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Evaluation of the genotoxicity of the food additive, gum ghatti

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ABSTRACT

Gum ghatti is a food additive in some parts of the world, serving as an emulsifier, a stabilizer, and a thickening agent. To evaluate its genotoxic potential, we conducted Good Laboratory Practice compliant *in vitro* and *in vivo* studies in accordance with the Organisation for Economic Co-operation and Development (OECD) guidelines. No evidence of toxicity or mutagenicity was detected in a bacterial reverse mutation assay using five tester strains evaluating gum ghatti at up to 6 mg/plate, with or without metabolic activation. Gum ghatti also did not induce chromosome structural damage in a chromosome aberration assay using Chinese hamster ovary cells. To assess the ability to induce DNA damage in rodents, a combined micronucleus/Comet assay was conducted in male B6C3F1 mice. Gum ghatti was administered at 1000, 1500, and 2000 mg/kg/day by gavage once daily for 4 days and samples were collected 4 h after the final dosing. No effect of gum ghatti was measured on micronucleated reticulocyte (MN-RET) frequency in peripheral blood, or DNA damage in blood leukocytes or liver as assessed by the Comet assay. Our results show no evidence of genotoxic potential of gum ghatti administered up to the maximum concentrations recommended by OECD guidelines.

1. Introduction

Gum products are used in the food, pharmaceutical and cosmetic industries, typically as stabilizers, gelling and thickening agents, emulsifiers, and color emulsions for pastes, creams, syrups, flavorings, and other food and beverage products. Additional uses as food additives include inhibition of ice and sugar crystal formation and the controlled release of flavors. The rapid increase in consumption of ready-made meals and the growing awareness of the benefit of dietary fiber has led to a steady increase in the use of gums in food products (Williams et al., 2003). Gum ghatti, an amorphous translucent exudate from wounds in the bark of the *Anogeissus latifolia* Wallich tree, found in the dry deciduous forests of India and Sri Lanka, is a high molecular weight complex polysaccharide that occurs in nature as a mixed calcium, magnesium, potassium, and sodium salt; upon hydrolysis it yields L-arabinose, D-galactose, D-mannose, D-xylose, L-rhamnose, and D-glucuronic acid (Aspinall et al., 1955, 1965; Tischer et al., 2002). It was developed originally as a substitute for gum arabic, but never became established as a major tree gum due to its batch to batch variability in solubility and viscosity (Al-Assaf et al., 2008; Jefferies et al., 1977). A new gum ghatti product, "GATIFOLIA", has been produced by a non-chemical physical process in which the gum is dissolved, filtered, sterilized, and spray-dried; this procedure results in an improved gum of consistent quality. Gum ghatti has some physical properties superior to other gum products including greater acid resistance, salt tolerance, and oil binding capacity, resulting in excellent emulsification properties, even at considerably lower concentrations than gum arabic (Al-Assaf et al., 2000, 2008). It is also more soluble in water than many other polysaccharides and can be used in some formulations for which gum arabic is not suitable (Al-Assaf et al., 2000).

Gum ghatti is marketed in Japan as an existing food additive (JMHLW, 2009), and was assigned "generally recognized as safe" (GRAS) status in the United States in 1965 by the Flavor and Extract Manufacturers Association (FEMA No. 2519; (Hall and Oser, 1965)), and by the US FDA in 1977 (21CFR184.1333; (FDA, 2010)). Although approved as a food additive in these and some other non-European countries, manufacturers are looking to expand the use of gum ghatti in products in both existing and new

Abbreviations: CHO, Chinese hamster ovary; FDA, US Food and Drug Administration; GLP, Good Laboratory Practice; ICH, International Conference on Harmonization; JaCVAM, Japanese Center for the Validation of Alternative Methods; JECFA, Joint FAO/WHO Expert Committee on Food Additives; JMHLW, Japan Ministry of Health, Labour, and Welfare; LMW, low molecular weight; MN, micronucleus or micronuclei; MN-RET, micronucleated reticulocytes; OECD, Organization for Economic Cooperation and Development; RET, reticulocyte(s); SD, standard deviation; SEM, standard error of the mean.

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markets. However, no acceptable dietary intake limits have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for gum ghatti due to the very limited toxicity and genotoxicity information available (http://whqlibdoc.who.int/trs/WHO_TRS_733.pdf), especially at concentrations exceeding currently accepted levels.

