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Tumor Induction with the *N'*-Acetyl Derivative of 4-Hydroxymethylphenylhydrazine, a Metabolite of Agaritine of *Agaricus bisporus*[1]

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ABSTRACT

***N'*-Acetyl-4-(hydroxymethyl)phenylhydrazine was administered as a 0.0625% solution in drinking water continuously for the life span of Swiss mice, from 6 weeks of age. Compared to that in untreated controls, in treated animals the lung tumor incidence rose from 15 to 34% in females and 22 to 48% in males, whereas the incidence of blood vessel tumors increased from 8 to 32% in females and from 5 to 30% in males. Histopathologically, the tumors were classified as adenomas and adenocarcinomas of the lungs and angiomas and angiosarcomas of the blood vessels.**

The commonly eaten mushroom *Agaricus bisporus* contains *β-N*-[γ-L(+)-glutamyl]-4-hydroxymethylphenylhydrazine, which under certain conditions yields 4-hydroxymethylphenylhydrazine and L-glutamic acid. Since 4-hydroxymethylphenylhydrazine is relatively unstable, its acetyl derivative was synthesized for this study. The possible environmental significance of the findings is discussed.

INTRODUCTION

The commercially cultivated mushroom *Agaricus bisporus* contains *β-N*-[γ-L(+)-glutamyl]-4-hydroxymethylphenylhydrazine (agaritine) (2, 5). Sporophores of the mushroom contain the enzyme γ-glutamyltransferase, which catalyzes the hydrolysis of agaritine to L-glutamate and 4-hydroxymethylphenylhydrazine (3, 5). This cleavage also occurs under mild acidic conditions (5). 4-Hydroxymethylphenylhydrazine is a relatively unstable compound; therefore it appeared promising to attach an acetyl group to it to render the compound feasible for biological experiments. The instability of 4-hydroxymethylphenylhydrazine has been attributed to the facile 1,4 elimination of water across the phenyl ring. The loss of water in agaritine is minimized by the electron-withdrawing γ-carbonyl group of the glutamic acid. The carbonyl group in AMPH[2] provides a similar stabilization. The similar substitution of the hydrazine moiety in agaritine and AMPH suggests a comparable electronic configuration and hence an analogous chemical reactivity for this portion of the molecule.

The present work is also part of the concentrated effort to reveal the possible tumorigenic potencies of hydrazine analogs. This class of chemicals, whether synthetic and/or naturally occurring, is apparently widely distributed in the environment (5, 6, 8, 10, 11, 14); therefore the human population is exposed to them to a considerable degree. This report records the tumorigenicity of AMPH administered continuously in drinking water for the life of albino Swiss mice.

MATERIALS AND METHODS

Albino Swiss mice from a colony randomly bred by us since 1951 were used in these experiments. They were housed in plastic cages with granular cellulose bedding, separated according to sex in groups of 10, and given Wayne Lab Blox diet in regular pellets (Allied Mills, Inc., Chicago, Ill.) and tap water or the chemical solution *ad libitum* as described below. The chemical used was AMPH (Chart 1; M.W., 180.21; m.p., 124.5-125.5°; purity; >97%), which was synthesized in this laboratory in the following way.

p-(*N'*-Acetylhydrazino)benzoic Acid

A suspension of 100 g (0.66 mole) *p*-hydrazinobenzoic acid in 700 ml ethyl acetate was magnetically stirred while 62 ml (0.67 mole) acetic anhydride was slowly added. After stirring for 20 min, the mixture was filtered, and the precipitate was washed with anhydrous ether. Recrystallization from methanol-water gave white needles. (m.p., 220-221°) in 75% yield.

Nuclear magnetic resonance ($CDCl_3$): δ7.78 and 6.72 (AB quartet, 4 aromatic hydrogen atoms, J, 8 Hz), 4.25 (broad shoulder, 3 H, NH, and OH), and 1.94 (shoulder, 3H, CH_3). Mass spectrum, *m/e*: 194 (m^+), 152 (m − C_2H_2O), and 136 (m − C_2H_4ON).

$C_9H_{10}N_2O_3$

Calculated: C 55.66, H 5.20, N 14.53
Found: C 55.86, H 5.26, N 14.34

N-Acetyl-*N'*-(*p*-hydroxymethyl)phenylhydrazine

A solution of 25 g (0.13 mole) of *p*-(*N'*-acetylhydrazino)-benzoic acid and 500 ml of tetrahydrofuran was mechanically stirred under nitrogen at 5°, while 400 ml of a 1 M solution (0.4 mole) of diborane in tetrahydrofuran (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was added dropwise at such a rate that the temperature did not rise above 10°. Stirring was continued for one-half hr after the addition was completed, and 400 ml of 1 N NaOH were added dropwise. The mixture was stirred overnight, while it was slowly warming to room temperature. Solid potassium carbonate was added until 2 distinct phases appeared

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[2] The abbreviation used is: AMPH, *N'*-acetyl-4-(hydroxymethyl)-phenylhydrazine.

