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A critical review of the genetic toxicity of steviol and steviol glycosides

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ABSTRACT

Extracts of the leaves of the stevia plant (*Stevia rebaudiana* Bertoni) are used to sweeten food and beverages in South America, Japan and China. The components responsible for the sweet properties of the plant are glycosides of steviol, primary stevioside (ent-13-hydroxykaur-16-en-18-oic acid), which is 250–300 times sweeter than sucrose and rebaudiosides A and C. Stevioside and steviol have been subjected to extensive genetic testing. The majority of the findings show no evidence of genotoxic activity. Neither stevioside nor its aglycone steviol have been shown to react directly with DNA or demonstrate genotoxic damage in assays relevant to human risk. The mutagenic activity of steviol and some of its derivatives, exhibited in strain TM677, was not reproduced in the same bacteria having normal DNA repair processes. The single positive *in vivo* study measuring single-strand DNA breaks in Wistar rat tissues by stevioside, was not confirmed in experiments in mice and appears to be measuring processes other than direct DNA damage. Neither stevioside nor steviol-induced clastogenic effects at extremely high dose levels *in vivo*. Application of a Weight-of-Evidence approach to assess the genetic toxicology database concludes that these substances do not pose a risk of genetic damage following human consumption.

1. Introduction

Stevia, a member of the Compositae family, is a plant native to South America, but has been distributed to Southeast Asia. Extracts of the leaves of the stevia plant (*Stevia rebaudiana* Bertoni) have been used for centuries to sweeten food and beverages in South America, Japan and China (Geuns, 2003). The primary components responsible for the sweet properties of the plant are glycosides of steviol. They are extracted from the leaves with hot water, followed by solvent purification of the water-soluble extract. The leaves of the stevia plant contain at least 10 different glycosides, the major constituents being stevioside and rebaudoside A. The primary glycoside is stevioside (ent-13-hydroxykaur-16-en-18-oic acid) which is 250–300 times sweeter than sucrose (JECFA, 1999). Stevioside is very stable (JECFA, 1999; Geuns, 2003). Other glycosides found in significant amounts include rebaudiosides A and C and dulcoside A. Fig. 1 shows the structures of the major glycosides found in stevia extracts.

Following oral administration, stevioside is poorly absorbed by the upper intestinal tract of rodents and humans; however, stevioside is almost completely metabolized by intestinal microflora in the lower intestinal tract to the aglycone, steviol (JECFA, 1999).

Metabolism studies of stevioside in humans found very low blood levels of stevioside or free steviol (JECFA, 2005). Male volunteers given a single dose of 375 mg of stevioside showed peak blood levels of stevioside to be 0.1 μg/ml at 60–180 min after dosing. No free steviol, epoxide or 15-oxosteviol was detected. As a result of metabolism of stevioside by bacterial flora in the lower intestine, free steviol was only found in the feces (Wingard et al., 1980). A second study was conducted with male and female volunteers. Each person received 250 mg of stevioside (>97% purity) at 8 h intervals (3X) for 10 consecutive days. Analyses for stevioside, free steviol and steviol metabolites were conducted with blood, urine and feces (Geuns et al., 2007). No free stevioside or steviol was detected in the blood or urine. Following hydrolysis with β-glucuronidase/sulfatase, steviol was detected at concentrations ranging from 0.7 μg/ml to 21 μg/ml (JECFA, 2005). In rats given a single oral dose of 500 mg/kg body weight of stevioside (95% purity), low concentrations of steviol were found after 8 h which increased up to 1 μg/ml at 24 h following administration (Wang et al., 2004). Geuns (2003) argues that only very small amounts of stevioside or steviol are likely absorbed from the human gut following oral administration and that no other metabolites are likely produced *in vivo*. Steviol metabolism by colon bacteria is similar in rats and humans, although the rate of metabolism and uptake in rats appears to be slightly faster (Koyama et al., 2003).

Toxicological assessments of stevioside suggest that it is a relatively safe compound. Stevioside has a very low acute oral toxicity

Abbreviations: bw, body weight; CHL, Chinese hamster lung; DNA, deoxyribonucleic acid; g, gram; kg, kilogram; JECFA, Joint FAO/WHO Expert Committee on Food Additives; mg, milligram; mL, milliliter; μg, microgram; NCE, normal chromatic erythrocyte; PCE, poly chromatic erythrocyte; rfa, deep rough; RTG, relative total growth; SCE, sister chromatid exchange; tk, thymidine kinase; WHO, World Health Organization.

