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Gardenia blue is not carcinogenic in the rasH2 mouse

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Abstract

Introduction: Gardenia blue is currently being considered as a naturally derived food colorant for use in the global marketplace.

Methods: To assess its carcinogenic potential, 100 female and 100 male CByB6F1-Tg (HRAS)2Jic (rasH2) mice were allocated to four dose groups and exposed to gardenia blue in the diet for 26 weeks at dose levels of 0.0% (control), 0.5%, 2.5%, or 5.0% (corresponding to 0.0, 664.8, 3341.0, and 6623.2 mg/kg/day in male mice and 0.0, 1182.7, 5561.1, and 10,440.3 mg/kg/day in female mice, respectively). An additional group of 10 males and 10 females was administered intraperitoneal N-methyl-N-nitrosourea (MNU) as a positive control. Clinical observations, body and organ weights, clinical chemistry, hematology, and hormone analyses were performed in addition to urinalysis and histopathology.

Results: The positive control elicited expected responses specific to rasH2 mice. There were sporadic background non-dose-related findings in clinical pathology parameters and anatomic pathology common to rasH2 mice in the absence of any gardenia blue induced dose-related changes.

Discussion: Under these study conditions, the no-observed-adverse-effect level was 5% gardenia blue (6623.2 mg/kg/day in male mice and 10,440.3 mg/kg/day in female mice).

Conclusions: Based on this study a high dietary level of gardenia blue was negative for carcinogenicity in the rasH2 mouse test system.

Keywords

Gardenia blue, carcinogenicity testing, rasH2 mouse

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Introduction

Gardenia blue, the principal coloring component of gardenia blue powder, is obtained from the fruit of *Gardenia jasminoides* Ellis. Following aqueous extraction from the fruit, the extraction liquid is passed through a reverse osmotic membrane to yield a high molecular weight fraction, gardenia yellow (i.e., crocin and crocetin), and a low molecular weight fraction consisting of iridoid glycoside (up to 45% geniposide). These iridoid glycosides are converted to their aglycones by treatment with β-glucosidase converting

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the geniposide to genipin. When genipin is treated with a protein hydrolysate in the presence of oxygen, polymerization occurs and a polymeric blue color, gardenia blue, is formed. The gardenia blue polymer is then heated to denature the enzyme and filtered. The color additive gardenia blue is prepared by mixing the gardenia blue polymer with food grade dextrin or maltodextrin. The amount of dextrin/maltodextrin is added to achieve a specific color value.

Gardenia blue colorant produced by San-Ei Gen, FFI, Inc., conforms to the specifications described in the current ninth edition of "Japan's Specifications and Standards for Food Additives".[1] Gardenia blue is permitted for use as a color additive for food and beverage in Japan, China, Taiwan, and Korea and has been used in Japan for over 30 years. Following leads from traditional Chinese medicine, genipin and geniposide extracts of gardenia fruit (*Gardenia jasminoides* Ellis) have been widely used based on pharmacological and physiological properties including anti-inflammatory properties,[2–4] neuroprotective effects[5,6] and anti-cancer activity.[7–9] Annual production in Japan is about 138 metric tons (as color value 50) with a per capita intake of about 3.0 mg/person/day.[10]

An extensive battery of GLP-compliant genotoxicity studies of gardenia blue and its precursor, genipin, including combined micronucleus/comet assays and "reverse" comet assays of gardenia blue, indicate gardenia blue in food presents no significant genotoxic concern for humans.[11] Early toxicity studies at dietary levels as great as 5% have been negative for adverse treatment-related effects.[12,13] Recent Good Laboratory Practices (GLP)-compliant studies at dietary doses up to 5% have demonstrated no treatment-related adverse effects for prenatal development,[14] in a 12-months rat toxicity study following in utero exposure,[15] or in a 2-years rat carcinogenicity study.[16]

While synthetic color additives still dominate the food coloring marketplace, identification and commercialization of natural source colorants is an ongoing effort to satisfy consumer demand for food to be as natural as possible and to capitalize on potential health benefits of natural pigments.[17–20] Identification of new colorants for foods and beverages has prompted the present rasH2 cancer bioassay as part of a safety assessment of the natural food colorant, gardenia blue, conducted under contemporary USFDA GLP.[21]

Methods

Identification of test articles

Gardenia blue (lot number 180,910; San-Ei Gen FFI, Inc., Osaka, Japan) consisting primarily of 32.3% gardenia blue color and 62.7% maltodextrin was mixed with Purina Certified 5002 meal diet at dose concentrations of 0.5%, 2.5%, and 5.0% every 2 months and kept at room temperature. N-methyl-N-nitrosourea (CAS 684-93-5) was obtained from Spectrum Chemicals, New Brunswick, NJ and delivered in pH 4.0 citrate buffer from Sigma-Aldrich, St Louis, MO. Dose formulation and analyses were performed at RTI International, Research Triangle Park, NC, and were consistently within acceptable specifications.

Animal husbandry and maintenance

CByB6F1-Tg (HRAS)2Jic (rasH2) mice of approximately 5 weeks of age were obtained from Taconic Biosciences, Inc. (Germantown, NJ), individually identified by transponder chip (BioMedic Data Systems, Inc., Seaford, DE), and housed individually in polycarbonate caging with microinsulator tops and absorbent heat-treated hardwood bedding (Northeastern Products Corp., Warrensburg, NY) that was changed weekly. Prior to assignment to treatment groups, animals were given Purina Certified 5002 meal diet (Ralston Purina Co, St Louis, MO) ad libitum as the carrier diet and reverse osmosis-treated tap water. The mice were allowed at least 7 days acclimation prior to inclusion in the study and were approximately 7–9 weeks of age at the start of dosing. Animal weights at start of dosing were 18.2–21.6 grams for females and 22.8–28 grams for males. Following approval from the Integrated Laboratory Systems (ILS), Inc. (Research Triangle Park, NC, USA) Animal Care and Use Committee, all procedures during the study were carried out in compliance with the Animal Welfare Act Regulations (9 CFR 1–4), and animals were handled and treated according to the *Guide for the Care and Use of Laboratory Animals*.[22]

