Original Article

www.ToxicolRes.org
Toxicological Research

Toxicol. Res. Vol. 34, No. 3, pp. 259-266 (2018)
https://doi.org/10.5487/TR.2018.34.3.259

Open Access

Genotoxicity Study of Glycopeptide (G-7%NANA)

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Abstract

Glycomacropeptide (GMP), a whey protein of milk, has functions including differentiation and development of nervous system, and anticancer and antiviral effects. To develop new functions, N-acetylneuraminic acid (NANA) containing 7% sialic acid was separated from GMP to produce G-7%NANA. N-glycolylneuraminic acid (Neu5Gc) is another type of sialic acid separated from GMP, which has been linked to immune disorders and chronic inflammation-mediated diseases. Therefore, safety was a concern in the use of G-7%NANA in functional foods. To ensure safety, in this study, three genetic toxicity tests on G-7%NANA were conducted. In the reverse mutation test using *Salmonella typhimurium* TA98, TA100, TA1535, TA1537, and *Escherichia coli* WP2uvrA, and in the chromosome aberration test using CHO-K1 cells, no significant differences from negative control were found at all dose levels. Similarly, no dose-related differences were evident compared to negative control in the micronucleus test using ICR mice. There was no evidence of G-7%NANA-related genetic toxicity.

Key words: Glycomacropeptide, N-acetylneuraminic acid, Genetic toxicity test, Sialic acid, N-acetylneuraminic acid

INTRODUCTION

Whey is an aqueous solution separated after the manufacture of cheese from milk. Whey protein (κ-casein) is the only milk protein containing sugar. Chymosin proteolytically cleaves κ-casein between residues 105 (phenylalanine) and 106 (methionine). The generated fragments comprising residues 1-105 and 106-169 are termed para-κ-casein and glycopeptide or glycomacropeptide (GMP), respectively. The latter contains sugar. GMP has 64 hydrophilic amino acids (1,2) and has been shown to have anticancer and antiviral functions (3-8). In addition, GMP induces the secretion of hormones related to satiety in the intestine, which induces a full feeling after food consumption (9,10).

The sugars in GMP include galactose (Gal), N-acetylgalactosamine (GalNAc), and N-acetylneuraminic acid (NANA or Neu5Ac) (11). In particular, a number of beneficial functions have been associated with GMP separated by NANA in the terminal part of GMP, or neuraminidase, of which sialic acid is a lyase (12-15). NANA is a type of sialic acid generated by N-acetylation. NANA is found in all mammalians including humans. Sialic acid (5-amino-3,5-dideoxy-D-glycero-D-galactononulsonic acid; neuraminic acid) has a sugar attached to carbon 9; the acid is an aggregate with 43 derivatives generated in animals and plants (16,17). NANA exists as a mixture with other forms of sialic acids in most mammalians, but in humans it is present as a single sialic acid (18-20).

NANA is the only type of sialic acid in the human body. Its content is enriched 20-fold or more in the cell membrane of nerve cells related to synaptogenesis and neurotransmission, compared with that in the cell membranes of other organs. In addition, sialic acid is the active ingredient for ganglioside, which has an important role in nervous system structure and activity (21). NANA is thought to be enriched in the brain, where nerve cells are dense, and has been proposed to have a significant role in the differentiation and development of the nervous system (22). Nutritionally, GMP and NANA lack the aromatic amino acid, phenylalanine, which makes them appropriate sources

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pISSN: 1976-8257 eISSN: 2234-2753

of protein for people with metabolic disorders, such as phenylketonuria (23).

These beneficial attributes of GMP and NANA have spurred the development of drugs and food supplements that contain these molecules (13,24,25). The synthesis and isolation of sialic acid have received particular focus. Of note, sialic acid is enriched in highly metastatic cells (26).

The most common method of producing sialic acid is to use N-acetyl-D-glucosamine synthesized and separated in *Escherichia coli* (27,28). Commercial-scale separation of sialic acid from the whey protein of milk has not been achieved. Considering this, a compound designated G-7%NANA was developed via the enzymatic separation of NANA, the typical form of sialic acid of GMP, from whey protein of milk. In this study, three tests of genetic toxicity were conducted to verify the safety of G-7%NANA.