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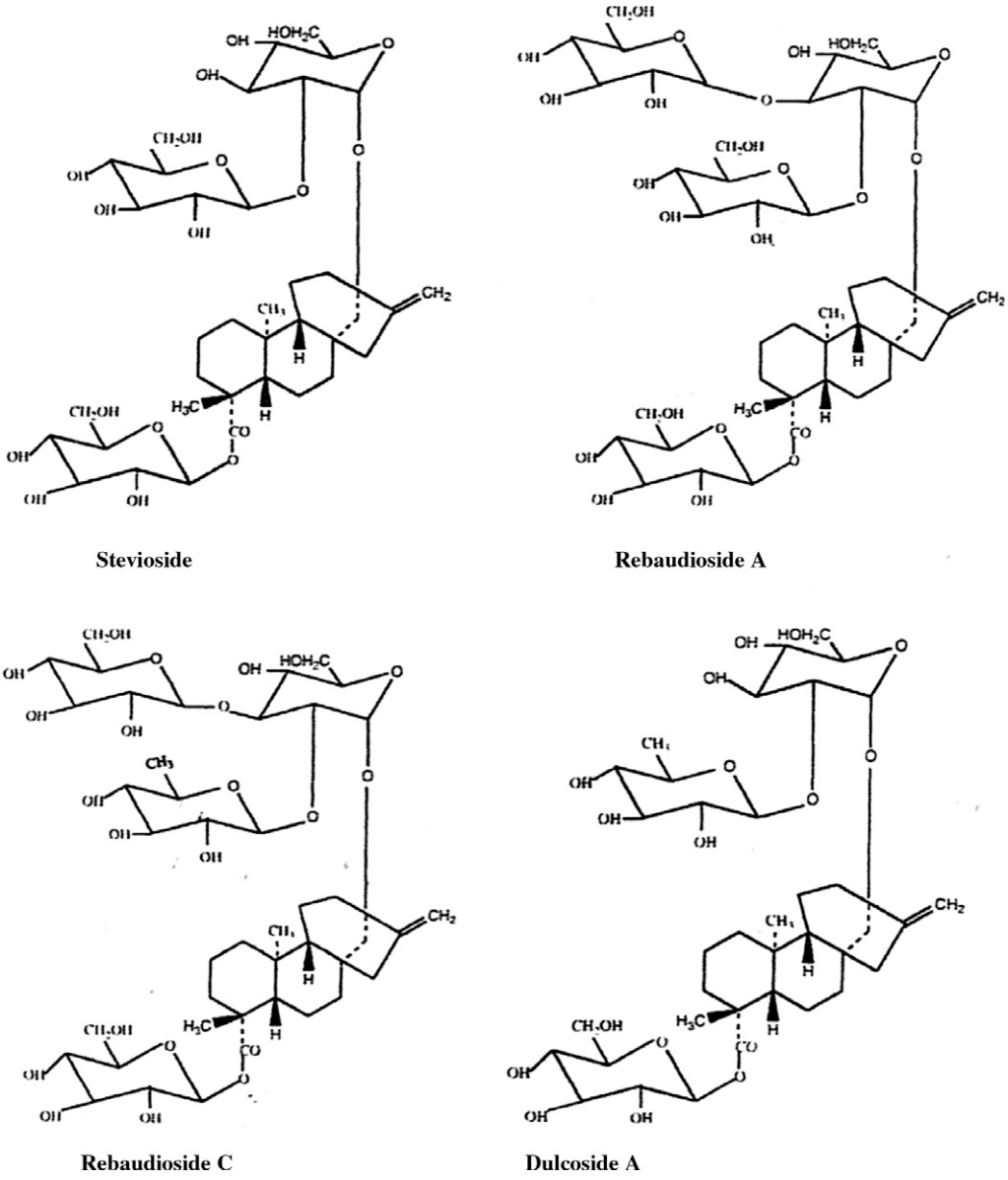

Fig. 1. Structures of the major stevia extract glycosides (from: WHO, 2006).

with oral LD_{50} values >15 g/kg bw in rodent species (JECFA, 1999). Long-term carcinogenicity studies of stevioside (5% in the diet) in Fischer 344 rats did not show any evidence of cancer after 108 weeks (Toyoda et al., 1997). Stevioside, at 5% in the diet, also failed to act as a promoter of bladder carcinogenesis in Fischer 344 rats (Hagiwara et al., 1984).

The majority of positive genetic toxicology studies involve bacterial mutation with steviol, particularly those using a forward-mutation method in strain TM667 (Pezzuto et al., 1985), but steviol has also been reported to induce chromosome breakage and gene mutation in mammalian cells (Matsui et al., 1996a).

JECFA (2005) concluded that stevioside and rebaudioside A have been subjected to adequate genetic testing using conventional methods and show no evidence of genotoxic activity. However, a recent publication by Nunes et al. (2007) reported that stevioside provided at 4 mg/ml in drinking water, produced DNA breakage in rat blood cells, spleen, liver and brain when administered for 45 days in the drinking water.

This review will attempt to examine the body of evidence available for steviol and steviol glycosides and provide perspective on the possible genetic hazard associated with their consumption.

2. Genetic toxicology testing

2.1. Stevioside

A listing of the genetic testing performed with stevioside and stevia extracts containing steviosides is shown in Table 1. Stevioside was reported to be non-mutagenic in Ames strains of *S. typhimurium*, and in strains of *E. coli* and *B. subtilis* (Tama Biochemical Co, Ltd., 1981). These studies included rat liver S9 supernatant. Stevioside was evaluated in the Ames strains TA98 and TA100 using a pre-incubation exposure technique (Suttajit et al., 1993). In these investigations, stevioside was purified to 99% and tested up to 50 mg/plate without S9 and with hepatic S9 made

Table 1
Genetic toxicology results for stevioside and stevia extracts containing stevioside

