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Lack of Tumorigenicity of Aminopyrine Orally Administered to B6C3F$_1$ Mice

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To test the tumorigenic potential of aminopyrine, an antipyretic analgesic, it was administered in drinking water at levels of 0 (control), 0.04 and 0.08% to 50 male and 50 female B6C3F$_1$ mice for 100 weeks, and the mice were subsequently maintained without aminopyrine for a further 4 weeks. The most frequent types of tumor, in both treated and control groups, were hepatocellular tumor in male mice and malignant lymphoma/lymphoid leukemia in female mice. No statistically significant differences were observed in the incidences of these tumors between treated and control groups. The incidences of several other tumors in male and female mice also showed no statistically significant differences between treated and control groups. Therefore, no tumorigenic effect of orally administered aminopyrine in B6C3F$_1$ mice was apparent in the present study.

Key words: Tumorigenicity — Aminopyrine — B6C3F$_1$ mouse

Aminopyrine (4-dimethylaminoantipyrine, AP) is an non-narcotic antipyretic analgesic which belongs to the category of pyrazolone derivatives, together with antipyrine and sulpyrine. Agranulocytosis, occasionally lethal, has been reported to occur in humans and, therefore, AP is scarcely used in the USA and Europe at present. In Japan, the use of AP was also forbidden in 1986, until which time AP had been one of the most widely used analgesics, because of the rarity of reported side effects.

Studies using the *Salmonella typhimurium* microsome test and the hypoxanthine guanine phosphoribosyl transferase (HGPRT) and Na^+/K^+ ATPase system in cultured mammalian cells have shown no demonstrable mutagenicity of AP.[1] However, nitrosamine, a well-known carcinogen, may be formed *in vivo* from nitrite and amine in an appropriate environment such as the acid condition in the stomach.[2] Many drugs were tested in combination with nitrite by oral application in order to investigate possible tumorigenic activity of their nitrosation products formed *in vivo*. AP is a well-known example having a proven capability to form nitrosamines with nitrite *in vivo*.[3]

In 1975, Taylor and Lijinsky demonstrated tumor induction in rats by combined administration of AP with nitrite,[4] and there were also reports on tumor induction in rats and Syrian hamsters by combined administration of AP and nitrite.[5, 6] However, no tumorigenicity of AP was noted when it was administered by itself in control animals.

So far, there has been no report on experimental studies of administration of AP alone. However, AP is generally used uncombined with nitrite in humans, and so it is necessary to examine the tumorigenicity of AP alone for evaluation of the tumorigenic risk to humans.

The present study was, therefore, conducted to evaluate the tumorigenicity and toxicity of AP alone in mice by oral administration, the route of AP intake in humans.

MATERIALS AND METHODS

Subacute toxicity study Seventy male and 68 female 4-week-old B6C3F$_1$ mice were purchased from Charles River Japan Inc. (Atsugi) and observed during a 4-week quarantine period. The mice were divided into five treatment groups, each consisting of 10 males and 10 females and one control group consisting of 20 male and 18 female mice. Mice in the treated groups were given *ad lib.* for 10 consecutive weeks, AP (Aldrich Chemical Company Inc., Milwaukee, Wis.) in drinking water at levels of 0.04, 0.08, 0.15, 0.3 and 0.6%. AP used in this study had a purity of 98% and the fresh solution used as drinking water was made from distilled water once every 3 days. Mice in the control group were given distilled water. All mice, both treated and control, had access *ad lib.* to a basal diet (CRF-1, Charles River Japan Inc.). Ten mice of the same sex were housed in a plastic cage, and all cages were kept in the same air-conditioned room. The room temperature and humidity ranged from 23°C to 26°C and from 60% to 70%, respectively. At the end of the 10-week treatment period, all surviving mice were anesthetized with ether and autopsied. Each mouse was weighed once a week throughout the treatment period. Amounts of drinking water consumed per cage over 3 consecutive days were measured at the 9th week.

The following organs of each mouse were weighed and the organ to body-weight ratios were calculated for brain, heart, lungs, liver, kidneys, spleen and thymus. Microscopic examination was performed of the following

organs and tissues: heart, trachea, lungs, salivary gland, esophagus, stomach, small and large intestines, liver, gallbladder, pancreas, kidneys, urinary bladder, testes, epididymis, seminal vesicles (ovaries, oviducts, uterus, vagina in females), pituitary gland, thyroid gland, adrenal glands, thymus, spleen, lymph nodes and bone marrows (vertebral, sternal and femoral), brain, spinal cord and skin.