Experimental design

This study was designed using the rasH2 transgenic mouse alternative in accordance with OECD testing guideline 451[23] and as an acceptable species for short-term carcinogenicity.[24] One hundred (100) female and 100 male rasH2 mice were allocated to one of four designated dose groups that stratified animals across groups by equivalent body weight with the number of animals per dose group sufficient to provide adequate statistical evaluation.[25] The animals were administered gardenia blue at dose levels of 0.0%, 0.5%, 2.5%, or 5.0% for at least 26 weeks (Table 1). Justification of dose levels was based on a dose range-finding study where rasH2 mice were exposed to gardenia blue in the diet at dose levels up to 5.0% for 28 days with no abnormal clinical observations or significant changes in body weight.[26] An additional ten females and ten males were administered 75 mg/kg MNU (N-methyl-N nitrosourea) via a single intraperitoneal injection on day 1 as a positive control.[25] Body weights and clinical observations were performed weekly with daily cage-side observations. Feed consumption for control and gardenia blue groups (but

Table 1. Group designation, animal identification, and dose levels.

Group	Test material	Dose level	No. of males	No. of females
1	Gardenia blue	0.0% (diet)[a]	25	25
2	Gardenia blue	0.5% (diet)[a]	25	25
3	Gardenia blue	2.5% (diet)[a]	25	25
4	Gardenia blue	5.0% (diet)[a]	25	25
5	MNU	75 mg/kg[b]	10	10

[a]MNU, N-methyl-N-nitrosourea.
[b]Groups 1–4 were dosed daily for 26 weeks.
Group 5 (positive control) was administered one intraperitoneal injection on Day 1.

not for the MNU groups) was measured weekly beginning at initiation of exposure. After at least 26 weeks of exposure, animals were humanely euthanized. Prior to termination, animals were fasted overnight and an 18-hour urine sample was collected.

Viability, clinical signs, body weight and food consumption

Cage-side observations for individual animals were performed daily following the initiation of dosing on day 1. Observations for morbidity and mortality were performed twice weekly on weekdays and once daily on weekends and holidays. Observations for clinical signs and body weight measurements were performed within 2 days of arrival, during allocation to an exposure group, prior to exposure on day 1, weekly thereafter, and at termination. Food consumption was measured at least once weekly with the exception of mice administered the positive control, MNU. Urine samples from 10 males and 10 females per dose group were collected prior to termination.

Survival, necropsy, blood collection, and tissue handling

All animals in the control and test article-treated groups (Groups 1–4) survived to scheduled sacrifice except for the following: one found dead male in the control group; 2 found dead or moribund females in the 0.5% group; 3 moribund males and 1 found dead female in the 2.5% group; and two moribund or found dead males and 4 moribund or found dead females in the 5.0% group. Lymphoma was determined to be the cause of death for 2 of the early mortality mice while cause of death for the remaining early deaths was not determined. The animals that did not survive to the terminal necropsy did not exhibit any toxicity or carcinogenicity effects attributable to the test article. Animals of both sexes in the MNU positive control group were euthanized at study termination or found dead prior to the terminal necropsy.

At study termination all remaining animals were euthanized by CO_2 asphyxiation and complete necropsies were performed at the end of the 6-months exposure period. Whole blood, blood smears, plasma, and serum samples were collected by cardiac puncture immediately prior to necropsy for hematology, biochemistry, and coagulation parameters, and hormone level measurements. All organs and tissues were examined for grossly visible lesions with attention given to any discoloration of the gastrointestinal tract, kidneys, and mesenteric lymph nodes based on previous observations in rat studies. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm thickness, and stained by hematoxylin and eosin. Tissues weighed, fixed, and examined microscopically in male and/or female animals are listed in Table 2. A grading scheme was used to evaluate pathologic lesions in the tissues as follows: no lesion (0), minimal (grade 1), mild (grade 2), moderate (grade 3), and marked (grade 4).[27] Following initial pathology evaluation by the study pathologist, an independent pathology peer review was performed to confirm initial histopathologic diagnosis.[28] The target tissues, thymus, and stomach of animals treated with MNU were examined histopathologically to confirm that the positive control induced the expected results.

Statistical analysis

Group means and standard deviations were calculated and reported. All data were analyzed using Statistical Analysis System version 9.2 (SAS Institute, Cary, NC), including final body weight, body weight gain, food consumption (g/kg/day), urinalysis endpoints, clinical pathology endpoints, absolute and relative organ weights, and histopathological endpoints. First, studentized residual plots were used to detect possible outliers in the data. Homogeneity of variance was then analyzed using Levene's test.[29] If the data were heterogeneous, then appropriate transformation of the data was performed (logarithm, square root, and/or multiplicative inverse), and the data were reanalyzed for homogeneity of variance. Data were then analyzed using a one-way analysis of variance (ANOVA)

Table 2. Tissues preserved for histopathology.[a]

Adrenals[b]	Muscle (skeletal)
Aorta	Nasal cavity
Bone/bone marrow (sternum and femur)	Ovaries[b]
Brain (cerebrum, cerebellum, and medulla/pons)[b]	Pancreas
Coagulating gland	Pituitary
Cecum, colon, and rectum (large intestines)	Prostate
Corpus and cervix uteri	Salivary glands
Duodenum, jejunum, and ileum (small intestines, including Peyer's patches)	Sciatic nerve
Epididymides[b]	Seminal vesicles
Esophagus	Skin
Eyes with optic nerve	Spinal cord (cervical, mid-thoracic, lumbar)
Harderian glands	Spleen[b]
Heart[b]	Stomach (non-glandular and glandular)
Kidneys[b]	Testes[b]
Lacrimal glands	Thymus
Liver with gall bladder[b]	Thyroid/parathyroid(s)[b]
Lungs with trachea	Urinary bladder
Lymph nodes (mesenteric and mandibular [cervical])	Uterus with cervix[b]
Mammary glands	Vagina