Test	Response	LED/HNED[a]	Conditions	Comment	Citation
Reverse mutation in *S. typhimurium, E. coli, B. subtilis*	Negative in all strains	Data not available	Tests conducted both with and without S9	Company sponsored testing program	Tama Biochemical Co, Ltd., Safety of stevia (Tama Report 1–20, 1981) cited in Medon et al. (1982)
SCEs in human fetal cells, *in vitro*	Negative	Data not available	Test conditions not available	(See above)	(See above)
Chromosome aberrations in cultured rat cells, *in vitro*	Negative	Data not available	Test conditions not available	(See above)	(See above)
Forward mutation in *S. typhimurium* TM677	Negative	Data not available	Tested both with and without S9 induced by Arochlor 1254	SOT Abstract for 1982 meeting presentation	Medon et al. (1982), SOT abstract publications
Reverse mutation in Ames strains TA98 and TA100	TA100 reported negative TA98 reported positive, without S9	TA100 = 50 mg/plate (HNED) TA98 = 50 mg/plate (LED)	Pre-incubation method used S9 produced from rats induced with combination of phenobarbital and 5,6-benzoflavone	Stevioside was 99% pure TA98 showed a four-fold increase; however, a 1% impurity would be 500 ug/plate at the high concentration	Suttajit et al. (1993)
Chromosome aberrations in human lymphocytes, *in vitro*	Negative	10 mg/ ml	Study conducted with and without S9 from rats induced by phenobarbital and 5,6 benzoflavone	No data were provided for this study in the publication	(See above)
Reverse mutation in Ames strains plus *E. coli* WP_2 uvrA/pKM101	Negative in all strains tested	5 mg/plate for all strains and treatment conditions	Pre-incubation method used, standard Ames strains plus TA102 and TA104	Stevioside purity was 83%, no toxicity was seen in the test at the highest concentration tested	Matsui et al. (1996a)
Umu-test	Negative	5 mg/ml	Performed according to the methods of Oda et al. (1985)ck.	S9 used was from rats treated with a combination of phenobarbital and 5,6 benzoflavone	(See above)
Rec-assay	Negative	10 mg/paper disk	Performed according to the methods of Hirano et al. (1982)	Used S9 from rats treated with Arochlor 1254	(See above)
Chromosome aberrations in CHL cells, *in vitro*	Negative	12 mg/ml	Treatments were for 6, 24 and 48 h without S9 and for 6 h with S9, maximum concentrations set at >50% toxicity	Used S9 from rats treated with Arochlor 1254	(See above)
Reverse mutation in Ames strains TA98 and TA100	Negative results in both strains for all treatment conditions	50 mg/plate	Pre-incubation method used, all S9s induced by a combination of Phenobarbital and 5,6-benzoflavone	Compared S9s from rat, mouse, hamster and guinea pig	Klongpanichpak et al. (1997)
Mouse lymphoma forward mutation assay	Negative	5000 μg/ml	Micro-titer method used, 3 h exposures with and without S9 plus 24 h treatment without S9	No toxicity observed at the maximum concentration under either treatment condition	Oh et al. (1999)
Mouse micronucleus assay, *in vivo*	Negative	250 mg/kg	Single dose with 24 harvests of bone marrow and hepatocytes	ICR mice treated at only one dose, no toxicity reported	(See above)
Comet assay, *in vivo*	Negative results in all tissues examined	2000 mg/kg oral administration to ddY mice	Tissues examined for DNA damage at 3 and 24 h post exposure	Organs included glandular stomach, colon, liver, kidney, bladder, lung, brain and bone marrow	Sasaki et al. (2002)
Comet assay, *in vivo*	Negative results in all tissues examined	2000 mg/kg administration to BD F_1	Tissues examined for DNA damage at 3 and 24 h post exposure	Organs included stomach, colon and liver	Sekihashi et al. (2002)
Comet assay, *in vivo*	Positive in all tissues examined	4 mg/ml in drinking water	Blood cells examined weekly, spleen, liver and brain tissues examined at exposure termination	Wistar rats given stevia extract for 45 days in their drinking water. No DNA effects were seen before week five	Nunes et al. (2007)

[a] LED: lowest concentration tested that shows a clearly positive response according to the criteria of the specific test; HNED: highest concentration tested for a study with negative results.

from rats induced with a combination of phenobarbital and 5,6-benzoflavone. Stevioside samples were also pre-treated with β-glucosidase before testing in strains TA98 and TA100. At a concentration of 50 mg/plate, stevioside produced a four-fold increase in revertants of TA98 in the absence of S9. A two-fold increase was also reported for 50 mg/plate in the presence of S9. The β-glucosidase-treated samples showed TA98 mutagenic responses in roughly the same ranges as those without glucosidase. TA100 did not respond to the mutagenic activity under any of the treatment conditions employed. At a concentration of 25 mg/plate, stevioside was not mutagenic in either TA98 or TA100. The need to use a concentration of 50 mg/plate to produce a response is indirect evidence that a low level contaminant, and not the pure glycoside, was responsible for the increase. For example, the sample used by Suttijat et al. was reported to be 99% pure. A contaminant of 1%, if present, could have achieved the relatively high concentration of 500 μg/plate at the tested concentration of 50 mg/plate. Additional tests using the standard Ames strains plus strains TA104 and TA102 and S9 made from rats induced by Kanechlor KC400, a mixture of PCBs, were reported by Matsui et al. (1996a). Stevioside, at concentrations of up to 5 mg/plate, was not toxic or mutagenic to any of the strains. In these studies the purity of the

stevioside was less than used by Suttijat, but the concentrations used in the tests by Suttijat were so great that stevioside preparations of 99% purity could still produce higher levels/plate of a minor contaminant. Finally, Klongpanichpak et al. (1997) studied the mutagenicity of stevioside in Ames strains TA98 and TA100 comparing hepatic S9s from rats, mice, hamsters and guinea pigs induced by a combination of phenobarbital and 5,6-benzoflavone. At concentrations as high as 50 mg/plate, mutagenicity was not observed in either strain. The data from this and previous studies suggest that stevioside does not induce mutation in the Ames test.

Other microbial tests reported negative responses for stevioside, including the Rec-assay, the *umu*-test (Matsui et al., 1996a), and a forward mutation assay in *S. typhimurium* TM677 (Medon et al., 1982). Rebaudioside A was also reported negative in the TM677 forward mutation assay (Pezzuto et al., 1985). All of these tests were conducted with and without S9.

