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Systematic review and evaluation of aspartame carcinogenicity bioassays using quality criteria

Lois Haighton[a,*], Ashley Roberts[a], Brandon Walters[a], Barry Lynch[a]

[a] Intertek Regulatory & Scientific Consultancy, 2233 Argentia Rd., Suite 201, Mississauga, ON L5N 2X7, Canada

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Abbreviations: DKP, diketopiperazine; GLP, good laboratory practices; NTP, National Toxicology Program; OECD, Organization for Economic Co-operation and Development; PKU, phenylketonuria

*Corresponding Author: Lois Haighton, Tel: +1 905-542-2900, Email: lois.haighton@intertek.com

Abstract

The current review assessed cancer studies of aspartame based on a quality appraisal using the Klimisch grading system. Nine studies having complete histopathology were included: three 2-year studies by Searle; three transgenic mice studies by the NTP; three lifetime studies by the Ramazzini Institute. A tenth study limited to brain tumors was not rated. None were determined as Klimisch Code 1 (reliable without restrictions). The Searle studies predated GLP standards but their methodology was comparable; transgenic mouse models are not validated, but are accepted as supporting data. These studies were rated Klimisch Code 2 (reliable with restrictions). The Ramazzini Institute used a lifetime model of their own design that has been questioned due to high rates of spontaneous tumors, issues with tumor type diagnosis and concerns about the impact of chronic infections. As many of these problems could be attributed to using animals that died or were terminated near end of life, along with the other problems noted, these studies were rated Klimisch Code 3 (not reliable). As the Klimisch Code 2 studies demonstrated a lack of carcinogenic potential, and as aspartame is hydrolyzed to common components and lacks genotoxic activity, a conclusion that aspartame is not carcinogenic is supported.

Keywords: aspartame; cancer; quality appraisal; Klimisch rating; bioassays

1. Introduction

The non-nutritive artificial sweetener, aspartame, or N-(L-α-Aspartyl)-L-phenylalanine, 1-methyl ester, is approximately 200-fold sweeter than sugar and has been in use as a table top sweetener and in a variety of foods and beverages for over 3 decades. The structure for aspartame is presented in Figure 1.

Aspartame, which is prepared from the two amino acids phenylalanine and aspartic acid, is stable under dry conditions but not under conditions of prolonged heating (Magnuson et al., 2007). The manufacturing process is described in Butchko et al. (2002) and Magnuson et al. (2007).

Foods containing aspartame must include the phrase “contains phenylalanine” on the label to inform individuals who have phenylketonuria (PKU). Since one of the metabolites of aspartame is phenylalanine, use of this non-caloric sweetener is not recommended for individuals with PKU since they have a greatly reduced capacity to metabolize phenylalanine. Individuals with PKU monitor dietary phenylalanine regardless of its food source. Also, the label of the table top aspartame product is to note that it is not recommended for cooking or baking (U.S. FDA, 1981).

Following the submission of the original food additive petition for aspartame (38 FR 5921), which was noted by the United States (U.S.) Food and Drug Administration (FDA) to contain voluminous amounts of safety data (U.S. FDA, 1973, 1981), aspartame was initially approved in 1974 for use in certain foods (sugar substitutes; cold cereals; chewing gum; beverages; gelatins; dairy products; toppings) and flavoring purposes (flavor enhancer and sweetener) (39 FR 27317; 21 CFR 172.804) (U.S. FDA ,1974, 2017). However, as this initial decision was challenged, further review of aspartame and data supporting safety was generated over a 6-year period to address concerns raised about potentially adverse effects of amino acids, including aspartic

acid, on the brain, including brain tumors (U.S. FDA, 1981; Butchko et al., 2002). The FDA audited three pivotal studies in support of aspartame and the Universities Associated for Research and Education in Pathology Inc. (UAREP) audited another 12 pivotal studies. The results of all studies were authenticated. The Commissioner of the FDA then conducted a review of the data and issued a final decision in 1981 concluding that aspartame was safe as intended for use (U.S. FDA, 1981). Presently, greater than 90 countries have approved aspartame as a sweetener in numerous products (Magnuson et al., 2007).

There continues to be interest in researching the toxicity of aspartame, particularly as it pertains to carcinogenic risk. In particular, authors of studies completed by the Ramazzini Institute have reported aspartame to be a multipotential carcinogen which is at odds with previous toxicology findings. The current research was undertaken to identify all animal cancer studies of aspartame and critically assess the findings of each study based on a quality appraisal of the study design, methodology, conduct, and reporting.

2. Methodology

2.1 Literature searches

The literature search was conducted in April 2017 using the electronic search tool ProQuest DialogTM. A total of 13 databases were searched and include AdisInsight: Trials, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, ToxFile®. The search terms used reflected the substance (aspartame; Methyl L-α-aspartyl-L-phenylalaninate; N-(L-α-Aspartyl)-L-phenylalanine, 1-methyl ester; L-Phenylalanine, L-alpha-aspartyl-, 2-methyl ester; L-Phenylalanine, N-L-alpha-aspartyl-, 1-methyl ester; Succinamic acid, 3-amino-N-(alpha-carboxyphenethyl)-, N-methyl ester, stereoisomer;

E951; E 951; 22839-47-0) and the test species (animal; rat; mouse; mice; dog; rabbit; pig; hamster; monkey; rodent; pig; piglet). The literature search was restricted to studies that were conducted *via* the oral route for animals (oral*; gavage; feeding; diet; dietary; intub*; drinking water; intragastric; administ*). The outcome terms used were those related to carcinogenicity (carcino*; tumor*; tumour*; neoplas*; oncogen*; cancer*). No restrictions with respect to language or year of publication were imposed for the literature search (asterisk indicates truncation).

In addition to cancer bioassays, any publications which contained the above search terms were obtained including review articles and discussion papers. The reference lists for these other types of articles were hand searched to determine if any other cancer bioassays existed that had not been identified from the electronic literature search. Two large reviews on the safety aspartame were identified which included Magnuson et al. (2007) which was published in a peer reviewed journal and a scientific opinion report on aspartame from the European Food Safety Authority (EFSA, 2013). The details of the pivotal unpublished Searle studies were included in these publications. The original Searle studies were then requested from and made available by the company Ajinimoto, which produces aspartame.

All studies were pre-screened to ensure that the study was conducted in whole animals and that histopathology evaluations were conducted for the purpose of assessing carcinogenicity potential. *In vitro* studies were not included nor were studies conducted in humans included; however, an appraisal of the available epidemiology studies was conducted and reported separately in the accompanying publication.

2.2 Quality Appraisal Tools

Standardized methods for conducting toxicology testing are maintained by the FDA "Redbook" and the Organization for Economic Co-operation and Development (OECD). The OECD test guideline (TG) 451 (carcinogenicity) and TG453 (combined chronic toxicity\carcinogenicity) are the accepted standards for assessing the carcinogenic potential of a chemical (OECD, 2009a, 2009b). Deviations from the OECD methodology need to be carefully considered as they can compromise the validity of the reported test results.

A systematic approach for evaluating the quality of toxicology studies, which included the means for establishing a reliability score, was developed by Klimisch et al. (1997). The Klimisch approach, which focused on the reliability of study data, was originally intended for use in assessing study reports for inclusion in the International Uniform Chemical Information Database or IUCLID. More recently, use of the Klimisch approach was promoted for evaluating study reports, usually unpublished, which were to be included in registration dossiers submitted under the EU REACH regulation (ECHA, 2011).

The Klimisch et al. (1997) approach defined a numerical code from 1 to 4 to separate studies available into categories where "Code 1" is "reliable without restriction", Code 2" is "reliable with restrictions", "Code 3" is "not reliable" and "Code 4" is "not assignable". Thus, studies for which the methodology was described in great detail and which were conducted in accordance with internationally accepted test guidelines and good laboratory practices (GLP) would be the most reliable (Code 1), studies mostly in compliance with GLP and generally conducted according to established methods would be reliable with restrictions (Code 2), studies carried out according to a methodology that is not acceptable and/or for which documentation of the method does not allow sufficient evaluation would be considered as not reliable (Code 3), and, if the data source

does not provide sufficient details of the methodology to allow for evaluation, it would be considered not assignable (Code 4).

