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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

SUMMARY

The European Food Safety Authority (EFSA) has been asked by the European Commission to assess the carcinogenicity study performed by the European Ramazzini Foundation of Oncology and Environmental Sciences (ERF) on the artificial sweetener aspartame, which was reported in publications in 2005 and 2006. The ERF considered that the results of their study indicate that aspartame is a 'multipotential carcinogenic agent', based on increases in malignant tumour-bearing animals, lymphomas/leukaemias (primarily in female rats), transitional cell carcinomas of the renal pelvis and ureter, also in female rats, and malignant schwannomas of peripheral nerves. EFSA asked its Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) to review the study as a matter of high priority.

Aspartame has been used as a sweetener in foods and as a table-top sweetener for more than 20 years in many countries throughout the world. In Europe, it was first authorised for use by several Member States during the 1980s and was approved for use throughout the European Union in 1994, following thorough safety evaluations by the European Commission (EC) Scientific Committee on Food (SCF).

Aspartame has undergone extensive testing in animals and studies in humans, including four animal carcinogenicity studies conducted during the 1970s and early 1980s. These studies, together with studies on genotoxicity, were evaluated by regulatory bodies worldwide and it

was concluded that they did not show evidence of genotoxic or carcinogenic potential for aspartame. Since its approval, however, the safety of aspartame has been repeatedly questioned, with discussions focusing not only on the safety of aspartame itself, but also on the safety of its breakdown products, aspartic acid, phenylalanine and methanol. All these substances occur naturally in the body. In response to such questions, the SCF undertook a further review of all the data on aspartame in 2002 and concluded that there was no need to revise the outcome of their earlier risk assessment or the previously established Acceptable Daily Intake (ADI) for aspartame, of 40 mg/kg body weight (bw).

The AFC Panel has assessed the new carcinogenicity study, using not only the ERF publications but also a more extensive report provided to EFSA by the ERF at the end of 2005 and further data from the same study provided by ERF in April 2006. The Panel noted that this lifetime study, using more dose groups and more animals per group than conventional carcinogenicity studies, represented a substantial effort and had the potential to be more sensitive to low incidence effects. After its evaluation the Panel considers that the study has flaws which bring into question the validity of the findings, as interpreted by the ERF. In particular, the high background incidence of chronic inflammatory changes in the lungs and other vital organs and tissues and the uncertainty about the correctness of the diagnoses of some tumour types were major confounding factors in the interpretation of the findings of the study.

The Panel's conclusions on the findings of the ERF study include the following:

- The increased incidence of lymphomas/leukaemias reported in treated rats was unrelated to aspartame, given the high background incidence of chronic inflammatory changes in the lungs and the lack of a positive dose-response relationship. It is well-known that such tumours can arise as a result of abundant lymphoid hyperplasia in the lungs of rats suffering from chronic respiratory disease. The most plausible explanation of the findings in this study with respect to lymphomas/leukaemias is that they have developed in a colony suffering from chronic respiratory disease. The slight increase in incidence of these tumours in rats fed aspartame is considered to be an incidental finding of the ERF study and can therefore be dismissed.

- The preneoplastic and neoplastic lesions of the renal pelvis, ureter and bladder occurring primarily in female rats along with renal calcification were most probably treatment-related, at least at the higher doses. It is widely accepted that the effect is a high dose effect of irritant chemicals or chemicals producing renal pelvic calcification as a result of imbalances in calcium metabolism, specific to the rat. The Panel considers that these effects are of no relevance for humans.

- The data on total malignant tumours do not provide evidence of a carcinogenic potential of aspartame. In the opinion of the Panel, the aggregation of all malignant tumour incidences or all malignant tumour-bearing animals for statistical purposes is not justified, given that, as explained above, the lymphomas/leukaemias and the renal tumours should have been excluded from the analysis.

- Concerning the malignant schwannomas, the Panel notes that the numbers of tumours were low, the dose-response relationship, while showing a positive statistical trend in males, was very flat over a wide dose range and there is also uncertainty about the diagnosis of these tumours. The Panel concludes that this finding can only be fully

evaluated following a histopathological peer-review of all relevant slides related to the nervous system in the ERF study and if necessary also from the historical controls.

The Panel takes note of the previous evaluations of aspartame by the SCF and other expert bodies, the negative results of recent carcinogenicity studies carried out by the US National Toxicology Program on aspartame in transgenic mice. The Panel was also informed about a recent epidemiological study carried out by the US National Cancer Institute in which no increase in brain or blood related cancers was reported to be associated with aspartame consumption. The Panel also takes note of the comprehensive studies on the substance indicating that aspartame does not have genotoxic activity.

Kinetic data in humans indicate that dose levels around the acceptable daily intake (ADI) (40 mg/kg bw/d), even when taken as a bolus dose, do not lead to systemic exposure to aspartame. Furthermore, exposure to any of its breakdown products, including methanol or formaldehyde, is negligible.

The Panel considers that no significant new data have emerged since 2002 on aspects other than carcinogenicity and there is therefore no reason to review the previous SCF opinion on aspartame.

The Panel notes that dietary exposure to intense sweeteners in the population has been assessed in a number of European countries. In all of these studies, dietary exposure to aspartame was well below the ADI of 40 mg/kg bw (up to 10 mg /kg bw), even in high consumers.

In summary, the Panel concludes, on the basis of all the evidence currently available from the ERF study, other recent studies and previous evaluations that there is no reason to revise the previously established ADI for aspartame of 40 mg/kg bw.

KEYWORDS
Aspartame, L-aspartyl-L-phenylalanine methyl ester, artificial sweetener, lifetime study, CAS No. 22839-47-0, E 951, intense sweetener

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BACKGROUND

In June 2005**,** the European Food Safety Authority (EFSA) was informed about the outcome of a long-term carcinogenicity study on the sweetener aspartame, carried out by the Cesare Maltoni Cancer Research Center, European Ramazzini Foundation of Oncology and Environmental Sciences, Bologna, Italy (the Ramazzini Foundation, ERF). The ERF considered that the results of their study indicate that aspartame is a multipotential carcinogenic agent, and recommended that a re-evaluation of the present guidelines on the use and consumption of aspartame should be undertaken.

EFSA, following a request from the European Commission, requested its Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) to review these findings, as a matter of high priority.

TERMS OF REFERENCE

In accordance with Article 29 (1) (a) and Article 31 of Regulation (EC) No. 178/2002, the European Commission requested the European Food Safety Authority

- to assess the published study and
- depending on the outcome of this assessment, to review the previous SCF opinion on the safety of this substance, in the light of the new study.

ASSESSMENT

1. Introduction

The sweetener aspartame has been authorised for use in foods and as a table-top sweetener for more than 20 years and in many countries throughout the world. The safety of aspartame has been extensively investigated through clinical and laboratory research, intake studies and post-marketing surveillance. In the European Union (EU), aspartame was first authorised for use by several Member States in the 1980s and European legislation harmonising its use in foodstuffs was introduced in 1994, following thorough safety evaluations (in 1984, 1988 and 1997) by the European Commission (EC) Scientific Committee on Food (SCF, 1985, 1989, 1997).

Since its approval, however, the safety of aspartame has been repeatedly questioned, with discussions focusing not only on the safety of aspartame itself but also on the safety of its breakdown products aspartic acid, phenylalanine and methanol. All these substances occur naturally in the body. A further review of all the original and more recent data on aspartame was carried out in 2002 by the SCF (SCF, 2002). Both published and unpublished data, including all information from genotoxicity studies, carcinogenicity studies in animals and studies in humans, were considered at that time and the SCF concluded that there was no need to revise the outcome of the earlier risk assessment or the previously established Acceptable Daily Intake (ADI) for aspartame, of 40 mg/kg body weight (bw).

Soffritti and co-workers, in publishing the results of the ERF long-term carcinogenicity study (Soffritti *et al*., 2005; Soffritti *et al.*, 2006), concluded that their results provided evidence that aspartame is a ´multipotential carcinogenic agent´, even at a dose (20 mg/kg bw/day) corresponding to half of the current ADI, and suggested that this would necessitate the

updating of current scientific advice on the safety of aspartame. They reported an increased incidence of:

- malignant tumour-bearing animals (both males and females),
- lymphomas/leukaemias in female rats, with a positive trend in both males and females,
- transitional cell carcinomas of the renal pelvis and ureter and their precursors (dysplasias) in female rats, and
- malignant schwannomas of peripheral nerves with a positive trend in male rats.

The study report of the ERF long-term carcinogenicity study on aspartame, containing individual animal tumour data and other information not presented in the Soffritti *et al.* publications, was provided to EFSA in December 2005 (Soffritti and Belpoggi, 2005). On the 19 April 2006 EFSA was provided with additional data on type and severity of non-neoplastic lesions in individual animals and tumour data in untreated historical controls of ERF (period 1984-1991).

EFSA's AFC Panel, in addressing the Terms of Reference provided by the European Commission, has assessed this unpublished report and the additional data provided in April 2006, along with the two recent publications on the study (Soffritti *et al.,* 2005; 2006), in the light of the previous opinions of the SCF. The Panel has also considered more recent genotoxicity and carcinogenicity studies which have become available since the publication of the most recent SCF opinion in 2002, in order to evaluate the relevance of these findings for human health.

2.1 Study design and conduct

The study design and conduct are described in Soffritti *et al.* (2006) and detailed in the unpublished study report (Soffritti and Belpoggi, 2005). The study was a large lifetime study using 7 dose groups and 100-150 rats/sex/group. The animals used were male and female Sprague Dawley rats from the in-bred colony of the ERF.

Aspartame was administered from 8 weeks of age at levels of 0, 80, 400, 2000, 10,000, 50,000 or 100,000 mg/kg in the diet, reported by the authors to be equivalent to an aspartame intake of 0, 4, 20, 100, 500, 2500 or 5000 mg/kg bw/day, based on average animal body weights of 400 g and an average feed consumption of 20 g/day for both sexes. The aspartame used was a food grade material produced by Nutrasweet, with a specification for aspartylphenylalanine diketopiperazine (DKP) of < 1.5% and free phenylalanine of < 0.5%. Purity was checked by infrared absorption spectrometry.

Animals were housed 5 per cage, corresponding approximately to an area of 200 cm^2 per rat. The in-life phase of the study was conducted from June 1997 to May 2000 and the in-life phase ended with the spontaneous death of the last rat (159 weeks old) in week 151. The study is reported to have been conducted in accordance with Good Laboratory Practice (GLP) and internal quality control procedures were stated to be implemented according to GLP.

Parameters measured in the study were as follows:

- water and feed consumption;

- body weights;
- mortality;
- clinical observations (status, behavior, toxic effects);
- general pathological lesions, macroscopic and microscopic;
- types of tumours and tumour precursors;
- number of benign and malignant tumours and tumour precursors for each tumour bearing animal;
- number of malignant tumours per 100 animals;
- number and percentage of animals bearing different types of tumours and tumour precursors;
- latency time of benign and malignant tumours and tumour precursors.

All organs and tissues were preserved in 70% ethyl alcohol, except for bones which were fixed in 10% formalin and then decalcified with 10% formaldehyde and 20% formic acid in water solution. Histopathology was routinely performed on a comprehensive range of organs and tissues of all animals from each group. Sections from all animals were examined and evaluated histopathologically, first by a junior pathologist and then by a senior pathologist. A working group of pathologists from the US National Toxicology Program (NTP) provided a second opinion for a limited number of tumours and their precursors selected by the ERF and considered by the ERF pathologists to be related to treatment with aspartame. A short report of the outcome of this NTP Pathology Working Group has been provided to EFSA (Hailey, 2004).

Two statistical tests were used to analyse neoplastic and non-neoplastic lesion incidence data. The Cochran-Armitage trend test (Armitage, 1971; Gart *et al*., 1979) was used to test for linear trends in tumour incidence. Also used was the poly-*k* test (Bailer and Portier, 1988; Portier and Bailer, 1989; Piegorsch and Bailer, 1997), a survival-adjusted quantal response modification of the Cochran-Armitage test.

