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25 January 2012
VIA E-MAIL (andrewsda@niehs.nih.gov)

Danica Andrews
Designated Federal Official
Office of Liaison, Policy and Review
Division of the NTP
NIEHS
PO Box 12233, MD K2-03
Research Triangle Park, NC 27709

RE: Comments on Draft NTP TR-580 (Beta Picoline)

Dear Ms. Andrews,

Vertellus Specialties Inc. has reviewed the draft *NTP Technical Report on the Toxicology and Carcinogenesis Studies of beta-Picoline (CAS No. 108-99-6) in F344/N Rats and B6C3F1/N Mice (Drinking Water Studies)*[i], made available to the public on 14 December 2011 as mentioned in 76 FR 77832. We appreciate the opportunity to submit written public comments on this draft.

Vertellus has several concerns regarding this report, which can be found in the attached "white paper" entitled *Critical Review of the Conclusions of the Draft Report of the NTP 2-year Bioassay on Beta-Picoline*, authored by Barbara Vogt, Ph.D., DABT. We would appreciate a full consideration and explanation of these points in the upcoming Peer Review Panel meeting on February 8-9, 2012. Vertellus plans to view the proceedings via webcast.

In the meantime, we welcome the opportunity to discuss these comments in further detail. Please contact me at mbogle@vertellus.com or by phone at 317-248-6548 if you have any questions.

Regards,

[Redacted]

Misty L. Bogle
Corporate Health, Safety & Product Stewardship Manager
Regulatory Management

MLB/mlb
Attachment (25 pages)

[i] NTP TR 580, NIH Publication No. 12-5922

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Critical Review of the Conclusions of the Draft Report of the NTP 2-year Bioassay on Beta-Picoline

By Barbara Vogt, Ph.D., DABT
Toxicologist, the Redstone Group
January 16, 2012

Executive Summary:

The National Toxicology Program has issued a draft report that beta-picoline has "clear evidence of carcinogenic activity", based on liver and lung tumors in female mice exposed to the substance in the drinking water, along with "some evidence" in female rats, based on lung tumors. There is a scientific basis for these designations to be challenged. A research agenda which includes a "mode of action" framework for this chemical should be considered. Additional mechanistic and exposure-based studies in animals are recommended.

Introduction:

The National Toxicology Program (NTP) has published its draft Technical Report (draft TR 580, NIH Publication No. 12-5922) on the results of subchronic and chronic toxicity, carcinogenicity, genotoxicity and reproductive toxicity of beta-picoline (3-Methyl Pyridine, CAS 108-99-6). Beta-picoline was administered for 2 years in the drinking water to male and female F344/N rats at doses of 156.25, 312.5 or 625 mg/L (equivalent to 6, 12 or 22 mg/kg bw/d (males) and 7, 14 or 26 mg/kg bw/d (females)) and to male and female B6C3F1 mice at 312.5, 625 or 1250 mg/L (equivalent to 26, 50 or 92 mg/kg bw/d (males) or 18, 37, 68 mg/kg bw/d (females)). The draft conclusions are that, under the conditions of the 2-year study:

a) There was no evidence of carcinogenic activity in male rats
b) There was some evidence of carcinogenic activity in female F344/N rats (increased incidence of alveolar/bronchiolar adenoma or carcinoma (combined))
c) There was equivocal evidence of carcinogenic activity in male mice (increased incidences of alveolar/ bronchiolar adenoma or carcinoma (combined))
d) There was clear evidence of carcinogenic activity in female mice (increased incidences of alveolar/bronchiolar adenoma or carcinoma (combined) in the lung and hepatocellular carcinoma and hepatoblastoma in the liver).
e) Exposure to beta-picoline caused increased incidence of nonneoplastic lesions of the lung in female mice and the nose in male and female mice.

The categorizations are based on the "Definition of Carcinogenicity Results" as defined by the NTP (See Appendix 1). Of note, the NTP emphasizes the dependency of the categorical results on the conditions of the study. The categories refer to the strength of the experimental evidence under these conditions, and not to either potency or mechanism. Elucidation of the Mode of Action is not a part of the NTP mission.

The draft TR 580 report has several omissions and inconsistencies. In its early draft phase, it has no report from the Pathology Working Group (PWG), which serves an important oversight mechanism to resolve questionable diagnostic calls and insure consistency, and to provide details on the observed pathology, especially between neoplastic and non-neoplastic tissue. There are several inconsistencies between the statistical significance values observed in the tables and the text of the Results and Discussion sections, and discrepancies with significant values and asterisked values.

In this review are addressed several overarching issues of scientific concern or interpretation. The first is a comparison of the findings of this study with those of a previous NTP study on pyridine. Secondly is a discussion on the strength of the tumor data supporting the conclusions of the NTP on the carcinogenic potential of this exposure to beta-picoline, especially considering the background incidence of the tumors. Alternative explanations are presented to explain several observations. Suggestions are made to undertake analyses of these and other data which would be helpful to understand the observed findings, or generate additional mechanistic data.

