Food and Chemical Toxicology 123 (2019) 162–168

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Genetic toxicity studies of glycolipids from *Dacryopinax spathularia*

Jens Bitzer[a], Thomas Henkel[a], Andrey I. Nikiforov[b,*], Marisa O. Rihner[b], C.M. Verspeek-Rip[c], Birol Usta[d], Michèle van den Wijngaard[d]

[a] IMD Natural Solutions GmbH, Dortmund, Germany
[b] Toxicology Regulatory Services, Charlottesville, VA, USA
[c] Charles River Laboratories (formerly WIL Research Europe B.V.), DD's-Hertogenbosch, the Netherlands
[d] Triskelion B.V. (formerly TNO Triskelion B.V.), Zeist, the Netherlands

ARTICLE INFO

Keywords:
Genetic toxicity
Jelly mushroom glycolipids
Natural preservative

ABSTRACT

A series of *in vitro* studies were conducted to assess the genetic toxicity of jelly mushroom glycolipids from *Dacryopinax spathularia* (herein referred to as "AM-1"). In the bacterial reverse mutation assay (Ames test), there was no evidence of mutagenic activity in any *Salmonella typhimurium* strains tested or in *Escherichia coli* strain WP2*uvr*A, at dose levels up to 5000 μg/plate. In the micronucleus (MN) 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 900 μg/ml of culture medium). 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 mouse lymphoma thymidine kinase assay (MLA). Therefore, AM-1 was concluded to be negative in all three assays performed both in the absence and presence of Aroclor 1254- or phenobarbital/ß-naphthoflavone-induced rat liver (S9 mix) for metabolic activation. 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 (CAS RN 2205009-17-0) are structurally-related glycolipid congeners, all sharing a long chain fatty acid (LCFA) backbone and the same trisaccharide moiety (Fig. 1). Due to the antimicrobial and preservative properties of AM-1, a series of studies and scientific assessment have been conducted to evaluate its safety for use as a food ingredient. The results of subchronic oral toxicity testing with AM-1 in rats and dogs have been reported by Bitzer et al. (2017a,b). In addition, the absorption, distribution, metabolism, and excretion (ADME) profiles of AM-1 and its major hydrolysis product, LCFA, have been studied both *in vitro* and *in vivo* as reported in Bitzer et al. (2017c). Reproductive and developmental toxicity studies of AM-1 have been conducted in rats via oral gavage and confirmed that AM-1 is not a developmental or reproductive toxicant (Bitzer et al., 2018).

The studies reported herein were conducted to evaluate the genetic toxicity potential of AM-1 using *in vitro* bacterial or mammalian cell systems. The Ames and micronucleus (MN) studies were conducted at TNO Triskelion B.V. (The Netherlands) during September to November 2011 (Study No. 9905/07) and November 2011 to January 2012 (Study No. 9917/05) and the Mouse Lymphoma Assay (MLA) was conducted at Charles River Laboratories (formerly WIL Research Europe B.V.) during September to December 2015 (Study No.

Abbreviations: 2-AA, 2-amino-anthracene; 2-NF, 2-nitrofluorene; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; 9-AA, 9-aminoacridine; ADME, absorption, distribution, metabolism, and excretion; OECD, Organisation for Economic Cooperation and Development; B[a]P, Benzo(a)pyrene; CE, cloning efficiency; CP, Cyclophosphamide; CBPI, Cytokinesis-Block Proliferation index; G-6-P, D-glucose-6-phosphate, disodium salt; DMSO, dimethyl sulfoxide; ELSD, evaporative light scattering detector; FDA, US Food and Drug Administration; GLP, Good Laboratory Practice; HPLC, high performance liquid chromatography; LCFA, long chain fatty acid; MS, mass spectrometry; MMS, methyl methanesulfonate; MN, micronucleus; MLA, mouse lymphoma assay; MF, mutation frequency; ENU, N-ethyl-N-nitrosourea; NADP, nicotinamide adenine dinucleotide phosphate, disodium salt; PHA-L, phytohaemagglutinin; RPMI, Roswell Park Memorial Institute; TAMC, total aerobic microbial count; TFT, trifluorothymidine

* Corresponding author. Toxicology Regulatory Services, 154 Hansen Rd., Suite 201, Charlottesville, VA, 22911, USA.
E-mail address: anikiforov@toxregserv.com (A.I. Nikiforov).

https://doi.org/10.1016/j.fct.2018.10.045
Received 18 May 2018; Received in revised form 15 October 2018; Accepted 16 October 2018
Available online 25 October 2018
0278-6915/

