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A 90-day oral toxicity study of glycolipids from *Dacryopinax spathularia* in CD® rats

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ABSTRACT

The subchronic toxicity of jelly mushroom glycolipids from *Dacryopinax spathularia* (herein referred to as "AM-1") was studied in Crl:CD(SD) rats. The test item was administered via the drinking water at concentrations of 1.5, 5.0 or 15 mg/mL for 90 days with an additional 4-week recovery period. No test article-related deaths, clinical observations or neurological effects were noted. Decreased drinking water consumption for mid- and high-dose groups was attributable to the reduced palatability of drinking water containing higher test article concentrations. Mean body weights of high-dose males were slightly reduced beginning study week 1 due to decreased food and drinking water intake, but were not statistically significant by week 7. No test article-related adverse effects were noted for hematological or clinical chemistry, or urinalysis parameters. Statistically significant changes in select parameters were within historical control data ranges, lacked histopathological correlates, and did not occur in a consistent pattern that would suggest biological significance. Microscopic examination did not reveal any test article-related morphological changes. The no-observed-adverse-effect level (NOAEL) was considered to be 15 mg/mL (1201 and 1423 mg AM-1/kg bw/day for male and female rats, respectively). These results support the safety assessment of jelly mushroom glycolipids for potential use in food.

1. Introduction

A mixture of glycolipids, herein referred to as "AM-1", was obtained via natural fermentation of the edible jelly mushroom *Dacryopinax spathularia* (Schwein.) (Martin, 1948), also referred to as *Cantharellus spathularius* (Schwein.) and "sweet osmanthus ear mushroom" in China. The components of AM-1 are structurally-related glycolipid congeners, all sharing a long chain fatty acid (LCFA) backbone and the same trisaccharide moiety. Due to the antimicrobial and preservative properties of AM-1, a series of studies and scientific assessments have been conducted to evaluate its safety for use as a food ingredient. Safety of the AM-1 production organism for use in food ingredient production was evaluated using the procedures outlined by Pariza and Johnson (2001). It was concluded that *Dacryopinax spathularia* does not have any toxigenic or pathogenic potential, nor is it expected to be present in the finished glycolipid material that would be used as a food ingredient. The potential for production of medically or clinically relevant antibiotics by the organism can also be excluded. AM-1 is produced in accordance with current Good Manufacturing Practices (cGMP) and the finished product must comply with established specifications for identity, composition, impurities, and contaminants.

AM-1 was previously demonstrated to be of low acute toxicity

Abbreviations: Absorption, distribution, metabolism, and excretion, (ADME); activated partial thromboplastin time, (aPTT); alanine amino-transferase, (ALAT); alkaline phosphatase, (AP); aspartate aminotransferase, (ASAT); calcium, (Ca); cholinesterase, (CHE); current Good Manufacturing Practices, (cGMP); Functional Observation Battery, (FOB); gamma-glutamyl transpeptidase, (gamma-GT); glutamate dehydrogenase, (GLDH); Good Laboratory Practice, (GLP); hematocrit value, (HCT); hematoxylin-eosin, (H-E); hemoglobin content, (HGB); human corneal epithelium, (HCE); Human Repeat Insult Patch Test, (HRIPT); lactate dehydrogenase, (LDH); large unstained cells, (LUC); long chain fatty acids, (LCFA); mean corpuscular hemoglobin, (MCH); mean corpuscular hemoglobin concentration, (MCHC); mean corpuscular volume, (MCV); Mean Photo Effect, (MPE); neutrophilic granulocytes, (Neut); no-observed-adverse-effect level, (NOAEL); Organisation for Economic Cooperation and Development, (OECD); periodic acid–Schiff, (PAS); platelets, (PLT); protein (total), (TP); red blood cells, (RBC); reticulocytes, (Reti); standard operating procedures, (SOPs); thromboplastin time, (TPT); U.S. Food and Drug Administration, (FDA); white blood cells, (WBC).

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via the oral and dermal routes of exposure, non-irritating to the skin and eyes, non-sensitizing, and non-genotoxic (*INS unpublished data on file*). There were no deaths or clinical signs of toxicity following a single oral gavage dose of 2000 mg AM-1/kg bw in female WISTAR Crl: WI(Han) rats. The body weight gain of the test animals was within the normal range of variation for this strain throughout the 14-day observation period, and no treatment-related macroscopic findings were observed in any animal at the scheduled necropsy. Based on the results of *in vitro* tests employing human skin (SkinEthic® Reconstructed Human Epidermis, and EpiDerm™) and 3D-tissue human corneal epithelium (HCE) models, AM-1 was classified as non-irritating (mean tissue viability > 50%) and non-corrosive (relative absorbance > 50% after the 3-min exposure and >15% after the 1-h exposure) to the skin and eye. AM-1 was also classified as non-phototoxic (Mean Photo Effect [MPE] = 0.03) in an *in vitro* assay using Balb/c 3T3 cells.

AM-1 is not considered to be a contact sensitizer based on the results of a dermal sensitization study in guinea pigs (Buehler Method) with 60% AM-1 (w/w) in distilled water and a Human Repeat Insult Patch Test (HRIPT) with 0.5% AM-1 (w/w) in distilled water. In the guinea pig study employing a much higher AM-1 test concentration, very faint to faint erythema was noted with decreasing incidence at 14 of 20, 6 of 20, and 1 of 20 test sites 24 h after the first, second, and third induction, respectively. Very faint erythema (0.5) was noted at 2 of 20 test sites 24 h after the challenge dose and all irritation cleared from the affected sites by 48 h. No skin irritation or adverse reactions were noted during the course of the human patch test.