An internationally adopted battery of tests has been established to evaluate the potential genetic risks to human somatic and germ cells associated with exposure to a chemical (ICH, 1996, 1997); the recommended tests provide for both *in vitro* and *in vivo* detection of diverse genotoxic effects ranging from point mutations to chromosomal aberrations that arise through a variety of mechanisms (Eastmond et al., 2009). According to current and new draft ICH and FDA guidances for industry on genotoxicity testing (FDA, 1997; ICH, 1997, 2008), a standard test battery should include a mutagenicity test using multiple strains of bacteria, an *in vitro* mutation assay or test for chromosome structural damage in mammalian cells, and a rodent erythrocyte MN assay (Heddle, 1973; MacGregor et al., 1980; Schmid, 1975) for initial assessment of *in vivo* genotoxic potential. Current opinion supports use of the alkaline (pH >13) Comet assay (Tice et al., 2000) as a follow-up or supplemental test to the *in vivo* MN assay as a secondary measure of genotoxicity in a tissue other than bone marrow, with special emphasis placed on liver (Brendler-Schwaab et al., 2005; ICH, 2008). The Comet assay can detect a broad spectrum of DNA damage including DNA strand breaks, apurinic sites, alkali-labile covalently bound DNA adducts, and a spectrum of DNA lesions induced by reactive oxygen/lipid peroxidation species (Fortini et al., 1996; Gedik and Collins, 2005; Glei et al., 2007). Its increasing prominence in genotoxicity profiling (Burlinson et al., 2007) is underscored by the extensive international effort currently underway, led by JaCVAM, to validate the Comet assay in rodents and develop an OECD test guideline. To reduce costs and minimize the use of experimental animals, a combined MN/Comet assay has been recommended as a comprehensive approach to assessing *in vivo* genotoxicity (Pfuhler et al., 2007; Rothfuss et al., 2011); currently, a combined 4-day testing protocol is being utilized for evaluation of chemicals of potential concern to US public health (Recio et al., 2010; Witt et al., 2008).

To obtain further safety assessment data, gum ghatti was evaluated in a battery of GLP tests compliant with ICH, OECD, and FDA guidances on genotoxicity testing (FDA, 2000a–c; ICH, 2008; OECD, 1997a–c). Mutagenicity was evaluated in a bacterial reverse mutation assay (Ames et al., 1975; Gatehouse et al., 1994; Maron and Ames, 1983) using one *Escherichia coli* and four *Salmonella* tester strains, in the presence and absence of metabolic activation (OECD 471). In a chromosome aberration test (Galloway et al., 1987), gum ghatti was assayed in the CHO–WBL cell line with and without metabolic activation (OECD 473). Gum ghatti was additionally tested for *in vivo* genotoxicity in male B6C3F1 mice using a 4-day protocol to simultaneously measure MN-RET frequencies by a highly reproducible flow cytometry-based method, and primary DNA damage in blood leukocytes and liver by the Comet assay (Recio et al., 2010; Witt et al., 2008). This combined MN/Comet assay protocol complies with regulatory requirements stipulated for the MN assay (OECD 474) while adhering to the most recent guidelines for the conduct of the Comet assay recommended by international working groups (Burlinson et al., 2007; Hartmann et al., 2003; JaCVAM, 2009).

2. Material and methods

2.1. Chemical analysis

All genotoxicity assays were conducted according to GLPs. Chemical formulations were analyzed at OpAns, LLC (Durham, NC). Samples removed from the top, middle, and bottom portions of each formulation were submitted for analytical testing. Gum ghatti (GATIFOLIA; CAS No. 9000-28-6; San-Ei Gen F.F.I., Inc., Osaka, Japan) prepared in deionized water and stored at room temperature was determined to be stable for at least 4 days. Acceptance criteria for concentration and homogeneity were ±15% of the theoretical concentration and a <5% percent coefficient of variation of the samples (top, middle, and bottom), respectively. Results of chemical analyses verified that the formulation solutions for all genotoxicity assays conducted were uniform and within these established accuracy limits for all concentration levels.

2.2. Bacterial reverse mutation assay

A mutagenicity assay of gum ghatti, with and without metabolic activation, was conducted as described previously (Ames et al., 1975; Maron and Ames, 1983; Mortelmans and Zeiger, 2000) using the following five *Salmonella* and *E. coli* strains as prescribed in the guideline for the bacterial reverse mutation assay (OECD 471): TA98, TA100, TA97a, TA1535, and *E. coli* WP2 *uvr*A pKM101. All strains (Moltox, Inc., Boone, NC) were maintained as frozen stocks and checked for maintenance of genetic markers prior to the study. The results of a 5-strain range-finding assay were negative; therefore, a top concentration of 6 mg/plate, with and without metabolic activation, was chosen to ensure testing of at least 5 mg/plate as recommended by OECD (OECD, 1997b) and Japanese guidelines (JMHLW, 1996) for non-cytotoxic compounds. Concentrations tested were 0, 125, 250, 500, 1500, 3000, and 6000 μg per plate. Strain specific positive controls tested without metabolic activation were 2-nitrofluorene (TA98), sodium azide (TA100 and TA1535), ICR191 (TA97a), and methyl methanesulfonate (*E. coli* WP2). 2-aminoanthracene was used as the positive control for all strains tested with metabolic activation. Bacterial cultures contained 3–4.5 $\times$ 10^9 bacteria/mL based on optical density determination. Metabolic activation was provided by a 10% phenobarbital/benzoflavone-induced rat liver S9 mix with added Regensys cofactors (Moltox, Boone, NC). Test solutions were prepared in sterile distilled water as serial dilutions to deliver the required concentration in a constant volume. The assay tubes were pre-incubated at 37 °C for 20 min before plating onto minimal agar. Three test plates per concentration were incubated at 37 °C for 48 h and then counted using the Sorcerer/Ames Study Manager System (Perceptive Instruments, Ltd., Suffolk, UK). To ensure accuracy of the results, reagent sterility and automated scoring checks were conducted. Criteria for a positive response were a $\geqslant$2-fold increase in the average plate count compared to the solvent control for at least one concentration level and a dose response over the range of tested concentrations in at least one strain with or without metabolic activation.