Pyridine (CAS 110-86-1) is a structurally similar heterocyclic aromatic compound, without a methyl constituent group on the 3 position to the nitrogen. Pyridine has been studied by the NTP in a chronic 2-year bioassay via the drinking water, and reported in Technical Report (TR) 470, in 2000. F344/N rats were exposed to doses ranging from 100 ppm (7 mg/kg bw/d) to 400 ppm (33 mg/kg bw/d) and B6C3F1 mice at doses ranging from 250 ppm (35 mg/kg bw/d) to 1000 ppm (110 mg/kg bw/d). Kidney pathology was observed in male rats associated with alpha-2-microglobulin induction, a condition which does not occur in humans. Therefore, the NTP added a second group of male rats of the Wistar strain, which is not sensitive to development of this nephropathology, and dosed at 100 ppm (8 mg/kg bw/d) to 400 ppm (36 mg/kg bw/d). The findings of this study are listed in the following table:

Table 1: Results of a 2-year Bioassay on Pyridine and Conclusions on Carcinogenic Activity in F344/N Rats, Wistar Rats and B6C3F1 mice

	Male F344 Rat	Female F344 Rat	Male Wistar Rat	Male B6C3F1 Mouse	Female B6C3F1 Mouse
Non-neoplastic Effects	Liver	Liver and bile duct	Liver, glandular stomach, parathyroid gland	None	None
Neoplasms	Renal tubule adenoma, renal tubule carcinoma	None	None	Hepatocellular adenoma, hepatocellular carcinoma, hepatoblastoma, combined hepatocellular adenoma or carcinoma or heptoblastoma	Hepatocellular adenoma, hepatocellular carcinoma, hepatoblastoma, combined hepatocellular adenoma or carcinoma or heptoblastoma
Uncertain Findings		**Mononuclear Cell Leukemia**	**Interstitial Cell Adenoma of Testis**		
Conclusion	**Some**	**Equivocal**	**Equivocal**	**Clear**	**Clear**

The conclusions are that, under the conditions of this study, pyridine displayed clear evidence of carcinogenic activity producing liver tumors, some evidence of producing kidney tumors in strains of rats susceptible to alpha-2-microglobulin induction, and equivocal evidence of inducing mononuclear cell leukemia in female rats and tumors of the testis in male Wistar rats.

The strongest finding, that of liver tumors in mice, is interpreted as having limited relevance to human cancer risk. Hepatoadenomas and hepatocarcinomas occur frequently in unexposed animals this strain of mouse, as the background incidence in males ranging up to 81%, and in females up to 82%. The incidence in controls was found to increase during the last 20 years, for unknown reasons (Haseman, et.al., 1996). Hepatoblastoma was relatively rare, with a 3% incidence in historical male B6C3F1 controls (range 0-12%), but is also thought to have little relevance to human cancer risk (Turusov, et.al, 2002). The mechanism of the high background rate of hepatic tumors in mice has been found to involve DNA hypomethylation, allowing transcription of genes conferring a growth advantage to spontaneously initiated cells (Goodman, 1998). Humans are less susceptible to this effect, as they have the capacity to maintain normal levels of methylation. Human exposure to chemicals which induce liver tumors in mice has carried a same degree of concern for cancer development as for chemicals where the background incidence in test species is low. The International Agency on Research on Carcinogens (IARC) classifies pyridine as a Group 3 substance, "Not classifiable" as to its carcinogenicity to humans (IARC, 2000).

The finding of kidney adenomas in male 344 rats is likewise considered not relevant to humans due to the induction of alpha-2-microglobulin, which does not occur in humans. There were no tumors noted in male Wistar rats in the absence of nephropathy associated with alpha-2-microglobulin.

In the years since the publication of the NTP TR 470 on pyridine, the consensus is that, although there was clear evidence that liver tumors occurred in mice and kidney tumors occurred in susceptible rats exposed to pyridine, pyridine is not considered a human carcinogen (IARC, 2000).

Discussion of the Study Design for the beta-picoline Bioassay:

The choice of study conditions has the potential to impact the results of the study, and this is the case with the current study. The NTP has the difficult task of deciding how to administer beta-picoline to test animals in a long term bioassay. The choices are: dermal, oral via gavage, dietary in food, oral via the drinking water, by inhalation, intravenously via pump, or intraperitoneally. All these choices have drawbacks and establishment of concurrent control groups of animals are essential for understanding the potential impact of the administration process.

The decision was made to administer beta-picoline to animals in drinking water, perhaps impacted by the same route of exposure as in the study with pyridine. This also appears to be based on the potential

for the substance to find its way into drinking water after environmental release from chemical manufacturing (see draft TR 580). Beta-picoline is a high production volume industrial intermediate and solvent, with a strong odor (sweetish, described as either unpleasant or not unpleasant), a low odor threshold (< 1 ppm, AIHA, 1988) and a Henry's Law Constant (7.73 E-6 at 20°C) which indicates that it volatilizes slightly out of water into the air space above. More importantly, the substance is corrosive to biological tissue, a fact which appears to have been known to NTP staff after the review of the toxicological literature by Zeiger, Tice and Brevard (1999) and specifically after the referenced Dutertre-Catella, 1989, where severe dermal and ocular irritation with histological findings consistent with corrosion and inflammation were documented. The manufacturer of the substance is in possession of studies indicating that beta-picoline is corrosive to the skin and is extremely irritating to the eye (Fitzgerald, 1991; Spear, 1984, 1985). The American Industrial Hygience Association Workplace Environmental Exposure Level Guide (WEEL, 1988) indicated that beta-picoline is an eye irritant and causes superficial necrosis and burns after prolonged contact with the skin.

