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alpha-Glycosyl Isoquercitrin (AGIQ) and its lack of carcinogenicity in rasH2 mice

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ABSTRACT

alpha-Glycosyl Isoquercitrin (AGIQ), is used in Japan as a food additive and was granted generally recognized as safe (GRAS) status in 2005 (FEMA) and 2007 (FDA). The safety and toxicity information for AGIQ is sparse and therefore, the carcinogenicity potential of AGIQ was examined in the CByB6F1-Tg(HRAS)2Jic (rasH2) model. One hundred female and male rasH2 mice, each, were allocated to one of four designated dose groups; 0 (control)%, 1.5%, 3.0% or 5.0% AGIQ. Animals were administered the diets for six months and an additional 10 females and 10 males, each, were administered a positive control, *N*-methyl-*N*-nitrosourea (MNU). Body weights and clinical observations were collected. A full screen necropsy, organ weights, clinical chemistry, urinalysis and histopathology were performed. The positive control animals elicited appropriate responses specific to this strain (rasH2) of mice. There were statistically significant sporadic non-dose-dependent changes in clinical chemistries without corresponding pathological correlation. No microscopic AGIQ-related findings were noted; the range of pathology observations were all considered background findings, either specific to rasH2 mice or common to inbred strains of mice. Therefore, under the study conditions, the no-observed-adverse-effect level (NOAEL) was determined to be more than 5.0% (7215.4 mg/kg BW/day in male mice and 14685.5 mg/kg/day in female mice).

1. Introduction

alpha-Glycosyl Isoquercitrin (AGIQ) or enzymatically modified isoquercitrin (EMIQ), is a water soluble flavanol obtained when multiple glucose moieties are conjugated to isoquercitrin. The latter is derived from enzymatic decomposition of rutin, a natural glycoside isolated from buds and flowers of Japanese Pagoda tree (*Sophora japonica*). Owing to its enhanced bioavailability and potent anti-oxidant properties, benefits from AGIQ include potent anti-inflammatory, anti-mutagenic, tumor suppressive, and cardio-protective functions (Manach et al., 1997; Yang et al., 2005; Makino et al., 2009; Amado et al., 2009; Li et al., 2011; Valentova et al., 2014; Wang et al., 2015; Shimada et al., 2010; Hara et al., 2014; Xiong et al., 2019).

The safety and toxicity of highly purified AGIQ have been evaluated in GLP compliant animal studies including a repeat dose 90-day toxicity and a single dose toxicokinetic (TK) study in rats (Nyska et al., 2016), a comprehensive genetic toxicity study (Hobbs et al., 2018), and a recent ten- and four-week toxicity and toxicokinetic study in juvenile Gottingen minipigs (Maronpot et al., 2019). Earlier reports have also shown AGIQ to be non-carcinogenic, although, these studies lack validated test article purity and were non-GLP compliant (Salim et al., 2004; Harwood et al., 2007). Given, these limitations, data on carcinogenic potential of AGIQ is still underreported.

Tg.rasH2 or transgenic CByB6F1-Tg(HRAS)2Jic is a hemizygous mouse carrying at least 3 copies of the human c-Ha-ras gene in tandem with its own promoter/enhancer element that codes for ras p21 protein. The over expression of this protein predisposes the mice to spontaneous and chemically induced neoplasia without mutations in the c-Ha-ras gene (Yamamoto et al., 1998; Usui et al., 2001; Tamaoki 2001; Morton et al., 2002).

Tg.rasH2 offers an edge over traditional 2-year carcinogenicity bioassays in terms of better predictability of human carcinogens, fewer false

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positives, shorter duration, and the number of animals used for testing (Alden et al., 2002). It also serves as a reliable alternative model for a 26-week short-term carcinogenicity assay for testing both genotoxic and non-genotoxic chemicals administered via oral and parenteral routes (Long et al., 2010). Since its official acceptance by the regulatory agencies in 2003 (Takaoka et al., 2003), a number of studies conducted over the years have generated sufficient historical control data, and reported a range of spontaneous neoplastic and non-neoplastic lesions in addition to chemically induced lesions in these mice (Nambiar et al., 2012; Paranjpe et al., 2013, 2016; Boyle et al., 2018; Kuroda et al., 2018).

The purpose of this study was to determine the carcinogenicity potential of AGIQ in transgenic rasH2 mice.

2. Methods

2.1. Animal husbandry and maintenance

rasH2 mice of approximately 5 weeks of age were obtained from Taconic Biosciences, Inc. (Germantown, NJ), housed individually in polycarbonate caging with microinsulator tops and absorbent heat-treated hardwood bedding (Northeastern Products Corps., Warrensburg, NY) that was changed once a week. All animals were given *ad libitum reverse* osmosis treated tap water (City of Durham, NC) that was tested by National Testing Laboratories Inc (Cleveland, OH) and Purina certified 5002 meal diet (Ralston Purina Co., St. louis, MO) that was blended with AGIQ (>97% purity, quercitrin: 0.13%) at concentrations of 0 (control), 1.5%, 3.0% and 5.0% (w/w) (Davis, 2018) by RTI International (Research Triangle Park, NC, USA). The stability of 0.5% and 5.0% AGIQ in dosed diets stored between 0 and 10 °C was determined to be at least 63 days (Moundous 2015). Treatment diets were batch analyzed and tested for homogeneity and concentration prior to administration. Dose formulations for all samples showed homogeneity with a coefficient of variability between 0.23 and 5.66% while the dietary AGIQ passed protocol specifications with recovery between 87.8% and 100.8%. The mice were allowed at least 7 days acclimation period prior to inclusion in the study and thereafter maintained in study exclusive housing equipped with a 12/12 h light/dark cycle at 20–25 °C and with a relative humidity ranging from 30 to 70%. Upon approval from Integrated Laboratory Systems (ILS), LLC. (Research Triangle Park, NC, USA) Animal Care and Use Committee, all procedures during the study were carried out in compliance with the United States Food and Drug Administration (FDA)'s Good Laboratory Practice (GLP) regulations (21 CFR Part 58) (GLP, 2019), 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* (ILAR, 2011).