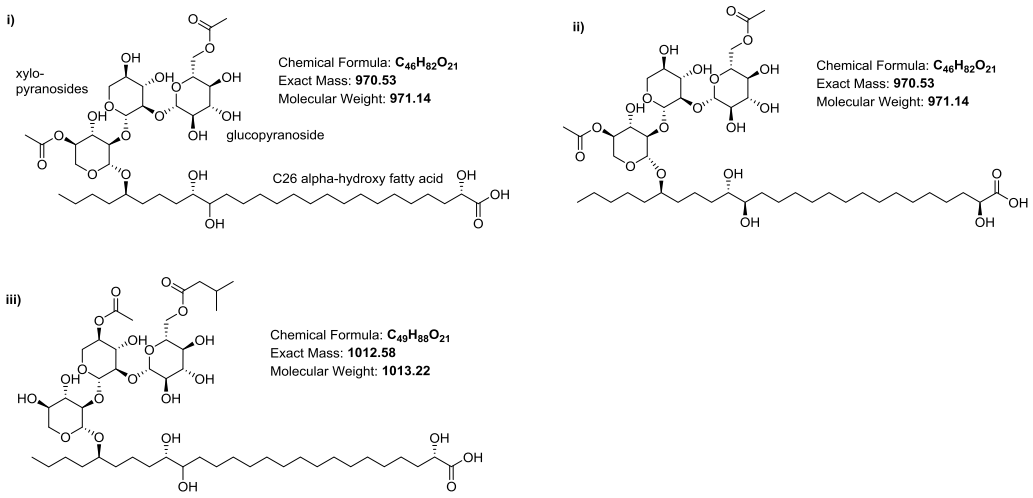

Fig. 1. Representative structure diagrams for main components of jelly mushroom glycolipids (AM-1) mixture.

510377). All studies were performed in compliance with the Organisation for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice (GLP) (OECD, 1998), which are compatible with the U.S. Food and Drug Administration (FDA) Good Laboratory Practice Regulations (21 CFR Part 58) (FDA, 1987). The study protocols were designed in general accordance with the relevant testing guidelines of OECD and/or the U.S. FDA, i.e. U.S. FDA Redbook Guideline IV.C.1. c. "Mouse Lymphoma Thymidine Kinase Gene Mutation Assay" (FDA, 2001) and OECD Testing Guideline No. 490 "Genetic Toxicology: In Vitro Mammalian Cell Gene Mutation Test Using the Thymidine Kinase Gene" (OECD, 2015), OECD Testing Guideline No. 487 "In Vitro Mammalian Cell Micronucleus Test (MNvit)" (OECD, 2010), and OECD Testing Guideline No. 471 "Genetic Toxicology: Bacterial Reverse Mutation Test" (OECD, 1997).

2. Materials and methods

2.1. Test article

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 for the Ames and MN studies was supplied by IMD Natural Solutions GmbH (formerly InterMed Discovery GmbH) (Dortmund, Germany) as a white solid powder with > 99% total glycolipid content as estimated via HPLC-ELSD analysis. Quantitative NMR analysis specified total glycolipid content more precisely as 92%. Residual protein by Kjeldahl method was determined as 0.5%, using conversion factor N x 6.25 from nitrogen content. .

The AM-1 test sample for the MLA study was supplied by IMD Natural Solutions GmbH (Dortmund, Germany) as an off-white powder with ca. 92% total glycolipid content as determined via HPLC-MS analysis. The remaining ca. 6–8% of the test article was comprised of water (2.1% determined by Karl-Fischer method), protein (2.7% determined by Kjeldahl method using conversion factor N x 6.25 from nitrogen content), sodium chloride (1.1% by potentiometric analysis of chloride) and total lipids (1.7% by gravimetric analysis determined by Weibull-Stoldt method). The presence of free monosaccharides was excluded (< 0.1% by GC-MS). The test article was confirmed to comply with established specifications for heavy metals content (Ni 1.6 ppm; As 0.13 ppm; Cd, Pb, Hg ≤ 0.05 ppm) and microbiological purity (total aerobic microbial count, TAMC < 10 CFU/g; absence of *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* in 1 g each; absence of *Salmonella* spp. in 10 g).

2.2. Test system, culture medium and S9 activation system

2.2.1. Ames test

Salmonella typhimurium strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* strain WP2*uvr*A were obtained from Trinova Biochem (Giessen, Germany) and originally from Moltox Molecular Toxicology Inc. (Boone, NC, USA). The strains were checked for histidine or tryptophan requirement and for sensitivity to ampicillin, crystal violet, and UV radiation.

Nicotinamide adenine dinucleotide phosphate, disodium salt (NADP) was obtained from Roche Diagnostics (Woerden, The Netherlands). Minimal glucose agar plates were partly obtained from Biotrading (Mijdrecht, The Netherlands) and partly made in-house. Biotine, L-histidine and L-Tryptophan were obtained from Merck KGaA, (Darmstadt, Germany); D-glucose-6-phosphate, disodium salt (G-6-P), 9-aminoacridine (9-AA), N-ethyl-N-nitrosourea (ENU), dimethyl sulfoxide (DMSO), and Benzo(a)pyrene (B[a]P) were obtained from Sigma Chemical Company (St. Louis, MO, USA); Aroclor 1254 was obtained from Monsanto Chemical Company (St. Louis, MO, USA); and 2-nitrofluorene (2-NF), 2-amino-anthracene (2-AA) and sodium azide (SA) were obtained from Aldrich (Brussels, Belgium).

