

Himematsutake (Iwade Strain 101) extract (ABM-FD): Genetic toxicology and a 3-month dietary toxicity study in rats

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Abstract

Agaricus blazei Murrill, an edible mushroom, is used as a functional food due to medicinal effects of (1→6)-β-D-glucan protein complex which has been shown to have anti tumour activity in mice. A 13 week oral subchronic study in rats performed at 500, 1000 or 2000 mg/kg/day caused, at the highest dose, reduced erythrocyte numbers and high mean cell volume in males, high creatinine and urea concentrations in both sexes and low spleen weights in females, but no histopathological change. The findings suggested low level chronic toxicity at 2000 mg/kg/day and a no observed adverse effect level (NOAEL) of 1000 mg/kg/day. Genotoxicity tests on the aqueous extract were negative in the bacterial reverse mutation test, either with or without S9 mix, up to 5000 μg/plate and in a rat bone marrow micronucleus test up to 2 g/kg bodyweight. The extract was positive at acceptable levels of toxicity in an L5178Y mouse lymphoma assay following 24 h exposure in the absence of S9 and this was associated with an increase in the number of small colonies, suggesting possible clastogenic activity or aneuploidy, rather than point mutation. The aqueous extract of *A. blazei* is therefore of low subchronic toxicity and did not cause any direct effect upon the DNA molecule and the weak positive in the L5178 mouse lymphoma test was likely due to large deletions or the loss of the whole chromosomes rather than to direct damage to the DNA.

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1. Introduction

Agaricus blazei Murrill, an edible mushroom belonging to the *Agaricaceae* family, is native to southern Brazil and is popularly known as “Himematsutake” in Japan. It is used widely as a medicinal and functional food (rather than for nutritional purposes) because it has potent medicinal properties. Aqueous extracts of the mycelium, fruiting bodies or culture solution of *A. blazei* Murrill have been approved in Japan as a food additive to give a bitter taste (Japanese MHLW, 1996a; JETRO, 2004). Anti-tumour polysaccharides have been isolated from a number of dif-

ferent mushrooms and their activity is attributed, principally, to potentiation of the immune response (Mizuno et al., 1998). An insoluble polysaccharide from the hot-water extract of the fruiting body of *A. blazei* Murrill, obtained by successive extractions with 1% ammonium oxalate solution and 5% sodium hydroxide solution, was shown to have significant anti-tumour activity (Mizuno et al., 1990a,b). This polysaccharide, a (1→6)-β-D-glucan protein complex (FIII-2-b) rich in aspartic and glutamic acid, alanine, leucine and proline, has been shown to have anti-tumour activity in mice, suppressing Sarcoma 180, Ehrlich ascites carcinoma, Meth A fibrosarcoma, Shionogi carcinoma 42 and Lewis lung carcinoma. The mode of action involves activation of natural killer cells/activated macrophages or helper/inducer lymphocyte T-cells (Mizuno et al., 1999; Ito, 2000; Ito et al., 2002). The studies

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described in this article were performed on extracts from the fruiting body of *A. blazei* Murrill (Himematsutake (Iwade Strain 101) extract) in order to support a GRAS Notice submission to the US Food and Drug Administration according to FDA Rule 62 FR 18938.

2. Material and methods

The studies described in this article, an *in vitro* bacterial mutation technique, a mouse lymphoma L5178Y cell mutation test and an *in vivo* micronucleus test, and one study to assess potential sub-chronic mammalian toxicity after repeated oral doses to rats, were designed to meet or exceed the requirements of the Office of Food Additive Safety (Redbook, 2000) Guidelines of the US Food and Drug Administration. All studies were conducted at Huntingdon Life Sciences Ltd, Suffolk, England in compliance with Good Laboratory Practice Regulations of the U.K., OECD and European Union.

The ABM-FD for these studies was drawn from Batch EX 200408 and was a brown solid provided in a number of bottles, each containing approximately 20 g of ABM-FD. The test material contained 2.9 g β -glucan/100 g and was stored in the dark at ambient temperature.

The chemical composition of a typical batch of Himematsutake extract is presented in Table 1.

2.1. Thirteen-week repeated dose toxicity study in the rat

Outbred albino Sprague-Dawley (CrI:CD® (SD) IGS BR) rats obtained from Charles River (UK) Limited, Margate, England, were distributed into four groups, each comprising 20 animals per sex. When the animals were approximately 7 weeks of age, three groups received ABM-FD orally, by gavage, at dosages of 500, 1000 or 2000 mg/kg/day whilst the fourth group received the vehicle (water for formulation prepared by reverse osmosis at Huntingdon Life Sciences) at the same volume–dosage (10 mL/kg bodyweight) and served as the Control.

Animals were observed for mortality and/or signs of severe toxic effect and there were certain observations (in a standard arena, assessment of sensory reactivity, grip–strength and motor activity) that were included to determine if ABM-FD was neurotoxic. Body weights and food consumption were recorded weekly during the treatment period. Ophthalmological investigations were performed before commencement of treatment and at the end of the treatment period. During week 2, 7 and

13, blood samples were collected under anaesthesia into EDTA anticoagulant for analysis of the cellular components of the blood, citrate anticoagulant for the measurement of clotting times and lithium heparin anticoagulant for the measurement of plasma levels of a range of enzyme markers and other plasma constituents.

At the end of the treatment period all animals were subjected to a detailed autopsy which included weighing of selected major organs and retention of an extensive list of tissues in an appropriate fixative. All retained tissues for Control and high dosage animals and the axillary and mesenteric lymph nodes, spleen, thymus and femoral/sternal bone marrow and grossly abnormal tissues of low and intermediate dosage animals were processed to slide and stained with haematoxylin and eosin and examined by light microscopy.

2.2. Bacterial reverse mutation test

This study was designed to assess the ability of ABM-FD to cause point (gene) mutation in *Salmonella typhimurium* and *Escherichia coli*. (Ames et al., 1975; Maron and Ames, 1983; Green, 1984; Mahon et al., 1989). These strains were used to detect base changes (*S. typhimurium* TA1535 and TA100, and *E. coli* WP2 *uvrA* (pKM101) and frameshift mutations (*S. typhimurium* TA1537, TA98 and TA100). The strains of *S. typhimurium* were obtained from the National Collection of Type Cultures, London, England and the strain of *E. coli* was obtained from the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland. Positive control chemicals (sodium azide, 9-aminoacridine, 2-nitrofluorene, 4-nitroquinoline-1-oxide, 2-aminoanthracene and benzo[a]-pyrene) were supplied by Sigma–Aldrich Chemical Co. All treatments were conducted in both the presence and absence of S9 mix.