2.2. Experimental design

This study was designed as an alternative using transgenic mice to the testing guideline 451:

Carcinogenicity Studies (OECD, 2009). One hundred female and one hundred male rasH2 mice were allocated to one of four designated dose groups. The animals were administered one of three dose levels of AGIQ via the feed, or the carrier diet, for at least six months (26 weeks). An additional ten females and ten males were administered MNU (*N*-methyl-*N*-nitrosourea), the positive control, via a single intraperitoneal injection (Table 1). Body weights and clinical observations were performed weekly with daily cage-side observations. After at least six months of exposure, animals were humanely euthanized by carbon dioxide inhalation followed by exsanguination. Prior to termination, animals were fasted overnight and an 18-h urine sample was collected. A full screen necropsy was performed with collection of selected tissue weights.

Table 1
Experimental design.

Dose Group	Sex	No. of animals	*alpha*-Glycosyl Isoquercitrin Dose Level (%)
1	M	25	0.0
	F	25	
2	M	25	1.5
	F	25	
3	M	25	3.0
	F	25	
4	M	25	5.0
	F	25	
5	M	10	MNU- 75 mg/kg
	F	10	

2.3. Viability, clinical signs, body weight and food consumption

Observations for morbidity and mortality was performed twice daily on weekdays and once daily on weekends and holidays following the initiation of dosing on day 1. Body weight measurements were performed within two days of arrival, during allocation to an exposure group, prior to exposure on day 1 and weekly, thereafter, and finally 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.

2.4. Hematology, biochemistry and coagulation

Whole blood, blood smears, plasma, and serum samples were collected and analyzed for hematology, biochemistry, coagulation parameters, and hormone levels from at least 10 animals/sex/AGIQ group. Blood was not collected from the MNU positive control animals.

2.5. Necropsy and tissue handling

All animals were euthanized by CO_2 asphyxiation followed by exsanguination and complete necropsies were performed after at least 6 months of exposure. All organs and tissues were examined for grossly visible lesions. Tissues were fixed in 10% neutral buffered formalin (NBF) or Davidson's solution, embedded in paraffin, sectioned at 5 μm thickness, and stained by hematoxylin and eosin. The following tissues were examined microscopically in males and/or female AGIQ treated animals: adrenals, brain (cerebrum, cerebellum, medulla/pons), bone/bone marrow (sternum and femur), epididymides, esophagus, eyes, Harderian glands, heart, intestines (small and large including Peyer's patches), kidneys, lacrimal gland, liver with gall bladder, lungs with trachea, lymph nodes (mesenteric and mandibular), mammary glands, muscle (skeletal), nasal cavity, ovaries, pancreas, salivary glands, sciatic nerve, seminal vesicles, skin, spinal cord (cervical, mid-thoracic, lumbar), spleen, stomach, testes, thymus, thyroid/parathyroid, urinary bladder, uterus with cervix, and vagina. Thymus, spleen, and stomach from MNU treated animals were evaluated microscopically to confirm the expected positive control response. A grading scheme derived from Schafer et al. (2018) 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). Following diagnosis by the study pathologist, a peer review was conducted to confirm the accuracy of histopathologic diagnosis as outlined by the Society of Toxicologic Pathology (STP) (Morton et al., 2010).

2.6. Statistical analysis

For this study, the group means and standard deviations were calculated and reported. All data from AGIQ treated animals were analyzed using Statistical Analysis System version 9.2 (SAS Institute, Cary NC) to include: final body weight; body weight gain; food

consumption (g/kg/day); absolute and relative tissue weights, urinalysis, clinical pathology and histopathology. Studentized residual plots were first used to detect possible outliers in the data. Then homogeneity of variance was analyzed using Levene's test. For heterogeneous data, appropriate log transformations were performed and the data was re-analyzed for homogeneity of variance. One-way analysis of variance (ANOVA) was then used to compare AGIQ treated groups to the appropriate control groups using Dunnett's test. Finally, dose-dependent changes were evaluated using the linear regression model. If data could not be transformed to be homogenous, data were evaluated using the non-parametric Dunn's test and dose-dependent changes were analyzed using the non-parametric Jonckheere's trend test.

3. Results

3.1. Survival, clinical observations, body and organ weights

All animals in the control and exposed groups survived to the scheduled sacrifice with the exception of thirteen animals sporadically found dead or moribund across all groups and sexes; no AGIQ-related macroscopic or microscopic effects were noted. There were no AGIQ-related abnormal clinical or cage-side observations.

There were no statistically significant differences in the final body weights or body weight gain in the male mice. Female mice exhibited a statistically lower final body weight and overall body weight gain in the 3% and 5% groups (Table 2B), when compared to control. These findings did not correlate with an overall increase or decrease in food consumption (Table 3B) and did not correlate with any histopathological changes. Males in the 5% dose group consumed significantly more feed when compared to the controls (Table 3A); however, there was no corresponding increase in body weight (Table 2A).