The S9 liver homogenate was obtained from Aroclor 1254-induced male Wistar rats and prepared at the testing facility according to Ames et al. (1975) and Maron and Ames (1983). The batch was checked for sterility, protein and cytochrome P450 content. Aliquots of the S9 liver homogenate were prepared and mixed with a NADPH generating system. The final concentrations were: $MgCl_2$ 8 mM, KCl 33 mM, G-6-P 5 mM, NADP 4 mM, sodium phosphate 100 mM (pH 7.4), NaCl 46 mM, and S9 10%.

2.2.2. MN test

Human blood samples were obtained from young healthy, non-smoking donors with no known recent exposures to genotoxic chemicals or radiation. The blood was collected in sterile, heparinized vacutainer tubes and gently mixed before use to prevent clotting. The medium for culturing the human peripheral blood lymphocytes consisted of Roswell Park Memorial Institute (RPMI)-1640 medium (with hepes and glutamax), supplemented with heat-inactivated (30 min, 56 °C) foetal calf serum (20%), penicillin (100 U/ml medium), streptomycin (100 µg/ml medium) and phytohaemagglutinin (2.4 µg/ml).

Foetal calf serum was purchased from Lonza, Verviers, Belgium; RPMI-1640 medium (with hepes and glutamax) and penicillin-streptomycin from Life Technologies (Invitrogen) Paisley, UK; NADP from Roche Diagnostics, Almere, The Netherlands; Aroclor 1254 from Monsanto Chemical Company, St. Louis, MO, USA; glacial acetic acid from Merck-Darmstadt, Darmstadt, Germany; methanol from Biosolve, B.V., Valkenswaard, The Netherlands; DMSO, G-6-P, mitomycin C, vinblastine sulphate, acridine-orange, and cytochalasin B from Sigma-Aldrich Chemie GmbH, Germany; phytohaemagglutinin (PHA-L) from BioChrom AG, Germany; and cyclophosphamide from Baxter B.V., Utrecht, The Netherlands.

The S9 liver homogenate was obtained and prepared as described above for the Ames test. For the MN test, the S9 mix consisted of $MgCl_2$ 8 mM, KCl 33 mM, G-6-P 5 mM, NADP 4 mM, sodium phosphate 100 mM (pH 7.4), and S9 40%.

2.2.3. MLA

L5178Y/TK$^{+/-}$-3.7.2C mouse lymphoma cells were obtained from American Type Culture Collection, (ATCC, Manassas, VA, USA) and stored in liquid nitrogen (−196 °C). The cultures were checked for mycoplasma contamination and none were found. Cell density was kept below 1×10^6 cells/ml.

Basic medium consisted of RPMI-1640 Hepes buffered medium (Dutch modification) (Life Technologies) containing penicillin/streptomycin (50 U/ml and 50 μg/ml, respectively) (Life Technologies), 1 mM sodium pyruvate (Sigma, Zwijndrecht, The Netherlands) and 2 mM L-glutamin (Life Technologies). Growth medium was comprised of basic medium, supplemented with 10% (v/v) heat-inactivated horse serum (Life Technologies; R10-medium). Prior to dose range finding and mutagenicity testing, the mouse lymphoma cells were grown for one day in R10-medium containing 10^{-4} M hypoxanthine (Sigma), 2×10^{-7} M aminopterine (Fluka Chemie AG, Buchs, Switzerland) and 1.6×10^{-5} M thymidine (Merck) (HAT-medium) to reduce the amount of spontaneous mutants, followed by a recovery period of 2 days on R10-medium containing hypoxanthine and thymidine only. After this period cells were returned to R10-medium for at least one day before starting the experiment. Exposure medium was comprised of basic medium supplemented with 5% or 10% (v/v) heat-inactivated horse serum for the 3-h and 24-h exposure, respectively. Selective medium consisted of basic medium supplemented with 20% (v/v) heat-inactivated horse serum (R20-medium) and 5 μg/ml trifluorothymidine (TFT) (Sigma). Non-selective medium consisted of basic medium supplemented with 20% (v/v) heat-inactivated horse serum (R20-medium).

Dimethyl sulfoxide (DMSO; Merck Darmstadt, Germany) was used as the vehicle and negative control. Methyl methanesulfonate (MMS; Sigma Aldrich GmbH, Steinheim, Germany) was used as a direct acting mutagen (positive control not requiring metabolic activation) and Cyclophosphamide (CP; Utrecht, The Netherlands) was used as an indirect acting mutagen (positive control requiring metabolic activation).

Rat liver microsomal enzymes (S9 homogenate), obtained from Trinova Biochem GmbH, Giessen, Germany, were prepared from male Sprague Dawley rats that had been dosed orally with a suspension of phenobarbital (80 mg/kg body weight) and β-naphthoflavone (100 mg/kg). S9-mix was prepared immediately before use and kept on ice. S9-mix components contained per ml of physiological saline: 1.63 mg $MgCl_2.6H_2O$; 2.46 mg KCl; 1.7 mg G-6-P; 3.4 mg NADP; 4 μmol HEPES. The above solution was filter (0.22 μm)-sterilized. To 0.5 ml S9-mix components, 0.5 ml S9-fraction was added (50% (v/v) S9-fraction) to complete the S9-mix. The concentration of the S9-fraction in the exposure medium was 4% (v/v).

