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Abstract

The Brazilian mushroom *Agaricus blazei* Murill has antimutagenic, antioxidant, immunostimulatory and antitumorigenic activities, and is increasingly consumed as a health food worldwide. We undertook the present study to evaluate the chronic toxicity and oncogenicity of *A. blazei* Murill in F344 rats. To establish a no-observed-adverse-effect level (NOAEL), four treatment groups of 100 rats each (50 males and 50 females) were fed a powder diet containing lyophilized *A. blazei* aqueous extract at 0, 6250, 12,500, and 25,000 ppm for up to 2 years. During this period, there was no remarkable change in mean body weight, body weight gain, hematologic or serum chemistry parameters, or absolute or relative organ weights in control or treatment groups. Mortality in male treatment groups (26%, 16%, and 30%), however, was significantly lower than in controls (48%). Histopathological studies showed no increased incidence of tumors in any treatment group, and total tumor incidence across all groups was comparable to historical data. In conclusion, an *A. blazei* Murill lyophilized powder diet even at 25,000 ppm (1176 mg/kg b.w./day for male rats and 1518 mg/kg b.w./day for female rats) resulted in no remarkable carcinogenic effects in F344 rats over a 2-year period. Therefore, the dietary NOAEL is 25,000 ppm.

Keywords: *Agaricus blazei* Murill; ABMK; Mushroom; Dietary supplement

1. Introduction

Agaricus blazei Murill is an edible mushroom belonging to the class *Basidomycete,* and has been traditionally used by natives of the Piedade region of San Paulo, Brazil as a health food because of its medicinal properties. The beneficial effects of edible and medicinal mushrooms have long been recognized, and mushrooms have become popular as ordinary foods and dietary supplements worldwide. *A. blazei* Murill consumption has increased significantly in Brazil, Japan, China, Korea, Canada, and the US, concomitant with increased production and marketing as a dietary supplement. In Japan alone, annual consumption of *A. blazei* Murill has increased to 300 tons annually.

Another mushroom species of *Agaricus, bisporus* is also consumed worldwide and has been reported to be carcinogenic in Swiss mice when fresh mushroom was fed at very high amounts (11 g/mouse/day) (Toth and Erickson, 1986). However, when baked and fed, *A. bisporus* mushroom (4.8 g/mouse/day) failed to show carcinogenicity in Swiss mice (Toth et al., 1997). In contrast to carcinogenic effects in Swiss mice, when Charles River Sprague–Dawley rats were fed with 30% *A. bisporus* mushroom dry powder in the diet for 500-days, *A. bisporus* mushroom failed to show carcinogenicity (Matsumoto et al., 1991).

Numerous pharmacological studies have demonstrated that *A. blazei* Murill has antioxidant (Izawa and Inoue,

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2004), antimutagenic (Osaki et al., 1994; Delmanto et al., 2001; Menoli et al., 2001; Luiz et al., 2003; Guterrez et al., 2004); antitumorigenic (Mizuno et al., 1999; Fujimia et al., 1998; Ebina and Fujimia, 1998); immunostimulatory activity in mice (Mizuno et al., 1998; Kaneno et al., 2004) and in humans (Ahn et al., 2004); and chemopreventive activity (Mizuno et al., 1999; Barbisan, 2003; Pinheiro et al., 2003; Lee et al., 2004). Kuroiwa et al. (2005) showed no remarkable adverse effects of *A. blazei* Murill in a 90-day subchronic toxicity study in F344 rats; however, few other toxicological studies have been reported.

The rapidly growing number of consumers of *A. blazei* Murill in many countries and the paucity of toxicological studies prompted us to conduct 2-year carcinogenicity bioassays of *A. blazei* Murill as part of a safety assessment of this widely consumed mushroom.

2. Materials and methods

2.1. Experimental animals and facility

The protocol for this 2-year carcinogenic bioassay was approved by the IACUC Committee of the Korea Institute of Toxicology Institute (KIT). Four hundred and ten SPF-derived, specific pathogen-free F344/DuCrj male and female rats (5-weeks of age) were purchased from Charles River Japan, Inc. (Yokohama, Kanagawa). Animals were quarantined, adapted to rooms for two weeks, and then randomly allocated to eight experimental groups. Stainless steel animal cages (with wire-mesh bottom, $220W \times 410L \times 200H$ mm) housed $\leqslant 3$ animals each. Animal room temperature and relative humidity were maintained at 23 ± 3 °C and $50 \pm 10\%$, respectively. The frequency of room air exchange, duration of light and dark cycles, and intensity of room light were 10–20 times/h, 12 h light cycle (07:00–19:00), 12 h dark cycle (19:00–07:00), and 150–300 lx (one lux is International System unit of illumination, equal to 1 lumen/m^2), respectively. During the entire experiment, all parameters were monitored and stored using a fully validated LABCAT automated data management system (ver. 4.65).

All animal caretakers, technicians, and toxicologists in contact with animals wore sterile coveralls as well as head covers, masks, and gloves. Food was sterilized at high pressure (121 °C for 20 min). All other necessary items in the animal rooms were sterilized with high pressure autoclave, UV irradiation, ethylene oxide gas, or 70% isopropanol.