Group organs weight data are listed in Table 4A and 5A for males and Table 4B and 5B for females. There was a significant decrease in absolute testicular weight in 1.5% group and a significant increase in the relative spleen weight in the 5% group compared to the control animals in the male mice. In female mice, there was a significant decrease in absolute kidney and liver weights in the 5% group, and a significant decrease in absolute heart weight in the 3% and 5% groups. The relative brain weight was also significantly increased in the 5% group compared to controls. These findings in either sex did not correlate with any pathologic findings.

3.2. Hematology, coagulation and clinical chemistry

Mean hematological parameters, coagulation values and clinical chemistry analysis are presented in Table 6A and 7A for males and Table 6B and 7B for females. Across dose groups and sexes, sporadic incidences of non-dose dependent statistically significant changes were observed without corresponding correlative? findings in histopathology. Male rats had a statistically significant decrease in HCT, MCH, absolute eosinophil counts, eosinophil percentage and absolute basophil counts in the 5% dose group and a decrease in the absolute basophil counts in the 3% dose group. Female rats had a statistically significant decrease in white blood cell count, MCH, and absolute neutrophil count in the 5% dose group and a decrease in the MCH in the 3% dose group. No significant changes were observed in the coagulation parameters in both sexes Table 7A and 7B

Table 2A
Body Weight Changes in Male rasH2 Mice Administered AGIQ.

Dose Level (percent)	Initial Group Mean Body Weight (g) ± SD	Final Group Mean Body Weight (g) ± SD	Mean Body Weight Gain (g) ± SD
0.0	24.8 ± 1.1	33.0 ± 4.7	9.4 ± 4.0
1.5	24.5 ± 1.2	34.7 ± 3.5	10.4 ± 3.1
3.0	24.8 ± 1.3	33.0 ± 5.2	9.1 ± 2.9
5.0	24.4 ± 1.2	31.9 ± 3.5	7.7 ± 2.3

Table 2B
Body Weight Changes in Female rasH2 Mice Administered AGIQ.

Dose Level (percent)	Initial Group Mean Body Weight (g) ± SD	Final Group Mean Body Weight (g) ± SD	Mean Body Weight Gain (g) ± SD
0.0	19.7 ± 1.0	26.2 ± 2.8	7.0 ± 1.8
1.5	19.5 ± 1.0	25.3 ± 3.2	6.1 ± 2.0
3.0	19.7 ± 0.9	**25.0* ± 1.7**	**5.3** ± 1.3**
5.0	19.6 ± 1.0	**24.1* ± 1.5**	**5.1** ± 0.8**

Statistically significant at $p < 0.05$ (*) and $p < 0.005$ (**) by linear trend test.

Table 3A
Food and Test Article Consumption in Male rasH2 Mice Administered AGIQ.

Dose Level (percent)	Food Consumption ± SD (g/kg body weight/day)	Test Article ± SD (mg/kg body weight/day)
0.0	131.16 ± 9.7	0.0 ± 0.0
1.5	134.80 ± 11.5	2022.0 ± 171.9
3.0	138.54 ± 12.5	4156.3 ± 373.8
5.0	**144.31** ± 15.3**	7215.4 ± 763.7

Statistically significant at $p < 0.005$ (**) by linear trend test.

Table 3B
Food and Test Article Consumption in Female rasH2 Mice Administered AGIQ.

Dose Level (percent)	Food Consumption ± SD (g/kg body weight/day)	Test Article (mg/kg body weight/day)
0.0	281.29 ± 45.6	0.0 ± 0.0
1.5	258.80 ± 58.6	3882.0 ± 878.8
3.0	300.25 ± 47.8	9007.4 ± 1434.4
5.0	293.71 ± 53.4	14685.5 ± 2672.2

3.3. Urinalysis

No significant changes were observed in any of the urinalysis parameters (volume, appearance, specific gravity, glucose, protein, occult blood, ketones, bilirubin, urobilinogen, and sodium). There was a significant increase in the urine pH in 3% group in males (Table 8A) and a significant decrease in urinary potassium in the 5% group in females (Table 8B). While the urine pH value was non-dose dependent, the urinary potassium was considered dose-dependent although both changes were unrelated to administration of the test article.

3.4. Macroscopic and microscopic observations

The only macroscopic finding that was considered test article related was the yellow discoloration of the femur and calvarium. Additionally, sporadic incidences of mass lesions, tissue discolorations, and organ enlargements observed at necropsy were documented. These lesions occurred across control, and AGIQ dose groups at similar frequencies and were not considered test article related.

Stained sections of all tissues from control and high dose animals, and select tissues (spleen, Harderian glands and lungs) from intermediate dose animals were evaluated by light microscopy (Table 9). No observed microscopic finding was considered to be test article related. No histologic correlate to the grossly observed yellow discoloration of the femur was apparent. A gamut of spontaneous histologic changes was observed in various tissues across dose groups and sexes including the control group.

Neoplastic lesions included hemangiomas, hemangiosarcomas, bronchiolo-alveolar adenomas, bronchiolo-alveolar carcinomas, Harderian gland adenoma, squamous cell carcinoma, lymphoma,

Table 4A
Organ Weights in Male rasH2 Mice administered AGIQ.