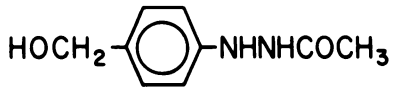

Chart 1. Chemical structure of AMPH.

(pH, ~10; aqueous layer). The aqueous layer was extracted 3 times with equivalent volumes of ether. The extracts were combined with the original organic phase and dried over sodium sulfate. Concentration *in vacuo* resulted in a tan residue, which was recrystallized from tetrahydrafuran-ether to yield 70% *N*-acetyl-*N'*-(*p*-hydroxymethyl)phenylhydrazine (m.p., 125–126°).

Nuclear magnetic resonance (CD_3OD): δ 7.16 and 6.74 (AB quartet, 4 aromatic hydrogens, J, 8 Hz), 4.73 (shoulder, 3H, NH and OH), 4.46 (shoulder, 2H, CH_2) and 2.00 (shoulder, 3H, CH_3).

$C_9H_{12}N_2O_2$

Calculated: C 59.98, H 6.66, N 15.54
Found: C 59.82, H 6.80, N 15.36

Toxicity studies were carried out with AMPH prior to the chronic experiment. Five dose levels of AMPH, 0.5, 0.25, 0.125, 0.0625, and 0.03125%, were administered in the drinking water daily for 35 days. Each group consisted of 8 animals, 4 females and 4 males. By taking into account 4 parameters, survival rates, body weights, chemical consumption figures, and histological changes, the 0.0625% level was found to be suitable for the lifelong treatment. Above the 0.0625% dose level, most of the animals died, their body weights decreased, and the chemical consumption figures were substantially lower than those of the controls. Histopathologically, vacuolated liver cells, congested lungs, and marked cellular activity in the glomeruli were observed. This toxicity technique was developed in this laboratory (12).

The solution was prepared 3 times weekly, and the total consumption of water containing AMPH was measured at the same intervals during the treatment period. The solution was placed in brown bottles because of the possible light sensitivity of the chemical. The chronic experimental group and the controls were the following.

Group 1. AMPH was dissolved in the drinking water as a 0.0625% solution and was given for the life spans of 50 female and 50 male mice that were 6 weeks (42 days) old at the beginning of the experiment. The average daily water consumption of the treated animals was 10.2 ml for the females and 10.6 ml for the males. Therefore, the average daily intake of AMPH was 6.4 mg for a female and 6.6 mg for a male.

Group 2. As untreated controls, 100 female and 100 male mice received tap water and were observed from weaning time (5 weeks of age).

The experimental and control animals were carefully checked and weighed weekly, and the gross pathological changes were recorded. The animals either were allowed to die or were killed with ether when found in poor condition. Complete necropsies were performed on all animals. All organs were examined macroscopically and were fixed in 10% buffered formalin. The liver, spleen, kidneys, bladder, thyroid, heart, pancreas, testes, ovaries, brain, nasal turbinal, at least 4 lobes of the lungs of each mouse, and organs showing gross pathological changes were studied histologically. Sections from these tissues were stained routinely with hematoxylin and eosin.

RESULTS

The survival rates after weaning are recorded in Table 1. As can be seen from the data, there is no significant difference in the survival of treated and control mice.

The number, percentages of animals with tumors, and their ages at death (latent periods) are summarized in Table 2. The 2 most important neoplasms are described in detail as follows.

Lung Tumors. Of the treated females, 17 (34%) developed 26 lung tumors; of these, 10 mice had 16 adenomas, 5 mice had 6 adenocarcinomas, and 2 mice had 2 adenomas and 2 adenocarcinomas. In the treated males, 24 (48%) developed 32 such neoplasms; of these, 16 mice had 22 adenomas, 7 had 7 adenocarcinomas, and 1 mouse had an adenoma and 2 adenocarcinomas. Macroscopically and histologically, all lung lesions were similar to those described earlier (15, 18).

Blood Vessel Tumors. Of the treated females, 16 (32%) developed blood vessel tumors; of these, 9 mice had angiomas, and 7 had angiosarcomas. The tissue distributions of angiomas were: liver, 4; ovaries, 4; lymph nodes, 3; whereas the distributions of angiosarcomas were: liver, 6; muscle, 1; fat, 1; spleen, 1; subcutis, 1. In the treated males, 15 (30%) developed such neoplasms; of these, 7 mice had angiomas, and 8 had angiosarcomas. The tissue distributions of angiomas were: liver, 5; lymph nodes, 2; whereas 8 angiosarcomas were found only in liver. Grossly and histologically, the blood vessel tumors were similar to those described earlier in this laboratory (13, 16, 20).

Other Tumors. A few other types of tumors were also found in the treated groups shown in Table 2. Since they occurred in low incidences, their appearance cannot be attributed to the treatment.