The entire study was carried out in compliance with standard guidelines for chronic carcinogenic bioassay (KFDA Regulatory Code No. 1998-17, April 16, 1998). Experimental facilities were certified by US AAALAC for performing the reported assays.

2.2. Test compound and dietary dose levels

Lyophilized aqueous extract of *A. blazei* Murill (ABMK), a light brown dry powder, was prepared from hot water extraction at 95 °C for 120 min. and was kindly supplied and funded to Korea Institute of Toxicology by Kyowa Engineering Co., Ltd., Tokyo, Japan. Quality assurance of lyophilized ABMK was monitored at various steps of the production process. A strict standardized culture bed was maintained without any pesticides. Each mushroom production lot was monitored by analyses of DNA sequence and chemical composition such as amino acid composition, protein, lipids, carbohydrates and total ashes. Agaritine assay was not included in the chemical assays between 1991 and 2003. However, since 2005, agaritine has been routinely determined and found to contain agarithin at an average concentration of 400 ppm after sterilization procedure. Heavy metals and microbial analyses of ABMK, feed water, and animal diet (Je IL Animal Feed Company), were all documented and recorded. Test doses in the basal diet were prepared weekly to contain 0, 6250, 12,500, and 25,000 ppm ABMK and stored in the dark at 4 °C until fed to animals *ad libitum* for a 2-year period. A 25,000 ppm-dose was selected as the highest dose (30-fold of daily human consumption), since a 104 week supply of lyophilized ABMK powder was cost prohibitive and thus supply was limited. Chemical stability testing showed that ABMK is stable at 4 °C in vacuum Retro-packs by chemical analysis of total carbohydrate, lipids, proteins and composition of total amino acids.

2.3. Observation and evaluation of toxicological parameters

Both food consumption and body weight were determined weekly for the first 13-weeks and subsequently, determined every 4 weeks. Daily food intake was determined per cage, and the average daily intake of food (g/rat/day) as well as the test material (g/rat/day) was calculated.

Animals were examined daily for clinical abnormalities. Ill or moribund rats were carefully autopsied. Eyes were checked visually every day, and more careful eye examinations were performed weekly. Formal ophthalmologic examination of all rats was performed during the animal room adaptation period using an ophthalmoscope (Genesis, Kyowa, Japan) following pupil dilation (via hydrobrominated formatropin, Sam Il Pharmaceutical Co.). One week prior to sacrifice, a similar ophthalmologic examination was repeated in controls and animals treated with the highest test dose (25,000 ppm).

Hematological parameters—WBC, RBC, Hgb, Hct, MCV, MCH, MCH, PLT count—were determined by a T-540 Coulter Counter. Differential leukocyte count was determined by microscopy following Wright staining. Serum chemistries—TP, ALB, A/G, T.Bil, T. Chol, BUN, CRE, Glu, HDL, TG, Ca, PO4, AST, ALT, ALP, CPK—were also determined in all animals at 104 weeks.

Autopsy was performed on all surviving rats after 104 weeks. Animals were fasted overnight and sacrificed by exsanguination under anesthesia. All rats were examined externally first, followed by careful examination of the internal organs, thoracic cavity, abdominal cavity and brains. Animals that died prior to 104 weeks were subjected to the same autopsy procedure. Eight major organs were weighed, and relative organ weights were calculated for individual rats using their respective body weight. Organs were fixed in 10% neutral formalin. Histopathological examination was performed for the control and 25,000 ppm test groups following paraffin embedding, sectioning, and hematoxylin and eosin staining of organ tissue of liver, spleen, thymus, pancreas, cerebrum, skin, lung, adrenal gland, thyroid gland, pituitary gland, rectum, testis, seminal vesicle, preputial gland, peritoneum, pleura, ovary, uterus, vagina, clitoral gland, and mammary gland. Histopatholgical nomenclature and terms used for describing non-malignant and malignant tumors were based on the "Standardized System of Nomenclature and Diagnostic Criteria (SSNDC)." If no appropriate term or nomenclature was found, an appropriate term was generated. General physiological alterations were not included in histopathological evaluations. In describing tumor burden, histopathologically identical, multiple tumors or metastases in the same organ were counted as one event. In contrast, multiple, histopathologically distinct tumors in the same organ were counted as multiple events. When hyperplasias, adenomas, or neoplasms of identical histological origin were found simultaneously, the most advanced neoplasm was counted as one event. Tumor burden was calculated as the mean number of tumors per animal per group divided by the total animals autopsied per group.

2.4. Statistical analysis

The data for body weights, food intake, test material intake, hematologic and serum chemistry parameters, and the absolute and relative organ weights were statistically analyzed by one way analysis of variance. When statistical significance was found to be less than $p < 0.05$, Dunnett's analysis was applied. If necessary, Fisher's exact test was also used to analyze statistical significant differences between the experimental groups (Fisher, 1958). Survival analysis of 2-year bioassay groups was performed by product limit procedure (Kaplan and Meir, 1958), and differences in survival rates among the experimental groups were analyzed using the

Log-Rank test (Peto and Peto, 1972; Tarone, 1975). When an animal died without any tumors, the date of death and survival time (days) was used.

