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Subject: Pyridate: 2-Year Dietary Carcinogenicity Study in Mice

Test Material: Pyridate Technical (CL-11344)

Accession Number: 072346

Sponsor: Chemie Linz AG, Austria

Testing Facility: Netherlands Organization for Applied Scientific Research

Study Number: B80-0731 Assay No. 277

Testing Period: January 1981 to January 1983

Report Submitted to Sponsor: December 1983

Materials and Methods:

Pyridate technical (CL-11344), a brown viscous liquid used in this study, had a purity of 90.3 percent. All impurities (9.7%) were identified and are shown in the attached (Annex 1). All samples were stored at 5 °C until use.

Male and female weanling mice (cpb: Swiss random) weighing 8 to 13 g, were obtained from the Central Institute for the Breeding of Laboratory Animals, TNO, Zeist, the Netherlands, and used throughout this study. Upon arrival, the animals were examined for good health status and acclimated to the laboratory conditions for 7 days prior to use. Male and female animals were randomly divided into 4 groups (50 animals/sex) and fed diets containing 0, 200, 1000, or 5000 ppm (equivalent to approximately 0, 24, 120, or 600 mg/kg/day) of Pyridate. All animals were identified individually by earmark and housed in macrolon cages, one per cage for males and five per cage for females. The animal cages were kept in a room ventilated with 8 to 10 air changes per hour. The room temperature was maintained at 20 to 24 °C, the relative humidity at 40 to 80 percent and a light/dark cycle of 12 hours was also maintained. Basal diets containing the test article were prepared every 6 to 8 weeks and stored at -20 °C until use. Animals were given fresh portions of the diet every day except for weekends. On Friday, a somewhat greater portion of diet was given to animals to cover the need for the weekend. Drinking water was available ad libitum. The test article concentrations in the diet were determined at irregular intervals (1-6 months). Homogeneity and stability of Pyridate in the diet

were established three times during the study. All animals were checked daily for clinical signs of toxicity or mortality and palpated for masses. Body weights were recorded once a week for the first 14 weeks and every 2 weeks thereafter for the remainder of the study. Food or water consumption was not recorded. Likewise, no hematology, clinical chemistry or urinalysis were conducted in this study. The authors reported that food and water consumption and hematological measurements were carried out only in a separate group of mice (15 animals/sex/dose level) which was treated with the same dose levels of Pyridate as in the present study and represented a satellite group intended for the evaluation of the chronic toxicity of Pyridate in mice (80-week feeding study).

At termination of the study (week 104) all surviving animals were killed by exsanguination and subsequently subjected to detailed gross examination. Animals that died during the study or were killed at a moribund condition were also necropsied. Samples of the following tissues from all animals were preserved in an aqueous, neutral, phosphate-buffered, 4 percent formaldehyde solution:

- aorta
- adrenals
- axillary lymph nodes
- bone (femur)
- brain (medullary, cerebellar and cortical sections)
- caecum
- cervix
- colon
- duodenum
- epididymides
- esophagus
- eyes
- gallbladder
- head
- heart
- ileum
- jejunum
- kidneys
- liver (at least two lobes)
- lungs (all lobes with main stem bronchi)
- mammary glands
- mesenteric lymph nodes
- ovaries
- pancreas
- parotid salivary glands
- pituitary
- prostate
- sciatic nerve
- skeletal muscle
- skin
- spinal cord (at least two levels)
- spleen
- sternum with bone marrow
- stomach (glandular and nonglandular)
- submaxillary salivary glands
- sublingual salivary glands
- testes
- thyroid with parathyroids
- trachea
- urinary bladder
- uterus
- thymus (if present)

All nodules, tissue masses, and otherwise macroscopically abnormal tissues were preserved, along with samples of adjacent tissue where appropriate.

For histopathological examination, samples from all organs and tissues listed above from all animals were embedded in Paraplast, sections were cut at 5 µm and stained with hematoxylin-eosin. The pituitary, adrenals, thyroid, and ovaries were sectioned at three levels. All organs and tissues were examined microscopically for the presence of hyperplastic, preneoplastic, and neoplastic lesions.

