Food and Chemical Toxicology 47 (2009) 1831–1836

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Genotoxicity studies on a high-purity rebaudioside A preparation

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ARTICLE INFO

Article history:
Received 18 March 2009
Accepted 26 April 2009

Keywords:
Rebaudioside A
Stevia
Genotoxicity

ABSTRACT

Rebaudioside A (Reb A) is a steviol glycoside isolated from the leaves of the *Stevia rebaudiana* plant. This non-nutritive, natural sweetener is reported to be 250–450 times sweeter than sucrose and has potential for wide use in the US diet, and is used in Japan and South America today. The safety of Reb A has been investigated in several recently published studies and information on genotoxicity is described herein. Reb A was investigated for its potential to induce genotoxicity in three *in vitro* and two *in vivo* assays (conducted according to OECD guidelines). Reb A was non-mutagenic in an Ames test using *Salmonella typhimurium* and *Escherichia coli*, in a chromosomal aberration test using Chinese Hamster V79 cells and in a mouse lymphoma assay using L5178Y+/− cells, all studies were conducted at concentrations up to 5000 μg/ml, with and without metabolic activation. Also, Reb A was non-genotoxic in a bone marrow micronucleus test in mice at doses up 750 mg/kg bw and in an unscheduled DNA synthesis test in rats at 2000 mg/kg bw. These studies provide additional evidence that Reb A is not genotoxic at the doses tested and further support the generally recognized as safe determination of Reb A.

1. Introduction

Due to increasing rates of obesity, sugar-free and reduced calorie foods and beverages are becoming increasingly popular in the United States and other countries (Kroger et al., 2006). Non-nutritive sweeteners duplicate the sweetness of sugar (sucrose) without the caloric intake.[1] In a recent study, it was reported that globally, the demand for alternatives to sugar is increasing at a rate of 8% *per* year (ScienceDaily, 2008). Currently, there are several synthetic non-nutritive sweeteners (e.g., aspartame, sucralose, neotame, etc.) approved for use as food ingredients by the United States Food and Drug Administration (FDA). These sweeteners range from being one hundred to several thousand times sweeter than sugar and can thus be used in small amounts to replace the sweetness of much larger amounts of sugar; for this reason, they are also often referred to as high-potency sweeteners (Kroger et al., 2006). However, for various technical and esthetic reasons, there has been a sustained consumer demand for natural low calorie sugar substitutes.

Stevia rebaudiana is a perennial herb that is a member of the Asteraceae family; the genus *Stevia* includes up to 300 other species (Kinghorn, 2002). The plant is native to northeastern Paraguay, but is also now cultivated in countries such as Mexico, Central America, Japan, China, Malaysia, South Korea, Spain, Belgium, the UK, and the US. The leaves of *S. rebaudiana* contain a complex mixture of at least eight different sweet *ent*-kaurene diterpene glycosides collectively known as steviol glycosides.[2] All of the steviol glycosides contain a common backbone called steviol and differ only in the glycosidic constituents attached at carbon-13 and/or carbon-19 (Kennelly, 2002). The most abundant of the steviol glycosides are stevioside and rebaudioside A (Reb A). The yields of stevioside and Reb A from dried leaves of *S. rebaudiana* can vary greatly from about 5–22% and 1.25–12% of the weight of dry leaves, respectively, depending on the cultivar and growth conditions (Kennelly, 2002). Reb A is the sweetest of the steviol glycosides and although non-caloric, is approximately 250–450 times sweeter than sucrose (Kinghorn, 2002). Extracts of *S. rebaudiana* (mainly stevioside) have been used extensively as sweeteners in several countries including Japan and Korea. In the United States, steviol glycosides, including Reb A, have been used as a dietary supplement since the passage of the Dietary Supplement Health and Education Act (DSHEA) in 1994 (Kroger et al., 2006), however, at that time they were not approved for use as food ingredients. Although Reb A is generally recognized as safe (GRAS) for use as a food ingredient in the United States, the objective of the current studies is to supplement the safety database on Reb A by describing the results of our genotoxicity studies. While some previous *in vitro* genotoxicity studies conducted with steviol glycosides have reported positive effects, in those studies the test materials utilized were either crude, not well-characterized extracts of *S. rebaudiana* or steviol and stevioside preparations of varying purity. Also, the test systems utilized were not standard assays and were not recommended in the Organization for Economic Co-operation

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[1] http://www.eatright.org/ada/files/Nutritivenp.pdf; site visited July 28, 2008.

