

Aspartame Administered in Feed, Beginning Prenatally Through Life Span, Induces Cancers of the Liver and Lung in Male Swiss Mice

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Background Aspartame (APM) is a well-known intense artificial sweetener used in more than 6,000 products. Among the major users of aspartame are children and women of childbearing age. In previous lifespan experiments conducted on Sprague–Dawley rats we have shown that APM is a carcinogenic agent in multiple sites and that its effects are increased when exposure starts from prenatal life.

Objective The aim of this study is to evaluate the potential of APM to induce carcinogenic effects in mice.

Methods Six groups of 62–122 male and female Swiss mice were treated with APM in feed at doses of 32,000, 16,000, 8,000, 2,000, or 0 ppm from prenatal life (12 days of gestation) until death. At death each animal underwent complete necropsy and all tissues and organs of all animals in the experiment were microscopically examined.

Results APM in our experimental conditions induces in males a significant dose-related increased incidence of hepatocellular carcinomas ($P < 0.01$), and a significant increase at the dose levels of 32,000 ppm ($P < 0.01$) and 16,000 ppm ($P < 0.05$). Moreover, the results show a significant dose-related increased incidence of alveolar/bronchiolar carcinomas in males ($P < 0.05$), and a significant increase at 32,000 ppm ($P < 0.05$).

Conclusions The results of the present study confirm that APM is a carcinogenic agent in multiple sites in rodents, and that this effect is induced in two species, rats (males and females) and mice (males). No carcinogenic effects were observed in female mice. Am. J. Ind. Med. © 2010 Wiley-Liss, Inc.

KEY WORDS: artificial sweeteners; aspartame; carcinogenicity; cancers; hepato-carcinomas; lung adenocarcinomas; prenatal exposure; Swiss mice; rats

INTRODUCTION

Aspartame (APM) was discovered by chance in 1965 by a chemist at G.D. Searle in the US, who was working on a new

drug to treat gastric ulcers [Mazur, 1984]. Nowadays, after saccharine, APM is the most used artificial sweetener in the world [Hazardous Substances Data Bank, 2005]. Each year over 34,000,000 pounds of APM are produced [<http://www.foodnavigator.com/>] and used in more than 6,000 products including over 500 pharmaceuticals. Hundreds of millions of people consume APM worldwide; for children and women of childbearing age, who are among the major consumers, the daily intake of APM is estimated to be 2.5–5 mg/kg b.w. [Butchko et al., 2002].

APM was approved by the U.S. Food and Drug Administration (FDA) in 1981 for dry food [FDA, 1981], in 1983 for soft drinks [FDA, 1983], and in 1996 for all foods [FDA, 1996]. In 1994 it was approved for use throughout the European Union [EFSA, 2006].

Abbreviations: APM, aspartame; CMCRC/RI, Cesare Maltoni Cancer Research Center/Ramazzini Institute; EFSA, European Food Safety Authority; EU, European Union; FDA, Food and Drug Administration.

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APM is metabolized in the gastrointestinal tract by esterases and peptidases into three components: the amino acids phenylalanine and aspartic acid, and methanol [Ranney et al., 1976]. APM can be also absorbed into the mucosal cells prior to hydrolysis and then metabolized within the cell to its three components which then enter circulation [Matthews, 1984]. Methanol is not subject to metabolism within the enterocyte and rapidly enters the portal circulation and is oxidized in the liver to formaldehyde, an highly reactive chemical which strongly binds to proteins [Haschemeyer and Haschemeyer, 1973] and nucleic acids [Metzler, 1977] forming formaldehyde adducts. In a study, in which APM, ^{14}C -labeled in the methanol carbon, was given orally to adult male Wistar rats for 10 days, it was shown that the carbon adducts of protein and DNA could have been generated only from formaldehyde derived from APM methanol. Moreover, it was suggested that the amount of formaldehyde adducts may be cumulative [Trocho et al., 1998]. Several reviews conclude that APM is digested in all species in the same way [Ranney et al., 1976]. Since APM is metabolized before entering the blood stream, there is no distribution of APM outside the gastrointestinal tract. Epidemiological studies conducted among users of artificial sweeteners (including APM) did not show an increased carcinogenic risk, except in one study which postulated an association of increased risk of brain cancer and use of APM [Olney et al., 1996].

Studies performed by the US National Toxicology Program (NTP) in which groups of 15 males and 15 females of transgenic mice, p53 haploinsufficient strain (p53) and Tg.AC homozygous strain (Tg.AC) dermal exposure model were treated with diets containing 0, 3, 125, 6,250, 12,500, 25,000, or 50,000 ppm of APM for 40 weeks and then sacrificed did not show any carcinogenic responses [NTP, 2005]. Overall there was no evidence of a positive response for tumors in animals treated with APM in feed up to 50,000 ppm.

Although the studies did not show carcinogenic response, it should be noted that altered genetic mice were evaluated by NTP with the intent to develop faster, less costly and more predictive *in vivo* models for identifying potential chemical carcinogenic agents and that APM was selected as a presumed non-carcinogen. Pritchard et al. [2003] evaluated the NTP findings regarding the potential of transgenic mouse models to identify carcinogenic agents. The authors concluded that the Tg.AC dermal exposure model and p53 oral exposure model had an overall accuracy of 74% in correctly predicting chemicals that are listed by the International Agency for Research on Cancer (IARC) and/or NTP in their respective lists of chemicals classified carcinogenic or probably carcinogenic in humans. The study concluded that the transgenic mouse models missed a number of known or probable human carcinogens, whereas long-term rodent bioassays missed none of these chemicals.

Indeed, the authors of the studies performed by NTP concluded that the negative findings were of uncertain value: “because this is a new model, there is uncertainty whether the (aspartame) study possessed sufficient sensitivity to detect a carcinogenic effect” [NTP, 2005]. In fact the P53 deficient transgenic model does not respond to non-genotoxic carcinogenic chemicals, and hence choosing that model confirmed this fact with APM. The NTP has since virtually discontinued the use of genetically modified models for identifying carcinogens.