2.3. Treatment, sampling and analysis

2.3.1. Ames test

Three bacterial reverse mutation tests were performed and DMSO was used as a solvent for the test article. In the first and second test, the test article was dissolved at 50 mg/ml based on a purity of 99% and a clear, slightly brown solution was obtained. Five different concentrations were tested ranging from 62 to 5000 μg/plate. The plate-incorporation test with strain TA1537 was repeated at dose levels of 19, 38, 75, 150, and 300 μg/plate due to excessive cytotoxicity at dose levels ≥185 μg/plate in the first test. In the third test the test article was dissolved at 200 mg/ml, based on a purity of 99% and a slight-turbid, brown solution was obtained. In every test the negative control (DMSO) and the positive controls were run simultaneously with the test article.

In the first test via the plate incorporation method (with and without S9 metabolic activation), each strain of bacteria together with the test article or control was added to molten top agar maintained at ca. 46 °C. In the second and third tests via the treat and plate method (without metabolic activation), strain TA1535 was incubated with the test article or control at ca. 37 °C for ca. 3 h (while shaking) prior to plating. All determinations were made in triplicate. The plates were incubated at ca. 37 °C for approximately 48–72 h. Subsequently, the his + and trp + revertants were counted. Toxicity was defined as a reduction (by at least 50%) in the number of revertant colonies and/or a clearing of the background lawn of bacterial growth as compared to the negative (vehicle) control and/or the occurrence of pinpoint colonies.

2.3.2. MN test

Based on test article solubility and the results of a dose range-finding cytotoxicity test, AM-1 was dissolved in the solvent, DMSO, and tested at concentrations of up to 700 and 1000 μg/ml in the absence and presence of metabolic activation (± S9), respectively, for the 4-hr (pulse treatment) incubation. In the 20-hr (continuous treatment) incubation assay without metabolic activation (-S9), AM-1 was tested at concentrations of up to 900 μg/ml.

The main *in vitro* MN test consisted of both pulse treatment groups (± S9) and the continuous treatment group (-S9) which were performed simultaneously. In all instances duplicate cultures were used. Solvent controls and positive controls were tested concurrently as detailed in Table 3. After 48 h the cells were harvested by low speed centrifugation and resuspended in fresh medium without fetal calf serum and PHA-L and the test or control article was added. Incubations were continued for 4 h (± S9, pulse treatment) or for 20 h (-S9, continuous treatment). All incubations were performed at 37 °C in humidified air containing 5% CO_2. After 4 h (pulse treatment) and 20 h (continuous treatment) the culture medium with the test articles and S9-mix were removed. The cells were washed twice with phosphate-buffered saline (pH 7.4) and supplied with 5 ml fresh culture medium enriched with fetal calf serum (20%), PHA-L and cytochalasin B (6 μg/ml; final concentration) as cytokinesis inhibitor. The cells were incubated for an additional 20 h (pulse treatment) and 28 h (continuous treatment) prior to being harvested (second cell-cycle). Based on the evaluation of cytotoxicity in all treatment groups (± S9 pulse and -S9 continuous), three dose levels of the test article (Table 3) together with the negative control and positive control, were analyzed for micronucleus induction in bi-nucleated lymphocytes. At the end of the recovery period the cells in each culture were harvested and processed. Prepared slides were stained with a fluorescence DNA-specific dye (acridine orange) for analysis. One slide per culture was analyzed for Cytokinesis-Block Proliferation index (CBPI) and two slides were analyzed for micronucleus formation. Two thousand (2000) binucleated cells per concentration (1000 per culture) were examined for the presence of micronuclei.

2.3.3. MLA

Based on test article solubility and the results of a dose range-finding cytotoxicity test, AM-1 was dissolved in the solvent, DMSO, and tested at concentrations of up to 450 and 800 μg/ml in the absence and presence of metabolic activation, respectively, for the 3-hr incubation (Table 4). In the 24-hr incubation assay without metabolic activation, AM-1 was tested at concentrations of up to 500 μg/ml (Table 5). The highest doses that were tested gave a cell survival of approximately 10–20% and the survival in the lowest doses was approximately the same as the cell survival in the solvent control.

In the main study, cultures (10^6 cells/ml for 3 h treatment;

1.25×10^5 cells/ml for 24 h treatment) were incubated with AM-1 or the negative or positive control article. The cell cultures for the 3 h treatment (+S9 and -S9) were placed in sterile 30 ml centrifuge tubes, and incubated in a shaking incubator at 37.0 ± 1.0 °C and 145 rpm. The cell cultures for the 24 h treatment (-S9) were placed in sterile 75 cm^2 culture flasks at 37.0 ± 1.0 °C. All incubations were carried out in a humid atmosphere (41–90%) containing 5.0 ± 0.5% CO_2 in air in the dark. After exposure, the cells were separated from treatment solutions by two centrifugation steps (216 g, 5 min) each followed by removal of the supernatant. The cells in the final suspension were counted with the coulter particle counter. The remaining cells were cultured for two days after the treatment period for expression of the mutant phenotype. Two days after the end of the treatment with the test article the cells were plated for determination of the cloning efficiency (CE_{day2}) and the mutation frequency (MF). For determination of the CE_{day2} value, the cell suspensions were diluted and seeded in wells of a 96-well dish. Once cell was added per well (2 × 96-well microtiter plates/concentration) in non-selective medium. For determination of the MF a total number of 9.6×10^5 cells/concentration were plated in five 96-well microtiter plates. The microtiter plates for CE_{day2} and MF were incubated for 10–12 days. After the incubation period, the plates for the TFT-selection were stained with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). The plates for the CE_{day2} and MF were scored with the naked eye or with the microscope.