MATERIALS AND METHODS

Test compound. GMP was purchased as NatraPep-GMP (Murry Goulburn Co-Operative Co., Melbourne, Australia). The product features GMP combined with 7% sialic acid. Sialic acid content was examined using a model 1260 high-performance liquid chromatography system (Agilent Technologies, Santa Clara, CA, USA). Sialic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA) and diluted to 0.1 ppm (w/w), 1 ppm, and 10 ppm for use as the standard solutions. G-7%NANA was prepared from the GMP of milk whey protein as detailed in Fig. 1.

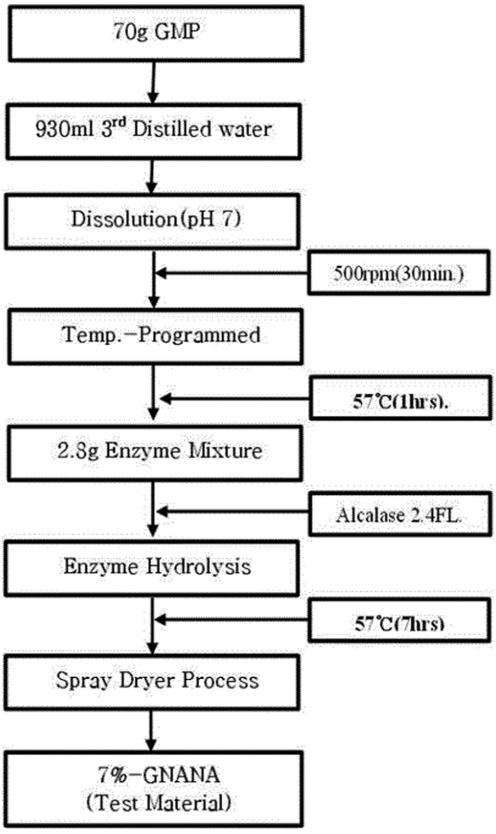

Fig. 1. Manufacturing process diagram for G-7%NANA, a test substance, as made through the enzyme (Alcalase 2.4 FL) separation and frozen drying process of sialic acid, the marker compound having the Glycomacropeptide (GMP) as a substrate.

Test guidelines. Mutagenic, *in vitro* clastogenic, and *in vitro* mammalian chromosome aberration assays were conducted under GLP conditions in accordance with the international guidelines of the Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 471 (Bacterial reverse mutation test), No. 473 (*in vitro* Mammalian Chromosome Aberration Test), and No. 474 (Mammalian Erythrocyte Micronucleus Test), respectively. All were adopted on July 21, 1997. The rodent phase of the in vivo micronucleus study was conducted in accordance with the protocol reviewed by the animal welfare officer of Catholic University of Daegu (Accreditation No. IACUC-2014-039).

Reverse mutation (RM) test. *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 and the *E. coli* strain WP2uvrA were used. All strains were used after determination of genetic characteristics, such as amino acid demand, ultraviolet (UV) sensitivity, *rfa* mutation, and R-factor maintenance. Dose determination was performed in triplicate with the highest dose being 5,000 μg. The dose determination and main tests involved metaphase analysis in the absence and presence of the S9 fraction. In the absence of S9, sodium azide (NaN_3), 9-aminoacridine (9-AA), 4-nitroquinoline-N-oxide (N-NQO), and 2-aminoanthracene (2-AA) were used as the positive controls. In the presence of the S9 fraction, 2-AA was the positive control. Each *S. typhimurium* strain was placed in a test tube and blended with 2 mL top agar, 0.1 mL fermentation solution, 0.05 mL G-7%NANA, and 0.5 mL phosphate buffered saline (PBS; 0.2 M, pH 7.4). For metaphase analysis, 0.5 mL of the S9 mix was used. The blended agar was applied in double on minimal glucose agar, and each plate was incubated at 37°C for 48 hr. Reverse mutated colonies were counted. The colony counts were the average values of triplicate determinations. When the reverse-mutated colonies increased in a dose-dependent manner or increased more than 2-fold, the compound was judged to be a mutation inducer (29,30). No statistical evaluations were conducted.

