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TOXICITY STUDY OF A RUBBER ANTIOXIDANT, 2-MERCAPTOBENZIMIDAZOLE, BY REPEATED ORAL ADMINISTRATION TO RATS

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ABSTRACT — The chemical structure of 2-mercaptobenzimidazole (2-MBI), which is widely used as a rubber antioxidant, is partially similar to those of thiourea (TU) and ethylenethiourea (ETU), both potent thyrotoxic compounds. In order to determine the oral toxicity of 2-MBI, a 28-day repeated dose toxicity study in Wistar rats followed by observation over a 14-day recovery period was conducted at dose levels of 2, 10 and 50 mg/kg 2-MBI administered by gavage. No toxic deaths occurred due to 2-MBI treatment. Decreases of body weight gain and food consumption in the 50 mg/kg dose group were observed during the second half of the treatment period. In addition, hematological examination and serum biochemical tests revealed decreased white blood cells and hemoglobin and increased serum urea nitrogen, cholesterol, phospholipid, γ-glutamyl transpeptidase and the Na^+/K^+ ratio in the 50 mg/kg dose group. Marked thyroid enlargement (to10 fold the control weight), histopathologically associated with diffuse hyperplasia of follicles with decreased colloid and thickening of the fibrous capsule, was found. Reduction in thymus weight was also observed in a dose-dependent manner without significant histopathological alteration.

The non-observed effect level (NOEL) of 2-MBI in this gavage study was found to be less than 2 mg/kg/day based on the significant decrease in thymus weight in the 2 mg/kg 2-MBI treatment group.

In an ancillary study, measurement of serum levels of T_3, T_4 and TSH, and thyroid weight after gavage treatment with 0.15 and 0.3 mmol/kg of three antithyroid compounds for 14 days revealed a more potent antithyroid effect for 2-MBI than for TU or ETU.

KEY WORDS : 2-mercaptobenzimidazole, Rats, Gavage administration, Thyroid toxicity, Thymus involution, Rubber antioxidant

INTRODUCTION

2-Mercaptobenzimidazole (2-MBI), widely used as a rubber accelerator and/or antioxidant, is structurally similar to ethylenethiourea, a carcinogen/teratogen, and methimazole, a hyperthyroid drug (IARC,1974; Paynter *et al.*,1988). Antithyroid effects of 2-MBI have been demonstrated with oral administration of 8.3 mg/ kg to rats causing thyroid enlargement and a decrease in circulating thyroxin (T_4) (Janssen *et al.*,1981) . A dose of 7.5 mg/kg inhibits iodine uptake into the thyroid by 95% (Searle *et al.*, 1950). 2-MBI has been also used to assess the effects of decreased thyroid function in carcinogenesis in rats (Kellen,1972) It is thought to block the biosynthesis of thyroxin by inhibiting thyroid peroxidase (Deorge,1986; Taurog,1976). This thioureylene compound has also been shown to be a potent inhibitor of deiodinase which catalyses the conversion of 3,3',5-triiodothyronine (T_3) to 3,3'-diiodothyronine (Visser *et al.*,1979). Administration of 2-MBI to rats has been

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shown to result in reproductive toxicity (Barilyak,1974) and chromosomal aberrations in rat fetal cells (Barilyak and Melnik,1979). Recently, Yamano *et al.* (1995) investigated the adverse effects of 2-MBI on pregnant rats and their fetuses and observed major fetal malformations but only at a dose lethal to most treated dams. They concluded that maternal toxicity preceded fetal toxicity.

In a 13-week inhalation toxicity study of 2-MBI in F344 rats, Gaworski *et al.* (1991) encountered thymic atrophy and adrenal cortex necrosis in addition to a potent antithyroid effect. Exposure to 2-MBI *via* other routes has also been demonstrated due to use of rubber products processed with this antioxidant and vulcanization accelerator (Airaudo *et al.*, 1990). Thus Airaudo *et al.*(1990) demonstrated some anesthetic drugs to be contaminated with 2-MBI in the range of 2.8 - 11.8 ppm, the contamination sources being rubber plunger-seals of syringes and /or drug packing containers. Recently, we have also detected 11.5- 67.7 ppm of 2-MBI in commercial farming rubber boots, a possible source of human exposure to 2-MBI (Isama *et al.*, submitted). 2-MBI is known to be a metabolite of the immunomodulator, 3-(p-chlorophenyl)-2,3-dihydro-3-hydroxythiazolo[3,2a]benzimidazole-2-acetic acid (Janssen *et al.*, 1981).