Group No.	Dose Level (percent)	Adrenals (paired)	Testes (paired)	Epididymides (paired)	Liver	Spleen	Kidneys (paired)	Heart	Brain
1	0.0	0.00764 ± 0.00271	0.28289 ± 0.01932	0.11483 ± 0.01685	1.42684 ± 0.14891	0.07933 ± 0.01790	0.60359 ± 0.06741	0.24621 ± 0.11927	0.49541 ± 0.01466
2	1.5	0.00683 ± 0.00243	**0.26824* ± 0.02233**	0.11398 ± 0.00938	1.46283 ± 0.20066	0.07900 ± 0.01357	0.59249 ± 0.05168	0.22265 ± 0.04025	0.49719 ± 0.04652
3	3.0	0.00685 ± 0.00221	0.28292 ± 0.04096	0.11058 ± 0.01571	1.45209 ± 0.26859	0.07707 ± 0.01642	0.58671 ± 0.09858	0.21693 ± 0.04015	0.48964 ± 0.01699
4	5.0	0.00722 ± 0.00262	0.27334 ± 0.02008	0.10932 ± 0.01236	1.43868 ± 0.18654	0.07903 ± 0.01091	0.56842 ± 0.05979	0.20746 ± 0.02316	0.49154 ± 0.03171

Weight provided as g ± SD. Statistically significant at $p < 0.05$ (*) by Dunnett's test.

Table 4B
Organ Weights in Female rasH2 Mice administered AGIQ.

Group No.	Dose Level (percent)	Adrenals (paired)	Uterus	Ovaries (paired)	Liver	Spleen	Kidneys (paired)	Heart	Brain
1	0.0	0.09941 ± 0.42206	0.30915 ± 0.10529	0.02375 ± 0.00593	1.31320 ± 0.13586	0.10658 ± 0.02010	0.43127 ± 0.03260	0.17658 ± 0.02715	0.52491 ± 0.02297
2	1.5	0.01202 ± 0.00224	0.27209 ± 0.09021	0.02088 ± 0.00638	1.43787 ± 0.80329	0.12879 ± 0.15060	0.42313 ± 0.05319	0.17237 ± 0.02213	0.52086 ± 0.01791
3	3.0	0.01170 ± 0.00229	0.26053 ± 0.08923	0.02305 ± 0.00527	1.26634 ± 0.14471	0.11168 ± 0.06067	0.41316 ± 0.03291	**0.16704* ± 0.02052**	0.51973 ± 0.01655
4	5.0	0.01128 ± 0.00191	0.30693 ± 0.08798	0.02098 ± 0.00388	**1.14397* ± 0.33462**	0.18987 ± 0.31220	**0.38913** ± 0.06954**	**0.15976* ± 0.02046**	0.51269 ± 0.04677

Weight provided as g ± SD. Statistically significant at $p < 0.05$ (*) and $p < 0.005$ (**) by Dunnett's test.

Table 5A
Relative Organ Weights in Male rasH2 Mice Administered AGIQ.

Group No.	Dose Level (percent)	Adrenals (paired)	Testes (paired)	Epididymides (paired)	Liver	Spleen	Kidneys (paired)	Heart	Brain
1	0.0	0.0239 ± 0.0105	0.866 ± 0.150	0.3470 ± 0.0256	4.323 ± 0.452	0.248 ± 0.079	1.835 ± 0.232	0.753 ± 0.409	1.530 ± 0.244
2	1.5	0.0193 ± 0.0073	0.777 ± 0.103	0.3334 ± 0.0369	4.114 ± 0.278	0.232 ± 0.043	1.701 ± 0.142	0.621 ± 0.080	1.440 ± 0.133
3	3.0	0.0211 ± 0.0081	0.883 ± 0.166	0.3408 ± 0.0397	4.388 ± 0.562	0.230 ± 0.029	1.779 ± 0.176	0.664 ± 0.122	1.528 ± 0.279
4	5.0	0.0219 ± 0.0067	0.870 ± 0.072	0.3504 ± 0.0360	4.444 ± 0.205	**0.246^ ± 0.033**	1.774 ± 0.106	0.655 ± 0.097	1.576 ± 0.262

Weight provided as % ± SD. ^Heterogenous data set with significant pair-wise difference by non-parametric Dunn's test.

Table 5B
Relative Organ Weights in Female rasH2 Mice Administered AGIQ.

Group No.	Dose Level (percent)	Adrenals (paired)	Uterus	Ovaries (paired)	Liver	Spleen	Kidneys (paired)	Heart	Brain
1	0.0	0.0507 ± 0.0103	1.1620± 0.4291	0.092 ± 0.029	4.978 ± 0.479	0.418 ± 0.133	1.641 ± 0.137	0.671 ± 0.087	1.999 ± 0.199
2	1.5	0.0477 ± 0.0088	1.0320± 0.3330	0.082 ± 0.024	5.627 ± 2.663	0.510 ± 0.517	1.675 ± 0.153	0.679 ± 0.093	2.061 ± 0.195
3	3.0	0.0477 ± 0.0096	1.0510± 0.3799	0.094 ± 0.023	5.014 ± 0.403	0.456 ± 0.242	1.644 ± 0.092	0.662 ± 0.069	2.090 ± 0.142
4	5.0	0.0468 ± 0.0077	1.2741± 0.3867	0.084 ± 0.016	4.799 ± 0.999	0.845 ± 1.400	1.567 ± 0.289	0.650 ± 0.082	**2.098*± 0.221**

Weight provided as % ± SD. Statistically significant at $p < 0.05$ (*) by Dunnett's test.

granulocytic leukemia, and thymoma. Among them, hemangiosarcomas had the highest incidence and commonly originated in the spleen and rarely elsewhere in organs such as the brain, testis, and uterus. Bronchiolo-alveolar adenomas had the second highest incidence followed by other tumors with minor occurrences. Squamous cell carcinoma, bronchiolo-alveolar carcinomas, lymphoma and granulocytic leukemia comprised the list of metastatic tumors. The incidences of the neoplastic lesions in this study are in range and consistent with the historical control data (Table 10) obtained from ILS's internal database and those published for this strain (Kanno et al., 2003, Paranjpe et al., 2018, Davis J. 2019(unpublished report)). The positive control (MNU-treated) group had incidences of malignant lymphomas in the stomach, thymus and spleen.