Long-term carcinogenicity bioassays performed on rats and mice in the early 1970s by industry did not show any carcinogenic effects. In female p53 haploinsufficient mice, the results of the micronucleus test were judged to be positive, based on a significant trend test and a small but statistically significant increased frequency of micronucleated erythrocytes in the 50,000 ppm group ($P=0.028$) [NTP, 2005]. A detailed review and comments on the genotoxicity, long-term carcinogenicity studies in rodents and epidemiological studies available today on APM has been reported previously [Soffritti et al., 2005, 2006, 2007]. Overall, we believe that the potential long-term toxic effects of APM, and in particular the carcinogenic effects, had not been adequately demonstrated by the long-term bioassays on rats and mice, mainly because of the small number of animals used per sex per group and the duration of the experiments (in which rodents were sacrificed at 110 weeks of age, corresponding to the two-thirds of the lifespan).

For these reasons we started a project encompassing several experiments on rats and mice in which APM was administered in feed at various doses to a large number of rats or mice per group per sex. Treatment started at different ages and lasted for different periods; rodents were always kept under observation until natural death to allow APM to express all its full carcinogenic potential.

In the first experiment we demonstrated that APM, administered from 8 weeks of age for the lifespan to Sprague–Dawley rats, induced a significantly increased incidence of lymphomas/leukemias and of neoplastic lesions of the renal pelvis and ureter in females, and a significantly increased incidence of malignant Schwannomas of the peripheral nerves in males [Soffritti et al., 2006]. In a second experiment we showed that APM, administered from fetal life until natural death, caused lymphomas/leukemias in male and female rats and, for the first time, cancers of the mammary glands in females [Soffritti et al., 2007]. Furthermore, this study demonstrated that when lifespan exposure starts during fetal life, the incidences of lymphomas/leukemias were increased in comparison to the treatment starting postnatally. Neither cranial Schwannomas nor neoplasms of the renal pelvis and ureter were observed in the second experiment. This result may be explained by the fact that the number of rats per sex per group in this study was

lower and therefore the sensitivity of the study for this type of tumors may have been reduced.

MATERIALS AND METHODS

The APM used for this experiment was supplied by the Azienda Chimica e Farmaceutica (A.C.E.F.) S.p.A. (Fiorenzuola D'Adda, PC, Italy). The certified grade of purity was 98.7% and the impurities were as follows: diketopiperazine 0.2%; L-phenylalanine 0.1%. APM was pulverized in a standard pelleted diet at concentrations of 0, 2,000, 8,000, 16,000, or 32,000 to simulate an assumed daily APM intake of 0, 250, 1,000, 2,000, and 4,000 mg/kg b.w., and was administered to groups of 62–122 male and female Swiss mice from the 12th day of fetal life until death. The dose levels of APM were chosen on the basis of available data reported in the literature. The standard “Corticella diet” was provided by Laboratorio Dottori Piccioni, Milan, Italy; the same diet used for more than 30 years at the laboratory of the Cesare Maltoni Cancer Research Center (CMCRC). Fresh tap water was provided daily. The major constituents of the diet were: water 12%; raw protein 24%; raw fat 3.50%; raw fibers 5.50%; ashes 10.50%; non-nitrogenous extracts 56.50%. The diet was analyzed for nutritional components, microorganisms, and possible contaminants (pesticides, metals, estrogen activity, nitrosamines, and aflatoxins) every 6 months, and disposed of if older than 3 months from the date of manufacture. The diet was formulated every 40–50 days. At room temperature APM is stable in food and liquid. The stability of APM in the feed was analyzed periodically during the experiment. Feed and water were supplied *ad libitum*.

Specific pathogen-free Swiss mice used to generate the experimental animals were provided by the Charles River Laboratories (Milan, Italy). After a period of quarantine of 40 days the breeders were randomly distributed by weight into three groups of 40 and two groups of 60, encompassing the same number of males and females (240 overall). Due to the external origin of the animals, the matching is considered out-bred. At 13 weeks of age, one male and one female breeder were placed in each cage for 5 days after which males were removed from the cages. The dietary exposures of the experimental animals started on day 12 of fetal life with the administration of APM in feed to female breeders. The 12th day was selected to begin exposure because at that time organogenesis is completed however organs and tissues still remain highly susceptible to the effects of toxic and carcinogenic agents [IARC, 1973].

All male and female pups of each litter were used in the experiment to reach the programmed number per sex per group. This results in an unequal distribution of animals among groups and “odd” number per group. The pups were weaned at 4–5 weeks of age in order to facilitate the distinction between males and females, then identified by ear

punch and assigned to the respective dose groups as determined by the dietary concentrations of APM administered to the breeder. After weaning the breeders were removed and APM treatment of the offspring continued until death. The animals of the control group received feed without APM.

The offspring were housed 10 per cage in polycarbonate cages (41 cm × 25 cm × 15 cm) with stainless still wire tops and a shallow layer of white wood shavings as bedding. Cages were changed once a month. All cages were housed in the same room, with a temperature of $23 \pm 2^\circ\text{C}$ and relative humidity of 50–60%. All offspring were observed until 130 weeks when remaining mice were killed and necropsied. The experiment was conducted in accordance with Italian law regulating the use and humane treatment of animals for scientific purposes [Decreto Legislativo 116, 1992].

Drinking water and feed consumption by cage were measured weekly for 13 weeks, and every 2 weeks thereafter, from 6 weeks of age until 110 weeks of age. Body weights of each animal were measured weekly for 13 weeks and after every 2 weeks until 110 weeks of age and after that, every 8 weeks until the end of the experiment. Clinical controls of the animals to monitor and record healthy signs and symptoms and gross pathological lesions were performed concurrently with the measurement of the body weight from 6 to 110 weeks of age and, after that, every 2 weeks until the end of the experiment. To monitor the behavior and the health of the animals and to avoid as much as possible postmortem modifications, a patrol was performed three times daily from Monday to Friday, and twice on Saturday, Sunday, and holidays. During the health control, unhealthy or moribund animals were isolated from the others to avoid cannibalization. Moribund animals were sacrificed to minimize autolysis. Deceased animals were kept in refrigerator at a temperature of 4°C and the necropsies were performed no later than 16–19 hr following detection.

At 130 weeks of age, since <10% of the mice were still alive (overall 67) and the animals were equally distributed per groups and sex, a final sacrifice was planned. The sacrifices were performed in 10 days and the animals were equally distributed per group and sex each day. Remaining animals were euthanized with CO_2 inhalation. Both femurs and a portion of liver, and of each neoplastic lesion, were frozen in liquid nitrogen and stored at -70°C for subsequent biomolecular analysis.