2.2 AFC Panel comments on study design and conduct

Although there is a statement in the report that the study was conducted in accordance with GLP, it was confirmed that neither the ERF nor the study in question has been inspected by the “Istituto Superiore di Sanità” (the National Institute of Health, the Italian GLP compliance monitoring authority). The author’s claim of GLP compliance of this study cannot, therefore, be confirmed at this stage (written correspondence of Dutch GLP Inspectorate to the EFSA Aspartame working group, 2006). EFSA has asked the ERF for written confirmation on the GLP but this has not been received at the time of the publication of the opinion.

The study report (Soffritti and Belpoggi, 2005) did not refer to a specific Test Guideline followed for conduct of a long-term carcinogenicity study. For the purpose of the current evaluation, the Panel compared the ERF study with the OECD Test Guideline 451, “Carcinogenicity Studies”. Deviations from Test Guideline OECD 451 were as follows:

- A complete analysis of the test substance was not provided in the report, as required by the Test Guideline. The Test Guideline recommends that “the composition of the

test substance, including major impurities, should be known prior to initiating the study".

- The report indicates that "at the start of the experiment, the various concentrations and the stability of aspartame in the feed was evaluated" but neither the analytical procedure nor the results of analysis were included in the report. The report did not provide clear information on (i) type and composition of the diet, (ii) the actual, as opposed to the nominal, concentrations of aspartame in the diet (and therefore it was not possible to calculate the actual exposure of the animals to aspartame), and (iii) the presence of e.g. contaminants in the feed.

- No clinical observations or macroscopic changes were described in the report

- No haematological (blood smears) assays were performed

- Although histopathology was routinely performed on a comprehensive range of organs and tissues from all animals in the study, tabulated histopathological findings in the study report were restricted to tumour pathology, inflammatory changes in the brain, meninges, lungs, pleura, pericardium, liver, renal pelvis and peritoneum, and hyperplastic or dysplastic changes in the renal pelvis, ureter, urinary bladder and nasal olfactory epithelium[1].

The life-span carcinogenicity bioassay design used in the study contrasts with "a study duration that covers the majority of the expected lifespan" as recommended in OECD Test Guideline 451. The Panel notes that, based on previous studies using the same protocol, the authors considered that extending the treatment period until the natural death of rats increased the sensitivity of the assay (Soffritti *et al*., 2002c).

However, disadvantages of lifetime treatment to natural death, compared to termination of the study at 104 or 110 weeks, include an increase in background pathology and higher probability of autolytic changes. While the authors have stated that care was taken to limit post mortem modifications, the occurrence of autolytic changes in tissues was noted in the NTP Pathology Working Group Chairperson's report (Hailey, 2004) which reviewed some of the histopathological lesions from the aspartame study.

In relation to the review of the NTP Pathology Working Group, the Panel also noted that it was not clear from the study report or the publications whether the conclusions from this review (Hailey, 2004) were taken into account and the pathological diagnoses in the study corrected accordingly.

Finally, the Panel notes that the size of the cages in which the animals were housed during the study might not sufficiently cover the need for space of adult animals (Dir. 86/609/EEC), and considers that this could have had an influence on the outcome of the study in that high-density housing could have contributed to the high incidence of infection seen in the study.

[1] EFSA was additionally provided with individual animal data on non-neoplastic histopathological changes and with some historical control data on 19th April 2006.

3. Results (as reported by the authors of the study)

During the in-life phase of the study no behavioural changes were observed among treated animals compared to the controls. Yellowing of the coat was observed in animals exposed to aspartame at the highest dose. The authors noted that this change was previously observed in their laboratory in one study in which rats were exposed to formaldehyde administered in drinking water.

A dose-related trend towards reduced food consumption was apparent in treated animals, particularly females, compared with controls, and body weight was slightly reduced (approximately 15%) in males at the highest dose level. The reduction in body weight was only apparent after 104 weeks of age. No substantial difference in survival was observed among the groups in males; in females, a slight decrease in survival was observed in the control group compared with treated groups, starting from 104 weeks of age.

In relation to non-neoplastic findings, the study report (Soffritti and Belpoggi, 2005) indicates that "*A range of inflammatory changes were observed in a variety of organs and tissues in both males and females. Acute and chronic inflammatory processes, particularly in the lungs and kidneys, were the most common. It must be noted that the high incidence of bronchopneumonia, observed in male and female treated and untreated rats, may be related to the spontaneous death of animals*".

In relation to preneoplastic and neoplastic changes observed in the study, the authors reported (Soffritti *et al.,* 2006) that administration of aspartame to rats in this study was associated with an increased incidence of:

1. lymphomas/leukaemias in female rats, with a positive significant trend in both male and female rats;
2. transitional cell carcinomas of the renal pelvis and ureter and their precursors (dysplasias), with a positive significant trend in female rats;
3. malignant schwannomas of peripheral nerves, with a positive significant trend in male rats;
4. malignant tumour-bearing animals, with a positive significant trend in both sexes.

The authors also noted that infrequent preneoplastic and neoplastic lesions of the transitional cell epithelium of the urinary bladder were observed in treated animals, with none in controls, and that 12 malignant brain tumours (10 gliomas, 1 medulloblastoma and 1 meningioma) were observed, without dose-response relationship, in male and female aspartame-treated groups, while none were observed in controls.

4. AFC Panel comments on observed effects in the ERF study

4.1 Assessment of non-neoplastic findings

The Panel notes that survival of rats in the study at 103 weeks of exposure was relatively poor, ranging from 22% (controls) to 31% in males and 27.3% (controls) to 45% in females. The non-tumour pathological findings in the rats indicated a very high incidence of infection, both in treated and untreated rats, which is likely to be linked to the poor survival. There was no treatment-related trend observable in the incidence of this infection. The incidences in treated and control groups for brain abscesses ranged from 7-11% in males and from 4-20% in

females, for pyelonephritis from 23-62% in males and from 31-83% in females, for pleuritis from 22-71% in males and from 47-94% in females, and for bronchopneumonia from 81-95% in males and from 69-97% in females. In addition, a relatively high incidence of peritonitis, liver abscesses and hepatitis, pericarditis and meningitis occurred in all groups. This very high incidence of infections is unusual for toxicology studies according to current standards.

4.2 Assessment of the pre-neoplastic and neoplastic findings

Each of the pre-neoplastic and neoplastic pathological changes reported by the authors (Soffritti *et al.*,2006) as being associated with administration of aspartame is described in more detail in the following sections, followed by the Panel's views on the findings.

4.2.1 Lymphomas/leukaemias

Soffritti and co-workers (Soffritti *and* Belpoggi, 2005) reported the following:

"The data indicate that APM causes a significant positive trend of lymphomas and leukemias in males ($p \leq 0.05$) and in females ($p \leq 0.01$). When compared to untreated control group, the increased incidence of lymphomas and leukemias in treated females was statistically significant at doses of 100,000 ($p \leq 0.01$), 50,000 ($p \leq 0.01$), 10,000 ($p \leq 0.05$), 2,000 ($p \leq 0.05$) and 400 ($p \leq 0.01$) ppm. The most frequent type of hemolymphoreticular neoplasias observed in the experiment were lymphoimmunoblastic lymphomas, mainly involving lung and mediastinal/peripheral nodes, and histiocytic sarcomas/monocitic leukemias, involving mainly thymus, lung, liver, spleen, nodes and, in the cases of leukemias, blood vessels. The differential diagnoses were based on the morphological criteria followed in our laboratory for several decades and are in line with the guidelines of the International Classification of Rodent Tumors (IARC 1993). Lymphomas and leukemias are considered together, since both solid and circulating phases are present in many lymphoid neoplasms, and distinction between them is artificial."

Table 1, reproduced in amended form from Soffritti and Belpoggi (2005), shows the incidences of lymphomas/leukaemias observed in female and male rats in the study

Table 1 - Incidence of lymphomas/leukemias in female and male Sprague-Dawley rats as reported in the ERF study

Group No./ Concentration of aspartame in the feed (mg/kg)	Animals		Total lymphomas/leukaemias[a]		
	Sex	No.	No.	%	
I	M	100	29	29	
(100,000)	F	100	25	25	##
	M+F	200	54	27	
II	M	100	20	20	
(50,000)	F	100	25	25	##
	M+F	200	45	22.5	
III	M	100	15	15	
(10,000)	F	100	19	19	#
	M+F	200	34	17	
IV	M	150	33	22	
(2,000)	F	150	28	18.7	#
	M+F	300	61	20.3	
V	M	150	25	16.7	
(400)	F	150	30 [b]	20	##
	M+F	300	55	18.3	
VI	M	150	23	15.3	
(80)	F	150	22	14.7	
	M+F	300	45	15	
VII	M	150	31	20.7	*#
(0)	F	150	13	8.7	**#
Control	M+F	300	44	14.7	

a Percentages refer to the number of animals at start
b One animal bore two hemolymphoreticular neoplasias: lymphoblastic lymphoma and histiocytic sarcoma
* Statistically significant ($p \leq .05$) using Cochran-Armitage test
** Statistically significant ($p \leq 0.01$) using Cochran-Armitage test
# Statistically significant ($p \leq 0.05$) using poly-k-test (k=3)
Statistically significant ($p \leq 0.01$) using poly-k-test (k=3)

The Panel notes that *in females* (Table 1), the incidence of lymphomas/leukaemias ranged from 8.7% (13/150) in the control group to 25% (25/100) in the 50,000 and 100,000 mg/kg diet group, the differences being statistically significant in the five highest dose-groups. In the lowest dose group, the lymphomas/leukaemias incidence (14.7% or 22/150) was not statistically different from that in the controls (8.7% or 13/150). Although there was an increasing dose-response in the two lowest dose-groups compared with controls (incidences 8.7, 14.7 and 20% in control, 80 and 400 mg/kg groups, respectively), no further increase in dose-response was apparent for the 400, 2,000 and 10,000 mg/kg diet groups (incidences 20, 18.7 and 19%, respectively). Moreover, the incidence in the 400 mg/kg diet group (20%) was only slightly lower than that in the highest dose group (25%) despite the fact that the high-dose animals were fed a diet containing 250 times more aspartame than the animals of the 400 mg/kg group. In addition, the lymphomas/leukaemias incidences in all groups of females were within the range of historical controls (average 13.3% with a range of 4-25%), the incidence in the group of concurrent controls (8.7%) being in the lowest quartile of the background incidences. However, the Panel notes that the historical control data provided to EFSA for both males and females related only to the years 1984 to 1991.

In males (Table 1), the incidence of lymphomas/leukaemias ranged from 15.0% to 29%. The difference in incidence between the control group of 20.7% (31/150) and the high dose group of 29% (29/100) is not statistically significant according to the Fisher exact test (p= 0.17, two-tailed) (statistical analysis carried out by the Panel). In males the incidences of lymphomas/leukaemias in both the 10,000 and 50,000 mg/kg groups (15 and 20%, respectively) were lower than the incidence in controls (20.7%). Also, the incidences of lymphomas/leukaemias in the various groups, including the high-dose group (29%) were within the range of the historical control incidences (8-30.9%).

In the view of the Panel the study does not provide evidence of a relationship between administration of aspartame and induction or enhancement of the development of lymphomas/leukaemias in rats. This conclusion is based on:

- the absence of a dose-response relationship for the tumour incidences, which is remarkable given the width of the dose range, if the assumption is made that this is a treatment-related response;

- the fact that the incidence of lymphomas/leukaemias in both female and male treated groups fell within the respective historical control range provided by ERF for each sex. Moreover the low incidence in the female control group had a significant influence on the outcome of the statistical analysis.