The draft TR 580 indicates that, in order for drinking water to be prepared for the study, the pH of the tap water solution with beta-picoline was adjusted to 6.0 - 7.5 by the addition of acetic acid. This suggests that the original solution was alkaline, and therefore potentially caustic. With an odor threshold of approximately 1 ppm (AIHA, 1988), solutions of beta-picoline in water ranging in concentration from 67 to 1250 mg/L (or 67 to 1250 ppm) is expected to have an odor and taste discernible to mammals. Acetic acid also possesses an odor which may be discernable to animals.

Chronic administration of a corrosive test material by the oral route has the potential to result in local irritation and corrosion effects in test animals, or refusal of the animals to voluntarily consume the test material in the drinking water. Both of these occurrences were documented to have taken place in this study. The ingestion of a potentially irritating solution in a chronic toxicity study requires careful selection of doses. This has been recognized by the Organization for Economic Cooperation and Development (OECD) in their 2010 Guidance No. 116, suggesting that chronic studies using irritating or corrosive materials *"may need to have the doses diluted to avoid severe irritant effects, and testing at concentrations which are likely to be corrosive or irritant to the (gastrointestinal tract) should be avoided."* (parentheses added). While the NTP selected the doses for the two-year bioassay based on

systemic maximally tolerated doses in the 14-week study (no more than 10% decrease in body weight), a more careful analysis of local effects in the mouth may have resulted in the choice of lower concentrations to insure that irritation was not present.

In summary, there is ample evidence that beta-picoline has corrosive or irritating properties. However, there is little information that the dose-range finding studies or the 14-week study considered the potential impact of the corrosivity of the test substance when monitoring animals, measuring food/water intake or assessing critical dosages.

The presence of local effects in the mouth, upper airways and eventually the lungs of rats and especially mice in this study suggests that irritation occurred as a consequence of exposure to beta-picoline via the drinking water. Findings after 105 weeks of exposure, relevant to a local, portal-of-entry effect, include those in the table.

Table 2: Nonneoplastic Pathology in the Mouth, Nose and Lung in Rats or Mice in the 2-year Bioassay

Observation	Species, sex	Incidence and Dose	Statistical Significance	Comment
Squamous cell papillomas and carcinomas, oral mucosa or tongue	Rat, male and female	1/50 in 312.5 and 1/50 in 625 mg/L	NS	occurred in less than 5% of any group, so not reported in detail.
Nasal olfactory epithelial metaplasia	Rats, females Mice, males and females	Female Rats: 0/50 in 0, 3/50 in 156.25, 1/50 in 312.5 and 1/50 of 625 mg/L . Male mice: 8/50 in 0, 12/50 in 312.5, 30/50 in 625, and 41/50 in 1250 mg/L. Female mice: 2/49 in 0, 2/44 in 312.5, 7/49 in 625, 14/47 in 1250 mg/L	NS S at mid and high dose; $p < 0.01$ S at high dose; $p < 0.01$	
Nasal olfactory epithelial atrophy	Mice, males and females	Male mice: 3/50 in 0, 4/50 in 312.5, 8/50 in 625, and 7/50 in 1250 mg/L. Female mice: 1/49 in 0, 2/44 in 312.5, 2/49 in 625, 7/47 in 1250 mg/L	NS S at high dose; $p < 0.01$	
Lung alveolar epithelial hyperplasia	Mice, males and females	Male mice: 4/50 in 0, 6/50 in 312.5, 6/49 in 625, and 7/50 in 1250 mg/L. Female mice: 2/50 in 0, 4/50 in 312.5, 3/49 in 625, 8/50 in 1250 mg/L	NS S at high dose; $p < 0.01$	Females in 625 mg/L group also had 3 bronchiolar hyperplasias, not included.

S: Statistically significant, $p < 0.05$
NS: Not statistically significant

These histological findings suggest the presence of chronic local irritation effects. Absolute and relative lung weights in the high dose group of female mice were significantly less than those of controls, despite the presence of tumors. This suggests that the choice of dose to be administered to mice could have been lowered, with the intention to avoid chronic irritation (See OECD, 2010). There was no suggestion of irritation to the esophagus, forestomach or stomach; this is not unexpected after ingestion of a neutral solution.

These data also provide information critical to understanding the etiology of the lung effects in this study. The local track of "mouth to nose to bronchiole to alveolus" suggests that the material entered the lung via the oral cavity. The most likely manner of this exposure is inhalation of a volatile fraction of the test material. This is suggested by the authors of the NTP draft report.

The dosing solutions were tested and found to be stable under the animal room conditions. They were made and stored in sealed polyethylene bottles. The water was administered to animals in glass bottles with stainless steel sipper tubes and Teflon® seals. Analytical data indicate that little or no volatization occurred under these conditions. However, this does not rule out the possibility that the substance may have volatilized in the warm humid conditions of the rat and mouse palate, which could give rise to *in situ* inhalation of volatile test material into the lung. This is more likely if the irritating nature of the solution caused pain or burning of the mucous membranes of the mouth, causing the animal to hold the solution on the tongue longer than is normally expected.