2.3. Chromosome aberration assay

Tissue culture reagents were purchased from Invitrogen (Carlsbad, CA). The CHO–WBL cell line was obtained from Dr. Sheila Galloway at Merck Research Laboratories (West Point, PA). Mycoplasma-free cultures were initiated from a frozen stock and expanded in McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM Glutamax, and penicillin–streptomycin [100 units/mL and 100 mg/mL, respectively], and incubated at 37 °C in a humidified atmosphere of 5% CO_2. Results of a toxicity test indicated no reduction in viable cell counts at concentrations of gum ghatti up to 6000 μg/mL; to ensure reaching a top concentration of 5000 μg/mL to meet OECD guidelines for non-cytotoxic chemicals (OECD, 1997a), 6000 μg/mL was selected as the target top concentration for the chromosome aberration assay. Duplicate cultures of exponentially growing cells seeded approximately 19 h earlier at 0.25 $\times$ 10^6 cells/mL in T25 flasks were exposed to various concentrations of gum ghatti, vehicle, or positive control chemical; exposure durations were 4 and 19 h in the absence of metabolic activation, and 4 h in the presence of metabolic activation provided by a 10% phenobarbital/benzoflavone-induced rat liver S9 mix with added Regensys cofactors (Moltox, Boone, NC). Test chemical solutions were formulated in distilled water and administered at 10% of the final culture volume. Gum ghatti was tested at 6000, 3000, 1000, 500, and 250 μg/mL for 4 h ±S9, and 6000, 3000, and 1000 μg/mL for 19 h in continuous exposure without S9. Mitomycin C and cyclophosphamide were used as the positive controls for cultures incubated in the absence and presence of S9, respectively. After 4 h, treatment medium was replaced with complete medium for the 4 h ±S9 treatment cultures which were then incubated for an additional 15 h; test chemicals remained on the continuous treatment cultures for the entire 19 h period. Colcemid™ (Sigma, St. Louis, MO) was added at 0.1 μg/mL for the final 2 h of incubation to arrest cells in metaphase. At the end of the culture period, spent medium from each flask was collected; cells were washed twice with PBS, harvested with 0.25% trypsin/EDTA, and collected back into the saved spent media. Aliquots were removed for determination of cell count and viability assessment via the trypan blue exclusion test (Strober, 2001). Remaining cells were pelleted by centrifugation, treated with warm hypotonic solution, fixed with methanol:acetic acid (3:1), and stored at least overnight. To prepare slides, cells were pelleted by centrifugation, resuspended in methanol:glacial acetic acid, dropped onto a microscope slide, air-dried, and stained with giemsa. For each test condition, the frequency of chromosome aberrations and mitotic index (percentage of metaphase cells) were determined at the top three concentration levels. The mitotic index was determined from 1000 cells per replicate. Structural and numerical chromosome damage (*e.g.*, chromatid and chromosome gaps and breaks, double minute, dicentric and ring chromosomes, and other

complex rearrangements) was determined from scoring 100 metaphase spreads per replicate on coded slides at 1000× magnification; replicates were analyzed independently by different scorers. The number of chromosome aberrations (with and without gaps) per cell, and the number of damaged cells (no gaps), were tabulated. If 10 metaphase cells with structural chromosomal damage (excluding gaps) were detected out of the first 25 cells scored, scoring for that culture was terminated. To assess numerical abnormalities, the number of polyploidy mitoses, including those with endoreduplicated chromosomes, per 100 metaphase spreads/replicate (polyploidy index, PI) was determined.

2.4. Animal husbandry

Male B6C3F1 mice (Taconic Farms, Inc., Germantown, NY), were 8–10 wk of age at the time of treatment. Animals were housed singly in polycarbonate cages with absorbent hardwood bedding in an AAALAC-accredited specific pathogen free facility with a 12 h light/12 h dark cycle. Temperature and humidity were monitored continuously. AIN-93M diet (Research Diets, Inc., New Brunswick, NJ) and water were provided *ad libitum*. These studies were approved by the ILS, Inc. Institutional Animal Care and Use Committee, and all procedures were completed in compliance with the Animal Welfare Act Regulations, 9 CFR 1-4, and the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996).