- the aggregation of the haemolymphoreticular tumour types for statistical purposes, involving a combination of tumours of different cellular origin which is not justified in the view of the Panel. In the report of the NTP Pathology Working Group (Hailey, 2004) it was noted that "*NTP does not routinely subdivide lymphomas into specific histological types as is done by the Ramazzini Foundation, however the PWG accepted their more specific diagnosis if the lesion was considered to be consistent with a neoplasm of lymphocytic, histiocytic, monocytic and/or myeloid origin*" (Hailey, 2004). The Panel agrees with NTP that subdivision of lymphomas into specific histological types can be scientifically sound, but should only be done when necessary (and possible). It is also scientifically sound to aggregate lymphoblastic lymphomas, lymphocytic lymphomas, lymphoimmunoblastic lymphomas and lymphoblastic leukaemias as malignant

lymphomas. However, in the opinion of the Panel myeloid leukaemias and histiocytic sarcomas (plus the one case of monocytic leukaemia) should be treated as separate malignancies and should not be combined with the lymphomas, since these types of tumours are of different cellular origin;

- the fact that in particular the lung was one of the major organs affected by the main subtypes (lymphoimmunoblastic lymphomas and histiocytic sarcomas) of the haemolymphoreticular tumours. This tumour type (alternatively named lymphosarcoma in other diagnostic classification systems such as IARC, 1993) occurs at a high incidence in some strains of rats, (not particularly Sprague-Dawley rats). However, this tumour normally occurs primarily in the spleen (IARC, 1993). In contrast, in the Ramazzini study, this tumour often occurred in the lungs. In addition, it is well established that this pulmonary type of lymphoreticular tumour may occur as a consequence of severe chronic respiratory disease (Innes *et al*., 1967, Nelson, 1967, Swaen and van Heerde, 1973), as occurred in the present study. Hyperplasia of bronchus-associated lymphoid tissue is characteristic of (chronic) murine respiratory mycoplasmosis, MRM, caused by *Mycoplasma pulmonis* (an organism that preferentially colonizes the luminal surface of the respiratory epithelium in rats); it is documented that mycoplasmas produce lymphokine-like substances that are mitogenic for B and T lymphocytes *in vitro* (Naot *et al.*, 1979; Davis *et al*., 1980; Lindsey *et al*., 1985; Comparative Pathology Laboratory Disease Data Sheets, University of California (at Davis), http://ccm.ucdavis.edu/cpl/index1.htm).

The data indicate that the incidence of lymphomas/leukaemias in any given group of ERF Ramazzini Sprague-Dawley rats depends on one or more confounding factors, including unknown (environmental) factors. Clearly, a high and highly variable incidence of background lymphomas/leukaemias will make toxicological data involving these tumour types often uninterpretable.

In summary, therefore, the Panel notes that there is a suggested correlation between the administration of aspartame and the increased incidences of lymphomas/leukaemias in some treated groups relative to the concurrent control group. The Panel considers however that the slight increase in incidence of these tumours in rats fed aspartame is an incidental finding of the ERF study and should therefore be dismissed.

4.2.2 Hyperplastic and neoplastic lesions of the epithelium of the renal pelvis, ureter and urinary bladder

Soffritti and co-workers (Soffritti *and Belpoggi*, 2005) reported the following:

"A dose-related increase of the incidence of dysplastic hyperplasias and dysplastic papillomas of the transitional cell epithelium of the renal pelvis and ureter were observed in females. Carcinomas in females occurred with a positive trend ($p \leq 0.05$) and the incidence in females exposed at 100,000 ppm was significantly higher ($p \leq 0.05$) compared to the controls. Carcinomas were also observed among males treated at 100,000, 50,000, 10,000, and 2,000 ppm. In females, dysplastic lesions and carcinomas combined, show a significant positive trend ($p \leq 0.01$) and a statistically significant increase among females treated at 100,000 ($p \leq 0.01$), 50,000 ($p \leq 0.01$), 10,000 ($p \leq 0.01$), 2,000 ($p \leq 0.05$) ppm."

In relation to tumours of the urinary bladder, they reported the following:

"Sparse preneoplastic and neoplastic lesions of the transitional cell epithelium of the bladder were observed in treated animals and none in the controls. In particular, 2 transitional cell carcinomas in males exposed to 10,000 ppm and 1 in females exposed to 2,000 ppm were observed. These lesions are extremely rare among the historical controls, both in males and females."

In the view of the Panel the hyperplastic and neoplastic lesions of the renal pelvis and ureter, observed mainly in females, and similar findings occurring at low frequency in the bladder of both sexes, were probably treatment-related. In previous long-term studies with aspartame focal hyperplasia of the urethelial epithelium was also reported, without progression to neoplasia. This was consistently noted in male Charles River rats administered high doses of aspartame (Hazelton Laboratories,1973), together with renal tubular and pelvic pigmentation and increased tubular degeneration, with some evidence also of an effect in lower dose males and in females. Ishii and coworkers reported renal pelvic mineralisation in Wistar rats administered high doses of aspartame (more marked in females), unaccompanied by hyperplasia but associated with high urinary calcium (Ishii *et al.,* 1981; JECFA, 1981). The third long-term toxicity/carcinogenicity study carried out with aspartame (Hazelton Laboratories, 1974a) also showed a slight increase in renal tubular pigmentation in male and female surviving rats, and an increase in renal pelvic mineralisation among treated females.

Hyperplasia of the epithelium of the renal pelvis, ureter and urinary bladder has been widely reported in the rat. It has been associated with pelvic calcification (Cohen *et al.*, 2002) and is also induced by a variety of chemicals that are cytotoxic to the urothelial epithelium, e.g high doses of sodium salts such as sodium ascorbate, succinate, aspartate, glutamate, bicarbonate, saccharin, although the phenomenon has been mainly studied in the urinary bladder, and notably in male rats (Cohen *et al*., 1995; Cohen *et al.*, 2002).

In the ERF study the urothelial changes were associated with kidney calcification (mineralisation) (Soffritti *et al*., 2006). The authors reported an increased incidence of calcification in females, in particular in those treated at 100,000 mg/kg diet (39%), 50,000 mg/kg (25%), or 10,000 mg/kg (19%), compared with controls (8%). Such an increase was not seen in males, see Table 2. The Panel notes however that kidney mineralisation was also present in the female lower dose groups, with incidences of 29%, 17% and 9% (see Table 2, compiled from the unpublished report and additional data provided to EFSA by ERF).

Table 2:
Type and incidence (%) of histopathological changes of the renal pelvis and ureter

Levels in diet (mg/kg)	Pyelonephritis	Calcification/Mineralisation [a)]	Hyperplastic and neoplastic changes of the renal pelvic and ureteral epithelium		
			Hyperplasia [b)]	Papilloma [b)]	Carcinoma
Males					
0	36	3	3	0	0
80	62	6	7	0	0
400	23	1	8	0.7	0
2,000	25	4	5	0	0.7
10,000	29	14	3	1	1
50,000	31	4	6	0	1
100,000	32	3	5	0	1
Females					
0	48	8	8	0.7	0
80	83	29	13	2	0.7
400	31	17	17	0.7	2
2,000	31	9	11	0.7	2
10,000	36	19	12	4	3
50,000	42	25	11	1	3
100,000	42	39	15	3	4

[a)] Designated as ´kidney calcification´ (Soffritti at al., 2006) and described as mineralisation in the additional data provided to EFSA in April 2006. The Panel notes that the site of the effect is not given (e.g. intercorticomedullary, papillary or pelvic)
[b)] With or without atypia (dysplasia)

Table 2 shows that the incidence of kidney calcification/mineralisation was higher in the 80 mg/kg diet group (lowest dose group) than in the 5,0000 mg/kg diet group. This observation seems to undermine the hypothesis by Soffritti *et al*., (2006) that kidney calcification played an important role in the development of hyperplastic and neoplastic renal pelvic lesions. However, since Soffritti *et al*., (2006) did not specify the localization of the kidney mineralization, it might well be that renal *pelvic* calcification was positively correlated with the hyperplastic and neoplastic urothelial lesions.

The Table also shows that there was a high incidence of pyelonephritis in both males and females, without a dose-response relationship. This may be an important predisposing factor for the development of hyperplastic and neoplastic renal pelvic lesions at the higher dose levels of aspartame.

The Panel notes that the NTP Pathology Working Group review (Hailey, 2004) revealed that a number of hyperplastic and neoplastic lesions listed in Table 2 were more severely classified by the study pathologists from the ERF, compared with the diagnoses of the NTP review group. For instance, from the 3 renal pelvis carcinomas reviewed, one was diagnosed as carcinoma (by majority vote), and two were diagnosed as hyperplasia, one unanimously and one by majority vote. With a total of 24 (renal pelvic, ureteral and urinary bladder) carcinomas reported by the study pathologist, this difference in view could have a significant impact on the outcome and interpretation of the study.

The Panel accepts the view of Soffritti *et al*., (2005, 2006) that hyperplastic and neoplastic lesions of the epithelium of the renal pelvis, ureter and bladder were likely to have been related to the administration of aspartame, especially at the higher doses, and notes that hyperplastic changes in this epithelium were reported in the earlier long-term studies with aspartame.

The Panel further notes that it is widely accepted that such effects are a high dose effect of some irritant chemicals or chemicals producing renal pelvic calcification as a result of imbalances in calcium metabolism, specific to the rat, and it is therefore considered to be of no relevance for humans (Cohen *et al*., 1995; Cohen *et al*., 2002; JECFA, 1981, IARC, 1999).

4.2.3 Preneoplastic and neoplastic lesions of the olfactory epithelium

Soffritti and co-workers (Soffritti *and Belpoggi,* 2005) reported the following:

"The data indicate that the incidence of hyperplasia of olfactory epithelium increased with a significant positive trend in males ($p \leq 0.01$) and females ($p \leq 0.01$). The differences were statistically significant ($p \leq 0.01$) at 100,000, 50,000 and 10,000 in both males and females and also statistically significant in males at 400 ppm. The hyperplasia was characterized by increased thickness of the epithelium. Sparse adenomas were observed among treated males and females and none among the control. It is noteworthy that one case of olfactory neuroblastoma was observed in a female treated at the highest dose."

The Panel concurs with the view of the study authors that the hyperplastic changes described in the olfactory epithelium were in some way related to the administration of aspartame to the rats. The underlying mechanism is unclear and the toxicological interpretation of the hyperplastic changes is difficult; one explanation may be that the changes are due to a local effect of inhaled diet particles containing high levels of aspartame. Inflammatory nasal changes may also have played a role in the development of the epithelial hyperplasia.

The Panel considers that the tumours of the olfactory epithelium reported by the ERF were isolated findings and unrelated to aspartame feeding. Moreover, the Panel notes that the tumours classified as adenoma might actually have been hyperplasias rather than tumours because the two cases of early adenoma reviewed by the NTP Pathology Working Group were unanimously diagnosed as hyperplasia (Hailey, 2004).

4.2.4 Malignant schwannomas of peripheral nerves

Soffritti and co-workers (Soffritti *and Belpoggi.*, 2005) reported the following:

"The data indicate that the incidence of malignant schwannomas of peripheral nerves occurred with a positive trend ($p \leq 0.05$) in males. In females, 9 malignancies were observed among treated animals of the different dosage groups and none among the controls. All lesions diagnosed as malignant schwannomas in males and females were positive for S100 staining. The most frequent site of origin of the malignant schwannomas was cranial nerves (72%); the other cases arose at the spinal nerve roots. Microscopically, malignant schwannomas invaded the soft tissues locally. Metastases of cranial nerve malignant schwannomas were observed in three males treated at the highest dose. The metastases were found in submandibular lymph nodes in two cases and in one case the tumor metastatized to

the lung and to the liver. Histologically the feature of malignant schwannomas was Antoni B."
The Panel notes that the incidences of malignant schwannomas in peripheral nerves in males were 0.7% (1/150), 0.7% (1/150), 2.0% (3/150), 1.3% (2/150), 2% (2/100), 3% (3/100) and 4% (4/100) in the 0 (control), 80, 400, 2,000, 10,000, 50,000 and 100,000 mg/kg diet groups, respectively. There was a positive dose-response relationship with a statistically significant trend ($P \leq 0.05$). The NTP Pathology Working Group confirmed the diagnosis of malignant schwannoma with invasion in the two cases reviewed, both by majority vote, one 6 to 1 and the other 4 to 3 (Hailey, 2004). The incidences in all groups of males, including the control group, were higher than the overall average, historical control incidence of 0.4% (range 0-2%) (Soffritti *et al*., 2006) while in the two highest dose groups the incidences were outside the range of historical controls.

In females, no malignant schwannomas were found in the control and 400 mg/kg groups while the incidences in the other groups ranged from 1-2%. The historical control incidences in females ranged from 0-2% with an average of 0.1% (Soffritti *et al*., 2006).