[a]Tissues were paraffin-embedded, sectioned, and stained with hematoxylin and eosin for histopathology from all control and high dose mice, except for spleen, harderian glands, and lungs, which were processed and examined from all gardenia blue-dosed groups.[15]
[b]Tissues were weighed at necropsy. Thyroids/parathyroids were weighed after fixation.

and treated groups were compared to the appropriate control group using Dunnett's test.[30] Finally, dose-dependent changes were evaluated using a linear regression model. If data could not be transformed to be homogeneous, data were evaluated using the non-parametric Dunn's test[31] and dose-dependent changes analyzed via the non-parametric Jonckheere's trend test.[32]

Results

Survival, clinical observations, body weights, and test article consumption

Animals surviving to termination in the gardenia blue-exposed and control groups consisted of 24, 23, 22, and 23 males and 25, 24, 24, and 21 females in the 0.0%, 0.5%, 2.5%, and 5.0% groups, respectively. As expected with the MNU positive control, 4 of 10 males and 6 of 10 females were euthanized or found dead prior to study termination. There were no gardenia blue-related abnormal clinical signs or cage-side observations nor effects on body weight gain in gardenia blue groups compared to controls for males or females (Tables 3 and 4). Food and test article consumption data for males and female are also provided in Tables 3 and 4.

Tissue weights

Group summary absolute organ weight data are provided in Table 5 for male mice and Table 6 for female mice. There was a statistically significant increase in heart weight in male mice administered 2.5% gardenia blue and in thyroid weight in the females administered 0.5% gardenia blue compared to concurrent controls. A statistically significant increasing linear trend was measured for liver weights in males (without any dose group-specific significance) administered gardenia blue without any correlating histopathological changes.

Group summary relative organ weight data are provided in Table 7 for male mice and Table 8 for female mice with occasional statistical effects in lower dose groups. In the males there was a significant increase in the relative weight of the brain and kidney at 0.5% gardenia blue, and in the heart at 0.5% and 2.5%; relative liver weight was increased at all dose levels with an increased linear trend. In female mice, a significant increase in the relative thyroid weight was measured in the 0.5% dose group compared to controls.

Clinical pathology

Group summary clinical pathology data for complete blood count, coagulation, clinical chemistry, and urinalysis

Table 3. Male body weight changes, food consumption, and gardenia blue consumption.

	Gardenia blue dietary dose level			
Parameter	0.0%	0.5%	2.5%	5.0%
Number of males at termination	24	23	22	23
Initial body weight (g)	25.70 ± 1.21	25.37 ± 1.09	25.29 ± 1.31	25.25 ± 1.26
Final body weight (g)	37.50 ± 2.76	36.27 ± 3.11	36.70 ± 2.86	37.28 ± 2.89
Body weight gain (g)	11.76 ± 2.32	10.84 ± 2.91	11.42 ± 2.10	11.93 ± 2.28
Food consumption (mg/kg body weight/day)	132.0 ± 18.9	133.0 ± 8.8	133.6 ± 8.6	132.5 ± 8.2
Gardenia blue consumption (mg/kg body weight/day)	0.0 ± 0.0	664.8 ± 44.1	3341.0 ± 214.9	6623.2 ± 408.3

Results presented are mean ± standard deviation.

Table 4. Female body weight changes, food consumption, and gardenia blue consumption.

	Gardenia blue dietary dose level			
Parameter	0.0%	0.5%	2.5%	5.0%
Number of females at termination	25	24	24	21
Initial body weight (g)	19.86 ± 0.76	19.73 ± 0.89	19.62 ± 0.74	19.77 ± 0.92
Final body weight (g)	26.84 ± 2.22	26.68 ± 1.71	26.07 ±1.85	26.48 ± 1.86
Body weight gain (g)	6.98 ± 2.12	6.93 ± 1.52	6.43 ± 1.67	6.75 ± 1.70
Food consumption (mg/kg body weight/day)	245.5 ± 39.0	236.5 ± 52.3	222.4 ± 37.8	208.3 ± 31.8
Gardenia blue consumption (mg/kg body weight/day)	0.0 ± 0.0	1182.7 ± 261.4	5561.1 ± 944.8	10440.03 ± 1626.9

Results presented are mean ± standard deviation.

Table 5. Male absolute organ weights.

	Gardenia blue dietary dose level			
Parameter	0.0%	0.5%	2.5%	5.0%
Number of males at termination	24	23	22	23
Adrenals (paired)	0.00715 ± 0.00287	0.00706 ± 0.00220	0.00689 ± 0.00184	0.00753 ± 0.00200
Brain	0.48711 ± 0.03715	0.49650 ± 0.02983	0.50132 ± 0.03949	0.49958 ± 0.02977
Epididymides (paired)	0.11924 ± 0.00891	0.11834 ± 0.01179	0.12060 ± 0.01181	0.12149 ± 0.01660
Heart	0.22555 ± 0.02115	0.23316 ± 0.03397	0.25303 ± 0.04412*	0.23226 ± 0.03560
Kidneys (paired)	0.62195 ± 0.05374	0.61492 ± 0.06128	0.65300 ± 0.07729	0.61847 ± 0.06555
Liver	1.45547 ± 0.13301	1.44190 ± 0.14602	1.52061 ± 0.14559	1.51267 ± 0.20924^
Spleen	0.08375 ± 0.02920	0.08680 ± 0.04008	0.08967 ± 0.03995	0.08009 ± 0.01176
Testes (paired)	0.30632 ± 0.03306	0.30784 ± 0.03164	0.32397 ± 0.06626	0.32243 ± 0.05125
Thyroid with parathyroid (paired)	0.00295 ± 0.00068	0.00278 ± 0.00075	0.00267 ± 0.00068	0.00310 ± 0.00090

Results presented are mean grams ± standard deviation; *statistically significant at $p < 0.05$; ^statistically significant increasing linear trend test.

Table 6. Female absolute organ weights.