[2] The steviol glycosides isolated from *S. rebaudiana* include: stevioside, rebaudiosides A–E, steviolbioside and dulcoside A.

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doi:10.1016/j.fct.2009.04.046

and Development (OECD) or FDA Redbook guidelines for genotoxicity studies (FDA, 2000; OECD, 2009). Therefore, we conducted a series of standardized, OECD and FDA Redbook compliant *in vitro* and *in vivo* studies, to investigate the potential genotoxicity of a high purity (>95%) Reb A preparation.

2. Methods and materials

2.1. Bacterial reverse mutation test (Ames test)

Reb A (batch 0703134, purity 95.6%) was evaluated for mutagenic activity in the Ames test using standard *Salmonella typhimurium* plate incorporation and preincubation methods (Ames et al., 1975; Maron and Ames, 1983) in accordance with OECD guideline number 471 entitled "Bacterial Reverse Mutation Test". The potential for mutagenicity was assessed in *S. typhimurium* tester strains TA98, TA100, TA1535, TA1537 and tester strain *Escherichia coli*. WP2uvrA incubated with Reb A at concentrations of 31.6, 100, 316, 1000, 2500 and 5000 μg/plate in the presence and absence of the postmitochondrial fraction of liver homogenates (S9) from rats pre-treated with Aroclor 1254. Toxicity was evaluated based on a decrease in the number of revertant colonies and/or thinning of the bacterial lawn. This study was conducted at BSL Bioscience, Scientific Laboratories, Behringstrasse 6, 82152 Planegg Germany.

2.2. In vitro mammalian chromosome aberration test

Reb A (batch 0703134, purity 95.6%) and control vehicle (0.9% sodium chloride) were evaluated for mutagenic potential in cultured human lymphocytes, in accordance with OECD guideline number 473 "Standard Chromosome Aberration Test." Genotoxicity was assessed in the presence and absence of postmitochondrial fractions of liver homogenates (S9) prepared from rats pre-treated with Aroclor 1254. The potential mutagenicity of Reb A was evaluated in two experiments in which the cells were exposed to the test material at short (4 h) term exposure at concentrations of 1000, 2500 and 5000 μg/ml and long term (20 h) exposure periods at concentrations of 1000, 2500 and 5000 μg/ml. This study was conducted at BSL Bioscience, Scientific Laboratories, Behringstrasse 6, 82152 Planegg Germany.

2.3. Mouse micronucleus test

Reb A (batch 0703134, purity 95.6%) was evaluated for mutagenic potential in mouse bone marrow cells and in accordance with the OECD guideline 474 entitled "Mammalian Erythrocyte Micronucleus Test".

2.3.1. Animals

Adult NMRI mice were obtained from Harlan Winkelmann (Borchen, Germany). Animals were housed in Macrolon Type III cages with standard bedding, and a controlled 12-h light/dark cycle. Temperature was maintained at 19–25 °C with a relative humidity of 45–65%. Potable water and standardized diet were provided *ad libitum*.

2.3.2. Experimental procedure

Preliminary toxicity tests were conducted to determine appropriate dose levels for the experiment. For the test material and controls, groups of 5 male and female Swiss NMRI mice were administered the test article via intraperitoneal injection (*i.p.*). For the Reb A treatment, groups of male and female mice were injected once with 150, 375, or 750 mg/kg bw and observed for 44 h. Two additional groups of male and female mice were administered the negative control (0.9% NaCl) or test material at the high dose (750 mg/kg bw) and observed for 68 h. A positive control group was administered cyclophosphamide (via *i.p.* injection) at 40/mg/kg. Samples of peripheral blood were collected from the tail vein of each animal 44 or 68 h after treatment. For each animal, the number of micronucleated polychromatic erythrocytes (MPE) was counted in 1000 polychromatic erythrocytes (PE). The polychromatic (PE) and normochromatic (NE) erythrocyte ratio was determined after scoring 1000 erythrocytes (E). The results were expressed as relative PCE (relative PCE = proportion of polychromatic (immature) erythrocytes among total erythrocytes). This study was conducted at BSL Bioscience, Scientific Laboratories, Behringstrasse 6, 82152 Planegg Germany.