For the analysis of tumor burden, when the Log-Rank test resulted in statistical significance, the Poly-3 statistical method was applied (Bailer and Portier, 1988; Portier and Bailer, 1989); when the results were not statistically significant, logistic regression analysis was applied for dose effects (Cox, 1972). The relative tumor burden among the 50 male and female rats in the control and 25,000 ppm dose groups was analyzed by Fisher's exact tests and the Cochran–Armitage Trend test (Armitage, 1971; Gart et al., 1979). All data entry and analyses were performed using Labcat module (ver. 4.65) and the SAS program. Statistical significance was set at the $p < 0.05$ and $p < 0.01$ levels.

3. Results

3.1. Clinical signs, food consumption, and whole body weight

Food consumption of control and treatment groups (males and females) are shown in Table 1. No difference in food intake was observed between control and any of the treatment groups during the 2-year study period. The average daily intake of food was 15.5 g in male and 11.5 g in female rats, respectively. The mean daily intake of *A. blazei* Murill dry powder in the 6250, 12,500, and 25,000 ppm test groups was 292, 567, and 1176 mg/kg b.w./day for males and 391, 805, 1518 mg/kg b.w./day for females, respectively. Total intake of *A. blazei* Murill dry powder during 104 weeks was 71, 143, and 281 g for males and 53, 108 and 204 g for females, in the 6250, 12,500, and 25,000 ppm treatment groups, respectively.

Body weight change in all test groups during the 2-year study period is shown in Fig. 1. Body weight of males and females at 104 weeks in control, 6250, 12,500, and 25,000 ppm groups were 389.3 ± 50.6 (SD), 393.8 ± 39.4 (SD), 400.8 ± 33.0 (SD), 410.0 ± 27.4 (SD), and 245.8 ± 26.1 (S.D), 245.4 ± 19.4, 243.7 ± 21.5, and 253.3 ± 20.4, respectively. There were no statistically significant differences between the body weights of control and any treatment groups at any time during the entire 104-week period.

Survival and the incidence of a variety of clinical findings are shown in Table 2. Percent survival in males at all treatment doses was significantly higher than in untreated males ($p < 0.006$). Survival in females in treatment groups remained unchanged. The incidence of moribund and dead animals in all male treatment group was significantly lower than that of controls ($p < 0.006$), while in all female treatment groups, no significant changes were observed. Likewise, the incidence of cataracts in all male treatment groups was lower than in controls ($p < 0.01$–0.03), but no such effect was seen in the female treatment groups.

Table 1
Food consumption and intake of *Agaricus blazei* Murill powder

Group (ppm)	Mean food consumption (g/rat/day)		Mean intake of *Agaricus blazei* Murill powder (mg/rat/day)		(mg/kg b.w./day)		Total intake of *Agaricus blazei* Murill powder (g/rat/104 weeks)	
	Male	Female	Male	Female	Male	Female	Male	Female
0	15.4	11.6	0	0	0	0	0	0
6250	15.5	11.6	97	73	292	391	71	53
12,500	15.6	11.4	195	149	567	805	143	108
25,000	15.5	11.2	386	281	1176	1518	281	204

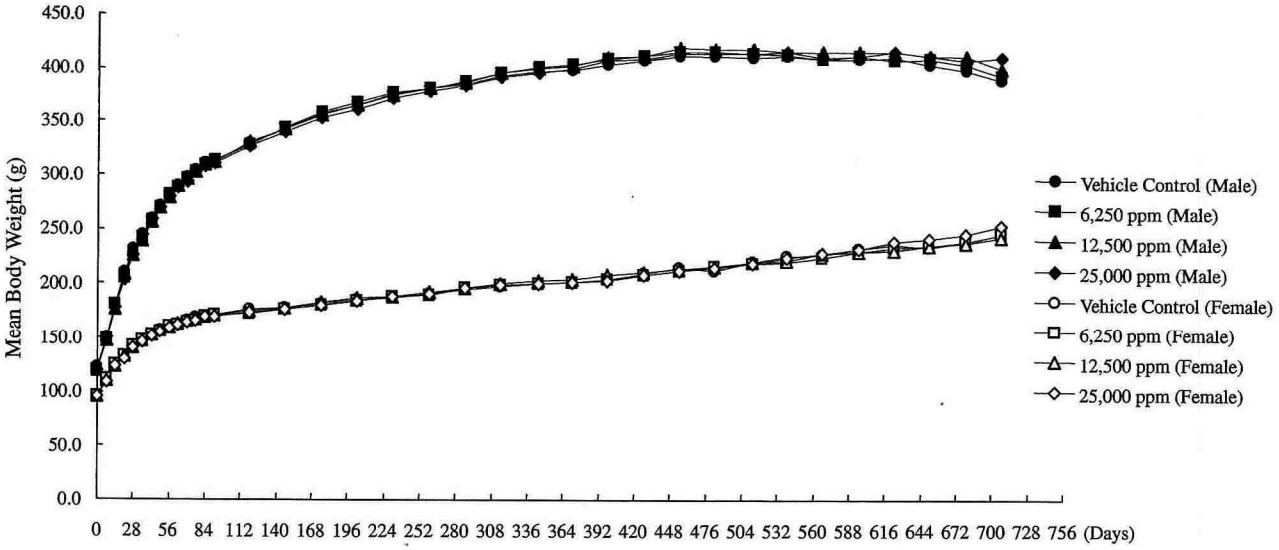

Fig. 1. Growth curve of male and female F344 rats treated with *Agaricus blazei* Murill in diet for 104 weeks.