Chromosome aberration (CA) test. Chinese hamster ovary fibroblast cells (CHO-K1; Korea Cell Line Bank, Seoul, Korea) were cultured on minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), penicillin, and streptomycin, and were subcultured in the

presence of 0.5% trypsin-EDTA every 2 or 3 days. The chromosomal number of the cells was 22 and the cell population doubled about every 15 hr. After removal of the culture medium, 0.05 mL G-7%NANA and 0.5 mL S9 mix were added to 4.45 mL fresh culture medium (37°C) to a total volume of 5 mL. For the metaphase analysis, the test material was cultured for 6 hr in normal medium followed by 18 hr for samples containing S9 and 24 hr in the absence of S9. Dose determination experiments involved doses of 0.08, 0.16, 0.32, 0.63, 1.25, 2.5, and 5.0 mg/mL. The inhibition of cell proliferation was calculated as the mitotic index from a chromosomal sample against the negative control (100%). The inhibitory rate was also determined by metaphase analysis with and without S9. Cyclophosphamide (CPA; 15 μg/mL) and mitomycin C (MMC; 0.15 and 0.5 μg/mL) were used as the positive controls. Sterile distilled water was used as the negative control. Cells treated with G-7%NANA were cultured for 24 hr and then treated with 0.2 mg/mL of Colcemid for 2 hr followed by centrifugation (160 × g, 5 min). The upper layer of the cell solution was removed and treated with 75 mM KCl stock solution (37°C) for 20 min and fixed in Carnoy's solution (acetic acid : methanol = 1 : 3). Chromosomes were stained with 3% Giemsa solution for 5 min and then the CA of a medium of 100 cells was determined. Two portions of each sample plate were used for determination at each concentration. CA was grouped by concentration-related structural and numerical aberration through microscopic observation of a medium of 200 cells. In cases of structural aberration, breakage and exchange of chromosome and chromatid were counted, and both cases with and without gap were recorded (31,32). Numerical aberration was defined from the frequency of diploid, polyploidy, and endo duplication classified by chromosomal number. The test group and the solvent control group were compared using the Chi-square and Fisher's exact tests at the significance level of 0.01. When significance was observed, the dose-response relationship regarding the incidence of chromosomal anomalies was evaluated with the Cochran-Armitage trend test. Adjudication of the test was based on the standard operating procedure of our center, and the incidence rate of chromosomal anomalies in CHO-K1 cells was adjudicated based on the following standards according to the background level. However, regarding chromosomal anomalies, only the frequency of gap mutations was recorded and was not included in the adjudication.

Micronucleus (MN) test. This test was performed in a specific pathogen-free area (room temperature 23 ± 3°C, relative humidity 50 ± 10%, ventilation frequency 10~20 times/hr, lighting duration 12 hr, and luminous intensity 150~300 lx). Mice were fed irradiation-sterilized pellet food (Altromin Spezialfutter GmbH & Co. KG. Inc., Lage, Germany) ad libitum. Water was disinfected by a UV sterilizer and ultrafiltration, and made available ad libitum using a water bottle. The main test was conducted in three treatment groups: 2,000, 1,000, and 500 mg/kg/day. MMC 2 mg/kg/day was used as the positive control. It was dissolved in sterile distilled water before use. Feeding up to 2,000 mg/kg did not result in any deaths in mice. Additionally, no pathogenic difference was found in male mice after feeding this dose. Therefore, male mice were used in the main test with the highest dose being 2,000 mg/kg/day and two additional lower doses of 1,000 and 500 mg/kg/day. G-7%NANA was fed for 24 and 48 hr. After feeding the G-7%NANA, the cervical vertebra was disarticulated, and the femur was extracted with no blood contamination. Myelocytes were washed with 500 μL FBS and centrifuged (160 × g, 5 min). After discarding the upper layer, the myelocytes were resuspended in FBS. The solution was smeared on a slide glass, dried at ambient temperature, and fixed with methanol for 5 min. The fixed sample was dyed with acridine orange (40 μg/mL) and covered with a cover glass. Bone marrow cell proliferation and micronucleated polychromatic erythrocytes (PCE) were observed. To determine proliferation, about 200 of total erythrocytes (polychromatic and normochromatic erythrocytes) that had been selected in a well-smeared area were observed and the rate of PCE was determined from the MN cells observed in 2,000 PCE examined. Significant differences in both the frequency of micronucleated PCE of the mice fed G-7%NANA, compared to the negative control, and the ratio of PCE against total erythrocytes were determined by one-way analysis of variance (ANOVA), Dunnett's *t*-test, and Duncan test ($p = 0.05$) of multiple comparison through SPSS 19.0 K statistical software (SPSS Inc., Chicago, IL, USA).