During necropsy, all tissues and organs were examined to detect all visible alterations. The following organs and tissues were collected: skin and subcutaneous tissue, mammary gland (four sites: axillary and inguinal, right and left), brain (three sagittal sections), pituitary gland, Zymbal glands, salivary glands, Harderian glands, cranium (five sections, encompassing oral and nasal cavity, external and internal ear ducts), tongue, thyroid and parathyroid, pharynx, larynx, thymus and mediastinal lymph nodes, trachea, lung

and mainstream bronchi, heart, diaphragm, liver, colecystis, spleen, pancreas, kidneys, adrenal glands, esophagus, stomach (fore and glandular), intestine (four levels), urinary bladder, prostate, vagina, other organs, and tissues with pathological changes. All organs and tissues were preserved in alcohol 70%, apart from bone tissues which were preserved in 10% formalin. Neoplastic lesions of >1 cm observed during necropsy were divided into two parts, one fixed in alcohol 70% and the other frozen in liquid nitrogen and stored at -70°C for subsequent biomolecular analysis.

All lesions were trimmed including a portion of normal adjacent tissue. The tissues were embedded in paraffin blocks and sections of $3\text{--}6\text{ }\mu\text{m}$ were cut for each specimen and stained with hematoxylin and eosin. All slides were evaluated by a junior pathologist (at least 4 years of experience) and all tumors and lesions of oncologic interest were reviewed by two senior pathologists (20 years of experience). The statistical analyses of the malignant neoplastic lesions and of survival were based on the Cox proportional hazard model which adjusts for possible differential survival among the experimental groups.

RESULTS

Treatment with APM did not affect the breeding of the offspring. The percentages of pregnant females among the various groups were as in the range of 90–95%, apart from the group treated at 8,000 ppm of APM in which only 80% of the females were pregnant. All pregnant females of each

group delivered normally. The average number of pups per litter was 12–13 among all groups and the mean of body weights of each pup measured 1 week after delivery were uniform among treated and control mice (4.1–4.8 g).

The first week after weaning no differences were observed in the individual consumption of feed and water, or in body weights, among treated and control animals. The feed consumption measured until 110 weeks of age did not show any substantial differences among the treated groups compared to the controls (Fig. 1A,B). Dietary concentration of 2,000, 8,000, 16,000, 32,000 ppm delivered average daily doses of approximately 247, 987, 1,919, 3,909 mg/kg b.w. to males and females. No differences were observed in body weight among the treated groups compared to the control group (Fig. 1C,D). There were no clinical findings related to APM exposure. Applying the Cox proportional hazard analysis to the survival data, there were no statistically significant differences in survival for any of the exposed groups of mice of either sex compared with their controls. Furthermore, there was no indication of a dose-related trend for survival (Fig. 1E,F). The oncologic results are reported in Tables I–IV. Multiple tumors of different type and site, of different type in the same site, of the same type in bilateral organs, of the same type in the skin, in the subcutaneous tissue, in mammary glands, or at distant sites of diffuse tissue (i.e., bones and skeletal muscle) were counted as single/independent tumors. Multiple tumors of the same type in the same tissue and organ, apart those listed above, were counted only once.

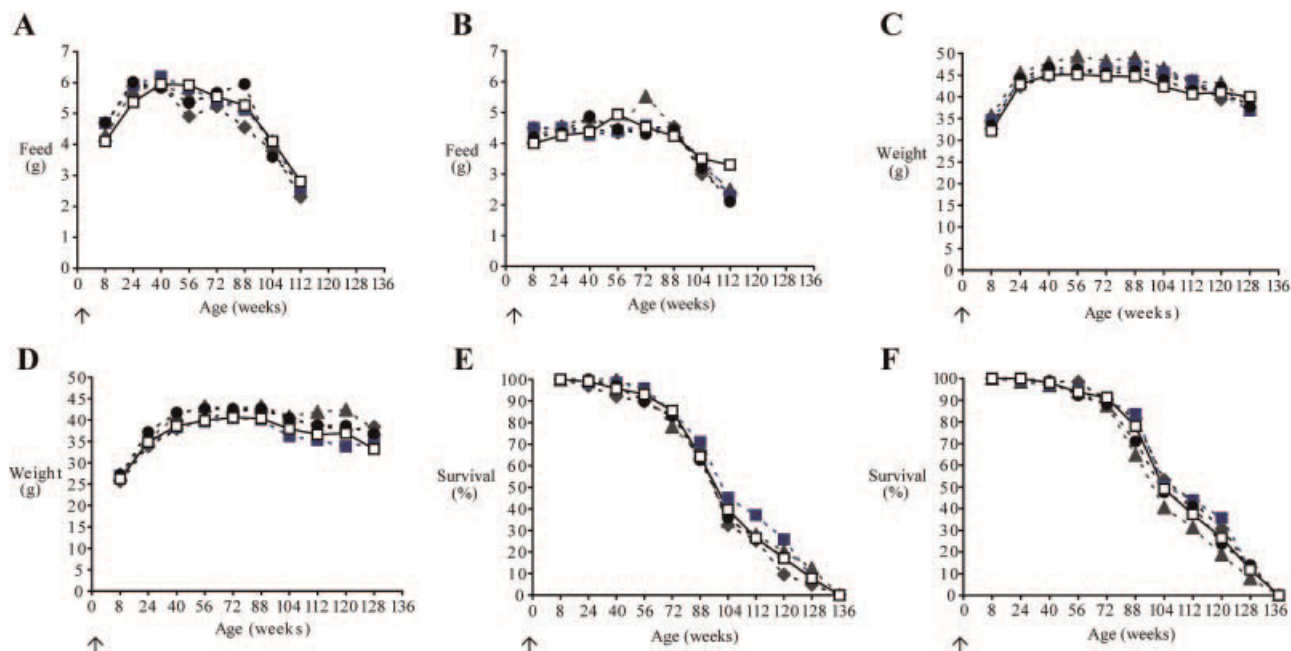


FIGURE 1. A: Mean daily feed consumption in males. B: Mean daily feed consumption in females. C: Mean body weights in males.