	Gardenia blue dietary dose level			
Parameter	0.0%	0.5%	2.5%	5.0%
Number of females at termination	25	24	24	21
Adrenals (paired)	0.01024 ± 0.00258	0.01022 ± 0.00363	0.01051 ± 0.00248	0.01130 ± 0.00239
Brain	0.52414 ± 0.01563	0.52706 ± 0.01356	0.52118 ± 0.02324	0.52478 ± 0.02008
Heart	0.17618 ± 0.02413	0.16938 ± 0.01565	0.17697 ± 0.02274	0.17541 ± 0.02446
Kidneys (paired)	0.44357 ± 0.02973	0.43819 ± 0.02867	0.43571 ± 0.02871	0.43888 ± 0.03415
Liver	1.26954 ± 0.14984	1.25520 ± 0.13760	1.30355 ± 0.18508	1.32347 ± 0.12000
Ovaries (paired)	0.02242 ± 0.00539	0.02278 ± 0.00578	0.02490 ± 0.00536	0.02138 ± 0.00639
Spleen	0.10168 ± 0.02698	0.09423 ± 0.01426	0.09533 ± 0.01840	0.09547 ± 0.00955
Thyroid with parathyroid (paired)	0.00301 ± 0.00075	0.00374 ± 0.00116*	0.00302 ± 0.00092	0.00299 ± 0.00053
Uterus with cervix	0.28043 ± 0.08310	0.27089 ± 0.08841	0.26210 ± 0.07206	0.25718 ± 0.06968

Results presented are mean grams ± standard deviation; *statistically significant at $p < 0.05$.

Table 7. Male relative organ weights.

	Gardenia blue dietary dose level			
Parameter	0.0%	0.5%	2.5%	5.0%
Number of males at termination	24	23	22	23
Adrenals (paired)	0.0194 ± 0.0081	0.0216 ± 0.0080	0.0191 ± 0.0055	0.0206 ± 0.0052
Brain	1.325 ± 0.142	1.498 ± 0.199*	1.379 ± 0.136	1.370 ± 0.138
Epididymides (paired)	0.3242 ± 0.0332	0.3577 ± 0.0623	0.3325 ± 0.0366	0.3326 ± 0.0463
Heart	0.613 ± 0.075	0.704 ± 0.137*	0.696 ± 0.115*	0.635 ± 0.087
Kidneys (paired)	1.686 ± 0.138	1.851 ± 0.249*	1.798 ± 0.211	1.692 ± 0.175
Liver	3.941 ± 0.304	4.339 ± 0.586*	4.178 ± 0.281*	4.116 ± 0.384*^
Spleen	0.228 ± 0.079	0.259 ± 0.105	0.245 ± 0.103	0.220 ± 0.036
Testes (paired)	0.830 ± 0.083	0.932 ± 0.172	0.890 ± 0.168	0.880 ± 0.117
Thyroid with parathyroid	0.0080 ± 0.0018	0.0082 ± 0.0021	0.0073 ± 0.0019	0.0084 ± 0.0021

Results presented are mean grams ± standard deviation; *statistically significant at $p < 0.05$; ^statistically significant increasing linear trend test.

parameters are provided in Tables 9–11 for males and Tables 12–14 for females. There were minimal changes noted in the clinical chemistry and urine values for both males and females administered gardenia blue. In males, basophils in blood and urobilinogen in urine were statistically increased at the top dose. In females, urine protein was elevated at the top dose. Statistical flags noted in the tables represented values that did not correlate with any histopathological findings.

Macroscopic and microscopic observations

The only noteworthy macroscopic changes in gardenia blue-treated males or females versus corresponding controls were dose-related gross blue discoloration of the gastrointestinal tract and mesenteric lymph nodes and dose-related darkening of the kidneys. Additionally, sporadic incidences of mass lesions, tissue discolorations, and organ enlargements occurred across control and gardenia blue dose groups and were not considered test article-related.

Stained sections of all tissues from control and high dose animals (Table 15), and spleen, Harderian glands, and lungs (Table 16) from intermediate dose animals were evaluated by light microscopy.[33] Gross lesions in all animals were examined and correlated microscopically when possible. None of the observed microscopic finding were considered to be test article related. No histologic findings were evident to correlate to the grossly observed blue or dark tissue

Table 8. Female relative organ weights.

	Gardenia blue dietary dose level			
Parameter	0.0%	0.5%	2.5%	5.0%
Number of females at termination	25	24	24	21
Adrenals (paired)	0.0375 ± 0.0110	0.0395 ± 0.0139	0.0397 ± 0.0093	0.0424 ± 0.0099
Brain	1.935 ± 0.279	2.034 ± 0.225	1.973 ± 0.151	1.958 ± 0.142
Heart	0.646 ± 0.106	0.653 ± 0.091	0.669 ± 0.091	0.653 ± 0.092
Kidneys (paired)	1.630 ± 0.210	1.690 ± 0.210	1.648 ± 0.130	1.635 ± 0.126
Liver	4.652 ± 0.616	4.834 ± 0.662	4.908 ± 0.569	4.933 ± 0.498
Ovaries (paired)	0.083 ± 0.024	0.088 ± 0.024	0.094 ± 0.018	0.080 ± 0.025
Spleen	0.380 ± 0.117	0.362 ± 0.059	0.357 ± 0.047	0.356 ± 0.034
Thyroid with parathyroid	0.0114 ± 0.0036	0.0145 ± 0.0048*	0.0113 ± 0.0032	0.0111 ± 0.0020
Uterus with cervix	1.0437 ± 0.3573	1.0466 ± 0.3600	0.9924 ± 0.2810	0.9621 ± 0.2755

Results presented are mean grams ± standard deviation; *statistically significant at $p < 0.05$.

Table 9. Male complete blood count and coagulation data.