RESULTS

RM test. For the main test, five doses (312, 625, 1,250, 2,500, and 5,000 μg/plate) and a negative control were examined. The number of reverse mutation colonies and growth were unaffected by dose in the *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 and the *E. coli* strain WP2uvrA, regardless of the presence of a metabolic activation system, compared to the negative control (Table 1). Thus, concentrations of 61.7, 185, 556, 1,670 and 5,000 μg/plate, representing the highest dose and ratios of three of successive lower doses, were examined. G-7%NANA did not show a significant difference in the tested strains at all doses regardless of the presence of metabolic activation systems, compared to the negative control (Table 2). The number of colonies were increased via reverse mutation by the positive control regardless of the presence of the metabolic activation system, indicating that the test conditions and the conclusion were appropriate (Table 1, 2).

Table 1. Dose-determination test of Reverse mutation with and without metabolic activation by G-7%NANA

		Dose (μg/plate)	No. of reverse mutation colonies/plate				
			Base-substitution type			Base-substitution type	
			TA100	TA1535	WP2urA	TA98	TA1537
w/o S9Mix		0	109.0 ± 3.61	19.0 ± 2.65	32.0 ± 2.00	46.0 ± 2.00	12.7 ± 3.06
		312	105.7 ± 7.51	20.3 ± 4.73	31.0 ± 1.00	46.0 ± 4.36	12.7 ± 1.15
		625	115.3 ± 9.29	21.0 ± 2.00	36.0 ± 2.00	44.7 ± 4.16	11.0 ± 1.00
		1250	116.0 ± 9.54	19.7 ± 2.52	31.0 ± 3.61	44.3 ± 4.04	14.0 ± 1.73
		2500	105.7 ± 7.77	21.3 ± 1.15	21.0 ± 1.73	46.7 ± 4.93	15.3 ± 1.15
		5000	114.3 ± 3.03	21.0 ± 4.00	34.0 ± 2.00	42.7 ± 1.15	11.7 ± 1.53
w/ S9Mix		0	117.0 ± 20.22	21.0 ± 2.65	29.7 ± 3.06	43.0 ± 5.20	13.0 ± 3.00
		312	113.0 ± 11.35	18.7 ± 3.79	33.3 ± 4.04	46.3 ± 3.06	13.0 ± 1.00
		625	121.1 ± 11.53	20.7 ± 4.04	30.0 ± 2.65	50.0 ± 1.00	14.3 ± 2.08
		1250	126.0 ± 6.56	21.3 ± 4.62	32.3 ± 4.73	44.0 ± 3.46	12.7 ± 0.58
		2500	133.3 ± 9.87	21.0 ± 4.00	31.0 ± 3.61	41.7 ± 2.89	12.3 ± 1.15
		5000	133.7 ± 11.93	19.7 ± 3.21	35.0 ± 3.46	45.3 ± 3.06	14.7 ± 1.15
Positive control	w/o S9Mix	Chemicals	NaN_3	NaN_3	4-NQO	4-NQO	9-AA
		Dose (μg/plate)	1.5	1.5	0.5	0.5	80
		No. of colonies/plate	327.7 ± 23.97*	228.7 ± 15.04*	220.7 ± 18.90*	269.3 ± 31.50*	151.0 ± 11.53*
	w/ S9Mix	Chemicals	2-AA	2-AA	2-AA	2-AA	2-AA
		Dose (μg/plate)	1.0	2.0	10	0.5	2.0
		No. of colonies/plate	441.7 ± 17.95*	189.3 ± 13.50*	258.3 ± 3.06*	265.3 ± 21.55*	218.0 ± 34.70*

*Significantly different from the control (0.0 dose) at $p < 0.05$.

