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Review

Steviol glycoside safety: Is the genotoxicity database sufficient?

J.D. Urban [a,*], M.C. Carakostas [b], D.J. Brusick [c]

[a] ToxStrategies, Inc., 9390 Research Blvd., Suite 250, Austin, TX 78717, United States
[b] ToxStrategies, Inc., 2 Reeve Court, Suite 200, St. Helena Island, SC 29920, United States
[c] Brusick Consultancy, 123 Moody Creek Road, Bumpass, VA 23024, United States

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ABSTRACT

The safety of steviol glycoside sweeteners has been extensively reviewed in the literature. National and international food safety agencies and approximately 20 expert panels have concluded that steviol glycosides, including the widely used sweeteners stevioside and rebaudioside A, are not genotoxic. However, concern has been expressed in recent publications that steviol glycosides may be mutagenic based on select studies representing a small fraction of the overall database, and it has been suggested that further *in vivo* genotoxicity studies are required to complete their safety profiles. To address the utility of conducting additional *in vivo* genotoxicity studies, this review evaluates the specific genotoxicity studies that are the sources of concern, and evaluates the adequacy of the database including more recent genotoxicity data not mentioned in those publications. The current database of *in vitro* and *in vivo* studies for steviol glycosides is robust and does not indicate that either stevioside or rebaudioside A are genotoxic. This, combined with a lack of evidence for neoplasm development in rat bioassays, establish the safety of all steviol glycosides with respect to their genotoxic/carcinogenic potential.

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1. Introduction

Stevia rebaudiana (Bertoni) Bertoni (Asteraceae), commonly known as stevia, is an herb native to certain regions of South America that for centuries has been cultivated for its sweet leaves. Stevia leaves contain more than 30 steviol glycosides, of which stevioside and rebaudioside A are the most abundant and have the best characterized sweetness profiles (Wölwer-Rieck, 2012). Rebaudioside A in particular has been developed into several commercially available non-caloric sweeteners.

Steviol glycosides are complex molecules comprised of a central 13-hydroxykaur-16-en-18-oic acid (steviol) bound to a variable number of glucose molecules. For example, there are three and four glucose molecules attached to the steviol moiety for stevioside and rebaudioside A, respectively. In both humans and rats, steviol glycosides are poorly absorbed in the stomach and upper intestine. It is not until steviol glycosides come in contact with the flora of

Abbreviations: ANZFSA, Australia–New Zealand Food Safety Authority; ADI, acceptable daily intake; bw, body weight; DNA, deoxyribonucleic acid; EFSA, European Food Safety Agency; gm, gram; GRAS, Generally Recognized as Safe; kg, kilogram; JECFA, Joint FAO/WHO Expert Committee on Food Additives; mg, milligram; ml, milliliter; µg, microgram; OECD, Organization for Economic Co-operation and Development; UDS, unscheduled DNA synthesis; WHO, World Health Organization.

* Corresponding author. Tel.: +1 512 351 7358; fax: +1 512 382 6945.
E-mail address: jurban@toxstrategies.com (J.D. Urban).

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the large intestine that they are metabolized. Intestinal flora hydrolyze steviol glycosides to the aglycone steviol, which is resistant to further catabolism and is readily absorbed by the intestine (Renwick and Tarka, 2008). It appears the number of glucose molecules attached to the steviol moiety dictate the rate at which the intestinal bacteria break them down. For example, the extra glucose moiety on rebaudioside A results in a slightly longer metabolism compared to stevioside, which has one less glucose moiety (Koyama et al., 2003; Roberts and Renwick, 2008; Wheeler et al., 2008). Once it enters the enterohepatic circulation, steviol is transported to the liver where it is conjugated with glucuronide. In rats, steviol glucuronide is excreted in the bile and returned to the intestine where it undergoes deconjugation prior to elimination in the feces (Roberts and Renwick, 2008). In humans, however, steviol glucuronide is mostly excreted in the urine, with small amounts of steviol in the feces following oral administration (Wheeler et al., 2008). The amount of steviol in the systemic circulation is exceedingly small in humans. No steviol was detected in the plasma of 14 of 16 human subjects who had ingested either rebaudioside A or stevioside (limit of quantitation 100 ng/ml plasma) (Wheeler et al., 2008). The two remaining subjects with detectable steviol had maximum plasma concentrations (C_{max}) just above the limit of detection.

The safety of steviol glycosides has been extensively reviewed in the published literature and by national and international food safety agencies. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) reviewed steviol glycoside safety on several occasions and concluded that stevioside and rebaudioside A were not genotoxic (JECFA, 2005). In 2008, JECFA established an acceptable daily intake (ADI) for steviol glycosides (0–4 mg/kg bw/day based on steviol equivalents) indicating that they considered the evidence presented on steviol glycosides sufficient for ensuring its safety at that level of daily exposure for a person's entire lifetime (JECFA, 2008). This conclusion by JECFA was based on a broad array of published and unpublished genotoxicity test results on individual steviol glycosides as well as their common metabolite steviol, considered to be a weak genotoxigen in some *in vitro* assays (reviewed by Brusick, 2008). Similar conclusions that steviol glycosides were safe at proposed levels of consumption have been announced by the European Food Safety Agency (EFSA, 2010), Australia–New Food Safety Authority (ANZFSA, 2008) and others (Carakostas et al., 2012). In the United States, approximately 20 expert panels have concluded that purified preparations of steviol glycosides meet the standard for being classified "Generally Recognized as Safe" (GRAS) and their conclusions successfully notified to the US FDA (USFDA, 2012a).