Table 2
Survival, clinical signs, and ophthalmoscopic findings of F344 rats of both sexes at 104 weeks (group summary)

	Gender	Dose (ppm)			
		Control	6250	12,500	25,000
Total initial number of F344 rats	Male	50	50	50	50
	Female	50	50	50	50
Total number of F344 rats surviving	Male	26	36**	42**	35**
	Female	45	41	43	45
Found moribund (sacrificed) and dead	Male	24	14**	8**	15**
	Female	5	9	7	5
Clinical findings					
Cataract	Male	10	2*	3*	2*
	Female	1	2	3	2
Decreased locomotor activity	Male	19	14*	6*	9*
	Female	42	36*	29*	33*
Dark periocular material	Male	14	8*	7*	6*
	Female	42	36*	29*	33*
Emaciation	Male	9	7	6	6
	Female	1	5	3	2
Lacrimation	Male	3	0	0	1
	Female	26	23	22	18
Paralysis	Male	3	2	0	1
	Female	3	2	0	1
Scars	Male	5	2	2	4
	Female	1	0	0	0
Paleness	Male	13	13	8	9
	Female	4	13	7	4

Statistical analysis by Cochran–Armitage and Fisher's exact test using SAS Ver. 9. *$p < 0.01$–0.03, **$p < 0.006$.

In addition, the incidence of both lethargy and dark periocular exudates was significantly lower in both male and female treatment groups than in controls ($p < 0.01$–0.03).

3.2. Hematological and serum chemistry parameters

The results of hematological tests serum chemistry parameters showed that no significant treatment-related changes were observed in either male or female groups (data not shown).

3.3. Organ weights and autopsy findings

The final body weights, and absolute and relative organ weights of animals are statistically evaluated. There was no treatment-related change in either absolute or relative organ weights in male or female treatment groups (the data not shown) with an exception of testes weights. Testicular atrophy is well known to occur over time, frequently associated with interstitial. Tumors in 2-year old F344 male rats. All testes weights in both control and treatment groups were well within the historical data of 2 year old F344 rats (Boorman, 1990; Mohr et al., 1992; Kang et al., 1994).

3.4. Histopathological findings

Histopathology findings in male and female animals are presented in Tables 3 and 4, respectively. Tumor burden in total animals, tumors burden in dead and moribund animals, and tumors burden in animals at terminal sacrifice are summarized in Tables 5.1–5.3. Tables 3 and 4 show the histopathology results of the listed organs with the nature of lesions in control and 6250, 12,500, and 25,000 ppm treatment groups. There were no significant treatment-related or dose-related increases in benign or malignant tumor incidence. Histopatholgical examination showed some incidence of large granular lymphocyte lymphoma in spleen, benign pheochromocyotoma in adrenal gland, C-cell adenoma in thyroid gland, adenoma of the pars distalis of the pituitary gland, fibroma/subcutis of skin, and a very low incidence of hepatocellular carcinoma in both sexes. Additionally interstitial adenoma of testes in all male groups, and endometrial stromal polyps in uterus, and fibroadenoma in mammary glands in all female groups were observed; however, the incidence of these lesions in all treatment groups was not statistically different from controls.

Tumors burden in dead and moribund animals prior to terminal sacrifice is summarized in Table 5.1. The total number of dead or moribund male rats in control, 6250, 12,500, and 25,000 ppm treatment groups was 24, 14, 8, and 15, respectively. The incidence of dead and moribund animals in all male treatment groups was significantly lower than that of controls. In contrast, the total number dead or moribund female rats in control, 6250, 12,500, and 25,000 ppm treatment groups was 5, 9, 7, and 5, respectively (no statistically significant difference). The total number of rats with tumor burden of any type in control, 6,250, 12,500, and 25,000 ppm treatment groups was 22 (92%), 13 (93%), 8 (100%), and 15 (100%) for male animals, and 3 (60%), 8 (89%), 5 (71%) and 4 (80%) for female animals, respectively. Percentage value in the parentheses represents % of rats with tumors over the total number of rats examined. On the other hand, the total number of rats with malignant tumors in control and treatment groups was 19 (79%), 12 (86%), 5 (63%), and 11 (73%) for male animals, and 2 (40%), 6 (67%), 3 (43%), and 4 (80%) for female animals, respectively. Furthermore, the total number of rats in control and treatment groups with benign tumors was 17 (71%), 9 (64%), 6 (75%) and 14 (93%) for male animals, and 2 (40%), 2 (22%), 2 (57%), and 0 (0%) for female animals, respectively.