CA test. Doses of 0.08, 0.16, 0.32, 0.63, 1.25, 2.5, and 5 mg/mL were administered to determine the administration dose for the main test. No dose was cytotoxic (Table 3). In line with this finding, concentrations of 0.08, 1.25, and 5 mg/mL were used to conduct the CA test. In the absence and presence of the metabolic activation system, no significant differences were evident at all concentrations of G-7%NANA with respect to occurrence frequency,

Table 2. Main test of reverse mutation with and without metabolic activation by G-7%NANA

		Dose (μg/plate)	No. of reverse mutation colonies/plate				
			Base-substitution type			Base-substitution type	
			TA100	TA1535	WP2urA	TA98	TA1537
w/o S9Mix		0	124.3 ± 2.31	25.7 ± 2.31	24.7 ± 1.53	32.0 ± 1.73	13.7 ± 2.08
		61.7	111.3 ± 7.77	25.7 ± 2.08	26.0 ± 5.00	34.0 ± 2.65	13.0 ± 1.00
		183	115.7 ± 12.66	25.0 ± 2.00	27.3 ± 2.08	35.3 ± 1.53	13.7 ± 0.58
		556	132.0 ± 13.00	27.7 ± 2.08	26.7 ± 3.21	39.0 ± 1.00	14.7 ± 2.89
		1670	119.3 ± 7.51	26.0 ± 5.00	26.7 ± 3.79	36.7 ± 0.58	13.3 ± 0.58
		5000	128.7 ± 0.97	28.3 ± 2.89	24.0 ± 1.00	37.3 ± 2.08	13.3 ± 0.58
w/ S9Mix		0	134.7 ± 0.58	24.0 ± 1.73	25.3 ± 1.53	35.7 ± 1.53	14.7 ± 2.52
		61.7	120.0 ± 2.65	23.3 ± 3.21	25.0 ± 6.93	38.3 ± 2.08	13.7 ± 1.53
		183	117.7 ± 1.15	24.0 ± 3.21	25.7 ± 3.79	37.0 ± 2.65	12.7 ± 3.06
		556	109.7 ± 8.96	22.0 ± 1.00	28.0 ± 4.00	37.0 ± 3.61	14.0 ± 1.00
		1670	125.7 ± 7.77	23.3 ± 4.93	29.3 ± 1.53	33.7 ± 4.73	13.0 ± 1.00
		5000	125.7 ± 3.20	27.0 ± 2.65	29.7 ± 4.16	36.7 ± 2.52	13.7 ± 0.58
Positive control	w/o S9Mix	Chemicals	NaN_3	NaN_3	4-NQO	4-NQO	9-AA
		Dose (μg/plate)	1.5	1.5	0.5	0.5	80
		No. of colonies/plate	442.3 ± 10.26*	237.0 ± 13.53*	241.3 ± 25.01*	287.3 ± 15.14*	272.7 ± 9.87*
	w/ S9Mix	Chemicals	2-AA	2-AA	2-AA	2-AA	2-AA
		Dose (μg/plate)	1.0	2.0	10	0.5	2.0
		No. of colonies/plate	406.7 ± 3.51*	241.0 ± 14.11*	344.7 ± 9.50*	265.7 ± 17.62*	290.0 ± 23.43*

*Significantly different from the control (0.0 dose) at $p < 0.05$.

Table 3. Cytotoxcity data for dose-determination and in the main study of CA test