The present 28-day repeated dose oral toxicity study of 2-MBI followed by 2-week recovery examination in Wistar rats was conducted to evaluate adverse effects and their reversibility, and the non-observed-effect level (NOEL). Considering its potential as an environmental endocrine disrupter, toxicological investigations of 2-MBI with various exposure routes appear to be important. In an ancillary 14-day repeated dose oral toxicity study, the antithyroid potency of 2-MBI was compared with those of thiourea (TU) and ethylenethiourea (ETU), both thyroid toxic and tumorigenic compounds (Paynter *et al*, 1988), employing circulating thyroid hormone and TSH levels as parameters.

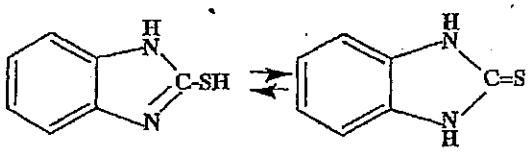

Fig. 1. Chemical structure of 2-mercaptobenzimidazole (2-MBI).

MATERIALS AND METHODS

Chemicals

2-MBI (MW:150.20, CAS No. 583-39-1, RTECS No. DE1050000) was obtained from Ouchi Shinko Chemical Ind., Ltd.(Osaka, Japan) as a slight yellow powder, soluble in methanol, ethanol and acetone and practically insoluble in water and chloroform, and was used without further purification. Reagents employed for hematological and biochemical analyses were purchased from Wako Pure Chemicals Industries (Osaka, Japan), Boeringer Mannheim-Yamanouchi (Tokyo, Japan), Shinotest Laboratory (Tokyo, Japan) and Midorijuji Co. Ltd. (Kobe, Japan). Corn oil was purchased from Sigma (MO, USA). Thiourea and ethylenethiourea were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Experimental animals and diets

Specific pathogen-free Wistar male and female rats (4 weeks old) were purchased from SLC Co. (Shizuoka, Japan) and acclimated for one week prior to the initiation of the study.The basal pellet diet (F-2) was purchased from Funabashi Farm (Funabashi, Japan). Food and tap water were available *ad libitum* throughout except in the acute toxicity study.

Housing Conditions

The animal room was maintained at 24±1℃ and 55±5% humidity with a 12 hr light/dark cycle. Rats were housed in aluminum hanging cages (3 rats/cage) for the acute toxicity study and in plastic cages (5 rats /cage) using chip bedding for the subacute toxicity study.

Experimental design

2-MBI was dissolved or suspended in corn oil and administered to rats by the i.g. route using disposable gavage tube (Fuchigami Kiki, Tokyo, Japan).

Acute oral toxicity study: The acute oral toxicity study of 2-MBI in male and female rats was conducted according to the method reported by Lorke (1983). 2-MBI was administered by gavage in 1 ml per 100 g body weight under fasted conditions. In the first stage test, 3 rats per group (mean body weights: males, 92 g, females, 80 g, fasted for 16 hr before administration) were treated with 10, 100 and 1000 mg/kg of 2-MBI . Clinical signs and mortality were monitored for 10 hr on the day of administration and then twice a day up to day 14 when the test was terminated. Based on the results of the mortality in the 1st stage test, the acute toxicity of 140, 225, 370 and 600 mg/kg of 2-MBI was

examined using 2 rats of each sex / dose group as a 2nd stage test (Lorke,1983) in which clinical signs and mortality were again monitored for 14 days.

Twenty-eight-day repeated dose oral toxicity study: For the dose-determining study, male and female rats (5 rats/group) were orally administered 80 mg (approximately 1/4 dose of the oral LD_{50}), 40 ,20, 10 and 5 mg/kg of 2-MBI for a consecutive two weeks. Taking into account the observed reduction in body weight gain in groups receiving more than 40 mg/kg and the 2 weeks longer treatment period for the 28-day repeated oral toxicity study, doses of 0 (control, corn oil alone), 2, 10 and 50 mg/kg of 2-MBI were administered by gavage to groups of 10 male and 10 female rats for 28 consecutive days. Half the rats in each group (5 males and 5 females) were used for blood clotting time tests. Additional sub-groups of 10 rats each of both sexes receiving 0 and 50 mg/kg were maintained without treatment for 14 days subsequent to termination of 2-MBI administration in order to assess recovery and /or appearance of delayed adverse effects.

