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A 90-day oral (dietary) toxicity study of the 2*R*,4*R*-isomer of monatin salt in Sprague–Dawley rats

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ABSTRACT

The root bark of *Sclerochitin ilicifolius* contains an intensely sweet substance analytically identified as isomers of 2-hydroxy-2-(indol-3-ylmethyl)-4-aminoglutaric acid and generically coined "monatin." Groups of male and female Crl:CD(SD) rats were fed 0 (control), 5000, 10,000, 20,000 or 35,000 ppm *R,R*-monatin salt in the diet for 90 days. There were no toxicologically relevant clinical or histopathological findings in any of the test article-treated groups. Significantly lower cumulative body weight gains were noted in the 35,000 ppm group. Mean body weights in the 35,000 ppm group males and females were 7% and 12% lower, respectively, than the control group at study week 13. In the absence of other observations associated with systemic toxicity and lower food consumption, the magnitude of the body weight difference in the 35,000 ppm group females relative to the control group exceeded 10%, which indicated attainment of a maximum tolerated dose (MTD) level. Based on the results of this study, and conservatively assuming the body weight observations at the MTD to be indicative of an adverse effect, the dietary no-observed-adverse-effect level (NOAEL) of *R,R*-monatin salt for 90 days was 20,000 ppm in female rats (approximately 1544 mg/kg bw/day) and 35,000 ppm in male rats (approximately 2368 mg/kg bw/day).

1. Introduction

The root bark of *Sclerochitin ilicifolius*, a spiny-leaved hardwood shrub native to South Africa (Archibald et al., 1956), is intensely sweet. The source of this sweetness was identified as an amino acid, 2-hydroxy-2-(indol-3-ylmethyl)-4-aminoglutaric acid, and assigned the coined name of monatin, based on indigenous reference to the plant in local dialect as *molomo monate*, which literally translates to "mouth nice" (Vahrmeijer, 2010; Vleggaar et al., 1992). To date, the term monatin has collectively referred to any of the four possible stereo-isomers (*R,R*-, *S,S*-, *R,S*- and *S,R*-) that have been identified (Bassoli et al., 2005). However, it is the *R,R*-isomer (Fig. 1) that is the most sweet, with a potency of approximately 2000–2500 times that of sucrose. Hence, the *R,R*-isomer garners commercial interest for potential use as a sweetener in foods.

Numerous publications have documented various ways of synthesizing monatin (Bassoli et al., 2005; Nakamura et al., 2000; Rousseau et al., 2011; Tamura et al., 2003) since Vleggaar et al. (1992) first isolated the *S,S*-isomer about 20 years ago and reported on the existence of only minute amounts (<0.002%) of this natural sweetener in the root bark of *S. ilicifolius*. The root bark of *S. ilicifolius*, a member of the Anathacaeae family (McDade et al., 2005), has been used historically by indigenous populations in South Africa as a sweetening agent in food and medicines without apparent adverse effects (Vahrmeijer, 2010).

While *S. ilicifolius* appears to be non-toxigenic and the roots continue to be used in a traditional manner, the toxicity profile of monatin, or any of its isomers, is generally unknown. As part of an overall program to evaluate the safety of this natural sweetener and any potential for general use in food as an ingredient, the present study was conducted to assess the 90-day subchronic toxicity, in Sprague–Dawley rats, of the predominantly sodium salt form of the 2*R,4R*-isomer of monatin (*R,R*-monatin salt). This study was conducted in compliance with the US Food and Drug Administration (FDA) Good Laboratory Practice Regulations (FDA, 1987) and the Organisation for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice (OECD, 2003).

Abbreviations: ANOVA, analysis of variance; APTT, activated partial thromboplastin time; FDA, United States Food and Drug Administration; FOB, functional observational battery; HDW, hemoglobin distribution width; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MTD, maximum tolerated dose; NOAEL, no-observed-adverse-effect level; OECD, Organisation for Economic Cooperation and Development; PT, prothrombin time; RANOVA, repeated measures of analysis of variance; RDW, red cell distribution width.