	Concentration (mg/mL)	Dose-determination	Main study
		Cell growth rate (% of negative control)	Cell growth rate (% of negative control)
		6 hr treatment	6 hr treatment
w/ S9Mix	0.0 (negative control)	100	100
	0.08	100	99
	0.16	100	NT[a]
	0.32	100	NT
	0.63	99	NT
	1.25	100	100
	2.5	97	NT
	5	100	100
	Concentration (mg/mL)	Dose-determination	Main study
		Cell growth rate (% of negative control)	Cell growth rate (% of negative control)
		6 hr treatment	6 hr treatment
w/o S9Mix	0.0 (negative control)	100	100
	0.08	100	100
	0.16	100	NT
	0.32	100	NT
	0.63	93	NT
	1.25	96	100
	2.5	100	NT
	5	100	100
	Concentration (mg/mL)	Dose-determination	Main study
		Cell growth rate (% of negative control)	Cell growth rate (% of negative control)
		24 hr treatment	24 hr treatment
	0.0 (negative control)	100	100
	0.08	100	97
	0.16	100	NT
	0.32	96	NT
	0.63	100	NT
	1.25	90	98
	2.5	91	NT
	5	100	100

[a]NT: not tested.

compared to the negative control ($p > 0.01$). In the positive control group, the number of cells with chromosome aberrations significantly increased ($p < 0.01$) regardless of the presence of the metabolic activation system, indicating that the test condition and conclusion were appropriate (Table 4).

MN test. To detect micronuclei, 2,000, 1,000, and 500 mg/kg/day doses were used for testing in ICR mice. No adverse symptoms including death were noted, and there was no significant weight change between the groups ($p > 0.05$; Table 5). In the micronucleus test using mouse marrow cells, no significant differences were observed at any concentration in the ratio of polychromatic erythrocytes against overall erythrocytes (the indicator for cytotoxicity) compared to the negative control ($p > 0.05$). In addition, the frequency of the appearance of polychromatic erythrocytes having micronuclei was similar at all concentrations, with no significant differences when compared to the frequency of micronuclei in the negative control ($p > 0.05$; Table 6). The positive control showed no significant difference in proliferation inhibition of polychromatic erythrocytes compared to the negative control ($p > 0.05$; Table 5). However, a significant difference was found in the frequency of appearance of polychromatic erythrocytes with micronuclei ($p < 0.05$; Table 6). The results indicated that the test condition and conclusion were appropriate.

DISCUSSION

The sialic acid form of milk GMP consists of 75% NANA and 23% N-glycolylneuraminic acid (Neu5Gc) (18). How-

Table 4. Effect of the G-7%NANA on chromosomal aberration of CHO-K1 cells

Group	hr	Dose (mg/mL)	No. of observed cells	Polyploid		No. of aberrant cells							Incidence (%) (−g)
						Gap	Chromatid type		Chromosome type		Others	Total no. of aberrant cells	
				No.	Incidence (%)	g[a]	ctb[a]	cte[a]	csb[a]	cse[a]		−g	+g
w/ S9mix	6	0	200	1	0.5 ± 0.71[b]	2	0	0	0	0	0	0	1	0.0 ± 0.00
		0.08	200	0	0.0 ± 0.00	1	0	0	0	0	0	0	1	0.0 ± 0.00
		1.25	200	0	0.0 ± 0.00	0	1	0	0	0	0	1	1	0.5 ± 0.71
		5	200	0	0.0 ± 0.00	2	0	0	1	0	0	1	3	0.5 ± 0.71
CPA		15 (μg/mL)	200	0	0.0 ± 0.00	1	11	16	4	4	0	25	36	17.5 ± 2.12
w/o S9mix	6	0	200	0	0.0 ± 0.00	2	1	0	0	0	0	1	3	0.5 ± 0.71
		0.08	200	0	0.0 ± 0.00	1	1	0	0	0	0	1	2	0.5 ± 0.71
		1.25	200	0	0.0 ± 0.00	0	1	0	0	0	0	1	1	0.5 ± 0.71
		5	200	0	0.0 ± 0.00	1	1	0	1	0	0	1	2	0.5 ± 0.71
MMC		0.5 (μg/mL)	200	0	0.0 ± 0.00	2	16	16	1	3	0	36	38	17.5 ± 2.12
w/o S9mix	24	0	200	0	0.0 ± 0.00	1	1	0	0	0	0	1	2	0.5 ± 0.71
		0.08	200	0	0.0 ± 0.00	2	1	0	0	0	0	1	3	0.5 ± 0.71
		1.25	200	0	0.0 ± 0.00	2	1	0	0	1	0	2	4	1.0 ± 0.00
		5	200	0	0.0 ± 0.00	3	1	0	0	0	0	1	4	0.5 ± 0.71
MMC		0.15 (μg/mL)	200	0	0.0 ± 0.00	1	14	16	3	1	0	34	35	17.0 ± 1.41

[a]g, gap; ctb, chromatid break; cte, chromatid exchange; csb, chromosome break; cse, chromosome exchange.
[b]Mean ± SD (n = 2).

