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Oral chronic toxicity and carcinogenicity study of *alpha*-glycosyl isoquercitrin (AGIQ) in Sprague Dawley rats

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ABSTRACT

alpha-Glycosyl isoquercitrin (AGIQ) is a flavonoid that possesses antioxidant and tumor suppressive capabilities and is marketed as a food additive in Japan. The aim of this study was to assess the potential for oral chronic toxicity and carcinogenicity of AGIQ in male and female Sprague Dawley rats following up to 5.0% dietary exposure. In the chronic toxicity study, rats were exposed to AGIQ or vehicle for one year with a 6-month interim termination point; for the carcinogenicity study, rats were treated for 24 months. No signs of AGIQ-related toxicity clinically or histologically were observed for up to one year except for yellow discoloration of bone. In the carcinogenicity study, a statistically significant increase in the incidence of malignant glioma of the brain or spinal cord was observed in female rats exposed to 5.0% AGIQ compared to those exposed to control feed. A Scientific Advisory Panel of experienced neuropathologists reviewed the gliomas (routine stains and glial cell markers) and concluded that the gliomas were a rare, spontaneous, rat-specific neoplasm: malignant microglial tumor. The lesions could not definitively be attributed to AGIQ exposure and have limited implications with respect to predicting human cancer risk.

1. Introduction

The natural flavonoid isoquercitrin (quercetin-3-*O*-β-D-glucoside) is beneficial to human health and its demonstrated benefits including anti-inflammatory, anti-oxidant, anti-mutagenic, anti-clastogenic, anti-depressant, hypotensive, hypolipidemic and anti-viral effects (Amado et al., 2009; Edenharder and Grunhage, 2003; Gasparotto Junior et al., 2011; Kim et al., 2010; Li et al., 2011; Valentova et al., 2014). Commercial isoquercitrin production involves enzymatic hydrolysis of rutin followed by transglycosylation using dextrin and cyclodextrin glucanotransferase to yield *alpha*-glycosyl isoquercitrin (AGIQ), also known as enzymatically modified isoquercitrin (Erlund, 2004; Manach et al., 1997). AGIQ contains a combination of isoquercitrins with one or more glucose moieties (Akiyama et al., 2000; FDA (2007)). After ingestion in humans, AGIQ is deglycosylated in part by salivary amylase, absorbed to some extent in the small intestine, and metabolized in the large intestine by anaerobic enterobacteria to form quercetin, which is absorbed and quickly glucuronidated (Day et al., 2001; Hollman et al., 1996; Nyska et al., 2016). The aromatic rings of quercetrin can also be metabolized by other large intestinal bacteria to phenyl and hydroxyphenylacetic acids and short chain fatty acids that are then absorbed (Valentova et al., 2014).

AGIQ is approved as an antioxidant additive in foods and beverages for use in Japan and has "generally recognized as safe" (GRAS) status by the Expert Panel of the Flavor and Extract Manufacturers Association (FEMA) (Smith et al., 2005a, 2005b) and by the U.S. Food and Drug Administraton (FDA, 2007). Like isoquercitrin, AGIQ has been found in F344 and Sprague Dawley rat studies to have antioxidant and tumor suppressive capabilities (Fujii et al., 2013; Hara et al., 2014; Kimura et al., 2013; Morita et al., 2011; Nishimura et al., 2010; Shimada et al., 2010).

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Abbreviations:

AGIQ	*alpha-glycosyl isoquercitrin*
FDA	U.S. Food and Drug Administration
GLP	Good Laboratory Practice
SAP	Scientific Advisory Panel

AGIQ has been reported as safe and non-carcinogenic in older non-Good Laboratory Practice (GLP)-compliant Wistar and F344 rat studies, including some which used test material of low purity (Engen et al., 2015; Hasumura et al., 2004; Salim et al., 2004; Tamano et al., 2001; Valentova et al., 2014). In a comprehensive GLP-compliant genotoxicity study, both isoquercitrin and AGIQ were positive in bacterial reverse mutations assays, and isoquercitrin caused chromosomal aberrations in CHO-WBL cells. However, additional in vitro mammalian micronucleus and chromosomal aberrations assays as well as in vivo micronucleus and comet assays in both sexes of B6C3F1 mice and Sprague Dawley rats were negative (Hobbs et al., 2018). Additionally, a Muta™ Mouse mutation assay evaluating multiple tissues was also negative for isoquercitrin and AGIQ (Hobbs et al., 2018), supporting that AGIQ is not genotoxic/mutagenic. Support for the safe use of AGIQ in food and beverages includes (a) a GLP-compliant 90-day toxicity and single-dose toxicokinetics study in Sprague Dawley rats with a no observable adverse effect (NOAEL) at a dietary level of up to 5% (3461 mg/kg/day and 3867 mg/kg/day for males and females, respectively) (Nyska et al., 2016); (b) lack of toxicity in juvenile Göttingen minipigs exposed to AGIQ in reconstituted milk supplement for 4 weeks at up to 1000 mg/kg/day (0.1%) (Maronpot et al., 2019); (c) negative teratogenicity in New Zealand White rabbits at gavage doses up to 1000 mg/kg/day (Maronpot et al., 2020); and (d) lack of carcinogenicity in rasH2 mice at up to 5% dietary exposures for 6 months (Mahapatra et al., 2021).