2.4. Mouse lymphoma assay

Mouse lymphoma L5178Y+/− cells were used to evaluate the mutagenicity of Reb A (batch 0703134, purity 95.6%) in a eukaryotic cell culture system in accordance with the OECD guideline 476 entitled "*In vitro* Mammalian Cell Gene Mutation Test". Established cultures in suspension were maintained in RPMI 1640 medium supplemented with 9 μg/ml hypoxanthine, 15 μg/ml thymidine, 22.5 μg/ml glycine and 0.1 μg/ml methotrexate. The potential mutagenicity of Reb A was evaluated in two experiments in which the cells were exposed to the test material at short (4 h) and long term (24 h) exposure periods. In experiment I, forward mutations in the thymidine kinase locus were evaluated in cells following exposure to Reb A for 4 h at concentrations of 10, 39, 156, 313, 625, 1250, 2500 and 5000 μg/ml with and without S9. In experiment II, forward mutations were evaluated in cells following exposure to Reb A for 24 h at concentrations of 100, 200, 400, 800, 1500, 2500, 3750 and 5000 μg/ml with S9 and 20, 39, 156, 625, 1250, 4000, 4500 and 5000 μg/ml without S9. This study was conducted at BSL Bioscience, Scientific Laboratories, Behringstrasse 6, 82152 Planegg Germany.

2.5. Unscheduled DNA synthesis (UDS) test

Reb A (batch 0703134, purity 95.6%) was evaluated for potential genotoxicity in mammalian liver cells and in accordance with the OECD guideline 486 entitled "Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo*".

Groups of fasted (6–18 h) male Wistar rats (four *per* group) were treated *via* gavage with a single dose of Reb A (batch 0702011, purity 95.6%) at 2000 mg/kg bw, the vehicle control (distilled water) or the positive controls, 2-acetylaminofluorene (100 mg/kg bw) and dimethylnitrosamine (5 mg/kg bw). Following the exposure periods, the rats were anaesthetized and hepatocytes were isolated from the liver of each animal by perfusion with Hank's balanced salt solution supplemented with collagenase type IV adjusted to pH 7.4 and maintained at 37 °C. The number of silver grains above the nucleus were counted. In addition, the number of grains of one nucleus-equivalent area over the cytoplasm adjacent to the nucleus was counted. At least two slides *per* animal and 50 cells *per* slide were evaluated. Three animals *per* group were evaluated as described above. The remaining animal *per* test group was evaluated if an animal died spontaneously or in case of technical problems concerning isolation of the hepatocytes. This study was conducted at BSL Bioscience, Scientific Laboratories, Behringstrasse 6, 82152 Planegg Germany.

3. Results

3.1. Bacterial reverse mutation test (Ames test)

There were no statistically significant increases in the number of revertant colonies or cytotoxicity in any *S. typhimurium* or *E. coli* strain exposed to Reb A, with or without metabolic activation at concentrations up to 5000 μg/plate. Under the conditions of this study, Reb A was non-mutagenic in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *E. coli* strain WP2uvrA, as indicated in Table 1.

3.2. In vitro mammalian chromosome aberration test

Reb A did not induce a statistically significant increase in the incidence of chromosomal aberrations or polyploidy in cultured Chinese Hamster V79 cells after 4-, and 20-h treatments at any of the doses tested, with or without S9 metabolic activation (Table 2). Under the conditions of this study, Reb A is non-mutagenic.

3.3. Mouse micronucleus test

All animals in the highest dose group exhibited signs of toxicity in the form of reduction of spontaneous activity, rough fur, prone position and cramps. Reb A did not induce any statistically significant increases in the incidence of polychromatic immature erythrocytes or micronucleated immature erythrocytes and was not cytotoxicity in NMRI mice administered Reb A at doses of 150, 375 or 750 mg/kg bw compared to the vehicle control (Table 3). Under the conditions of this study, Reb A was non-mutagenic.

3.4. Mouse lymphoma assay

Reb A did not induce any treatment-related increases in the incidence of mutations or clastogenic effects in mouse lymphoma L5178Y+/− cells after 4- and 20-h treatments at concentrations of 10, 39, 156, 313, 625, 1250, 2500 and 5000 μg/ml, with and without metabolic activation, when compared to the negative control. Based on the results observed in this study, Reb A was non-mutagenic (Table 4).