Tumorigenicity study $B6C3F_1$ mice (150 male and 150 female, 4-week-old) were also purchased from Charles River Japan Inc. and after the 4-week quarantine period the mice were divided into three groups, each consisting of 50 males and 50 females. Mice in the first two groups were given *ad lib.*, for consecutive 100 weeks, AP (Aldrich Chemical Company Inc.) in drinking water at levels of 0.04 and 0.08%. A dose of 0.08% was determined to be the maximum tolerated dose (MTD) based on the results of the subacute toxicity study described above. The fresh solution used as drinking water was made from distilled water once every 3 days. Mice in the third group (a control group) were given distilled water *ad lib.* during the same period. All mice had access *ad lib.* to a basal diet (CRF-1). Ten mice of the same sex were housed in a plastic cage and all cages were kept in the same air-conditioned room. After the 100-week treatment period, all surviving mice were maintained without AP in drinking water for a further 4 weeks.

Amounts of drinking water consumed per cage over 3 consecutive days were measured weekly for the first 10 weeks, and then once every 4 weeks until the 100th week. Each mouse was weighed once a week for the first 10 weeks and once every 4 weeks until the 104th week.

Any mouse found dead or moribund during the experiment was autopsied. Mice (79 males and 102 females) which survived until the end of the 104-week experimental period were anesthetized with ether and autopsied. Weighing and pathological examination of organs and tissues were performed in the same manner as in the subacute toxicity study.

Statistical assessment of differences in tumor incidence was performed by the chi-square test and that of differences in mean values for survival time and body or organ weights was by the Student's *t* test.

RESULTS

Subacute toxicity study The average values of AP intake per mouse at the 9th week, obtained by calculation from amounts of drinking water were 18.6 mg (0.6% group), 23.4 mg (0.3% group), 10.7 mg (0.15% group), 4.3 mg (0.08% group) and 2.5 mg (0.04% group) for male mice, and 17.4 mg (0.6% group), 9.3 mg (0.3% group), 5.4 mg (0.15% group), 3.2 mg (0.08% group) and 1.3 mg (0.04% group) for female mice. The male mice of the highest dose group drank a rather small amount of water, and the intake of AP was less than the value expected. No mice in any treated or control group died during the experimental period. The average weekly gain in body weight of male and female mice given 0.15, 0.3 or 0.6% AP was less than 90% of the corresponding control. Based on these results, the MTD of AP in drinking water was estimated to be 0.08% for mice for both sexes.

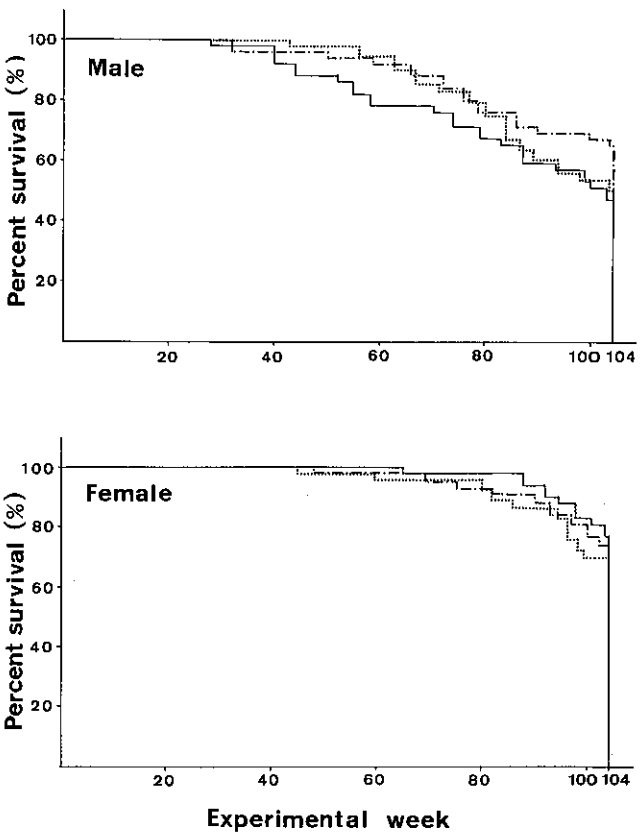

Fig. 1. Percent survivals of mice of the 0% group (——), 0.04% group (·····) and 0.08% group (—•—).

At autopsy the average body weight for male mice given 0.15, 0.3 or 0.6% AP and female mice given 0.6% AP was lower than that for the control mice, but the average organ weight for mice given AP was not significantly different from that of mice in the control group. Microscopic examination showed no morphologically significant evidence of toxicity of AP in the mice given AP.