To assist with evaluating studies for quality of design, conduct and reporting the European Commission developed ToxRTool (Schneider et al., 2009), which was developed around the principles of Klimisch. Other appraisal systems have been developed which focus on proper documentation of findings or risk of bias, and while there is overlap between the systems, only Klimisch and ToxRTool include a scoring framework (Lynch et al., 2016). More recent guidance for animal studies include the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) developed in 2010 which includes much of the same reporting requirements from OECD 34 updated in 2005 (OECD, 2005; Kilkenny et al., 2010).

The available carcinogenicity studies on aspartame with complete histopathological examinations were evaluated against the essential criteria for reliability from the ToxRTool and coded based on the original Klimisch grading system. If the original laboratory study reports were not available, the scientific publications were used.

3. Results

3.1 Carcinogenicity studies

3.1.1 Overview

In total, 10 distinct carcinogenicity or combined chronic toxicity/carcinogenicity studies which assessed aspartame were identified from the electronic and manual searches. These studies are described in Table 1. It was noted that for some *in vivo* studies, the results were presented in more than one publication. The details of the original toxicology studies that were submitted by Searle to the FDA as part of the FDA food additive petition are from a comprehensive

publication on the safety of aspartame (Magnuson et al., 2007) and a European Food Safety Authority (EFSA) opinion report (EFSA, 2013). Of the studies which Searle completed, 15 chronic studies including studies in dogs and monkeys were re-evaluated by a Public Board of Inquiry formed following the formal objection to the original FDA approval of aspartame. The complete findings are discussed elsewhere (U.S. FDA, 1981; Magnuson et al., 2007; EFSA, 2013). However, for the purpose of this carcinogenicity assessment, of the many Searle studies available, only the 2-year studies in rats (2) and mice (1) are considered further.

The carcinogenicity studies in which full histopathology examinations were conducted were the three 104-week (one from *in utero* exposure) cancer studies completed by Searle, which were the basis of the original food additive petition in the U.S. Subsequent to these studies, given spurious intracranial tumor findings which were a subject of the challenge to the original approval of aspartame which lead to the FDA audit of the studies. The potential for aspartame to induce brain tumors, specifically (the tumor type requiring further scrutiny in the Searle studies) was investigated in Wistar rats fed aspartame in amounts corresponding to 0, 1, 2, or 4 g/kg body weight/day (Ishii, 1981). The results of the Ishii (1981) study did not indicate that aspartame has carcinogenic potential. As detailed in the Federal Register, the Public Board of Inquiry convened by the FDA to audit the original Searle studies had some reservations about the spontaneous brain tumor findings, which they deemed as bizarre; however, after their decision, the results of this long-term Wistar rat study were made available the Commissioner (U.S. FDA, 1981). The Commission disagreed with the Board and agreed with the Bureau of Foods that there were reliable data to support that the brain tumor incidence rates observed in the Searle studies were within expected spontaneous rates for the type and strain of rats and given the study size. It was noted that the Commissioner did not use the additional data, including the Wistar rat study, to form the “central basis” of his decision, but the data did “confirm the large body of evidence” from the hearing. In addition, an evaluation of the brain tumor types

and incidences observed in the Searle studies, in comparison to the spontaneous occurrence of central nervous system (CNS) tumors in rats, and to the findings with known neurocarcinogenic agents was conducted (Koestner, 1984). Koestner (1984) concluded the incidence of brain tumors in the rats from the Searle studies was well within the range of background incidences, and that aspartame was not considered to be neurocarcinogenic to rats. These results also were published later in a peer-reviewed journal (Koestner, 1986) although aspartame was identified as a “test substance”.

Subsequent to the Searle and Ishii (1981) studies, the National Toxicology Program (NTP) assessed the carcinogenic potential of aspartame in three transgenic animal models (NTP, 2005). No evidence of carcinogenic activity was found. More recently, the three lifetime (two from gestation day 12) exposure studies completed by the Ramazzini Institute have been published each of which the authors concluded that aspartame has carcinogenic potential on the basis of reported incidence of several tumor types, including lymphoreticular tumors and liver tumors (Soffritti et al., 2005, 2006, 2010; Belpoggi et al., 2006). Finally, a study was identified which assessed effects of aspartame treatment on the incidence of pancreatic acinar carcinoma (Dooley et al., 2017). Aspartame was not found to exhibit carcinogenic potential in this study.

In addition to the whole animal studies noted above, two tumor promotion studies were identified (Ito et al., 1983; Hagiwara et al., 1984). Aspartame was not found to be a tumor promotor in either of these bioassays.

Tumor incidence data from the Searle, Ishii (1981), NTP and Ramazzini Institute studies are summarized in Table 2. The results of each of these studies are discussed in greater depth below.

196 *3.1.2 Searle Studies in Mice*

197 Searle conducted a 104-week study in ICR Swiss Mice (Searle, 1974a) the details of which were
198 summarized in EFSA (2013). Groups of mice (36/sex/group), which were approximately 28 days
199 of age at the beginning of the study, were administered diets providing approximately 0, 1000,
200 2000, or 4000 mg aspartame/kg body weight/day for 104 weeks (Searle, 1974a). Parameters
201 evaluated included physical appearance, behavior, body weight gain, feed consumption,
202 survival, clinical chemistry, ophthalmoscopic examination, organ weights, and gross and
203 histopathology examination including tumor incidences. There were no differences reported
204 between the controls and the treatment groups with respect to physical appearance, behavior,
205 mean terminal body weights, survival, gross pathology, or tumor incidence. Survivorship in
206 males in the control through 4000 mg/kg body weight/day groups, was 32.5%, 27.8%, 25.8%,
207 and 25.0%, respectively. Corresponding values in females were 41.7%, 38.9%, 41.7%, and
208 41.7%, respectively. It is not uncommon to see sex differences in mortality in long-term
209 toxicology studies, and, for some laboratory species under standardized conditions, the median
210 lifespan of males has consistently been shorter than that of females (Austad and Fischer, 2016).

211 In the first year of the study, a reduction in body weight gain among treated males was
212 accompanied by a reduction in feed consumption and did not exceed 3% at week 52, and
213 terminal body weight gain was comparable to controls. Hematological and blood chemistry
214 results were generally comparable to controls; sporadic differences reaching statistical
215 significance were noted to be within historical control ranges. Organ weights changes were
216 comparable to controls with the exception increased relative heart weight in high-dose females
217 only, increased relative thyroid weight in low-dose females, increased relative thyroid weight in
218 low-dose males, decreased relative prostate weight in low- and mid-dose males, but not high-
219 dose males. Histopathology examinations were conducted on all gross lesions from all animals,
220 the brain and urinary bladder of all animals, and a further 20-27 organs without gross lesions for

all control and high-dose animals, for two-thirds of mid-dose and for one-third of low-dose animals. No treatment related neoplastic or non-neoplastic changes were observed. The authors and the EFSA Panel concluded that the high dose of 4000 mg/kg body weight/day was the no-observed-adverse-effect level and that aspartame had no carcinogenic effect.

3.1.3 NTP Studies in Transgenic Mice

The NTP (2005) conducted 9-month feeding studies in three strains of genetically modified mice which are predisposed to the development of cancer: Tg.AC hemizygous mice; p53 haploinsufficient mice; and, CDKN2A deficient mice. Since the genetic alterations make the mice much more susceptible to chemical carcinogens, a smaller number of animals and shorter duration of exposure is required to detect a carcinogenic response. However, at the time of the study, the p53 haploinsufficient model was considered new and sensitivity to detect a tumorigenic response was uncertain. Presently, such studies are considered suitable as supporting data for assessing tumorigenic potential.