The presence of irritation in the upper airways and lungs may be unique to this study of a test material administered in the drinking water. Explanations other than inhalation of *in situ* volatilzed material include aspiration of the liquid into the respiratory tract, although this would result in pathology of the lung and inhibition of lung function, which was not seen in the study. The alternative is that systemic exposure resulted in respiratory tissue toxicity. However, as these striking irritation effects follow a local, spatially progressive track, direct contact from the mouth to the lung is the most likely explanation.

Discussion of Results:

The primary finding of the carcinogenesis study concerns lung tumors in mice. It is critical to appreciate that lung tumors in B6C3F1 occur at a moderately high background rate (Haseman, et.al., 1998). It is known that there are genetic factors, including CYP1A1/CYP1B1 inducibility and strain-specific mutations in the K-ras oncogene, which give rise to different rates of alveolar/bronchiolar adenomas and carcinomas in mice (Jennings-Gee, et.al., 2006, Hoenerhoff, et.al., 2009). This suggests that chemically-induced lung tumors in mice are highly dependent on genetics and chemical exposures may not be relevant to humans having a different mutational profile. The current historical control incidence of lung tumors in mice and rats is given in the following two tables. The first table provides the background incidence of lung tumors in NTP studies where the test material is administered in the drinking water. The incidence of alveolar/bronchiolar adenomas or carcinomas (combined) in male mice is 28 to 32%, and in female mice, up to 22%.

Table 3: Historical Incidence of Alveolar/Bronchiolar Adenomas or Carcinomas (combined) in NTP Drinking water studies

	Incidence	Range
Male Rats	7%	6-8%
Female Rats	4%	0-8%
Male Mice	30%	28-32%
Female Mice	13%	4-22%

The background incidence of tumors was also reported in studies regardless of the route of administration. These data may include effects of handling or stress associated with administration of the test material and different laboratory locations. For alveolar/bronchiolar adenomas or carcinomas (combined) in mice, the incidence is slightly lower but the range broader (up to 40% in male mice) than in those studies where the route of administration was oral via the drinking water.

Table 4: Historical Incidence of Alveolar/Bronchiolar Adenomas or Carcinomas (combined) in NTP studies (All routes of exposure)

	Incidence	Range
Male Rats	3.6%	0-10%
Female Rats	2.3%	0-8%
Male Mice	26.2%	14-40%
Female Mice	8.4%	2-22%

In the paragraphs below, for the present study, the incidence of lung tumors in each species and sex will be discussed in terms of single and combined groupings (adenomas, carcinomas or combined), dose-response, multiplicity, latency (time to first tumor), comparison with control incidences, and effects on lifespan. These elements comprise the basis for the evaluation of whether there is biological significance to support the statistical significance of the findings.

Table 5. Incidence of Alveolar/Bronchiolar Tumors (Lung) and Liver in Rats and Mice Exposed to beta-Picoline

Observation	Species, sex	Incidence and Dose	Statistical Significance	Comment
Alveolar/bronchiolar adenoma, carcinomas or combined	Rat, male	No difference from controls	NS	
Alveolar/bronchiolar adenoma	Rats, females	0/50 in 0, 3/50 in 156.25, 2/50 in 312.5 and 5/50 of 625 mg/L .	S, $p < 0.05$	
Alveolar/bronchiolar Carcinoma		No difference from controls	NS	
Alveolar/bronchiolar adenoma or carcinoma (combined)		0.50 in 0, 4/50 in 156.25, 2/50 in 312.5, and 5/50 in 625 mg/L	S, $p < 0.05$	
Alveolar/bronchiolar adenoma	Mice, males	6/50 in 0, 11/50 in 312.5, 16/50 in 625, and 8/50 in 1250 mg/L.	S at mid dose; $p < 0.05$	
Alveolar/bronchiolar Carcinoma		No difference from controls	NS	
Carcinomas or combined		No difference from controls	NS	
Alveolar/bronchiolar adenoma	Mice, females	5/50 in 0, 6/50 in 312.5, 4/49 in 625, 11/50 in 1250 mg/L	S dose trend, $p < 0.05$	
Alveolar/bronchiolar Carcinoma		No difference from controls	NS	
Alveolar/bronchiolar adenoma or carcinoma (combined)		11/50 in 0, 13/50 in 312.5, 13/49 in 625, 21/50 in 1250 mg/L	S at high dose; $p < 0.05$	
Hepatocellular adenoma	Mice, females	38/49 in 0, 46/50 in 312.5, 46/50 in 625, 39/50 in 1250 mg/L	S at low and mid dose, $p < 0.05$	
Hepatocellular carcinoma		11/49 in 0, 20/50 in 312.5, 26/50 in 625, 23/50 in 1250 mg/L	S, all doses and dose trend, $p < 0.05$	
Hepatocellular adenoma or carcinoma (combined)		12/49 in 0, 21/50 in 312.5, 28/50 in 625, 24/50 in 1250 mg/L	S, all doses and dose trend, $p < 0.05$	

S: Statistically significant, $p < 0.05$
NS: Not statistically significant

Analysis of Tumor Incidence for Rats: In considering the lung tumor data in rats, there are no differences in males, and only limited findings in females.