Non-neoplastic lesions included a range of spontaneous hyperplastic, hypertrophic, degenerative, infiltrative, and inflammatory lesions that either occurred with similar incidences in control and high dose (or

Table 6A
Changes in Clinical Pathology in Male rasH2 Mice Administered AGIQ.

	Group No.	Dose Level	WBC	RBC	HB	HCT	MCV	MCH	MCHC	Platelet
		(percent)	(1000/μL)	(1000000/μL)	(g/dL)	(%)	(fL)	(pg)	(g/dL)	(1000/μL)
Mean ± SD	1	0.0%	4.29 ± 1.36	12.38 ± 0.75	17.08 ± 0.72	60.05 ± 3.86	48.48 ± 0.78	13.81 ± 0.71	28.51 ± 1.44	526.85 ± 23.72
	2	1.5%	4.67 ± 1.35	12.00 ± 0.29	16.51 ± 0.50	58.15 ± 1.81	48.46 ± 0.65	13.77 ± 0.25	28.43 ± 0.48	682.07 ± 59.49
	3	3.0%	4.37 ± 1.59	11.97 ± 0.30	16.34 ± 0.57	58.03 ± 2.07	48.48 ± 1.23	13.68 ± 0.41	28.20 ± 0.76	698.33 ± 78.35
	4	5.0%	4.04 ± 1.38	11.82 ± 0.64	16.14 ± 0.74	**57.14* ± 3.12**	48.35 ± 0.76	**13.66* ± 0.26**	28.29 ± 0.58	719.14 ± 45.68

	Group No.	Dose Level	NEU	NEU	LYM	LYM	MONO	MONO	EOS	EOS	BASO
		(percent)	(%)	(1000/μL)	(%)	(1000/μL)	(%)	(1000/μL)	(%)	(1000/μL)	(%)
Mean ± SD	1	0.0%	17.92 ± 5.94	0.74 ± 0.31	74.56 ± 6.11	3.22 ± 1.07	1.50 ± 0.53	0.07 ± 0.03	2.60 ± 1.78	0.12 ± 0.09	0.82 ± 0.62
	2	1.5%	14.99 ± 7.17	0.63 ± 0.17	79.00 ± 8.04	3.76 ± 1.21	1.29 ± 0.32	0.06 ± 0.02	2.44 ± 1.36	0.11 ± 0.08	0.75 ± 0.76
	3	3.0%	16.68 ± 3.44	0.71 ± 0.22	77.26 ± 3.52	3.39 ± 1.30	1.38 ± 0.89	0.06 ± 0.04	2.67 ± 2.40	0.13 ± 0.18	0.44 ± 0.57
	4	5.0%	18.99 ± 5.65	0.75 ± 0.28	76.88 ± 5.50	3.13 ± 1.14	1.09 ± 0.69	0.05 ± 0.04	**1.39* ± 0.93**	**0.05* ± 0.04**	0.39 ± 0.44

	Group No.	Dose Level	BASO	LUC	LUC
		(percent)	(1000/μL)	(%)	(1000/μL)
Mean ± SD	1	0.0%	0.04 ± 0.03	2.64 ± 02.28	0.11 ± 0.10
	2	1.5%	0.04 ± 0.05	1.51 ± 1.01	0.07 ± 0.06
	3	3.0%	**0.02* ± 0.04**	1.60 ± 1.57	0.06 ± 0.05
	4	5.0%	**0.01* ± 0.01**	1.26 ± 0.88	0.05 ± 0.03

Statistically significant at $p < 0.05$ (*) by Dunnett's test.

Table 6B
Changes in Clinical Pathology in Female rasH2 Mice Administered AGIQ.

	Group No.	Dose Level	WBC	RBC	HB	HCT	MCV	MCH	MCHC	Platelet
		(percent)	(1000/μL)	(1000000/μL)	(g/dL)	(%)	(fL)	(pg)	(g/dL)	(1000/μL)
Mean ± SD	1	0.0%	5.25 ± 1.37	11.38 ± 0.65	16.63 ± 0.90	57.52 ± 3.43	50.51 ± 1.09	14.61 ± 0.45	28.92 ± 0.97	426.8 ± 201.72
	2	1.5%	10.08 ± 8.30	10.95 ± 1.33	15.66 ± 1.58	54.86 ± 5.59	50.25 ± 1.89	14.34 ± 0.49	28.55 ± 0.68	556.4 ± 126.98
	3	3.0%	5.47 ± 1.56	11.38 ± 0.46	16.17 ± 0.60	57.09 ± 2.53	50.13 ± 0.97	**14.23* ± 0.32**	28.38 ± 0.60	567.7 ± 120.22
	4	5.0%	**3.49^ ± 1.01**	11.45 ± 0.93	16.52 ± 0.57	57.13 ± 5.55	49.82 ± 1.17	**14.49* ± 1.09**	29.17 ± 2.99	569.9 ± 204.93