The following organs of all surviving animals were weighed:

brain	liver	spleen
heart	lungs	testes
kidneys		

The organ weight to body weight ratios (relative organ weight) were also calculated.

Statistical Analysis: Data on body weights and organ weights were evaluated by one-way analysis of (co-)variance, followed by Dunnett's multiple comparison test. The gross and histopathological findings as well as the data on mortality were analyzed by the Fisher exact probability test. Tumor data of the liver were analyzed statistically using trend and homogeneity analysis of proportions and life table data and according to the method of Peto.

The authors reported the following deviations from the original protocol (abstracted from the original report):

- By mistake the head and bone were not collected from animals that died or were killed in extremis in the course of the study.
- A number of organs were not weighed since they showed a large tumorous lesion.
- Mammary glands were examined histopathologically in females only.
- Not all organs of each animal could be examined microscopically because of:
  - (a) autolysis or cannibalism;
  - (b) loss during autopsy: especially small and/or soft organs such as the axillary lymph nodes, clitoral glands, ovaries, pituitary, thyroid, and adrenals; and
  - (c) loss during fixation or processing.

Results:

Pyridate analytical concentrations in the diet (determined at irregular intervals, ranging from 1 to 6 months - not bimonthly as reported by the authors) showed considerable variation between the various batches of diet tested. On the average, however, concentrations were found to be 16 to 29 percent lower than the target levels of 200, 1000, or 5000 ppm before adjustments for recovery (71-84%) were made. Thus, actual diet concentrations for the low- and mid-dose groups appeared to be slightly lower than the target concentrations when corrected for recovery (80-85%, reported by authors). For the high dose, actual diet concentrations were approximately the same as the intended level. Pyridate appeared to be homogeneously distributed in the diet as shown by the fairly low coefficient of variation of 1.2, 0.9, and 2.1 percent for the low-, mid-, and high-dose groups, respectively. Stability tests indicated that when Pyridate is stored at 23 °C, 0 to 29 percent is lost in 24 hours and 12 to 48 percent in 72 hours. Losses were inversely proportional to the concentrations in diet. Storage of Pyridate (mixed with diet) at -20 °C for 3 months resulted in 0 to 18 percent losses.

Clinical observations did not reveal any significant differences in toxic symptoms between treated and control groups. Similarly, the number of mice with grossly visible masses (observed by palpation of animals throughout the study) was approximately the same in treated and control groups. The total number of grossly visible masses was statistically significantly higher in females of the low-dose group compared to controls and lower in males of the high-dose group.

The mortality incidence was fairly low during the first year of study ranging from 6 to 24 percent in males and 4 to 12 percent in females. Increasing mortality was recorded in all groups between 12 and 24 months of study with no statistically significant difference between treated and control groups in both sexes. The mortality in the male and female high-dose groups was relatively lower than the other groups throughout the study. By the termination of the study, 62 to 74 percent of males and 50 to 68 percent of females died.

Mean body weights of male mice of the high-dose group were statistically significantly lower than controls from day 35 to day 532 and remained lower (numerically) for the remainder of the study. Statistically significantly lower mean body weights were also recorded in the high-dose group females from day 196 to day 574 and remained lower (numerically) than controls for the duration of the study.

The mean absolute or relative organ weights were comparable between treated and control groups in both sexes.

Gross pathology performed on all male and female mice that died spontaneously, were killed at moribund condition or sacrificed at the termination of the study revealed that the incidence of lesions was for the most part comparable between treated and control groups. Statistically significantly higher incidence of lesions was seen with the mid-dose group in female kidneys (granular surface) compared to controls and the mid-dose group in male liver (tumors or suspected tumors), Table 1. Lesions that appeared to be of somewhat higher frequency in treated than control groups are listed in Table 1.