3.5. Unscheduled DNA synthesis (UDS) test

Reb A did not cause any signs of toxicity in male Wistar rats administered a single oral dose of Reb A at 2000 mg/kg bw when

Table 1
Reverse mutation assay of Reb A in *Salmonella typhimurium* and *Escherichia coli*: mean number of revertants/plate.

Concentration (μg/plate)	TA98		TA100		TA1535		TA1537		WP2uvra	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Panel A										
0	28	28	78	90	8	12	9	8	43	51
31.6	22	28	87	80	9	6	10	12	51	52
100	22	33	95	93	10	13	7	9	58	50
316	22	26	77	80	5	9	8	10	57	50
1000	22	29	76	95	5	11	6	13	45	55
2500	26	37	80	78	7	10	7	10	47	31
5000	30	30	87	95	6	9	5	13	40	42
Positive control	522[a]	1205[b]	748[c]	574[b]	79[c]	872[b]	48[a]	297[b]	386[d]	170[b]
Panel B										
0	19	26	90	88	8	10	7	11	46	48
31.6	18	24	88	87	7	8	11	12	48	52
100	23	23	90	103	11	6	7	9	47	53
316	21	29	101	102	8	13	8	14	47	49
1000	19	27	109	90	6	11	7	11	53	45
2500	20	25	97	90	6	9	10	13	54	53
5000	26	28	93	80	6	9	7	11	46	52
Positive control	551[a]	1987[b]	773[c]	1450[b]	545[c]	107[b]	146[a]	183[b]	578[d]	144[b]

Reb A was tested using the standardized plate incorporation assay (Panel A) and the modified preincubation method (Panel B).
[a] 4-Nitro-o-phenylenediamine.
[b] 2-Aminoanthracene.
[c] Sodium azide.
[d] Methylmethanesulfonate.

Table 2
Chromosomal aberrations formed after treatment with Reb A (μg/ml) in the absence (A) or presence (B) of S9 mix.

	Structural chromosome aberrations		Mean *percent* aberrant cells	
Treatment group	Total (+gap)	Total (−gap)	Mean% (+gap)	Mean% (−gap)
Group A. −S9 mix				
4-h treatment, 20 h fixation period				
0	8	4	4.0	2.0
1000	11	6	5.5	3.0
2500	9	7	4.5	3.5
5000	8	3	4.0	1.5
900 EMS	25	19	12.5	9.5
20-h treatment, 20 h fixation period				
0	7	2	3.5	1.0
1000	4	1	2.0	0.5
2500	11	4	5.5	2.0
5000	7	4	3.5	
400 EMS	29	21	2.0	
Group B.+S9 mix			14.5	10.5
4-h treatment, 20 h fixation period				
0	9	3	4.5	1.5
1000	6	3	3.0	1.5
2500	5	3	2.5	1.5
5000	5	2	2.5	1.0
900 CPA	25	21	12.5	10.5
4-h treatment, 20 h fixation period				
0	7	3	3.5	1.5
1000	6	3	3.0	1.5
2500	6	4	3.0	2.0
5000	5	2	2.5	1.0
900 CPA	25	18	12.5	9.0

CPA – cyclophosphamide (positive control); EMS – ethyl methanesulfonate (positive control).

observed for 2 and 16 h post-dose. Analysis of hepatocytes revealed no UDS induction in the hepatocytes of the animals administered Reb A or the negative control (Table 5). In contrast, the positive controls 2-acetylaminofluorene and dimethylnitrosamine caused a significant increase in the net nuclear grain counts and the percentage of cells with net grain counts >5%. Under the condition of this study, Reb A was non-genotoxic.

4. Discussion

Due to concern over reported positive *in vitro* genotoxic effects of steviol, the potential genotoxicity of steviol glycosides has been in question. Steviol, tested *in vitro,* had been reported to exhibit positive results in the *umu*-test, chromosomal aberration assay, gene mutation assay, plasmid mutagenesis assay and the forward mutation assay. In the *umu*-test, which measures the induction of the *umu*-operon as an indirect measure of gene mutation, *S. typhimurium* strain TA1535/pSK1002 was incubated with steviol at concentrations ranging from 313–2500 μg/ml with and without metabolic activation (S9). It was reported that steviol exhibited a positive response in the absence of S9 at 625 and 1250 μg/ml, but not at 2500 μg/ml, the highest dose tested. In the presence of S9, it was reported that steviol was positive at 1250 and 2500 μg/ml, but was negative at 625 μg/ml (Matsui et al., 1996). The authors

Table 3
Micronucleus frequency in bone marrow cells from NMRI mice administered a single oral dose of saline (vehicle control), Reb A or positive control.