	Gardenia blue dietary dose level			
Parameter	0.0%	0.5%	2.5%	5.0%
Number of males at termination	24	23	22	23
White blood cells (1000/μl)	4.19 ± 1.72	3.46 ± 1.21	4.40 ± 1.50	5.44 ± 2.49
Red blood cells (1000000/μl)	11.55 ± 0.59	11.66 ± 0.58	11.24 ± 0.84	11.31 ± 0.56
Hemoglobin (g/dl)	16.22 ± 0.66	16.43 ± 0.74	15.93 ± 0.98	16.02 ± 0.69
Hematocrit (%)	56.72 ± 3.33	57.56 ± 3.23	55.68 ± 3.56	56.08 ± 2.88
Mean corpuscular volume (fl)	49.09 ± 1.37	49.37 ± 0.85	49.64 ± 1.73	49.59 ± 0.80
Mean corpuscular hemoglobin (pg)	14.05 ± 0.37	14.07 ± 0.25	14.18 ± 0.35	14.17 ± 0.27
Mean corpuscular hemoglobin concentration (g/dl)	28.63 ± 0.71	28.56 ± 0.52	28.61 ± 0.49	28.58 ± 0.53
Platelets (1000/μl)	892.27 ± 171.69	868.00 ± 229.71	1057.27 ± 231.39	888.18 ± 201.58
Neutrophils (%)	21.82 ± 4.26	28.50 ± 10.57	21.10 ± 6.05	24.56 ± 5.51
Neutrophils (1000/μl)	0.90 ± 0.43	0.94 ± 0.37	0.88 ± 0.28	1.29 ± 0.66
Lymphocytes (%)	70.30 ± 6.67	66.49 ± 10.13	74.24 ± 5.85	67.46 ± 9.59
Lymphocytes (1000/μl)	3.00 ± 1.31	2.36 ± 1.03	3.31 ± 1.27	3.77 ± 1.93
Monocytes (%)	1.91 ± 1.00	1.29 ± 0.66	1.44 ± 0.38	1.86 ± 0.95
Monocytes (1000/μl)	0.08 ± 0.05	0.05 ± 0.03	0.06 ± 0.03	0.12 ± 0.11
Eosinophils (%)	4.63 ± 4.31	2.15 ± 0.92	2.00 ± 0.61	3.86 ± 5.47
Eosinophils (1000/μl)	0.16 ± 0.08	0.07 ± 0.04*	0.09 ± 0.04	0.15 ± 0.11
Basophils (%)	0.36 ± 0.18	0.36 ± 0.33	0.41 ± 0.22	0.65 ± 0.27**^
Basophils (1000/μl)	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.04 ± 0.02**^
Large unstained cells (%)	0.97 ± 0.70	1.19 ± 1.70	0.82 ± 0.49	1.54 ± 1.28
Large unstained cells (1000/μl)	0.04 ± 0.03	0.04 ± 0.04	0.03 ± 0.03	0.07 ± 0.06
Prothrombin time (seconds)	9.6 ± 0.5	10.6 ± 0.9	10.4 ± 0.9	10.1 ± 0.7
Activated partial thromboplastin time (seconds)	26.3 ± 0.0	29.1 ± 0.0	28.3 ± 2.3	30.7 ± 5.8

Results presented are mean ± standard deviation; *statistically significant at $p < 0.05$; **statistically significant at $p < 0.01$;^statistically significant increasing linear trend test.

Table 10. Male clinical chemistry data.

	Gardenia blue dietary dose level			
Parameter	0.0%	0.5%	2.5%	5.0%
Number of males at termination	24	23	22	23
Blood urea nitrogen (mg/dl)	18.4 ± 3.2	20.7 ± 6.4	19.1 ± 5.2	19.6 ± 4.9
Creatinine (mg/dl)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Glucose (mg/dl)	181.0 ± 28.7	184.3 ± 45.6	199.5 ± 36.8	173.8 ± 35.4
Sodium (mmol/L)	161.4 ± 4.8	163.4 ± 5.4	160.9 ± 4.0	160.1 ± 3.7
Potassium (mmol/L)	9.0 ± 0.4	9.1 ± 0.7	9.4 ± 0.8	9.4 ± 0.7
Chloride (mmol/L)	111.1 ± 2.8	113.4 ± 2.6	111.8 ± 2.7	110.7 ± 2.6
Alkaline phosphatase (U/L)	65.2 ± 11.1	67.6 ± 7.8	67.0 ± 6.7	70.8 ± 6.3
Alanine aminotransferase (U/L)	77.6 ± 106.2	88.1 ± 179.4	37.4 ± 21.7	42.3 ± 23.9
Aspartate aminotransferase (U/L)	121.3 ± 118.5	139.8 ± 115.8	92.9 ± 42.6	100.9 ± 43.3
Total bilirubin (mg/dl)	0.2 ± 0.3	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1
Gamma-glutamyl transferase (U/L)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Total protein (g/dl)	6.0 ± 0.2	6.1 ± 0.2	5.9 ± 0.2	5.8 ± 0.3 v
Albumin (g/dl)	3.6 ± 0.2	3.6 ± 0.1	3.5 ± 0.2	3.5 ± 0.1
Globulin (g/dl)	2.5 ± 0.2	2.5 ± 0.1	2.4 ± 0.1	2.4 ± 0.2
Calcium (mg/dl)	10.5 ± 0.3	10.3 ± 0.9	10.8 ± 0.5	10.4 ± 0.5
Phosphorus (mg/dl)	12.0 ± 0.7	11.7 ± 2.6	12.4 ± 0.9	11.8 ± 1.4
Cholesterol (mg/dl)	111.9 ± 12.2	111.7 ± 13.7	115.8 ± 17.8	112.5 ± 20.9
Triglycerides (mg/dl)	174.9 ± 49.1	181.5 ± 56.5	188.6 ± 35.3	188.9 ± 50.1
Glutamate dehydrogenase (U/L)	12.7 ± 3.5	13.3 ± 2.9	10.3 ± 2.5	15.4 ± 2.8

Results presented are mean ± standard deviation; vstatistically significant decreasing linear trend test.

Table 11. Male urinalysis data.