2.5. In vivo MN/Comet assay experimental design

A dose range finding study was conducted to identify the most sensitive gender and appropriate dose levels. Two male and two female B6C3F1 mice at 8–10 wk of age were administered gum ghatti at dose levels of 0, 500, 1000, 1500, and 2000 mg/kg, once daily for four consecutive days by oral gavage. The highest dose (2000 mg/kg) conforms to OECD guidelines for limit dose (OECD, 1997c). No treatment related clinical or cage-side observations were noted during the dose setting study. At the 2000 mg/kg dose, there was 34% and 14% suppression in reticulocyte (RET) levels in male and female mice, respectively, as measured by flow cytometry. Therefore, for the definitive study, male B6C3F1 mice (five animals/dose group) were administered gum ghatti at 2000, 1500, or 1000 mg/kg/day, vehicle (deionized water), or the positive control compound, ethyl methanesulfonate (EMS; Sigma–Aldrich, St. Louis, MO) in 0.9% saline (Ricca Chemical Company, Arlington, TX) at 150 mg/kg/day, daily for 4 days by oral gavage. Four hr after the final dose, peripheral blood was collected for flow cytometric analysis of MN, and blood and liver tissue were collected, frozen in liquid N_2, and stored at −80 °C until analysis by the Comet assay. There were no adverse clinical observations for any of the dose groups throughout the course of the study.

2.6. Erythrocyte micronucleus assay

Peripheral blood samples were processed for flow cytometric evaluation of micronucleated reticulocytes (MN-RET) as described previously (Witt et al., 2008). Briefly, cells were fixed and labeled using a MicroFlow^{PLUS} Kit (Litron Laboratories, Rochester, NY) according to manufacturer's directions and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Sunnyvale, CA). Aggregates were excluded based on forward and side scatter, and platelets were gated out based on staining with an anti-CD61-phycoerythrin antibody. Nucleated leukocytes in the peripheral blood were gated out based on intense propidium iodide staining. Immature reticulocytes (RET) were identified by staining with an anti-CD71-FITC antibody. For each peripheral blood sample, 20,000 RET were analyzed to determine the frequency of MN-RET (Kissling et al., 2007), defined as propidium iodide-positive RET. More than 10^6 mature normochromatic erythrocytes (NCE) were enumerated concurrently during MN-RET analysis, and the percentage of RET (%RET) among total erythrocytes was calculated as a measure of bone marrow toxicity.

2.7. Comet and neutral diffusion assays

Blood samples, 50 μL per animal collected during exsanguination, were placed into tubes containing 1 mL of mincing solution (Mg^{+2} and Ca^{+2} free Hank's Balanced Salt Solution (Invitrogen, Carlsbad, CA) containing 10% v/v DMSO, and 20 mM EDTA pH 7.4–7.7) and held on ice. For each animal, a portion of the left lobe of the liver was placed into a tube containing 1 mL of mincing solution and rapidly minced. Blood and liver samples were flash frozen in liquid nitrogen and stored at −80 °C until processed. For processing, cells were partially thawed in a warm water bath and placed on ice until slide preparation. Cell samples were empirically diluted with 0.5% low melting point agarose (Lonza, Walkersville, MD) at 37 °C, layered onto each well of a 2-well CometSlide™ (Trevigen, Gaithersburg, MD), and placed in cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, containing freshly added 10% DMSO and 1% Triton X-100) overnight. After rinsing in 0.4 M Trizma base (pH 7.5), slides were treated with alkali (300 mM NaOH, 1 mM Na_2EDTA, pH >13) for 20 min, then electrophoresed at 4 °C for 20 min at ~1.0 V/cm, 300 mA. After electrophoresis, slides were neutralized with 0.4 M Trizma base (pH 7.5) for 5 min, incubated for 5 min in ice-cold 100% ethanol (McCormick Distilling Co., Inc., Weston, MO) and allowed to air-dry. Slides were stored at room temperature in a desiccator until stained and scored. After staining slides with SYBR Gold™ (Molecular Probes, Invitrogen, Carlsbad, CA), 100 cells were scored per sample at 20× magnification without knowledge of their identity using Comet Assay IV Imaging Software, Version 4.11 (Perceptive Instruments, Ltd., Suffolk, UK). The extent of DNA migration was characterized using the % tail DNA endpoint measurement (intensity of all tail pixels divided by the total intensity of all pixels in the comet, expressed as a percentage). To assess for the presence of cells with low molecular weight (LMW) DNA, potentially indicative of apoptosis or necrosis (Burlinson et al., 2007; Tice et al., 2000), one slide for each sample was removed from lysis buffer after 1–2 h, neutralized with 0.4 M Trizma base (pH 7.5), and fixed. After staining slides with SYBR Gold™, 100 cells per slide were scored microscopically as having either condensed DNA (type I) or diffused LMW DNA (type II). NaCl, Na_2EDTA, Triton X-100, and Trizma base were purchased from Sigma–Aldrich (St. Louis, MO); NaOH and DMSO were purchased from Fisher Scientific (Pittsburgh, PA).