D: Mean body weights in females. E: Survival in males. F: Survival in females (- ◆ - 32,000 ppm; - ▲ - 16,000 ppm; - ■ - 8,000 ppm;

- ● - 2,000 ppm; - □ - Control). ↑ Start of the treatment.

TABLE I. Incidence of Benign Tumors in Male (M) and Female (F) Swiss Mice in a Transplacental Life-Span Feed Carcinogenicity Study of APM*

Dose ppm (mg/kg b.w.) ^a	Benign tumors					
	Animals at start (N)		Tumor-bearing animals, N (%)		Total N (per 100 animals)	
	M	F	M	F	M	F
0 (control)	117	102	42 (35.9)	39 (38.2)	53 (45.3)	56 (54.9)
2,000 (242)	103	122	35 (34.0)	53 (43.4)	49 (47.6)	81 (66.4)
8,000 (987)	62	73	20 (32.3)	30 (41.1)	26 (41.9)	49 (67.1)
16,000 (1,919)	64	64	26 (40.6)	30 (46.9)	33 (51.6)	51 (79.7)
32,000 (3,909)	83	62	22 (26.5)	32 (51.6)	29 (34.9)	64 (103.2)

*Tumor rates are based on the number of animals examined (necropsied).

^aThe dose estimates are based on the actual feed consumption and body weight data collected during the study.

Total Benign and Malignant Tumors

The incidence and the total number of benign and malignant tumors per 100 animals are reported in Tables I and II. A marginal increasing in incidences and in the number of malignant tumors were observed in treated males but not females. In the controls, the major tumor types that contribute to the incidences were skin fibrosarcomas (35.9%) in males and mammary carcinomas (13.7%) and lymphomas/leukemias (38.2%) in females.

Neoplastic Lesions of the Liver

The incidences of hepatocellular carcinomas (HCC) and adenomas (HCA) in males and females are reported in Table III. Males showed a significant dose-related increase in the incidence of HCC ($P < 0.01$) with, specifically, a significant increased incidence of HCC among the animals treated with APM at the dose levels of 32,000 ppm ($P < 0.01$) and 16,000 ppm ($P < 0.05$). In males there was a significant increase ($P < 0.05$) in the incidence of HCA and HCC (combined) at 16,000 ppm. The HCC usually were grossly described typically as nodules, varying in size and color, with

irregular borders. Microscopically, they appeared most frequently with a trabecular growth composed of well-differentiated hepatocytes forming trabecular of three or more layers thick (Fig. 2A,B). In a few cases acinar and solid patterns were also observed. The cumulative prevalence curves of male mice bearing HCC show an earlier onset of death among the animals treated at the highest doses (Fig. 3A). The majority of HCA were grossly visible. Microscopically they appeared as well demarcated and compressing the surrounding parenchyma. There was typically loss of the normal lobular architecture, cells occurred in irregular plates, 1–3 cell layers thick, disposed perpendicular or obliquely on the surrounding parenchyma. Increased incidences, albeit not significant, of liver angiosarcoma was observed among male mice with a doubling at the top three exposures: 4.3% controls versus 4.9%, 8.1%, 7.8%, and 9.6%.

Neoplastic Lesions of the Lung

The incidences of male mice bearing alveolar/bronchiolar carcinoma (A/BC) and alveolar/bronchiolar adenoma (A/BA), and the number of animals bearing multiple lung tumors, are reported in Table IV. A significant dose-related

TABLE II. Incidence of Malignant Tumors in Male (M) and Female (F) Swiss Mice in a Transplacental Life-Span Feed Carcinogenicity Study of APM*

Dose ppm (mg/kg b.w.) ^a	Malignant tumors					
	Animals at start (N)		Tumor-bearing animals, N (%)		Total N (per 100 animals)	
	M	F	M	F	M	F
0 (control)	117	102	66 (56.4)	69 (67.6)	94 (80.3)	92 (90.2)
2,000 (242)	103	122	58 (56.3)	90 (73.8)	76 (73.8)	117 (95.9)
8,000 (987)	62	73	45 (72.6)	47 (64.4)	66 (106.5)	61 (83.6)
16,000 (1,919)	64	64	39 (60.9)	44 (68.8)	63 (98.4)	61 (95.3)
32,000 (3,909)	83	62	57 (68.7)	40 (64.5)	87 (104.8)	55 (88.7)

*Tumor rates are based on the number of animals examined (necropsied).

^aThe dose estimates are based on the actual feed consumption and body weight data collected during the study.

TABLE III. Incidence of Hepatocellular Neoplasms in Male Swiss Mice in a Transplacental Life-Span Feed Carcinogenicity Study of APM*

Dose ppm (mg/kg b.w.) ^a	Animals at start (N)		HCA, N (%)		HCC, N (%) ^b		HCA plus HCC, N (%)	
	M	F	M	F	M	F	M	F
0 (control)	117	102	9 (7.7)	1 (1.0)	6 (5.1) ^c	0 (—)	15 (12.8)	1 (1)
2,000 (242)	103	122	10 (9.7)	6 (4.9)	12 (11.7)	2 (1.6)	22 (21.4)	8 (6.5)
8,000 (987)	62	73	4 (6.5)	2 (2.7)	9 (14.5)	0 (—)	13 (21.0)	2 (2.7)
16,000 (1,919)	64	64	6 (9.4)	0 (—)	10 (15.6)*	2 (3.1)	16 (25.0) [#]	2 (3.1)
32,000 (3,909)	83	62	2 (2.4)	0 (—)	15 (18.1)**	0 (—)	17 (20.5)	—

*Tumor rates of hepatocellular adenomas (HCA) and of hepatocellular carcinomas (HCC) are based on the number of animals necropsied.

^aThe dose estimates are based on the actual feed consumption and body weight data collected during the study.

^bIn male historical controls out of 1,047 Swiss mice, the overall incidence of HCC is 3.2% (range: 0–26.3%); in females, out of 999 controls, the overall incidence is 0.2% (range: 0–2.1%).

^cTrend test is significant ($P < 0.01$).

Significant ($P < 0.05$) using Cox proportional hazard model.