Tumorigenicity study Percentages of animals which survived are shown in Fig. 1. Excluded from survival analysis were 4 male and 13 female mice, which died accidentally during the early experimental period. Survival of male mice given AP was better than that of the

control mice, while female mice given AP showed worse survival than the control mice. At the end of the experiment (104th week), the percent of surviving male mice was 65% for high dose (0.08%), 50% for low dose (0.04%) and 47% for control. For female mice, the survival rate was 74% for high dose (0.08%), 70% for low dose (0.04%), and 77% for control.

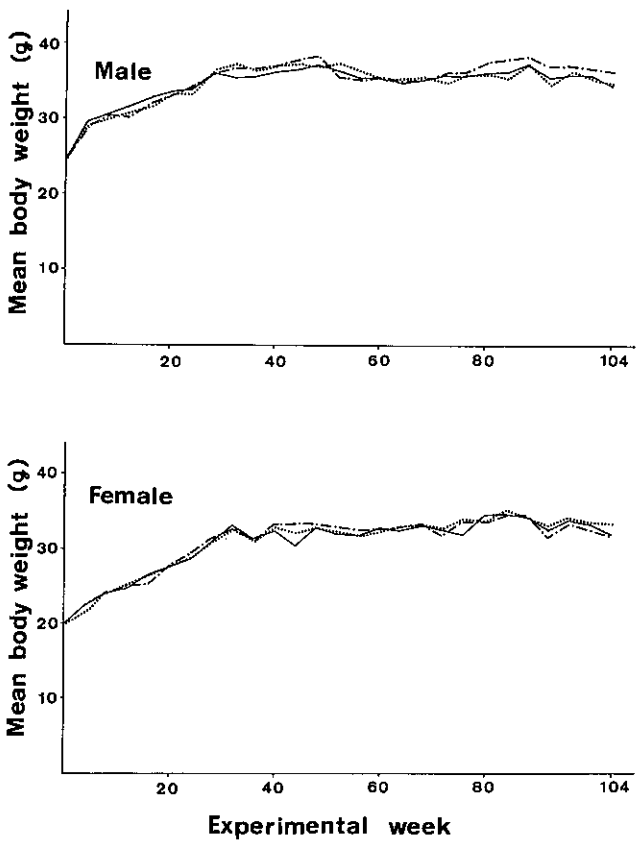

Fig. 2. Growth curves of mice of the 0% group (——), 0.04% group (·····) and 0.08% group (—•—).

The average body weight of mice given AP remained fairly stable throughout the treatment period and was not different from that of the control mice (Fig. 2).

The average values of daily intake (per mouse) of drinking water and AP are shown in Table I. The daily intake of drinking water by male mice given AP was slightly less than that for the control male mice, but the difference between the male mice given high-dose AP and those given low-dose AP was not statistically significant. The daily intake of drinking water by female mice given AP was not different from that for the control female mice. On calculation, the daily intake of AP was estimated to be 5.1 mg by male mice and 3.5 mg by female mice at the high dose (0.08%), and 2.7 mg by male mice and 1.8 mg by female mice at the low dose (0.04%). The ratio of daily intake of AP adjusted for body weight at the high dose (0.08%) level compared to that at the low dose (0.04%) level was 1.9:1 for both sexes.

Mice were regarded as "effective" for data analysis if they survived beyond the 28th week, the time at wich the first death from malignant lymphoma occurred among the male mice. As shown in Table II, there was no significant difference in the percentage of "effective" mice between mice given AP and control mice for both sexes.

Numbers of mice with tumors and the survival time for mice with tumors are also shown in Table II. The incidence of tumors in male mice given AP at the high dose level was significantly increased ($P<0.01$) compared with the control mice, but the survival time for tumor-bearing mice with tumors was not statistically significantly different between the two groups. Among female mice, the incidence of tumor and the survival time of tumor-bearing mice were not statistically significantly different between the mice given AP and the control mice.