TABLE 1. Summary of Macroscopical Observations

Macroscopical Observations	Males				Females			
	Dose (ppm)				Dose (ppm)			
	0	200	1000	5000	0	200	1000	5000
Axillary Lymph Nodes								
- enlarged	5/40[1,2]	2/37	1/44	0/39	1/41	4/37	1/43	6/46
Kidneys								
- granular surface	2/49	1/49	2/50	2/50	0/49	0/46	5*/50	3/49
Small Intestines								
- tumor or suspected tumor	0/45	0/49	3/46	0/45	2/45	4/41	1/45	4/48
- prominent Peyers patches	0/45	1/49	3/46	0/45	1/45	2/41	1/45	1/48
Mesenteric Lymph Nodes								
- enlarged	1/43	5/42	5/39	0/46	2/43	3/39	3/39	5/44
Ovaries								
- tumor or suspected tumor					0/40	3/32	3/42	2/37
- hemorrhagic					1/40	2/32	5/42	3/37
Liver								
- tumor or suspected tumor	4/49	7/49	14**/50	6/50	4/49	4/44	5/47	0/49
Lungs								
- spotted	4/48	5/47	5/49	7/50	3/49	2/46	7/48	7/49
- discolored (pale hyalin)	4/48	1/47	1/49	3/50	1/49	4/46	3/48	1/49
Pituitary								
- enlarged/swollen	1/31	0/32	1/37	0/31	0/41	0/36	0/39	4/42

[1]Number of mice with specified observations/total number of tissues examined.

[2]The total number of tissues examined was abstracted by the reviewer from the histopathology Tables. The authors did not report the number of tissues examined (for each specified observation) for macroscopical lesions.

*Significantly different from control; Fischer's exact test, *P < 0.05; **P < 0.01.

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Histopathological examinations revealed a variety of preneoplastic and neoplastic lesions in several tissues of male and female mice. In most instances, the incidence of these lesions was comparable between treated and control groups and were presumably due to aging of the animals. Lesions found to be of higher incidence (numerically or statistically) in treated than control groups are listed in Table 2. Statistically significantly higher incidence of foci of cellular alterations (clear cell type) was observed in the pituitary of female mice of the low-dose group compared to control. In the small intestine of female mice the incidence of hyperplastic lesions (in patches of Peyer) in the mid-dose group was statistically significantly higher than controls. In the spleen of male mice the incidence of nodular lymphoid hyperplasia was absent in control mice but present in treated mice (4/47, 3/48, and 4/49 in low-, mid-, and high-dose groups, respectively); in female mice the incidence was high in all treated and untreated groups.

Although none of the neoplastic lesions (Table 2) present in tissues of treated mice were statistically significantly higher than the corresponding controls, the incidence of liver neoplastic nodules (single and multiple) was increasingly higher with higher Pyridate concentrations indicating possibly a dose-response relationship (7/49, 6/49, 11/50, and 14/50 in control, low-, mid-, and high-dose groups, respectively). The incidence of hepatocellular carcinomas was slightly higher in mid- and high-dose groups of male mice. The combined incidence of neoplastic nodules and hepatocellular carcinomas in male mice (9/49, 7/49, 13/50, and 14/50 in control, low-, mid-, and high-dose groups, respectively) also suggests a dose-response trend. In female mice, the incidence of liver neoplastic nodules or hepatocellular carcinomas was very low in both treated and control groups.

The incidence of various tumors in other organs (listed in Table 2), although usually higher in treated than control animals, did not show evidence of a treatment-related trend.