Overall, the Panel considers that the biological relevance of the schwannomas in the ERF study is unclear, despite the positive statistical trend in males. The number of tumours was low, the dose-response relationship was flat over a wide dose range and there is also uncertainty about the diagnosis of these tumours.

4.2.5 Total malignant tumour-bearing animals

Soffritti and co-workers (Soffritti and Belpoggi, 2005) reported the following:

"The data indicate an increased incidence of benign tumor-bearing animals occurred with a significant positive trend in females ($p<0.05$). A statistically significant increase of the incidence of benign tumors was observed in males treated at 10,000 ppm ($p<0.01$) compared to the control group. The data [also] *indicate that the incidence of malignant tumor-bearing animals occurred with a significant positive trend in males ($p<0.05$) and in females ($p<0.01$). A statistically significant increase of the incidence of malignant tumors was observed in females treated at 50,000 ppm ($p<0.01$) compared to the control group."*

The Panel considers that overall, in both males and females, a toxicologically relevant dose-response relationship for total tumour incidence was lacking for malignant tumours. Moreover, there are uncertainties regarding the diagnoses of several types of tumours. The NTP Pathology Working Group diagnosed cases of adenocarcinomas of the mammary glands as fibroadenomas, cases of early squamous cell carcinomas of the ear duct or oral cavity as hyperplasias, a case of adenocarcinoma of the pituitary gland as cystic change, and cases of early transitional cell carcinomas of the renal pelvis as hyperplasia. Thus, the ERF study pathologists appeared to apply more severe classifications than the NTP Pathology Working Group with respect to diagnosis of histopathological changes. Possible adjustment for overdiagnosis would also have impact on the quantitative assessments presented in the ERF publications, particularly because the incidences of several of the reported tumour types were low.

The Panel considers, therefore, that the data on total malignant tumours as presented in the ERF publications cannot be considered an indication of a carcinogenic potential of aspartame.

The Panel also considers that the aggregation of all malignant tumour incidences or all malignant tumour-bearing animals for statistical purposes is not justified, given that, as explained above, at least the lymphomas/leukaemias and the malignant tumours of the renal pelvis and the ureter should have been excluded from the analysis. In the one case the finding is considered to be an incidental finding of the ERF study and in the other, the finding is considered to be of no relevance for humans.

5. Previous assessments of the safety of aspartame, other recent studies of relevance and review of genotoxicity and kinetic data

The safety of aspartame has previously been evaluated by the EC Scientific Committee for Food (SCF), in three separate evaluations (SCF, 1985; SCF, 1989; SCF, 2002), and the SCF also evaluated an alleged connection between aspartame and an increase in incidence of brain tumours in people in the USA (SCF, 1997). Other expert bodies have also evaluated the safety of aspartame, including the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 1975, 1980, 1981), the US Food and Drug Administration (FDA, 1984), the UK Committee on Toxicity (COT, 1993) and the Agence Française de Sécurité Sanitaire des Aliments (AFSSA, 2002). These evaluations included consideration of the long-term toxicity and carcinogenicity studies available on aspartame at the time of evaluation, as summarised in Table 3. No new carcinogenicity studies were published after 1981 until the NTP studies of 2003.

Table 3
Summary of carcinogenicity/chronic toxicity studies carried out on aspartame 1970 – 1981

Study and Reference	Numbers of animals and dose levels	Outcome
2-year chronic toxicity/carcinog-enicity study in Charles River rats (Hazelton Laboratories, 1973)	40 males, 40 females/ dose group, 0, 1, 2, 4 and 6-8 g/kg bw	No evidence of a treatment-related effect on incidence of neoplasms other than a low incidence of brain tumours, scattered among the 4 test groups, with none in controls. An in-depth re-evaluation of brains from this study identified a total of 12 neoplasms, scattered in a non-dose-related manner among the 4 test groups. Following consideration of the results of the lifetime chronic toxicity/ carcinogenicity study reported below, in which there was a random distribution of brain tumours in all groups including controls, it was concluded that the results of both studies taken together did not provide consistent evidence of an intracranial tumourigenic effect (Hazelton Laboratories, 1973). There was no evidence of a treatment-related effect on non-neoplastic changes, with the exception of renal changes in higher dose males, including focal hyperplasia of the renal pelvic epithelium and appearance of pigment deposits in tubular or pelvic epithelial cells.
Lifetime chronic toxicity/carcinog-	40 males, 40 females/ dose group, 0, 2, 4	There was a higher incidence of pituitary hyperplasia in high dose males and low dose

enicity study involving pre- and postnatal administration of aspartame in Charles River rats (Hazelton Laboratories, 1974a)	g/kg bw. Treated rats were F1A weanlings from parent animals that had been treated for 60 days prior to mating and which continued to receive aspartame through gestation and weaning	females, nodular hyperplasia of adrenal cortex in treated males, hyperplastic nodules in liver of treated females, attributed to lower than historical incidence in controls. Brain tumours were randomly distributed in all groups including controls. It was concluded that the hyperplastic findings in the rats were within the historical control range and were not treatment related (Kommineni, 1973). There was no evidence of a treatment-related effect on incidence of non-neoplastic changes, other than an increase in renal cortical pigmentation in male and female surviving rats, and an increase in renal pelvic mineralisation among treated females
2-year chronic toxicity/carcinogenicity study in Wistar rats (Ishii *et al.,* 1981; Ishii, 1981)	86 males, 86 females/ dose group, 0, 1, 2, 4 g/kg bw and an additional group fed 4 g/kg aspartame + DKP (3:1)	The authors concluded that there was no evidence of a treatment-related effect on incidence of neoplasms. One atypical astrocytoma was found in a control rat, and two astrocytomas, two oligodendrogliomas and one ependymoma were found in the exposed groups, with no statistical difference between the groups. There was no evidence of a treatment-related effect on incidence of non-neoplastic changes other than a dose-related increase in focal mineralisation of the renal pelvis in both males and females, associated with a dose-related increase in urinary calcium.
2-year chronic toxicity/ carcinogenicity study in Swiss mice (Hazelton Laboratories, 1974b)	36 males, 36 females, 1, 2, 4 g/kg bw. 72 males, 72 females as controls.	No evidence of a treatment-related effect on incidence of neoplasms or on incidence of non-neoplastic changes.
46-week study in hamsters (Rao *et al*., 1972)	5 males and 5 females per group were fed aspartame containing less than 1% DKP at dietary levels of 1000, 2000, 4000 and 12 000 mg/kg bw	Gross lesions were attributed to an unidentified infection in the colony. Neoplastic changes were not reported.
56 week bladder implant study in the Swiss albino mouse (Bryan, 1974).	200 females/dose group. Pellets of 80% cholesterol (20-22g) plus 20% aspartame (4.0-4.4g) implanted into the bladder. Negative controls cholesterol only, positive controls cholesterol plus 8-methyl ether of zanthurenic acid	No evidence of a treatment-related effect on incidence of neoplasms or on incidence of non-neoplastic changes. The following incidence of bladder neoplasia was recorded in mice surviving 175 days or more: negative control 17/155 (11%), aspartame 13/123 (10.6%), and positive control 40/111(36%). No bladder neoplasia were observed in animals dying or killed prior to 175 days of the study.

The toxicological evaluation of aspartame carried out at the 1980 and 1981 JECFA meetings (JECFA, 1980; JECFA, 1981) provides a detailed evaluation of the above studies. JECFA (1981) noted that the brain lesions (tumours) seen in some test animals in the 1973 chronic toxicity/carcinogenicity study in Charles River rats (Hazelton Laboratories, 1973) were investigated by a detailed pathology evaluation of the brains from both this study and the second lifetime study in Charles River rats (Hazelton Laboratories, 1974a). JECFA noted that the same brain lesions were seen in untreated controls as well as test animals in this latter study, and concluded that there was no evidence of tumorigenic activity of aspartame in any of the rat studies, in the 2-year chronic toxicity/carcinogenicity study in Swiss mice or in the 56-week bladder implant study in the mouse.

The SCF's 1985 critical review of the long-term studies concluded that the scattered findings of significant deviations from control values showed no consistent relationship to treatment, sex of animals or histopathology, so that 4000 mg/kg bw, taken from several studies, could be considered as a no-adverse-effect level for these studies. The 1989 SCF opinion did not reconsider the issue of carcinogenicity.

In 1997 the SCF, in evaluating the alleged connection between aspartame and an increase in incidence of brain tumours in humans in the USA, concluded that the data did not support the proposed biphasic increase in the incidence of brain tumours (with a suggested link to use of aspartame as a sweetener) in the USA during the 1980s, and advised the European Commission at that time that there was no new evidence to justify a re-examination of aspartame by the Committee. In 2002, the SCF issued an additional opinion on aspartame taking into account all new scientific information available. It was concluded that aspartame administration does not induce changes in behaviour, cognition, mood or learning. Aspartame was no more likely than placebo to be associated with headaches. The SCF concluded that there was no evidence to suggest a need to revise the outcome of the earlier risk assessment or the previously established ADI for aspartame, of 40 mg/kg bw.

The US Food and Drug Administration (FDA, 1984) and the UK Committee on Toxicity (COT, 1993) reached similar conclusions regarding the lack of evidence for a carcinogenic effect of aspartame.

The AFSSA report (2002) concluded that "*Taking into account all the studies that have been conducted, the frequency of spontaneous tumours in laboratory rats, the types of tumours observed and the absence of a dose-response relationship, it was concluded that aspartame had no carcinogenic potential on the brain in experimental animals (FDA FR, 1981-1984; Koestner, 1984; Cornell et al., 1984; Flamm, 1997).*" It also concluded that "*In France, the epidemiological data from the cancer registers do not enable a definitive indication to be given on a possible aspartame-brain tumour relationship, but they do show that, at the present time, the sale of this food additive in France is not being accompanied by an increase in the frequency of brain tumours or increased mortality from this disease in the general population*." It additionally concluded that "*The epidemiological study by Olney et al., which suggested a link between the placing on the market of aspartame and a possible increase in the frequency of brain cancers in humans, did not provide any scientific evidence to justify or demonstrate a basis for this suggestion; to date it has not been confirmed*." The SCF in its opinion of 2002 agreed with this view, and the SCF's conclusions were supported by the UK Food Standards Agency (FSA, 2002).

5.1 Recent studies of relevance to the assessment of the safety of aspartame.

Since these evaluations were carried out, the US National Toxicology Program (NTP) has carried out several 9-month carcinogenicity studies with aspartame in genetically modified mouse models (NTP, 2003), details of which are provided in Table 4. These mouse strains are DNA repair deficient and therefore more sensitive to (in particular genotoxic) carcinogens.

According to NTP there was no evidence of treatment-related neoplastic or non-neoplastic lesions in any of these studies. The Panel independently evaluated these studies and concurs with this view. The overall number of animals with primary neoplasms, with benign neoplasms or with malignant neoplasms in aspartame-exposed groups of both sexes was not significantly increased compared to the respective controls.

Table 4
Summary of the NTP 9-month carcinogenicity studies with aspartame in transgenic mice

Study and Reference	Numbers of animals and dose levels	Outcome
9-month study in transgenic mice, TgAC hemizygous strain, 2003	15 male and 15 female mice fed diets containing 0; 3,125; 6,250; 12,500; 25,000; or 50,000 mg aspartame per kg diet (equivalent to average daily doses of approximately 490; 980; 1,960; 3,960; or 7,660 mg aspartame/kg body weight to males and 550; 1,100; 2,260; 4,420; or 8,180 mg/kg to females)	There were no neoplasms or non-neoplastic lesions that were attributed to exposure to aspartame.
9-month study in transgenic mice, p53 haplo-insufficient strain, 2003	15 male and 15 female mice fed diets containing 0; 3,125; 6,250; 12,500; 25,000; or 50,000 mg/kg aspartame (equivalent to average daily doses of approximately 490; 970; 1,860; 3,800; or 7,280 mg/kg to males and 630; 1,210; 2,490; 5,020; or 9,620 mg/kg to females)	There were no neoplasms or non-neoplastic lesions that were attributed to exposure to aspartame.
9-month study in transgenic mice, Cdkn2a deficient strain, 2003	15 male and 15 female mice fed diets containing 0; 3,125; 6,250; 12,500; 25,000; or 50,000 mg/kg aspartame (equivalent to average daily doses of approximately 490; 960; 1,900; 3,700; or 7,400 mg/kg to males and 610; 1,200; 2,390; 4,850; or 9,560 mg/kg to females)	There was no evidence of enhanced tumour formation in male and female Cdkn2a deficient mice exposed to aspartame.