The purpose of this study was to assess the potential for chronic toxicity and carcinogenicity of AGIQ in male and female Sprague Dawley rats at dose levels up to 5% dietary exposure. The study was performed in anticipation of expanded marketing of AGIQ. At study termination, the unexpected occurrence of gliomas in the high-dose females prompted a follow-up evaluation of these lesions by an independent Scientific Advisory Panel (SAP). This paper describes principal conclusions supported by the study and SAP review.

2. Materials and methods

2.1. Regulatory compliance

The study was approved by the Integrated Laboratory Systems, Inc. (ILS, Research Triangle Park [RTP], NC, USA) Animal Care and Use Committee. All procedures, handling, and treatment were in compliance with the U.S. Animal Welfare Act Regulations (9 CFR 1–4) and the U.S. National Research Council "Guide for the Care and Use of Laboratory Animals" (NRC, 2011). The study was conducted in accordance with GLP regulations (FDA, 2018).

2.2. Test additive

AGIQ (purity >97%) containing 0.13% quercetrin was obtained from San-Ei Gen, F.F.I., Inc., Osaka, Japan. Dose formulations were prepared at RTI International (RTP, NC) in Purina Certified 5002 meal diet (Ralston Purina Co, St. Louis, MO, USA). Dietary analysis at RTI confirmed AGIQ concentrations of 0 (carrier control), 1.5%, 3.0%, and 5.0%.

2.2.1. Animal husbandry and maintenance

Male and female Hsd:Sprague Dawley (SD) rats used in this study were approximately 5 weeks of age and were obtained from Envigo Laboratories (Indianapolis, IN, USA) and housed singly in polycarbonate cages with micro-isolator tops throughout the 7-day acclimation period and study. Diet was provided ad libitum along with free access to reverse osmosis-treated tap water via polycarbonate water bottles. Heat-treated hardwood bedding (Northeastern Products Corp., Wattensburg, NY) was changed weekly. Environmental conditions were 20–25°C and 30–70% relative humidity.

2.3. Experimental design

The study consisted of 3 cohorts. The interim (6-month) cohort had 10 animals per sex for the 5.0% AGIQ and 0% (control) groups. The chronic toxicity (12-month) cohort consisted of 20 animals per sex per group with dietary exposures of 0%, 1.5%, 3.0% or 5.0% AGIQ. The carcinogenicity (24-month) cohort had 50 animals per sex per group with dietary exposures of 0%, 1.5%, 3.0% or 5.0% AGIQ.

2.4. Mortality, clinical signs, body weight, and food consumption

Each animal was observed twice daily on weekdays and once daily on weekends/holidays for morbidity and mortality. Clinical observations and body weights were recorded on Day 1, weekly thereafter, and at termination. Feed consumption (g/kg body wt/day) was calculated weekly for all study phases.

2.5. Ophthalmology, neurotoxicity, and clinical pathology screening

For the 12-month cohort, ophthalmological examinations were performed once prior to initial AGIQ exposure and again once more before termination. After at least 6 months of exposure, neurotoxicity screening including a functional observational battery (FOB, equivalent to published procedures (Moser, 2000)) and motor activity assessment (open field automated photobeam activity system) was performed. Blood and urine were collected from 10 animals per sex per group following exposure for 2 weeks (blood only), 6 months, and 12 months for clinical pathology (hematology, clinical chemistry, and coagulation from blood; urinalysis for urine). Blood volume was insufficient for 2-week-old females due to low body weights, so 10 additional female rats were included for the control and all treatment groups for blood collection at this time point. In the 24-month cohort, male rats were terminated after approximately 22.5 months when survival of controls fell to 25%. Female control rats survived in sufficient number to permit termination at 24 months. Blood was collected at 24 months for hematology, clinical chemistry, and coagulation assays.