Incidence: There is a significant increase in adenomas in high-dose females ($p < 0.05$). There is no increase in the rate of carcinomas; the combined incidence of adenomas or carcinomas is also statistically significant ($p < 0.05$). Lung epithelial hyperplasia is increased, but is not significantly different from controls.

Dose-Response: There is no linear dose-response for adenomas in female rates, even though there is a significant dose trend test, $p < 0.05$, for adenomas. This may be due to the absence of tumors in the concurrent group, when the average incidence from historical controls (drinking water) is 4. There is also a borderline significant trend test for the combined adenomas and carcinomas ($p=0.050$), derived from the absence of tumors in the control group.

Multiplicity: For adenomas in female mice, there is no data suggesting multiple tumors in single animals.

Latency: There were 638 days to appearance of first adenoma in the high dose group in females, compared to 0 days (no tumors) in control rats. This is longer than the 526 days when the first tumor appeared in the low dose group.

Historical control values: This is only slightly higher (10% rather than up to 8% in historical controls (drinking water).

Lifespan: The absence of any carcinomas at the mid and high doses, after 2 years, argues that these benign tumors in rats may not progress to malignant neoplasms, and carry no appreciable morbidity. There was no difference in survival of control and exposed animals.

Analysis of Tumor Incidence for Male Mice: There were limited findings concerning lung tumors in male mice.

Incidence: In this study, male mice were found to have alveolar/bronchiolar adenomas which were significantly increased only in one dose group, the mid dose (625 mg/L, $p< 0.05$). There was no significant increase in alveolar/bronchiolar carcinomas among exposed males, nor was there a significant increase in the combined endpoint of alveolar/bronchiolar adenomas or carcinomas.

Dose-Response: The lack of a dose response relationship is clear; the high dose group of 1250 mg/L showed a lower incidence of adenomas than the group exposed to 312.5 mg/L (low dose).

Multiplicity: In each of the two highest dose groups, there were 2/50 instances of multiple adenomas per animal. For carcinomas, the low dose group had 2/50 multiples, mid dose had 0, and high dose had 3/50 multiple tumors per animal. These figures do not suggest high multiplicity.

Latency: In the mid dose group with the significant increase in incidence of adenomas, there is a latency of 470 days, compared with 730 for controls and 709 for high-dose animals. This suggests tumors formed earlier than in the other dose groups.

Historical control values: The incidence in mid-dose males is only slightly higher (32% rather than 30% in historical controls (drinking water).

Lifespan: Survival of all exposed groups was similar to that of the control groups. There was a positive trend in the survival of males exposed to beta-picoline.

Analysis of Tumor Incidence for Female Mice: The most significant effect of beta-picoline is in development of lung tumors of female mice, coincident with an increased incidence of nonneoplastic lesions of the upper airway.

Incidence: There was no statistical difference in the incidence of adenomas between the exposed and control groups, nor was there a statistical difference observed for alveolar/bronchiolar carcinomas. However, when these two endpoints were combined, the high dose group of 1250 mg/L females showed a statistically significant increase in adenomas or carcinomas ($p < 0.05$), along with a significant dose trend.

Dose-Response: The incidence of alveolar/bronchiolar adenomas showed a borderline positive trend in female mice ($p = 0.046$), technically called "positive". The dose trend for carcinomas was not significant. The dose-response for combined neoplasms is relatively flat, with the high dose showing a small spike in incidence; the trend, however, is statistically significant ($p < 0.05$).

Multiplicity: There is 1/50 each multiple adenomas in the low and high dose groups of female mice. For the carcinoma, there are 2/50 in the low dose, 2/49 in the mid dose group, and 4/50 in the high dose group.

Latency: For the carcinoma (and combined tumors), the days to first tumor in the high dose females is lower (509 days) compared to 669 days in the controls.

Historical control values: For the combined adenomas and carcinomas, the historical range in unexposed females is up to 22%. The value in the high dose exposed females is 42%.

Lifespan: There was no effect of exposure to beta-picoline on survival, compared with controls.

The NTP's summary of the 2-Year Carcinogenesis and Genetic Toxicity Studies of beta-Picoline classifies lung tumors in female rats as "some evidence" of carcinogenicity, the lung tumors in male mice as "equivocal evidence", and lung and liver tumors in female rats as "clear evidence". Each of these can be somewhat repudiated, breaking down the concept of multispecies, multisex and perhaps multisite carcinogenesis. The NTP may be evaluating the lung adenomas/carcinomas in both male and female mice, and in female rats, along with a finding of nasal epithelial metaplasia and atrophy, in a weight-of-evidence approach across species and sexes supporting a hypothesis of lung tumorigenicity.

In female rats, only the high dose group displayed a statistically significant increase in adenoma and combined tumors, at a rate (10%) which was very close to historical control values (up to 8%). The significance may well be due to the absence (0 adenomas) in the control group, which may have occurred by chance. There occurred 3/50 adenomas in male rat controls. It is also interesting that, in rats, the control females displayed alveolar epithelial hyperplasia, but no tumors. This may suggest an irritating effect of the beta-picoline in the alveolar area. The multiplicity data is unimpressive, and the assessment is that the group effect is not biologically different from control values.