Dose (mg/kg)	Sampling time (h)	Sex	No. of animals	Relative PCE*	Relative MN**
0	44	F	5	2.35	0.14 ± 0.02
		M	5	2.91	0.20 ± 0.07
150	44	F	5	2.23	0.16 ± 0.04
		M	5	3.12	0.23 ± 0.05
375	44	F	5	2.14	0.25 ± 0.08
		M	5	3.38	0.25 ± 0.09
750	44	F	5	2.82	0.16 ± 0.05
		M	5	4.15	0.33 ± 0.28
40 (CPA)	44	F	5	1.44	1.27 ± 0.55
		M	5	1.39	2.13 ± 0.42
0	68	F	5	2.81	0.18 ± 0.06
		M	5	3.42	0.21 ± 0.06
750	68	F	5	2.96	0.17 ± 0.06
		M	5	5.22	0.22 ± 0.05

CPA – cyclophosphamide (positive control).
* Relative PCE is the quotient of polychromatic (immature) erythrocytes to total erythrocytes.
** Relative mean is the (quotient of micronucleated polychromatic (immature) erythrocytes to total PCE) × 100.

Table 4
Mutation frequencies and viabilities of L5178 $tk^{+/-}$ mouse lymphoma cells after 4 or 24 h exposure to Reb A in the presence and absence of S9.

Incubation condition	Concentration (μg/ml)	RSG (%)	RCE (%)	RTG (%)	Mutants/10^6 cells*
4 h −S9	0	100.00	100.00	100.00	42.62
	0	100.00	100.00	100.00	49.09
	10	100.35	101.73	102.09	44.91
	39	97.61	90.17	88.02	63.82
	156	102.15	98.84	100.97	46.13
	313	104.60	95.95	100.37	58.22
	625	104.87	94.22	98.81	59.04
	1250	106.26	99.42	105.64	54.68
	2500	105.95	104.05	110.24	36.82
	5000	95.40	103.47	98.71	46.83
EMS	500	76.33	76.30	58.24	1096.29
MMS	10	84.67	87.86	74.39	364.24
24 h −S9	0	100.00	100.00	100.00	51.91
	0	100.00	100.00	100.00	90.04
	20	91.81	103.01	94.58	61.42
	39	93.88	91.57	85.96	68.10
	156	90.57	105.42	95.48	58.11
	625	86.10	101.20	87.13	58.30
	1250	81.05	86.75	70.31	68.53
	4000	60.28	92.77	55.92	61.56
	4500	54.19	100.00	54.19	53.43
	5000	54.55	101.81	55.53	58.28
EMS	200	53.60	77.71	41.65	1555.30
MMS	10	59.45	95.18	56.58	522.37
4 h +S9	0	100.00	100.00	100.00	48.66
	0	100.00	100.00	100.00	86.90
	10	103.97	94.12	97.86	65.07
	39	102.94	110.84	114.09	44.13
	156	97.57	100.93	98.47	66.71
	313	98.58	103.41	101.93	61.86
	625	105.25	105.26	110.79	50.41
	1250	93.73	104.64	98.08	51.47
	2500	95.30	108.36	103.27	47.02
	5000	94.74	102.17	96.79	49.63
B[a]P	3.5	54.88	113.31	62.19	227.23
24 h +S9	0	100.00	100.00	100.00	71.23
	0	100.00	100.00	100.00	78.97
	100	105.31	99.38	104.66	93.92
	200	102.03	109.88	112.10	57.62
	400	111.13	104.32	115.93	81.92
	800	99.72	106.79	106.49	71.81
	1500	105.47	116.05	122.39	35.11
	2500	94.76	98.15	93.00	78.59
	3750	103.57	91.98	95.26	92.49
	5000	88.26	99.38	87.72	91.09
B[a]P	3.5	55.54	95.68	53.14	490.23

B[a]P – benz(a)pyrene; EMS – ethylmethanesulfonate; MMS – methylmethanesulfonate; RCE – relative cloning efficiency = [(mean value positive cultures/mean value positive cultures of corresponding controls) × 100]; RSG – relative suspension growth = [(value total suspension growth/value total suspension growth or corresponding controls) × 100]; RTG – relative total growth = (RSG × RCE)/100.
* Mutation frequency = [−ln(negative cultures/total wells (selective medium))/−ln(negative cultures/total wells (non-selective medium))] × 800.