2.6. Moribund/unscheduled termination

Beginning on the first day of exposure, animals found moribund or dead were necropsied to determine the cause of death, if possible. Moribund animals were euthanized by carbon dioxide (CO_2) inhalation followed by cervical dislocation. The gross necropsy included evaluation of external surfaces; orifices; cranial, thoracic and abdominal cavities; carcass; all organs; and gross lesions.

2.7. Scheduled termination

Animals were fasted overnight prior to necropsy. At the scheduled ages, animals were euthanized by CO_2 inhalation. Blood was collected via cardiac puncture from 10 males and 10 females per dose group (6- and 12-month cohorts) and from all surviving animals (24-month cohort). Death was confirmed by exsanguination.

The gross necropsy included evaluation of external surfaces; orifices; cranial, thoracic and abdominal cavities; carcass; all organs; and gross

lesions. The following organs were weighed at necropsy: adrenals (paired), brain, epididymides (paired), heart, kidneys (paired), liver, ovaries (paired), spleen, testes (paired), thyroid with parathyroid (paired), and uterus and cervix. Organs were immersed in neutral buffered 10% formalin containing ~1% methanol as a stabilizing agent.

2.8. Pathology

Organs preserved for histopathological evaluation included adrenals, aorta, brain (cerebrum, cerebellum and medulla oblongata/pons), bone and bone marrow (sternum and femur), cecum and colon, uterus and cervix, coagulating gland, epididymides, esophagus, eyes, Harderian glands, heart, intestines (small and large including Peyer's patches), kidneys, lacrimal gland, liver, lungs with trachea, lymph nodes (mesenteric and mandibular), mammary glands, ovaries, pancreas, pituitary, prostate, salivary glands, sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord (cervical, mid-thoracic, and lumbar divisions), spleen, stomach, testes, tongue, thymus, thyroid/parathyroid(s), urinary bladder, vagina, and gross lesions. Brain was examined at 7 levels for histopathological examination according to contemporary published recommendations (Bolon et al., 2013). Protocol-specified organs and gross lesions from the control and the high-dose animals were trimmed of adherent tissue and submitted for conventional histological processing to permit paraffin embedding. Five-μm-thick sections were stained routinely with hematoxylin and eosin (H&E). Tissues from animals in intermediate dose groups were also trimmed of adherent tissue, histologically processed, and embedded, but blocks were archived without sectioning. H&E-stained sections from animals fed 0% or 5% AGIQ were evaluated by a board-certified pathologist (Diplomate, American College of Veterinary Pathologists) using bright-field light microscopy. Lesions were assigned a severity grade consistent with current industrial practice (Schafer et al., 2018). A significant increase in the incidence of malignant glioma was observed in females dosed with 5% AGIQ, so brains and spinal cords were also evaluated for females exposed to 1.5% and 3.0% AGIQ as well as for 1.5% and 3.0% males in addition to the 5% males. An independent contemporaneous pathology peer review was conducted by a board-certified toxicologic pathologist (Diplomate, European College of Veterinary Pathologists) to confirm diagnoses on all tumors, all proliferative lesions, and on all tissues from a 20% random subset of treated and control animals.

2.9. Scientific Advisory Panel review of gliomas

An increased incidence of "Glioma, not otherwise specified (NOS)" documented in the brain and/or spinal cord of high-dose female rats led to convening an SAP of 7 subject matter experts (medical and veterinary neuropathologists) to better characterize the tumors in sections stained with H&E or by special neurohistological methods. The remit of the SAP was to assess the possibility that the gliomas resulted from AGIQ exposure. For the SAP review, serial sections of all brain and spinal cord cases with a diagnosis of "glioma, NOS" using H&E were labeled by conventional chromogenic immunohistochemistry (IHC) with a battery of glial cell-specific markers to characterize the glioma lineage as astrocytes (anti-glial fibrillary acidic protein, GFAP), microglia (anti-ionized calcium-binding adaptor molecule 1, Iba1—which also labels related cells of the macrophage/monocyte lineage), or oligodendrocytes (anti-oligodendrocyte transcription factor 2, Olig2). Digitized whole slide scans of tumor sections were reviewed independently by each SAP member. To minimize bias, each SAP member's diagnoses were transmitted to ILS for tabulation of the majority/consensus diagnosis. Thereafter, SAP members discussed the diagnoses and developed a consensus opinion regarding lesion interpretation and the implications for humans exposed to AGIQ.