2.8. Statistical analyses

Results of the chromosomal aberration assay were evaluated for statistical significance using Analyse-it® software for Microsoft Excel and Statistical Analysis System version 9.1 (SAS Institute, Cary, NC; Cochran Armitage Test). Viable cell counts and mitotic indexes were analyzed for a decrease over negative controls using a one-tailed pair wise independent samples *t*-test, and for a dose response using linear regression analysis. Percent structurally damaged cells (without gaps) and polyploidy indexes were analyzed for an increase over negative controls using a one-tailed pair wise Fisher's Exact test and for linear trend using a one-tailed Cochran–Armitage test. A test was considered positive for inducing chromosomal aberrations if both a statistically significant concentration dependent increase and a statistically significant increase for at least one concentration of chemical were detected, or if a statistically significant increase for two or more concentrations was measured in the absence of a significant trend test. The *in vivo* MN/Comet assay data were analyzed using Statistical Analysis System version 9.2 (SAS Institute, Cary, NC). Homogeneity of the data was assessed using the Levene's test with a 95% confidence level. For final body weight gain, MN-RET frequency, and the % of cells with LMW DNA, variance and linearity among treatment groups were evaluated using one way ANOVA and linear trend analyses. The Dunnett's multiple comparison test was used to determine if a treatment group is significantly different from vehicle controls. Consistent with the criteria outlined in the current version of the Comet assay protocol undergoing international validation (JaCVAM, 2009), significance of changes in the extent of DNA migration (% tail DNA endpoint) were analyzed using one way ANOVA and a two-tailed Dunnett's test; dose dependent changes were evaluated using a linear trend analysis. For neutral diffusion assay data that were not homogeneous, the Kruskal–Wallis and Dunn's tests were used to compare each dose level to the concurrent control, and a one-sided Jonckheere–Terpstra test was used to determine the presence of a dose response. Criteria for a positive result in the MN and Comet assays were at least one statistically significant dose group ($p < 0.05$) and a statistically significant trend test ($p < 0.05$). For all *in vivo* endpoints, a one-tailed *t*-test was used to verify a positive response to the reference compound, EMS ($p < 0.025$ for % tail DNA (JaCVAM, 2009), $p < 0.05$, otherwise).

3. Results

3.1. Results of the bacterial reverse mutation assay

A mutagenicity assay was conducted to assess the potential of gum ghatti to induce gene mutations in bacteria. Exposure to gum ghatti in both the absence and presence of metabolic activation was assessed up to 6000 μg, a concentration exceeding the recommended maximum for non-cytotoxic chemicals. No evidence of general toxicity or precipitation was noted in any of the test plates. Average plate counts for each set of replicate plates are provided in Table 1. A positive mutagenic response to gum ghatti was not produced in any of the five *Salmonella* or *E. coli* strains tested either with or without metabolic activation. Average revertant values for positive control chemicals, both with and without metabolic activation, were at least 2-fold above concurrent solvent controls. The lack of induction of an increase in revertants or any apparent concentration-dependent response, indicates that, under the assay conditions tested, gum ghatti is not mutagenic in the bacterial reverse mutation assay.

3.2. Results of the in vitro chromosome aberration assay

A study was conducted to assess the potential for gum ghatti to cause chromosome aberrations in Chinese hamster ovary cells.

Table 1
Bacterial reverse mutation assay results.

Dose (μg/plate)	Mean revertants/plate (±SD)	
	Without metabolic activation	With metabolic activation
TA97a		
0	90.7 ± 9.2	98.3 ± 6.8
125	97.7 ± 15.4	101.0 ± 3.6
250	82.0 ± 7.9	86.0 ± 2.6
500	98.7 ± 15.8	93.3 ± 17.0
1500	70.7 ± 9.5	92.0 ± 15.4
3000	86.0 ± 14.8	90.0 ± 16.8
6000	91.7 ± 6.0	88.7 ± 11.8
TA100		
0	114.3 ± 14.6	92.3 ± 12.5
125	77.0 ± 9.6	90.7 ± 6.7
250	101.7 ± 12.2	89.0 ± 3.6
500	109.7 ± 4.5	97.7 ± 8.5
1500	105.3 ± 5.5	98.0 ± 8.7
3000	116.7 ± 23.1	82.3 ± 9.7
6000	99.7 ± 8.5	83.0 ± 25.2
TA98		
0	17.3 ± 4.9	21.7 ± 5.1
125	17.7 ± 6.5	18.3 ± 7.5
250	12.0 ± 6.6	17.0 ± 3.6
500	11.0 ± 2.0	21.0 ± 5.3
1500	16.3 ± 3.2	25.0 ± 5.3
3000	15.7 ± 2.5	22.3 ± 3.8
6000	14.0 ± 1.0	22.0 ± 2.6
TA1535		
0	6.7 ± 1.2	11.0 ± 3.5
125	8.3 ± 2.5	10.3 ± 3.2
250	7.0 ± 1.7	14.0 ± 3.6
500	10.0 ± 1.7	11.7 ± 2.3
1500	12.7 ± 3.2	13.0 ± 3.6
3000	7.0 ± 1.7	11.3 ± 1.5
6000	12.3 ± 2.1	11.0 ± 0
E. coli WP2 uvrA pkM101		
0	114.7 ± 8.5	160.3 ± 4.0
125	113.7 ± 12.7	135.7 ± 11.2
250	131.3 ± 22.5	144.7 ± 5.1
500	124.3 ± 8.1	146.7 ± 5.8
1500	127.0 ± 3.6	137.3 ± 7.6
3000	106.0 ± 4.4	135.0 ± 22.6
6000	126.0 ± 5.3	148.0 ± 13.7
Positive controls		
TA97a	2101.0 ± 143.7	1992.7 ± 262.9
TA100	759.7 ± 47.1	1757.7 ± 181.8
TA98	361.0 ± 28.5	551.0 ± 77.3
TA1535	502.0 ± 26.6	309.7 ± 19.0
E. coli WP2 *uvr*A pkM101	944.3 ± 25.4	1070.7 ± 137.2