A preliminary test was performed using seven concentrations of ABM-FD (5, 15, 50, 150, 500, 1500 and 5000 μ g/plate) in water and on the vehicle and positive controls in the absence of presence of S9 mix (supplied by Moltox, North Carolina, USA). Based on the findings from this test, the first main mutation test was conducted using five concentrations of ABM-FD (50, 150, 500, 1500 and 5000 μ g/plate) but after obtaining a clear negative response, the test was repeated after a variation to the test procedure where the test mixtures were pre-incubated at 37 °C for 30 min.

Treatment was required to produce a reproducible increase in revertant colony numbers of at least twice (three times in the case of strains TA1535 and TA1537) the concurrent vehicle controls, with some evidence of a positive dose–response relationship, before it was considered to exhibit mutagenic activity in this test system.

2.3. L5178 mouse lymphoma assay

The *in vitro* mammalian cell mutation test in mouse lymphoma L5178Y cells is designed to assess the ability of ABM-FD to cause a forward mutation in mouse lymphoma cells heterozygous at the thymidine kinase (tk) locus (TK^{+/-}) (Amacher et al., 1979; Amacher et al., 1980; Clive et al., 1979; Cole et al., 1990; Moore et al., 2000ab, 2003a, in press; Robinson, 1989). The L5178Y mouse lymphoma (3.7.2c) cells (Clive and Spector, 1975) were obtained from the MRC Cell Mutation Unit, University of Sussex, Brighton. Some substances do not exert a mutagenic effect until they have been metabolised by enzyme systems not available in the bacterial or isolated mammalian cell. Therefore, in the *in vitro* test systems, the cells and test substance were incubated in both the absence and presence of a supplemented liver homogenate fraction (S9 mix). The positive controls chemicals (methyl methanesulphonate and 3-methylcholanthrene) were supplied by Sigma–Aldrich Chemical Co.

Three-hour treatments were conducted in both the presence and absence of S9 mix, and 24-h continuous treatments were conducted in the absence of S9 mix. In a preliminary test, ABM-FD was tested at 10 concentrations ranging from 9.77 to 5000 μ g/mL. In the main mutation tests, ABM-FD was tested at six concentrations from 156.25 to 5000 μ g/mL for Test 1 (3 h) and from 500 to 4000 μ g/mL for Test 2 (24 h). In the preliminary test, cell suspensions were incubated for approximately 30 min at 37 °C and dispensed into aliquots before adding S9 mix, if appropriate,

Table 1
Composition of Himematsutake fruiting body extract per 100 g extract

Component	Result	Component	Result
Moisture	58.0 g	Amino acids	
Protein	16.4 g	Arginine	0.36 g
Fat	0.3 g	Lysine	0.43 g
Ash	4.9 g	Histidine	0.16 g
Available carbohydrate	16.1 g	Phenylalanine	0.36 g
Energy	133 kcal	Tyrosine	0.27 g
Dietary fibre	4.3 g	Leucine	0.61 g
Sodium	398 mg	Isoleucine	0.36 g
Potassium	1.93 g	Methionine	0.13 g
Phosphorus	547 mg	Valine	0.48 g
Iron	2.28 mg	Alanine	0.73 g
Thiamine (hydrochloride)	0.08 mg	Glycine	0.47 g
Riboflavin	1.35 mg	Proline	0.47 g
Lead	0.09 ppm	Glutamic acid	1.27 g
Cadmium	0.14 ppm	Serine	0.43 g
Mercury	0.03 ppm	Threonine	0.43 g
Copper	1.78 mg	Aspartic acid	0.77 g
Zinc	1.18 mg	Tryptophan	0.11 g
		Cystine	0.06 g

AMD-FD solutions (at 10 times the desired final concentration) or vehicle. Cultures were incubated at 37 °C for 3-h in the absence and presence of S9 mix and for 24 h in the absence of S9 mix. At the end of the exposure period, the cells were washed, incubated and sampled after 24 and 48 h to assess growth in suspension. After sampling at 24 h the cell density was adjusted to 2×10^5 cells/mL. The relative suspension growth (RSG) was used to determine the concentrations of ABM-FD used in the main mutation test.

For the first main mutation test, cells were exposed to the test substance for 3 h in the absence and presence of S9 mix. Following exposure, the cells were washed once, resuspended in medium and incubated for a further 48 h to allow for expression of mutant phenotype. The cultures were sampled after 24 and 48 h to assess growth in suspension. After sampling at 24 h the cell density was adjusted to 2×10^5 cells/mL. After 48 h, cultures with a density greater than 1×10^5 cells/mL were assessed for cloning efficiency (viability) and mutant potential. Cells were also similarly exposed to the test substance for 24 h in the absence of S9 mix, the only exception being that after sampling at 24 h, the cell density was readjusted to 2×10^5 cells/mL.

The test was regarded as negative if the mean mutant frequency of all test concentrations was less than the sum of the mean concurrent solvent control mutant frequency and the Global Evaluation Factor (GEF), which is 126×10^{-6} (Moore et al., 2006). If the mutant frequency of any test concentrations exceeded the sum of the mean concurrent solvent control mutant frequency and the GEF, a linear trend test was applied and, if positive, this indicated a positive, biologically relevant response.

2.4. Micronucleus test in the rat

The rat micronucleus test assesses the potential of the test substance to induce an increase in micronucleated erythrocytes following acute oral administration (Boller and Schmid, 1970; MacGregor et al., 1987; Mavournin et al., 1990).

Outbred male albino Sprague-Dawley (CrI:CD[®] (SD) IGS BR) rats, obtained from Charles River (UK) Limited, were used in this investigation. The animals were distributed into four groups of 7 males and one group (positive control) of 5 males. Three groups received ABM-FD orally, by gavage, at dosages of 500, 1000 or 2000 mg/kg/day for two days, and a similarly constituted control group received the vehicle, water for formulation, at the same volume–dosage (10 mL/kg bodyweight). Positive control animals received a single oral dose of cyclophosphamide (supplied by Sigma–Aldrich Chemical Co.) at 20 mg/kg approximately 24 h before necropsy. All animals were killed approximately 24 h after their final dose and both femurs dissected, cleared of extraneous tissue and the proximal epiphysis removed. The bone marrow of both femurs from each animal was flushed out and pooled in filtered foetal calf serum. After centrifugation, the cell pellet was resuspended in a small volume of foetal calf serum to facilitate smearing in the conventional manner on glass microscope slides (Schmid, 1976). Each smear was fixed in methanol, air-dried, rinsed in purified water, stained in acridine orange solution for 3 min, washed and rinsed with water and stored in the dark at approximately 4 °C for a minimum of 1 h until being scored by fluorescence microscopy for the proportion of immature erythrocytes and the number of micronucleated mature erythrocytes.

A positive response is normally indicated by a statistically significant increase in the incidence of micronucleated immature erythrocytes for the treatment group compared with the vehicle control group ($p < 0.01$) and/or individual and/or group mean values exceeding the laboratory historical control range (Morrison and Ashby, 1995). Bone marrow cell toxicity (or depression) is normally indicated by a substantial and statistically significant decrease in the proportion of immature erythrocytes ($p < 0.01$).