Table 5
UDS in rat hepatocytes isolated 2 or 16 h after administration of negative control, Reb A, or positive control.

Treatment	Net grain count	*Percentage* with > 5 net grains
2 h post treatment		
Reb A 2000 mg/kg	1.41	3.33
DMN 1000 mg/kg	8.70	85.7
16 h post treatment		
Control (distilled water)	0.98	1.33
Reb A 2000 mg/kg	1.08	0.67
2-AAF 5 mg/kg	13.90	96.0

2-AAF – 2-acetylaminofluorene; DMN – dimethylnitrosamine; UDS – unscheduled DNA synthesis.

concluded that steviol was only weakly positive as there was only a 2-fold increase in *umu*-operon induction. However, the reported positive responses were not dose-dependent and in the case of the 625 μg/ml concentration, a positive response was observed without metabolic activation but not with, which suggests that the reported positive responses were not related to treatment. In addition, this assay is not a standard gene mutation assay and is not listed in the OECD or FDA Redbook guidelines form genotoxicity studies.

In another gene mutation assay and in a chromosomal aberration assay; both conducted in Chinese hamster lung (CHL) cells, positive results were only observed with metabolic activation and only at doses that were also cytotoxic (400–1000 μg/ml) which suggests that the positive findings were a result of cytotoxicity as opposed to genotoxicity (Matsui et al., 1996). In forward mutation assays it was reported that steviol induced mutagenicity in *S. typhimurium* strain TM677 in the presence of metabolic activation (Pezzuto et al., 1985; Matsui et al., 1996). In a subsequently study, it was reported that 15-oxosteviol, a possible metabolite of steviol, was responsible for the mutagenicity observed in *S. typhimurium* TM677 treated with steviol (Pezzuto et al., 1985). To further investigate the potential genotoxicity of steviol and possible metabolites, Procinska et al. (1991) conducted a series of preliminary forward mutation, reverse mutation and histidine-independence assays with 15-oxosteviol. It was reported that there was no evidence of mutagenicity in any of the test systems. Procinska et al. (1991) also repeated the forward mutation assay conducted by Pezzuto et al. (1985); using *S. typhimurium* TM677 and 15-oxosteviol. In this study, 15-oxosteviol did not induce a genotoxic response. It was also stated by Procinska et al. (1991) that further examination of the positive results reported by Pezzuto et al. (1985) revealed that the results were not supported by the data and that the data was misinterpreted due to a common error in interpreting data generated from the *S. typhimurium* TM677 system.

The positive results reported in some of the previous studies were not corroborated in a variety of OECD and FDA Redbook recommended *in vitro* and *in vivo* assays in which steviol did not exhibit genotoxicity at significantly higher concentrations. These studies included an Ames test with *S. typhimurium* strains TA97, TA98, TA100, TA102, TA104, TA1535, TA1537 and *E. coli* WP2uvrA/pKM101 at concentrations up to 5000 μg/plate (Suttajit et al., 1993; Matsui et al., 1996), a chromosomal aberration assays in Chinese hamster lung fibroblasts and human lymphocytes at 0.1–1.5 mg/ml and a *rec* (gene mutation) assay with *Bacillus subtilis* at 10 mg/disk (Suttajit et al., 1993; Matsui et al., 1996). Under *in vivo* conditions, steviol did not exhibit genotoxicity in a comet assay in BDFI mice at up to 2000 mg/kg bw (Sekihashi et al., 2002) or in bone marrow micronucleus tests in rats, mice and hamsters at dose ranging from 250–8000 mg/kg bw (Matsui et al., 1996; Temcharoen et al., 2000). In addition, the US Environmental Protection Agency (EPA) indicates in its Guidelines for Mutagenicity Risk Assessment (EPA, 1986) that more weight should be placed on *in vivo* mutagenicity studies as compared to *in vitro* mutagenicity studies. Further, in the FDA Toxicological Principles for the Safety Assessment of Food Ingredients (FDA, 1982) it is noted that positive genotoxicity results that may not be relevant *in vivo*, may arise *in vitro* due to changes in pH, osmolality or high levels of cytotoxicity. Therefore, the negative *in vivo* results observed in the rat micronucleus tests should outweigh the positive results obtained in the *in vitro* chromosome aberration study. Hence, when taken together, these studies provide strong evidence that steviol has a low order of genotoxic potential.