Additionally, the US National Cancer Institute (NCI) has recently presented, in abstract form only, the results of a study in which they examined aspartame-containing beverage consumption and the incidence of hematopoietic and brain cancers among 340,045 men and 226,945 women aged 50-69 years in the NIH-AARP Diet and Health Study (NCI, 2006). They have concluded that *"Our findings from this epidemiologic study suggest that consumption of aspartame-containing beverages does not raise the risk of hematopoietic or brain malignancies."*

The Panel thus concludes that the results of these previous evaluations of aspartame, recent negative carcinogenicity studies with transgenic mice and the new epidemiological study carried out by NCI do not indicate that aspartame has a carcinogenic potential.

5.2 Review of genotoxicity data

The Panel also reviewed the genotoxicity studies on aspartame and a summary of this evaluation is provided in Appendix I to this opinion. The overall conclusion of this evaluation is that in a battery of *in vitro* genotoxicity assays, aspartame was found to have no mutagenic or DNA damaging potential. At non-toxic concentrations, the compound had no *in vitro* clastogenic activity. Aspartame was assayed for genotoxicity in various recent or older, published or unpublished, *in vivo* studies. Aspartame was not clastogenic in mice and rats after single and repeated oral administration. The isolated weak increase in blood micronucleated erythrocytes, occurring in female p53 haploinsufficient transgenic mice treated for 90 days with the highest dose of aspartame (average daily doses of approximately 9,620 mg/kg bw), is considered by the Panel of uncertain biological significance because of the unusual study design and the unknown relevance of this particular animal model for risk assessment.

Overall the data strongly indicate that aspartame is devoid of any genotoxic potential.

5.3 Review of kinetic data

The Panel notes the suggestion of Soffritti and co-workers that the effect of aspartame on the incidence of lymphomas/leukaemias was due to its transformation into methanol in the gastrointestinal tract (Soffritti *et al.*, 2006). For this reason, the Panel undertook an evaluation of the toxicokinetics of aspartame in order to examine the plausibility of this suggestion. The results of this evaluation are provided in Appendix II, and a short summary of the evaluation is provided in the following paragraphs.

After oral intake of aspartame in humans, the molecule is quantitatively hydrolysed, either within the lumen of the gastrointestinal (GI) tract or in the GI tract mucosal cells to yield the amino acids aspartic acid, phenylalanine and methanol.

Aspartic acid is rapidly converted into other amino acids or metabolised to carbon dioxide and urea. An increase in blood levels of aspartic acid and related amino acids occurs only at high dose levels (> 100 mg aspartame/kg bw as a bolus dose), but these changes are similar to those found after consumption of a normal amount of protein via the diet.

An increase in blood phenylalanine and phenylalanine-derived amino acids can be observed at dose levels at the bolus-equivalent of the ADI, but this will remain within the range that can be found after consumption of a normal amount of protein via the diet.

Methanol arising from the hydrolysis of aspartame is rapidly metabolised further to formaldehyde and then via formic acid to carbon dioxide. Kinetic studies in humans using doses ranging from 0 – 200 mg/kg bw aspartame/day (Tephly and McMartin, 1984, see Appendix II for further details) have shown that a single bolus dose of 34 mg aspartame/kg bw (slightly below the ADI of 40 mg/kg bw) did not result in a detectable increase in blood methanol level, while a dose of 50 mg/kg bw resulted in a just detectable level of 3.4 mg/L. A dose of 100 mg/kg bw aspartame resulted in blood methanol levels of 11 mg/L and methanol

levels increased further with higher doses. However even 200 mg/kg bw aspartame did not increase the blood level of formic acid, although urinary formate excretion increased significantly. When it is taken into consideration that in monkeys exposed to 3 g methanol/kg bw (750 times higher than the amount of methanol released from aspartame at the ADI level) formaldehyde was only detectable in the blood at low levels of 27 - 45 µM (~ 0.81 – 1.35 mg/L) it is clear that aspartame would not yield any toxicologically relevant systemic exposure to formaldehyde (McMartin *et al*., 1979; Tephly and McMartin, 1984, see Appendix II for further details).

This information shows that at intake levels around the ADI of aspartame there is no toxicologically relevant systemic exposure to methanol, its metabolites or the amino acid moieties. The Panel had no information about plasma levels of the hydrolysis products of aspartame in rats given very high doses of the sweetener, as in the ERF study, but it is possible that at the highest dose levels in the ERF studies systemic exposure to these substances may have occurred.

The Panel also notes that methanol is present as such in food (e.g. fruits, fruit juices, vegetables, roasted coffee, honey and alcoholic beverages), or is released from e.g. methyl esters. It is also liberated by micro-organisms from methoxylated polysaccharides (e.g. pectin) or from these polysaccharides during cooking (IPCS, 1997). Concentrations in fruit juices can vary widely (1 - 640 mg methanol/L) and a wide range of methanol concentrations have also been reported in alcoholic beverages.

DISCUSSION

The long-term rat study carried out by the ERF to evaluate the potential carcinogenic effects of aspartame (Soffritti *et al*., 2005, 2006; Soffritti and Belpoggi, unpublished report provided to EFSA in December 2005) was a life-span study with a large number of animals and a wide range of dose levels. In the view of Soffritti *et al.* (2002c, 2006), this design increases the sensitivity of the study as compared to conventional OECD carcinogenicity studies for the following reasons: life-span studies are more likely to detect tumours that occur late in life; the large number of animals increased the chance to find treatment-related "rare tumours"; and will increase the statistical sensitivity; and the wide dose range would facilitate the detection of a possible compound and dose-related effect.

However, disadvantages of lifetime treatment to natural death compared to termination of the study at 104 or 110 weeks include an increase in background pathology and higher probability of autolytic change. While the authors have stated that care was taken to limit post mortem modifications, the occurrence of autolytic changes in tissues was noted in the NTP Pathology Working Group Chairperson’s report (Hailey, 2004) which reviewed some of the histopathological lesions from the aspartame study.

The high background incidence of chronic inflammatory changes due to infections was a major confounding factor in the interpretation of the findings of the study, including hyperplastic and neoplastic changes. The Panel notes the high incidence of bronchopneumonia (81-95% in males and 69-97% in females), pyelonephritis, (23-62% in males and 31-83% in females) and pleuritis (22-71% in males and 47-94% in females) and a relatively high incidence of brain abscesses (7-11% in males and 4-20% in females), peritonitis, liver abscesses and hepatitis, pericarditis and meningitis that occurred in all groups

(Soffritti and Belpoggi, unpublished report, 2005, and confirmed in further data from the study provided by ERF to EFSA in April 2006). It appears, therefore, that chronic inflammation is a problem in the ERF colony. The Panel notes that no serology was undertaken during the ERF study to confirm the presence of infection, as would be normal practice.

Infection and disease were common in conventional reared (non Specific-Pathogen-Free, SPF) rats and mice used in toxicology studies some 30 years ago. The causative organism in many rodent colonies has been demonstrated to be *Mycoplasma pulmonis,* (Lindsey *et al*., 1985). Infection with *Mycoplasma pulmonis* is considered to shorten the lifespan of the rat, may affect xenobiotic metabolism and the response to carcinogens (Comparative Pathology Laboratory Disease Data Sheets, University of California (at Davis) (http://ccm.ucdavis.edu/cpl/index1.htm); Everitt and Richter, 1990; Lynch *et al*., 1984, cited in Everitt and Richter, 1990), and is thus considered to lead to experimental results of dubious value. Sites of predilection for the organism in the host are the lungs, nasopharynx and the middle ears.

In the ERF study, the lungs were one of the main organs affected by lymphoimmunoblastic lymphomas and histiocytic sarcomas/monocytic leukaemias, the most frequently observed types of haemolymphoreticular tumours. Since it is well-known that lymphoreticular tumours may develop from abundant peribronchial, peribronchiolar and perivascular lymphoid hyperplasia (lymphoid cuffing) in the lungs of rats suffering from chronic respiratory disease, the relatively high incidences of lymphomas/leukaemias found in aspartame-treated groups are most likely to be related to chronic inflammatory changes in the lungs. This conclusion is supported by an observation of Paget and Lemon (1965; cited and discussed by Nelson, 1967) that when they eliminated chronic respiratory disease from a particular strain of rats, they also eliminated a form of intrathoracic lymphosarcoma.

Soffritti *et al.* suggested however that the increase in lymphomas/leukaemias in aspartame-treated female rats could be related to the metabolite methanol, which is in turn metabolised to formaldehyde in both humans and rats (Soffritti *et al.,* 2002a). Methanol administered in drinking water induced a statistically significant increase in the incidence of lymphomas/leukaemias in female rats in a study carried out by the ERF (Soffritti *et al.,* 2002a). However, with the exception of the ERF drinking water study no other studies have been reported in the peer-reviewed literature on the potential carcinogenicity of methanol in laboratory animals (IPCS, 1997). The Panel notes that, even if the lymphoreticular tumours in rats were related to methanol, that at dose levels in humans around the ADI (40 mg/kg bw/d); even when taken as a bolus dose, no changes in the blood levels of methanol or its metabolites are to be expected. It is thus highly unlikely that similar events could occur in humans as a result of regular intake of aspartame.

A dose-related increase in the incidence of lymphomas/leukaemias was also observed in female rats treated with formaldehyde administered in drinking water (Soffritti *et al.,* 1989; Soffritti *et al.,* 2002b); However, the World Health Organization (WHO) noted certain limitations in the Soffritti *et al.* drinking water study with formaldehyde (Soffritti *et al.,* 1989; Soffritti *et al.,* 2002b), including the "pooling" of tumour types, the lack of statistical analysis, and limited examination of non-neoplastic end-points (WHO, 2002) and that, in contrast, other formaldehyde drinking water studies have given negative results (Tobe *et al*., 1989; Til *et al.,* 1989).

Soffritti and co-workers also observed an increase in lymphomas/leukaemias in female rats treated in a life-time gavage study with the gasoline oxygenated additive methyl-*tertiary*-butyl-ether (MTBE), which is metabolised to formaldehyde and *tert*-butanol (Belpoggi *et al*., 1995; 1998). In the EU-Risk Assessment Report (EU-RAR) for this substance (Hansen *et al.*, 2002) it was noted that these tumours were not reported in other carcinogenicity studies on MTBE (Hansen *et al.*, 2002). Based on limitations in the particular study, the EU-RAR states that: "*there are several factors that set considerable restrictions to the proper assessment of the relevance of these tumours to man. Therefore, the level of confidence in these results is not very high."* In addition, when discussing the carcinogenic properties of MTBE metabolites, the EU-RAR concluded that formaldehyde was not likely to be a relevant factor in the tumour formation seen with MTBE.

The Panel notes that according to the description in the EU-RAR, the origin of the lymphoreticular tumours observed with MTBE was the pulmonary lymphoid tissue, which is similar to what was observed in the ERF study with aspartame in the same colony of rats.

For these reasons, the increased incidence of lymphomas/leukaemias in females which was considered by the authors as the most important observation in the ERF study, is considered by the Panel to be unrelated to the treatment with aspartame, given the high background incidence of chronic inflammatory changes and the lack of a positive dose-response relationship. The Panel notes that the incidence of these tumours was similar (at approximately 20%) over the dose range of 400 to 10,000 mg/kg diet (20 to 500 mg/kg bw) and only slightly increased, to 25%, at the dose levels of 50,000 and 100,000 mg/kg diet (2500 and 5000 mg/kg bw). The Panel considers that, for a compound-related effect to be plausible, the large increase in dose over this range (x 250) should show more proportional impact. The most likely explanation of the findings in this study with respect to lymphomas/leukaemias is that they have developed in a population of rats suffering from to chronic respiratory disease. The slight increase in incidence in rats fed aspartame is considered to be an incidental finding of the ERF study, and should therefore be dismissed in relation to assessment of its implications for humans.