Stevioside has also been subjected to three *in vitro* tests measuring the induction of chromosome aberrations using mammalian cells. Stevioside was evaluated in cultured rat cells (Tama Biochemical Co, Ltd., 1981), human lymphocyte cultures (Suttajit et al., 1993) and Chinese hamster lung (CHL) cells (Matsui et al., 1996a). These tests indicated that stevioside, either in the presence or absence of S9 was not clastogenic at high concentrations (up to 12 mg/ml in the study by Matsui et al., 1996a). Stevioside also failed to induce sister chromatid exchange (SCE) in human fetal fibroblast cultures (Tama Biochemical Co, Ltd., 1981). Oh et al. (1999) reported results from a microtiter test for mutation in cultured mammalian cells. The test detects gene mutation generated by chromosome deletion or point mutation in the thymidine kinase (*tk*) gene in mouse lymphoma L5178Y *tk*+/− cells. Treatment conditions used in this assay included cells exposed to stevioside at concentrations up to 5 mg/ml both with and without S9 for a 3 h period as well as cultures continuously exposed to 5 mg/ml without S9 for 24 h. Stevioside was not mutagenic.

Two single dose studies in mice and one multiple administration study in rats assessing the *in vivo* activity of stevioside in the Comet assay have been published. This assay is capable of detecting single-strand DNA breaks, alkali labile sites and certain types of DNA cross-linking lesions in treated animals or cells (Tice et al., 2000). In the two single dose studies, several organs were sampled after 3 and 24 h including the stomach, colon, liver, kidneys, bladder, lung, brain and bone marrow in two independent studies with mice (Sasaki et al., 2002; Sekihashi et al., 2002). Neither study produced evidence indicating DNA breakage at doses of 2 gm/kg administered orally. Sekihashi et al. (2002) also reported the results of *in vitro* exposures of stevioside to two different cell lines (TK6 and WTK1) both with and without S9 mix. No evidence of DNA breakage was seen at concentrations up to 500 μg/ml in either cell line. A more recent study conducted in Wistar rats exposed to a solution of stevioside (4 mg/ml) for 45 days, reported significant increases in DNA breakage from multiple organs, including blood cells, spleen, liver and brain (Nunes et al., 2007). In this study, groups of five male rats each were provided either normal drinking water (controls) or an aqueous solution of stevioside (4 mg/ml or roughly 400 mg/kg per day) for 45 days. During the 45 day exposure period, blood samples were collected weekly from each animal and examined for DNA breakage. At the end of the 45 day period, animals were killed and additional samples collected from the livers, brains and spleens. The number of nuclei scored per animal was 50. The results, however, were based on the total number of cells analyzed for a group of animals (i.e., 250 nuclei). Unfortunately, this study did not include a positive control. Results from a known DNA-breaking agent would have provided a useful gauge of the results reported by stevioside in this publication. Of interest was the fact that the results from this study showed significant elevations in the number of blood cell nuclei only after 5 weeks of exposure. Responses from weeks 1–4 were negative. Other organs sampled showed an even greater level of DNA damage with a significant number of the DNA tails for treated animals showing class three length which is twice the diameter of the nucleus head, or longer.

Stevioside was tested *in vivo* and did not induce micronuclei in either bone marrow cells or liver hepatocytes of mice dosed orally at 250 mg/kg in a single dose (Oh et al., 1999). After a single dose, bone marrow and liver cells were harvested at 24 h, only. No later target cell harvests were reported.

Among the 16 individual assays reported in Table 1, only two tests reported some evidence of genotoxic activity (Suttajit et al., 1993; Nunes et al., 2007). The results reported by Stuttajit et al. for TA98 can best be explained by the presence of contamination in their sample when tested at a concentration of 50,000 μg/plate. The results of Nunes et al. were unexpected in light of the extensive amount of negative studies reported for stevioside. Concerns about the methodology employed in this study will be discussed in a later section.

2.2. Steviol

Molecular structures for steviol and some of its metabolites are shown in Fig. 2.

A listing of the genetic testing performed with steviol, in what might be classified as standard types of genetic toxicology tests, is shown in Table 2. Steviol did not induce reverse mutation in the Ames test or in *E. coli* WP_2 *uvr*A pKM101. Among the 15 studies listed, four indicate genotoxic activity for steviol. Two of these positive responses are from the *umu*-test and mutation studies in plasmid DNA (Matsui et al., 1989, 1996a). The *umu*-test is an indirect measure of mutation detecting the induction of the *umu*-operon which appears to be involved in the SOS-type mutation pathway. In addition to tests with microbial organisms, studies measuring clastogenicity and gene mutation in CHL cells produced marginally positive responses that appear to be associated with toxic and not purely genotoxic effects (Matsui et al., 1996a). The results from these two tests will be discussed in more detail below. The most extensive set of positive data generated for steviol was derived from a single assay detecting mutation at the 8-azaguanine resistance gene in *S. thyphimurim.* Results from studies using the TM677 forward mutation system are listed in Table 3.

Strain TM677, used in the forward mutation assay, was initially derived from a revertant of the Ames strain TA1535 followed by incorporation of the R-factor plasmid pKM101 (Skopek et al., 1978). The mutation test is conducted as a quantitative suspension assay in which mutants are selected by their resistance to the toxin 8-azaguanine (8-AG). Interestingly, steviol was not mutagenic in a different forward mutation system in bacteria measuring resistance to rifampicin (Procinska et al., 1991). A mechanism has not been determined to explain the unique sensitivity of strain TM677 to the mutagenic effects of steviol.