3. Results

3.1. Ames test

The results of the bacterial mutation tests with AM-1 are presented in Tables 1 and 2. There were no test article-related increases in the mean number of revertant colonies compared to the background spontaneous reversion rate (negative control) observed in any strain

Table 2
Results of Bacterial Reverse Mutation Assay with AM-1, Repeat Tests with Strain TA1535 using Treat-and-Plate Method.

Dose (µg/mL)	Treatment Method	Mean ± SD revertants per plate	
		TA1535 (*First repeat*)[a]	TA1535 (*Second repeat*)[b]
Solvent Control:	Treat-and-plate (-S9)		
DMSO, 0		11 ± 3	15 ± 3
Test Article, AM-1:			
62		24 ± 6	14 ± 1
185		36 ± 5	13 ± 5
556		37 ± 7	13 ± 5
1667		32 ± 21	20 ± 6
5000		38 ± 3	16 ± 2
Positive Control:			
MNNG, 5.0		4100 ± 179	2155 ± 249

MNNG = 1-methyl-3-nitro-1-nitrosoguanidine; SD = Standard Deviation; Mean = Average number of revertants per plate (n = 3 plates per treatment).

[a] The first repeat test with strain TA1535 was performed according to the treat-and-plate method due to a non dose-related increase in the mean number of revertant colonies observed at several concentrations in the initial plate incorporation test, concurrent with a slightly more dense background lawn of bacterial growth.

[b] The second repeat test with strain TA1535 was performed according to the treat-and-plate method due to a non dose-related increase in the mean number of revertant colonies observed at several concentrations in the first treat-and-plate test, concurrent with low negative control counts [vehicle control acceptable range: 10–75; vehicle control historical range: 11–20].

Table 1
Results of bacterial reverse mutation assay with AM-1, plate-incorporation test.

Dose (µg/plate)	Treatment Method	Mean ± SD revertants per plate					
		TA1535	TA1537	TA1537 (*Repeat*)[a]	TA98	TA100	WP2*uvr*A
Solvent Control:	Plate-incorporation (-S9)						
DMSO, 0		20 ± 2	11 ± 4	*7 ± 3*	25 ± 4	193 ± 5	54 ± 6
Test Article, AM-1:							
62/19[a]		64 ± 19	13 ± 6	*11 ± 2*	31 ± 10	175 ± 18	50 ± 11
185/38[a]		26 ± 1	15 ± 3[b]	*13 ± 5*	18 ± 5	152 ± 56	44 ± 6
556/75[a]		53 ± 22	15 ± 5[b]	*9 ± 2*	23 ± 3	94 ± 20	47 ± 9
1667/150[a]		28 ± 6	10 ± 3[b]	*5 ± 1*	14 ± 3	159 ± 18	24 ± 2
5000/300[a]		81 ± 24	14 ± 3[b]	*6 ± 3*[a]	24 ± 10	97 ± 17[c]	48 ± 5
Positive Control:							
SA, 1.0		706 ± 44				846 ± 110	
9AA, 80			2595 ± 1341	*2495 ± 603*			
2NF, 2.0					635 ± 55		
ENU, 100							707 ± 73
Solvent Controls:	Plate-incorporation (+S9)						
DMSO, 0		21 ± 1	17 ± 4	*15 ± 4*	45 ± 7	124 ± 10	62 ± 5
Test Article, AM-1:							
62/19[a]		17 ± 8	14 ± 3	*21 ± 3*	45 ± 4	142 ± 6	53 ± 14
185/38[a]		18 ± 6	15 ± 4[b]	*16 ± 6*	43 ± 9	123 ± 17	57 ± 8
556/75[a]		14 ± 3	24 ± 5[b]	*15 ± 6*	36 ± 5	100 ± 14	61 ± 5
1667/150[a]		17 ± 4	15 ± 6[b]	*16 ± 3*	28 ± 10	79 ± 6	48 ± 17
5000/300[a]		20 ± 3	19 ± 7[b]	*10 ± 1*[a]	26 ± 4	81 ± 19	55 ± 9
Positive Controls:							
2AA, 2.0		431 ± 29			1542 ± 106	2997 ± 153	
BaP, 4.0			318 ± 23	*309 ± 23*			
2AA, 80							588 ± 34

DMSO = dimethyl sulfoxide; SA = sodium azide; 9AA = 9-aminoacridine; 2NF = 2-nitrofluorene; ENU = N-ethyl-N-nitrosourea; 2AA = 2-aminoanthracene; BaP = benzo(*a*)pyrene; SD = Standard Deviation; Mean = Average number of revertants per plate (n = 3 plates per treatment).
-S9 = cells cultured without metabolic activation system; **+S9** = cells cultured **with** metabolic activation system (S9 mix).