Duplicate cultures of CHO–WBL cells were exposed to gum ghatti at 250, 500, 1000, 3000, and 6000 μg/mL for 4 h in the presence and absence of metabolic activation followed by a 15 h recovery period, or continuously exposed to 1000, 3000, and 6000 μg/mL gum ghatti for 19 h without metabolic activation, and arrested in metaphase. No precipitate or pH changes were noted in any of the cultures during the treatment period. Viability and various measures of structural and numerical chromosome abnormalities were determined for each duplicate culture at the top three concentration levels. The results of the chromosome aberration assay for CHO–WBL cultures exposed to gum ghatti are summarized in Table 2. No decrease in viable cell counts or in mitotic index, or increase in chromosome aberrations as represented by percent damaged cells, was observed at any of the analyzed concentrations of gum ghatti. In contrast, the positive control chemicals, mitomycin C and cyclophosphamide, induced significant increases in the percentage of metaphase cells with chromosome aberrations when compared to concurrent negative controls for all exposure conditions. With one exception, no increase in the number of polyploidy mitoses was observed at any tested concentration of gum ghatti, either in the absence of presence of activated metabolism. A significant increase in polyploidy index was only measured in cells undergoing S9-induced metabolism and treated with the lowest concentration of gum ghatti evaluated (1006 μg/mL) for 4 h; this increase was marginal ($p = 0.0484$), was not accompanied by a statistically significant concentration-dependent trend, and was not considered to be biologically relevant. Likewise, the marginally significant positive trend ($p = 0.0477$) in the polyploidy index for the continuous exposure cultures, without an associated significant increase in a single treatment group, is not considered to be indicative of a true positive response. These data indicate that, under the conditions tested in this study, gum ghatti was not toxic and did not cause structural chromosomal damage in CHO–WBL cells.

3.3. Results of the in vivo MN/Comet assay

In a preliminary dose setting study, male and female B6C3F1 mice were administered gum ghatti by oral gavage for four consecutive days. Although no treatment related clinical observations were noted during the study, analysis of peripheral blood samples by flow cytometry determined a 34% and 14% suppression in RET levels in male and female mice, respectively, at the highest tested dose (2000 mg/kg/day; data not shown). Based on these results, a combined MN/Comet assay was conducted in which male B6C3F1 mice were administered gum ghatti at 1000, 1500, and 2000 mg/kg/day for four consecutive days. No treatment-related clinical observations were noted during the course of the study. Initial and final body weight data for animals dosed with gum ghatti for 4 days are summarized in Table 3. There were no statistical differences in final body weight or body weight gain between the animals administered gum ghatti and the concurrent control animals. Additionally, no dose-dependent change was observed. The positive reference chemical, EMS, did induce a statistically significant loss in mean body weight as compared to the vehicle control group.

Results of flow cytometric analysis of MN-RET and RET frequencies are summarized in Table 4. Under the conditions used in the MN/Comet study, no increase in the frequency of MN-RET was observed for male mice administered gum ghatti. No decrease in the % RET was measured in treated mice, indicating a lack of cytotoxicity at the tested doses. In contrast, there was a statistically significant ($p = 0.0045$) 5.9-fold increase in MN-RET with a corresponding 48.4% suppression of RET in animals treated with EMS.

The results of the assessment of DNA damage and cytotoxicity in the blood and liver of mice administered gum ghatti as measured by the Comet assay and neutral diffusion assay, respectively, are provided in Tables 5 and 6. With one exception, the liver samples for all of the study animals had ⩽2% type II cells containing low molecular weight DNA. However, one animal in the vehicle control group had an exceptionally high percentage (20%) of type II cells and the diameter of the type I cells was observed to be generally larger than that of the type I cells in the other samples, suggesting that something was abnormal about either the animal or the sample preparation. Consistent with this result, no Comet assay data could be collected for the liver from that animal as essentially all of the cells on the slide were unscoreable "hedgehogs", which are thought to reflect the highly fragmented DNA characteristic of cytotoxicity and/or necrosis/apoptosis. An animal in the lowest dose group (1000 mg/kg/day) was also noted as having larger than normal type I cells, although in this case, only 1% of the cells were categorized as type II. Nevertheless, very few scoreable cells were identified on the liver Comet slide for this animal, and therefore, the Comet data for this sample were excluded from the analysis. Although it is not uncommon to encounter a Comet

Table 2
Summary of chromosome aberration assay results.