3. Results

In the 13-week repeated dose toxicity study (Horne et al., 2005), ABM-FD did not cause any clinical finding

and there were no treatment-related deaths. Two females receiving 1000 mg/kg/day died or were killed due to an accidental mis-administration of the test formulation, since there was trauma to the oesophagus and consequential changes in the thorax, and a Control male also died due to changes in the thorax, though there was no oesophageal trauma in this animal.

There was no effect of treatment upon weight gain and food consumption (Fig. 1 and 2, Table 2).

There were no test article-related ocular abnormalities in this study.

Haematological investigations indicated a reduction of haematocrit, haemoglobin concentration and erythrocyte count in week 13 in males receiving 2000 mg/kg/day and these animals also showed a slight reduction of erythrocyte count in week 7. At all investigations, there was a small, but consistently increased mean cell volume in males receiving 2000 mg/kg/day. Erythrocyte counts were also low in week 13 in males receiving 500 or 1000 mg/kg/day but there was no dosage-relationship and no effect upon any of the other erythrocytic characteristics and, consequently, these changes were not attributed to treatment. Variations of erythrocytic parameters in females were minor and transient and were attributed to normal biological variation. At 2000 mg/kg/day, females showed a consistent reduction of lymphocyte and large unstained cell counts, when compared with the Controls, though this was statistically significant only in week 7 and 13. There was also a trend towards low lymphocyte count in females receiving 1000 mg/kg/day, with those receiving 500 mg/kg/day showing a similar finding in week 2 only but these differences did not attain statistical significance. As a consequence of these changes, total leucocyte counts were slightly low in females receiving 1000 or 2000 mg/kg/day, and in week 2 in females receiving 500 mg/kg/day, though statistical significance was attained only in week 7 and 13 in high dosage females. Haematology data are summarised in Table 3.

There were several changes in the blood plasma during the course of the study (Table 4). In week 13 there was an increased urea concentration in males receiving 1000 mg/kg/day and in males and females receiving 2000 mg/kg/day which, at 2000 mg/kg/day, was associated with an increased of plasma creatinine. Urea and creatinine concentrations were also increased slightly, compared with the Controls, in week 7 in females receiving 2000 mg/kg/day. Glucose concentrations were consistently higher than the Controls in males receiving 2000 mg/kg/day, though the difference lacked statistical significance in week 7. Females were not affected; the increase of glucose concentration in week 2 in females receiving 2000 mg/kg/day was, because of its transient nature, less clearly attributable to treatment. There were a number of variations of electrolytes at each investigation. Potassium concentrations were consistently high at all dosages in males and were also high in week 2 and 7 in females receiving 1000 or 2000 mg/kg/day and in week 7 in those receiving 500 mg/kg/day. The difference from controls was not always dose-related. In

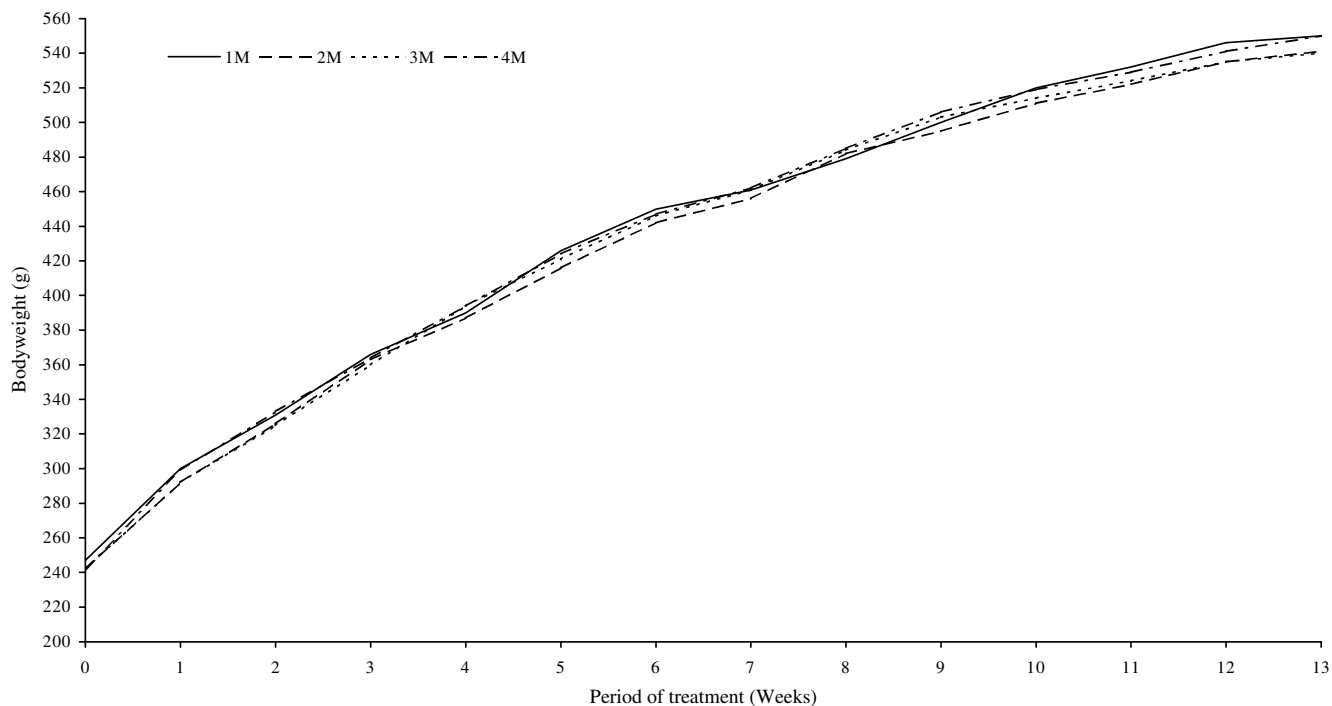


Fig. 1. Bodyweight – group mean values (g) for males.

