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A two-year dietary carcinogenicity study of (2*R*,4*R*)-monatin salt in mice

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ABSTRACT

Groups of Crl:CD-1 (ICR) mice (60/group/sex) were fed 0 (2 control groups), 5000, 20,000, or 40,000 ppm of enzymatically sourced (2*R*,4*R*)-monatin salt ("*R,R*-monatin") in the diet for up to two years. There were no adverse effects on survival, incidence of palpable masses and tumors, feed consumption, hematology or serum chemistry parameters, organ weights, or ophthalmic, macroscopic, and microscopic examinations. The only notable effect was statistically significantly lower mean body weights and body weight gains in all treated groups, which generally occurred throughout the study and were most likely a result of caloric dilution of the test diets and not considered adverse. There were no test article-related changes in the incidence or occurrence of neoplastic diseases in mice on this study. The no-observed-effect-level (NOEL) for carcinogenicity of *R,R*-monatin fed to mice for 24 months was 40,000 ppm, the highest dietary concentration tested, which was equivalent to approximately 6502 and 7996 mg/kg bw/day in males and females, respectively.

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1. Introduction

(2*R*,4*R*)-Monatin salt (hereafter *R,R*-monatin; Fig. 1) is an enzymatically-sourced high-potency sweetener that is chemically identical to the non-proteinogenic α-amino acid identified in the root bark of a South African shrub, *Sclerochitin ilicifolius* (Archibald et al., 1956; Vleggaar et al., 1992) and is about 3000 times sweeter than sucrose (Fry et al., 2012). No known adverse effects have been reported from the traditional consumption of *S. ilicifolius* by populations indigenous to the regions in South Africa (Vahrmeijer, 2010); however, interest in the use of *R,R*-monatin as a high-potency sweetener (Abraham et al., 2005) warranted a scientific evaluation of its safety.

The only consistent test article-related findings in previous studies with enzymatically-sourced *R,R*-monatin fed to rats and mice up to 35,000 ppm in the diet for a period of 90 days were lower body weights and body weight gains, as well as increased serum chloride levels (Hlywka et al., 2011, 2013). Decreased serum potassium levels were also observed in rats. The no-observed-adverse-effect level (NOAEL) for female rats fed *R,R*-monatin was 20,000 ppm (approximately 1544 mg/kg bw/day) based on statistically significantly reduced body weights at the highest dietary concentration of 35,000 ppm. The NOAEL for male rats, and male and female mice was the highest dietary concentration tested, 35,000 ppm, which is equivalent to approximately 2368 mg/kg bw/day for male rats and approximately 5764 and 8013 mg/kg bw/day for male and female mice respectively (Hlywka et al., 2011, 2013). Similarly, in a developmental study in which pregnant rats were fed

Abbreviations: AAALAC, Association for Assessment and Accreditation of Laboratory Animal Care; ADME, absorption, distribution, metabolism and excretion; ALT, alanine aminotransferase; FDA, US Food and Drug Administration; GGT, gamma-glutamyl transferase; GLP, Good Laboratory Practice; HPLC/MS/MS, high performance liquid chromatography tandem mass spectrometry; IACUC, Institutional Animal Care and Use Committee; ICH, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; NOAEL, no-observed-adverse-effect level; NOEL, no-observed-effect-level; OECD, Organisation for Economic Co-operation and Development; SD, standard deviation.

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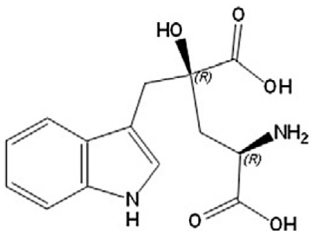

Fig. 1. Structure of (2*R*,4*R*)-monatin.

up to 50,000 ppm *R,R*-monatin during gestation days 6–21, the only significant findings were reductions in maternal and fetal body weights at the highest dietary concentration (Brathwaite et al., 2013). There were no fetal malformations, developmental variations, or effects on intrauterine survival and therefore, the NOAEL was 30,000 ppm (equivalent to 2564 mg/kg bw/day) for maternal and embryo/fetal developmental toxicity based on the reduced body weight changes. In a dietary two-generation reproductive toxicity study of *R,R*-monatin in rats, no effects on spermatogenic endpoints, reproductive performance, mean gestation length, or the process of parturition were observed at any exposure level in the F_0 and F_1 generations (Crincoli et al., 2015a). A conservative NOAEL for systemic, reproductive, and neonatal effects of *R,R*-monatin was 15,000 ppm, the second highest dietary concentration tested, on the basis of test article-related effects on body weight and feed efficiency, a slight decrease in maternal implantation sites and corresponding reduction in live litter size, and the magnitude of the reductions in pre-weaning pup body weights at 35,000 ppm.