Pezzuto et al. (1985) investigated the mutagenicity of steviol and several related compounds in the TM677 forward mutation assay and found that steviol was both toxic and mutagenic in this assay at concentrations of 100 μg/ml in the presence of liver S9 obtained from Arochlor 1254 – induced rats. Liver S9 preparations from rats induced by phenobarbital and 3-methlycholanthrene did not provide optimal activation of steviol to its mutagenic intermediate. Other related compounds tested (isosteviol, ent-kaurenoic acid, steviol-16α, 17-epoxide and dihydrosteviol A&B) were not reported to be mutagenic in strain TM677 (Pezzuto et al., 1985). In further TM677 mutation studies with steviol and related compounds, Pezzuto et al. (1986) showed that the 13-hydroxy group of steviol is required for the expression of mutagenic activity and that the epoxide metabolite is not responsible for the mutagenic

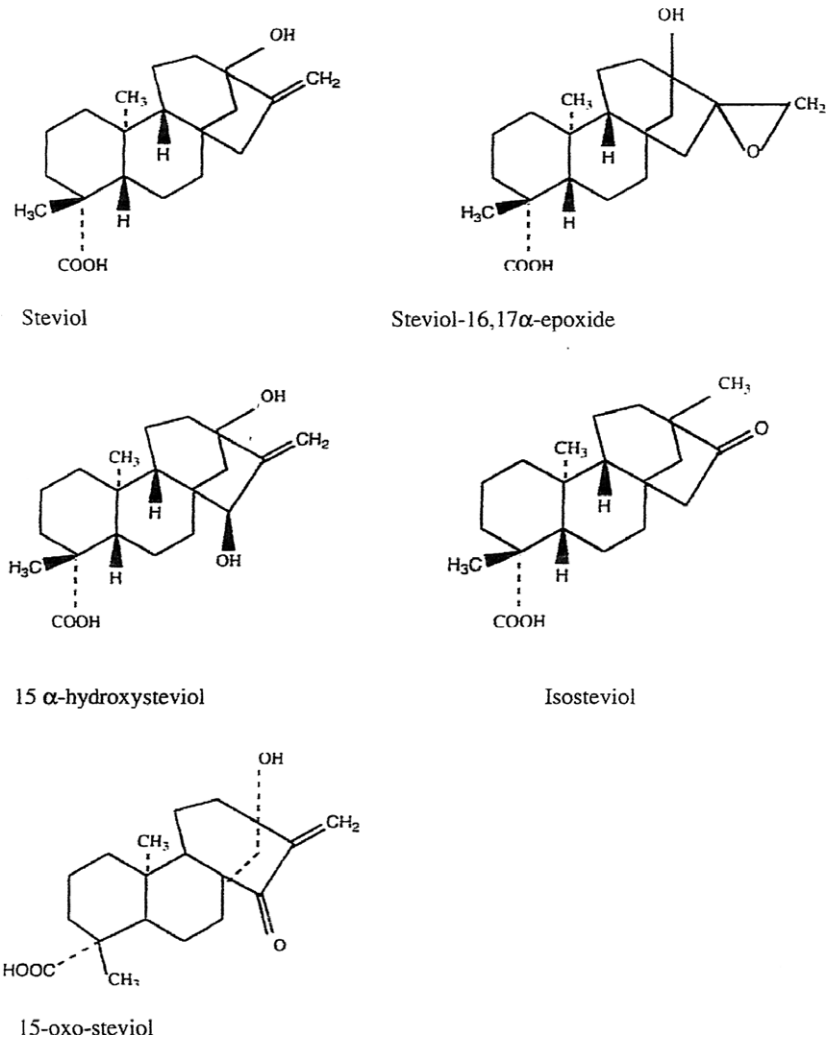

Fig. 2. Structures for steviol and some of its metabolites (from: WHO, 2006).

activity associated with steviol in this assay). *In vitro* metabolism of steviol, using Arochlor-induced rat liver S9 mix, identified the presence of nine compounds not present in the control samples (Compadre et al., 1988). Most compounds were present at very low concentrations, but 7β-hydroxysteviol, 15α-hydroxysteviol and 17-hydroxyisosteviol were identified. 15α-hydroxysteviol represented about 70% of total metabolite concentrations, but was not found to be mutagenic. Compadre et al. hypothesized that 15-oxosteviol, might be a possible short-lived intermediate of 15α-hydroxysteviol and tested it in strain TM677. The results were reported as positive without S9 mix and interpreted by Compadre et al. that this compound is the likely ultimate mutagenic agent. Later, Procinska et al. (1991) argued that Compadre et al. incorrectly analyzed the data for 15-oxosteviol and concluded that the compound appeared positive due to selective cell killing and was not actually mutagenic. However, Terai et al. (2002) reported that several steviol derivative compounds tested for mutation in the TM677 strain were weakly mutagenic including 15-oxosteviol, but only in the presence of S9 mix made from livers of rats induced with Arochlor 1254. The inconsistencies among the results for 15-oxosteviol including the possibility that it requires metabolic activation suggest that it may not be the ultimate DNA reactive structure. The data published by Terai et al. (2002) were given only as mutation frequencies without underlying colony counts and may not have been corrected for toxicity. Therefore, it is possible that they differed from other published results due to artifacts similar to the Compadre et al. (1988) publication.

Although steviol was mutagenic at concentrations of approximately 100 μg/ml in the forward mutation assay in strain TM677 with S9 mix from Arochlor 1254-induced rats, steviol was not mutagenic in any of the standard Ames reverse mutation strains as well as two strains, TA102 and TA104, believed to specifically detect oxidative mutagens (Suttajit et al., 1993; Matsui et al., 1996a; Klongpanichpak et al., 1997). The reverse mutation studies employed various modifications in attempts to demonstrate mutagenicity by steviol including the use of S9 from various mammalian species following induction by different chemicals such as phenobarbital and 5,6-benzoflavone, Kanechlor KC-400 and 3-methylcholanthrene. A paper by Matsui et al. (1989) in which plasmid DNA mutants of the 7 *xgprt* gene were analyzed suggested that the active metabolite of steviol induces small deletions which could explain why it was not active in the standard Ames strains, which do not easily detect multiple base-pair deletions. A subsequent publication by this group (Matsui et al., 1996b) sequenced 24 steviol-induced and 16 spontaneous mutants induced in the *gpt* gene of strain TM677 and found that in addition to deletions, steviol-induced single base-pair substitutions as well. Consequently, the mutagenic mechanism of steviol in bacteria cannot be explained by small deletions and remains somewhat elusive. Procinska et al. (1991) suggested that the mutagenicity of steviol