In relation to the malignant schwannomas of the peripheral nerves observed in the study, the Panel notes the statistically significant positive trend ($p \leq 0.05$) in males, and the pattern of incidence in females, in which nine malignancies were observed among treated animals of the different dosage groups and none among the controls. However, the numbers of tumours were low, the dose-response relationship, while showing a positive statistical trend in males, was very flat over a wide dose range and there is also uncertainty about the diagnosis of these tumours.

The Panel notes that 12 malignant brain tumours (10 gliomas, 1 medulloblastoma and 1 meningioma) were observed in male and female aspartame-treated groups, whereas none were observed in controls. Since there was no dose-response relationship for these tumours the Panel does not consider the administration of aspartame responsible for their occurrence.

The hyperplastic and neoplastic lesions of the renal pelvis and ureter, observed mainly in females, and similar reported findings occurring at low frequency in the bladder of both sexes, were considered by the Panel to be probably treatment-related. Renal effects including focal hyperplasia of the renal pelvic epithelium and focal mineralisation of the renal pelvis associated with a dose-related increase in urinary calcium have also been reported in previous long-term studies with aspartame. Such effects in the renal pelvis, ureter and bladder appeared

to be rat-specific and not relevant for humans. The Panel also notes that, according to the NTP Pathology Working Group review, two of the four checked slides were more severely classified, as neoplasia rather than hyperplasia, which has a significant impact on the overall finding.

The ERF study shows an increase in hyperplastic changes in the rat nasal olfactory epithelium, with a significant positive trend in both males and females. This finding appeared to be related to the administration of aspartame to the rats, although the authors did not comment on its significance in their discussion of the critical findings of the study. The presence of inflammatory change in the nasal cavities might have played a role in the development of these lesions, as could the inhalation of particles of diet containing aspartame.

The Panel notes that dietary exposure to intense sweeteners in the population has been assessed in a number of European countries including Finland (Salminen and Penttila, 1999), France (Garnier Sagne *et al*., 2001), Sweden (Ilback *et al*., 2003), UK (Food Standard Agency, 2003) and Italy (Arcella, 2004). These studies were designed to assess high level exposure to intense sweeteners per kg body weight and therefore focused on children, teenagers and diabetics. In two of these studies, exposure was also assessed under a hypothetical scenario of substitution of all regular food products with sugar-free products and of all discretionary sugar with table top sweeteners (Arcella *et al*., 2004; Ilback *et al*., 2003). In all of these studies, dietary exposure to aspartame was well below the ADI of 40 mg/kg bw (up to 10 mg /kg bw), even in high consumers.

CONCLUSIONS AND RECOMMENDATIONS

The Panel has assessed the two recent publications on the ERF study (Soffritti *et al.,* 2005; 2006) together with the unpublished study report made available to EFSA and further data from the study provided by ERF to EFSA in April 2006, and has noted the conclusions of the authors, that the results of their study provided evidence that aspartame is a ´multipotential carcinogenic agent´, even at a dose corresponding to half of the current ADI of 40 mg/kg bw. The authors based this conclusion on an increased incidence of lymphomas/leukaemias, transitional cell carcinomas of the renal pelvis and ureter and their precursors (dysplasias), malignant schwannomas of peripheral nerves and an increase in the number of malignant tumour-bearing animals.

The Panel concludes, on the basis of its assessment, that the increased incidence of lymphomas/leukaemias reported in the study was unrelated to the treatment with aspartame, given the high background incidence of chronic inflammation and the lack of positive dose-response relationship. The most plausible explanation of the findings in this study with respect to lymphomas/leukaemias is that they have developed in a population of rats suffering from chronic respiratory disease. The slight increase in incidence in rats fed aspartame is considered to be an incidental finding and should therefore be dismissed.

Concerning the malignant schwannomas, the Panel notes that the numbers of tumours were low, the dose-response relationship, while showing a positive statistical trend in males, was very flat over a wide dose range and there is also uncertainty about the diagnosis of these tumours. The Panel concludes that this finding can only be fully evaluated following a histopathological peer-review of all relevant slides related to the nervous system in the ERF study and if necessary also from the historical controls.

The Panel concludes that the preneoplastic and neoplastic lesions of the renal pelvis, ureter and bladder occurring primarily in female rats along with renal calcification were most probably treatment-related at least at the higher doses. It is widely accepted that the effect is a high dose effect of irritant chemicals or chemicals producing renal pelvic calcification as a result of imbalances in calcium metabolism, specific to the rat. The Panel considers that these effects are of no relevance for humans.

The Panel concludes that the data on total malignant tumours do not provide evidence of a carcinogenic potential of aspartame. The Panel considers that the aggregation of all malignant tumours or all malignant tumour-bearing animals for statistical purposes in a risk assessment is not justified because the lymphomas/leukaemias and the renal tumours should have been excluded from the analysis. In the one case the finding is considered to be an incidental finding of the particular study and in the other, the finding is considered to be of no relevance for humans.

The Panel takes note of the previous evaluations of aspartame by the SCF and other expert bodies, the negative results of recent carcinogenicity studies carried out on aspartame in transgenic mice and a recent epidemiological study carried out by the US National Cancer Institute in which no increase in brain or blood related cancers was reported. The Panel also takes note of the comprehensive studies on the substance indicating that aspartame does not have genotoxic activity.

Kinetic data in humans indicate that dose levels around the ADI (40 mg/kg bw), even when taken as a bolus dose, do not lead to systemic exposure to aspartame. Furthermore, exposure to any of its breakdown products, including methanol or formaldehyde, is negligible.

The Panel considers that no significant new data have emerged since 2002 on aspects other than carcinogenicity and there is therefore no reason to review the previous SCF opinion on aspartame.

The Panel notes that dietary exposure to intense sweeteners in the population has been assessed in a number of European countries. In all of these studies, dietary exposure to aspartame was well below the ADI of 40 mg/kg bw (up to 10 mg /kg bw), even in high consumers.

In summary, the Panel concludes, on the basis of all the evidence currently available from the ERF study, other recent studies and previous evaluations, that there is no reason to revise the previously established ADI for aspartame of 40 mg/kg bw.

DOCUMENTATION PROVIDED TO EFSA

Soffritti, M., and Belpoggi, F. (2005). Long-term carcinogenicity bioassay to evaluate the potential biological effects, in particular carcinogenic, of aspartame administered in feed to Sprague-Dawley rats. (Protocol No.: BT 6008), Unpublished report of the European Foundation of Oncology and Environmental Sciences “B. Ramazzini”, December 2005, Bologna.

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ABBREVIATIONS

ADI	Acceptable Daily Intake
AFSSA	Agence Française de Sécurité Sanitaire des Aliments
bw	body weight
COT	UK Committee on Toxicity
DKP	aspartylphenylalanine diketopiperazine
ERF	Cesare Maltoni Cancer Research Center, European Ramazzini Foundation of Oncology and Environmental Sciences, Bologna, Italy
EU-RAR	EU-Risk Assessment Report
FDA	US Food and Drug Administration
GI	gastrointestinal
GLP	Good Laboratory Practice
IARC	International Agency for Research on Cancer
JECFA	Joint FAO/WHO Expert Committee on Food Additives
MTBE	methyl-*tert*-butyl-ether
MRM	murine respiratory mycoplasmosis
NCI	US National Cancer Institute
NTP	US National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
ppm	parts per million
PWG	Pathology Working Group
SCF	EC Scientific Committee on Food
WHO	World Health Organization

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APPENDIX I

Assessment of possible genotoxic activity of aspartame

The genotoxicity of aspartame was evaluated in several *in vitro* and *in vivo* assays.

In vitro assays

Aspartame was considered negative in three Ames Salmonella mutagenicity tests in the presence or in the absence of metabolic activation. In the first assay (JECFA 1980), aspartame was tested at doses ranging from 10 to 5,000 µg/plate in strains TA1535, TA1537, TA1538, TA98 and TA100. In a second assay (NTP 2003), no mutagenicity was detected in strains TA98, TA100, TA1535, TA1537 or TA97 for doses up to 10,000 µg/plate. In a third assay (Rencuzogullari *et al*., 2004) aspartame was tested on TA98 and TA100 at doses ranging from 50 to 2,000 µg/plate in the presence and in the absence of metabolic activation.

Aspartame was tested for DNA damaging activity in the *in vitro* primary rat hepatocyte/DNA repair assay at concentrations of 5 and 10 mM (Jeffrey and Williams, 2000). The compound was found negative in this assay.

Aspartame was evaluated *in vitro* in a chromosomal aberration test, a sister chromatid exchange test (SCE test) and a micronucleus test on human lymphocytes (Rencuzogullari *et al*., 2004). In each test, cells were exposed for 24 and 48 hours to concentrations of 500, 1,000 and 2,000 µg/mL. The tests were conducted in the absence of metabolic activation.

- in the chromosomal aberration test, statistically significant increases (2.5 to 4.2 fold the control values) in the percentage of aberrant cells or in the number of chromosomal aberrations per cell were observed at all doses. There were no relationships between the effects and the doses or the duration of exposure to aspartame. The chromosomal effects were observed in the presence of moderate cytotoxicity (18-24% at 24h and 11-56% at 48h).

- aspartame was found negative in the SCE test.

- in the micronucleus test, a statistically significantly increased incidence in micronucleated cells (2 fold the control values) was recorded at the dose of 2,000 µg/mL, at each treatment time. The effects were observed in the presence of high cytotoxicity (50% at 24 h and 70% at 48 h).

Taken together, the results of Rencuzogullari *et al*. indicate no direct interaction of aspartame with DNA, and suggest that the increase in chromosomal aberrations and micronuclei observed are related to an indirect mechanism such as cytotoxicity. Furthermore, the moderate dose-unrelated increase in the incidence of chromosomal aberrations in cultured human lymphocytes was observed in the absence of metabolic activation. The effect is regarded of no relevance for the *in vivo* situation.

In vivo assays

Aspartame was found negative in a dominant lethal assay when administered as a single oral dose of 2000 mg/kg bw to Sprague Dawley rat (JECFA 1980).

In a host mediated assay in rats, aspartame was administered orally at four dose levels, 250, 500, 1000 and 2000 mg/kg bw, for 5 days. In this test, the recoveries of bacteria from the peritoneal cavity were extremely low. In these experimental conditions, the mutation frequencies in the treated groups were not statistically significantly different from those in the control group (JECFA 1980).

The potential clastogenic activity of aspartame was evaluated in several *in vivo* chromosomal aberration assays in rat and mice.

- aspartame was administered orally for 5 days to Holtzman rats at doses of 440, 800, 1,200 or 1,600 mg/kg bw The compound did not induce chromosomal aberration levels higher than in control rats, in bone marrow cells or in spermatogonia (JECFA 1980).

- aspartame was administered orally for 5 days to rats at doses of 500, 1,000, 2,000 or 4,000 mg/kg bw No significant increases of chromosomal aberrations were observed in bone marrow cells at any tested doses of aspartame (JECFA 1980).

- when aspartame was administered orally for 5 days to C57Bl/6 mice at doses of 40 and 400 mg/kg bw, no clastogenic activity (chromosomal aberrations) was found in bone marrow (Durnev *et al*., 1995).

- in another published study (Mukhopadhyay *et al*., 2000), male Swiss mice were treated with single oral doses of aspartame at 3.5, 35 and 350 mg/kg bw in combination with acesulfame-K (1.5, 15 and 150 mg/kg bw, respectively). Bone marrow chromosomal analysis was performed on samples collected 18 h after administration. In these experimental conditions, aspartame did not induce statistically significant increases in the percentage of damaged cells or chromosomal aberrations per cell.

An acute bone marrow micronucleus test was conducted with aspartame administered orally to male Fisher 344 rats at 3 daily doses of 500, 1,000 or 2,000 mg/kg bw No increase in micronucleated polychromatic erythrocytes was observed at any dose level (NTP 2003).