The tumors burden in male and female rats at terminal sacrifice is shown in Table 5.2. The total number of surviving rats in control and 6250, 12,500, and 25,000 ppm treatment groups at terminal sacrifice was 26, 36, 42, and 35 for male animals, and 45, 41, 43, and 45, for female animals, respectively. The number of males surviving at the 104-week endpoint was significantly greater in all treatment groups compared to controls, while the number of surviving female animals in the treatment groups was not statistically different from controls. Total number of male and female rats with tumors in control, 6250, 12,500, and 25,000 ppm treatment groups was 25 (96%), 35 (97%), 41 (98%), 35 (100%), and 28 (62%), 24 (59%), 24 (56%), and 28 (62%), respectively. On the other hand, the total number

Table 3
Incidence of neoplastic microscopic findings in F344 male rats fed *Agaricus blazei* Murill for 104 weeks

Organs	Lesions	Dose (ppm)				Historical data ranges
		Control	6250	12,500	25,000	
		Tumor burden (% of rats bearing tumors)[e]				
Liver	Hepatocellular carcinoma	0(0.0)	1(2.0)	1(2.0)	2(4.3)	0–12[a]
Spleen	Large granular lymphocyte lymphoma	19(40.4)	17(34.7)	20(40.0)	15(31.3)	10–72[a]
	Lymphoblastic lymphoma	2(4.3)	4(8.2)	1(2.0)	2(4.2)	0–5.6[b]
	Fibroma	0(0.0)	1(2.0)	0(0.0)	0(0.0)	0–2[a]
	Granulocytic leukemia	0(0.0)	0(0.0)	0(0.0)	1(2.1)	0–1[c]
Thymus	Thymoma	1(2.5)	0(0.0)	0(0.0)	0(0.0)	0–2[a]
Pancreas	Islet cell adenoma	1(2.1)	1(2.1)	3(6.1)	1(2.2)	0–11[a]
	Islet cell carcinoma	0(0.0)	0(0.0)	0(0.0)	1(2.2)	0–8[a]
Cerebrum	Anaplastic glioma	0(0.0)	0(0.0)	0(0.0)	1(2.0)	0–2[a]
Skin	Keratoacanthoma	0(0.0)	1(2.0)	0(0.0)	1(2.0)	0–14[a]
	Squamous cell carcinoma	0(0.0)	1(2.0)	0(0.0)	0(0.0)	0–61[a]
	Basal cell carcinoma	1(2.0)	0(0.0)	1(2.0)	0(0.0)	0–4[a]
	Fibroadenoma/mammary gland	0(0.0)	1(2.0)	0(0.0)	0(0.0)	0–12[a]
	Adenocarcinoma/mammary gland	0(0.0)	0(0.0)	0(0.0)	1(2.0)	0–2[a]
	Fibroma/subcutis	6(12.0)	0(0.0)	1(4.0)	8(16.0)	0–12[a]
	Malignant fibrous histiosarcoma/subcutis	0(0.0)	0(0.0)	0(0.0)	1(2.0)	0–2[a]
Lung	Bronchiolo-alveolar adenoma	0(0.0)	0(0.0)	1(2.1)	1(2.1)	0–6[a]
	Bronchiolo-alveolar carcinoma	0(0.0)	0(0.0)	0(0.0)	1(2.1)	0–6[a]
Adrenal gland	Pheochromocytoma, benign	4(8.7)	3(6.1)	4(8.5)	0(0.0)	26.2[b]
	Adrenal cortical adenoma	0(0.0)	0(0.0)	1(2.1)	0(0.0)	0–8[a]
	Pheochromocytoma, malignant	1(2.2)	0(0.0)	0(0.0)	1(2.2)	2.4[b]
Thyroid gland	C-cell adenoma	4(9.5)	12(25.0)	6(13.0)	8(18.6)	0–22[a]
	C-cell carcinoma	2(4.8)	1(2.1)	0(0.0)	1(2.3)	0–12[a]
	Follicular cell carcinoma	0(0.0)	1(2.1)	0(0.0)	0(0.0)	0–2[a]
	Follicular cell adenoma	0(0.0)	0(0.0)	2(4.3)	0(0.0	0–5[a]
Pituitary gland	Adenoma, pars distalis	10(23.8)	4(9.1)	8(17.0)	7(16.3)	5–52[a]
Rectum	Fibrosarcoma	0(0.0)	0(0.0)	0(0.0)	1(2.4)	0–2[a]
Testis	Interstitial cell adenoma	38(82.6)	37(82.2)	38(97.4)	41(83.7)	64–98[a]
	Interstitial cell carcinoma	0(0.0)	0(0.0)	0(0.0)	1(2.0)	0–10[d]
Seminal vesicle	Adenoma	0(0.0)	0(0.0)	0(0.0)	1(2.3)	0.2[a]
Preputial gland	Adenoma	1(2.0)	0(0.0)	0(0.0)	0(0.0)	0–16[a]
Peritoneum	Mesothelioma	2(4.0)	0(0.0)	1(2.0)	3(6.0)	0–10[a]
	Osteosarcoma	0(0.0)	0(0.0)	0(0.0)	1(2.0)	0–4[a]
	Histiocytic sarcoma	0(0.0)	0(0.0)	0(0.0)	1(2.0)	0–2[a]
Pleura	Mesothelioma	0(0.0)	1(2.0)	0(0.0)	0(0.0)	0–10[a]

[a] Boorman et al. (1990).
[b] Tashima, (1989).
[c] Mohr et al. (1992).
[d] Derelanko and Hollinger (1995).
[e] Percentage = Numbers of animals observed with tumor(s)/total number of animals necropsied.

of male and female rats with malignant tumors in control and 6250, 12,500, and 25,000 ppm groups was 5 (19%), 13 (36%), 17 (40%), 15 (43%) and 5 (11%), 8 (20%), 6 (14%), and 5 (11%), respectively. However, the total number of male and female rats with benign tumors in control and the treatment groups was 24 (92%), 33 (92%) 39 (93%), 35 (100%). and 26 (58%), 21 (51%) 19 (44%), and 27 (60%), respectively.