The finding of a statistically significant increase in alveolar/bronchiolar adenomas in mid-dose male mice (32%), without a dose response and an incidence very near the historical control value (up to 30%), provides a weak argument for biological significance of this finding. Furthermore, there is no mortality associated with the exposure in male mice; in fact, exposed mice had a slightly longer lifespan than did control males. Taken alone, the incidence effect is weak and is not supported by other adverse effects in male mice.

In female mice, the argument is stronger for a statistical effect, based on an incidence of combined adenomas or carcinomas of 42% in mice exposed to the highest dose compared to a concurrent and background historical control value of up to 22%. These data are significant, along with a marginally-significant trend test for a dose response. Coincidently, there was a significant elevated incidence of

alveolar epithelial hyperplasia and metaplasia in the nasal epithelium suggesting a site of contact effect of the test material. However, there was no significant increase in adenomas alone, nor in carcinomas alone, in any of the female exposed groups. This makes a difficult argument for a biological effect, even though the combined tumors showed statistical significance at the high dose.

Before considering the full biological significance of the lung tumors in female mice, it is important to study the other significant finding in female mice, hepatocellular adenomas, carcinomas and blastomas. These tumor incidences were elevated in female mice, and this could impact the increased incidence of lung tumors in females. Hepatocellular tumors could have impacted the nutritional, immunological and hormonal status of these female animals.

Hepatic Tumors in Mice:

In this study, hepatocellular carcinoma was significantly increased in all exposed groups of female mice. Adenomas were significantly increased in female mice given 312.5 mg/L ($p < 0.05$) and 625 mg/L (marginal, $p = 0.052$) but not at the higher dose. The incidence of 92% was higher than the historical and concurrent control values of up to 78%. Multiplicity of tumors (10/50) was increased in the 625 mg/L group, but again comparable to control values at the high dose group. Thus, there was not a true dose-response effect for adenomas.

Hepatoblastoma is a relatively rare tumor in mice, especially in females, and is considered part of the spectrum of hepatic adenomas and carcinomas (Turasov, et.al., 2002). It is interesting that pyridine also displayed an exposure-related increase in hepatoblastoma (NTP TR 470). In the present study, the combined incidences of hepatocellular carcinoma or hepatoblastoma were significantly increased in all exposed females, with a highly significant dose trend test ($p < 0.01$). The observed incidence of 56% was higher than the historical and concurrent control values of up to 46%. There appears to be no published calculation of hepatocellular adenomas and carcinomas (combined, with or without hepatoblastoma) in this report. Furthermore, metastatic hepatocellular carcinomas were found in the lungs of treated female mice (though not statistically significant).

Male mice generally have a higher incidence of hepatic tumors than female mice (Haseman, et.al, 1998, Toh, 1973, Grassio and Hardy, 1975). It has been observed that ovariectomized females have an

increased incidence of spontaneous and initiated liver tumors than intact females (Vesselinovitch, et.al, 1982, Moser, et. al., 1997), thus resembling the incidences seen in males. Steroid hormones are suggested to play a role in the incidence of liver tumors in mice. The evidence suggests that estrogen inhibits mouse liver carcinogenesis, and exposure to anti-estrogenic compounds increases the incidence of liver tumors in females (Moser, et.al., 1997).

In the present study, it is of interest that exposed male mice showed no significant increase in liver tumor incidences, arguing against beta-picoline being a hepatocarcinogen. However, the fact that the incidence of liver tumors in exposed females is higher than that in males is suggestive of an endocrine-linked promotion of liver tumors. As estrogen antagonizes liver tumor development, the putative hypothesis appears to be that beta-picoline is an anti-estrogen which decreases estrogen levels or its effectiveness, similar to other female hepatocarcinogens such as methylene chloride (NTP TR 306), hexachloroethane (NCI TRS 68), and chlordane (NCI TRS 8). These chemicals present a profile of a tumor promotor of already initiated cancer cells. In the current study, the data provided in support of this anti-estrogen hypothesis is the significantly higher probability of extended estrus in the reproductive cycle (Markhov transition matrix analysis) in 312 and 625 mg/L female rats (not 1250 mg/L rats), and a significant increase in the pentoxy resorufin-O-dealkylase (PROD) activity of liver tissue (a measure of CYP2B1 activity), in male rats given 312 mg/L and higher doses, and in females given 156 mg/L and higher doses, in the 3 month study. While these measures were performed in rats or rat tissue, there was no effect of the test material on hepatocellular neoplasms in rats. Measures of this activity were not presented for mice, the species displaying the increased rates of liver cancer. Furthermore, these are isolated measures of potential metabolic effects of the test substance, and they do not present an integrated picture of classic anti-estrogen effects. While not specifically mentioned, there appears to be no data on reproductive organ weights (ovaries and uterus), no analysis of vaginal smears and lavages, no measure of estrogen-receptor binding, no changes in fertility indices, and no data on hepatocyte proliferation or quantitation of altered hepatic foci in an initiated cell model. A full analysis of hepatic Phase 1 liver enzymes should be presented rather than data on a single isozyme (CYP2B1 as PROD), or at a minimum, a survey of all enzymes associated with estrogen metabolism. Increases in the length of the female estrus cycle have also occurred with decreased body weight gain and stress (Koisumi, et.al., 1993, Masoro, 1995, Bitman and Cecil 1967, Finn and Martin, 1973). The suggestion that beta-picoline may be a reproductive toxicant is premature and scientifically unwarranted.