For each of the 9 month feeding studies, groups of transgenic mice (15/sex/group) were administered dietary concentrations of aspartame (>98% purity) of 3125, 6250, 12,500, 25,000, or 50,000 ppm with controls receiving unsweetened feed. At necropsy, complete histopathology examinations were conducted on all control and high-dose animals, and tissues from at least 15 sites (including brain, liver, kidneys, lungs, mammary glands and lymph nodes) for other treatment groups were examined for neoplastic and non-neoplastic effects for all animals. Survival was comparable to controls for all strains and there were no increases in tumors in any of the mouse strains. The only non-neoplastic effect noted was an increase in cytoplasmic perioportal vacuolization in hepatocytes which was not accompanied by any neoplastic effects in the liver. This effect was observed in male Cdkn2a deficient mice. The NTP concluded that aspartame did not cause cancer in any of these genetically modified strains of mice.

Soffritti et al. (2010) conducted a study in Swiss mice for which the duration of exposure extended from day 12 of gestation until death. The parental animals were separated into three groups of 40 animals and two groups of 60 (assumed to be the control and lowest dose group based on the greater number of pups in these groups at the beginning of the study) and mated, one male and one female per cage, at 13 weeks of age. After 5 days the males were removed from the cages and the dams were administered aspartame (98.7% pure; 0.2% diketopiperazine [DKP]) in the feed at doses of 0 (feed only), 2000, 8000, 16,000, and 32,000 ppm starting from 12 days of gestation until the pups were weaned at 4-5 weeks of age. The authors noted that the average number of pups per litter and the mean body weights 1 week after delivery were comparable between the treated and control groups.

At weaning, the pups were designated to receive the same aspartame doses as their dams. The number of pups in each group varied (control group: 117 males and 102 females; 2000 ppm: 103 males and 122 females; 8000 ppm: 62 males and 73 females; 16,000 ppm: 64 males and 64 females; 32,000 ppm: 83 males and 62 females). The concentrations in the feed corresponded to average daily doses of 0, 242, 987, 1919, and 3909 mg/kg body weight/day (males and females combined). The animals remaining at 130 weeks of age, which were noted to be equally distributed per group and sex, were sacrificed over a 10-day period and final necropsies conducted. Organs and tissues were collected from the skin, mammary gland, brain, cranium, pituitary gland, Zymbal glands, tongue, salivary glands, Harderian glands, thyroid, parathyroid, pharynx, larynx, lung, bronchi, thymus, mediastinal lymph nodes, trachea, heart, diaphragm, liver, colecystis, spleen, pancreas, kidneys, adrenal glands, esophagus, stomach, intestine, bladder, prostate, vagina, and from other organs and tissues noted to have pathological changes for histopathological evaluation.

No significant differences between control and treated animals were observed with respect to feed consumption, body weight gain or survival, and no treatment related clinical findings were

observed. No significant differences in the incidence of tumors between the control and treated animals were noted for the female mice. For male mice, the authors reported a significant dose-related increase in the incidence of hepatocellular carcinomas with the incidence at 16,000 ppm (15.6%) and 32,000 ppm (18.1%) being significantly different from the concurrent control group (5.1%). However, the hepatocellular tumor incidences for all male groups including the controls were within the historical control range of 0-26.3%. For hepatocellular adenomas and carcinomas combined, only the incidence for males at the 16,000 ppm dose (25%) was significantly different from controls (12.8%). The authors also reported a significant dose-related increase in the incidence of alveolar/bronchiolar carcinomas in male mice, with the incidence at 32000 ppm (13.3%) significantly different from controls (6.0%); however, the incidences for all treated groups were within the historical control range of 0-14.3%.

3.1.4 Searle Studies in Rats

As described by EFSA (2013) Searle conducted two carcinogenicity studies in rats. In the first study (Searle, 1973), Sprague-Dawley albino rats (40/sex/group) were administered aspartame in feed at concentrations corresponding to dose levels of 0, 1000, 2000, 4000, or 6000-8000 mg/kg body weight/day. The age of the animals at the start of treatment was not reported; however, initial body weights of 75-108 g for males and 80-102 g for females were cited. The high dose was increased from 6000-8000 mg/kg body weight/day by week 44. The DKP content was reported to range from 0-1.5% per batch. Parameters evaluated included physical appearance and behavior, body weight gain, feed consumption, mortality, clinical chemistry, ophthalmoscopic examination, organ weights, and gross pathology. Gross lesions for animals in all groups were subjected to histopathological examinations. An additional 20-25 organs (including brain, heart, liver, lungs, urinary bladder, stomach, small and large intestines, testes, mammary gland, uterus, vagina, pituitary gland, spinal cord, peripheral nerves, thyroid, adrenal gland, salivary gland, skin, skeletal muscle, mesenteric lymph node, and bone marrow) were

examined for animals in control and 4000 and 8000 mg/kg body weight/day dose groups, and for about one-quarter of the animals in the two lowest-dose groups.

Animals receiving the higher doses of aspartame had a significantly lower growth rate than controls which may have been due to significantly reduced feed consumption for the first 52 weeks. The reduced growth rate was observed in males given 8000 mg/kg body weight/day and for females in the 4000 and 8000 mg/kg body weight/day dose groups. Survival was notably reduced in high-dose females, but comparable to controls for all other treatment groups, after 104 weeks. Survivorship for males for the control through 8000 mg/kg body weight/day doses was 38.4%, 45.0%, 52.5%, 57.5%, and 52.5% respectively. Mean survival times ranged from 569 to 666 days. Survivorship in females for the control through 8000 mg/kg body weight/day doses was 46.7%, 57.5%, 50.0%, 35.0%, and 25.0% respectively. Mean survival times ranged from a low of 423 days in the high dose females to 663 days in the low-dose females. Relative organ weight changes (not otherwise specified) were not deemed to be of biological significance since there were no corresponding gross or histopathology findings. Pneumocyte hyperplasia was noted to be slightly increased in high-dose females and an increased incidence of cystic follicles of the ovaries was observed at all dose levels; however, EFSA (2013) noted that the incidence of the cystic follicles in the controls was unusually low. A slightly increased incidence of slight to moderate seminal vesicle atrophy was observed in the 4000 and 8000 mg/kg body weight/dose groups. Focal pancreatic fibrosis and mild atrophy was noted to be inconsistently observed in treated groups, but affected primarily high-dose animals. High-dose females, but not males, also were observed to have a higher incidence of nodular hyperplasia of the pancreas but there was no increase in the incidence of pancreatic tumors. The incidences of iron-containing hemosiderin deposits in renal tubular and pelvic epithelial cells, focal hyperplasia of pelvic epithelial cells, and tubular degeneration were increased in high-dose male rats but not in females or other male dose groups. Other organ changes were found to be inconsistent and

lacked a clear dose-response relationship. No effects on other parameters and no treatment related increases in the incidences of neoplasms were observed. Treatment related non-neoplastic changes were limited to the renal effects noted in high-dose males. The no-observed-adverse-effect level (NOAEL) was determined by EFSA to be 4000 mg/kg body weight/day.

In the second *in utero* Searle rat study (Searle, 1974b) as described in EFSA (2013), exposures to aspartame occurred from gestation, through lactation to 104 weeks post-weaning. Parental rats were administered diets corresponding to dose levels of 0, 2000, or 4000 mg aspartame/kg body weight for 60 days prior to mating, and through gestation and lactation for the dams. The DKP content was reported to range from 0.40-1.50% per batch. The weanling rats (60/sex/control group and 40/sex/group) were administered the same diets as their dams for 104 weeks. The same parameters as in the previous rat study were evaluated. There were no differences in survival compared to controls. Survivorship for males for the control through 4000 mg/kg body weight/day doses was 41.7%, 50.0%, and 57.5% respectively. Survivorship in females for the control through 4000 mg/kg body weight/day doses was 48.4%, 45.0%, and 52.5% respectively. At week 91 a minimum of 34 and 27 rats remained alive in the control and aspartame treated groups, respectively.

A significantly reduced growth rate among high-dose males was accompanied by a significantly reduced feed consumption. No biologically significant organ weight changes were observed (given the absence of accompanying histopathological findings).

There were no effects of treatment on the incidence of neoplasms or non-neoplastic lesions. A variety of non-neoplastic histopathological changes in the stomach, pancreas, ovaries, pituitary, adrenal cortex, kidney, livers, were not considered to be treatment related since there were no dose-response relationships, or findings were not common to both sexes, or incidences were

within historical control ranges. The NOAEL was determined to be 4000 mg/kg body weight per day, the highest dose tested.