Based on results of a 90-day subchronic toxicity study of *R,R*-monatin in Beagle dogs, the NOAEL for females was determined to be 35,000 ppm (1101 mg/kg bw/day), which was the highest dietary concentration tested, and for males was <5000 ppm (<151 mg/kg bw/day) based on significantly decreased testicular weights with microscopic correlates in all treated males (Crincoli et al., 2015b).

Results of *in vitro* (Ames and L5178Y/TK$^{+/-}$ mouse lymphoma mutagenesis) and *in vivo* (mouse bone marrow erythrocyte) assays showed no mutagenic potential for *R,R*-monatin (Casterton et al., 2014a). Within 48 h of a single oral gavage dose of 10 mg/kg bw, *R,R*-monatin was rapidly absorbed and eliminated primarily unchanged in the urine and feces of Beagle dogs (Casterton et al., 2014b).

This two-year feeding study investigated the potential carcinogenicity of *R,R*-monatin in mice.

2. Materials and methods

This study was conducted at WIL Research, Ashland, Ohio, USA with animal facilities that are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. The study was performed in compliance with the United States (U.S.) Food and Drug Administration (FDA) Good Laboratory Practice (GLP) Regulations (FDA, 1987) and the Organisation for Economic Co-operation and Development (OECD) Principles of GLP (OECD, 1997), and in general accordance with the FDA Redbook 2000 (FDA, 2000a,b,c) and the OECD Guidelines for the Testing of Chemicals (OECD, 2009).

2.1. Test article

Enzymatically-sourced (2*R*,4*R*)-monatin salt [sodium/potassium (2*R*,4*R*)-2-amino-4-carboxy-4-hydroxy-5-(3-indolyl) pentanoate] used in this study was an off-white powder produced in two batches with purities of 98.1% and 96.4% (corrected for potassium, sodium, and diastereomeric purity) and supplied by Cargill, Incorporated. The test article, hereinafter referred to as "*R,R*-monatin", was administered orally *via* the diet.

2.2. Diet preparation

PMI Nutrition International, LLC, Certified Rodent LabDiet® 5002 (meal) was used for preparation of the control (untreated) and test diets. The test diets were prepared weekly as weight/weight (*R,R*-monatin/diet) mixtures with basal diet, without any further correction for purity.

2.3. Test animals

Crl:CD-1 (ICR) mice (340 males and 340 females) were received in good health from Charles River Laboratories, Inc., Raleigh, NC and acclimated for approximately 15 days. During the exposure phase, all animals were housed individually in clean, wire-mesh cages suspended above cage-board at a room temperature of 22 ± 3 °C with a relative humidity of 50 ± 20% and a 12-h light/12-h dark photoperiod. The study protocol was approved by WIL Research's Institutional Animal Care and Use Committee (IACUC) and complied with all applicable sections of the Final Rules of the Animal Welfare Act regulations (9 CFR), the Guide for Care and Use of Laboratory Animals (NRC, 1996), and the PHS Policy on Humane Care and Use of Laboratory Animals. Drinking water and the basal or test diets were provided to the test animals *ad libitum* throughout the study. Animals were maintained in compliance with the "Guide for the Care and Use of Laboratory Animals" (National Research Council, 1996).

2.4. Analysis of R,R-monatin in the test diet

Stability of the test article in diet formulations for up to 10 days at room temperature storage was verified in previous studies (data not shown). Test diet formulation samples for homogeneity and concentration determination were collected on a regular basis and analyzed using a validated high performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS) method in the positive electrospray ionization mode.

2.5. Assignment of animals to treatment groups

Animals were assigned to control and test groups (60/sex/group) using a validated computerized randomization procedure based on body weight stratification in a block design. Individual body weights at randomization were within ±20% of the mean for each sex. One male in control group 2 was found dead on study day 9 and one male in the 20,000 ppm group was euthanized *in extremis* on study day 12. These animals were replaced on study days 9 and 13, respectively. Replacement animals were arbitrarily assigned based on body weight. The animals were approximately six weeks old and individual body weights ranged from 24.1 to 33.5 g (mean = 28.3) for males and from 18.4 to 26.7 g (mean = 21.9) for females at the initiation of test diet administration.

2.6. Administration

Mice were offered test diets containing 5000, 20,000, or 40,000 ppm *R,R*-monatin (groups 3–5; Table 1) *ad libitum* for up to 734 consecutive days based on the results of a 90-day dietary toxicity study of *R,R*-monatin in mice (Hlywka et al., 2013). Concurrent control groups (groups 1 and 2) were fed standard basal

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