Beta-picoline is not genotoxic, and so is not anticipated to be an initiator of DNA damage. If beta-picoline is inhaled into the lung, its irritancy toward mucous membranes may result in a state of chronic cell sloughing and proliferation of cells in the bronchial or alveolar area. The constant stimulation of cell turnover will allow cells with spontaneously damaged DNA to replicate and result in tumors (Ames and Gold, 1990).

Toxicity and Dehydration Effects:

Poor palatability of beta-picoline resulted in an aversion of animals to drinking, as stated in the Results section of the 14-week study. In the 625 mg/L and 1250 mg/L groups of rats and mice, there occurred decreased water consumption, dehydration, hemoconcentration, and significantly decreased body weight at the end of the study (10% decrease or more). This, along with the finding of nephropathy associated with alpha-2-microglobulin and formation of hyaline droplets, contributed to the decision to abandon the 1250 mg/L dose as too toxic to employ in the two-year bioassay for rats. Some individual animals may be more susceptible to the palatability issue and may have experienced the metabolic effects of dehydration, while taking in a smaller dose of the test article or experiencing a bolus dosing pattern. Conversely, other individual animals may have been more tolerant of the taste/odor, consumed more test material and potentially displayed greater toxicity (local and systemic).

As individual water intake may vary, one study design flaw in the bioassay is allowing water consumption to be measured in groups of 2-3 male rats, and groups of 5 female rats and mice, housed together rather than individually. Group water/test material consumption data fails to identify individual animals which may have consumed more test material and those which consumed less. Statistical analysis of dose may better be a continuous variable based on water consumption, rather than a categorical one.

Decreased water consumption can have metabolic consequences. Decreased water consumption in high-dose rats (male and female), and in female mice after 13 weeks, occurred during the 2-year bioassay. Rats (male and female) demonstrated decreased body weights (10%) compared with controls; male and female mice showed decreased body weights after 57 weeks (males) or 13 weeks (females). Rat clinical chemistry and hematology supports the observation of dehydration. Absolute liver weight in the mid and high dose group of male rats, and relative liver weights in the mid and high dose females

were significantly reduced. Other relative organ weights were reduced. This suggests an "altered nutritional status" at these dose groups, as stated in the NTP report.

The effects of decreased water consumption can be a significant factor in the manifestation of toxicity. Dehydration, as measured by even a 2% loss in body weight, can alter physiologic and functional homeostasis in rats (Hohnegger et al, 1986). Simple decreases in water consumption by laboratory animals, coincident with decreased urine production, do not necessarily qualify as meeting criteria for "dehydration" (Campbell, et al, 2009). The critical factor which qualifies water restriction as dehydration is the emergence of clinically relevant changes in blood and urine parameters (Bobreck, 1973). In studies by the NTP of developmental toxicity of bromochloroacetic acid and tribromoacetic acid in the drinking water, hematology and clinical chemistry parameters were considered as potential indicators of dehydration (NTP, 1998a and b). While most dehydration data are based on short term periods (up to two weeks) of water restriction, the assessment of dehydration in longer term studies, such as 13-week studies, is less well understood.

There is considerable understanding of the behavior of rats concerning consumption of both palatable and unpalatable solutions of fluids (Rolls,et al, 1978 and Scalera, 2000). "Primary" water consumption is intake of the amount required for survival or homeostatic balance, whereas "secondary" consumption is intake of the amount above the minimum required. Secondary water consumption is elective and discretionary. Laboratory rodents are known to preferably drink selected flavored solutions (i.e. sucrose), while shunning other flavors (i.e. quinine). After a period of total water restriction, rats will consume palatable fluids quickly for an initial thirst-quenching period of 10-15 minutes, then steadily until their total normal intake is achieved. When water-restricted rats are presented with unpalatable solutions, they also quickly imbibe for the initial thirst-quenching period. This "override" of taste aversion occurs only for the first 10 minutes upon re-exposure, not in the following 50 minutes after their initial thirst has been satiated (Scalera, 2000). Thus, rats forego consumption of the secondary or discretionary fluid intake and remain less well hydrated than controls consuming unflavored water. It appears that rats do not "notice" the aversive taste when they are driven to drink their "primary" amount; but they later become sensitive to unpalatable flavors by refusing to drink "secondary" amounts of fluid.

The aversive taste or odor, or the consequential dehydration, may have resulted in an added stress in addition to the metabolic stress of the test material. This may impact the development of tumors in the high dose group by stressing the animals, promoting the development of tumors by cortisol-related mechanisms.

Additional Studies which may be Helpful in Assuaging Concern for the Safety of beta-Picoline:

It is advisable to enter into discussions with NTP and regulators to understand interpretations of studies and plans for additional studies and actions. Scientific studies will be needed to elucidate the mode of action involving biological pathways. Duplication of some selected endpoints may be advisable, in bioassay formats. Understanding and characterizing the exposure, and differentiating between oral exposure in drinking water and inhalation of volatile compound derived from drinking water is essential, followed by assessment of the corrosivity of that inhalation exposure.

Conclusion:

The National Toxicology Program has issued a draft report stating that there is evidence that beta-picoline has carcinogenic activity. After evaluating the findings of tumor incidences in this bioassay, each incidence can be analyzed for the relative strength of each species/gender effect, along with the overall integrity of the scientific hypotheses of carcinogenic activity.