Table 2
Genetic toxicology results for steviol in standard genetic toxicity tests

Test	Response	LED/HNED[a]	Conditions	Comment	Citation
Reverse mutation in Ames strains TA98 and TA100	Negative	20 mg/plate	Pre-incubation modification using S9 from rats induced by a combination of phenobarbital and 5,6-benzoflavone	Steviol was prepared by periodae oxidation of stevioside followed by acid hydrolysis and recrystallization	Suttajit et al. (1993)
Chromosome aberrations, *in vitro*	Negative	200 μg/ml	Studies conducted in human lymphocyte cultures with and without S9	No actual data provided to support author's conclusions	(See above)
Reverse mutation in Ames strains plus *E. coli* WP_2 *uvr*A/pKM101	Negative	5000 μg/plate	Pre-incubation modification using S9 from rats induced by Kanechlor KC-400	Negative in all standard strains plus strains TA102 and TA104	Matsui et al. (1996a)
Umu-test	Positive	2500 μg/plate	Performed according to methods of Oda et al. (1985) with S9 from rats induced by a combination of phenobarbital and 5,6-benzoflavone	Approximate two-fold increase at the high concentration considered a weak positive	(See above)
Rec-assay	Negative	10 mg/paper disk	Performed according to methods of Hirano et al. (1982)	Used S9 from rats induced by PCBs	(See above)
Chromosome aberrations, *in vitro*	Positive	1000 μg/ml	Studies conducted in CHL cells, Cells sampled at 6, 24 and 48 h without S9 and at 6 h with S9	Used S9 from rats induced by PCBs Positive response only with S9	(See above)
Gene mutation in mammalian cells, *in vitro*	Positive	400 μg/ml	Studies conducted in CHL cells and assessed by resistance to diphtheria toxin	Used S9 from rats induced by PCBs Positive response only with S9 at highly toxic treatments (3% survival)	(See above)
Mouse micronucleus assay, *in vivo*	Negative	500 mg/kg	MS/Ae mouse strain used, compound administered i.p. with 24 and 48 h harvests	Toxicity seen at 1000 mg/kg	(See above)
Reverse mutation in Ames strains TA98 and TA100 Klongpanichpak et al. (1997)	Negative	2000 μg/plate	Pre-incubation method using S9 from animals induced by a combination of phenobarbital and 5,6-benzoflavone	Authors compared S9s from rat, mouse, hamster and guinea pig All tests negative	
Gene mutation in mammalian cells, *in vitro*	Negative	341 μg/ml	Study conducted in mouse lymphoma cells L5178Y at the *TK* gene (with and without S9)	Toxicity did not exceed a RTG of 40%	Oh et al. (1999)
Mouse micronucleus assay, *in vivo*	Negative	200 mg/kg	Single oral dose with only a 24 h harvest of liver hepatocytes	No harvest at 48 h	(See above)
Micronucleus assay, *in vivo*	Negative	4 gm/kg For hamsters and 8 gm/kg for rats and mice	Study conducted using single oral dose in mice, rats, hamsters (both sexes), bone marrow cells harvested at 24, 30, 48 and 72 h post exposure	Toxicity seen in all species at high dose with females appearing to be more sensitive	Temcharoen et al. (2000)
Comet assay, *in vivo*	Negative	2 gm/kg	Mice were exposed by a single oral dose and tissues collected at 3 and 24 h post exposure	Stomach, colon, liver, kidney, and testis tissues evaluated for DNA damage	Sekihashi et al. (2002)
Comet assay, *in vitro*	Negative	500 μg/ml	Studies conducted in TK6 and WTK1 cell cultures both with and without S9		(See above)
Plasmid mutagenesis	Positive	Not reported	Induction of *xgprt* mutants in plasmid pSV2-*gpt* in the presence of S9	Mutants analyzed and shown to be small deletions which was offered as an explanation why steviol was not mutagenic in the Ames strains	Matsui et al. (1989)

[a] LED: lowest concentration tested that shows a clearly positive response according to the criteria of the specific test; HNED: highest concentration tested for a study with negative results.

in the TM677 mutation system might be caused by an impurity as the compound appears to saturate at ~1.0 mg/ml yet the mutagenicity increases up to concentrations of 10 mg/ml.

Results from genetic toxicity studies in cultured mammalian cells have not provided better insight into the genotoxic properties of steviol. A study of the clastogenic activity of steviol in cultured Chinese hamster lung (CHL) cells appeared to show an increase in cells with aberrations at 1000 μg/ml (Matsui et al., 1996a). Treatment conditions for the positive response were 6 h exposure with Arochlor 1254-induced S9. Steviol was not clastogenic in the absence of S9 following continuous exposures of 6, 24 or 48 h. Toxicity at 1000 μg/ml with S9 was slightly greater than 50%. At the next lower dose (750 μg/ml), the clastogenic response was marginal-to-negative. Steviol was not clastogenic in cultured human lymphocytes when tested at 200 μg/ml with S9 (Suttajit et al., 1993). The toxicity of steviol to the lymphocytes was not reported in this publication preventing a comparison of these data to the CHL data at equivalent levels of toxicity.

Using the CHL cell line, Matsui et al. (1996a) also evaluated the potential of steviol to induce diphtheria toxic resistant mutations. A significant increase in mutation was observed at a steviol concentration of 400 μg/ml with S9 (three-fold over background); however, the only concentration of steviol that showed increased mutation also produced 97% cell killing indicating that the effect was due to toxicity to the CHL cells and not true mutagenic activity. Additional support for this conclusion is provided by the results of an independent gene mutation assay conducted in L5178Y mouse lymphoma cells at the *tk* gene (Oh et al., 1999). The mouse lymphoma study was conducted using a micro titer method and included both non-activation and S9 exposure conditions. Steviol was tested up to 341 μg/ml (RTG of 40%) without evidence of induction of (*tk*−/−) mutants.