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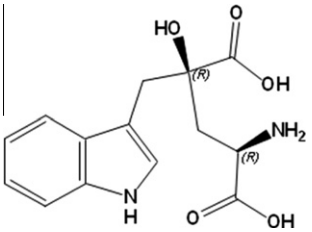

Fig. 1. The 2*R*,4*R*- isomer of 2-hydroxy-2-(indol-3-ylmethyl)-4-aminoglutaric acid.

2. Materials and methods

The safety of 2*R*,4*R*-monatin, as sodium/potassium salt, was evaluated in a 90-day feeding study with male and female Crl:CD(SD) rats conducted at WIL Research Laboratories, Ashland, Ohio, USA. This study was designed and conducted in general accordance with the FDA Redbook 2000 testing guidelines (FDA, 2003) and the OECD Guidelines for the Testing of Chemicals (OECD, 1998).

2.1. Test article

Enzymatically sourced *R,R*-monatin salt (sodium/potassium 2*R*,4*R*-2-amino-4-carboxy-4-hydroxy-5-(3-indolyl) pentanoate) (98% UV purity; 5.9% moisture) used in this 90-day toxicity study was supplied by Cargill, Incorporated. The basal diet, PMI Nutrition International LLC Certified Rodent LabDiet® 5002 (meal), was used as the control diet and in the preparation of the *R,R*-monatin salt/dietary admixes (test diet).

2.2. Test animals

One hundred and ten (110) male and 110 female Crl:CD(SD) rats approximately 35 days of age were received in good health from Charles River Laboratories, Inc., Raleigh, North Carolina and acclimated for 20 days. Upon arrival, all animals were housed individually in clean, stainless steel, wire-mesh cages suspended above cage-board at a room temperature of 22 ± 3 °C, with a relative humidity of approximately 30–70% and a 12-h light/12-h dark photoperiod. Reverse osmosis-treated drinking water was provided *ad libitum* throughout the study period. Animals were maintained in accordance with the "Guide for the Care and Use of Laboratory Animals" (National Research Council, 1996).

Individual body weights were recorded and detailed physical examinations were performed during the acclimation/pretest period to ensure the use of healthy animals. Individual feed and water consumption were measured and ophthalmic examinations were conducted during the pretest period. Functional observational battery and locomotor activity data were also recorded during the pretest period.

2.3. Preparation of diets

The test diets were prepared weekly as weight/weight (*R,R*-monatin salt/diet) mixtures with basal diet, without any further correction for purity. Diets were stored at room temperature.

2.4. Analysis of R,R-monatin salt in rat diet

R,R-Monatin salt mixtures in rodent diet at 10,000, 20,000 and 35,000 ppm were determined to be stable at ambient temperatures for up to 10 days (data not shown). Samples collected from the dietary admixes prepared for the first week of dosing were analyzed for ambient temperature stability at 5000 ppm, as well as, for concentration and homogeneity at all inclusion levels (5000 through 35,000 ppm). Additionally, samples were collected from dietary admixes prepared for study weeks 6 and 12 to confirm concentration. Control group diet (basal diet) samples were also collected at all intervals to verify the absence of *R,R*-monatin salt. All analyses were performed by a validated high-performance liquid chromatography method using ultraviolet detection.

2.5. Assignment of animals to treatment groups

Group assignments were generated based on body weight stratification in a block design using a validated computerized randomization procedure. Each group consisted of 20 males and 20 females. Ten animals/sex/group were randomly selected for clinical pathology evaluations, with the remaining 10 animals/sex/group selected for behavioral evaluation and blood collection. Animals were approximately 8 weeks old at the initiation of test diet administration; body weight values ranged from 215 to 305 g (mean = 258 ± 19.3 g) for the males and 161–225 g (mean = 188 ± 13.8) for the females. Individual body weights at randomization were within ±20% of the mean for each sex.

