laboratory differences in incidence among 4 NTP labs
  - Inhalation vs diet studies have lower MNCL incidences

- **Other factors affecting incidence of MNCL**
  - Splenectomy
  - Splenic toxicity
  - X-irradiation
  - Hypophysectomy
  - Liver foci and tumors
  - Genetic drift
  - Diagnostic criteria

Among nutritional factors, diet restriction has been most studied. Basically, diet restriction reduces the spontaneous and treatment-induced incidences and progression of F344 MNCL within the timeframe of conventional two-year carcinogenesis bioassays (Lipman et al., 1999; Stefanski et al., 1990). The reduction of MNCL by dietary restriction has been shown to be modulated through suppression of the GH:IGF-1 axis as well as by enhancement of host defenses against tumor cells (Hursting et al., 1993). In lifetime feed-restriction studies, F344 rats live longer, have a delay in development of MNCL but eventually a significant percentage of these older F344 rats develop MNCL (Shimokawa et al., 1993; Thurman et al., 1994). Corn oil gavage studies have a reduced incidence of MNCL (Haseman et al., 1985) and this oil effect has also been shown for safflower oil and tricaprylin gavage studies (NTP TR-426). Early studies have shown markedly reduced occurrence of MNCL follow early splenectomy (Moloney & King 1973) and X-irradiation (Hellman et al., 1982). A treatment-related reduction in the incidence of MNCL in two-year carcinogenic bioassays has been associated with splenic toxicity in 90-day toxicity studies (Elwell et al., 1996), although several studies with treatment-related reduced MNCL are not associated with splenic toxicity. Other influencing factors or associations with the occurrence of MNCL include an inverse relationship with proliferative hepatic lesions (Harada et al., 1990; Haseman 1983) and hypophysectomy (Ward et al., 1990; Stefanski et al., 1990). While all the rats used in NTP carcinogenicity bioassay come from the same supplier, differences in background incidences among 4 laboratories carrying out NTP bioassays have been shown (Haseman 1983). Some less defined factors influencing MNCL include genetic drift, and changing diagnostic criteria. Interestingly, the incidence
and progression of MNCL in F344 rats is not influenced by administration of genotoxic carcinogens (Casewell 1999).

570 F344 Rat NTP Bioassays

- **24 bioassays classified as positive for carcinogenicity at various tissue sites with MNCL also classified as positive (4.2%)**
  - 8 in both sexes, 8 in females, 8 in males
- **15 bioassays classified as equivocal for carcinogenicity at various tissue sites with MNCL also classified as equivocal (2.6%)**
  - 4 in both sexes, 6 in females, 5 in males
- **26 bioassays classified as positive for carcinogenicity at various tissue sites with a decreased incidence of MNCL (4.6%)**
  - 24 in both sexes, 1 in females, 1 in males

NTP Bioassays in F344 rats that include a MNCL response

Among 570 carcinogenicity bioassays conducted in F344 rats, MNCL has been identified in 24 studies and has been classified as being positive, having some evidence, or having clear evidence of carcinogenicity. All but 3 of these 24 studies were classified as positive based on tumor responses in major organ systems but also included MNCL as one of the tumor responses. Another 15 studies were judged to have an equivocal tumor response in one or more tissues in addition to an equivocal MNCL response. In both the positive and equivocal studies the MNCL response was seen with equal frequency in males, females and in both sexes. In 26 carcinogenicity bioassays there was a treatment-related decreased frequency of MNCL seen in both sexes. Overall the majority of bioassays with a treatment-related clear or equivocal increase or a decrease in MNCL represents a small percentage of the 570 carcinogenicity bioassays conducted in F344 rats.

With respect to the 24 studies implicating MNCL as part of a positive carcinogenicity response, the MNCL response was seen in either male or female rats as often as it was present in both sexes. It is important to keep in mind that the tumor responses in these studies were seen in tissues other than the hematopoietic system while a MNCL response was also noted as being present. When the response in a non-hematopoietic tissue was robust, there was generally no significant comment or discussion made regarding the MNCL. However, in at least half of the 24 studies, there was discussion and challenge during the formal peer review regarding whether MNCL should be included as part of the evidence of carcinogenicity. Many of these peer review discussions resulted in a downgrading of the positive call on MNCL from positive to equivocal or from positive to “may have been related to treatment.” On the other hand, when the positive response involved multiple tissue sites and was very clear, the inclusion of MNCL as part of the positive response typically received no
significant discussion and was simply listed along with the multi-site tumor response.