Table 1
Treatment and survival rate in AMPH-treated and control Swiss mice

Group	Treatment	Initial no. and sex	No. of survivors at the following wk of age												
			10	20	30	40	50	60	70	80	90	100	110	120	130
1	0.0625% AMPH in drinking water daily for life	50, F	50	50	50	44	42	39	35	27	22	16	6	1	
		50, M	50	49	48	45	42	38	29	20	8	2	1	1	
2	Untreated control	100, F	100	100	99	96	95	91	78	66	45	28	13	2	
		100, M	100	98	92	88	80	62	36	17	11	3	2	1	

Table 2

Tumor distribution in AMPH-treated and control Swiss mice

Group	Treatment	Effective no. and sex	Animals with tumors of: Lungs No.	Lungs %	Lungs Age at Death [a] (wk)	Blood vessels No.	Blood vessels %	Blood vessels Age at death [a] (wk)	Other tissues No.	Other tissues Type of tumor	Other tissues Age at death (wk)
1	0.0625% AMPH in drinking water daily for life	50, F	17	34	90 (47–121)	16	32	92 (49–116)	7	Malignant lymphomas	45, 102, 106, 106, 109, 116, 121
									2	Adrenocortical adenomas	106, 118
									2	Polypoid adenomas of glandular stomach	111, 121
									1	Fibrosarcoma, s.c.	108
									1	Adrenocortical carcinoma	109
									1	Squamous cell carcinoma of skin	99
									1	Adenocarcinoma of breast	79
									1	Adenocarcinoma of ovary	105
									1	Adenocarcinoma of cecum	81
		50, M	24	48	79 (34–121)	15	30	78 (47–97)	5	Malignant lymphomas	48, 77, 82, 90, 121
									1	Carcinoma of pituitary	72
									1	Adrenocortical carcinoma	87
									1	Polypoid adenoma of cecum	82
2	Untreated controls	100, F	15	15	90 (67–116)	8	8	92 (74–119)	20	Malignant lymphomas	31, 33, 47, 76, 79, 85, 86, 87, 89, 89, 91, 91, 94, 99, 102, 103, 104, 111, 114, 116
									2	Fibrosarcomas, s.c.	101, 102
									2	Sex cord mesenchyma tumors	107, 114
									1	Adenoacanthoma of ovary	71
									1	Adenoma of ovary	110
									1	Adenoma of thyroid	88
									1	Adenocarcinoma of breast	127
									1	Adenocarcinoma of duodenum	86
									1	Carcinoma of glandular stomach	64
		100, M	22	22	70 (40–124)	5	5	75 (57–92)	8	Malignant lymphomas	28, 62, 67, 78, 84, 91, 92, 112
									2	Fibrosarcomas, s.c.	69, 92
									2	Adenomas of thyroids	69, 92
									2	Hepatomas	68, 94
									1	Adenoma of parathyroid	78

[a] Average; numbers in parentheses, age range at death.

DISCUSSION

The current study shows for the first time that the lifetime administration of 0.0625% AMPH in the drinking water of randomly bred albino Swiss mice, from 6 weeks of age, gave rise to lung and blood vessel tumors. The lung tumor incidence rose from 15 to 34% in females and from 22 to 48% in males, whereas the blood vessel tumor incidence increased from 8 to 32% in females and from 5 to 30% in males, compared with that in the untreated controls. The statistical analysis carried out by using Fisher's exact test (1) for 2 × 2 tables shows that in females ($p < 0.007$) and males ($p < 0.001$) the lung tumor incidence was significantly higher in the treated groups. The incidence of blood vessel tumors is also significantly higher in treated females ($p < 0.0002$) and males ($p < 0.00004$), compared with that in control mice. Light microscopic examination of the lesions revealed the characteristic appearances of adenomas and adenocarcinomas of lungs and angiomas and angiosarcomas of blood vessels.

The commonly eaten cultivated mushroom *A. bisporus* contains up to 0.04% agaritine. The latter is a stable compound; however, enzymatically or under acidic conditions it breaks down to 4-hydroxymethylphenylhydrazine and L-glutamic acid (3). 4-Hydroxymethylphenylhydrazine is, on the other hand, an unstable product; therefore its acetylated form was synthesized, which is much more suitable for chronic tumorigenesis studies. Also, it has been shown *in vitro*, but not *in vivo*, that 4-hydroxymethylphenylhydrazine undergoes further changes and yields 4-methylphenylhydrazine (3, 4). Interestingly, in a parallel study in this laboratory 4-methylphenylhydrazine hydrochloride was shown to induce tumors in Swiss mice (19). The estimated United States *A. bisporus* consumption totaled approximately 360,000,000 pounds in 1975 (21). In view of the carcinogenicity of these compounds, further studies obviously are needed to clarify this field of interest.

The present investigation is also part of the systematic approach to identify tumor-inducing hydrazine compounds in the environment and to study their mechanisms of action. Several of these chemicals are used in medicine, agriculture, and industry (14). In addition, a few were found to occur in nature in considerable quantities in tobacco and in some edible mushrooms (5–9, 11). Because of the carcinogenicity of this widely used class of hydrazine compounds, it seems advisable to warn against their use.

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