The tumor burden in total number of animals is shown in Table 5.3. The total number of males and females with all tumors in control, 6250, 12,500, and 25,000 ppm treatment groups was 47 (94%), 48 (96%), 49 (98%), 50 (100%), and 31 (62%), 32 (64%), 29 (58%), and 32 (64%), respectively. The total number of male and female rats with malignant tumors in control and the treatment groups was 24 (48%), 25 (50%), 22 (44%), and 26 (52%), and 7 (14%), 14 (28%), 9 (18%), and 9 (18%), respectively. Furthermore, the total number of male and female rats with benign tumors in control and the treatment groups was 41 (82%), 42 (84%), 45 (90%), 49 (98%), and 28 (56%), 23 (46%), 23 (46%), and 27 (54%), respectively. The tumor burden of benign or malignant of all types in the total number of animals was not significantly different between control and 6250, 12,500, or 25,000 ppm treatment groups, indicating no apparent treatment-related effects.

4. Discussion

In the present two-year carcinogenicity bioassay of *A. blazei* Murill lyophilized powder fed to F344 rats, no

Table 4
Tumor burden of microscopic finding in F344 female rats fed *Agaricus blazei* Murill for 104 weeks

Organs	Lesions	Dose (ppm)				Historical data ranges
		Control	6,250	12,500	25,000	
		Tumor burden (% of tumor bearing animals)[e]				
Liver	Heaptocellular adenoma	2(4.0)	1(2.0)	0(0.0)	0(0.0)	0–10[a]
Spleen	Large granular lymphocyte lymphoma	3(6.0)	8(16.0)	4(8.2)	4(8.9)	6–31[a]
	Leiomyosarcoma	1(2.0)	0(0.0)	0(0.0)	0(0.0)	0–2[a]
	Lymphoblastic lymphoma	0(0.0)	3(6.0)	2(4.2)	0(0.0)	0–3.7[b]
Thymus	Thymoma	1(2.2)	0(0.0)	0(0.0)	0(0.0)	0–2[a]
Pancreas	Islet cell adenoma	0(0.0)	0(0.0)	0(0.0)	1(2.0)	0–7[a]
Skin	Squamous cell carcinoma	1(2.0)	0(0.0)	0(0.0)	0(0.0)	0–4[a]
	Basal cell carcinoma	0(0.0)	1(2.0)	0(0.0)	0(0.0)	0–2[a]
	Trichoepithelioma	0(0.0)	0(0.0)	0(0.0)	1(2.0)	0–2[a]
	Carcinoma/modified sebaceous gland	0(0.0)	0(0.0)	0(0.0)	2(4.0)	0–6[a]
	Fibroma/subcutis	0(0.0)	1(2.0)	0(0.0)	0(0.0)	0–6[a]
	Lipoma/subcutis	1(2.0)	0(0.0)	0(0.0)	0(0.0)	0–2[a]
	Rhabdomyosarcoma/subcutis	0(0.0)	0(0.0)	1(2.0)	0(0.0)	0–0.5[b]
Lung	Bronchiolo-alveolar adenoma .	0(0.0)	0(0.0)	1(2.1)	0(0.0)	0–4[a]
Kidney	Renal tubule carcinoma	0(0.0)	0(0.0)	1(2.0)	0(0.0)	0–2[a]
Adrenal gland	Pheochromocytoma, benign	1(2.0)	1(2.0)	2(4.1)	0(0.0)	6.5[b]
	Pheochromocytoma, malignant	0(0.0)	1(2.0)	0(0.0)	0(0.0)	1.5[b]
Thyroid gland	C-cell adenoma	7(14.0)	5(10.9)	5(10.4)	5(10)	0–34[a]
	C-cell carcinoma	0(0.0)	1(2.2)	0(0.0)	0(0.0)	0–10[a]
Pituitary gland	Adenoma, pars distalis	11(23.9)	13(28.9)	11(24.4)	15(32.6)	18–70[a]
	Craniopharyngioma	1(2.2)	0(0.0)	0(0.0)	0(0.0)	0–2[d]
Rectum	Leiomyosarcoma	0(0.0)	0(0.0)	0(0.0)	1(2.1)	0–2[c]
Uterus	Endometrial stromal polyp	10(20)	6(12.2)	5(10.2)	10(20.0)	8.37[a]
	Leiomyoma	0(0.0)	0(0.0)	1(2.0)	0(0.0)	0–2[a]
	Endometrial adenoma	0(0.0)	2(4.1)	0(0.0)	1(2.0)	0–4[a]
	Endometrial adenocarcinoma	1(2.0)	0(0.0)	0(0.0)	3(6.0)	0–6[a]
	Endometrial stromal sarcoma	1(2.0)	1(2.0)	0(0.0)	0(0.0)	0–6[a]
	Mesothelioma	0(0.0)	1(2.0)	0(0.0)	0(0.0)	0–2[a]
Vagina	Vaginal polyp	0(0.0)	0(0.0)	1(2.0)	0(0.0)	0–2[a]
Clitoral gland	Squamous cell papilloma	0(0.0)	1(2.0)	0(0.0)	1(2.0)	0–2[a]
Mammary gland	Adenoma	0(0.0)	0(0.0)	1(2.0)	0(0.0)	0–6[a]
	Fibroadenoma	3(6.1)	2(4.3)	3(6.4)	4(8.5)	10–49[a]
	Adenocarcinoma	1(2.0)	0(0.0)	0(0.0)	0(0.0)	0–8[a]
Peritoneum	Mesothelioma	0(0.0)	1(2.0)	0(0.0)	0(0.0)	0–2[a]
	Fibrosarcoma	0(0.0)	0(0.0)	1(2.0)	0(0.0)	0–2[a]
Mammary gland	Adenoma	0(0.0)	0(0.0)	1(2.0)	0(0.0)	0–6[a]
	Fibroadenoma	3(6.1)	2(4.3)	3(6.4)	4(8.5)	10–49[a]
	Adenocarcinoma	1(2.0)	0(0.0)	0(0.0)	0(0.0)	0–8[a]
Peritoneum	Mesothelioma	0(0.0)	1(2.0)	0(0.0)	0(0.0)	0–2[a]
	Fibrosarcoma	0(0.0)	0(0.0)	1(2.0)	0(0.0)	0–2[a]