2.10. Statistical analysis

Group mean and standard deviations were calculated and analyzed using Statistical Analysis System software (version 9.2; SAS Institute, Cary, NC, USA). Data that could not be transformed to be homogeneous were evaluated using the non-parametric Dunn's test while dose-dependent changes were analyzed via the non-parametric Jonckheere's trend test. Depending upon homogeneity of variance data using Levene's test, FOB data were analyzed by the analysis of variance test, Dunnett's test, the Kruskal-Wallis test, and/or Dunn's test. Endpoints in the FOB which used graded or count scales were analyzed employing a non-parametric strategy using the Kruskal-Wallace test, Dunn's test, Fisher's Exact test, and/or Dunnett's test. Survival curves were estimated using the Kaplan and Meier procedure separately for each sex of rat. Animals found dead from reasons other than natural causes and those animals surviving to the terminal time point were censored from the survival analysis. Tumor incidence data were analyzed using the Cochran-Armitage Trend test (which ignores survival) and the post-hoc Poly-K test (which considers survival).

3. Results

3.1. 6-Month toxicity cohort (5.0% AGIQ and control)

All animals survived to termination. No treatment-related clinical abnormalities, body weight effects, differences in food consumption, or hematologic values were present. Statistically significant increased prothrombin and decreased cholesterol alterations in males and increased gamma-glutamyl transpeptidase (GGT) in females were within normal limits (Supplementary Tables 1a and 1b). AGIQ-treated males had statistically increased absolute and relative kidney weights compared to controls while absolute brains weights in females were statistically lower than control brain weights (Supplementary Tables 2a and 2b). AGIQ-related macroscopic or microscopic kidney or brain findings were absent, so these weight changes were considered incidental.

Pathology findings were limited to macroscopic yellow discoloration of femurs in AGIQ-treated male and female rats and sporadic incidental background microscopic tissue changes in treated and control animals (Supplementary Table 3 a-e).

3.2. 12-Month toxicity cohort

All animals survived to termination at 12 months except for 1 control male, 2 low-dose males, and 3 mid-dose males. Sporadic cage-side observations, including alopecia and scabbing, were present in treated as well as control rats, and final body weights and body weight gains were comparable in males for all groups (Supplementary Table 4 a-c). Females in the 5% AGIQ group had lower final body weights and body weight gains compared to controls with a statistically significant linear trend in these parameters associated with AGIQ exposure. Considering no overall changes in food consumption in females or males (Supplementary Table 5 a-c) and the lack of correlative anatomic changes (Supplementary Table 6 a-f), the body weight changes in females were not considered adverse.

3.2.1. Neurotoxicity screening

Ten males and 10 females from the 12-month cohort underwent neurotoxicity screening after 6 months of exposure. There was some variation in response during the FOB, but no clear AGIQ-related effects were identified using the FOB, motor activity, or pathology assessment (Supplementary Tables 7a and b).

3.2.2. Two-week clinical pathology

At the 2-week timepoint, clinical pathology for Groups 3–6 and 11–14 exhibited some statistically significant hematology values

(Supplementary Tables 8a and b) that fell within the normal reference range and thus were incidental. Clinical chemistry analytes were largely similar between treated and control groups but with high variability in female serum bile acid levels at all doses. Coagulation parameters were unaffected at this time point.

3.2.3. Six-month clinical pathology

Clinical pathology for Groups 3–6 measured at 6 months (Supplementary Table 9 a & b) identified variable and sporadic differences in thrombocyte numbers in males and a positive trend in females without any pairwise group differences. Prothrombin time (PT) and activated partial thromboplastin time (APTT) were within normal limits despite a statistically significant increase in PT in females exposed to 1.5% AGIQ. Males exposed to 5% AGIQ had increased bile acids with a positive trend associated with increased exposure (Supplementary Table Appendices 9 a & b). Females exposed to 5% AGIQ at 6 months had increased bile acid levels (mean = 30.4 units, vs. 12.9 in controls) and altered serum GGT (1 vs 0), alanine aminotransferase (ALT, 34 vs 25) and glutamate dehydrogenase (GLDH, 13 vs 6) activities. Although males exposed to 5% AGIQ had statistically higher levels of serum calcium and phosphorus, these levels were within normal control limits.