In the ancillary 14-day repeated dose oral toxicity study, 5 male rats were treated with either 25 mg (0.15 mmol) or 50 mg (0.3 mmol)/kg of 2-MBI by gavage for 2 weeks to examine the effect of 2-MBI on circulating thyroid hormone and TSH levels. For comparison, TU and ETU, both well characterized antithyroid agents, were also administered to rats following the same protocol.

Clinical signs were monitored throughout the study. The male and female animals were weighed and randomly allocated to 12 groups (n=5) three days prior to the initiation of the treatment. On the first day of treatment, and then twice weekly throughout the study, body weights were measured and the most recently obtained body weight values were used for the calculation of administration doses per kg body weight. Food consumption was measured once a week.

Stability test of 2-MBI in corn oil

The stability of 2-MBI in corn oil(2%) was examined by HPLC after extracting the test compound with methanol. The HPLC conditions applied were as follows: a Shimadzu LC-6A liquid chromatograph (Shimadzu Co. Ltd., Kyoto, Japan) attached to a Shimadzu SPD-M6A photodiode array UV-VIS detector (Kyoto, Japan) with a Wako Wakosil-II 5C18 HG Prep (4.6 mm i.d. ×250 mm) column were used with a mobile phase of 0.1% phosphoric acid/methanol (50/50, v/v). The flow rate and the detection wavelength were 1ml/min and 304 nm, respectively.

2-MBI in corn oil (2%) was confirmed to be stable for at least one week at room temperature. Thus, test samples for gavage administration were prepared once a week. The purity of 2-MBI used was >95%.

Clinical parameters

In the 28-day repeated oral dose toxicity study, blood was collected from the orbital plexus under ethylether anesthesia, and the hematological parameters, red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean hemoglobin concentration (MHC), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT) and white blood cells (WBC) were assessed with a Sysmex M-2000 System (Toa Medical Electronics Co., Kobe, Japan). Differential white blood cell counts were performed using a Microx (Tateishi Electric Co., Japan).

Serum biochemical analyses were conducted for 24 items; total protein (TP), albumin (ALB), blood urea nitrogen (BUN), creatinine (CRN), glucose (GLC), non-esterified fatty acid (NEFA), phospholipid (PL), triglyceride (TG), total cholesterol (T-CHO), free cholesterol (F-CHO), alkaline phosphatase (ALP), amylase (AMY), cholinesterase (CHE), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), leucine aminopeptidase (LAP), lactate dehydrogenase (LDH), calcium (Ca^{2+}), magnesium (Mg^{2+}), inorganic posphorus (Pi), sodium (Na^{+}), potassium (K^{+}), and chlorine (Cl^{-}) using an Auto Clinical Analyzer, Hitachi Model 7150 (Hitachi Ltd., Tokyo, Japan) . T_3/T_4 and TSH levels in serum were measured by RIA using analytical kits.

At autopsy, the weights of the brain, heart, lungs, liver, kidneys, spleen, adrenals, testes, ovaries, pituitary, thymus, submaxillary glands and thyroid glands of each animal were measured. These organs and the esophagus, stomach, small and large intestine, pancreas, ischiatic nerve, urinary bladder, seminal vesicles, uterus, prostate and, mesenteric lymph nodes as well as samples of spinal cord, skeletal muscle, and bone marrow (femur and sternum) were fixed in 10% buffered formalin solution for routine histological processing. Paraffin sections were stained with hematoxylin-and eosin for histopathological examination.

Statistical analysis

All quantitative data, except for the histopathological findings, were statistically analyzed by one-way analysis of variance (ANOVA) techniques with Dunnett's or Scheff's multiple comparison procedures. Significance was established at the $p<0.05$ level.