The brains from all animals in the two rat studies were subjected to an additional histopathological evaluation (E87). A total of 12 (out of 440; E33-34 - Searle, 1973) and 9 (out of 280; E70 - Searle, 1974b) rats were found to have intracranial tumors of different types (astrocytoma, oligodendroglioma, ependymoma, meningeal sarcoma, unclassified glioma, meningioma) following the additional histopathological evaluations. The distribution of the tumors is presented in Table 2. It is noted that the brain tumors originated from different tissues, and there were no-dose responses observed.

3.1.5 Brain Tumor Study in Rats

The brain tumorigenicity of aspartame was further evaluated in a 2-year feeding study with SLC Wistar rats (Ishii, 1981). Starting at 6 weeks of age, groups of rats (86/sex/group), were provided aspartame in the diets corresponding to doses of 0 (control), 1000, 2000, or 4000 mg aspartame/kg body weight/day (purity not reported). A subsequent group was fed diets which provided 4000 mg aspartame+ DKP (3:1)/kg body weight/day. Within each group, 10 males and 10 females were terminated after 26 weeks, 16 males and 16 females were terminated after 52 weeks, and the remaining survivors were terminated at 104 weeks. All animals killed in extremis, found dead or killed at termination were subjected to macroscopic evaluation. External surfaces and the cut surfaces of the transverse slices of the brain (which was cut in six sections) were examined under a dissecting magnifying glass. Two of the middle transverse brains sections, as well as sections which were found to contain gross abnormalities were fixed for histological examinations. At 26 and 52 weeks, none of the animals terminated (10/sex/group and 16/sex/group, respectively) were found to have brain tumors. At the end of the 104-week treatment phase, remaining animals, 59-60/sex/group, were terminated and the brains of each

368 animal was assessed for the presence of brain tumors. One control female, terminated at week
369 99 was observed to have an atypical astrocytoma, one low-dose male terminated at 75 weeks
370 had an oligodendroglioma, one high-dose male, terminated at 93 weeks, and one mid-dose
371 female both had an astrocytoma, and a mid-dose female had an ependymoma. The incidence
372 (males and females combined) of brain tumors ranged from 0.8-1.7%. The historical incidence
373 for spontaneous CNS tumors in the rat, as cited from other sources, was reported by Ishii (1981)
374 to range from 0.09-5.8%. As the findings in the test animals were sporadic and not significantly
375 different from controls, it was concluded by the author that aspartame did not cause brain tumors
376 in the rat. In a concurrent group administered aspartame (4000 mg/kg) and diketopiperazine in
377 a 3:1 ratio, of which one female, terminated at 51 weeks, had an oligodendroglioma, aspartame
378 also was considered not to cause brain tumors.

379 The reported deaths and incidence of tumors were not statistically significantly different between
380 the control group and the test groups (based on Fischer's exact test with a significance level of
381 20%; it is noted that a significance level of 5% is usually applied rather than 20%; however, if the
382 authors were not able to identify a significant effect at 20% then they would not be able to obtain
383 a significant effect at the more stringent significance level of 5%). Ishii concluded from this study
384 that there was no evidence to support an effect of aspartame on brain tumor incidence. In a re-
385 evaluation of the neoplastic and non-neoplastic tumors identified in this study, Iwata (2006)
386 concluded that the tumors identified all could have occurred spontaneously.

387 Additional studies in male F344 rats were identified which investigated the potential for
388 aspartame to act as a promoter of bladder cancer using N-butyl-N-(4-hydroxybutyl) nitrosamine
389 as the initiator (Ito et al., 1983; Hagiwara et al., 1984). In the Ito et al. (1983) study, male rats
390 (n=36) were pretreated with 0.01% of the initiator in drinking water for 4 weeks, followed by 36
391 weeks with 0 or 5% aspartame in the diet. In the second study (Hagiwara et al., 1984), pre-
392 treatment conditions were the same but the exposure of the male rats (n=25 to 30/group) to

diets containing 0 or 5% aspartame was for 32 weeks. Each study also included a control group that did not receive N-butyl-N-(4-hydroxybutyl) nitrosamine. Aspartame was not found to be a bladder tumor promoter in either study.

3.1.6 Ramazzini Institute Rat Studies

A lifetime study was conducted by Soffritti et al. (2005) in Sprague-Dawley rats administered diets which provided 0, 80, 400, 2000, 10,000, 50,000, or 10,0000 ppm aspartame (reported to correspond to 0, 4, 20, 100, 500, 2500, or 5000 mg aspartame/kg body weight/day, respectively; purity >98%; DKP <1.5%) from 8 weeks of age until spontaneous death (the last animal died at the age of 159 weeks). Groups of 150 male and 150 female rats were randomly allocated to the control and 3-lowest dose groups (*i.e.* 0, 80, 400, and 2000 ppm) and groups of 100 male and 100 female rats were randomly allocated to the three highest dose groups. Hemolymphoreticular neoplasia observed included lymphoblastic lymphoma and leukemia, lymphocyctic lymphoma, lymphoimmunoblastic lymphoma, histiocytic sarcoma and monocytic leukemia, and myeloid leukemia. The incidence of lymphomas and leukemias in female rats was reported to be statistically significantly greater in the 400-10,0000 ppm dose groups, relative to the control group, furthermore, the increase in incidence of lymphomas and leukemias was reported to be dose-related. The incidence rate of lymphomas and leukemias in females in the control group was less than one-half the incidence rate of lymphomas and leukemias in male rats in the control group. While the incidence rate in control females was low, it was still within the historical range reported by the study authors for their laboratory; however, considering the historical data for lymphomas and leukemias for this laboratory, it is possible that had historical averages been considered, the incidence in the aspartame groups for this endpoint would not have been statistically significant. Moreover, it appeared that the incidence rates of the specific tumor types were quite low and that trends and significant findings were only observed following aggregation.

There was no significant difference in the incidence of lymphomas and leukemias in the aspartame groups compared to the control groups for males or for the combination of males and females.

Within the above study by Soffritti et al. (2005), the incidences of malignant brain tumors (malignant gliomas, mixed gliomas, medulloblastoma, and malignant meningioma) were reported and were generally sparse, not dose-related, and within the historical range for this laboratory.

In a subsequent article by Belpoggi et al. (2006), additional carcinogenicity data from the lifetime rat study by Soffritti et al. (2005) were presented and discussed. These additional data included evaluations of skin, subcutaneous tissue, interscapular fat, mammary glands, Zymbal glands, ear ducts, nasal cavities, oral cavity and lips, pharynx, lung, pleura, stomach (forestomach and glandular), intestine, salivary glands, liver, pancreas, kidneys, pelvis, bladder, prostate, seminal vesicles, testes, ovaries, uterus, vagina, peritoneum, pituitary gland, thyroid gland, parathyroid gland, adrenal glands, pheochromocytoma, meninges, cranial nerves, other peripheral nerves, bones, soft tissues, heart, myxoma, thymus, spleen, lymph nodes, and evaluations for squamous cell carcinoma and osteosarcoma. The study authors reported that there was a significant positive trend for the occurrence of malignant tumor-bearing animals and increasing doses of aspartame in both males and females; however, when the incidence of tumor-bearing animals in each group was compared to that of the control group, only female rats in the 50,000 ppm group had a significantly increased incidence rate. Moreover, it is not standard practice to assess neoplastic potential through the simple cumulative incidence rates of all malignant neoplasms (Albert, 1985; Haseman et al., 1986; McConnell et al., 1988; Brix et al., 2010). Normally, related tumor types are aggregated and analyzed separately.

441 The incidence of hyperplasia of the olfactory epithelium was significantly increased in the 400
442 ppm group in males and the 10,000, 50,000, and 100,000 ppm groups in males and females
443 relative to the control group. A significant positive trend was reported for this endpoint.

444 A significant positive trend was reported for carcinomas in the renal pelvis and ureter in female
445 rats administered aspartame; however, a statistically significant difference in the incidence rate
446 was only observed in the 100,000 ppm group relative to the control group. Belpoggi et al. (2006)
447 reported that there was a statistically significant increase in the total incidence of dysplastic
448 hyplerplasias, dysplastic papillomas, and carcinomas of the renal pelvis and ureter in female rats
449 in the 400, 2000, 10,000, 50,000, and 100,000 ppm groups relative to the control groups. No
450 significant effect or trend was reported in male rats.