With respect to the equivocal MNCL responses, there was often significant debate during the peer review process with concern about how much any particular MNCL incidence exceeded the historical control incidence. In most of these instances the evidence for carcinogenicity was based on a clear tumor response in one or more non-hematopoietic tissues with interpretation of the MNCL response being complicated by early mortality. Interestingly, several of these equivocal MNCL responses involved female rats in situations where only a single dose exceeded the historical control incidence. The discussions during the peer review meeting involving the MNCL responses often did not result in any change in interpretation of the MNCL response. The point to be made here is that acceptance of an equivocal MNCL was rarely unanimous among the peer review panel.

With respect to the 26 studies in which there was a treatment-related decrease in MNCL, the effect seen was typically dramatic and involved both sexes with incidences less than 10% in the treated groups. Splenic toxicity was implicated as influencing the decreased MNCL in 16 of the 26 studies based primarily on increase splenic extramedullary hematopoiesis and/or increase splenic hemosiderin pigmentation seen in prechronic studies. For the remaining 10 studies with treatment-related decreases in MNCL, there was no evidence of splenic toxicity. In all 26 studies the treatment-related decreased in MNCL was not associate with body weight reduction or reduced survival.

**MNCL as the Only Evidence of a Potential Response Positive MNCL response**
- 2-Amino-5-nitrothiazole
- Butyl benzyl phthalate
- Dimethymorpholinophosphoramide

**Equivocal response**
- Acetaminophen
- Chlorinated & chloraminated water
- Dialyl phthalate

*MNCL as the only evidence of a positive tumor response in an NTP bioassay* 
MNCL was observed as the only evidence of potential carcinogenicity in three F344 rat studies. 2-Amino-5-nitrothiazole (NTP TR-053) was considered positive in males. However, this is an older bioassay and the positivity was based on lymphoma and granulocytic leukemia and not on confirmed MNCL. This bioassay was completely negative for carcinogenicity in females.
A positive MNCL call was made in female F344 rat in a butyl benzyl phthalate study (NTP TR-213). However, a repeat butyl benzyl phthalate study (NTP TR-458) failed to confirm the prior positive female response.

Dimethylmorpholinophosphoradmidate (NTP TR-323) was the first NTP study in which MNCL was considered a response in both sexes. While there was some debate about the MNCL response during the peer review process the classification of ‘some evidence for carcinogenicity’ was not changed.

MNCL as the only evidence of an equivocal tumor response in an NTP bioassay MNCL was identified as an equivocal response and was the only response in three studies. In the acetaminophen study (NTP TR- 394) the response in female F344 rats was classified as equivocal even though there was uncertainty as to whether the MNCL was related to treatment.

In the chlorinated and chloraminated water study (NTP TR- 537) there was extensive debate during the peer review but a call for ‘no evidence’ was defeated during the final voting.

In the diallyl phthalate study (NTP TR–284) there was documented debate regarding an equivocal response in female F344 rats. However, there was more emphasis in the discussion on the allyl group rather than on the MNCL response. It was mentioned that there were a lot of borderline diagnoses of MNCL in this study, casting some doubt on the final classification of an equivocal response in female rats.

**Recommendation**

- **F344 response not a suitable endpoint for assessing a carcinogenic response with confidence in rat bioassays**
- **In retrospect, selection of the F344 rat for NCI/NTP bioassays not ideal**
- **NTP has switched to Harlan Sprague-Dawley rats**

Given the high and variable background incidence and known as well as unknown factors that variably influence the occurrence of MNCL in F344 rat, this endpoint is not ideal for assessing a carcinogenic response in a carcinogenesis bioassay. The fact that in 2006 NTP made a decision to switch to a different rat was based on several factors, the most important being the variable MNCL response in the F344 rat and the difficulty in interpreting this response. The good news is that we will probably not be faced with interpreting the MNCL response in the future, as most organizations will no longer be using the F344 rat. In the meantime, the MNCL response in past F344 carcinogenicity studies is not a suitable endpoint for assessing a carcinogenic response with confidence in F344 rat bioassays.
References


splenectomy in the rat. *Cancer Research,*** 33, 573-574.