3.2.4. Twelve-month clinical pathology and histopathology

Blood cell counts were sporadic and not related to treatment while coagulation times related to both intrinsic and extrinsic coagulation pathways were significantly elevated in comparison with concurrent control values but within normal control limits in the 5% AGIQ males (Supplementary Tables 10a and b). Minimal to mild dose-related increases were detected in ALT, aspartate aminotransferase (AST), alkaline phosphatase (ALP), GGT and GLDH activities in females, with pairwise differences noted in the 5% AGIQ group for all analytes except ALP; while this pattern of changes is suggestive of possible liver (hepatocyte and biliary epithelial) injury, all values were within normal limits. Minor increases in glucose, calcium and phosphorus concentrations were present in the male 5% AGIQ group with all values within normal historical control limits.

Macroscopic yellow discoloration of the femur and calvarium was the only macroscopic finding related to AGIQ administration. Microscopic examination of tissues including special attention to liver revealed no AGIQ-related abnormalities (Supplementary Tables 11a–f).

3.3. 24-Month cohort

3.3.1. Survival

Excessive attrition of control males due to a large number of early deaths and moribund sacrifices resulted in termination of all surviving males approximately 5 weeks early to ensure a sufficient sample size for statistical analysis, in accordance with OECD (Organisation for Economic Co-operation and Development) Test Guideline 453. AGIQ did not decrease tumor latency despite some decreased survival in 5% males. Survival data for males and females are provided in Table 1.

3.3.2. Clinical pathology

Aside from statistically significant trends in occasional serum analytes, pairwise comparison demonstrated no alterations in hematological or clinical chemistry values in terminally sacrificed males and females (Supplementary Table 12 a & b).

3.3.3. Body and organ weights

Statistically significant decreases in absolute but not relative weights of brain, kidney, and adrenal gland were recorded in the 5% AGIQ females (Supplementary Table 13 a & b). Body weights and body weight gains also were decreased in the 5% AGIQ females with final body weights averaging 9.5% lower than controls (Supplementary Table 14 a-c). No correlating histopathological changes were observed to explain these statistically significant differences.

3.3.4. Macroscopic observations

The only treatment-related macroscopic finding was a dose-related, diffuse, yellow discoloration of the femurs and calvaria in most AGIQ-exposed animals at all dose levels (Table 2). The yellow discoloration of bone was not apparent microscopically. Several other gross findings typical of aged rats were scattered sporadically across all groups including controls and thus were considered to be incidental.

3.3.5. Histopathology evaluation of the brain and spinal cord

Protocol-required tissues from the 0% (control) and 5% (high-dose) groups were examined microscopically. Malignant gliomas were observed microscopically in the brain (N = 4) and/or spinal cord (N = 1) for the high-dose (N = 5 of 49 [10.2%]) females as well as in a single control male (N = 1 of 51 [2%]). A read-down evaluation of the brain and spinal cord on all males and females in the 1.5% and 3% groups identified two malignant gliomas in the brains of male rats exposed to 1.5% AGIQ. This read-down was completed after the SAP was concluded. No gliomas were observed in control or 1.5% and 3% females or 3% and 5% males. Non-neoplastic glial proliferative lesions were not observed in any control or treated animals. All tumors were initially assigned the INHAND (International Harmonization of Nomenclature and Diagnostic Criteria) diagnosis “glioma, NOS” based on microscopic features in H&E-stained sections (Bradley et al., 2020) and were subsequently changed to “microglial tumor, malignant’ based on serial sectioning, immunohistochemical staining, and the consensus diagnoses of the SAP. Primary brain and spinal cord neoplasms are presented in Table 3.

All malignant gliomas evaluated in this study were similar in appearance on H&E-stained sections. Neoplasms were unencapsulated, composed of cells with fusiform nuclei, had indistinct poorly demarcated margins, were moderately to densely cellular, and were localized to one major area of the central nervous system. Anisocytosis (variable cell shape and size) and anisokaryosis (variable nuclear shape and size) were mild, mitotic figures were rare but present in almost all lesions, and tumor cell aggregates surrounded many blood vessels (perivascular cuffing) at the lesion periphery. The presence of mitoses and absence of other changes (e.g., parenchymal degeneration and necrosis) warranted the interpretation that these lesions were glial neoplasms and not reactive glial responses. An IHC battery of glial markers demonstrated that essentially all neoplastic cells expressed Iba1 strongly to intensely but did not label with GFAP or Olig2. Accordingly, the consensus diagnosis of the SAP was that all these tumors were of microglial origin; a suitable INHAND term for this lesion is “microglial tumor, malignant”

Table 1
Survival data for male and female Sprague Dawley rats in the 24-month carcinogenicity study.