**Significant ($P < 0.01$) using Cox proportional hazard model.

[#]Significant ($P < 0.05$) using logistic analysis.

trend ($P < 0.05$) for A/BC was observed. When compared to the controls, a significant increase of the incidence of A/BC was observed among the males exposed to 32,000 ppm ($P < 0.05$). A significant dose-related trend ($P < 0.05$) for A/BA and A/BC combined was observed in males. When controls were compared to treated males, a significantly increased incidence ($P < 0.05$) occurred at 32,000 ppm. At necropsy A/BC were described usually as gray-white colored nodules, varying in size from 2 to 15 mm (in some cases they occupied an entire pulmonary lobe), with irregular borders. Microscopically A/BC presented different patterns of growth, namely papillary, tubular, and solid, with invasive growth into interstitial, vessels, bronchi, and pleura surfaces. The tumor cells were pleomorphic and with oval/cuboidal shape. Nuclei of the tumor cells were atypical and altered

mitotic figures were present (Fig. 2C,D). Also in this case the cumulative prevalence curves of male mice bearing A/BC show an early onset of death among the animals treated at the highest doses (Fig. 3B). It is noticeable that, also in this case, among the animals treated at 32,000 ppm, 5/55 (9.1%) died due to lung A/BC during the period between 0 and 98 weeks of age. Two of 60 (3.3%) A/BC were observed among the controls deceased in the same period.

Grossly, A/BA also appeared circumscribed gray-white, 1–8 mm nodules, in some cases multiple and with peripheral location. Histomorphologically, the tumor margins were usually well demarcated, neoplastic epithelial cells were relatively uniform, cuboidal with oval nuclei, and mitotic figures were rare. Small foci of mild atypia were present in a few cases. Three histological types, based on the pattern of

TABLE IV. Incidence of Lung Neoplasms in Male Swiss Mice in a Transplacental Life-Span Feed Carcinogenicity Study of APM*

Dose ppm (mg/kg b.w.) ^a	Animals at start (N)		A/BA, N (%)		A/BC, N (%) ^b		A/BA plus A/BC, N (%)	
	M	F	M	F	M	F	M	F
0 (control)	117	102	8 (1) (6.8) ^c	4 (3.9)	7 (6.0) ^d	7 (6.9)	15 (12.9) [#]	11 (10.8)
2,000 (242)	103	122	9 (2) (8.7) ^c	9 (7.4)	6 (5.8)	10 (8.2)	15 (14.6)	19 (15.6)
8,000 (987)	62	73	7 (1) (11.3) ^c	3 (4.1)	7 (11.3)	6 (8.2)	14 (22.6)	9 (19.2)
16,000 (1,919)	64	64	7 (1) (10.9) ^c	2 (3.1)	8 (3) (12.5) ^c	7 (10.9)	15 (23.4)	9 (23.4)
32,000 (3,909)	83	62	6 (7.2)	3 (4.8)	11 (13.3)**	2 (3.2)	17 (20.5)*	5 (8.06)

*Tumor rates of lung alveolar/bronchiolar adenomas (A/BA) and of lung alveolar/bronchiolar carcinomas (A/BC) are based on the number of animals necropsied.

^aThe dose estimates are based on the actual feed consumption and body weight data collected during the study.

^bIn male historical controls out of 1,047 Swiss mice, the overall incidence of A/BC is 1.45% (range: 0–14.3%); in females, out of 999 controls, the overall incidence is 0.2% (range: 0–2.1%).

^cBetween parentheses are reported the numbers of animals bearing multiple tumors.

^dTrend test is significant ($P < 0.05$).

**Significant ($P < 0.01$) using Cox proportional hazard model.

Significant ($P < 0.01$) using Cox proportional hazard model.

[#]Significant ($P < 0.05$) using logistic analysis.

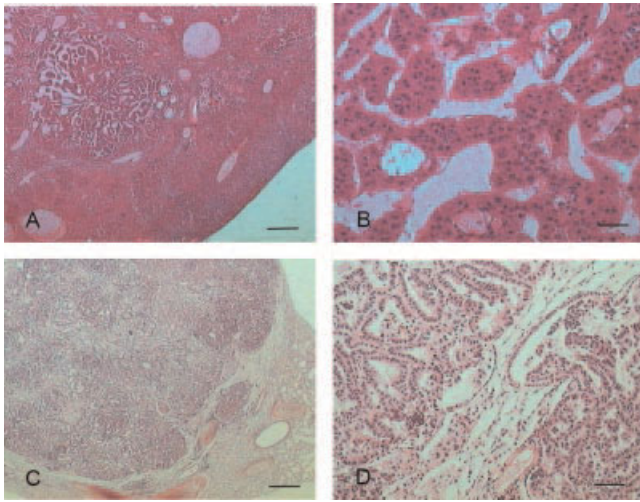


FIGURE 2. **A:** Hepatocellular carcinomas, trabecular pattern, in a male mice administered 32,000 ppm aspartame in feed; H&E; magnification, 2.5 $\times$; bar = 12.5 μ m. **B:** Details of the hepatocarcinoma shown in (A); H&E; magnification, 10 $\times$; bar = 50 μ m. **C:** Lung alveolar/bronchiolar carcinomas, in a male mice administered 32,000 ppm aspartame in feed; H&E; magnification, 2.5 $\times$; bar = 12.5 μ m. **D:** Details of the alveolar/bronchiolar carcinoma shown in (C); H&E; magnification, 20 $\times$; bar = 100 μ m.

cellular growth, were observed: solid, papillary (including tumors containing tubular structures), and mixed.

The overall incidences of historical controls of HCC and alveolar/bronchiolar carcinomas in male and female Swiss mice in the CMCRC laboratory over the last 20 years are reported in the footnotes of Tables III and IV, respectively.