451 A significant positive trend was reported for the incidence of malignant Schwannomas of
452 peripheral nerves in male rats; however, this was not reported in female rats and there were no
453 significant differences in the incidence rate of this endpoint between any aspartame group and
454 the control group.

455 Soffritti et al. (2007) summarized a second lifetime carcinogenicity study in Sprague-Dawley rats
456 exposed to diets containing 0, 400, or 2000 ppm aspartame (corresponding to 0, 20, or 100
457 mg/kg body weight/day, respectively; purity >98.7%; DKP <0.3%) with exposure starting from
458 the 12th day of fetal life until spontaneous death (the last animal died at the age of 144 weeks).
459 In this study, groups of 95 male and 95 female rats were allocated to the control group and
460 groups of 70 male and 70 female rats were allocated to each of the aspartame groups.

461 A “seemingly dose-related” decrease in the survival of rats in the aspartame groups was noted.
462 The authors reported a significant increase in the incidence of total malignant tumors in males in
463 the 2000 ppm group relative to the control group; a numeric, but non-significant increase was

464 also reported in female rats. The total number of malignant tumors per 100 animals was not
465 statistically significantly different between the control and aspartame groups. Similarly,
466 Chiozzotto et al. (2011) when reporting on this same study, reported that there was a significant
467 increase in the incidence of benign-tumor bearing animals in females in the 2000 ppm group
468 relative to the control group; however, the total number of tumors per 100 animals were
469 comparable.

470 The incidence of animals bearing lymphomas/leukemias in male and female rats in the 2000
471 ppm group was significantly increased compared to the control group (Soffritti et al., 2007). The
472 incidence rates of lymphomas/leukemias for males was within the historical control range for this
473 laboratory; while the incidence rate of lymphomas/leukemias in females (31.4%) in the 2000
474 ppm group was outside of the reported historical control range (4-25%). Among female rats, the
475 incidence of animals bearing mammary carcinomas was significantly increased in the 2000 ppm
476 group relative to the control group.

477 In a subsequent article by Chiozzotto et al. (2011), additional carcinogenicity data from the study
478 by Soffritti et al. (2007) were presented and discussed. These additional data included
479 evaluations of skin, subcutaneous tissue, mammary glands, Zymbal glands, ear ducts, nasal
480 cavities, oral cavity and lips, tongue, salivary glands, lung, stomach (forestomach and glandular),
481 intestine, liver, pancreas, soft tissues, peritoneum, kidneys, pelvis, testes, ovaries, pituitary
482 gland, thyroid gland, parathyroid gland, adrenal glands, the central nervous system (brain,
483 meninges), the peripheral nervous system (cranial nerves, other peripheral nerves), bones,
484 heart, thymus, spleen, hemolymphoreticular tissues, and evaluations for osteosarcoma. A
485 significant increase in the incidence of mammary adenocarcinoma bearing animals was reported
486 in females in the 2000 ppm group relative to the control group and a significant positive trend
487 was reported for this outcome; however, there was no difference in the total number of tumors
488 per 100 animals for this endpoint.

3.2 Quality Evaluation

The ToxRTool criteria are separated into 5 groups [(I) test substance identification; (II) test system characterization; (III) study design description; (IV) study results documentation; (V) plausibility of study design and data] with some of the criteria have greater importance and needing to be achieved in order for the study to be considered reliable.

The essential criteria and the findings for the seven studies are summarized in Table 3. As demonstrated in Table 3, the studies as conducted are all very comparable with respect to meeting the validation criteria but with the most critical difference being the age at which the animals would have been necropsied. The current validated standards for the assessment of carcinogenicity are the OECD TG 451or, for a combined chronic toxicity study, the OECD TG 453 (OECD 2009a, 2009b). In the U.S., the FDA also has published the guidance "Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food" more commonly referred to as the "Redbook 2000" (U.S. FDA, 2000) which also includes information for conducting a valid carcinogenicity study and combined chronic toxicity/carcinogenicity studies.

OECD TG 451 & 453(2009) states that: "*The duration of the study will normally be 24 months for rodents, representing the majority of the normal life span of the animals to be used. Shorter or longer study durations may be used, dependent on the lifespan of the strain of the animal species in the study, but should be justified. For specific strains of mice, e.g., AKR/J, C3H/J or C57BL/6J strains a duration of 18 months may be more appropriate.*" (OECD, 2009a, 2009b)

Likewise, in the Redbook (U.S. FDA, 2000-2007), the recommended duration is 2 years and for the *in utero* exposure phase for addition to carcinogenicity studies, the Redbook indicates that "*exposure should be continued throughout pre-mating, mating, gestation, and lactation until the*

F1 animals have been weaned. Dosing of all test and control F1 animals should begin at weaning, and continue for 7 days per week for the duration of the study (e.g., 1-year or longer in chronic study and 2-years in bioassay)." (U.S. FDA, 2000).

Thus, the Ramazzini Institute studies which were lifetime studies where the animals were exposed until natural death do not meet the current standards. A flaw with a lifetime exposure approach relates to high background incidence of spontaneous tumors in aging rodents (Arnold et al., 1983; Swenberg 1985; Gad et al., 2007; Hayes et al., 2011) and a higher probability of autolytic tissue changes in animals found dead (EFSA, 2006; Magnuson et al., 2007; NTP-EPA, 2011). For the mouse studies, an increased incidence of liver and lung tumors were reported by Soffritti et al. (2010); however, the species used, Swiss mice, have a high background incidence of both tumor types and it was noted that the incidence in the aspartame study fell with the Institute's historical control ranges.

For the first rat study reported on by the Ramazzini Institute researchers, where exposures to aspartame were from 8 weeks of age to natural death (Soffritti et al., 2005, 2006; Belpoggi et al., 2006), there was a high background incidence of chronic inflammatory changes in vital organs; also questions were raised about the diagnosis of some tumor types (EFSA, 2013). Additionally, there is a high background incidence of spontaneous mammary tumors in female rats (EFSA, 2013). These same flaws applied to the rat study with exposures to aspartame from 12 days of gestation until natural death (Soffritti et al., 2007; Chiozzotto et al., 2011). The only tumor type noted to be present at an increased incidence in both rat studies was the increased incidence of lymphomas/leukemias. Independent evaluations of the data were undertaken by the NTP and by EFSA (2006), who requested additional information on the studies to be able to evaluate the validity of the findings (Magnuson et al., 2007). Criticisms noted were the lack of randomization of the animals into control and test groups and the use of the institute's own rat strains which were noted to have a very high incidence of infection (Magnuson et al., 2007; Schoeb et al.,

2009; Ward et al., 2009). Survival was also noted to be poorer for some of the control groups. The NTP and U.S. Environmental Protection Agency sponsored an independent pathological review of data from five other studies conducted at the institute (NTP-EPA, 2011) and found many of the malignant tumors and lymphoid dysplasias diagnosed by the Ramazzini institute to be attributable to chronic infections in the animals. Given the flaws in these studies, the Expert Panel convened to assess aspartame in 2006 (Magnuson et al., 2007) and the UK Committee on Carcinogenicity of Chemicals in the Food Consumer Products and the Environment (COC, 2006; reported in EFSA, 2013), found the Ramazzini institute studies to not be credible.

The transgenic models, which would include genetically modified animal strains that are more susceptible to develop cancer (*e.g.* knockout mouse models), were developed with the intention of allowing for the detection of carcinogens earlier so that fewer animals could be used, and followed for a shorter duration. However, these studies are not currently considered by regulatory agencies to be substitutes for the 2-year standard rodent carcinogenicity studies. As reported in the FDA (2000-2007) Redbook, the Office of Food Additive Safety will consider a transgenic study as supplemental data while the Center for Drug Evaluation and Research will accept 6-month studies with transgenic mice as a substitute for one of the rodent carcinogenicity studies (U.S. FDA, 2000). The OECD also comments on the utility of conducting a transgenic mouse model in place of the bioassay in a second species but in addition to the full standard cancer bioassay in rats (OECD, 2012). Thus, the NTP (2005) aspartame studies in transgenic models are considered to be supportive of a lack of carcinogenic potential for aspartame.