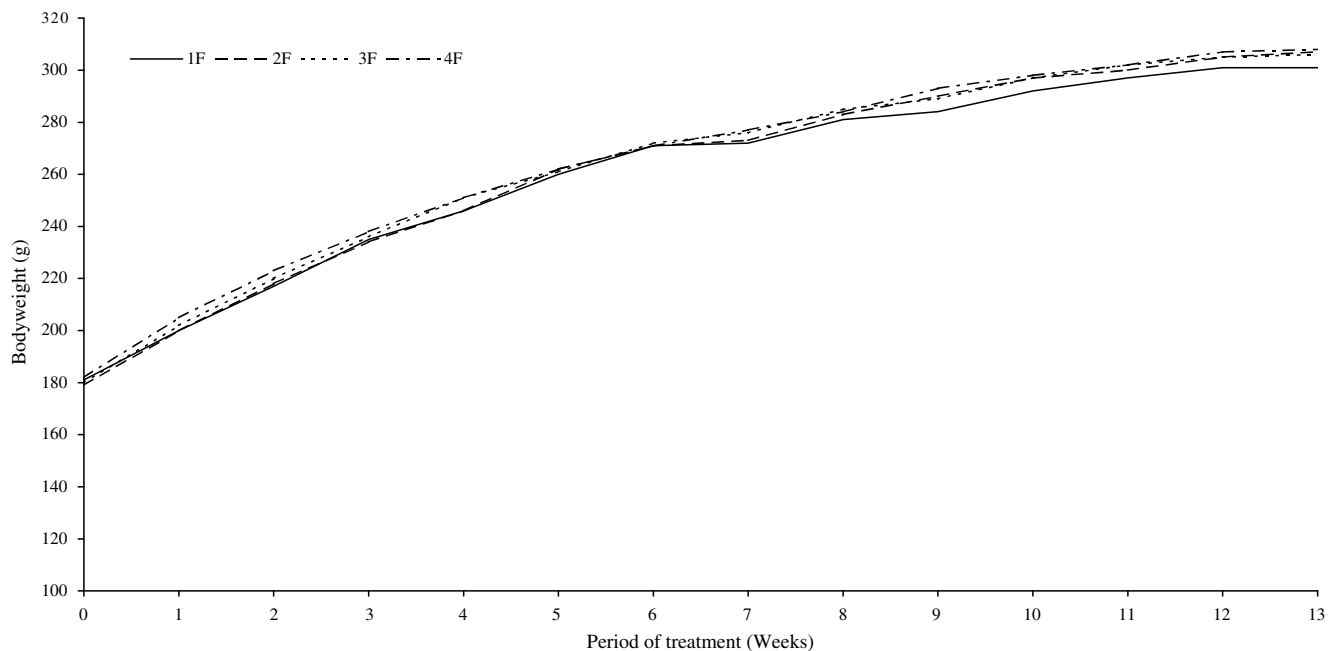


Fig. 2. Bodyweight – group mean values (g) for females.

week 13 there was a slight reduction of calcium concentration at all dosages, in a broadly dose-related manner, with a similar change occurring in week 2 in treated females. In females in week 13 there was also a slight reduction of phosphorus concentration but this was not dose-related. None of the other differences from Controls was considered toxicologically significant. In females receiving

2000 mg/kg/day there was a small, but consistently low alanine amino-transferase activity compared with the Controls but this trend was not present in males and such reductions of transaminase activity are not considered toxicologically significant.

The composition of the urine was not affected by treatment.

Table 2
Bodyweight (g) and food consumption (g/animal/week)

Group/sex	1 M	2M	3M	4M	1F	2F	3F	4F
Dose (mg/kg/day)	0	500	1000	2000	0	500	1000	2000
<i>Overall weight change</i>								
Week 0–13	303 ± 48	299 ± 41	298 ± 55	308 ± 42	120 ± 21	129 ± 24	127 ± 21	126 ± 17
<i>Overall mean food intake</i>								
Week 1–13	199	196	194	194	140	140	140	139

Table 3
Summary of treatment-related haematological findings (mean ± standard deviation)

Group			1	2	3	4
Dose (mg/kg/day)			0	500	1000	2000
Haematocrit (L/L)	Week 13	Males	0.453 ± 0.020	0.441 ± 0.015	0.440 ± 0.015	0.436 ± 0.018*
		Females	0.430 ± 0.016	0.418 ± 0.013	0.428 ± 0.020	0.426 ± 0.015
Haemoglobin (g/L)	Week 13	Males	15.7 ± 0.6	15.4 ± 0.6	15.3 ± 0.5	15.0 ± 0.6*
		Females	15.2 ± 0.5	14.8 ± 0.5	15.1 ± 0.7	15.0 ± 0.4
Erythrocytes (10 ¹² /L)	Week 7	Males	8.13 ± 0.37	7.87 ± 0.17	7.89 ± 0.28	7.72 ± 0.47*
		Females	7.63 ± 0.16	7.68 ± 0.26	7.90 ± 0.40	7.79 ± 0.40
	Week 13	Males	8.70 ± 0.39	8.28 ± 0.29*	8.32 ± 0.28*	8.14 ± 0.51**
		Females	7.72 ± 0.33	7.55 ± 0.32	7.81 ± 0.33	7.79 ± 0.47
Mean cell volume (fL)	Week 2	Males	59.4 ± 1.6	60.2 ± 1.8	59.6 ± 1.6	61.3 ± 2.1*
		Females	58.7 ± 1.0	58.5 ± 1.0	58.2 ± 1.5	58.5 ± 1.8
	Week 7	Males	56.1 ± 1.7	57.1 ± 1.3	56.8 ± 0.9	57.7 ± 2.5*
		Females	57.7 ± 1.1	57.4 ± 0.9	56.9 ± 1.8	56.9 ± 1.6
Total leucocytes (10 ⁹ /L)	Week 13	Males	52.1 ± 1.4	53.4 ± 1.6	52.9 ± 1.0	53.6 ± 1.7*
		Females	55.7 ± 1.2	55.4 ± 1.3	54.8 ± 2.0	54.8 ± 1.8
	Week 2	Males	11.09 ± 2.70	12.90 ± 2.24	12.14 ± 3.10	11.41 ± 1.66
		Females	10.99 ± 2.03	9.78 ± 1.74	9.58 ± 5.11	8.72 ± 2.02
Lymphocytes (10 ⁹ /L)	Week 7	Males	11.31 ± 2.91	15.09 ± 2.87	13.63 ± 4.07	13.70 ± 3.03
		Females	9.96 ± 1.74	10.24 ± 1.47	8.54 ± 2.74	7.56 ± 1.89*
	Week 13	Males	13.20 ± 4.37	16.11 ± 2.35	14.64 ± 3.80	12.49 ± 2.21
		Females	9.63 ± 1.60	9.58 ± 1.54	8.25 ± 1.73	7.35 ± 2.65*
Large unstained cells (10 ⁹ /L)	Week 2	Males	8.82 ± 2.31	10.54 ± 1.95	10.08 ± 2.49	9.07 ± 1.33
		Females	9.15 ± 1.73	7.92 ± 1.40	7.69 ± 3.64	7.06 ± 1.66
	Week 7	Males	9.19 ± 2.33	11.56 ± 1.91	10.60 ± 3.54	9.91 ± 1.69
		Females	8.26 ± 1.57	8.34 ± 1.44	6.84 ± 2.29	5.85 ± 1.45**
Week 13	Males	Males	9.97 ± 3.00	12.10 ± 1.52	11.36 ± 2.97	9.97 ± 1.85
		Females	7.91 ± 1.78	7.98 ± 1.27	6.82 ± 1.50	5.70 ± 1.86**
	Week 2	Males	0.23 ± 0.09	0.31 ± 0.10	0.29 ± 0.15	0.25 ± 0.10
		Females	0.20 ± 0.06	0.17 ± 0.04	0.21 ± 0.20	0.14 ± 0.06
Week 7	Males	Males	0.31 ± 0.15	0.40 ± 0.12	0.42 ± 0.17	0.34 ± 0.16
		Females	0.23 ± 0.07	0.23 ± 0.06	0.19 ± 0.09	0.15 ± 0.06*
	Week 13	Males	0.37 ± 0.15	0.37 ± 0.12	0.33 ± 0.09	0.27 ± 0.09
		Females	0.23 ± 0.08	0.18 ± 0.04	0.17 ± 0.03	0.14 ± 0.10**

Significantly different from the Controls.