	Group No.	Dose Level	NEU	NEU	LYM	LYM	MONO	MONO	EOS	EOS
		(percent)	(%)	(1000/μL)	(%)	(1000/μL)	(%)	(1000/μL)	(%)	(1000/μL)
Mean ± SD	1	0.0%	15.09 ± 8.03	0.80 ± 0.49	78.50 ± 5.24	4.11 ± 1.06	1.22 ± 0.75	0.06 ± 0.04	2.72 ± 2.23	0.15 ± 0.15
	2	1.5%	15.68 ± 12.25	0.84 ± 0.82	77.6 ± 11.41	7.99 ± 14.76	0.90 ± 0.75	0.05 ± 0.04	2.15 ± 2.40	0.10 ± 0.09
	3	3.0%	13.81 ± 6.19	0.72 ± 0.29	79.79 ± 4.83	4.38 ± 1.30	1.50 ± 0.68	0.08 ± 0.04	2.91 ± 2.59	0.17 ± 0.19
	4	5.0%	11.89 ± 4.21	**0.40* ± 0.14**	80.95 ± 6.82	2.84 ± 0.91	1.59 ± 0.94	0.05 ± 0.04	1.87 ± 0.97	0.07 ± 0.04

	Group No.	Dose Level	BASO	BASO	LUC	LUC
		(percent)	(%)	(1000/μL)	(%)	(1000/μL)
Mean ± SD	1	0.0%	0.40 ± 0.31	0.02 ± 0.02	2.09 ± 2.24	0.12 ± 0.15
	2	1.5%	1.19 ± 2.65	0.55 ± 1.77	3.31 ± 4.90	1.09 ± 3.30
	3	3.0%	0.47 ± 0.19	0.03 ± 0.01	1.54 ± 0.77	0.09 ± 0.05
	4	5.0%	0.69 ± 0.45	0.02 ± 0.01	3.14 ± 2.74	0.11 ± 0.11

Statistically significant at $p < 0.05$ (*) by Dunnett's test.
^Heterogenous data set with significant pair-wise difference by non-parametric Dunn's test.
WBC, white blood cell; RBC, red blood cell; HB, haemoglobin; HCT, haematocrit; MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular haemoglobin concentration; NEU, neutrophil; LYM, Lymphocytes; MONO, Monocytes; EOS, Eosinophils; BASO, Basophils; LUC, Large Unstained Cells.

Table 7A
Changes in Coagulation parameters in Male rasH2 Mice Administered AGIQ.

	Group No.	Dose Level (percent)	PT (seconds)	APTT (seconds)
Mean ± SD	1	0.0%	9.2 ± 0.3	31.2 ± 3.2
	2	1.5%	9.1 ± 0.5	29.5 ± 1.1
	3	3.0%	9.6 ± 1.0	29.6 ± 2.6
	4	5.0%	9.2 ± 0.6	30.6 ± 4.5

Table 7B
Changes in Coagulation parameters in Female rasH2 Mice Administered AGIQ.

	Group No.	Dose Level (percent)	PT (seconds)	APTT (seconds)
Mean ± SD	1	0.0%	11.58 ± 3.69	28.03 ± 2.40
	2	1.5%	10.66 ± 2.77	29.30 ± 3.64
	3	3.0%	13.10 ± 9.63	30.39 ± 5.00
	4	5.0%	9.86 ± 0.68	34.14 ± 2.34

APTT, activated partial thromboplastin time; PT, prothrombin time.

treated) animals or sporadically across groups. These lesions included but were not limited to myopathy in the skeletal muscle, subcapsular hyperplasia in the adrenal glands, cystic endometrial hyperplasia in the uterus, hyperplasia of bronchiolo-alveolar epithelium in the lungs, and sperm granulomas in the epididymides, chronic progressive nephropathy in the kidneys, peri-insular hypertrophy in the pancreas and mononuclear infiltrates in salivary and lacrimal glands.

4. Discussion

The potential of AGIQ to cause neoplasia was investigated in a GLP-compliant 6-month carcinogenicity study in transgenic rasH2 mice. Similar to the findings in rats reported by Nyska et al., (2016), there was no microscopic correlate to the grossly observed yellow discoloration of the femur and calvarium in rasH2 mice in the present study. Though this macroscopic examination was AGIQ treatment related it is not considered adverse as the yellow discoloration likely results from the accumulation of one or a combination of three metabolites: quercetin, quercetin 3-*O*-glucuronide, and 3-*O*-methylquercetin (Davis et al., 2020).

Based on the recommendations made by Paranjpe et al., (2014), only control and high dose and select tissues from mid and low dose groups were evaluated microscopically without compromising the quality and integrity of the study. The effects of alkylating carcinogen (MNU) were assessed in the positive control (group 5) animals for the presence of neoplasia. Animals in this group had incidences of lymphomas in hematopoietic organs and forestomach (Morton et al., 2002). The range of neoplastic and non-neoplastic findings observed across groups in this study were all considered background/spontaneous in nature. The majority of these findings (myopathy of skeletal muscle and subcapsular hyperplasia of adrenal glands) are specific to rasH2 strain of mice and have been described previously in the literature (Nambiar et al., 2012; Paranjpe et al., 2013; Boyle et al., 2018; Kuroda et al., 2018). The remainder of the findings (i.e chronic progressive nephropathy in the kidneys) are well characterized spontaneous lesions in inbred strains of mice (Seely 1999; Seely and Brix 2014; Maronpot RR, 2014; Nolte et al., 2016). Males had a dose dependent increase in food consumption with statistical significance in the 5% AGIQ animals without corresponding body weight changes; therefore, these changes were not considered AGIQ-related. There was a dose dependent decrease in body weight gain in females with statistical significance at 3.0 and 5.0% AGIQ but was not considered AGIQ-related since the body weight gains did not correlate with the absolute body weights and food consumption. Additionally, there was no histologic correlate to the observed variations in the absolute and relative organ weights, clinical pathology, and urinalysis values obtained at necropsy in both sexes. Hence, these findings were not considered to be AGIQ-related.