Finally, it is noted that the Searle Studies predate both the OECD guidance documents and the FDA Redbook, however, the methodology followed is in keeping with the principle guidance. The main methodological difference for the studies is that the number of animals used was 40 animals per group rather than the recommended 50 animals at the start. The question about the number of animals used had been raised by the Public Board of Inquiry as discussed in the

562 Commissioner's Final Decision (U.S. FDA, 1981). In the Commissioner's Final Decision on
563 aspartame, the following is noted: "*One additional point which needs to be addressed briefly is*
564 *study size. As noted above, the Board suggested that this study should have included more*
565 *animals. The protocol used in E-70 called for 40 rats/sex in each of the two treated groups and*
566 *60 rats/sex in the control group. Searle has demonstrated that this allocation of treated and*
567 *control animals is comparable to the Bureau's current allocation standard (50 animals/sex for*
568 *both treated and control groups) in terms of its ability to detect an increased tumor rate (Searle's*
569 *Exceptions at 40. Chart 1). Thus. I do not share the Board's concern about study size*."

570 In any case, regardless of the number at the start of treatment, survivorship in the Searle study
571 E70 was such that more than 20 animals per group remained alive at least through weeks 80-
572 90, a guideline for valid interpretation of results from currently conducted 2-year rodent
573 carcinogenicity studies (U.S. FDA, 2001).

574 Furthermore, the results for both rat studies were comparable with no differences noted for
575 animals with an *in utero* exposure period included compared to those for which exposure started
576 after weaning. Thus, the number of animals in both Searle rat studies (E33/34 and E70 –
577 Searle, 1973, 1974b) combined allowed for an evaluation of tumors in 80 rats/sex in each of the
578 2000 and 4000 dose groups compared to 120 rats/sex from the control groups.

579 In applying the Klimisch categories for reliability, the Searle studies can be concluded to be
580 reliable with restrictions, or Code 2, as the methodology was generally in compliance with GLP.
581 The transgenic studies also were considered to be reliable with restrictions, Code 2; while there
582 is, as of 2017, no standard OECD method for conducting a study in transgenic mice, regulatory
583 agencies have been accepting such studies as the second species study in combination with a
584 full rat bioassay to support new drugs. The Ramazzini Institute studies are considered to be not

reliable (Code 3) on the basis of an inappropriate study design given the many flaws that have been documented in several publications and authoritative regulatory reviews.

4. Discussion and conclusions

Several studies in rats and mice were identified which investigated the carcinogenic potential of aspartame. These included standard cancer animal bioassays (Searle E33-34, Searle E70 (Searle, 1973, 1974b), transgenic mouse studies (NTP, 2005), and lifetime studies from the Ramazzini Institute (Soffritti et al., 2005, 2007, 2010). Three of the studies, one rat study from Searle and one rat and one mouse study from Ramazzini, included an *in utero* exposure phase. It was noted that individual results of the three Ramazzini cancer in life bioassays were discussed in more than one publication (Belpoggi et al., 2006; Soffritti et al., 2006, 2014; Chiozzotto et al., 2011). This could be a confusing factor if an evaluation on the basis of a weight of evidence approach were undertaken, as the number of Ramazzini Institute studies available may appear to be greater than three.

Also identified were tumor promotion studies (Ito et al., 1983; Hagiwara et al., 1984) and studies investigating specific cancers: brain tumors in rats (Ishii, 1981) and pancreas acinar carcinoma development in the mouse (Dooley et al., 2017).

One other cancer "study" identified in the literature search, but not previously discussed herein, was a meta-analysis of aspartame rodent studies (Mallikarjun and Sieburth, 2015). For this review, Mallikarjun and Sieburth (2015) included the data from the three Searle studies, the three NTP studies, the three Ramazzini Institute studies and also the brain tumor Wistar rat study conducted by Ishii (1981). The range of dose employed in the different studies were categorized as regular, (2000-3125 ppm diet), high (4000-10,000 ppm diet) and very high (32,000-50,000 ppm diet). Six independent aggregate effect sizes were determined and the

608 authors used an odds ratio measure to determine cancer risk. The number of animals having
609 one or more malignant neoplasms was the outcome variable assessed. Overall, the authors
610 concluded that, while the finding of the individual studies were conflicting, the results of this
611 meta-analysis based on aggregate effect sizes indicated that aspartame was not related to an
612 increase in the risk of malignant tumors. The study is interesting, but it is questionable in terms
613 of validity since combining studies with very different methodologies and for which the age of the
614 animals at necropsy was varied, may not yield meaningful results.

615 Some of the studies included in the analysis, notably the Searle studies and the Ishii (1981)
616 brain tumor study, Mallikarjun and Sieburth (2015) appeared to only include animals having
617 malignant brain tumors and not the total number of rodents having any malignant tumor as had
618 been reported in the Ramazzini Institute studies. A meta-analysis of the results of a number of
619 cancer bioassays in animals (*i.e.* number of animals with a malignant tumor compared to the
620 controls) would likely be more reliable in situations where all the studies being compared had
621 followed the same OECD guidance and in same strains and using the same pathology
622 assessment criteria. Moreover, the evaluation of specific tumor types or groups of tumor types
623 would have been preferable to the assessment of the number of animals having a malignant
624 tumor.

625 For this evaluation, the available carcinogenicity studies on aspartame with complete
626 histopathological examinations, which included the original Searle studies, the NTP transgenic
627 model studies, and the Ramazzini Institute studies, were evaluated against the essential quality
628 criteria for reliability from the ToxRTool and coded based on the original Klimisch grading
629 system.

630 None of the studies were judged to be Klimisch Code 1, or reliable without restrictions, for
631 several reasons. The Searle studies predated the GLP OECD and FDA Redbook standards,

and 40 animals/sex per treatment group were used instead of 50. With respect to the NTP (2005) studies, there is no validated OECD guideline or standardized protocol for transgenic mouse models, although such studies are routinely accepted by regulatory authorities as supporting data. The Searle studies and NTP transgenic mouse studies were of sufficient quality for a Klimisch Code 2, reliable with restrictions.

The Ramazzini Institute studies used a lifetime model of their own design and questions about the interpretation of the aspartame studies, as well as issues raised about the impact of chronic infections on pathology in other institute studies, have been documented (Magnuson et al., 2007; Schoeb et al., 2009; Ward et al., 2009; NTP-EPA, 2011; EFSA, 2013) which limit both the credibility and reliability of these three studies.

As many of the problems noted in the Ramazzini Institute studies could be attributed directly to the practice of conducting the necropsies in animals that died or were terminated near end of life, and given that other problems noted were significant enough to impact the overall results, these studies were concluded to be Klimisch Code 3, not reliable. The aspartame studies and many other studies conducted at the Ramazzini Institute have been scrutinized by regulatory agencies and found to be deficient (COC, 2006; EFSA, 2009a, 2009b; NTP-EPA, 2011; EFSA, 2013). It is notable that the Ramazzini Institute has a history of publishing results of cancer bioassays on materials (*e.g.* sucralose and Coca-Cola in addition to aspartame), for which there is no other evidence of carcinogenicity, including those materials extensively studied for carcinogenic potential in Guideline bioassays.

Finally, while biological plausibility has not been directly addressed in this publication, aspartame is noted to be used in foods and beverages in very low amounts and following ingestion, aspartame is hydrolyzed in the gut to the aspartic acid, phenylalanine and methanol which are metabolized *via* normal well-characterized endogenous metabolic pathways

(Magnuson et al., 2007; EFSA, 2013). As a result, there would seem to be no reasonable mode of action that could be invoked to infer that aspartame has carcinogenic potential. This, along with a lack of genotoxic activity (Magnuson et al., 2007; EFSA, 2013), supports the conclusion, which can also be drawn from the available studies that are considered at least Klimisch Code 2 ("reliable with restrictions"), that aspartame is without carcinogenic potential. The recent studies conducted by the Ramazzini Institute were found to be unreliable, and therefore, have no impact on this conclusion.