In vivo tests capable of detecting chromosome breakage were performed with mice, rats and hamsters producing negative results (Oh et al., 1999; Temcharoen et al., 2000). The test animals were administered steviol orally at doses up to 4 gm/kg for hamsters, and up to 8 gm/kg for mice and rats. Toxicity was evident in the animals as shown by reduced PCE/NCE ratios, but no increases in micronucleated PCEs were reported. Steviol at a dose level of 2 gm/kg administered orally to mice in a Comet assay failed to

Table 3
Genetic toxicology results for steviol and structurally related compounds in the TM677 forward mutation assay

Test	Response	LED/HNED[a]	Conditions	Comment	Citation
Forward mutation in *S. typhimurium* TM677	Steviol-positive negative compounds were isosteviol, dihydrosteviol A&B, ent-kaurenoic acid, steviol-16a,17-epoxide	LED for steviol was 100 μg/ml HNED for the other compounds was 10 mg/ml	Pre-incubation suspension assay, S9 mix from rats treated with Arochlor 1254	Authors tested S9s from animals induced by Arochlor as well as phenobarbital (marginal response) and 3-methyl-cholanthrene (no response)	Pezzuto et al. (1985)
Forward mutation in *S. typhimurium* TM677	Steviol-positive 19-0-B-D glycopyranosyl steviol-positive negative compounds were ent-kaurenoic acid and steviol acetate	LED for Steviol was 100 μg/ml and for glycopyranosyl steviol ug/ml, negative compounds tested up to 7500 μg/ml	Pre-incubation suspension assay, S9 mix from rats treated with Arochlor 1254	Authors showed that 13-hydroxy group is essential, acylation at this site blocks mutagenicity Authors suggest epoxide not the active mutagenic agent	Pezzuto et al. (1986)
Forward mutation in *S. typhimurium* TM677	15-Oxosteviol- positive (-S9), 15a-hydroxysteviol-negative	LED for 15-oxosteviol was 150 μg/ml HNED for 15a-hydroxysteviol was 7500 μg/ml	Pre-incubation suspension assay, S9 mix from rats treated with Arochlor 1254	15-Oxosteviol was not identified as a metabolite using mass spectral analysis but was hypothesized to be a short-lived derivative of 15a-hydroxysteviol, known metabolite	Compadre et al. (1988)
Forward mutation in *S. typhimurium* TM677	15-Oxosteviol- negative	122 μg/ml	Pre-incubation suspension assay, without and with S9 mix from rats treated with Arochlor 1254	15-Oxosteviol was not mutagenic in 4 trials leading the authors to conclude that the data of Compadre et al. (1988) was a technical artifact of selective toxicity	Procinska et al. (1991)
Forward mutation in *S. typhimurium* TM677	Steviol-positive	Not reported	Pre-incubation suspension assay, without and with S9 mix from rats treated with Arochlor 1254	Sequence analysis of steviol-induced mutants indicated that mechanism for steviol involves DNA synthesis disruption	Matsui et al. (1996a)
Forward mutation in *S. typhimurium* TM677	15-Oxosteviol and 5 other derivatives of steviol were positive in the presence of S9	Not reported	Pre-incubation suspension assay, without and with S9 mix from rats treated with Arochlor 1254	Results in this manuscript contradicts other tests with 15-oxosteviol, because it was positive only with S9	Terai et al. (2002)

[a] LED: lowest concentration tested that shows a clearly positive response according to the criteria of the specific test; HNED: highest concentration tested for a study with negative results.

induce evidence of DNA strand breakage in several organs sampled stomach, colon, liver, testes, and kidney (Sekihashi et al., 2002).

3. Interpretation of the published data from genetic testing of stevioside and steviol

The major constituents of stevia extract (stevioside and rebaudioside A) are relatively un-reactive compounds without clear evidence of DNA binding or mutagenicity. Rebaudioside A was evaluated for genotoxicity with a set of *in vitro* and *in vivo* assays covering mutation, chromosome damage and DNA strand breakage with consistent and uniformly negative results (Pezzuto et al., 1985; Nakajima, 2000a,b; Sekihashi et al., 2002). High concentrations of stevioside failed to either induce mutations in bacteria or in cultured mammalian cells or chromosome breakage *in vitro* and *in vivo*. The only report of DNA damage from stevioside was reported by Nunes et al. (2007) in rats following subchronic exposures in drinking water at roughly 400 mg/kg. One explanation offered for the increase in DNA damage was the conversion of stevioside to steviol, which is both more toxic and was reported to induce genotoxicity in certain types of tests (Pezzuto et al., 1985; Matsui et al., 1996a). However, steviol, when administered orally to mice at concentrations up to 2000 mg/kg, did not induce measurable DNA breakage in liver, stomach, kidney or testis tissues (Sekihashi et al., 2002) raising doubts about this explanation for DNA breakage as the daily dose in the Nunes study would be approximately 400 mg/kg per day. It also appears unlikely that other steviol glycosides (e.g., rebaudioside A) would be responsible for the DNA breakage reported by Nunes et al. as they have not shown genotoxicity (JECFA, 2005). Two other studies of stevia extracts, containing high proportions of stevioside, were negative in Comet assays conducted in mice (Sasaki et al., 2002; Sekihashi et al., 2002). These studies sampled tissues for damage at 3 and 24 h post exposure. The reasons for selecting short-term sampling times in the Comet assay is that DNA lesions detected by this method are short-lived events (they undergo rapid DNA repair) and therefore, have to be captured before they disappear (Brendler-Schwaab et al., 2005). Consequently, if DNA damage reported by Nunes et al. (2007) is due to conversion of stevioside to steviol or some other genotoxic metabolite followed by systemic absorption, one would expect to see evidence of DNA damage in the rat blood cells long before week 5. The observations that there was no positive control and that no damage is observed before week 5 raises serious concerns about the technical conduct and, therefore validity, of the results in this assay.