[a] Test with strain TA1537 repeated at dose levels of 19, 38, 75, 150, and 300 µg/plate due to excessive cytotoxicity at dose levels ≥185 µg/plate in the first test. Cytotoxicity (thinning of the background lawn) was observed only at 300 µg/plate in the repeat test.

[b] Cytotoxicity (thinning of the background lawn) was observed at dose levels ≥185 µg/plate in strain TA1537 in the absence and presence of metabolic activation.

[c] Cytotoxicity (thinning of the background lawn) was observed at 5000 µg/plate in strain TA100 in the absence of metabolic activation.

Table 3
Summary of *In vitro* micronucleus assay results.

Dose Level (μg/mL)	Treatment/Recovery Time (hrs)	BN (%)	Cytotoxicity (%)[a]	MNBN/1000 BN	MNBN/2000 BN (%)
Solvent Control:	**4/20 (-S9)**				
DMSO, 0		53.7	0	8	17
		54.8		9	(0.85)
Test Article, AM-1:					
100		53.4	7	10	17
		48.4		7	(0.85)
200		41.8	25	9	16
		42.4		7	(0.80)
400		25.8	55	6	12
		26.6		6	(0.60)
Positive Controls:					
MMC, 0.4		27.6	51	91	172
		28.8		81	(8.60)***
VB, 0.025		29.4	46	71	128
		31.0		57	(6.40)***
Solvent Control:	**4/20 (+S9)**				
DMSO, 0		54.0	0	7	14
		52.7		7	(0.70)
Test Article, AM-1:					
300		53.2	2	12	22
		52.8		10	(1.10)
500		40.8	25	10	18
		38.2		8	(0.90)
700		23.4	59	11	19
		22.6		8	(0.95)
CP, 20		27.0	54	30	68
		25.2		38	(3.40)***
Solvent Control:	**20/28 (-S9)**				
DMSO, 0		54.0	0	8	16
		50.9		8	(0.80)
Test Article, AM-1:					
600		49.4	16	7	17
		53.6		10	(0.85)
800		44.8	25	12	18
		47.6		6	(0.90)
900		31.6	52	6	18
		28.8		12	(0.90)
Positive Controls:					
MMC, 0.1		40.8	34	199	366
		36.6		167	(18.30)***
VB, 0.025		36.6	31	61	126
		33.8		65	(6.30)***

DMSO = Dimethyl sulfoxide, MMC = Mitomycin C; VP = Vinblastin sulphate; CP = Cyclophosphamide; BN = binucleated cells; MNBN = micronucleated binucleated cells.
-S9 = cells cultured **without** metabolic activation system; **+S9** = cells cultured **with** metabolic activation system (S9 mix).
***Significantly greater than the vehicle control, $p \leq 0.001$ (Fisher's exact probability test, one-sided).
[a] Quantitative evaluation of cytotoxicity was performed on at least 500 cells per slide (1000 cells per dose level) using the CBPI (Cytokinesis-Block Proliferation Index) and associated Replication Index in treated versus control cultures (data not shown).

tested either in the absence or presence of S9-mix. All criteria for a valid test were met in each experiment.

In the first test, in strain TA1537, in the absence and presence of S9-mix, cytotoxicity was observed at dose levels ≥185 μg/plate. Therefore, a second test with strain TA1537 was performed via the plate-incorporation method at dose levels of 19, 38, 75, 150, and 300 μg/plate. Cytotoxicity was observed only at 300 μg/plate in the repeat test and there was no increase in the mean number of revertant colonies compared to controls (Table 1).

In the first test, in strain TA1535, in the absence of S9-mix, a more than 2-fold increase in the mean number of revertant colonies was observed at several concentrations of the test article compared to controls; however, this increase was not dose-related and occurred in conjunction with a slightly more dense background lawn of bacterial growth observed at all concentrations in strain TA1535 (Table 1). These results were attributed to a possible growth stimulation effect of the test article (Gatehouse, 1987; Verhagen et al., 1994) and were not evaluated for the final conclusion. Thus, a second test with strain TA1535 in the absence of S9 metabolic activation was performed according to the treat and plate method, which was developed in order to discriminate between a positive effect or a 'false positive' effect due to growth stimulation of the bacterial tester strains by test articles containing proteins (Ashby et al., 1987; Greenough et al., 1991). In the second test with strain TA1535 (treat and plate method), a non-dose-related increase in the mean number of revertant colonies was observed at several concentrations, concurrent with low negative control counts (Table 2). A third test with strain TA1535 was therefore performed according to the treat and plate method in the absence of S9 metabolic activation including additional negative control plates and the test article dissolved at 200 mg/ml. In the third test, AM-1 did not induce a more than 2-fold and/or dose-related increase in the mean number of revertant colonies compared to controls. As the third test was clearly negative, the findings of the first and second tests with TA1535 were not considered biologically relevant.

It was concluded that the results obtained with the test article in *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100, and

in the *Escherichia coli* strain WP2*uvr*A, in both the absence and the presence of the S9-mix, indicate that AM-1 is not a base pair substitution or frameshift mutagen under the experimental conditions employed.