The first "call" on carcinogenic activity is that there is "some evidence" in female F344/N rats, based on alveolar/bronchiolar adenomas in the lung in animals exposed to the high dose of 625 mg/L. The call meets the NTP criteria for "some evidence" based on the existence of at least one group effect which is statistically significant. There are valid scientific reasons to challenge this call. The relevance for this call impacting human cancer risk is moderate, as lung cancer in rats occurs relatively rarely and could therefore be chemically-related; however the overall strength of this call is impacted by the lung cancer incidence in mice, which is not highly relevant to human risk assessment.

The second call on carcinogenic activity is "equivocal evidence" in male B6C3F1 mice, based on alveolar/bronchiolar adenomas in the lung in animals exposed to the mid dose of 625 mg/L. The call is based on one group effect which is statistically significant. There are valid scientific reasons to challenge

this call, primarily based on the absence of a dose-response relationship. The relevance for this call impacting human cancer risk is low, as lung cancer in male B6C3F1 mice occurs at a relatively high background rate.

The third call on carcinogenic activity is "clear evidence" in female B6C3F1 mice, based on lung tumors, in animals exposed to beta-picoline at the high dose. The call meets the NTP criteria for "clear evidence" based on the existence of at least one group effect which is statistically significant. There are valid scientific reasons to challenge this call. The NTP has suggested that the substance may have anti-estrogenic activity, which results in tumor incidences resembling those of male mice. The relevance for this call impacting human cancer risk is low, as lung cancer in male mice occurs at a relatively high background rate, but it occurs less frequently in female B6C3F1 mice.

The next call on carcinogenic activity is "clear evidence" in female B6C3F1 mice, based on liver tumors, in animals exposed to beta-picoline at the high dose. The call meets the NTP criteria for "some evidence" based on the existence of at least one group effect which is statistically significant. There are not many scientific reasons to challenge this call, other than the absence of effect in male mice. The relevance for this call impacting human cancer risk is low, as liver cancer in B6C3F1 male and female mice occurs at a relatively high background rate.

The statement that exposure to beta-picoline caused increased incidence of nonneoplastic lesions of the lung in female mice and the nose in male and female mice is difficult to challenge, but the cause and impact of this conclusion can be challenged. The pathology observed may be due to the corrosive nature of the test material, rather than representing preneoplastic lesions which are an early part of the spectrum of cell changes culminating in tumors. Thus, the impact of these lesions is not as an early stage of cell dysfunction, but rather as a physical insult which promotes proliferative responses in spontaneously initiated cells.

The NTP has made the statement that beta-picoline may be a reproductive toxicant. There are many scientific reasons to challenge this call, although it is consistent with and helps explain the findings of significant increases in the rates of tumors in females closer to those in male animals. The relevance for this call impacting human cancer risk is unknown, but may be of high impact for reproductive toxicology.

Bioassay studies by the NTP do not include investigation of the mechanism of action. There is ample scientific evidence of the presence of other factors, such as the potential corrosivity of the test material, two routes of exposure (inhalation and oral) occurring simultaneously, and stressors such as water

restriction and dehydration. An analysis of a "mode of action" framework for this chemical is recommended for submission to the NTP and for product defense, along with additional mechanistic and exposure-based studies in animals.

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APPENDIX 1.

The National Toxicology Program's Definition of Carcinogenic Activity

The National Toxicology Program describes the results of individual experiments on a chemical agent and notes the strength of evidence for conclusions regarding each study. Negative results, in which the study animals do not have a greater incidence of neoplasia than control animals, do not necessarily mean that a chemical is not a carcinogen, inasmuch as the experiments are conducted under a limited set of conditions. Positive results demonstrate that a chemical is carcinogenic for laboratory animals under the conditions of the study and indicate that exposure to the chemical has the potential for hazard to humans. Five categories of evidence of carcinogenic activity are used in the Technical Report series to summarize the strength of the evidence observed in each experiment: two categories for positive results ("Clear Evidence" and "Some Evidence"); one category for uncertain findings ("Equivocal Evidence"); one category for no observable effects ("No Evidence"); and one category for experiments that because of major flaws cannot be evaluated ("Inadequate Study"). These categories of interpretative conclusions were first adopted in June 1983 and then revised in March 1986 for use in the Technical Reports series to incorporate more specifically the concept of actual weight of evidence of carcinogenic activity. For each separate experiment (male rats, female rats, male mice, female mice), one of the following quintet is selected to describe the findings. The categories refer to the strength of the experimental evidence and not to either potency or mechanism.

Clear Evidence of Carcinogenic Activity is demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.

Some Evidence of Carcinogenic Activity is demonstrated by studies that are interpreted as showing a chemical-related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.

Equivocal Evidence of Carcinogenic Activity is demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemically related.

No Evidence of Carcinogenic Activity is demonstrated by studies that are interpreted as showing no chemical-related increases in malignant or benign neoplasms.

Inadequate Study of Carcinogenic Activity is demonstrated by studies that because of major qualitative or quantitative limitations cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity.

(http://ntp.niehs.nih.gov/index.cfm?objectid=07027D0E-E5CB-050E-027371D9CC0AAACF)