664 **Figure Caption**

665 **Figure 1.** Structure of N-L-alpha-Aspartyl-L-phenylalanine 1-methyl ester or aspartame

666

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Declaration of interest

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Table 1 Summary of carcinogenicity studies of aspartame

Type of study	Strain & species (number per group)	Route/dose/duration	Parameters evaluated	Carcinogenic Findings	Comments	Reference
Searle Studies						
Oral feeding carcinogenicity study in mice	ICR Swiss Mice (36/sex/group) (72/sex/control)	Diet / 0, 1000, 2000, 4000 mg/kg bw/day/104 week	Appearance, behavior, body weight, food consumption, survival, hematology, blood chemistry, urine analysis, ophthalmoscopic exam, gross necropsy, organ weights, histopathology, tumor incidence	No increases in tumor incidences	NOAEL highest dose tested	Searle E75, (Searle, 1974a)
Oral feeding carcinogenicity study in rats	Charles River Albino rats (40/sex/group) (60/sex/controls)	Diet / 0, 1000, 2000, 4000 or 6000 to 8000 mg/kg bw/day /104 weeks [high dose was given 6000 mg/kg bw/day from week 0 (of study) to 16, 7000 mg/kg bw from week 16 to 44 and 8000 mg/kg bw/day from week 44 to 104]	Appearance, behavior, body weight, food consumption, survival, hematology, blood chemistry, urine analysis, ophthalmoscopic exam, gross pathology, organ weights, histopathology, tumor incidence	No increases in tumor incidences	Sporadic findings of intracranial neoplasms: astrocytoma; astrocytoma + ependymal; oligodendroglioma; ependymoma; meningeal Sarcoma; unclassified glioma	Searle E33-34 (Searle, 1973)
Oral feeding carcinogenicity rat study from fetal life to 104 weeks	Charles River Albino rats (40/sex/group; 60/sex/controls)	Diet / 0, 2000, 4000 mg kg/day / *in utero*, lactation, 104 weeks	Appearance, behavior, body weight, food consumption, survival, hematology, blood chemistry, urine analysis, ophthalmoscopic	No increases in tumor incidences	Sporadic findings of intracranial neoplasms: astrocytoma, ependymoma, menigeoma	Searle E70 (Searle, 1974b)

Type of study	Strain & species (number per group)	Route/dose/duration	Parameters evaluated	Carcinogenic Findings	Comments	Reference
			exam, gross pathology, organ weights, histopathology, tumor incidence & liver phenylalanine hydroxylase activity			
Ishii						
Oral feeding study in rats – limited to brain cancer	SLC Wistar rats (86/sex/group) 6 weeks of age	Diet / 0, 1, 2, 4 g/kg/day Interim sacrifice 10/sex/group at 26 weeks 16/sex/group at 52 weeks 59 or 60/sex/group at 104 weeks	Survival , gross pathology, and histopathology focused on brain tumors	No increase in brain tumor incidence	Sporadic findings of astrocytoma, atypical astrocytoma, oligodendroglioma, ependymoma. Tumors were primarily identified in rats that had died during the study or were terminated in extremis.	Ishii, 1981
NTP transgenic mouse						
Oral feeding carcinogenicity study in transgenic mice	Genetically modified Tg.AC Hemizygous mice (15/sex/group)	Diet /0, 3125, 6250, 12500, 50000 ppm/40 weeks	Clinical observations, body weight, feed consumption, survival, organ weights, gross necropsy, histopathology	NTP concluded that aspartame did not cause cancer	Transgenic mouse model is more susceptible to develop tumors and at an early stage	NTP, 2005
Oral feeding carcinogenicity study in transgenic mice	Genetically modified [B6.129-Trp53^{tm1Brd} (N5) haploinsufficient] mice (15/sex/group)	Diet /0, 3125, 6250, 12500, 50000 ppm/40 weeks	Clinical observations, body weight, feed consumption, survival, organ weights, gross necropsy, histopathology	NTP concluded that aspartame did not cause cancer	Transgenic mouse model is more susceptible to develop tumors and at an early stage	NTP, 2005
Oral feeding carcinogenicity study in transgenic mice	Genetically modified [B6.129-Cdkn2a Deficient] mice (15/sex/group)	Diet /0, 3125, 6250, 12500, 50000 ppm/40 weeks	Clinical observations, body weight, feed consumption,	NTP concluded that aspartame did not cause cancer	Transgenic mouse model is more susceptible to develop tumors and at an early	NTP, 2005

Type of study	Strain & species (number per group)	Route/dose/duration	Parameters evaluated	Carcinogenic Findings	Comments	Reference
			survival, organ weights, gross necropsy, histopathology		stage	
Ramazzini Institute						
Oral feeding carcinogenicity study in rats	Sprague-Dawley Rats (100-150/sex/group) 8 weeks old at study initiation	Diet – ad libitum/0, 80, 400, 2000, 10000, 50000, 100000 ppm (~0, 4, 20, 100, 500, 2500, 5000 mg/kg bw/day) Lifetime (until spontaneous death; last animal died at 159 weeks).	Clinical observation, body weight, drinking water and feed consumption, complete necropsy, histopathology, survival	carcinomas of renal pelvis and ureter (positive trend in F); malignant Schwannomas of peripheral nerves (positive trend in M – observed in 9 treated F vs. 0 F control); lymphomas-leukemias (positive trend in M; sign. increase in high dose F)		Soffritti et al., 2005, 2006, 2008 Belpoggi et al., 2006
Oral feeding carcinogenicity rat study from fetal life to natural death	Sprague-Dawley rats (70 to 95/sex/group)	Diet /0, 400, 2000 ppm (0, 20, 100 mg/kg bw/day) /Prenatal (from GD 12) to spontaneous death (last animal died at 144 weeks)	Clinical observation, body weight, drinking water and feed consumption, complete necropsy, histopathology, survival	Lymphomas/leukemia (sign. increase high dose M; sign. trend in F; sign. increase high dose F) Mammary cancer (sign. trend; sign increase high dose F)		Soffritti et al., 2007, 2008 Chiozzotto et al., 2011
Oral feeding carcinogenicity mouse study from fetal life to natural death	Swiss mice (62 to 122/sex/group)	Diet/ 0, 2000, 8000, 16000, 32000 ppm (0, 242, 987, 1919, 3909 mg/kg bw/day) /Prenatal (from GD 12) to 139 weeks	Clinical observation, body weight, drinking water and feed consumption, complete necropsy, histopathology, survival	Hepatocellular carcinomas (sign. trend in M; sign. increase at two highest doses M) Alveolar/bronchiolar carcinomas (sign. trend M; sign. increase high dose M); no sign. findings in F		Soffritti et al., 2010
Oral drinking water carcinogenicity mouse study from fetal life	C57BL/6 Ela1-Tag Mice (12 M aspartame; 13 M controls)	Drinking water/ 0 or 0.035%)/ utero (GD no specified) to 21 weeks of age	Focused on pancreatic cancer as time to first tumor; tumor growth rate; assessment based on MRI	No effect on pancreatic acinar carcinoma development		Dooley et al., 2017

Research papers from the Ramazzini lab also tallied total number of benign and malignant tumors regardless of origin or site, to report on total tumor bearing animals.
bw = body weight; F = female; GD = gestation day; M = male; NOAEL = no observed adverse effect level

Table 2 Summary of tumor incidence data from oral carcinogenicity studies of aspartame