Dose (μg/mL)	Viable cells/mL ($\times 10^5$)		Mitotic Index (%)		% Damaged cells		Polyploidy index (%)	
	Mean	*p*-Value[a]	Mean	*p*-Value[a]	Mean	*p*-Value[b]	Mean	*p*-Value[b]
4 h Exposure in the absence of metabolic activation								
0	6.18	–	4.40	–	3.0	–	4.0	–
1000	6.94	0.9937	3.65	0.1226	1.0	0.9662	3.5	0.6999
3000	7.68	0.9948	4.50	0.6091	0.5	0.9926	1.5	0.9691
6000	7.51	0.9980	4.45	0.5382	2.5	0.7285	3.5	0.6999
Trend test	$P = 0.4896$[c]		$P = 0.2618$[c]		$P = 0.5317$[d]		$P = 0.3677$[d]	
MMC (0.15)	6.2	0.6092	5.05	0.9655	20.8	<0.0001[e]	3.0	0.7922
4 h Exposure in the presence of metabolic activation								
0	7.71	–	6.05	–	2.0	–	4.0	–
1000	6.28	0.1112	4.85	0.1495	2.0	0.6381	8.5	0.0484[e]
3000	6.94	0.1900	5.10	0.2615	5.5	0.0558	7.0	0.1363
6000	6.37	0.0932	4.95	0.3283	3.0	0.3754	7.0	0.1363
Trend test	$P = 0.1304$[c]		$P = 0.3002$[c]		$P = 0.1880$[d]		$P = 0.2661$[d]	
CP (10)	5.25	0.0456[e]	1.25	0.0162[e]	34.0	<0.0001[e]	1.5	0.9691
Continuous exposure in the absence of metabolic activation								
0	10.30	–	5.10	–	2.0	–	2.5	–
1000	9.12	0.1473	4.05	0.2337	3.0	0.3754	3.0	0.5000
3000	8.56	0.0803	3.80	0.2119	4.0	0.1902	5.0	0.1462
6000	9.21	0.1208	4.40	0.3568	2.5	0.5000	5.5	0.1004
Trend test	$P = 0.1625$[c]		$P = 0.3722$[c]		$P = 0.4211$[d]		$P = 0.0477$[d,e]	
MMC (0.075)	8.82	0.1797	5.0	0.4668	22.0	<0.0001[e]	4.5	0.2078

Mitotic index (%) = percent of cells in metaphase.
% Damaged cells = percent of metaphase cells with at least one structural chromosomal aberration, excluding gaps.
Polyploid index = percent of polyploid metaphase cells, including endoreduplicated metaphase cells.
MMC = mitomycin C.
CP = cyclophosphamide.
[a] One-tailed independent samples *t*-test.
[b] Fisher's exact test.
[c] Linear regression.
[d] One-tailed Cochran Armitage trend test.
[e] Significant at $p < 0.05$ by a one-tailed independent samples *t*-test.

Table 3
Summary of body weight changes during the *in vivo* MN/Comet assay.

Dose (mg/kg/day)	Number of animals	Initial body weight (g) ± SD		Final body weight (g) ± SD		Body weight change[a] (g) ± SD	
		Mean	SD	Mean	SD	Mean	SD
0	5	27.2	1.3	27.2	1.8	0.1	0.6
1000	5	27.4	1.5	27.8	1.8	0.4	1.0
1500	5	26.8	1.5	27.2	1.2	0.4	1.2
2000	5	27.2	1.7	27.1	1.8	0.0	0.4
	Linear trend test	–		$p = 0.9402$		$p = 0.5840$	
EMS (150)	5	27.0	1.4	25.5	1.3	−1.5[b]	0.6

[a] Calculated on the basis of individual animal data.
[b] Significant at $p < 0.05$ by a *t*-test.

sample with many or all unscoreable cells, the reason for the poor quality of the liver samples from these two vehicle and low dose group animals is not clear as the animals did not exhibit any abnormal clinical signs and no technical issues during sample handling were identified. No unusual observations in either the Comet or neutral diffusion assay were noted for blood samples for any of the animals on study.

Under the conditions of the Comet assay, no increase in DNA damage, as assessed by the % tail DNA endpoint, was measured in any of the animals administered gum ghatti. In contrast, a significant increase ($p < 0.0001$) in DNA damage was measured in mice administered EMS. There was no evidence of cytotoxicity in the blood and liver of the animals administered gum ghatti or EMS, as assessed by the neutral diffusion assay. In this study, the time interval from obtaining tissue until the lysing step of the Comet assay was controlled by flash freezing the tissue samples in a buffered solution containing DMSO, thereby minimizing a potential source of sample variation that could become significant over the course of a necropsy. Mean values for % tail DNA and % LMW DNA cells in treated animals fell within laboratory historical control data for blood and liver samples frozen in this manner.

4. Discussion

Assessment of genotoxic potential is required for the registration of certain food additives intended for human use. To support regulatory acceptance of higher concentration levels of gum ghatti in food products in existing and new markets, the ability of gum ghatti to induce mutations, chromosomal abnormalities, and DNA damage was evaluated using a battery of standard GLP-compliant *in vitro* and *in vivo* genetic toxicology assays.

In a bacterial reverse mutation assay, gum ghatti did not produce a positive response in any of the five test strains, nor was there any evidence of toxicity in any strain at any dose, either with

Table 4
Summary of *in vivo* MN assay results.