2.6. Administration

Dietary concentration levels of 5,000, 10,000, 20,000 or 35,000 ppm *R,R*-monatin salt, which were selected based on results of a 14-day dietary dose range-finding study and a 28-day dietary toxicity study in Crl:CD(SD) rats (data not shown), were fed *ad libitum* to groups of 20 Crl:CD(SD) rats/sex (Groups 2–5; Table 1) for 90 days. The concurrent control group (Group 1) received the standard basal diet on the same treatment regimen. The selected route of administration was dietary because *R,R*-monatin is consumed as a constituent of *S. ilicifolius* and its sensory attribute of sweetness offers potential for use as a food ingredient. The mean amount of *R,R*-monatin salt consumed (mg/kg bw/day) by each sex and dose group was calculated from the mean feed consumption (g/kg bw/day) and the appropriate target concentration of the test article in the diet (Table 1).

2.7. Parameters evaluated

2.7.1. Clinical observations and survival

All animals were observed twice daily for mortality and moribundity and received a daily clinical examination. Detailed physical examinations were conducted on all animals weekly, beginning approximately one week prior to test article administration and prior to the scheduled necropsy.

2.7.2. Body weights

Individual body weights were recorded weekly, beginning during the pretest period (i.e., approximately one week prior to randomization, or about 3 weeks prior to start of exposure). Final body weights were recorded prior to the scheduled necropsy.

2.7.3. Feed and water consumption

Individual feed and water consumption were recorded weekly, beginning during the pretest period (i.e., approximately one week prior to randomization, or about 3 weeks prior to start of exposure). Feed efficiency (*i.e.*, body weight gained as a percent of feed consumed) was also calculated.

2.7.4. Functional observational battery

Functional observational battery (FOB) observations were recorded for 10 animals/sex/group during pretest (week -1) and near the end of the dosing period (week 12). Testing was performed without knowledge of the animal group assignment and evaluations were conducted in a sound-attenuated room equipped with a white noise generator set to operate at 70 ± 10 db with one exception; home cage observations were performed in the animal room. All animals were observed for parameters based on the published criteria for neurotoxicology evaluations (Gad, 1982; Haggerty, 1989; Irwin, 1968).

2.7.5. Locomotor activity

Locomotor activity, recorded for 10 animals/sex/group after the completion of the FOB, was measured automatically using the Kinder Scientific Motor Monitor System (Kinder Scientific, LLC, Poway, CA). This personal computer-controlled system utilizes a series of infrared photobeams surrounding a clear plastic, rectangular cage to quantify each animal's motor activity. Each animal was tested separately in a sound-attenuated room, as described above for the FOB. Data were collected in 5-min epochs and the test session duration was 60 min. These data were compiled as six 10-min sub-sessions for tabulation. Data for ambulatory and total motor activity were tabulated. Total motor activity was defined as a combination of fine motor skills (*i.e.*, grooming, interruption of one photobeam) and ambulatory motor activity (interruption of two or more consecutive photobeams).

2.7.6. Clinical pathology

Blood samples for clinical pathology evaluations (hematology and serum chemistry) were collected from 10 animals/sex/group during weeks 2 and 6. Blood and urine samples for clinical pathology evaluations (hematology, coagulation, serum chemistry and urinalysis) were collected from the same animals prior to the scheduled necropsy (week 13). The animals were fasted overnight prior to blood collections. Blood samples for hematology and serum chemistry were collected *via* the retro-orbital sinus of animals anesthetized by inhalation of isoflurane. Blood samples for coagulation parameters were collected at the time of necropsy *via* the vena cava of animals euthanized by the inhalation of carbon dioxide. Urine was collected overnight using metabolism cages.

Serum chemistry (albumin, total protein, globulin, A/G ratio, total bilirubin, urea nitrogen, creatinine, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyltransferase, sorbitol dehydrogenase, glucose, total cholesterol, calcium, chloride, phosphorus, potassium, sodium and triglycerides), hematological [total leukocyte count, red blood cell count, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), hemoglobin distribution width (HDW), red cell distribution width (RDW), platelet count, reticulocyte count (percent and absolute), percent and absolute leukocyte count (neutrophil, lymphocyte, monocyte, eosinophil and basophil)], coagulation, [activated partial thromboplastin time (APTT) and prothrombin time (PT)], and standard urinalysis