	Gardenia blue dietary dose level			
Parameter	0.0%	0.5%	2.5%	5.0%
Number of males at termination	24	23	22	23
Ketones (mg/dl)	15.0 ± 0.0	15.0 ± 0.0	15.0 ± 0.0	15.0 ± 0.0
Protein (mg/dl)	47.5 ± 35.0	30.0 ± 0.0	30.0 ± 0.0	30.0 ± 0.0
pH	6.8 ± 0.3	6.8 ± 0.3	6.8 ± 0.4	6.6 ± 0.2
Urobilinogen (mg/dl)	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.3	0.4 ± 0.4**^
Specific gravity	1.03 ± 0.02	1.03 ± 0.02	1.04 ± 0.02	1.03 ± 0.02
Bile (μmol/L)	2.5 ± 0.4	3.3 ± 0.4	2.7 ± 2.0	3.3 ± 1.7

Results presented are mean ± standard deviation; **statistically significant at $p < 0.01$; ^statistically significant increasing linear trend test; NS: not enough sample submitted.

discoloration. Spontaneous neoplasms commonly found in transgenic rasH2 mice[34] in various organs in the control and gardenia blue-treated animals included the following: hemangioma, hemangiosarcoma, bronchiolo-alveolar adenoma, Harderian gland adenoma, lymphoma, hepatocellular adenoma, and squamous papilloma. Non-neoplastic lesions comprising a range of background lesions either occurred with similar incidences in control and treated animals or sporadically across all groups. Lesions such as skeletal muscle myopathy,[35] subcapsular cortical hyperplasia of the adrenal glands,[36] and epithelial hyperplasia in the thymus are typical of the transgenic rasH2 mice, whereas lesions such as chronic progressive nephropathy (CPN), cardiomyopathy, and mononuclear cell infiltrates in the lacrimal and salivary glands are common background findings in inbred mice. Neoplastic lesions observed in the stomach and thymus of animals in the positive control group confirmed the efficacy of MNU.

Table 12. Female complete blood count and coagulation data.

	Gardenia blue dietary dose level			
Parameter	0.0%	0.5%	2.5%	5.0%
Number of females at termination	25	24	24	21
White blood cells (1000/μl)	3.62 ± 2.78	3.84 ± 1.43	5.15 ± 1.84	5.62 ± 2.45^
Red blood cells (1000000/μl)	11.48 ± 0.49	10.99 ± 0.45	11.44 ± 0.56	11.21 ± 0.56
Hemoglobin (g/dl)	16.63 ± 0.64	16.03 ± 0.50	16.52 ± 0.76	16.33 ± 0.67
Hematocrit (%)	57.51 ± 2.87	54.81 ± 1.96	57.58 ± 3.71	56.91 ± 3.37
Mean corpuscular volume (fl)	50.10 ± 0.81	49.91 ± 1.02	50.28 ± 1.00	50.76 ± 0.69
Mean corpuscular hemoglobin (pg)	14.48 ± 0.25	14.59 ± 0.40	14.45 ± 0.17	14.56 ± 0.22
Mean corpuscular hemoglobin concentration (g/dl)	28.93 ± 0.67	29.25 ± 0.79	28.73 ± 0.65	28.71 ± 0.58
Platelets (1000/μl)	719.42 ± 197.14	821.00 ± 128.61	773.17 ± 183.74	861.09 ± 120.76
Neutrophils (%)	20.45 ± 7.85	26.22 ± 12.25	19.65 ± 6.45	19.14 ± 11.94
Neutrophils (1000/μl)	0.65 ± 0.36	0.94 ± 0.45	1.00 ± 0.41	0.91 ± 0.51
Lymphocytes (%)	71.24 ± 7.51	65.60 ± 12.76	73.67 ± 7.13	76.10 ± 11.30
Lymphocytes (1000/μl)	2.63 ± 2.20	2.59 ± 1.29	3.83 ± 1.47	4.43 ± 2.07^
Monocytes (%)	1.23 ± 0.46	1.42 ± 0.53	1.47 ± 0.85	1.40 ± 0.73
Monocytes (1000/μl)	0.05 ± 0.04	0.05 ± 0.02	0.08 ± 0.06	0.08 ± 0.05^
Eosinophils (%)	4.74 ± 3.95	4.03 ± 5.15	3.35 ± 2.07	1.69 ± 1.00^v^
Eosinophils (1000/μl)	0.19 ± 0.26	0.15 ± 0.17	0.17 ± 0.10	0.10 ± 0.07
Basophils (%)	0.73 ± 0.38	0.57 ± 0.31	0.61 ± 0.38	0.62 ± 0.29^
Basophils (1000/μl)	0.03 ± 0.02	0.02 ± 0.01	0.03 ± 0.02	0.04 ± 0.02
Large unstained cells (%)	1.62 ± 0.97	2.16 ± 1.85	1.21 ± 1.09	1.04 ± 0.60
Large unstained cells (1000/μl)	0.07 ± 0.10	0.09 ± 0.09	0.06 ± 0.04	0.06 ± 0.04
Prothrombin time (seconds)	10.2 ± 0.6	11.4 ± 1.1	11.0 ± 1.2	10.3 ± 05
Activated partial thromboplastin time (seconds)	26.4 ± 1.8	NS	35.0 ± 8.2	NA

Results presented are mean ± standard deviation;^statistically significant increasing linear trend test; ^v^statistically significant decreasing linear trend test; NS: not enough sample submitted; NA: not analyzed.

Discussion

The rasH2 model (CByB6F1-Tg (HRAS)2Jic) was originally developed to ascertain if in vivo expression of the human c-Ha-ras gene driven by its own promoter would induce tumors.[37,38] Introduction of the rasH2 genetically manipulated mouse model for short-term carcinogenicity testing[39,40] was quickly followed with interlaboratory comparative studies[41–43] and its subsequent regulatory approval in the EU, Japan, and the U.S. as an acceptable bioassay alternative to the 2-years mouse carcinogenicity model.[43–45] The rasH2 model has been well characterized with respect to spontaneous neoplastic and non-neoplastic lesions.[46–48] Its originally defined bioassay study structure included a positive control, a high dose determined in a 30-days range-finding study, and two lower doses, resulting in general adoption of this model for genotoxic and non-genotoxic agents, notwithstanding some concerns and limitations of the model.[45] The rasH2 bioassay of gardenia blue reported here was carried out with a study design including a positive control, a maximum tolerated dose, and two lower doses.