Although, the carcinogenic potential of AGIQ has been tested earlier in rats in our facility and published in the literature and has been reported to be safe, a similar study in mice has not been reported. Hence, this study was conducted to complement the findings in another rodent species (i.e. mice) and evaluate the carcinogenic potential following prolonged and repeated exposures (>90 days) in accordance with OECD Test Guidelines 451: Carcinogenicity Studies (OECD 2009). Furthermore, a negative study such as this is important and has been reported to comprise majority of FDA submissions (Boverhoff et al., 2011). This is because the rasH2 model has been found to test positive for fewer compounds of carcinogenic potential compared to rats and is better able to predict risk of carcinogenicity relevant to humans (Friedrich and Olejniczak 2011).

Taken together, there was no increased incidence of neoplasms, proportion of malignant neoplasms, or reduction in the time to appearance of neoplasms in animals exposed to AGIQ compared with the concurrent control group.

Therefore, under the study conditions, the no-observed-adverse-

Table 8A
Changes in Urinalysis in Male rasH2 Mice Administered AGIQ.

	Group No.	Dose Level (percent)	Glucose (mg/dl)	Protein (mg/dl)	Bilirubin	pH	UBIL (mg/dL)	Specific Gravity	Ketones (mg/dL)	UK (mmol/L)
Mean ± SD	1	0.0%	NEG	30.0 ± 0.00	NEG	6.58 ± 0.20	0.33 ± 0.33	1.03 ± 0.01	15.00 ± 0.00	126.00 ± 4.86
	2	1.5%	NEG	30.0 ± 0.00	NEG	6.60 ± 0.42	0.36 ± 0.36	1.04 ± 0.02	15.00 ± 0.00	145.80 ± 3.41
	3	3.0%	NEG	30.0 ± 0.00	NEG	**7.06* ± 0.32**	0.29 ± 0.27	1.03 ± 0.02	15.00 ± 0.00	107.30 ± 7.58
	4	5.0%	NEG	30.0 ± 0.00	NEG	6.94 ± 0.42	0.68 ± 1.34	1.02 ± 0.01	20.0 ± 11.2	128.5 ± 91.3

Statistically significant at $p < 0.05$ (*) by Dunnett's test.

Table 8B
Changes in Urinalysis in Female rasH2 Mice Administered AGIQ.

	Group No.	Dose Level (percent)	Glucose (mg/dl)	Protein (mg/dl)	Bilirubin	pH	UBIL (mg/dL)	Specific Gravity	Ketones (mg/dL)	UK (mmol/L)
Mean ± SD	1	0.0%	NEG	NEG	NEG	6.89 ± 0.33	0.20 ± 0 .00	1.02 ± 0.00	15.00 ± 0.00	82.04 ± 22.50
	2	1.5%	NEG	30.0 ± 0.00	NEG	6.60 ± 0.21	0.20 ± 0.00	1.02 ± 0.01	15.00 ± 0.00	82.71 ± 46.39
	3	3.0%	NEG	NEG	NEG	6.78 ± 0.26	0.20 ± 0.00	1.02 ± 0.00	15.00 ± 0.00	73.98 ± 22.48
	4	5.0%	NEG	NEG	NEG	6.88 ± 0.23	0.20 ± 0.00	1.02 ± 0.01	15.00 ± 0.00	**53.29* ± 7.81**

Statistically significant at $p < 0.05$ (*) by Dunnett's test.
UBIL= Urine Bilirubin; UK, Urine Potassium.

Table 9
Histopathological findings in Tg:rasH2 mice administered AGIQ.