* $p < 0.05$.

** $p < 0.01$.

At necropsy after completion of 13 weeks of treatment there was a reduction of absolute and bodyweight-relative spleen weight in females given 2000 mg/kg/day (see Table 5). There were no treatment-related macroscopic findings and the histopathological examination did not indicate any treatment-related change. Findings occurred with the expected incidence and severity in the control and test article-treated groups or they occurred sporadically. The findings seen in these animals have been observed in control, untreated rats of this age and strain.

3.1. Bacterial reverse mutation test

There was no evidence of toxicity following exposure to ABM-FD in the preliminary test. A maximum exposure concentration of 5000 µg/plate was, therefore, selected for use in the main mutation tests (May, 2005a).

There was no evidence of toxicity in the first and second mutation tests following exposure to ABM-FD (Figs. 3 and 4, Tables 6 and 7). No substantial increases in revertant colony numbers over control counts were obtained with

Table 4
Summary of treatment-related blood biochemistry findings (mean \pm standard deviation)

Group			1	2	3	4	
Dose (mg/kg/day)			0	500	1000	2000	
Urea (mmol/L)	Week 7	Males	4.34 \pm 0.70	4.11 \pm 0.36	4.49 \pm 0.28	4.67 \pm 0.81	
		Females	5.79 \pm 0.68	5.70 \pm 0.59	6.22 \pm 1.01	6.58 \pm 0.91*	
	Week 13	Males	4.74 \pm 0.65	5.15 \pm 0.36	5.69 \pm 0.67**	6.07 \pm 0.76**	
		Females	6.25 \pm 0.63	6.78 \pm 0.89	6.30 \pm 0.46	7.20 \pm 0.86**	
Creatinine (mmol/L)	Week 13	Males	53 \pm 6	58 \pm 3	60 \pm 7	69 \pm 10**	
		Females	59 \pm 3	60 \pm 4	61 \pm 4	71 \pm 8**	
	Week 2	Males	4.90 \pm 0.70	4.74 \pm 0.36	5.28 \pm 0.60	5.53 \pm 0.41*	
		Females	5.48 \pm 0.82	5.50 \pm 0.24	5.44 \pm 0.41	6.06 \pm 0.57*	
Glucose (mmol/L)	Week 7	Males	5.99 \pm 0.75	5.87 \pm 0.76	5.71 \pm 0.72	6.78 \pm 1.31	
		Females	6.06 \pm 0.66	5.99 \pm 0.96	5.76 \pm 0.56	6.56 \pm 0.68	
	Week 13	Males	7.91 \pm 1.57	7.54 \pm 0.51	7.61 \pm 0.51	9.43 \pm 1.16*	
		Females	7.80 \pm 0.86	7.46 \pm 0.76	7.42 \pm 0.40	7.73 \pm 0.79	
Potassium (mmol/L)	Week 2	Males	4.3 \pm 0.3	4.8 \pm 0.3*	4.6 \pm 0.4*	4.9 \pm 0.4**	
		Females	3.9 \pm 0.2	4.1 \pm 0.2	4.2 \pm 0.3**	4.3 \pm 0.2**	
	Week 7	Males	4.3 \pm 0.2	5.0 \pm 0.4**	5.1 \pm 1.0**	4.9 \pm 0.3**	
		Females	4.0 \pm 0.3	4.4 \pm 0.3**	4.3 \pm 0.3**	4.5 \pm 0.2**	
	Week 13	Males	4.8 \pm 0.5	5.3 \pm 0.3*	5.3 \pm 1.0*	5.8 \pm 0.5**	
		Females	4.5 \pm 0.4	4.7 \pm 1.0	4.5 \pm 0.3	4.7 \pm 0.4	
	Calcium (mmol/L)	Week 2	Males	2.65 \pm 0.08	2.69 \pm 0.07	2.63 \pm 0.08	2.66 \pm 0.04
			Females	2.70 \pm 0.23	2.62 \pm 0.05*	2.62 \pm 0.05*	2.61 \pm 0.07**
Week 13		Males	2.88 \pm 0.05	2.81 \pm 0.07*	2.76 \pm 0.08*	2.76 \pm 0.06*	
		Females	2.92 \pm 0.12	2.83 \pm 0.08*	2.81 \pm 0.06*	2.80 \pm 0.08**	
Phosphorus (mmol/L)	Week 13	Males	2.24 \pm 0.12	2.26 \pm 0.12	2.25 \pm 0.17	2.32 \pm 0.16	
		Females	1.89 \pm 0.28	1.57 \pm 0.26*	1.69 \pm 0.25*	1.72 \pm 0.14*	

Significantly different from the Controls.

* $p < 0.05$.

** $p < 0.01$.

Table 5
Summary of organ weight changes related to treatment (mean \pm standard deviation)

Group			1	2	3	4
Dose (mg/kg/day)			0	500	1000	2000
Spleen	Males	Absolute (g)	0.84 \pm 0.15	0.86 \pm 0.16	0.84 \pm 0.11	0.79 \pm 0.14
		Relative (%)	0.155 \pm 0.024	0.159 \pm 0.023	0.157 \pm 0.022	0.146 \pm 0.025
	Females	Absolute (g)	0.60 \pm 0.09	0.65 \pm 0.45	0.57 \pm 0.13	0.54 \pm 0.098*
		Relative (%)	0.200 \pm 0.023	0.212 \pm 0.153	0.186 \pm 0.033	0.172 \pm 0.017**

Significantly different from the Controls.

* $p < 0.05$.

** $p < 0.01$.

any of the tester strains following exposure to ABM-FD at any concentration up to 5000 $\mu\text{g}/\text{plate}$ in either the presence or absence of S9 mix. Consequently, it was concluded that ABM-FD showed no evidence of mutagenic activity in this bacterial system under the test conditions employed.

3.2. Mouse lymphoma test

In the preliminary test, toxicity was observed only after a 24-h exposure. A maximum exposure concentration of 5000 $\mu\text{g}/\text{mL}$ was therefore selected for use in the main mutation tests (May, 2005b).