3.2. MN test

In the main *in vitro* MN test, in the presence and absence of a metabolic activation system (S9-mix), at all time points and at any of the concentrations analyzed, the test article did not show a statistically significant increase in the number of binucleated cells containing micronuclei, when compared to the numbers found in the concurrent control cultures (Table 3). Results observed for the negative and positive control treated cultures demonstrated that all criteria for a valid test were met. A dose-related cytotoxicity was observed in both the pulse treatment (± S9) and continuous treatment (-S9) groups, and the highest concentrations selected for micronuclei analysis were within the testing guideline specified range of 55 ± 5% cytotoxicity (OECD, 2010).

From the results obtained in the *in vitro* MN test it was concluded that, under the experimental conditions employed, AM-1 was not clastogenic and/or aneugenic to cultured human lymphocytes.

3.3. MLA

Summary data for the mouse lymphoma assay are presented in Tables 4 and 5

In the absence of S9-mix, AM-1 did not induce a significant increase in the mutation frequency in the first experiment (3-h exposure). This result was confirmed in an independent experiment with modification in the duration of treatment (24-h exposure). In the presence of S9-mix (3-h exposure), AM-1 did not induce a significant increase in the mutation frequency. Results observed for the negative and positive control treated cultures demonstrated that all criteria for a valid test were met.

It was concluded that AM-1 is not mutagenic in the TK mutation test system, both with and without metabolic activation, under the experimental conditions employed.

Table 4
3-Hour exposure results for L5178Y/TK $^{\pm}$ mouse lymphoma cells treated with AM-1.

Dose (μg/mL)	Treatment Time (hrs)	RSG (%)[a]	CE_{day2} (%)[b]	RS_{day2} (%)[c]	RTG (%)[d]	Total Mutation Frequency per 10^6 cells[e]
Solvent Control:	**3 (-S9)**					
DMSO, 0		100	95	100	100	132
DMSO, 0			95			98
Test Article, AM-1:						
50		107	83	87	93	88
100		106	108	114	120	101
200		99	90	94	94	118
250		63	115	120	76	74
275		44	93	97	43	102
350		54	98	103	56	83
375		36	88	92	33	97
450		16	86	91	15	134
Positive Control:						
MMS, 15		90	54	57	51	802
Solvent Control:	**3 (+S9)**					
DMSO, 0		100	107	100	100	53
DMSO, 0			120			55
Test Article, AM-1:						
100		92	105	93	86	61
200		87	81	72	63	89
300		86	98	87	75	57
400		79	85	75	60	76
500		58	111	98	57	65
600		45	141	124	56	52
700		34	129	114	39	61
800		15	137	121	18	49
Positive Control:						
CP, 7.5		45	63	56	25	1194

MMS = Methyl methanesulfonate; CP = Cyclophosphamide; RSG = Relative suspension growth; CE = Cloning efficiency; RS = Relative survival; RTG = Relative total growth; **-S9** = cells cultured **without** metabolic activation system; **+S9** = cells cultured **with** metabolic activation system (S9 mix).
**Or appropriate cell concentration.*

[a] RSG = SG (test)/SG (mean of two control values) x 100; where SG (Suspension Growth) = [Day 0 cell count/(1.25 × 10^5)*] x [Day 1 cell count/(1.25 × 10^5)*] x [Day 2 cell count].

[b] CE = -ln P(0)/number of cells plated per well; where P(0) = number of empty wells/total number of wells.

[c] RS_{day2} = CE_{day2} (test)/CE_{day2} (mean of two control values) x 100.

[d] RTG = RSG x RS_{day2}/100.

[e] Mutation frequency = {-ln P(0)/number of cells plated per well}/CE_{day2} x 10^6.

Table 5
24-Hour exposure results for L5178Y/TK $^{\pm}$ mouse lymphoma cells treated with AM-1.

Dose (μg/mL)	Treatment Time (hrs)	RSG (%)[a]	CE_{day2} (%)[b]	RS_{day2} (%)[c]	RTG (%)[d]	Total Mutation Frequency per 10^6 cells[e]
Solvent Control:	**24 (-S9)**					
DMSO, 0		100	115	100	100	50
DMSO, 0			90			73
Test Article, AM-1:						
150		92	116	114	104	42
200		84	93	91	76	62
250		80	115	112	90	74
300		60	88	86	51	82
350		53	95	93	49	59
400		30	94	92	28	60
450		26	78	76	20	80
500		10	84	82	9	68
Positive Control:						
MMS, 5		81	81	80	65	629

MMS = Methyl methanesulfonate; RSG = Relative suspension growth; CE = Cloning efficiency; RS = Relative survival; RTG = Relative total growth; **-S9** = cells cultured **without** metabolic activation system.
**Or appropriate cell concentration.*
[a] RSG = SG (test)/SG (mean of two control values) x 100; where SG (Suspension Growth) = [Day 0 cell count/(1.25 × 10^5)*] x [Day 1 cell count/(1.25 × 10^5)*] x [Day 2 cell count].
[b] CE = -ln P(0)/number of cells plated per well; where P(0) = number of empty wells/total number of wells.
[c] RS_{day2} = CE_{day2} (test)/CE_{day2} (mean of two control values) x 100.
[d] RTG = RSG x RS_{day2}/100.
[e] Mutation frequency = {-ln P(0)/number of cells plated per well}/CE_{day2} x 10^6.