NTP Technical Reports can be accessed at: [http://ntp.niehs.nih.gov](http://ntp.niehs.nih.gov)


F344 Predilection for MNCL

• Causative mechanisms unknown
• Age-related susceptibility
• Low incidence in other strains/stocks
• No treatment-related increase in MNCL in other strains/stocks
• Not being driven by a dominant genetic factor
• Incidence influenced by a wide variety of modifying factors
Factors Affecting Variability of Control F344 MNCL Incidences

• Nutritional Factors
  – Diet (NIH-07 vs NTP-2000)
  – Diet restriction
  – Oil gavage

• Environmental Factors
  – Laboratory differences (4)
  – Inhalation vs diet study

• Other Factors
  – Liver lesions
  – Splenectomy
  – Splenic toxicity
  – X-irradiation
  – Hypophysectomy
  – Germfree vs conventional
  – Genetic drift
  – Diagnostic criteria
570 F344 Rat NTP Bioassays

• 24 Positive, clear or some evidence of carcinogenicity (4.2%)
  – 8 in both sexes, 8 in females, 8 in males

• 15 Equivocal response (2.6%)
  – 4 in both sexes, 6 in females, 5 in males

• 26 Decreased incidence (4.6%)
  – 24 in both sexes, 1 in females, 1 in males
MNCL as Only Evidence of Potential Response

• Positive response
  – 2-Amino-5-nitrothiazole
  – Butyl benzyl phthalate
  – Dimethylmorpholinophosphoramidate

• Equivocal response
  – Acetaminophen
  – Chlorinated & cholaminated water
  – Diallyl phthalate
Recommendation

• F344 MNCL response not a suitable endpoint for confidently assessing a carcinogenic response in rat bioassays

• In retrospect, selection of the F344 rat for NCI/NTP bioassays not ideal

• NTP has switched to Harlan Sprague Dawley
February 25, 2014

WRAP UP & PROPOSED ALTERNATIVE CALCULATION OF AN NSRL FOR DINP

R. McKee
Perspective on DINP-Induced Rodent Tumors and NSRL Calculations

MNCL is not relevant to humans
- As documented in previous comments and further amplified by Drs. Maronpot and Irons at today’s meeting

Liver Tumors in rodents treated with DINP are the consequence of a PPARα-mediated process
- As documented in previous comments and further supported by the recent publication by Wood et al. (2010).¹

Other tumors observed in chronic rodent studies of DINP are reported at < statistically significant levels and/or are due to processes that are not relevant to humans.
- As documented in previous comments

In summary, exposure to DINP does not cause human relevant tumors and should not be listed as carcinogenic under Proposition 65

If an NSRL is to be derived, the method used for DEHP should be followed

The calculation should be based on liver tumor data
- As shown, the liver tumor data are comparable across studies and provide a good basis for statistical evaluation

A scaling factor (10x) should be applied to account for quantitative differences between rodents and humans
- Previously applied because of differences in receptor (PPARα) density,
- Further supported by evidence that MINP² is a more avid agonist for rodent than human receptors. ²

Liver Tumor Percentage as a Function of Dose of DINP in F344 Rats

Circles indicate data points significantly different from controls.

Data are most compatible with a threshold model.
Potency Estimates

- cancer potency estimates determined for liver tumor responses
  - determined using linearized multistage model from USEPA
    - first degree multistage and second degree multistage models
  - \( P(d) = 1 - \exp[-(q_0 + q_1d + q_2d^2 + \ldots + qjdj)] \), where \( P \) is probability of cancer mortality induced at a daily dose \( (d) \)

- most conservative potency estimates from male mouse liver tumor data

<table>
<thead>
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<th>Species</th>
<th>Sex</th>
<th>Organ</th>
<th>Potency</th>
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NSRL Calculation

- Animal potency estimates converted to human potency estimates by allometric scaling using Cal OEHHA method
  - The potency estimate was scaled 10x in DEHP NSRL to reflect species differences in activity of PPARα receptor concentration and activity

- Equation
  \[
  \text{human cancer potency} = q^1 \times \left( \frac{bw_{human}}{bw_{mouse}} \right)^{1/4} \times \frac{1}{10}
  \]
  - Human potency estimate: 0.00026 (mg/kg d)-1

- Potency estimate inserted into NSRL equation
  - Equation
    \[
    I = \frac{R \times bw_h}{q_{human}}
    \]
    \[
    I = \frac{10^{-5} \times 70 \, kg}{0.00026}
    \]
    - NSRL: 2664 µg/day
In Summary

• The rodent tumors reported in DINP studies are not relevant to humans

• If a no significant risk level is calculated, we recommend that the calculation be based on rodent liver tumors and follow the calculation method used in the previous DEHP assessment
  • There is a consistency of liver tumor response across species/genders
  • The liver tumor dose response relationships are similar across studies
  • Calculating the NSRL in this way would be consistent with historical practice

• As shown, using this method and following the methodology used previously for DEHP resulted in an NSRL of 2664 ug/day