Several *in vitro* and *in vivo* studies have also been conducted with the steviol glycoside, stevioside. The test systems utilized in these studies included bacterial and mammalian cell culture as well as intact rats, mice and hamsters. The genotoxicity endpoints evaluated in these studies included gene mutation, chromosome alterations as well as DNA damage. With the exception of one Ames test that reported positive results in *S. typhimurium* strain TA98 (and then only at the highest concentration of 50 mg/plate) (Suttajit et al., 1993; Temcharoen et al., 2000), stevioside was negative in all *in vitro* assays. In *in vivo* studies conducted in hamsters, rats and mice, stevioside did not exhibit genotoxicity at doses up to 2000 mg/kg bw/day (Sasaki et al., 2002). However, in a Comet assay, it was reported that stevioside caused DNA damage in rats administered an aqueous stevioside solution at 4 mg/ml (approximately 400 mg/kg bw/day) for 45 days (Nunes et al., 2007). However, the validity of this study has been questioned due to several flaws in the methodology and data analysis (Williams, 2007; Geuns, 2007).

In the current study, the potential genotoxicity of Reb A was investigated *in vitro* and *in vivo* in a series of studies, which included an Ames test, chromosomal aberration test, mouse lymphoma assay, unscheduled DNA synthesis test and a bone marrow micronucleus test. In the *in vitro* studies, *S. thyphimurium*, *E. coli*, mouse lymphoma and Chinese Hamster V79 cells were exposed to Reb A at concentrations up to 5000 μg/ml for 4–48 h. Reb A did not exhibit any mutagenic activity in any of the strains utilized at any of the concentrations tested. Also, in an *in vivo* mouse micronucleus test at doses up to 750 mg/kg bw and an unscheduled DNA synthesis test in rats at doses up to 2000 mg/kg bw, Reb A did not cause any genotoxic effects at any of the doses tested.

The results of the studies described in this manuscript are in agreement with the conclusion of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which conducted a comprehensive review of the available genotoxicity data on steviol and steviol glycosides. In the final report from the JECFA review, it was concluded that Reb A and stevioside have not shown conclusive evidence of genotoxicity *in vitro* and *in vivo* in a variety of standardized assays (JECFA, 2004). In addition, Brusick (2008) conducted a literature review of the genotoxicity studies conducted on steviol and steviol glycosides and concluded that based on a Weight-of-Evidence approach that these substances do not pose a risk of genetic damage following human consumption. Therefore, based on the totality of evidence, including our empirical observations presented herein and reports in the scientific literature, we have concluded that rebaudioside A is non-genotoxic. Further, these findings support the generally recognized as safe status of rebaudioside A.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

Ames, B.N., McCann, J., Yamasaki, E., 1975. Methods for detecting carcinogens and mutagens with the salmonella/mammalian-microsome mutagenicity test. Mutat. Res. 31, 347–364.

Brusick, D.J., 2008. A critical review of the genetic toxicity of steviol and steviol glycosides. Food Chem. Toxicol. 46, S83–S91.

EPA, 1986. Guidelines for Mutagenicity Risk Assessment. Published on September 24, 1986, Federal Register 51(185):34006–34012. Risk Assessment Forum, US Environmental Protection Agency, Washington, DC. Report Number: EPA/630/R-98/003.

FDA, 1982. Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food ('Redbook'). US Food and Drug Administration, Washington, DC.

FDA, 2000. Redbook 2000. Toxicological Principles for the Safety Assessment of Food Ingredients. IV.C.1. Short-term Tests for Genetic Toxicity. Office of Food Additive Safety. Center for Food Safety and Applied Nutrition. US Food and Drug Administration (FDA). Available from: <http://www.cfsan.fda.gov/redbook/red-ivc1.html>.