	Males				Females			
Exposure	0%	1.5%	3.0%	5.0%	0%	1.5%	3.0%	5.0%
Number	50	50	50	50	50	50	50	50
Number of animals to scheduled termination (24 months)					18	22	17	24
Percentage of animals to scheduled termination (24 months)					36	44	34	48
Number of animals to early scheduled termination (23 months)	13	10	16	6				
Percent of animals to early scheduled termination (23 months)	26	20	32	12				

Table 2
Incidences and severities of yellow discoloration in femurs of male and female Sprague Dawley rats in the 24-month carcinogenicity study.

	Males				Females			
AGIQ Exposure Group	0%	1.5%	3.0%	5.0%	0%	1.5%	3.0%	5.0%
Number Examined (Bone, Femur)	50	50	50	50	50	50	50	50
Number with Discoloration	0	38	38	47	1	34	47	46
Minimal	0	7	0	1	1	10	3	1
Mild	0	20	2	1	0	23	11	2
Moderate	0	10	23	14	0	1	28	14
Marked	0	1	13	31	0	0	5	29
Average Severity[a]	0	2.1	3.3	3.6	1	1.7	2.7	3.5

[a] Average severity was calculated by assigning numerical grades as follows: 1 = Minimal, 2 = Mild, 3 = Moderate, 4 = Marked. The average severity grades were determined using the number of animals affected. Animals without the lesion were not included in the average severity grade determination.

Table 3
Incidences of selected brain and spinal cord neoplasms in male and female Sprague Dawley rats in the 24-month carcinogenicity study.

	Males				Females			
AGIQ Exposure Group	**0.0%**	**1.5%**	**3.0%**	**5.0%**	**0.0%**	**1.5%**	**3.0%**	**5.0%**
Group Size	**51[a]**	**50**	**50**	**51[a]**	**50**	**50**	**50**	**49[b]**
Brain								
Number Examined	51	50	50	51[c]	50	50	50	49
Glioma, NOS	1	2	0	0	0	0	0	4
Meninges, Granular Cell Tumor, Malignant	0	0	0	0	0	0	0	1
Spinal Cord								
Number Examined	50	50	50	52	50	50	50	49
Glioma, NOS	0	0	0	0	0	0	0	1

[a] Extra males above the originally designed group size (n = 50) resulted from additions to replace early losses attributed to spontaneous disease.
[b] One animal died early during dosing and was not replaced.
[c] The brain of one animal was not found after necropsy.

(Bradley et al., 2020). Enlarged GFAP-positive glial cells scattered within and encircling the neoplasms were interpreted as non-neoplastic astrocytes, a reactive response to the malignant microglial tumors.

A malignant granular cell tumor was recorded in the meninges of the brain in one 5% AGIQ female. This neoplasm was characterized by proliferation of polygonal cells characterized by abundant eosinophilic granular cytoplasm and round to oval nuclei. The cells exhibited mild to moderate anisocytosis and anisokaryosis and rare mitotic figures. Compression and invasion of the adjacent brain was extensive.

3.3.6. *Histopathology of thyroid gland*

C-cell carcinoma was diagnosed when proliferation of C-cells that infiltrated the thyroid gland capsule and extended into the extra-thyroidal tissue. C-cell adenoma was characterized by a focal, sometimes compressive proliferation of C-cells that was larger than the diameter of five average-sized follicles but that did not infiltrate the thyroid gland capsule or extend into extra-thyroidal tissue. Focal C-cell hyperplasia was diagnosed when there was a focal proliferation of interfollicular C-cells that was no larger than the diameter of five average-sized follicles.

The incidences of unilateral and bilateral C-cell adenomas were slightly increased in the 5% AGIQ males and females (Table 4) but within historical control incidence. The increase was not statistically significant when compared with the concurrent controls. The incidences of C-cell carcinoma were similar across groups with the exception of a decrease in the 5% AGIQ males. The incidences and severities of C-cell hyperplasia were similar across groups including controls. The incidences and severity of proliferative C-cell lesions in males and females are presented in Table 4.

Table 4
Incidences and severity of selected neoplastic and non-neoplastic thyroid gland lesions in male and female Sprague Dawley rats in the 24-month carcinogenicity study.