The incidences of various histological types of tumors are shown in Table III. In male mice, the tumor with the highest frequency was hepatocellular tumor, including adenoma and carcinoma. Hepatocellular lesions seen in the present study were classified as described by Frith and Ward.[7] A dose-response relationship was observed

Table I. Average Daily Intake of Water and AP per Mouse

Sex	Group	Average water intake ml/day	Average AP intake mg/day (mg/kg body wt./day)
Male	0%	7.7	0
	0.04%	6.8	2.7 (76)
	0.08%	6.4	5.1 (140)
Female	0%	4.5	0
	0.04%	4.5	1.8 (56)
	0.08%	4.4	3.5 (110)

for the incidence of hepatocellular tumor in male mice when the high-dose and low-dose groups were compared. However, there was no significant difference when the incidence of hepatocellular tumor in the male treated groups was compared to that in the control group. When time-adjusted analysis as described by Peto *et al.*[8] was performed on incidence data for hepatocellular tumor in male mice, there was no positive trend of dose-related tumorigenicity for hepatocellular tumors.

Hemangiomas or angiosarcomas were found in the liver of male mice given AP, although the incidence was lower than 10%. Hemangiomas were also seen in the heart of male mice given AP at the high dose level. However, there seemed to be no significant evidence of vascular tumorigenesis by AP. Other tumors which showed an incidence of higher than 10% in male mice given AP were malignant fibrous histiocytoma of the integumentary tissue and adenoma or adenocarcinoma of the lung, but the incidence was not statistically significantly different from that of the control mice for each of these tumors.

In female mice, the incidence of malignant lymphoma/lymphoid leukemia was the highest in control mice and a dose-related decrease appeared to be present in treated mice. However, there was no statistically significant difference in the incidence, and therefore, it was suspected that AP had no suppressive effect on the occurrence of malignant lymphoma/lymphoid leukemia. Tumors of the uterus showed a dose-related increase, but the histological types of the uterine tumors were varied and no significant evidence of tumorigenesis of AP in the uterus was apparent.

DISCUSSION

In the present experiment, the highest tumor incidences were those of hepatocellular tumor in male mice and of malignant lymphoma/lymphoid leukemia in female mice. The reported incidence of hepatocellular tumor in non-treated $B6C3F_1$ male mice was 21.6%,[9] 31.3%[10] or 27.8%,[11] and in previous experiments performed in our laboratory the incidence was 32%[12] or 38%.[13] Therefore, the incidences of hepatocellular tumor in male treated mice and control mice were considered to be comparable to those found in previous studies. Hemangioma or angiosarcoma of the liver was noted only in the treated male mice, but the incidence was so low that no significant vascular tumorigenic effect of AP was considered to exist.

The incidence of malignant lymphoma/lymphoid leukemia in non-treated female $B6C3F_1$ mice has been reported to be 16.8%,[9] 27.2%[10] or 21.4%.[11] Results

Table II. Number of Effective Mice and Mice with Tumors

Sex	Group	No. of effective mice[a] with average survival time (wk)[b] and final body weight (g)[b]	No. of mice with tumor, incidence of tumor (%) and average survival time (wk)
Male	0%	46 87±23 33.8±2.1	22 (48) 89±20
	0.04%	44 91±18 34.5±2.0	19 (43) 97±12
	0.08%	48 93±19 38.7±4.6	36 (75)[c] 93±18
Female	0%	45 102±6 32.0±2.1	22 (48) 101±8
	0.04%	41 101±10 31.8±1.9	21 (51) 101±5
	0.08%	42 100±11 31.4±1.8	20 (48) 96±14

a) Mice that survived beyond the 28th week.
b) Values are average±standard deviation.
c) Significantly different from the 0% group ($P<0.01$) by the chi-square test.

Table III. Incidence of Mice with Specific Histological Types of Tumors

Site	Type of tumor	No. of tumor-bearing mice and incidence of tumor (%)					
		Males			Females		
		0%	0.04%	0.08%	0%	0.04%	0.08%
Liver	Hepatocellular adenoma	10 (22)	6 (14)	16 (33)	1 (2)		2 (5)
	Hepatocellular carcinoma	6 (13)	4 (9)	7 (15)	1 (2)		
	Hemangioma		1 (2)	4 (8)	1 (2)		1 (2)
	Angiosarcoma			1 (2)			2 (5)
Hematopoietic system	Malignant lymphoma	5 (11)	4 (9)	6 (13)	12 (27)	9 (22)	6 (14)
	Myelogenous leukemia		2 (5)	1 (2)			
	Myeloma				1 (2)		
Integumentary system	Fibroma					1 (2)	1 (2)
	Malignant fibrous histiocytoma	3 (7)	6 (14)	2 (4)	1 (2)		
	Round cell sarcoma			1 (2)			
Uterus	Adenocarcinoma						1 (2)
	Leiomyoma					1 (2)	1 (2)
	Leiomyosarcoma					1 (2)	
	Stromal sarcoma				1 (2)	2 (5)	2 (5)
	Hemangioma						1 (2)
Lung	Alveolar/bronchiolar adenoma	1 (2)		5 (10)	2 (4)	2 (5)	1 (2)
	Alveolar/bronchiolar carcinoma	1 (2)	1 (2)	1 (2)			1 (2)
Heart	Hemangioma			2 (4)			
Stomach	Adenoma				1 (2)		
	Squamous papilloma					2 (5)	2 (5)
Small intestine	Adenoma				1 (2)		
	Adenocarcinoma	1 (2)		1 (2)			
Pituitary gland	Chromophobe adenoma				1 (2)		
Thyroid gland	Papillary carcinoma				1 (2)		
Adrenal gland	Cortical adenoma	1 (2)					
	Pheochromocytoma			1 (2)			
Ovary	Cystadenoma						1 (2)
Breast	Fibroadenoma					1 (2)	
	Carcinoma					1 (2)	
Spleen	Hemangioma				2 (4)		
Spinal cord	Glioma					1 (2)	
Skin	Squamous cell carcinoma			1 (2)			
	Angiosarcoma	1 (2)					