In the first main mutation test (3-h exposure, Table 8), whether S9 was present or not, there were no increases in induced mutation frequency that exceeded the Global

Evaluation Factor following treatment with ABM-FD at concentrations within acceptable levels of toxicity.

In the second main mutation test (24-h exposure, Fig. 5, Table 9), treatment with ABM-FD at a concentration of 4000 $\mu\text{g}/\text{mL}$ was associated with an increase in induced mutation frequency that exceeded the Global Evaluation Factor. This increase represented a 3.8-fold increase in mutation frequency compared to the mutation frequency for the concurrent solvent control. There was evidence of a concentration–response relationship, which gave a significant positive linear trend ($p \leq 0.01$) and an increase in the number of small colony mutants. This finding occurred where there was significant toxicity (the relative suspension growth at 4000 $\mu\text{g}/\text{mL}$ was only 18% of that of the Control).

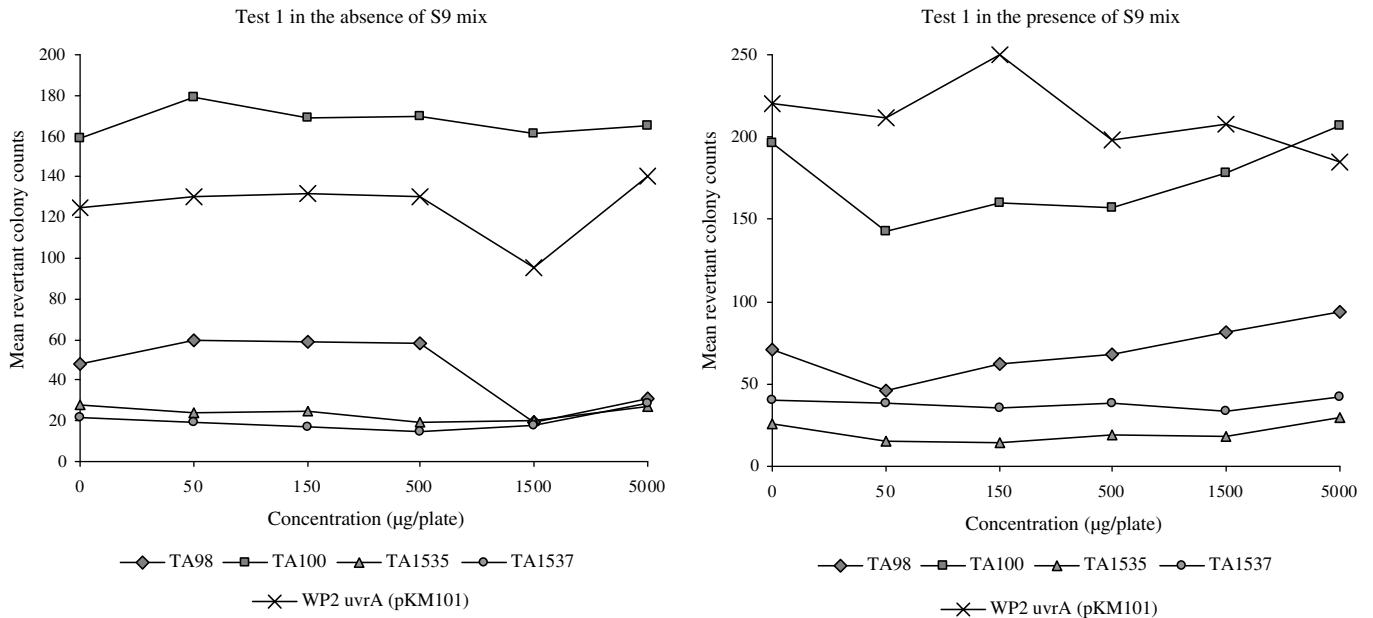


Fig. 3. Mean revertant counts in the first bacterial mutation test.

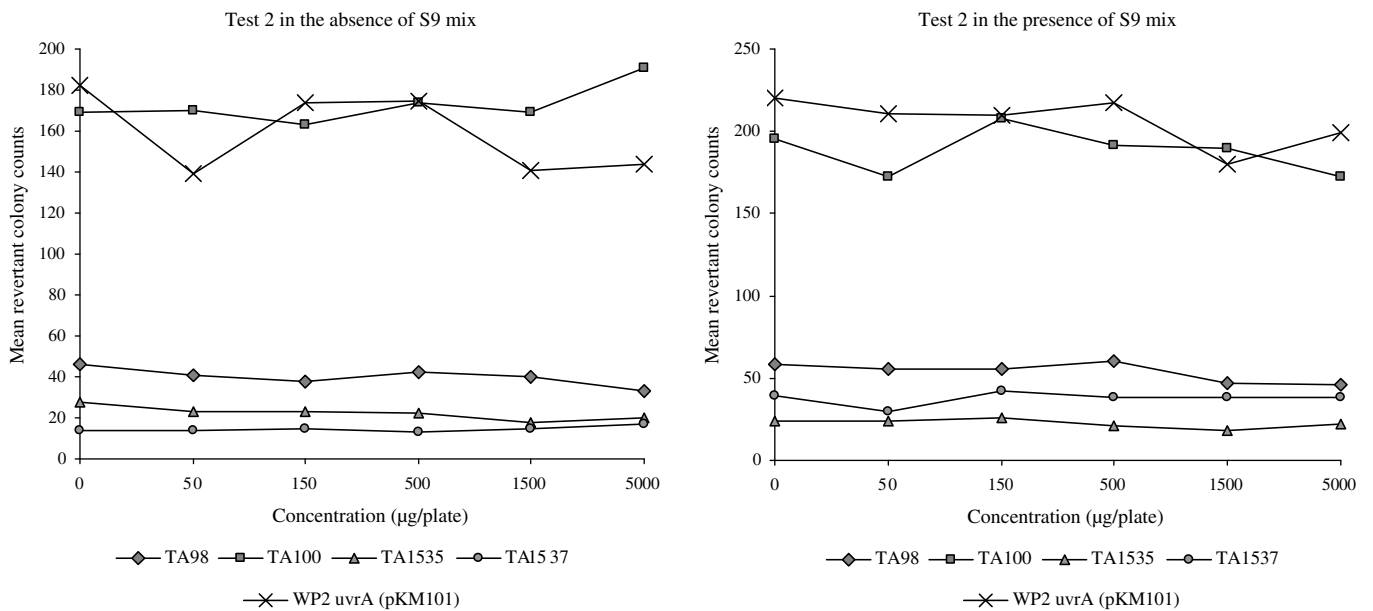


Fig. 4. Mean revertant counts in the second bacterial mutation test.