TABLE 2

Summary of Histopathological Observations

Histopathological Observations	Males Dose (ppm) 0	200	1000	5000	Females Dose (ppm) 0	200	1000	5000
Hyperplastic and/or Preneoplastic Lesions								
Mammary Gland: Lobular hyperplasia					4[1]/43	1/45	6/47	1/47
Ovaries: Granulosa-theca cell proliferation					1/40	0/32	0/42	3/37
Pituitary: Focus of Cellular alteration - clear cell type					0/41	5*/36	0/39	2/42
Small Intestine: Hyperplasia of patches of Peyer	0/45	2/49	0/46	0/45	0/45	2/41	6*/45	1/48
Spleen: Nodular lymphoid hyperplasia	0/44	4/47	3/48	4/49	8/48	6/42	7/47	8/48
Thyroid: Focal epithelial proliferation					0/43	0/39	2/38	0/41
Neoplastic Lesions								
Liver: Neoplastic nodules (single + multiple)	7/49	6/49	11/50	14/50	1/49	3/44	1/47	1/49
Hepatocellular carcinoma	2/49	2/49	3/50	4/50	0/49	0/44	1/47	1/49
Total (benign + malignant)	9/49	7/49	13/50	14/50	1/49	3/44	2/47	2/49
Lungs: Alveologenic tumor (multiple)	3/48	5/47	8/49	6/50	8/49	5/46	6/48	3/49
Malignant alveologenic tumor (multiple)	5/48	0/47	1/49	3/50	1/49	3/46	3/48	1/49
Mammary Gland: Adenocarcinoma (multiple)					1/43	6/45	0/47	1/47
Ovaries: Granulosa-theca cell tumor (multiple)					1/40	1/32	1/42	3/37
Pituitary: Hemorrhagic tumor					0/41	0/36	1/39	3/42
Solid tumor					0/41	0/36	1/39	1/42
Spleen: Lymphoma	0/49	1/47	1/48	0/49	0/48	0/42	2/47	2/48

[1]Number of mice with specified lesion/total number of tissues examined.
*Significantly different from control; Fisher's exact test, $p < 0.05$.

Discussion:

The present study has investigated the oncogenic potential of Pyridate in male and female mice. Although the target concentrations for the 3 dose levels used were specified as 200, 1000, and 5000 ppm, due to the unstable nature of Pyridate and the slightly lower actual concentrations measured in the diet (immediately after mixing), it is estimated that animals of the low- and mid-dose groups received 15 to 20 percent lower concentrations than reported. However, for the high dose, actual diet concentrations were about the same as the intended level.

Mortality rates were relatively high in the control, low-, and mid-dose groups and slightly lower in the high-dose groups in both sexes. Overall, the rate of mortality in female animals was approximately 10 percent lower than in males. There is no evidence that Pyridate had any effect on survival of male or female mice after 2 years on study. The high mortality observed was mostly due to aging.

A treatment-related effect, considered to be of some toxicological significance, was the slightly lower (6-13%) mean body weight observed throughout the study in male and female mice of the high-dose groups as compared to controls. [Comparable depression in the growth rate was also observed in the high-dose group of female mice (but not in males) in a chronic toxicity study (15 mice/sex/dose level for 80 weeks) carried out simultaneously with this study under identical conditions and dosage regimens.] Thus, it appears that the high dose tested (5000 ppm) was sufficiently high to approximate the maximum tolerated dose (MTD) in both sexes.

Mean absolute and relative organ weights were comparable between control and treated groups in both sexes and there was no evidence of treatment-related effects. Other parameters such as water and food consumption and hematology measurements (differential blood counts) were not examined in the present study. [Measurement for these parameters conducted with the 80-week chronic toxicity (satellite group) study in mice did not reveal any treatment-related effects.]

Gross pathology data revealed the presence of a variety of lesions in different tissues but there was no evidence of a dose-response relationship for any of the observed lesions.

Histopathological examinations revealed an increased incidence of preneoplastic and/or neoplastic lesions in several tissues of treated mice as shown in Table 2. Statistically significant increase, however, was seen only in pituitary gland (foci of cellular alteration) of female mice of the low-dose group and in small intestine (hyperplasia of patches of Peyer) of female mice of the mid-dose group. With the exception of liver neoplastic lesions, none of the preneoplastic or neoplastic lesions in

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other tissues (whether statistically or numerically higher than controls) showed any evidence of a dose-related trend. Concerning liver lesions the following can be said about the oncogenic potential of Pyridate in mice:

1. There was an increased incidence in liver neoplastic nodules (hepatocellular benign tumors) in male mice of the mid- and high-dose groups (7/49, 6/49, 11/50, and 14/50 in control, low-, mid-, and high-dose groups, respectively). These data show an apparent dose-related trend in the formation of benign liver tumors in male mice. Historical control data submitted by the sponsor upon request (see Appendix "B") indicate that the incidence of neoplastic nodules for liver in eight chronic studies with other SPF (Swiss Random) male mice was 5.9 percent (16/273) with a range of 0 to 9 percent (note that no neoplastic nodules were present in 6 out of 8 studies). The incidence of neoplastic nodules in the control group males of the current study was 14.3 percent (7/49). This relatively higher incidence of neoplastic nodules in the concurrent controls as compared to the historical controls, complicates further the evaluation of data concerning the oncogenic potential of Pyridate in mice.