Finally, peripheral blood micronucleus tests were conducted in male and female transgenic mice (Tg.AC hemizygous, p53 haploinsufficient and Cdkn2a deficient) after 9 months of exposure to aspartame at doses ranging from 3,125 to 50,000 mg/kg of food. The highest dose tested was equivalent to about 7500 and 8500 mg/kg bw in males and in females, respectively. Negative results, indicative of an absence of clastogenic activity of aspartame, were obtained in male and female Tg.AC hemizygous and Cdkn2a deficient mice and in male p53 haploinsufficient mice. In female p53 haploinsufficient mice, the results of the test were judged to be positive based on a statistically significantly increased frequency (x 2.3) of micronucleated erythrocytes seen in the 50,000 mg/kg group (NTP 2003).

In this respect the Panel noted that

- there is limited experience with the use of genetically modified rodents in genetic toxicity testing, with lack of information on variation of spontaneous mutation incidences;

- the reported increase in micronucleated erythrocytes was only observed in animals of one gender, in coincidence with a lower spontaneous control value;

- the effect described was observed after 9 month administration of a daily dose exceeding about 10-fold the highest recommended dose level for genotoxic toxicity testing according to OECD guidelines;

- no concurrent increase in tumour incidence was observed in the same group of animals.

On this basis the Panel concluded that the result of this study is of little if any biological significance.

Conclusion

In a battery of *in vitro* genotoxicity assays, aspartame was found to have no mutagenic or DNA damaging potential. At non-toxic concentrations, the compound had no *in vitro* clastogenic activity.
Aspartame was assayed for mutagenicity and clastogenicity in various recent or older, published or unpublished, *in vivo* studies. In all but one of these studies, aspartame was found to be negative. The isolated weak increase in blood micronucleated erythrocytes, occurring in female p53 haploinsufficient transgenic mice treated with the highest dose of aspartame, is considered of little if any biological significance.

Overall data strongly indicate that aspartame is devoid of any genotoxic potential in mice and rats.

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APPENDIX II

Kinetic data on aspartame

Introduction

The data presented in the sections below have been taken from the wealth of literature on aspartame and related substances, including information from an extensive review, edited by Stegink and Filer (1984). As the carcinogenicity study performed by the European Ramazzini Foundation (ERF; Soffritti *et al.*, 2006;) was in normal, post-pubertal animals, the discussion below focuses on the interpretation of these findings in the light of kinetic data from adult animals and normal human adults.

A major difference between the available data on aspartame kinetics, whether obtained in humans or experimental animals, and the aspartame carcinogenicity study by the ERF is that much higher dose levels were used in the ERF study. Only the lower end of the dose range used in the ERF study is covered by the available kinetic data, at least in humans,. Some data on kinetics are available in monkeys and dogs given aspartame at half the ADI (Oppermann, 1984). A study in pigs (Burgert *et al*., 1991) has been done at fairly high dose levels (up to ca. 18 times the ADI). Despite the availability of extensive reviews (e.g. Stegink and Filer, 1984), information on the kinetics of high dose levels of aspartame in rodents is not available in the open literature.

For methanol (MeOH), one of the hydrolysis products of aspartame (see below), studies of high dose kinetics have been performed, e.g. in relationship to the toxicity that has been observed with this substance (e.g. MAK, 2001). In monkeys the kinetics of MeOH were studied at bolus doses up to 750 times the amount released from aspartame at the ADI level. (McMartin *et al.* 1979)

Stability

Aspartame is not stable in aqueous solutions. It is converted into a diketopiperazine ((5-benzyl-3,6-dioxopiperazin-2-yl) acetic acid; DKP), especially at pH > 5 (see figure 1), which is hydrolysed at the lower end of the pH-range. Conversion of aspartame into DKP results in the loss of the sweet taste. Under dry conditions, conversion of aspartame into DKP is slow (5% per 100 hrs; at 105^{o}C) (Homler, 1984). It may be assumed that this implies there was sufficient stability of aspartame in the pelleted diet in the ERF study, despite the fact that regular checks of food quality and analysis were not documented. DKP can also be present in aspartame as a residue from the synthetic production process. ADIs of 40 and 7.5 mg/kg bw respectively have been set for both aspartame and DKP by JECFA and confirmed by the SCF (JECFA, 1980; SCF, 1985).

Hydrolysis

After oral ingestion, aspartame is hydrolysed, either within the lumen of the gastro-intestinal (GI) tract, or within the mucosal cells lining the inside of the GI-tract. Hydrolysis may be facilitated by specialised enzymes in the intestines (esterases, peptidases), rather than by the acidic conditions in the stomach (Oppermann, 1984). The products that result from these reactions are MeOH and the amino acids aspartic acid (Asp) and phenylalanine (Phe) (see figure 1). Hydrolysis is very efficient; the amount of aspartame that enters the bloodstream has been reported as undetectable in several studies (Oppermann, 1984; Burgert *et al*., 1991). A single report has suggested that around up to about 10% of the dose might be absorbed as

aspartame (Creppy *et al,* 1998), but this observation has not been mentioned in other reviews and would need confirmation.

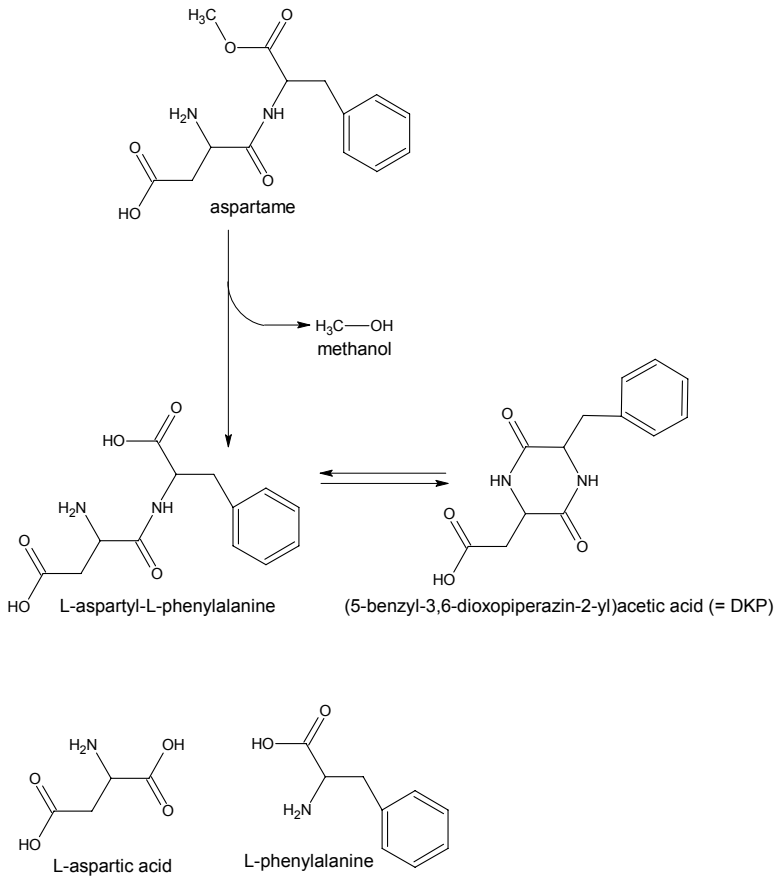

Figure I: Hydrolysis and cyclisation products of Aspartame.

Exposure estimates for the ERF study

Because for the ERF study no data on the actual aspartame contents of the animal feed was provided, accurate exposure estimates cannot be calculated. However, based on the actual feed intake and body weight data over weeks 0-113. The Panel calculated intakes of 0, 4.2, 20.6, 103, 513, 2439 or 4578 mg/kg bw./day in males and 0, 4.9, 23.8, 121, 578, 2755 or 5340 mg/kg bw./day in females, using the nominal concentrations of aspartame of 0, 80, 400, 2000, 10,000, 50,000 or 100,000 mg/kg in the diet. According to the authors the nominal concentrations are equivalent to aspartame intakes of 0, 4, 20, 100, 500, 2500 or 5000 mg/kg bw/day, based on average animal body weights of 400 g and an average feed consumption of 20 g/day for both sexes.

At the highest dose levels these aspartame intakes would result in doses of *ca.* 2400 mg Asp, 3000 mg Phe and 580 mg methanol/kg bw/d. These values may be compared to the intake of aspartame and its hydrolysis products in humans, when consumed at the ADI, at which level humans would be exposed to *ca.* 18.1 mg Asp, 22.5 mg Phe and 4.4 mg methanol/kg bw/d, or for a 60 kg person, *ca.* 1100 mg Asp, 1350 mg Phe and 260 mg methanol/d.

Non-aspartame sources of aspartame hydrolysis products

The hydrolysis products Phe, Asp and MeOH are normal constituents of the diet. The amino acids may be present as such or as part of the dietary proteins. The exposure to these amino acids depends strongly on the amount and type of the ingested dietary proteins. The Health Council of the Netherlands (Gezondheidsraad, 1999) estimated the daily intake of Phe and Asp (+ asparagine) at 4.9 and 8.4 g/day, respectively, based on a daily intake of 100 g of a standardised protein mixture (~30% soy protein and 60% casein); i.e. about 3.5 and 7.5 times higher than the intake of the Phe and Asp, respectively, from aspartame at the ADI level.

MeOH may also be present as such or is released from methyl-esters in foods such as fruits, fruit juices, vegetables, roasted coffee, honey and alcoholic beverages. It is also liberated by micro-organisms from methoxylated polysaccharides, such as pectin, or from these polysaccharides during cooking (IPCS, 1997). In fruit juices, concentrations may vary widely (1 - 640 mg MeOH/L) and also for alcoholic beverages a wide range of methanol concentrations has been reported.

Methanol is also a normal endogenous metabolite. It is released from S-adenosyl methionine when proteins are methylated by the enzyme protein carboxyl methylase. In a subsequent reaction, this methylated protein is de-methylated by protein methylesterases, and the methyl group is released as methanol (Stegink, 1984b).

Fate of the hydrolysis products

Asp and Phe

Immediately after hydrolysis and uptake, the amino acid fragments, Asp and Phe, which are no different from the ones that are liberated from the digestion of dietary proteins, can be incorporated in the normal physiological processes that are available for amino acids, i.e. protein synthesis, or degradation and conversion to other endogenous substances. In addition, Asp is also an important amino acid for shuttling reductive equivalents into mitochondria. Interestingly, the larger part of a dose of Asp does not enter the blood stream as such. Already in the mucosal cells 85% of a dose of Asp is converted into alanine, by decarboxylation, or by transfer of the amino group to pyruvate with concommitant production of oxaloacetic acid (i.e. the carbon skeleton of Asp) (Oppermann, 1984).

Methanol (MeOH)

Unlike the amino acids, Asp and Phe, methanol is not a primary nutritional substance, but it can be metabolised nevertheless and it can be incorporated into endogenous substances.

The metabolism of MeOH is fairly simple: it is oxidised stepwise via formaldehyde to formic acid and carbon dioxide. Metabolism of formaldehyde is extremely efficient. Even after intravenous infusion, it is difficult to find formaldehyde in blood, in which it has a half-life of about 1 minute (McMartin *et al*., 1979; Tephly and McMartin, 1984).

Plasma levels of aspartame and hydrolysis products in humans:

Stegink and Filer (1984) have reported on a sequence of experiments in humans to investigate the dose relationships between oral intake of aspartame and systemic blood levels of Asp, Phe and MeOH. The relationships were studied for doses ranging from 0 - 200 mg/kg bw/day, and the studies focussed on blood levels that occurred following a dose of 34 mg/kg bw/day, because at that time, this was the 99-percentile of the "projected" daily ingestion.

In some of the sections below, peak plasma levels of amino acids after aspartame intake are compared to peak plasma levels after ingestion of dietary proteins. It is noted that amino acids from proteins in meals are released slower than the amino acids in small peptides such as aspartame. Hence, it may be expected that peak plasma levels of e.g. Asp or Phe after protein ingestion are lower than peak plasma levels of these amino acids after ingestion of an equivalent amount of aspartame.