In the present 26-weeks carcinogenicity study in transgenic rasH2 mice, no microscopic finding was noted that could be attributed to the dietary administration of gardenia blue. The range of neoplastic and non-neoplastic lesions reported in this study are well-documented and are all considered spontaneous/background findings, either specific to the transgenic rasH2 mice[34,36–38,46,47,49,50] or common to inbred strains of mice.[51,52] Additionally, incidences of neoplastic lesions observed in the control group in this study were consistent with historical control data published in the literature.[34,39–41,53] There was no microscopic correlate to the treatment-related gross blue discoloration of internal organs (i.e. gastrointestinal tract and mesenteric lymph nodes). Additionally, there were no correlating histopathological changes to explain the statistically significant clinical pathology findings or the

Table 13. Female clinical chemistry data.

	Gardenia blue dietary dose level			
Parameter	0.0%	0.5%	2.5%	5.0%
Number of females at termination	25	24	24	21
Blood urea nitrogen (mg/dl)	21.7 ± 10.4	18.9 ± 9.0	21.4 ± 10.4	20.2 ± 9.4
Creatinine (mg/dl)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Glucose (mg/dl)	198.2 ± 34.9	195.4 ± 67.8	163.4 ± 27.9	187.4 ± 19.7
Sodium (mmol/L)	160.8 ± 3.2	153.9 ± 10.4	158.3 ± 9.2	162.6 ± 4.4
Potassium (mmol/L)	9.4 ± 0.6	8.7 ± 0.4	8.1 ± 0.7	9.5 ± 0.4
Chloride (mmol/L)	114.0 ± 2.8	107.9 ± 7.1	111.4 ± 6.5	113.4 ± 4.7
Alkaline phosphatase (U/L)	99.3 ± 15.4	101.0 ± 12.1	103.2 ± 18.6	105.0 ± 15.9
Alanine aminotransferase (U/L)	38.3 ± 15.9	67.5 ± 71.6	46.2 ± 27.8	67.6 ± 47.0
Aspartate aminotransferase (U/L)	155.1 ± 101.4	172.1 ± 103.3	149.8 ± 97.6	180.5 ± 93.8
Total bilirubin (mg/dl)	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Gamma-glutamyltransferase (U/L)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	NA
Total protein (g/dl)	5.8 ± 0.2	5.7 ± 0.2	5.6±0.3	5.6 ± 0.3
Albumin (g/dl)	3.6 ± 0.1	3.5 ± 0.2	3.4 ± 0.2	3.4 ± 0.2$^{\vee}$
Globulin (g/dl)	2.2 ± 0.1	2.2 ± 0.1	2.2 ± 0.2	2.2 ± 0.1
Calcium (mg/dl)	10.6 ± 0.1	10.4 ± 0.5	10.3 ± 0.7	10.2 ± 05
Phosphorus (mg/dl)	10.6 ± 1.9	11.1 ± 0.8	9.4 ± 1.5	9.7 ± 2.0
Cholesterol (mg/dl)	77.7 ± 9.0	74.8 ± 4.9	74.7 ± 15.3	73.7 ± 10.2
Triglycerides (mg/dl)	158.1 ± 48.9	159.4 ± 42.1	163.9 ± 54.9	194.5 ± 55.9
Glutamate dehydrogenase (U/L)	10.0 ± 0.0	10.0 ± 0.0	NS	NA

Results presented are mean ± standard deviation; $^{\vee}$: statistically significant decreasing linear trend test; NS: not enough sample submitted; NA: not analyzed.

Table 14. Female urinalysis data.

	Gardenia blue dietary dose level			
Parameter	0.0%	0.5%	2.5%	5.0%
Number of females at termination	25	24	24	21
Ketones (mg/dl)	15.0 ± 0.0	15.0 ± 0.0	15.0 ± 0.0	17.5 ± 7.9
Protein (mg/dl)	NS	30.0 ± 0.0	30.0 ± 0.0	100.0 ± 0.0*$^{\wedge}$
pH	6.6 ± 0.2	6.6 ± 0.2	6.5 ± 0.3	6.6 ± 0.2
Urobilinogen (mg/dl)	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.3	0.4 ± 0.5
Specific gravity	1.02 ± 0.0	1.02 ± 0.01	1.02 ± 0.01	1.03 ± 0.01
Bile (μmol/L)	3.9 ± 0.0	NA	NA	NA

Results presented are mean ± standard deviation; *statistically significant at $p < 0.05$; $^{\wedge}$statistically significant increasing linear trend test; NS: not enough sample submitted; NA: not analyzed.

Table 15. Tissues with histopathological changes (control and high dose groups).

	Male		Female	
Dietary percentage of gardenia blue	0.0%	5.0%	0.0%	5.0%
Number of animals	25	25	25	23
Adrenal glands				
Cortex, subcapsular hyperplasia, bilateral, minimal	2	3	4	6
Cortex, subcapsular hyperplasia, unilateral, minimal	15	11	1	0
Cortex, accessory structure	0	0	2	0
Bone marrow, femur				
Lymphoma	1	0	0	0
Cavity, nasal				
Olfactory epithelium, hyaline droplet accumulation, minimal	0	0	4	1
Respiratory epithelium, hyaline droplet accumulation, minimal	0	0	5	0
Respiratory epithelium, goblet cell hyperplasia, minimal	1	0	0	0
Olfactory epithelium, acute inflammation, minimal	0	0	1	0
Epididymides				
Interstitium, inflammation, chronic, minimal	1	0	—	—
Infiltration, mononuclear cell, minimal	0	1	—	—
Sperm granuloma, mild	0	2	—	—
Sperm granuloma, moderate	0	2	—	—
Eyes with optic nerve				
Periorbital infiltration, mononuclear cell, mild	0	0	0	1
Heart				
Inflammation, chronic-active, minimal	0	0	1	0
Mineralization, minimal	0	1	0	0
Mineralization, mild	0	1	0	0
Cardiomyopathy, minimal	23	22	19	23
Cardiomyopathy, mild	2	0	0	0
Intestine, ileum				
Hemangiosarcoma	0	1	0	0
Crypt, necrosis, minimal	1	0	0	0
Intestine, jejunum				
Paneth cell, hypertrophy, minimal	0	1	0	0
Kidneys				
Hemangiosarcoma	1	0	0	0
Chronic progressive nephropathy, minimal	13	16	9	10
Chronic progressive nephropathy, mild	0	1	0	0
Pelvis, infiltration, lymphocytic, mild	0	1	0	0
Lacrimal glands				
Infiltration, mononuclear cell, minimal	9	1	12	2
Infiltration, mononuclear cell, mild	1	1	1	0
Liver				
Hepatocellular adenoma	0	1	0	0
Basophilic focus	1	0	1	0
Clear cell focus	1	1	0	0
Inflammation, focal, minimal	10	8	14	13
Hepatocyte necrosis, minimal	2	0	0	1
Mineralization, vascular, minimal	1	0	0	0
Skeletal muscle				
Myopathy, minimal	3	6	1	2
Myopathy, mild	17	17	21	20
Myopathy, moderate	4	1	3	0