2. There was a slightly increased incidence in hepatocellular carcinomas in the mid- and high-dose groups of male mice (2/49, 2/49, 3/50, and 4/50 in control, low-, mid-, and high-dose groups, respectively).

3. The combined incidence of hepatocellular benign and malignant tumors in male mice (9/49, 7/49, 13/50, and 14/50 in control, low-, mid-, and high-dose groups, respectively) shows an apparent dose-related trend. This higher incidence of liver tumors in male mice of the mid- and high-dose groups cannot be explained by the slightly better survival of mice of the mid- and high-dose groups as the authors suggest in their report. In male mice the mortality rates were 70, 72, 74, and 62 percent for the control, low-, mid-, and high-dose groups, respectively.

   In spite of the apparent dose-related trend observed in male mice as far as the incidence of hepatocellular adenomas or the combined incidence of the hepatocellular adenomas and carcinomas is concerned, further appropriate statistical tests carried out by the sponsor and upon consultation with the Toxicology Branch Statistician (B. Litt), the tumor response seen was not considered to be a statistically significant trend.

4. No proliferative (hyperplastic) changes were observed in the liver of male or female mice in treated or control

groups. In view of the relatively high incidence of liver tumors in male mice, this finding is considered unusual. It is understood that when the tumor incidence is high, the incidence of hyperplastic changes is also high.

5. There was no substantial increase in the combined incidence of hepatocellular benign and malignant tumors (1/49, 3/44, 2/47, and 2/49 in control, low-, mid-, and high-dose groups, respectively), in female mice.

6. Based on the critical review of the available data, we do not consider Pyridate to be an oncogen in male or female mice at the dose levels tested.

The aforementioned conclusions for the oncogenic potential of Pyridate were also discussed with Dr. Louis Kasza, the Toxicology Branch Pathologist.

To complete the assessment of this study, additional data are requested as follows:

1. The attached analytical data on Pyridate (Annex 1) should be translated into English.

2. The authors should describe the procedure employed in estimating the recovery ("80-85%") of Pyridate in diet preparations.

3. The authors stated in the text of the report (page 8) that "analysis of the diets for the test substance was conducted bimonthly." However, dates appearing in Table 1, page 27 (Apprendix C) of the original report show that in some cases more than 5 months elapsed between analyses (i.e., June 1, 1981 - November 19, 1981; February 11, 1982 - July 23, 1923). If analyses were done we request that the authors supply the Agency with the missing values. If analyses were not done then give justification.

4. Individual histopathology incidence tables for male and female mice should be submitted.

5. Complete Table 12 (page 60) by providing the number of animals examined for lesions for: abdominal cavity, nasal cavity, hematopoietic system, and mediastinal lymph nodes.

6. Complete Table 9 (pages 42-50) by providing the number of animals examined for each specified observation.

7. A number of tissues were lost according to the authors, due to "autolysis or cannibalism, loss during autospy; loss during fixation or processing." Thus fewer types of some tissues were available for macroscopic and microsopic examination. These findings suggest that good quality control and/or good laboratory practices were not practiced. This study is considered to be a major study in support of the registration of this chemical and as such is included in those major studies recommended for data audit.

Conclusions:

The NOEL for Pyridate oncogenicity in male and female mice was greater than 5000 ppm (the highest dose level tested). The NOEL for toxicity (depressed body weights) was 1000 ppm for both sexes and the LEL for toxicity was 5000 ppm (the highest dose tested). The highest dose tested of 5000 ppm was considered to be an MTD.

Classification: Core-Supplementary.

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