[a] Boorman et al. (1993).
[b] Tashima, (1989).
[c] Mohr et al. (1992).
[d] Derelanko and Hollinger (1995).
[e] Percentage = Numbers of animals observed with tumor(s)/total numbers of animals necropsied.

consistent treatment-related changes in food consumption, body weight, or body weight gain were observed. Low incidence of mortality, cataract, lethargy, and dark periocular exudates in *A. blazei* Murill treated males may be attributable to antimutagenic (Menoli et al., 2001; Guterrez et al., 2004), antioxidant (Izawa and Inoue, 2004), anti-inflammatory (Lull et al., 2005), and/or immunostimulatory activity observed both in mice (Mizuno et al., 1998; Kaneno et al., 2004) and in humans (Nakane et al., 2001; Ahn et al., 2004). Interestingly, epidemiological data from the Nagano prefecture in Japan indicate a significantly lower cancer death rate among farmers producing mushroom as a main occupation compared to others in the prefecture (97.1 versus 160.1 deaths/yr, respectively, $p < 0.01$; Ikekawa et al., 1989).

Histopathological examination of control and all treatment animals of both sexes showed no remarkable increase in tumor incidence. Tumor burden in all treatment groups of both sexes were neither dose-dependent, nor treatment-related and were all within the normal range (Tashima, 1989; Boorman et al., 1990; Mohr et al., 1992; Kang et al., 1994; Derelanko and Hollinger, 1995).

Mortality of male animals treated with *A. blazei* Murill for 2-years was significantly lower than controls; however

Table 5.1
Summary of tumor burden in dead and moribund animals (% of tumor bearing animals)

Animal numbers/lesions	Gender	Control	6250	12,500	25,000	Historical data range[a] (%)
Total number of animals/Group	Male	24	14**	8**	15**	
	Female	5	9	7	5	
Total number of animals with tumors	Male	22(92%)	13(93%)	8(100%)	15(100%)	90–100
	Female	3(60%)	8(89%)	5(71%)	4(80%)	64–98
Total number of animals with malignant tumors	Male	19(79%)	12(86%)	5(63%)	11(73%)	38–82
	Female	2(40%)	6(67%)	3(43%)	4(80%)	18–56
Total number of animals with benign tumors	Male	17(71%)	9(64%)	6(75%)	14(93%)	82–100
	Female	2(40%)	2(22%)	2(57%)	0(0%)	58–86%

** Statistical analysis by Cochran–Armitage and Fisher's exact test using SAS ver. 9. $p < 0.01$ and $p < 0.006$, respectively.
[a] Boorman et al. (1990).

Table 5.2
Summary of tumor burden in animals at terminal sacrifice (% of tumor bearing animals)

Animal numbers/lesions	Gender	Control	6250	12,500	25,000	Historical data range[a] (%)
Total number of animals/Group	Male	26	36**	42**	35**	
	Female	45	41	43	45	
Total number of animals with tumors	Male	25(96%)	35(97%)	41(98%)	35(100%)	90–100
	Female	28(62%)	24(59%)	24(56%)	28(62%)	64–98
Total number of animals with malignant tumors	Male	5(19%)	13(36%)	17(40%)	15(43%)	38–82
	Female	5(11%)	8(20%)	6(14%)	5(11%)	18–56
Total number of animals with benign tumors	Male	24(92%)	33(92%)	39(93%)	35(100%)	82–100
	Female	26(58%)	21(51%)	19(44%)	27(60%)	58–86

** Statistical analysis by Cochran–Armitage and Fisher's exact test using SAS Ver.9. $p < 0.01$ and $p < 0.006$, respectively.
[a] Boorman et al. (1990).