Table 5. Body weights change after administration

Treatment	Dose (mg/kg/day)	Body weights (g)	
		Grouping & Administration	Sacrifice
Negative control	0	36.9 ± 1.05	36.0 ± 1.08
Test control	500	36.7 ± 0.96	36.5 ± 0.93
	1000	36.9 ± 0.88	36.2 ± 1.56
	2000	36.7 ± 1.11	36.2 ± 1.10
Positive control	2	36.6 ± 1.36	36.1 ± 1.43

ever, Neu5Gc, which comprises 23% of the GMP, is induced by antibodies including N-glycolylneuraminic-IgA, N-glycolylneuraminic-IgM, and N-glycolylneuraminic-IgG, and has been suggested to be the cause of immune diseases (33,34). Additionally, the coexistence of Neu5Gc and anti-Neu5Gc in the human body is suggested to be factor contributing to chronic inflammation-mediated disorders including cancer (35). These observations raise concern about the toxicity of G-7%NANA. Specifically, technological limitations in detecting the separation of NANA from GMP make it conceivable that Neu5Gc may be present. Sialic acid increases in the serum of cancer patients and has been suggested to be related to carcinogenesis. Thus, it is essential to conduct genetic toxicity testing (36,37).

In the present study, the results of three types of genetic toxicity test were negative for G-7%NANA bacterial reverse mutation test, *in vitro* mammalian chromosome aberration test, and mammalian erythrocyte micronucleus test. To obtain an appropriate toxicity range, a preliminary range-finding assay over a broad range of concentrations was used. As cytotoxicity increases, mechanisms other than

Table 6. *In vivo* micronucleus test

Treatment	Dose (mg/kg/day)	MNPCE observed	MNPCE/2,000 PCE (%)	PCE counted	PCE/200 RBC (%)
Negative control	0	2.6 ± 0.89	0.13 ± 0.045	82.8 ± 2.17	41.4 ± 1.08
Test substance	500	2.6 ± 1.14	0.13 ± 0.057	81.8 ± 2.68	40.9 ± 1.34
	1000	2.6 ± 1.52	0.13 ± 0.076	82.6 ± 2.41	41.3 ± 1.20
	2000	2.4 ± 1.52	0.12 ± 0.097	82.6 ± 3.13	41.3 ± 1.57
Positive control (MMC)	2	26.8 ± 1.10	1.34 ± 0.055	78.6 ± 3.71	39.3 ± 1.86

MNPCE: Micronucleated polychromatic erythrocyte, PCE: polychromatic erythrocyte, RBC: Red blood cells (polychromatic erythrocyte + normochromatic erythrocyte), MMC: Mitomycin C.

direct DNA damage by a compound or its metabolites can lead to "positive" results related to cytotoxicity and not genotoxicity. Such indirect induction of DNA damage secondary to damage to non-DNA targets is more likely to occur above a certain concentration threshold (38).

The data provide sample evidence of a lack of genetic toxicity. These results corroborate the prior unpublished findings of a lack of abnormality in the indexes related to immunology that were identified in a 90-day repeated dose toxicity test for G-7%NANA. Especially, any indicators and symptoms for antibody induction were not identified, inferring that G-7%NANA by itself and by co-existing with Neu5Gc does not cause immune-mediated diseases. There was no evidence of mutagenicity caused by G-7%NANA in the genetic toxicity analyses, and there was no possibility for the coexistence of Neu5Gc. Thus, new physiological functional products incorporating G-7%NANA can be developed.

ACKNOWLEDGMENTS

This work was supported by Hanil Bio Med Inc. and the Ministry of Environment in Korea suggesting 2018 education program for the management of information on the hazard and risk of chemical substances.

Received April 5, 2018; Revised June 1, 2018; Accepted June 12, 2018

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