Dose (mg/kg/day)	Number of animals	% RET			MN-RET/1000		
		Mean	SEM	*p*-Value[a]	Mean	SEM	*p*-Value[a]
0	5	1.57	0.20	–	2.57	0.30	
1000	5	1.59	0.13	0.7160	1.85	0.18	0.9983
1500	5	1.58	0.18	0.7229	2.05	0.21	0.9912
2000	5	1.66	0.09	0.5748	2.48	0.21	0.8376
	Linear trend test		$p = 0.9423$			$p = 0.1305$	
EMS (150)	5	0.81	0.05	0.0169[b]	15.13	2.23	0.0045[b]

[a] ANOVA/Dunnett's test.
[b] Significant at $p < 0.05$ by a *t*-test.

Table 5
Summary of Comet assay results.

Dose (mg/kg/day)	Number of animals	% Tail DNA		
		Mean	SEM	*p*-Value[a]
Blood				
0	5	2.8	0.09	–
1000	5	2.8	0.11	0.9988
1500	5	3.1	0.17	0.7522
2000	5	3.4	0.40	0.1889
	Linear trend test		$p = 0.4239$	
EMS (150)	5	23.4	0.80	<0.0001[b]
Liver				
0	4	13.7	1.17	–
1000	4	12.4	1.80	0.9296
1500	5	12.7	1.27	0.9530
2000	5	15.2	2.43	0.8921
	Linear trend test		$p = 0.6888$	
EMS (150)	5	30.2	0.35	<0.0001[b]

[a] ANOVA/two-tailed Dunnett's test.
[b] Significant at $p < 0.025$ by a *t*-test.

or without metabolic activation. Our study corroborated the findings of a previous report in which gum ghatti was tested at concentrations $\leqslant$0.1 mg/plate (Prival et al., 1991) and extended the analysis up to 6 mg/plate. The negative results indicate that, under the conditions tested, gum ghatti is not mutagenic in the Ames test. In an *in vitro* chromosome aberration assay using CHO–WBL cells, no decrease in viable cell counts or in mitotic index, or an increase in chromosome aberrations, was observed at any of the analyzed doses, indicating that gum ghatti is not toxic nor causes structural chromosomal damage in CHO cells. These cytogenetic results are consistent with those of studies conducted by the FDA in which no treatment-related abnormalities were observed in anaphase chromosomes in cultured WI-38 human embryonic lung cells exposed to up to 16 μg/mL gum ghatti, nor in metaphase chromosomes in the bone marrow of rats dosed with gum ghatti up to 5000 mg/kg (Newell and Maxwell, 1972). In a combined MN/Comet assay in which male B6C3F1 mice were administered gum ghatti for four consecutive days, there was no effect on animal body weight, no increase in MN-RET in peripheral blood as evaluated by a sensitive flow cytometry-based assay, and no evidence of DNA damage in blood or liver as assessed by the Comet assay. No indication of cytotoxicity was observed by any of the measured endpoints.

In summary, gum ghatti, evaluated up to concentrations exceeding the maximum levels recommended by OECD guidelines, tested uniformly negative under the conditions tested in the entire battery of GLP genotoxicity assays that was conducted. These results are not surprising given the overall lack of evidence of genotoxic and carcinogenic potential observed for other gum products, including gum arabic, guar gum, locust bean gum, gum guggul extract, and tara gum (http://ntp-apps.niehs.nih.gov/ntp_tox/index.cfm; Zeiger et al., 1992). These results suggest that gum ghatti can be used in products manufactured for human consumption at concentrations higher than currently acceptable limits without posing increased risk of genotoxic effects.

Table 6
Summary of neutral diffusion assay results.

Dose (mg/kg/day)	Number of animals	% Of cells with LMW DNA		
		Mean	SEM	*p* –Value[a]
Blood				
0	5	1.2	0.20	–
1000	5	0.4	0.24	–[b]
1500	5	0.2	0.20	–[b]
2000	5	0.8	0.37	–[b]
	Trend test[c]		$p = 0.4601$	
EMS (150)		4.4	1.47	0.0947[d]
Liver				
0	5	4.4	3.91	–
1000	5	0.8	0.20	0.9811
1500	5	0.8	0.37	0.9811
2000	5	0.4	0.24	0.9868
	Linear trend test		$p = 0.2143$	
EMS (150)		0.00	0.00	0.3230[d]

[a] ANOVA/Dunnett's test.
[b] Not significant by Kruskall–Wallis/Dunn's test (no *p*-value).
[c] Jonckheere–Terpstra test.
[d] *t*-Test.

Conflict of Interest

This work was conducted at ILS, Inc. and funded by San-Ei Gen, F.F.I., Inc., a manufacturer of gum ghatti. Maronpot Consulting LLC is a paid consultant for ILS, Inc. and San-Ei Gen, F.F.I., Inc.

Role of the funding source

ILS, Inc. was responsible for the study design, the collection, analysis, and interpretation of data, and the writing of the final study report and manuscript. The decision to submit the paper for publication was made by San-Ei Gen, F.F.I., Inc.

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