Findings	Male				Female			
	0.0%	1.5%	3.0%	5.0%	0.0%	1.5%	3.0%	5.0%
Adrenal glands	*24*			*25*	*23*			*25*
Hyperplasia; subcapsular, bilateral, cortex	8			7	21			21
Hyperplasia; subcapsular, unilateral, cortex	12			9	2			3
Accessory structure; cortex	0			2	3			2
Aorta	*25*			*25*	*25*			*25*
Bronchiolo-Adenocarcinoma; metastatic	1*			0	0			0
Bone marrow					*1*			
Hematopoiesis; increased					1			
Brain	*24*			*25*	*24*			*25*
Hemangiosarcoma				1				
Nasal cavity	*25*			*25*	*24*			*25*
Accumulation; hyaline, olfactory epithelium					4			4
Accumulation; hyaline, respiratory epithelium				1	7			10
Hyperplasia; respiratory epithelium	1				1			
Epididymides	*25*			*25*				
Granuloma; chronic	1			1				
Infiltration; mononuclear cell				1				
Harderian glands	*25*	*25*	*23*	*25*	*25*	*23*	*25*	*25*
Adenoma							1	
Hyperplasia					1	1		
Infiltration; mononuclear cell	4	4	4	5				2
Heart	*25*			*25*	*25*			*25*
Cardiomyopathy					1			
Infiltration; mononuclear cell					1			
Mineralization				1				
Intestine, Ileum	*24*			*25*	*25*			*25*
Necrosis; crypt mucosa	1							
Kidneys	*23*			*25*	*24*			*25*
Hemangioma								1
Chronic progressive nephropathy	2			4	4			1
Infarction; acute								1
Infiltration; mononuclear cell, corticomedullary junction	3			4	1			
Thrombus								1
Lacrimal glands	*25*			*25*	*25*			*25*
Infiltration; mononuclear cell	10			12	8			5
Liver	*25*			*25*	*24*			*24*
Basophilic focus	1				1			
Eosinophilic focus				1	1			1
Inflammation; focal	12			11	15			9
Necrosis	2			1	1			
Mixed cell focus	1							
Fatty change								1
Extramedullary hematopoiesis					1			
Lungs	*25*	*25*	*24*	*25*	*25*	*24*	*25*	*25*
Bronchiolo-alveolar Adenoma	1	2			2	1	1	2
Bronchiolo-alveolar Adenocarcinoma	1							1
Hemangioma		1						
Lymphoma; malignant					1			
Hyperplasia; alveolar epithelium	3	3			3		1	1
Inflammation; acute					1			
Lymph node, Mesenteric	*25*			*25*	*25*			*25*
Squamous cell carcinoma					1*			
Skeletal muscle	*25*			*25*	*25*			*25*
Myopathy; chronic	23			23	25			24
Pancreas	*25*			*25*	*25*			*25*
Squamous cell carcinoma					1*			
Vacuolation; acinus						1		
Hypertrophy; acinus	3			7	13			11
Parathyroid glands								
Cyst; unilateral	1			1				
Salivary glands	*25*			*24*	*24*			*25*
Infiltration; mononuclear cell	7			5	5			6
Seminal vesicles	*25*			*25*				
Hyperplasia; epithelium				1				
Spleen	*23*	*25*	*24*	*25*	*24*	*23*	*25*	*24*
Hemangiosarcoma	2	1	2		1		2	1
Lymphoma, malignant						1		
Bronchiolo-alveolar Adenocarcinoma								1*
Hyperplasia; white pulp						4		
Extramedullary hematopoiesis					2		2	1
Mineralization; arteriole					1			
Stomach	*25*			*25*	*24*			*25*
Squamous cell carcinoma					1			

(*continued on next page*)

Table 9 (*continued*)

Findings	Male				Female			
	0.0%	1.5%	3.0%	5.0%	0.0%	1.5%	3.0%	5.0%
Testes	*25*			*24*				
Hemangiosarcoma	1							
Spermatozoa; reduced, unilateral	1							
Thymus	*24*			*25*	*24*			*24*
Thymoma					1			
Cyst				1				
Depletion; lymphoid					1			
Hemorrhage; acute	1							
Urinary bladder	*24*			*25*	*25*			*25*
Infiltration; lymphocytic					6			
Uterus					*21*			*25*
Hemangiosarcoma					1			
Hyperplasia; cystic, endometrium					19			16
Inflammation; acute					1			

* = Metastatic lesions.

Table 10
Historical control data of neoplastic lesions in rasH2 mice collected from ILS internal database and published sources.

Neoplastic findings	Present study incidence (%)				Historical control data from ILS (Davis J. 2019) incidence (%)		Historical control data range from Literature (%)		References
	Male (Control) N = 25	Male (Dose groups) N = 75	Female (Control) N = 25	Female (Dose groups) N = 75	Male (Control) N = 25	Female (Control) N = 25	Male Range	Female Range	
Hemangiosarcoma (Spleen)	2/25 (8%)	3/75 (4%)	1/25 (4%)	3/75 (4%)	1/25 (4%)	0	0–12%	0–12%	Paranjpe et al., 2018
Hemangiosarcoma (Uterus, Testes, Brain)	1/25 (4%)	1/75 (1.3%)	1/25 (4%)	0	1/25 (4%) (Testes)	0	0–4% (Testes)	0–4% (Uterus)	Paranjpe et al. (2018)
Bronchiolo-alveolar Adenoma	1/25 (4%)	2/75 (2.6%)	2/25 (8%)	4/75 (5.3%)	1/25 (4%)	1/25 (4%)	0–20%	0–20%	Paranjpe et al. (2018)
Bronchiolo-alveolar Adenocarcinoma	1/25 (4%)	0	0	1/75 (1.3%)	0	0	0–12%	0–12%	Paranjpe et al. (2018)
Harderian gland Adenoma	0	0	0	1/75 (1.3%)	0	1/25 (4%)	0	0–8%	Paranjpe et al. (2018)
Hemangioma (Kidney, Lung)	0	1/75 (1.3%)	0	1/75 (1.3%)	0	0	1%	0	Kanno et al. (2003)
Lymphoma (Lymphoreticular)	0	0	1/25 (4%)	1/75 (1.3%)	1/25 (4%)	0	0–4%	0–8%	Paranjpe et al. (2018)
Squamous cell carcinoma (Stomach)	0	0	1/25 (4%)	0	0	0	0	0–4%	Paranjpe et al. (2018)
Thymoma	0	0	1/25 (4%)	0	0	0	0	0–4%	Paranjpe et al. (2018)

effect level (NOAEL) was determined to be more than 5.0% (7215.4 mg/kg/day in male mice and 14685.5 mg/kg/day in female mice).

CRediT authorship contribution statement

Debabrata Mahapatra: Writing – original draft. **Douglas A. Donahue:** Writing – review & editing. **Abraham Nyska:** Writing – review & editing. **Shim-mo Hayashi:** Writing – review & editing. **Mihoko Koyanagi:** Writing – review & editing. **Robert R. Maronpot:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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