The stevioside aglycone, steviol, appears to have a highly specific mutagenic mechanism that is not detected by most commonly employed tests used to determine genotoxicity (e.g., Ames, mouse lymphoma, Comet, micronucleus). The majority of data indicating mutagenic activity for steviol was generated in a forward mutation assay in *S. typhimurium* strain TM677. This test uses a *his+* revertant of strain TA1535 that incorporates the pKM101 plasmid responsible for increased sensitivity in the Ames test (Skopek et al., 1978a). Skopek et al. (1978b) demonstrated that while TM677 may be slightly more sensitive to 16 chemical mutagens, all 16 of the mutagens were also detected by one or more of the

standard Ames strains. The presence of the pKM101 plasmid, the *rfa* mutation and loss of excision repair in TM677 are absolute requirements for the detection of steviol-induced mutation in this method (Matsui et al., 1996a). An independent forward mutation in bacteria measuring resistance to rifampicin was not able to detect any mutagenic activity induced by steviol (Procinska et al., 1991). Pezzuto et al. (1985) showed that optimal activation of steviol to its mutagenic intermediate requires S9 from animals induced by PCBs rather than Phenobarbital or 3-methylcholanthrene, two other commonly used hepatic enzyme inducers. The genotoxic mechanism of steviol and steviol derivatives may be so specific to this assay that effects produced in TM677 bacteria cannot be extrapolated to systems with normal DNA repair and replication processes.

Activation of steviol by hepatic enzymes has produced few clues as to the identity of the ultimate mutagen. Such studies have shown that the 13-hydroxy group of steviol is required for expression of mutagenic activity in the TM677 strain. A hypothetical metabolite, 15-oxosteviol, was proposed as the ultimate mutagen based on studies by Compadre et al. (1988), but this proposal has been challenged by data from two other investigators (Procinska et al., 1991; Klongpanichpak et al., 1997). Another proposed explanation for the lack of steviol-induced reverse mutation in assays such as the Ames test or *E. coli* $WP_{2,}$ was that the compound's predominant DNA lesions are multiple base-pair deletions not recognized in reverse mutation systems. This explanation was contradicted by findings of Matsui et al. (1996b). DNA sequences of 24 steviol-induced mutations in TM677 showed that most (20/24) were single base-pair changes and not multiple base-pair deletions. The authors suggested that steviol, or its active metabolite(s) may interact with gene products that sensitize DNA polymerase to produce mismatch sites. This property of steviol may be unique to bacterial DNA metabolism and, for that reason, the compound does not produce evidence of DNA damage in mammalian cells *in vitro* or *in vivo*.

4. Genetic toxicity hazard assessment for steviol glycosides

Two key elements are necessary for an assessment of the likely risk of genetic damage following exposure to stevia extracts or pure stevioside. These are: (a) demonstrated DNA reactivity potential; and (b) relevant exposure levels.

(a) Based on the analyses described previously, neither stevioside nor the aglycone steviol have been shown to react directly with DNA. Neither compound has demonstrated genotoxic damage in organisms that are relevant to humans. The mutagenic activity of steviol and some of its derivatives exhibited in strain TM677 cannot be produced in the same bacteria with normal DNA repair processes.
(b) Consumption of 250–375 mg of stevioside by human volunteers showed the majority of free steviol to be eliminated in the feces with virtually no free stevioside, steviol or 14-oxosteviol in the blood several hours after exposure.

The only positive *in vivo* study, one measuring the induction of single-strand DNA breaks in Wistar rat tissues by stevioside, cannot be confirmed with similar experiments in mice and appears to be measuring a process or processes other than direct DNA damage. Neither stevioside nor steviol-induced clastogenic effects at extremely high dose levels *in vivo*.

Evidence of mutagenic potential of steviol is limited to *in vitro* assays. Even, if a small fraction of the steviol potentially generated from the hydrolysis of rebaudioside A were to be absorbed, studies in rats, mice and hamsters indicate that steviol is non-genotoxic *in vivo* up to doses of 8000 mg/kg body weight. Therefore, there should be no risk to humans at anticipated exposure levels.

Long-term studies of the carcinogenic potential of stevioside in male and female Fischer 344 rats failed to demonstrate any carcinogenic or pre-carcinogenic activity after 104 weeks on diets of up to 5% stevioside (Toyoda et al.,1995, 1997). Absence of carcinogenic activity in life-time exposures to high concentrations of stevioside support the conclusion that the highly specific genotoxic responses produced in TM677 have no relevance to human risk.

The following statements are supported by the evidence provided or cited in this review:

- Steviol glycosides rebaudioside A and stevioside are not genotoxic *in vitro*.
- Steviol glycosides rebaudioside A and stevioside have not been shown to be genotoxic *in vivo* in well-conducted assays.
- A report indicating that stevioside produces DNA breakage *in vivo* appears to be flawed and was improperly interpreted as a positive response.
- Steviol genotoxicity in mammalian cells is limited to *in vitro* tests that may be affected by excessive concentrations of the compound.
- The primary evidence for steviol genotoxicity is from very specific bacteria tests or purified plasmid DNA that lack DNA repair capabilities.
- Stevioside is not a carcinogen or cancer promoter in well-conducted rodent bioassays.

Application of an objective weight-of-evidence approach to assess the complete genetic toxicology database for steviol glycosides concludes that these substances do not pose a risk of genetic damage following human consumption.

Conflict of interest statement

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