	Male		Female	
AGIQ Exposure Group	0.0%	5.0%	0.0%	5.0%
Number Examined	*50*	*50*	*49*	*49*
C-cell Carcinoma	3	1	3	3
C-cell Adenoma	1	4	6	10
C-cell Adenoma, Multiple	1	1	0	1
C-cell Hyperplasia, Focal	6 (1.3)[a]	7 (1.3)	15 (1.3)	17 (1.9)
Minimal	5	6	12	8
Mild	0	0	1	4
Moderate	1	1	2	4
Marked	0	0	0	1

[a] Average severity (in parentheses) was calculated by adding the grades of all affected animals in the group and dividing by the number affected. Numerical grades were assigned as follows: 1 = Minimal, 2 = Mild, 3 = Moderate, 4 = Marked.

3.3.7. *Histopathology of kidneys*

The severity of chronic progressive nephropathy (CPN) in this study was graded as follows: minimal = low numbers of small cortical foci of basophilic tubules with thickened basement membranes and nuclear crowding; mild = slightly larger and more numerous foci with several per kidney section; moderate = larger and more numerous, coalescing foci with hyaline casts and dilated tubules throughout the renal parenchyma but with intervening areas of normal kidney; or marked = total involvement of the renal parenchyma.

CPN was recorded in almost all 24-month-old males and females in the control and 5% AGIQ groups. The incidences and severity of CPN in males and females are presented in Table 5. The severity ranged from minimal to marked. Marked CPN in many males was sufficiently severe to represent end-stage kidney disease and was likely responsible for the high number of early deaths in males of all groups. The incidences and average severity were similar between the control and high-dose males. Early lesions consisted of small numbers of basophilic tubules with nuclear crowding, thickened basement membranes, and infiltration of mononuclear inflammatory cells. With progression of the disease, increased severity of the aforementioned features was accompanied by the presence of hyaline casts, tubular dilation, glomerular changes, and

Table 5
Incidences and severity of chronic progressive nephropathy in kidneys of male and female Sprague Dawley rats in the 24-month carcinogenicity study.

	Male		Female	
AGIQ Exposure Group	0.0%	5.0%	0.0%	5.0%
Number Examined	*50*	*50*	*50*	*49*
Chronic Progressive Nephropathy	49 (3.7)[a]	50 (3.4)	50 (1.6)	49 (1.8)
Minimal	1	2	26	20
Mild	2	6	18	19
Moderate	7	12	6	8
Marked	39	30	0	2

[a] Average severity (in parentheses) was calculated by adding the grades of all affected animals in the group and dividing by the number affected. Numerical grades were assigned as follows: 1 = Minimal, 2 = Mild, 3 = Moderate, 4 = Marked.

variable interstitial fibrosis. The incidences and severity of CPN in males and females were similar between their respective control and high-dose groups, and the overall severity in males was notably increased in comparison with the females.

3.3.8. *Histopathology of other tissues*

A variety of other neoplastic and non-neoplastic findings were observed in various tissues and either occurred with similar incidences in control and high-dose animals or were spread sporadically across all groups. These findings were considered to be spontaneous, age-associated background lesions unrelated to AGIQ exposure.

Polyarteritis nodosa (INHAND diagnosis: "necrosis/inflammation, media or wall, artery") was observed in numerous males and a smaller number of females. The site distribution of this lesion varied, and no organs were consistently affected. For this reason, the incidences among groups for specific organs were variable.

Lung alveolar histiocytosis was diagnosed in numerous animals and across all groups. The incidence was decreased in the 5% AGIQ males in comparison with the concurrent controls.

Thymus epithelial hyperplasia was recorded in numerous animals across all groups. The incidences were increased in 5% AGIQ animals of both sexes and were considered a spontaneous age-related change associated with thymic involution.

4. Discussion

The present study was GLP-compliant. Early termination of males one month before the scheduled final necropsy was necessary due to excessive mortality in control males secondary to spontaneous CPN. For the interim clinical pathology evaluations, some statistically significant increases in serum enzyme activities in high-dose females at 6 and 12 months were reflective of possible hepatic effects. However, these significant effects were well within normal reference ranges and were not associated with alterations in hepatic microanatomy. Neither clinical pathology alterations nor hepatic histopathology were evident at the 24-month time point.

The yellow discoloration observed in bone is a well-known finding in animal toxicity studies with AGIQ and has been reported several times before in shorter duration animal studies (Davis et al., 2020; Nyska et al., 2016; Tamano et al., 2001). This finding is believed to result from deposition of yellow pigment associated with AGIQ, which is supplied as a yellow to yellow-orange powder. Yellow pigmentation of the bones was not accompanied by histological findings or altered bone growth in the prior or current AGIQ studies, so this finding is considered to be non-adverse.