Geuns, J.M.C., 2007. Letter to the editor: comments to the paper by Nunes et al., 2007. Food Chem. Toxicol. 45, 2601–2602.

JECFA, 2004. Steviol glycosides. Sixty-third Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). World Health Organization (WHO), Geneva, Switzerland. Report Number: JECFA/63/SC.

Kennelly, E.J., 2002. Sweet and non-sweet constituents of *Stevia rebaudiana*. In: Kinghorn, A.D. (Ed.), Stevia: The Genus Stevia. Taylor and Francis Inc., London, England, pp. 68–85.

Kinghorn, A.D., 2002. Overview. In: Kinghorn, A.D. (Ed.), Stevia: The Genus Stevia. Taylor and Francis Inc., London, England, pp. 1–17.

Kroger, M., Meister, K., Kava, R., 2006. Low-calorie sweeteners and other sugar substitutes: a review of the safety issues. Comprehensive Reviews in Food Science and Food Safety 5, 35–47.

Maron, D.M., Ames, B.N., 1983. Revised methods for the *Salmonella* mutagenicity test. Mutat. Res. 113, 173–215.

Matsui, M., Matsui, K., Kawasaki, Y., Oda, Y., Noguchi, T., Kitagawa, Y., Sawada, M., Hayashi, M., Nohmi, T., Yoshihira, K., Ishidate, M., Sofuni, T., 1996. Evaluation of the genotoxicity of stevioside and steviol using six *in vitro* and one *in vivo* mutagenicity assays. Mutagenesis 11, 573–579.

Nunes, A.P.M., Ferreira-Machado, S.C., Nunes, R.M., Dantas, F.J.S., De Mattos, J.C.P., Caldeira-de-Araujo, A., 2007. Analysis of genotoxic potentiality of stevioside by comet assay. Food Chem. Toxicol. 45, 662–666.

OECD, 2009. OECD Guidelines for the Testing of Chemicals. Section 4: Health Effects. Organisation for Economic Co-operation and Development (OECD). Available from: <http://titania.sourceoecd.org/vl=785010/cl=26/nw=1/rpsv/cw/vhosts/oecdjournals/1607310x/v1n4/contp1-1.htm>.

Pezzuto, J.M., Compadre, C.M., Swanson, S.M., Nanayakkara, N.P.D., Kinghorn, A.D., 1985. Metabolically activated steviol, the aglycone of stevioside, is mutagenic. Proc. Natl. Acad. Sci. USA 82, 2478–2482.

Procinska, E., Bridges, B.A., Hanson, J.R., 1991. Interpretation of results with the 8-azaguanine resistance system in *Salmonella typhimurium*: no evidence for direct acting mutagenesis by 15-oxosteviol, a possible metabolite of steviol. Mutagenesis 6, 165–167.

Sasaki, Y.F., Kawaguchi, S., Kamaya, A., Ohshita, M., Kabasawa, K., Iwama, K., Taniguchi, K., Tsuda, S., 2002. The comet assay with 8 mouse organs: results with 39 currently used food additives. Genetic Toxicology and Environmental Mutagenesis 519, 103–119.

ScienceDaily, 2008. Alternative Sweeteners are Increasing by 8% a Year. Available from: <http://www.sciencedaily.com/releases/2008/05/080512140912.htm>.

Sekihashi, K., Saitoh, H., Sasaki, Y.F., 2002. Genotoxicity studies of stevia extract and steviol by the comet assay. J. Toxicol. Sci. 27, 1–8.

Suttajit, M., Vinitketkaumnuen, U., Meevatee, U., Buddhasukh, D., 1993. Mutagenicity and human chromosomal effect of stevioside, a sweetener from *Stevia rebaudiana* Bertoni. Environ. Health Perspect. 101 (Suppl. 3), 53–56.

Temcharoen, P., Suwannatrai, M., Klongpanichpak, S., Apibal, S., Glinsukon, T., Toskulkao, C., 2000. Evaluation of the effect of steviol on chromosomal damage using micronucleus test in three laboratory animal species. J. Med. Assoc. Thai. (Chotmaihet thangphaet) 83, S101–S108.

Williams, G.M., 2007. Letter to the editor. Food Chem. Toxicol. 45, 2597–2598.