from our laboratory indicated the incidence to be 16%[12] or 46%.[13] Therefore, the incidence obtained in the present study appeared to be within the highly variable range estimated from other studies. On the basis of the results in the present study neither an inductive nor a suppressive effect of AP was suspected in female mice.

There have been several studies in the literature on the tumorigenicity of combined oral administration of AP and sodium nitrite (SN). In the early report by Taylor and Lijinsky,[4] 97% (29/30) of Sprague-Dawley rats of both sexes given 0.1% (1000 ppm) of AP and SN for 30 weeks died with hemangioendothelial sarcomas of the liver, compared with 87% (26/30) of animals given 0.025% (250 ppm) of AP and SN for 50 weeks. None of the animals given 0.1% AP alone for 30 weeks showed sarcoma in the liver. Scheunig *et al.*[5] demonstrated that the administration of 0.56% AP and nitrite by gastric intubation to Wistar rats of both sexes for 29 months induced different types of liver tumors in 96% of females and 97% of males; these percentages were significantly higher than those for control rats. Bergman and Wahlin[6] reported that 60% of Syrian hamsters, administered 0.1% AP and SN in drinking water for 20 weeks, developed cholangiocarcinoma in the liver, whereas control animals, administered AP or SN alone, showed no liver tumor.

When AP and SN were administered by gastric intubation to pregnant hamsters on the 11 or 12th gestational

day, increased numbers of colonies of mutated embryonic cells were observed and cells from the transformed colonies produced tumors when implanted into cheek pouches of young golden hamsters. Treatment of pregnant animals with 25–50 mg/kg each of AP and SN induced gene mutation and morphological transformation of cells from their embryos.[14]

Lijinsky and Greenblatt[15] showed the hepatotoxicity of 1- to 3-day oral administration of AP in combination with nitrite. Hepatic necrosis was also observed. Rusu *et al.*[16] reported that Wistar male rats given 10 mg of AP and SN daily for 20 days showed centrilobular necrosis. From these observations it would appear that the administration of AP and SN can induce hepatic necrosis as an acute toxic effect and tumorigenesis as a late effect. It is suspected that necrosis is induced by nitrosamines formed *in vivo*. Our subacute toxicity study provided no morphological evidence of hepatic necrosis and, therefore, the doses of AP used in the present experiment might not have been sufficient to form nitrosamines *in vivo*.

Gombar *et al.*[17] estimated the amount of dimethylnitrosamine (DMN) formed *in vivo* by administration of AP and nitrite to male Wistar rats. The administration of [^{14}C]AP at a dose of 140 mg/kg and SN at 80 mg/kg resulted in a formation of DMN at a dose of 4.6–6.7 mg/kg in urine and 5.4–10.6 mg/kg in liver, while no DMN was detected when AP at the same dose alone was administered. Nitrites are known to be present in cured meats and fishes and they are consistently found in human saliva at levels of usually 6–10 ppm.[18] However, the results reported by Gombar *et al.*[17] indicated that the amount of nitrites present in nature appears to be insufficient to form nitrosamine.

In the present study the maximum daily intake of AP by male mice was 140 mg/kg, a level which was unexpectedly similar to that in the experiment of Gombar *et al.*[17] Accordingly it may be suspected that orally administered AP in the present study did not form a detectable amount of nitrosamine *in vivo*. On the basis of these results, it appears that the usual level of human intake of AP may not pose a substantial tumorigenic hazard.

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