The most important finding in the current study was identification of gliomas in the brain and spinal cord of high-dose females at the 24-month time point. Based on cytological features and IHC staining, the Iba1-expressing neoplastic cells were identified as "microglial tumor, malignant," consistent with INHAND descriptions as a diagnostic option for tumors with this labeling pattern (Bradley et al., 2020).

The statistically significant increased incidence of microglial tumors in the high-dose females (10.2%) in the present study exceeded the 0% incidence in concurrent control females and the <1% incidence in published historical control data for animals of this rat strain and sex (Koestner, 1986). In subsequent examination of brain and spinal cord tissues from low dose females and middle and low dose males in the present study, 2 microglial tumors were identified in male rats exposed to 1.5% AGIQ in addition to the glioma previously identified in a control male rat. Recent historical data for the same strain of rats compiled from seven 24-month studies (4 of which included 6 levels of brain sectioning) conducted at Labcorp (Madison, WI, USA) from January 2012 through January 2022 revealed a total incidence of gliomas in female SD rats of 4/550 (0.7%) with a range of 0–3.3% (A. Sharma, personal communication). These historical data indicate that gliomas occur spontaneously in female rats, thus supporting the interpretation that the gliomas developing in the current study are unrelated to treatment. This assumption is based on the biological evidence approach (Keenan et al., 2009). Arguments for discounting a treatment as a potential cause for an effect are: the absence of a dose response; lack of non-neoplastic proliferation (in this case, microglial hyperplasia) or other pre-neoplastic morphologic changes in neural tissues; the relatively marginal increase in the incidence of brain tumors seen only in high-dose females; and the fluctuating incidence of glioma in historical data from Labcorp, suggesting that small variations in incidence of gliomas may occur and influence study interpretation (especially when a treatment effect is based on a low number of tumors).

Given the recent change in the routine brain trimming procedure in rodent toxicity studies from 3 to 7 levels for histopathologic examination (Bolon et al., 2013), the odds for detecting tumors is higher because the amount of tissue available for screening is increased (Eustis et al., 1994). Moreover, a growing body of evidence suggests that GFAP-negative brain glial tumors in rodents that have been diagnosed historically as "astrocytoma" or "glioma, NOS" are actually microglial tumors. Importantly, microglial tumors have been described with some frequency in rats (Kolenda-Roberts et al., 2013; Nagatani et al., 2009) but not in other laboratory animal species. Furthermore, human reports of "microglioma" are extremely rare (Hulette, 1996; Mathews et al., 2016), and in human diagnostic neuropathology practice "[t]he existence of true microgliomas as an entity continues to be doubtful" (Graeber et al., 2002). Accordingly, the SAP concluded that microglial tumors observed in this 24-month AGIQ carcinogenicity study in rats represent a rat-specific neoplasm and therefore are unlikely to inform the human risk assessment for AGIQ.

To further evaluate the potential human carcinogenicity risk from AGIQ, we have reviewed the Addendum to the ICH (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use) guideline S1B that was published in 2021 (EMA/CHMP/ICH, 2021). This guideline lists the weight-of-evidence attributes by which results of a 2-year rat study would imply that a substance poses a human carcinogenicity risk. Analysis of data from the current study against the listed S1B parameters indicates that AGIQ does not represent a potential carcinogenic risk to humans. Particular attributes that are relevant to the current study are the absence of hyperplasia, hypertrophy, atypical cellular alterations, or degenerative/regenerative changes in any organ, including the brain, as well as the absence of a dose response. Furthermore, the overall assessment of genotoxic potential for AGIQ is negative based on criteria from ICH S2(R1) guidance.

In summary, the current chronic toxicity and carcinogenicity data taken together with prior literature reports indicate that the food additive AGIQ does not induce neoplasia in rats. Instead, we conclude that the malignant microglial tumors observed in female rats in this study are a species-specific microglial response. Because this finding is usually spontaneous but may be influenced by exposure to certain chemicals (Kolenda-Roberts et al., 2013; Nagatani et al., 2009), the microglial

tumors observed in this study cannot definitively be attributed to AGIQ exposure. The essential absence of microglial tumors in humans indicates that rat malignant microglial tumors have limited implications with respect to predicting human cancer risk.

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CRediT authorship contribution statement

Robert Maronpot: Supervision, Writing – review & editing. **Yuval Ramot:** Writing – original draft. **Christopher Sproul:** Project administration. **Shim-mo Hayashi:** Funding acquisition, Supervision.

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yrtph.2023.105343.

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