Table 5.3
Summary of tumor burden in total number of animals (% of tumor bearing animals)

Animal numbers/lesions	Gender	Control	6250	12,500	25,000	Historical data range[a] (%)
Total number of animals/Group	Male	50	50	50	50	
	Female	50	50	50	50	
Total number of animals with tumors	Male	47(94%)	48(96%)	49(98%)	50(100%)	90–100
	Female	31(62%)	32(64%)	29(58%)	32(64%)	64–98
Total number of animals with malignant tumors	Male	24(48%)	25(50%)	22(44%)	26(52%)	38–82
	Female	7(14%)	14(28%)	9(18%)	9(18%)	18–56
Total number of animals with benign tumors	Male	41(82%)	42(84%)	45(90%)	49(98%)	82–100
	Female	28(56%)	23(46%)	23(46%)	27(54%)	58–86

[a] Boorman et al. (1990).

the incidence of benign, malignant, or total tumors in treated males was not significantly different from controls. The tumor burden of benign and malignant types in both moribund and dead animals appeared to be lower in the male treatment groups; however, these differences were all within the reported, historical range. In contrast, no mortality differences were observed between the female control and treatment groups, and there were no remarkable differences in the incidence of benign or malignant tumors in female moribund and dead or surviving animals. The sum of benign tumors, malignant tumors, and the total sum of tumors in both male and female treatment groups did not appear to be dose dependent, and all tumor incidences were found to be within the reported, historical range.

Previously, it was reported that the most widely consumed edible mushroom, *A. bisporus* was carcinogenic in Swiss mice, when uncooked *A. bisporus* mushroom was fed *ad libitum* for the first 3 consecutive days and followed by 4-days of semisynthetic diet per week for life (the estimated average daily consumption was 11 g/mouse or 440 g/kg b.w./day). A carcinogen was postulated to be agarithine (β-*N*-[γ-L(+)-glutamyl]-4-hydroxymethylphenylhydrazine) in this mushroom (Toth and Erickson, 1986). In contrast to the fresh *A. bisporus* mushroom study, the 2-year carcinogenicity study with baked *A. bisporus* mushroom (220–230 °C for 10 min; a feeding cycle of 12 h baked *A. bisporus* and 12 h of semisynthetic diet/daily) failed to demonstrate carcinogenicity in Swiss mice (Total et al., 1997). However, the same baked *A. bisporus* was fed *ad libitum* for 3 consecutive days followed by semisynthetic diet for 4 days per week for life, resulted in positive carcinogenic results (Toth et al., 1997). Another long term dietary carcinogenicity study (30% of *A. biosporus* powder in diet for 500 days) in the Sprague–Dawley rats with lyophilized dry powder of *A. bisporus* failed to demonstrate carcinogenicity (Matsumoto et al., 1991). Thus, both negative and

positive chronic carcinogenic bioassay results appeared to be dependent upon different feeding regimens. Clearly, it appears that the nutritional imbalance of feeding on either fresh *A. bisporus* or baked *A. bisporus* alone for 3 consecutive days probably played a role in positive carcinogenic results. Species difference may also play a role in *A. bisporus* carcinogenicity.

Although mutagenicity results of postulated agaritine metabolites, 4-(hydroxymethyl)-phenylhydrazine, 4-(hydroxymethyl)benzene diazonium ion were Ames' test positive, no such mutagenic metabolites were detected by Ame's mutagenic test in either plasma or urine in mice or rats after repeated exposure to agaritine (Walton et al., 2000). Furthermore, agaritine was not responsible for the mutagenicity of *A. bisporus* mushroom extracts (Papaparaskeva et al., 1991). Likewise, the mutagencity and genotoxic studies with the extract of *A. bisporus* was negative in both Ame's test and micronuclei test (Pool-Zobel et al., 1990). Two-year carcinogenicity studies with high doses of agaritine via drinking (daily consumption of agaritine in drinking water for male and female Swiss mice were 158 mg/kg b.w./day and 120 mg/kg b.w./day, respectively) or subcutaneous administration of agaritine (100 mg/kg b.w./day for 5 days per week) failed to demonstrate carcinogenicity and therefore provide further evidence that agaritine is not carcinogenic (Toth et al., 1981; Toth and Sorenson, 1984).

Our previous Ame's mutagencity test with ABMK produced positive results. However, the positive Ame's test results were attributed to the presence of L-histidine present in the ABMK test materials. Thus, positive Ame's test results with ABMK were false positives and not attributed to the presence of agaritine. Genotoxic studies with ABMK yielded negative results (Lee and Kirby, 2003, and Zheng et al., 2005).

In conclusion, there was no evidence that dietary treatment with *A. blazei* Murill dry powder enhanced any specific tumor type. Furthermore, total tumor incidence was similar across all groups. Since dietary intake of *A. blazei* Murill dry powder appears to enhance survival in males, some slight (but statistically insignificant) increases in tumor incidence may be attributed to the increased survival in the male treatment groups. These results indicate that dietary *A. blazei* Murill fed at 6250, 12,500, and 25,000 ppm for 2 years under the described experimental conditions did not appear to be carcinogenic in F344 rats. Thus, the dietary no observed-carcinogenic effect level (NOAEL) is 25,000 ppm (1176 mg/kg b.w./day for male rats and 1518 mg/kg. b.w./day for female F344 rats).

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