Plasma levels of MeOH

When human volunteers were given a single dose of 34 mg aspartame/kg bw, no detectable increase in the blood MeOH concentration could be observed (limit of detection: 3.5 -4 mg/L = *ca.* 120 μmol/L). When adults were given a dose of 50 mg aspartame/kg bw, mean peak blood levels of 3.4 mg/L were observed (i.e. just detectable). With doses of 100, 150 or 200 mg aspartame/kg bw, peak blood levels of 13, 21 and 26 mg MeOH/L blood were observed between 1 - 2 hrs post dosing. After 100 mg/kg bw, the blood MeOH returned to undetectable levels at 8 hrs post dosing. With the 150 and 200 mg/kg bw doses, MeOH persisted longer in the blood, but at 24 hrs post dosing blood MeOH was below the limit of detection. Both blood peak levels and blood AUCs were proportional to the dose (Tephly and McMartin, 1984).

Plasma levels of formate and the (reactive) formaldehyde intermediate metabolite have also been examined.

After giving aspartame to human volunteers at dose levels up to 200 mg/kg bw, no increase in the blood concentration of formic acid could be observed, not even after the highest dose of 200 mg aspartame/kg bw. Over the observation period of 0-24 hrs post dosing, the blood formic acid levels ranged from 10 - 20 mg/L, with no signs of any dose-related alterations, although renal excretion of formic acid was strongly enhanced during the 0-8 hrs period following aspartame intake (Stegink, 1984b).

Formaldehyde is an intermediate in the formation of formic acid from MeOH, but it is extremely rapidly eliminated from the blood. Even after intravenous infusion of formaldehyde, it is difficult to find this substance in the blood, because of a very short half-life (~ 1 minute). Formaldehyde formation was studied in older publications on acute toxicity of MeOH, but this intermediate could not be detected in animals or in humans (Tephly and McMartin, 1984). In monkeys given 3 g of MeOH/kg bw (750 times higher than available from aspartame at it's ADI level) formaldehyde could be detected in the blood at a level of 27 - 45 μM over the period of 18 hrs following dosing with methanol. No formaldehyde could be found in liver, kidney, urine or several neural tissues (LOD 25 μM; ~ 0.75 mg/L or 0.75 mg/kg tissue). Formic acid was readily detected in all tissues and body fluids studied, in particular in urine, liver, blood, and kidney (McMartin *et al.,* 1979).

Plasma levels of Asp

After a bolus dose of 34 mg aspartame/kg bw in humans, no rise in Asp levels in blood plasma could be observed and also plasma levels of some other amino acids (Glu, Gln, Asn), which might be derived from aspartate, were not elevated. Also at 50 mg/kg bw no changes in plasma levels of these amino acids were observed. In fasted adults, intakes of 100, 150 or 200 mg aspartame/kg bw caused a rise in the plasma Asp level of about 3-4 times the level before dosing (Stegink, 1984b). However, peak levels did not show any difference after the 150 or 200 mg/kg dose. The highest average peak concentration observed was 10 μmol Asp/L plasma (~ 1.3 mg/L) after 150 mg/kg bw, which was similar to the peak plasma level of *ca.* 1.1 mg/L, observed after the consumption of a meal containing 1 g protein/kg bw, providing

an amount of 82 mg Asp + Asn/kg bw (or *ca.* 46 mg Asp/kg bw; Stegink, 1984a). Also at these higher dose levels of aspartame, plasma Glu concentration showed a slight increase (up to 3-fold as compared to the pre-exposure concentration; max. 72.3 μmol/L (~10 mg/L) after 150 mg aspartame/kg bw) and again no proportionality to the dose of aspartame was observed (Stegink, 1984b). Glu levels did not increase above post-prandial peak levels (16 mg/L after 1 g protein/kg bw providing 162 mg Glu + Gln/kg bw or 94 mg Glu/kg bw; Stegink, 1984a).

Plasma levels of Phe

After a bolus dose of 34 mg aspartame/kg bw, a rise in Phe levels in blood plasma could be observed from ca. 50 to ca. 110 μmol/L (~ 8 – 18 mg/L). In erythrocytes a rise was observed from ca. 4 to ca. 7 μmol/100 g (~ 0.7 – 1.2 mg/100 g). Peak levels occurred at about 1 hr post administration and returned to normal within approximately 4 hrs. In addition a small increase in tyrosine levels was observed. The peak plasma Phe levels were more or less similar to those observed in humans postprandially (80 μmol Phe/L; ~ 13 mg/L; Stegink *et al*., 1991). With an aspartame dose of 50 mg/kg bw, the plasma peak was ca. 150 μmol/L (~ 25 mg/L). Phe levels were also determined after bolus doses of 100, 150 or 200 mg aspartame/kg bw. The following average peak levels of Phe were seen, respectively: ca. 200, 350 or 500 μmol/L (~ 33, 58 or 83 mg/L); and in all cases these fell back to normal levels within less than a day (Stegink, 1984b).

Metabolism of aspartame hydrolysis products

To study the fate of aspartame in animals, the substance has been given with ^{14}C labels in the various structural sub-fragments (i.e. MeOH, Asp and Phe). For reason of comparison, the same radioactivity was also administered in equimolar doses of the free main aspartame hydrolysis products.

^{14}C label in MeOH moiety:

When ^{14}C-MeOH or [^{14}C-methyl]-aspartame were given orally to rats or monkeys in equimolar doses, in both species, the radioactivity was rapidly eliminated via exhaled air (~60% of the dose in rats; ~ 70% in monkeys) within 8 hrs post dosing. With methanol a slightly higher rate of exhalation was observed, probably because hydrolysis and absorption are required when the radioactivity was administered as labelled aspartame. Virtually no radioactivity appeared in the faeces and 2 - 5% of the dose was eliminated via urine. In rats, ~40% and in monkeys about 30% of the dose was not accounted for, but it is noted that residual radioactivity in the carcass was not further studied. These studies were done with dose levels of 10 to 20 mg aspartame/kg bw (Oppermann, 1984).

Recently it has been demonstrated that when [^{14}C-methyl]-aspartame is administered to rats in a single oral dose level of 0.68 mmol/kg bw (~ 20 mg/kg bw) in liver, kidney and plasma about 0.1 – 0.4% of the dose/g tissue. In particular in blood (0.1-0.2%/g tissue) about 98% of the radioactivity was bound to protein and in liver about 78% was bound to protein / nucleic acids. Radioactivity was also found in protein of other tissues such as kidney (level about the same as in plasma), muscle, brain, cornea, retina and white and brown adipose tissue (each one order of magnitude less than plasma). The nature of the bound radioactivity was further studied in the liver. The authors speculated that the bound radioactivity resulted from the formation of formaldehyde from the ^{14}C-MeOH, because neither with MeOH nor with formate could covalent binding be demonstrated in direct labelling experiments. The exact nature of the radioactivity incorporated in proteins and nucleic acids was not elucidated. The chromatographic properties of their hydrolysis products (i.e. free amino acids and free DNA and RNA bases) were compared to those of normal amino acids and based on observed

differences, it was speculated that the incorporated radioactivity did not result from 1-carbon pathways (i.e. tetrahydrofolate pathways) but rather from direct formaldehyde adduct formation (Trocho *et al.,* 1998).

The study of Trocho *et al.* (1998) has been heavily criticised by Tephly (1999), who argues that neither methanol itself nor formaldehyde will reach the systemic circulation after oral exposure. The adducts in the study by Trocho *et al*. were not identified with certainty and adduct formation of ^{14}C-methanol or ^{14}C-formaldehyde were not studied *in vivo* for reasons of comparison. The very low amount of incorporation of ^{14}C from [^{14}C-methyl]-aspartame into tissue macro molecules was thought to be totally accounted for by the 1-carbon tetrahydrofolate pathway. However, Tephly (1999) did not comment on Trocho *et al.*'s finding of the aberrant chromatographic properties of the protein and nucleic acid hydrolysis products as compared to those of corresponding standards (i.e. free amino acids and nucleic acid bases).

It has also been argued that formaldehyde forms DNA protein cross-links (MAK, 2002) and that it reacts spontaneously with albumin (Tephly, 1999). This would at least explain findings of radioactive "adducts" in liver and plasma of animals dosed with aspartame (Trocho *et al.,* 1998). In addition, MAK (2002) have also argued that it is very difficult to study adduct formation of formaldehyde because of the efficient incorporation of this substance in 1-carbon pathways.

^{14}C label in Asp moiety:
In similar studies as described above for methyl-labelled aspartame, in rats, after [U-^{14}C-Asp]-aspartame, elimination of ^{14}C in the form of carbon dioxide in exhaled air accounted for 68% of the dose after [U-^{14}C-Asp]-aspartame and for 59% after [U-^{14}C-Asp], within 8 hrs post dosing. About 3-4% were eliminated via urine and < 2% via the faeces within 3 days post dosing. At the end of the observation period, some 10% of the radioactivity was still present in the carcass. With monkeys, at 12 hrs post dosing, about 77% of the dose was recovered as ^{14}C-CO_2 after aspartame and 67% after Asp. Also in monkeys urinary elimination of Asp or aspartame radioactivity was slightly more important (~ 3% of the dose) than faecal elimination (~ 1.5% of the dose). The authors mentioned that it was likely that the ~ 25% of the dose that was not recovered was still present in the animals' carcasses because of incorporation in normal body constituents (Oppermann, 1984).

^{14}C label in Phe moiety:
With [U-^{14}C phenylalaninyl]-aspartame, in rats and dogs, but not in monkeys, less radioactivity was exhaled as carbon dioxide than after administration of an equimolar amount of [U-^{14}C] Phe. The difference in rats and dogs, as compared to monkeys could not be explained. In all three species, exhalation accounted only for up to ca. 20% of the dose within 8 hrs of dosing. Some 5% were found in the urine and <1 to 3% of the dose was found in faeces in rats and monkeys. Dogs had a slightly greater faecal elimination (~6% of the dose). The author did not further comment on the reason for the overall low recovery of radioactivity from [^{14}C-Phe]-aspartame (Oppermann, 1984).

In the dogs after oral administration of [^{14}C-Phe]-aspartame, the nature of the plasma radioactivity was studied at various time points post dosing. Already 15 minutes after dosing, radioactivity was present as tyrosine or Phe but not as aspartame. The same results were found at later time points. In addition, it was demonstrated that plasma proteins contained the major

part of the plasma radioactivity already at 2 hrs post dosing, either as incorporated Phe, or as other amino acids with ^{14}C transferred from Phe to them (Oppermann, 1984).

Conclusion

After oral intake of aspartame in humans, the substance is readily hydrolysed either within the GI tract lumen or in the GI mucosal cells to yield the amino acids phenylalanine (Phe) aspartic acid (Asp) and methanol (MeOH). At dose levels which do not greatly exceed the ADI (40 mg/kg bw/d), even when taken as a bolus dose, no changes in the blood levels of MeOH or Asp occur, but some rise in Phe may be found.

MeOH can normally not be found in the blood and its metabolites (formaldehyde and formate) are also undetectable (limit of detection e.g. 25 μM for formaldehyde). This is also the case after intake of aspartame at the bolus-dose equivalent of the ADI. Asp is rapidly converted into other amino acids or metabolised to carbon dioxide and urea. A change in blood levels of Asp and related amino acids can only be observed at very high dose levels of ASP (> 100 mg/kg bw as a bolus dose), but these changes are similar to those found after consumption of (a fairly normal amount of) protein via the diet. At dose levels at the bolus-equivalent of the ADI, an increase in blood Phe can be observed but this remains within the range that can be found after consumption of a (a fairly normal amount of) protein via the diet. Phe-derived amino acids, in particular tyrosine, may show elevated blood levels, but these are also within the normal range.

It has been speculated that intake of aspartame may result in DNA adducts in particular in the liver of rats. However, it has not been demonstrated convincingly that these are real adducts or that the aspartame-related material is in fact derived from 1-carbon metabolism of the MeOH moiety and its primary metabolite formaldehyde. Similar incorporation of 1-carbon units would be expected from normal dietary exposure to MeOH, unrelated to aspartame. In addition the relevance of the alleged adducts may be questioned, because in none of the toxicity studies with aspartame has hepatotoxicity been observed, even at very high levels of exposure.

It is therefore concluded that at intakes at or below the current ADI in humans, no relevant systemic exposure to aspartame or its metabolites has been observed or should be expected.

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