(continued)

Table 15. (continued)

	Male		Female	
Dietary percentage of gardenia blue	0.0%	5.0%	0.0%	5.0%
Number of animals	25	25	25	23
Ovaries				
Angiectasis, minimal	—	—	2	4
Pancreas				
Infiltration, mononuclear cell, minimal	1	0	0	0
Acinar cell hypertrophy, minimal	4	2	9	1
Acinar cell hypertrophy, mild	2	0	1	1
Acinar cell hypertrophy, moderate	0	0	1	1
Salivary glands				
Infiltration, mononuclear cell, minimal	8	6	12	11
Infiltration, mononuclear cell, mild	4	3	1	2
Stomach				
Hemangiosarcoma	0	0	1	0
Glandular epithelium, hyperplasia, mild	0	0	1	0
Non-glandular epithelium, hyperplasia, mild	0	0	1	0
Non-glandular epithelium, hyperplasia, moderate	0	0	0	1
Non-glandular epithelium, hyperkeratosis, mild	0	0	1	2
Glandular epithelium, squamous metaplasia, mild	0	0	1	0
Testes				
Hemangiosarcoma	1	0	—	—
Tubule, degeneration, bilateral, minimal	0	1	—	—
Tubule, degeneration, unilateral, minimal	1	0	—	—
Tubule, degeneration, unilateral, mil	0	2	—	—
Thymus				
Hemangiosarcoma, metastatic	0	0	1	0
Cyst	1	0	0	0
Hyperplasia, epithelial, mild	1	0	0	1
Hyperplasia, epithelial, moderate	0	0	0	1
Hyperplasia, epithelial, marked	0	0	1	0
Uterus				
Papilloma, squamous	—	—	0	1
Endometrium, hyperplasia, cystic, mild	—	—	14	7
Endometrium, hyperplasia, cystic, moderate	—	—	19	14
Endometrium, hyperplasia, cystic, marked	—	—	2	0
Polyp, glandular	—	—	0	2
Head				
Adenocarcinoma	1	—	—	—
Thyroid glands				
Cyst, follicular	1	1	0	0

Table 16. Histopathological changes in harderian glands, lungs, and spleen.

	Male				Female			
Dietary percentage of gardenia blue	0.0%	0.5%	2.5%	5.0%	0.0%	0.5%	2.5%	5.0%
Number of animals	25	25	25	25	25	24	25	23
Harderian glands								
Adenoma	0	0	0	0	1	1	0	0
Hyperplasia, minimal	0	0	0	2	0	0	0	0
Hyperplasia, mild	1	0	0	0	0	0	0	0
Hyperplasia, moderate	0	0	0	0	0	0	0	1
Hyperplasia, marked	0	0	0	0	0	1	0	0
Infiltration, mononuclear cell, minimal	3	1	0	3	1	0	0	1
Lungs								
Bronchiolo-alveolar adenoma	1	1	2	2	1	0	1	0
Hemangiosarcoma	0	0	0	0	1	0	0	0
Lymphoma	0	0	1	0	0	0	0	0
Alveolar epithelium, hyperplasia, mild	0	0	0	0	1	0	0	0
Alveolar epithelium, hyperplasia, moderate	0	0	1	0	1	0	1	0
Alveolar epithelium, hyperplasia, marked	0	0	0	0	1	0	0	0
Metaplasia, osseous, minimal	0	0	0	0	1	0	0	0
Spleen								
Hemangioma	0	1	0	2	2	1	0	0
Hemangiosarcoma	1	0	2	0	0	0	0	0
Lymphoma	1	0	1	0	0	0	0	0
Hyperplasia, lymphoid, marked	0	0	0	0	1	0	0	0

relative and absolute organ weight changes in both sexes. Consistent with recently reported gardenia blue toxicity studies,[14–16] the present study with up to 5% dietary exposure of rasH2 mice to gardenia blue produced macroscopic tissue color changes that were not correlated with microscopic tissue changes and did not result in treatment-induced neoplastic or non-neoplastic lesions.

Author contributions

R Maronpot wrote the study protocol, the first manuscript draft, and edited the final submission. M Koyanagi, S Hayashi, M Nishino and M Iniwa reviewed and approved the study protocol and edited all draft versions of the manuscript. D Mahapatra conducted the gross and microscopic pathology and reviewed and edited the manuscript.

Declaration of conflicting interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: R Maronpot is a paid consultant to San-Ei Gen (SEG) and monitored all aspects of the study and drafted the manuscript. M Koyanagi, S Hayashi, M Nishino and M Iniwa are employees of SEG, provided the funding to Inotiv for the study conduct, and reviewed and edited the manuscript. D Mahapatra is an employee of the contract laboratory where the study was done and served as the study pathologist who supervised the necropsy and diagnosed all tissue changes and reviewed and edited the manuscript.

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Ethical approval

Ethical approval to conduct the animal studies for this project was obtained by the Inotiv Animal Welfare Ethics Committee prior to commencement of the study.

Informed consent

Patient data is not part of the current submission. Therefore, informed consent is not applicable.

Data availability statement

The datasets generated and/or analysed during the current study are available from Inotiv, Inc., Keystone Park Drive, Suite 200, Morrisville, North Carolina 27560 USA.

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