DISCUSSION

The present study, in which APM was administered in feed at the dose levels of 0, 2,000, 8,000, 16,000, or 32,000 ppm to Swiss mice from prenatal life until death, further confirms that APM induces carcinogenic effects in

rodents. The study shows: (a) significant dose-related increase of hepatocellular carcinomas in males ($P < 0.01$). Incidences were also significantly increased at the two top dietary concentrations of 32,000 ppm ($P < 0.01$) and 16,000 ppm ($P < 0.05$); (b) a significant dose-related increase of the incidence of lung alveolar/bronchiolar carcinomas ($P < 0.01$), and at 32,000 ppm ($P < 0.05$). Since the survival of the males was not affected by APM exposure, we used logistic analysis to evaluate the combined adenoma/carcinoma results of the liver and of the lung. The incidence of HCA and HCC combined resulted significantly increased ($P < 0.05$) in the group treated at 16,000 ppm. No significant dose-response was observed. The reason for the lack of significance is that the dose-response is flat over the exposure groups while the controls are lower (i.e., 12.8, 21.4, 21.0, 25.0, and 20.5). It is noticeable that until 98 weeks of age 8/55 deceased males (14.6%) treated at 32,000 ppm had HCC and no HCA. On the contrary, three HCA and no HCC were observed among the 60 controls deceased in the same period. This may depend on a more rapid progression of preneoplastic lesions to HCC. However, others suggest that the response to carcinogens differ, and that both HCA and HCC may develop de novo, without going through the stage of foci of cellular alterations [Frith et al., 1979].

A significant dose-related trend ($P < 0.05$) of A/BA and A/BC combined was observed among males. Moreover, the incidence of A/BA plus A/BC in males treated at 32,000 ppm was significantly increased ($P < 0.05$) compared to controls.

Both liver and lung carcinomas in all exposure groups of males were within the historical control range of these neoplasms in the CMCRC laboratory.

Concerning the HCC, the concurrent control (5.1%) falls within the lower range of our historical controls (0–26.3%) and because the incidences of HCC in the groups treated at 32,000 (18.1%) and 16,000 (15.6%) were over three and two times the concurrent control, we considered this effect related to the treatment. Concerning A/BC, the concurrent

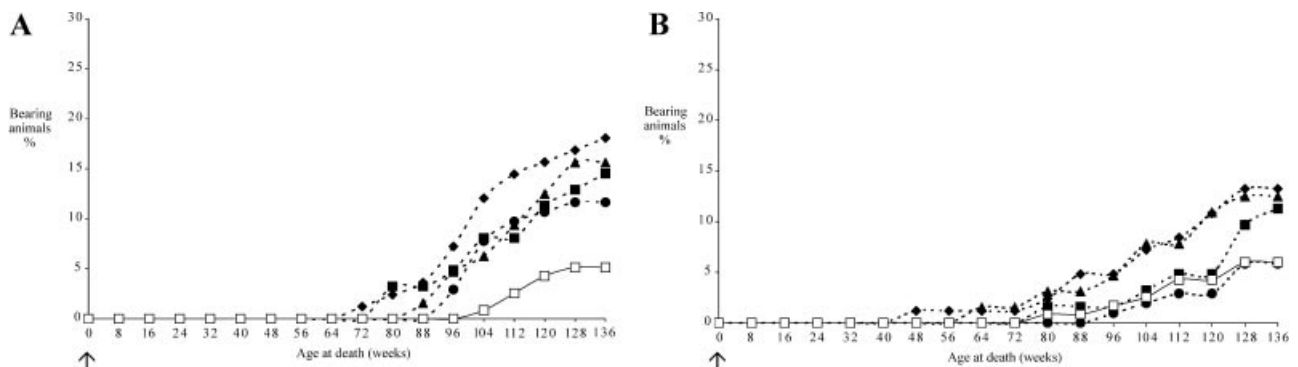


FIGURE 3. **A:** Life-span feed carcinogenicity study of aspartame administered to Swiss mice, from fetal life until natural death: cumulative prevalence of male mice bearing hepatocarcinomas, by age at death. **B:** Cumulative prevalence of male mice bearing lung alveolar/bronchiolar carcinomas, by age at death (—◆— 32,000 ppm; —▲— 16,000 ppm; —■— 8,000 ppm; —●— 2,000 ppm; —□— Control). [†]Start of the treatment.

control (6.0%) falls also within the lower range of our historical controls (0–14.3%) and because the incidence observed at the highest dose was more than double the concurrent control we considered these effects to be related to APM exposure [Haseman et al., 1984; Haseman, 1992, 1995].

No differences were observed in the incidences of liver and lung tumors among the females of treated and control groups. It has been reported that both spontaneously occurring and treatment induced hepatocellular tumors occur with significantly greater frequency and multiplicity in males than in females even though occasionally exceptions do occur [Maronpot, 2009]. Male mice are also more susceptible to develop A/BA and A/BC than females [Hahn et al., 2007; Dixon et al., 2008].

The carcinogenic effects observed in our mouse bioassay do not support the negative outcome obtained with the CD-1 mouse study performed at the Searle Laboratory in 1974 [Molinary, 1984]. In that experiment one group of 72 male and female CD-1 mice (control) and three groups of 32 males and 32 females were treated, respectively, with APM in feed at the dose levels of 0, 1, 2, 4 g/kg from prenatal life for 2 years. These studies are not comparable for two reasons: (a) the number of the treated animals per sex per group is smaller in comparison to the number in our experiment and to the number requested by the current standard for carcinogenic bioassays (at least 50 animals per sex per group) used by NTP and most others and (b) the length of observation is much shorter (110 weeks compared to 130 weeks). Both of these factors result in a loss of sensitivity for detecting a carcinogenic effect.

As already reported [Soffritti et al., 1999; Haseman et al., 2001; Huff et al., 2008; Soffritti et al., 2008], in long-term carcinogenicity bioassays the number of animals per sex/group and life span observation are critical points for identification and assessment of diffuse carcinogenic risks, defined as the exposure to a single or multiple agents or to mixtures that are expected to have limited carcinogenic

potential because of the agent type (weak carcinogen) and/or dose/concentration (low), but that involve large group of the population (as is the case with APM). Concerning the prolonged (over 110 weeks of age) or lifespan duration of the experiment, we must consider that neoplastic response depends not only on the chemical–physical characteristics of the agent and its toxicological properties, the mode of exposure, and the type of animals, but also, to a greater extent, on the latency of the tumor which varies and may be very long.

Truncating an experiment after 2 years (more or less two-thirds of the natural life of rodents) as requested by several regulatory agencies (and as practiced by NTP), may mask a possible carcinogenic response. This has been shown by us in experiments on benzene, Mancozeb (a widely used fungicide), vinyl acetate, toluene, and xylenes [Soffritti et al., 2002]. It should be noted that in the experiment on toluene and xylenes performed by NTP, in which the rats were sacrificed after 104 weeks of treatment, no carcinogenic effects were found [Huff, 2002, 2003; Huff et al., 2010], whereas lifetime studies conducted in the CMCRC showed unequivocal carcinogenicity after 104 weeks [Maltoni et al., 1997; Soffritti et al., 2004]. These two factors in our opinion makes the Searle study less sensitive than ours.