It was concluded that ABM-FD demonstrated mutagenic potential, in the absence of S9 mix when exposed for 24 h in this *in vitro* cell mutation assay under the experimental conditions described. The increased mutant frequency was obtained only after 24-h continuous exposure to the test substance and it was noted that the mutant colonies obtained were almost exclusively small, indicating that ABD-FM does not cause point mutations. Any genotoxicity could be due to possible clastogenic activity or the loss of a chromosome (aneuploidy), rather than to a direct effect upon DNA.

3.3. Micronucleus test in the rat

Clinical signs related to treatment at all doses comprised underactivity and nervous behaviour, flattened posture, fast and irregular respiration, prominent eyes, partially closed eyelids and unsteady gait. On Day 3, animals treated at 1000 or 2000 mg/kg/day showed occasional extension and stretching of the hind limbs. All animals survived until scheduled termination and no effect on bodyweight occurred (Pritchard, 2005).

ABM-FD did not cause any statistically significant increases in the number of micronucleated immature

Table 6
Summary of first bacterial reverse mutation test

Substance	Conc. ($\mu\text{g}/\text{plate}$)	S9	Mean count \pm SD				
			TA98	TA100	TA1535	TA1537	<i>E. coli</i>
Water		–	48 \pm 5	159 \pm 3	28 \pm 7	22 \pm 3	125 \pm 6
ABM-FD	50	–	60 \pm 15	179 \pm 8	24 \pm 3	19 \pm 9	130 \pm 14
	150	–	59 \pm 2	169 \pm 16	25 \pm 3	17 \pm 6	132 \pm 3
	500	–	58 \pm 8	170 \pm 5	19 \pm 5	15 \pm 5	130 \pm 16
	1500	–	19 \pm 8	161 \pm 16	20 \pm 8	18 \pm 3	95 \pm 9
	5000	–	31 \pm 4	165 \pm 15	27 \pm 8	29 \pm 6	140 \pm 30
2-NF	2	–	424 \pm 90	–	–	–	–
SA	2	–	–	724 \pm 122	966 \pm 112	–	–
9AA	50	–	–	–	–	330 \pm 170	–
4N-1-O	2	–	–	–	–	–	1140 \pm 171
Water	0	+	71 \pm 3	196 \pm 6	26 \pm 4	40 \pm 2	220 \pm 14
ABM-FD	50	+	46 \pm 13	143 \pm 7	15 \pm 5	38 \pm 9	212 \pm 27
	150	+	62 \pm 11	160 \pm 13	14 \pm 6	35 \pm 3	250 \pm 35
	500	+	68 \pm 6	157 \pm 9	19 \pm 3	38 \pm 8	198 \pm 19
	1500	+	81 \pm 4	178 \pm 4	18 \pm 3	34 \pm 6	208 \pm 36
	5000	+	94 \pm 12	207 \pm 23	30 \pm 2	42 \pm 4	185 \pm 11
B(a)P	5	+	216 \pm 25	–	–	217 \pm 7	–
2AA	5	+	–	1509 \pm 81	312 \pm 47	–	–
2AA	20	+	–	–	–	–	549 \pm 4

2-NF: 2-nitrofluorene; SA: Sodium azide; B(a)P: Benzo(a)pyrene; 2-AA: 2-aminoanthracene; 9AA: 9-aminoacridine; 4N-1-O: 4-nitroquinoline-1-oxide.

Table 7
Summary of second bacterial reverse mutation test

Substance	Conc. ($\mu\text{g}/\text{plate}$)	S9	Mean count \pm SD				
			TA98	TA100	TA1535	TA1537	<i>E. coli</i>
Water		–	46 \pm 3	169 \pm 11	28 \pm 2	14 \pm 3	182 \pm 15
ABM-FD	50	–	41 \pm 6	170 \pm 10	23 \pm 4	14 \pm 1	139 \pm 9
	150	–	38 \pm 3	163 \pm 6	23 \pm 5	15 \pm 2	174 \pm 14
	500	–	42 \pm 6	174 \pm 15	22 \pm 4	13 \pm 2	175 \pm 14
	1500	–	40 \pm 4	169 \pm 6	18 \pm 3	15 \pm 3	141 \pm 15
	5000	–	33 \pm 4	191 \pm 24	20 \pm 2	17 \pm 3	144 \pm 28
2-NF	2	–	409 \pm 59	–	–	–	–
SA	2	–	–	1173 \pm 38	1261 \pm 116	–	–
9AA	50	–	–	–	–	582 \pm 129	–
4N-1-O	2	–	–	–	–	–	1090 \pm 95
Water	0	+	58 \pm 2	195 \pm 18	24 \pm 3	39 \pm 2	220 \pm 25
ABM-FD	50	+	56 \pm 4	172 \pm 22	24 \pm 5	30 \pm 2	211 \pm 36
	150	+	56 \pm 7	208 \pm 11	26 \pm 2	42 \pm 4	210 \pm 27
	500	+	60 \pm 11	192 \pm 20	21 \pm 1	38 \pm 2	217 \pm 15
	1500	+	47 \pm 8	190 \pm 19	18 \pm 5	38 \pm 5	180 \pm 19
	5000	+	46 \pm 5	172 \pm 27	22 \pm 6	38 \pm 3	199 \pm 18
B(a)P	5	+	609 \pm 43	–	–	147 \pm 14	–
2AA	5	+	–	1628 \pm 197	283 \pm 42	–	–
2AA	20	+	–	–	–	–	559 \pm 64

2-NF: 2-nitrofluorene; SA: Sodium azide; B(a)P: Benzo(a)pyrene; 2-AA: 2-aminoanthracene; 9AA: 9-aminoacridine; 4N-1-O: 4-nitroquinoline-1-oxide.

erythrocytes or any decrease in the proportion of immature erythrocytes (Table 10). An increased incidence of micronuclei was observed in animal No. 221, receiving 1000 mg/kg/day. Even though both the individual and group values exceeded the laboratory historical control range, no statistical significance was observed. This individual result was, therefore, considered artefactual and of no biological relevance. Cyclophosphamide caused a

statistically significant increase in the frequency of micronucleated immature erythrocytes ($p < 0.01$) but did not affect the proportion of immature erythrocytes.

It was concluded, therefore, that ABM-FD did not cause an increase in the induction of micronucleated immature erythrocytes or bone marrow cell toxicity in rats, when administered orally by gavage in this *in vivo* test procedure.

Table 8
Summary of main mouse lymphoma test (3-h exposure)

ABM-FD Conc. ($\mu\text{g/mL}$)	3-h exposure							
	With S9				Without S9			
	RSG	MF	RCE	% small colonies	RSG	MF	RCE	% small colonies
0	100	0.000117	100	59	100	0.000169	100	71
156.25	102	0.000131	89	–	92	0.000132	125	–
312.5	92	0.000128	98	–	102	0.000101	134	–
625	109	0.000111	83	–	111	0.000140	118	–
1250	99	0.000111	82	–	109	0.000155	110	–
2500	93	0.000159	85	–	103	0.000131	126	–
5000	104	0.000158	93	–	89	0.000202	118	–
MMS	55	0.001454	63	82	77	0.001080	92	84

RSG: Relative suspension growth; MF: Mean mutation frequency; RCE: Relative cloning efficiency; MMS: Methyl methanesulphonate.
– Not determined.