Regardless, two recent publications (Brahmachari et al., 2011; Tandel, 2011) have questioned the safety of steviol glycosides. Notably, Brahmachari et al. (2011) echoed a suggestion made 15 years prior by Matsui et al. (1996a) that further studies should be conducted to investigate the genotoxic potential of steviol *in vivo*. Likewise, Tandel (2011), in penning a general review of sugar substitutes, repeated a statement from an online source that speculated an unspecified steviol metabolite was mutagenic, and therefore could promote cancer by "causing mutations in the cells' DNA". However, the information cited in both papers is from old studies that were well known to JECFA, EFSA, ANZFSA and all the expert panels who have reviewed the safety of stevia. Given that concerns of genotoxicity related to intake of steviol glycosides continue to be perpetuated in the literature, the goals of this review are threefold: (1) to revisit the genotoxicity studies that seem to be the sources of continued uncertainty, (2) review additional studies published since 2008 that may further elucidate potential genotoxicity in humans, and using these findings as context, (3) address the utility of conducting additional *in vivo* genotoxicity studies.

2. Studies reporting potential stevioside or steviol genotoxicity

The potential genotoxicity of steviol glycosides has been an area of intense study since steviol was reported to elicit genotoxic effects *in vitro*. Though negative in the majority of *in vitro* assays conducted, steviol induced positive results in a few select *in vitro* gene mutation and chromosomal aberration assays (Table 1).

Steviol is negative in standard Ames assays, but positive in a forward mutation assay not routinely used in genetic testing that employs *Salmonella typhimurium* TM677 (Pezzuto et al., 1985, 1986; Matsui et al., 1996a,b; Terai et al., 2002). This unique bacterial strain is a prototrophic revertant of strain TA1535, deficient in DNA repair proteins and containing both the plasmid pKM101 and rfa mutations (Brusick, 2008). Furthermore, an S9 mix from rats exposed to polychlorinated biphenyls is required for steviol to produce positive mutagenic effects. Though the positive result is reproducible, the highly specific strain and conditions necessary to elicit this outcome indicate that the TM677 results are not appropriate for human health safety evaluation and risk assessment (Terai et al., 2002). Steviol was also reported to be mutagenic and clastogenic in cultured mammalian cells (Chinese hamster lung cells) in the presence of the liver S9 fraction (Matsui et al., 1996a). However, the doses of steviol required to elicit positive results produced excessive cytotoxicity (nearly 100% in the gene mutation assay, and >50% cell death in the chromosome aberration assay). These levels of cytotoxicity virtually eliminate any validity of the studies and argue that cytotoxicity – rather than a distinct chemical DNA reaction – was responsible for the positive results observed in both assays (Brusick, 2008).

Matsui et al. (1989, 1996a,b) also reported positive results for steviol in the *umu*-test and a mutation study in plasmid DNA. Technical and interpretive issues with these results have been reviewed previously (Brusick, 2008). The results of these two tests have not been directly linked to any known adverse genetic effects and therefore are not useful as predictors of risk.

Mutation and genotoxicity data for rebaudioside A and stevioside overwhelmingly affirm the genetic safety of stevia extracts. The results for rebaudioside A in a number of *in vivo* and *in vitro* assays, including those that indicated activity by steviol, have been consistently negative (Pezzuto et al., 1985; Nakajima, 2000a,b; Williams and Burdock, 2009). The only *in vitro* test in which stevioside was reported mutagenic was by Suttajit et al. (1993), who reported positive results in *S. typhimurium* strain TA98 at a concentration of 50 mg/plate. This finding is inconsistent with other studies, and occurred at a concentration 10 times higher than the recommended maximum test concentration for this assay (USFDA, 2000). Even at 99% purity, a positive result could easily have been due to the presence of an unidentified mutagenic contaminant or contaminants present at upto 500 μg/plate (Brusick, 2008; Williams and Burdock, 2009).

The only *in vivo* positive result for stevioside was reported by Nunes et al. (2007), who conducted comet assays to study the genotoxic effects of stevioside. They reported that stevioside induced DNA damage in blood cells, spleen, liver and brain tissues of rats administered stevioside in drinking water at 4 mg/ml (or approximately 400 mg/kg bw/day) for 45 days. However, this study suffered from a number of issues and has been the focus of several published evaluations critical of the technical conduct and data interpretation of the study (Geuns, 2007; Williams, 2007; Brusick, 2008). For example, the DNA damage was not observed until week 5 of the study, even though the comet assay is designed to detect short-lived genetic damage that is quickly resolved by normal repair processes (Brusick, 2008). Also, this particular study did not include a positive control, nor was an evaluation of cytotoxicity presented (Geuns, 2007; Williams, 2007). Moreover, the results presented by Nunes et al. (2007) contradict those of