Overall, the results of our integrated project of lifespan carcinogenic bioassays on APM conducted on Sprague–Dawley rats and Swiss mice are consistent in showing that under our experimental conditions APM must be considered a trans-species carcinogenic agent in multiple sites (Table V), inducing a significantly increased incidence of malignant tumors in: (a) multiple tissues in male and female rats; (b) multiple organs in male mice; (c) an earlier occurrence in treated animals and an higher incidence and an anticipated onset of cancers when the treatment starts from fetal life [Soffritti et al., 2007]. Finally, the carcinogenic effects of APM in rats were shown also at dose levels of 100 and 20 mg/kg b.w. to which humans could be exposed [Soffritti et al., 2006, 2007].

TABLE V. Summary of the Carcinogenic Effects of APM in Life-Span Carcinogenicity Bioassays in Sprague–Dawley Rats and Swiss Mice

Species (start of exposure)	Lymphomas/leukemias		Kidneys ^a		Nervous system ^b		Mammary carcinomas		Liver HCC ^c		Lung A/BC ^d	
	M	F	M	F	M	F	M	F	M	F	M	F
Sprague–Dawley rats (8 weeks)		DR ⁺		DR ⁺	DR ⁺							
Sprague–Dawley rats (fetal)	+	DR ⁺						DR ⁺				
Swiss mice (fetal)									DR ⁺		DR ⁺	

DR, dose related.

^aCarcinomas of the pelvis or ureter.

^bCranial nerve malignant Schwannomas.

^cHepatocellular carcinomas.

^dAlveolar/bronchiolar carcinomas.

⁺Significantly increased.

CONCLUSIONS

The present study demonstrates for the first time that APM administered in feed to Swiss mice at doses of 32,000, 16,000, 8,000, 2,000, or 0 ppm, starting the dietary exposure on day 12 of gestation and lasting until death, induces significant dose-related increases of hepatocellular carcinomas ($P \leq 0.01$) and of alveolar/bronchiolar carcinomas ($P < 0.05$) in males. In particular, the significant increased incidences of hepatocellular carcinomas were observed at the dietary levels of 32,000 ppm ($P < 0.01$) and 16,000 ppm ($P < 0.05$) and of lung alveolar/bronchiolar carcinomas at 32,000 ppm ($P < 0.05$). HCA and HCC (combined) resulted significantly increased ($P < 0.05$) in the male group treated at 16,000 ppm. A/BA and A/BC (combined) resulted significantly increased ($P < 0.05$) in the male group treated at 32,000 ppm. A significant dose-related trend ($P < 0.05$) was also observed.

Given that APM is completely metabolized in the gastrointestinal tract to phenylalanine, aspartic acid, and methanol, it may be concluded that the observed carcinogenic effects were caused not by APM itself but rather by its metabolites. In particular, it cannot be disregarded that the conversion of APM methanol into formaldehyde in the liver may result in a generation of formaldehyde adducts [Trocho et al., 1998], which could explain the plausibility of hepatocarcinogenic effects of APM in male mice. The fact that females did not develop a significantly increased incidence of liver tumors may be explained by the gender resistance, as already reported.

On the basis of these results, together with previous carcinogenicity bioassays conducted on rats in our laboratories, APM should be considered a multiple site, trans-species carcinogenic agent. A re-evaluation of the current regulations on APM remains, in our opinion, urgent.

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tumors) there were no discrepancies between NTP and IR pathology evaluation. A special thanks to Luana De Angelis and to all the CRCCM staff who were involved in the study.

REFERENCES

- Butchko HH, Stargel WW, Comer CP, Mayhew DA, Benninger C, Blackburn GL, de Sonneville LMJ, Geha RF, Hertelendy Z, Kostner A, Leon AS, Liepa GU, McMartin KE, Mendnhall CL, Munro IC, Novotny EJ, Renwick AG, Schiffman SS, Schomer DL, Shaywitz BA, Spiers PA, Tephly TR, Thomas JA, Trefz FK. 2002. Intake of aspartame vs the acceptable daily intake. *Regul Toxicol Pharmacol* 35:S13–S16.
- Decreto Legislativo 116. 1992. Attuazione della direttiva n. 86/609/CEE in materia di protezione degli animali utilizzati a fini sperimentali o ad altri fini scientifici [in Italian]. *Suppl Ordinario Gazz Ufficiale* 40: 5–25.
- Dixon D, Herbert RA, Kissling GE, Brix AE, Miller RA, Maronpot RR. 2008. Summary of chemically induced pulmonary lesions in the National Toxicology Program (NTP) toxicology and carcinogenesis studies. *Toxicol Pathol* 36:428–439.
- European Food Safety Authority (EFSA). 2006. Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in contact with Food (AFC) on a request from the Commission related to a new long-term carcinogenicity study on aspartame. Question number EFSA-Q-2005-122. Adopted on 3 May 2006. *EFSA J* 356:1–44.
- Food and Drug Administration (FDA). 1981. Aspartame: Commissioner's final decision. *Fed Reg* 46:38285–38308.
- Food and Drug Administration (FDA). 1983. Food additives permitted for direct addition to food for human consumption: Aspartame. *Fed Reg* 48:31376–31382.
- Food and Drug Administration (FDA). 1996. Food additives permitted for direct addition to food for human consumption: Aspartame. *Fed Reg* 61:33654–33656.
- Frith CH, Kodell RL, Littlefield NA. 1979. Biologic and morphologic characteristics of hepatocellular lesions in BALB/c female mice fed 2-acetylaminofluorene. In: Staffa JA, Mehlman MA, editors. *Innovations in cancer risk assessment (ED₀₁ study)*. Park Forest South, IL: Pathotox Publishers, Inc., p 121–138.
- Hahn FF, Gigliotti A, Hutt JA. 2007. Comparative oncology of lung tumors. *Toxicol Pathol* 35:130–135.
- Haschemeyer RH, Haschemeyer AEV. 1973. *Proteins*. New York: John Wiley & Sons, p 11–30.
- Haseman J. 1992. Value of historical controls in the interpretation of rodent neoplasm data. *Drug Inf J* 26:191–200.
- Haseman J. 1995. Data analysis: Statistical analysis and use of historical control data. *Regul Toxicol Pharmacol* 21:52–59.
- Haseman J, Huff JE, Boorman GA. 1984. Use of historical control data in carcinogenicity studies in rodents. *Toxicol Pathol* 12:126–135.
- Haseman J, Melnick R, Tomatis L, Huff J. 2001. Carcinogenesis bioassays: Study duration and biological relevance. *Food Chem Toxicol* 39(7):739–744.
- Hazardous Substances Data Bank. 2005. TOXNET: Toxicological Data Network. Available: <http://toxnet.nlm.nih.gov/cgi-bin/sis/search/f?/temp/~mobZuL:1> [accessed August 3, 2005].
- Huff J. 2002. Chemicals studied and evaluated in long-term carcinogenesis bioassays by both the Ramazzini Foundation and the National