In an *in vitro* bacterial reverse mutation assay (Ames test) with AM-1, there was no evidence of mutagenic activity in *Salmonella typhimurium* strains TA1535, TA1537, TA98, and TA100, or *Escherichia coli* strain WP2 *uvr*A, at any non-cytotoxic dose level (up to 5000 μg/plate) in both the absence and the presence of Aroclor 1254-induced rat liver (S9 mix) for metabolic activation. In the *in vitro* micronucleus test using human lymphocytes, AM-1 did not show a statistically significant increase in the number of binucleated cells containing micronuclei when compared to concurrent control cultures at all time points and at any of the concentrations analyzed (up to 5000 μg/mL of culture medium). Therefore, AM-1 was concluded to be non-clastogenic and/or non-aneugenic in cultured human lymphocytes both in the presence and absence of metabolic activation (S9 mix). No increase in mutation frequency or numbers of small and large colonies were noted for AM-1 (up to 800 μg/mL) compared to concurrent controls when tested in the *in vitro* mouse lymphoma thymidine kinase assay (MLA) both in the presence and absence of metabolic activation (S9 mix).

The subchronic toxicity of AM-1 was evaluated in a 90-day oral (capsule administration) study in Beagle dogs as reported in Bitzer et al. (2017a). AM-1 was well tolerated at all dosages up to 1000 mg/kg/day and there were no test article-related effects on survival, clinical observations, neurological screening (functional observational battery) parameters, clinical pathology parameters, organ weights, macroscopic or microscopic evaluations. Test article-related changes were limited to minimal effects on food consumption (not statistically significant) and cumulative body weight gains in the 1000 mg/kg/day group females. Therefore, the no-observed-adverse-effect level (NOAEL) was considered to be 1000 mg/kg/day, the highest dosage level tested.

In addition to the acute toxicity, dermal toxicity (skin and eye irritation, sensitization), subchronic oral toxicity and genotoxicity studies described above, the absorption, distribution, metabolism, and excretion (ADME) of AM-1 and its major hydrolysis product, long chain fatty acids (LCFA), has been studied both *in vitro* and *in vivo* as reported in Bitzer et al. (2017b). The oral bioavailability of AM-1 and LCFA or their metabolites was determined to be approximately 11% demonstrating that AM-1 and LCFA are not well-absorbed by the oral route and are primarily eliminated in the feces without absorption. At the oral C_{max}, <0.2% of administered doses of AM-1 or LCFA was present in plasma. The pharmacokinetic, tissue distribution, and excretion balance data derived in this study support the conclusion that following ingestion, AM-1 is partially hydrolyzed to its components, glucose, xylose, acetate, isovalerate and LCFA. [^{14}C]-AM-1 and [^{14}C]-LCFA radioactivity was highest in the GI tract tissues, as expected following oral administration (Bitzer et al., 2017b).

In the present study, the subchronic toxicity of AM-1 was evaluated after administration in the drinking water to CD® rats for 90 days. This study was conducted at LPT Laboratory of Pharmacology and Toxicology (Hamburg, Germany) during March to July 2014 (Study No. 29803). The study was performed in compliance with the Organisation for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice (GLP) (OECD, 1998a,b), which are compatible with the U.S. Food and Drug Administration (FDA) GLP Regulations (21 CFR Part 58) (FDA, 1987). The study protocol was designed in general accordance with U.S. FDA Redbook Guideline IV.C.4.a. "Subchronic Toxicity Studies with Rodents" (FDA, 2003) and the OECD Testing Guideline No. 408 "Repeated Dose 90-day Oral Toxicity Study in Rodents" (OECD, 1998a,b).

2. Materials and methods

2.1. Test article, vehicle, and dose formulations

AM-1 is a natural mixture of congeneric glycolipids, as produced by fermentation of glucose with the jelly fungus *Dacryopinax spathularia*. The three main glycolipid components of AM-1 are depicted in Fig. 1. Components i) and ii) together account for ca. 30–40% of the glycolipids, and component iii) accounts for 15–25% as determined by HPLC-MS analysis. The balance of other glycolipids present in the fermentation product mixture are congeners of the parent components, sharing the same fatty acid and trisaccharide moiety but differing in the acylation pattern.

The AM-1 test sample was supplied by IMD Natural Solutions, GmbH (INS) (Dortmund, Germany) as an off-white powder with >92% total glycolipid content as determined via HPLC-MS analysis. The remaining ca. 6–8% of the test substance was comprised of water (1.8% determined by Karl-Fischer method), protein (2.1% determined by Kjeldahl method using conversion factor N x 6.25 from nitrogen content), sodium chloride (2.0% by potentiometric analysis of chloride) and total lipids (1.5% by gravimetric analysis determined by Weibull-Stoldt method). The presence of free monosaccharides was excluded (<0.1% by GC-MS). The test substance was confirmed to comply with established specifications for heavy metals content (Ni 0.7 ppm; As, Cd, Pb, Hg $\leq$ 0.05 ppm) and microbiological purity (total aerobic microbial count, TAMC $\leq$ 100 CFU/g; absence of *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* in 1 g each; absence of *Salmonella* spp. in 10 g).

The test article was diluted with the vehicle, drinking water (tap water) at target concentrations of 0.15%, 0.5%, and 1.5% (1.5, 5.0, and 15 g/L, respectively). Dose formulations were prepared once per week and stored refrigerated (2–8 °C) until use. Prior to use for dose administration, the test article-drinking water formulations were warmed to room temperature and stirred for a minimum of 15 min. The stability and concentration of the test article in the vehicle were evaluated by analysis of samples collected at appropriate intervals throughout the study (i.e. concentration analysis was conducted on the first and last dose formulation preparations in study weeks 1 and 13; stability analysis was conducted after 7