Study (dose)	Intracranial neoplasms[a]	Cranial Schwannomas of peripheral nerves	Total lymphomas/ leukemia[c]	Carcinomas renal pelvis & ureter carcinomas	Mammary	Hepatocellular adenomas + carcinomas	Alveolar bronciolar adenomas + carcinomas
Searle studies							
104 week ICR Swiss Mice (0, 1, 2, 4 g/kg/day)	NS	NS	NS	NS	NS	NS	NS
104 week Sprague-Dawley Rats (0, 1, 2, 4 or 6 to 8 g/kg/day)	M: Control: 0 or 1/60[c] 1: 2/40 2: 1/40 4: 4/40 8: 0/40; F: Controls: 0/60 1: 2/40 2: 0/40 4: 1/40 8: 2/40	NS	NS	NS	NS	NS	NS
104 week Sprague-Dawley Rats from GD 0[d] (0, 2, 4 g/kg/day)	M: Control: 3/60 2: 2/40 4: 1/40; F: Control: 1/60; 2: 1/40 4: 1/40	NS	NS	NS	NS	NS	NS
NTP Transgenic mouse study							
40 week Carcinogenicity study in genetically modified Tg.AC Hemizygous mice (0, 3125, 6250, 12500, 50000 ppm diet)	NS	NS	NS	NS	NS	NS	NS

Study (dose)	Intracranial neoplasms[a]	Cranial Schwannomas of peripheral nerves	Total lymphomas/ leukemia[c]	Carcinomas renal pelvis & ureter carcinomas	Mammary	Hepatocellular adenomas + carcinomas	Alveolar bronciolar adenomas + carcinomas
40 week Carcinogenicity study in genetically modified B6.129-Trp53^{tm1Brd} (N5) haploinsufficient mice (0, 3125, 6250, 12500, 50000 ppm diet)[e]	NS	NS	NS	NS	NS	NS	NS
40 week Carcinogenicity study in genetically modified B6.129-Cdkn2a Deficient mice (0, 3125, 6250, 12500, 50000 ppm diet)	NS	NS	NS	NS	NS	NS	NS
Ishii Study							
104-week with 26-week and 52-week satellite groups. Wistar rats (0, 1000, 2000, or 4000 mg/kg body weight/day)	0: 1/120 1000: 1/120 2000: 2/120 4000: 1/120 26-week satellite: 0/20 52-week satellite: 0/32	NR	NS 1 male in 4000 mg/kg bw/day group (co-occurred with reported incidence of a small cerebral astrocytoma)	NR	NR	NR	NR
Ramazzini Institute Studies							
Lifetime Swiss mice from GD12 (0, 2000, 8000, 16000, 32000 ppm)	NS	NS	NS	NS	NS	M: Control: 15/117 (12.8) 2000: 22/103 (21.4%) 8000: 13/62 (21.0%) 16000: 16/64 (25%)SS 32000: 17/83	M: Control: 15/117 (12.9%) 2000: 15/103 (14.6%) 8000: 14/62 (22.6%) 16000: 15/64 (23.4%) 32000: 17/83

Study (dose)	Intracranial neoplasms[a]	Cranial Schwannomas of peripheral nerves	Total lymphomas/ leukemia[c]	Carcinomas renal pelvis & ureter carcinomas	Mammary	Hepatocellular adenomas + carcinomas	Alveolar bronciolar adenomas + carcinomas
						(20.5%) F (NS): Control: 1/102 (1%) 2000: 8/122 (6.5%) 8000: 2/73 (2.7%) 16000: 2/64 (3.1%) 32000: 0/62	(20.5%) F (NS): Control: 11/102 (10.8%) 2000: 19/122 (15.6%) 8000: 9/73 (12.3%) 16000: 9/64 (14.1%) 32000: 5/62 (8.06%)
Lifetime Sprague-Dawley Rats (0, 80, 400, 2000, 10000, 50000, 100000 ppm diet)	M: Control: 0/150 80: 2/150 400: 0/150 2000: 2/150 10000: 1/100 50000: 2/100 10000: 1/100 F: Control: 0/150 80: 1/150 400: 0/150 2000: 1/150 10000: 1/100 50000: 1/100 10000: 1/100	M: Control: 1/150 80: 1/150 400: 1/150 (+2 not cranial) 2000: 2/150 10000: 2/100 50000: 3/100 100000: 3/100 (+1 not cranial) F: Control: 0/150 80: 1/150 (+1 not cranial 400: 0/150 2000: 1/150 (+2 not cranial) 10000: 1/100 50000: 1/100 100000: 1/100 (+1 not cranial)	M: Control: 31/150 80: 23/150 400: 25/150 2000: 33/150 10000: 15/100 50000: 20/100 100000: 29/100 F: Control: 13/150 80: 22/150 400: 30/150 2000: 28/150 10000: 19/100 50000: 25/100 100000: 25/100	M: Control: 0/150 80: 0/150 400: 0/150 2000: 1/150 10000: 1/100 50000: 1/100 100000: 1/100 F: Control: 0/150 80: 1/150 400: 3/150 2000: 3/150 10000: 3/100 50000: 3/100 100000: 4/100	NS	NS	NS
Lifetime Sprague-Dawley rats from GD12 (0, 400, 2000 ppm)	NS	NS	M: Control: 9/95 400: 11/70 2000: 12/70** F: Control: 12/95 400: 12/70	NS	F: Control: 5/95 400: 5/70 2000: 11/70	NS	NS

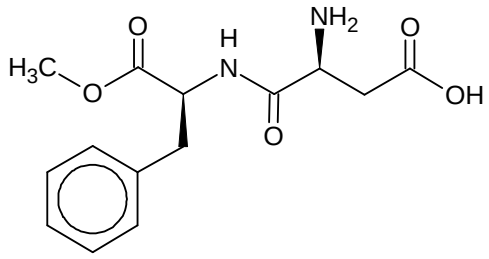
Study (dose)	Intracranial neoplasms[a]	Cranial Schwannomas of peripheral nerves	Total lymphomas/ leukemia[c]	Carcinomas renal pelvis & ureter carcinomas	Mammary	Hepatocellular adenomas + carcinomas	Alveolar bronciolar adenomas + carcinomas
			2000: 22/70				

NR = not conducted; NS = not observed to be different from controls; M = males; F = females; GD = gestational day; SS = statistically significant
[a] result encompasses several different types of intracranial neoplasms including astrocytoma, astrocytoma with ependymal components, oligodendroglioma, ependymoma, meningeal sarcoma, glioma unclassified, meningeoma
[b] result encompasses several hystocytotypes including lymphoblastic lymphoma, lymphoblastic leukemia, lymphocytic lymphoma, lymphoimmunoblastic lymphoma, histiocytic sarcoma, monocytic leukemia, and myeloid leukemia. The majority were lymphoimmunoblastic lymphoma and histiocytic sarcoma.
[c] reported to be 0/60 in Searle study and 1/60 in the authentication review conducted by the FDA when their decision to approve aspartame was challenged
[d] parental group pre-treated with aspartame for 60 days prior to mating.
[e] the NTP questioned the sensitivity of the strains p53 haploinsufficient mice for detecting a carcinogenic response
** significant compared to concurrent control but noted to be within historical range.

Table 3 Assessment of aspartame carcinogenicity studies against essential criteria for reliability based on Klimisch

	Searle mouse study	Searle rat study	Searle rat study from *in utero*	NTP transgenic mouse studies	Ramazzini mouse study from GD12	Ramazzini rat study	Ramazzini rat study from GD12
Criteria							
Criteria Group I: Test substance identification							
Test substance is identified?	yes	yes	yes	yes	yes	yes	yes
Criteria Group II: Test organism characterization							
Species is given?	yes	yes	yes	yes	yes	yes	yes
Criteria Group III: Study design description							
Administration route is given?	yes	yes	yes	yes	yes	yes	yes
Application media for dose or concentration is appropriate?	yes	yes	yes	yes	yes	yes	yes
Frequency and duration of exposure, and time-points of observations are explained?	yes	yes	yes	yes	yes	yes	yes
Negative controls were included?	yes	yes	yes	yes	yes	yes	yes
The number of animals per group was given?	yes	yes	yes	yes	yes	yes	yes
Criteria Group IV: Study results documentation <u>(no essential criteria under this group)</u>							
Criteria Group V: Plausibility of study design and results							
Is the study design chosen appropriate?	yes	yes	yes	supporting data only[a]	no	no	no

GD = gestational day; NTP = National Toxicology Program
[a] Transgenic studies are supporting but have not been recommended as a replacement of the OECD standard



- Aspartame cancer bioassay methods evaluated using quality criteria.
- Pivotal studies evaluated against the Klimisch grading system.
- Studies with highest rating support that aspartame is not carcinogenic in rodents.