- Toxicology Program. In tribute to Cesare Maltoni and David Rall. *Ann N Y Acad Sci* 982:208–230.
- Huff J. 2003. Absence of carcinogenic activity in Fischer rats and B6C3F1 mice following 103-week inhalation exposures to toluene. *Int J Occup Environ Health* 9(2):138–146.
- Huff J, Jacobson MF, Davis DL. 2008. The limits of two-year bioassay exposure regimens for identifying chemical carcinogens. *Environ Health Perspect* 116(11):1439–1442.
- Huff J, Hejtmancik M, Eastin W. 2010. Carcinogenic activity of industrial grade mixed xylenes. *Environ Health Perspect* (in press).
- International Agency for Research on Cancer (IARC). 1973. Transplacental induction of tumors and malformations in rats treated with some chemical carcinogens. *IARC Sci Publ* 4:100–111.
- Maltoni C, Ciliberti A, Pinto C, Soffritti M, Belpoggi F, Menarini L. 1997. Results of long-term experimental carcinogenicity studies of the effects of gasoline, correlated fuels and major gasoline aromatics on rats. *Ann N Y Acad Sci* 837:15–52.
- Maronpot RR. 2009. Biological basis of differential susceptibility to hepatocarcinogenesis among mouse strains. *J Toxicol Pathol* 22: 11–33.
- Matthews DM. 1984. Absorption of peptides, amino acids, and their methylated derivatives. In: Stegink LD, Filer LJ, Jr., editors. *Aspartame physiology and biochemistry*. New York: Dekker, p 29–46.
- Mazur RH. 1984. Discovery of aspartame. In: Stegink LD, Filer LJ, Jr., editors. *Aspartame physiology and biochemistry*. New York: Dekker, p 3–9.
- Metzler DE. 1977. *Biochemistry*. New York: Academic Press, p 90–107.
- Molinary SV. 1984. Preclinical studies of aspartame in nonprimate animals. In: Stegink LD, Filer LJ, Jr., editors. *Aspartame physiology and biochemistry*. New York: Dekker, p 289–306.
- National Toxicology Program (NTP). 2005. Genetically Modified Model Report. Toxicology Studies of Aspartame (CAS NO. 22839-47-0) in Genetically Modified (FVB Tg.AC Hemizygous) and B6.129-Cdkn2atm1Rdp (N2) Deficient Mice and Carcinogenicity Studies of Aspartame in Genetically Modified [B6.129-Trp53tm1Brd (N5) Haploinsufficient] Mice (Feed Studies). NTP GMM1. National Toxicology Program, Research Triangle Park. Available at: <http://ntp.niehs.nih.gov/files/GMM1-Web.pdf>.
- Olney JW, Farber NB, Spitznagel E, Robins LN. 1996. Increasing brain tumor rates: Is there a link to aspartame? *J Neuropathol Exp Neurol* 55:1115–1123.
- Pritchard JB, French JE, Davis BJ, Haserman JK. 2003. The role of transgenic mouse models in carcinogen identification. *Environ Health Perspect* 111:444–454.
- Ranney RE, Opperman JA, Maldoon E, McMahon FG. 1976. Comparative metabolism of aspartame in experimental animals and humans. *J Toxicol Environ Health* 2:441–451.
- Soffritti M, Belpoggi F, Minardi F, Bua L, Maltoni C. 1999. Mega-experiments to identify and assess diffuse carcinogenic risks. *Ann N Y Acad Sci* 895:34–55.
- Soffritti M, Belpoggi F, Minardi F, Maltoni C. 2002. Ramazzini Foundation cancer program: History and major projects, life-span carcinogenicity bioassay design, chemicals studied, and results. *Ann N Y Acad Sci* 982:26–45.
- Soffritti M, Belpoggi F, Padovani M, Lauriola M, Degli Esposti D, Minardi F. 2004. Life-time carcinogenicity bioassays of toluene given by stomach tube to Sprague–Dawley rats. *Eur J Oncol* 9:91–102.
- Soffritti M, Belpoggi F, Degli Esposti D, Lambertini L. 2005. Aspartame induces lymphomas and leukaemias in rats. *Eur J Oncol* 10:107–116.
- Soffritti M, Belpoggi F, Degli Esposti D, Lambertini L, Tibaldi E, Rigano A. 2006. First experimental demonstration of the multipotential carcinogenic effects of aspartame administered in the feed to Sprague–Dawley rats. *Environ Health Perspect* 114:379–385.
- Soffritti M, Belpoggi F, Tibaldi E, Esposti DD, Lauriola M. 2007. Life-span exposure to low doses of aspartame beginning during prenatal life increases cancer effects in rats. *Environ Health Perspect* 115:1293–1297.
- Soffritti M, Belpoggi F, Esposti DD, Falcioni L, Bua L. 2008. Consequences of exposure to carcinogens beginning during developmental life. *Basic Clin Pharmacol Toxicol* 102(2):118–124.
- Trocho C, Pardo R, Rafecas I, Virgili J, Remesar X, Fernandez-Lopez JA, Alemany M. 1998. Formaldehyde derived from dietary aspartame binds to tissue components *in vivo*. *Life Sci* 63:337–349.