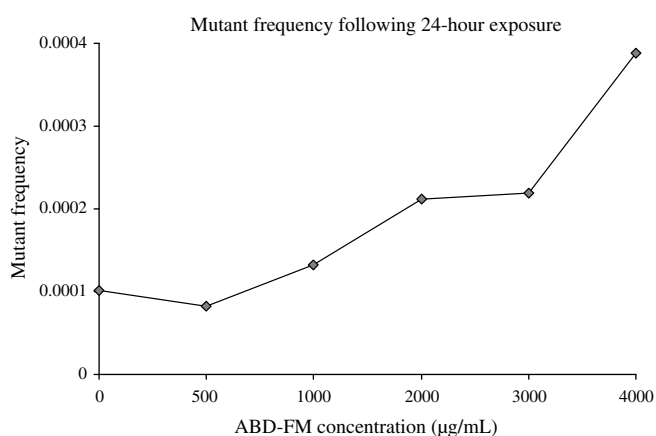


Fig. 5. Mutation frequency in the Mouse lymphoma test.

Table 9
Summary of main mouse lymphoma test (24-h exposure)

ABM-FD conc. ($\mu\text{g/mL}$)	24-h exposure			
	Without S9			
	RSG	MF	RCE	% small colonies
0	100	0.000102	100	60
500	88	0.000082	126	–
1000	86	0.000132	111	–
2000	60	0.000212	99	–
3000	34	0.000219	111	–
4000	18	0.000388	106	84
5000	7	–	–	–
MMS	60	0.001422	46	84

RSG: Relative suspension growth; MF: Mean mutation frequency; RCE: Relative cloning efficiency; MMS: Methyl methanesulphonate.
– Not determined.

4. Discussion

The 13-week subchronic toxicity study of the aqueous extract of *A. blazei* in CD rats showed that there were no overt toxicity but some treatment-related changes were reported at 2000 mg/kg/day. The cause of the low haematocrit, haemoglobin concentration and erythrocyte

Table 10
Summary of findings on the rat micronucleus test 24 h after the second dose

Treatment	Dosage (mg/kg/day)	Proportion of immature erythrocytes (%)	Incidence of micronucleated cells ¹
Vehicle	0	43	3.3
ABM-FD	500	40	2.4
ABM-FD	1000	48	5.9
ABM-FD	2000	43	3.6
Cyclophosphamide ²	20	36	44.8**

** $p < 0.001$.

¹ Per 2000 immature erythrocytes examined.

² Positive control; dosed once, 24 h before termination.

count in week 13 in males receiving 2000 mg/kg/day and the slight, but consistently high mean cell volume in these males was not identified since there was no evidence for any change in erythrocyte turnover (such as reticulocytosis or high plasma bilirubin) or of blood loss. High plasma creatinine and urea concentrations, when observed together, are indicative of an effect upon the kidney, and in particular upon glomerular filtration. There was, however, no alteration of urinary composition or any histopathological finding in the kidney. Consequently, any effect upon the kidney is considered likely to be an adaptive response to treatment which is of no toxicological importance. Low spleen weights in females given 2000 mg/kg/day and the dark areas on the thymus of males given 2000 mg/kg/day did not associate with any histopathological lesion. With the exception of the findings attributed to renal adaptation to treatment, these findings are indicative of a low level of subchronic toxicity, though they were not of a sufficient magnitude to cause any sign or change in weight gain or food consumption.

The aqueous extract of *A. blazei* is known to modulate the immune system and therefore the reason for the low lymphocyte and large unstained cell counts, reported in females receiving 1000 or 2000 mg/kg/day and transiently in those given 500 mg/kg/day, is unknown. The differences

from Controls were, however, generally small and not of a toxicologically significant magnitude as the individual values were still within the normal background range.

The cause of an increased motor activity in week 12 (as indicated by high beam breaks, suggesting increased rearing activity) in females receiving 2000 mg/kg/day was unclear as there was no increase in activity reported at the routine signs investigations and there were no other signs indicative of an effect upon the central nervous system. The consistently high glucose concentrations in males and variations of plasma electrolytes were likely to be consequences of the composition of the *A. blazei* extract as they did not correlate with any other findings.

Taken overall, the conclusion from this study was that the lowest-observed-effect level (LOEL) for ABM-FD after oral administration to rats was 2000 mg/kg/day and the no-observed-adverse-effect level (NOAEL) was at least 1000 mg/kg/day.

ABM-FD was clearly negative in the bacterial reverse mutation (Ames) test, both in the presence and absence of an exogenous metabolising system, S9 mix, and was also negative in a rat bone marrow micronucleus assay when tested up to the maximum dose level of 2 g/kg bodyweight. However, when tested in an L5178Y mouse lymphoma assay, ABM-FD gave a weak positive response, but only after 24-h exposure in the absence of S9 mix, at an acceptable level of cytotoxicity. The short term, 3-h exposures, either in the presence or absence of S9, were clearly negative. It was interesting to note that the positive response, which occurred only at the highest concentration tested, was also associated with an increase in the number of small colony mutants. This is significant in that it suggests that the mechanism of action is one of large deletions or the loss of the whole chromosome, i.e. aneuploidy, rather than a point mutation and therefore not due to any direct effect upon the DNA molecule. Further evidence for this mode of action is given by the negative Ames test and, to a lesser extent, the fact that the positive was only seen following 24-h exposure and at a level where there was clear evidence of toxicity. Further work would be required to test this hypothesis, such as the use of an *in vitro* micronucleus assay followed, in the event of a positive response, by fluorescent *in situ* hybridisation (FISH) staining for centromeres (Kirsch-Volders et al., 2003). A positive signal from the centromeric probe would indicate that the micronuclei were whole chromosomes and would then confirm aneugenic activity.

The results from these studies indicate that the aqueous extract of *A. blazei* is of low sub-chronic toxicity when administered orally at very high doses to rats, and did not cause any direct effect upon the DNA molecule, as indicated in the Bacterial Reverse Mutation and mouse lymphoma tests and *in vivo* micronucleus test. The fact that the weak positive in the L5178 mouse lymphoma test occurred only after 24-h incubation at the highest concentration tested (where there was toxicity) in the absence of S9 suggest that the mode of action in this test concerned

large deletions or the loss of the whole chromosomes, i.e. aneuploidy, rather than to any direct damage to the DNA.

Conflict of interest statement

This is to confirm there are no conflicts of interest relating to this submission.

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