4. Discussion

The objective of this work was to obtain information on the genetic toxicity potential of AM-1 in three *in vitro* test systems, including the bacterial reverse mutation assay (Ames test), the micronucleus test using human lymphocytes, and the mouse lymphoma thymidine kinase assay. Negative results in all three studies demonstrate that AM-1 is without any significant genotoxic activity under the conditions of the assays performed. As previously reported by Bitzer et al. (2017a,b; 2018), AM-1 was confirmed to have low systemic toxicity potential in rats and dogs and is not a developmental or reproductive toxicant. Thus, the results reported herein add to the safety database developed for jelly mushroom glycolipids (AM-1) supporting its potential use in food.

Conflicts of interest

Authors Bitzer and Henkel are employees of IMD Natural Solutions GmbH (INS), the funding sponsor of this research. Authors Rihner and Nikiforov are consultants to INS, the research sponsor. Author Verspeek-Rip is employed by Charles River Laboratories (formerly WIL Research Europe B.V.) which received funding to conduct the MLA study. Authors van den Wijngaard and Usta are employed by Triskelion B.V. (formerly TNO Triskelion B.V.) which received funding to conduct the Ames and MN studies.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2018.10.045.

References

Ames, B.N., McCann, J., Yamasaki, E., 1975. Methods for detecting carcinogens and mutagens with the Salmonella mammalian microsome mutagenicity test. Mutat. Res. 31, 347–365.
Ashby, R., Hjortjkaer, R.K., Stavnsbjerg, M., Gurtler, H., Pedersen, P.B., Bootman, J., Hodson-Walker, G., Tesh, J.M., Willoughby, C.R., West, H., Finn, J.P., 1987. Safety evaluation of Streptomyces murinus glucose isomerase. Toxicol. Lett. 36, 23–25.
Bitzer, J., Henkel, T., Nikiforov, A., Rihner, M.O., Leuschner, J., 2017a. A 90-day oral toxicity study of glycolipids from *Dacryopinax spathularia* in CD® rats. Food Chem. Toxicol. 109, 569–584.
Bitzer, J., Henkel, T., Nikiforov, A., Rihner, M., Henderson, K.A., 2017b. A 90-day oral toxicity study of glycolipids from *Dacryopinax spathularia* in Beagle dogs. Food Chem. Toxicol. 109, 544–551.
Bitzer, J., Henkel, T., Nikiforov, A., Rihner, M.O., Thomas, J.A., 2017c. Pharmacokinetics, excretion balance, and tissue distribution of [^{14}C]-labeled glycolipids and long chain fatty acids from *Dacryopinax spathularia* in rats. Food Chem. Toxicol. 109, 552–568.
Bitzer, J., Henkel, T., Nikiforov, A., Rihner, M.O., Herberth, M., 2018. Developmental and reproductive toxicity studies of glycolipids from *Dacryopinax spathularia* in CD® rats. Food Chem. Toxicol. 120, 430–438.
FDA, 1987. Good Laboratory Practice Regulations. Final Rule. 21 Code of Federal Regulations (CFR) Part 58. Food and Drug Administration, US (FDA) Department of Health and Human Services, Washington, DC.
FDA, 2001. U.S. FDA Office of Food Additive Safety, Redbook 2000, Toxicological Principles for the Safety Assessment of Food Ingredients, October 2001 (Guideline IV.C.1.C. Mouse Lymphoma Thymidine Kinase Gene Mutation Assay).
Gatehouse, D., 1987. Guidelines for the testing of environmental agents - critical features of bacterial mutation assays. Mutagenesis 2 (5), 397–409.
Greenough, R.J., Everett, D.J., Stavnsbjerg, M., 1991. Safety evaluation of alkaline cellulase. Food Chem. Toxicol. 29, 781–785.
Maron, D.M., Ames, B.N., 1983. Revised methods for the Salmonella mutagenicity test. Mutat. Res. 113, 173–215 + ERRATUM, Mutation Res. 113: 533.
Martin, G.W., 1948. Lloydia 11 (2), 116.
OECD (Organisation for Economic Co-operation and Development), 1998. OECD Principles on Good Laboratory Practice. ENV/MC/CHEM(98)17.
OECD (Organisation for Economic Co-operation and Development), 2015. Testing Guideline No. 490 "Genetic Toxicology: in Vitro Mammalian Cell Gene Mutation Test Using the Thymidine Kinase Gene". Adopted 28 July 2015.
OECD (Organisation for Economic Co-operation and Development), 2010. Testing Guideline No. 487 "In Vitro Mammalian Cell Micronucleus Test (MNvit)". Adopted 22 July 2010.
OECD (Organisation for Economic Co-operation and Development), 1997. OECD Testing Guideline No. 471 "Genetic Toxicology: Bacterial Reverse Mutation Test". Adopted 21 July 1997.
Verhagen, H., Bruijntjes-Rozier, G.C., Coenen, T.M., Oosterom, J., 1994. Modified suspension Ames test for testing proteinaceous substances: an initial step. Food Chem. Toxicol. 32 (12), 1161–1166.