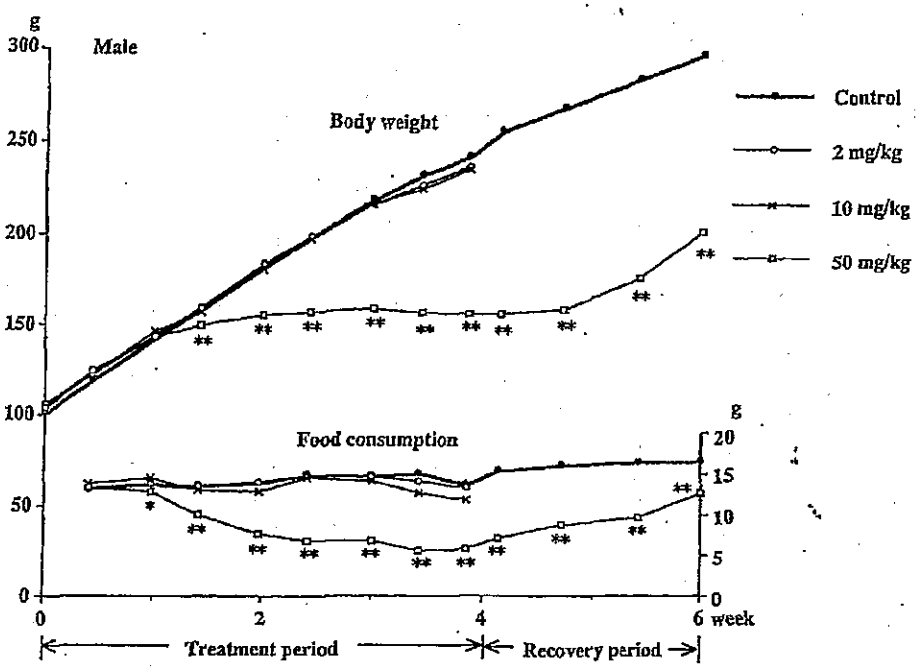

Fig. 2. Body weight and food consumption curves for male rats treated with 2-mercaptobenzimidazole.

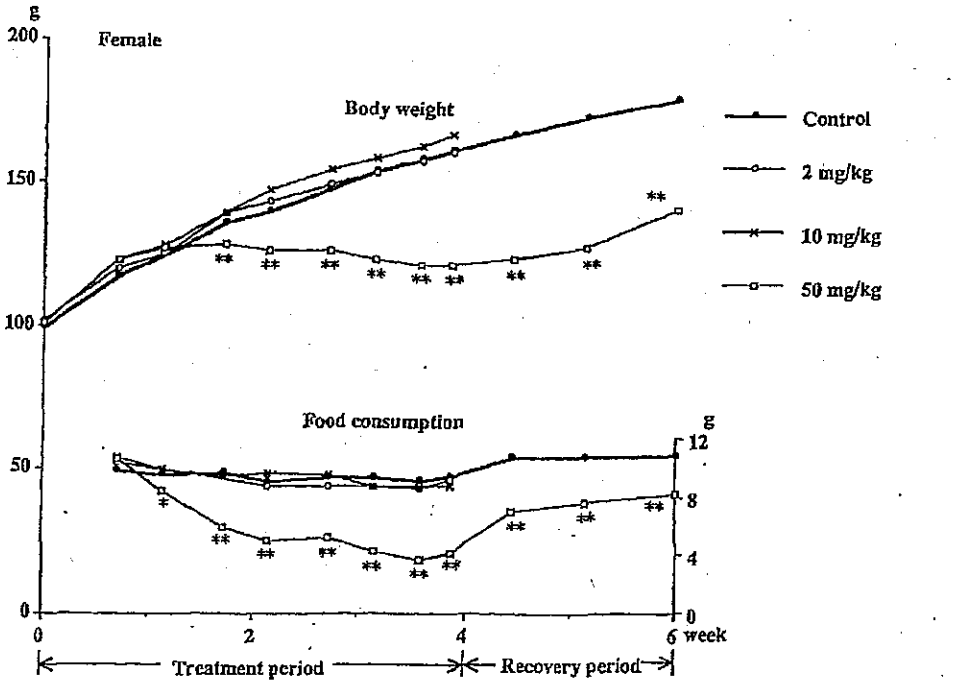

Fig. 3. Body weight and food consumption curves for female rats treated with 2-Mercaptobenzimidazole.

Table 1. Hematological findings for rats after 28 days of treatment with 2-mercaptobenzimidazole and a 2-week recovery period.

Groups(Dose)		Treatment				Recovery	
No. of animals		Control 5	2 mg/kg 5	10 mg/kg 5	50 mg/kg 5	Control 5	50 mg/kg 5
Male							
RBC	$x10^{12}/l$	9.00 ± 0.10	9.07 ± 0.37	8.61 ± 0.19	8.66 ± 0.25	9.86 ± 0.19	7.49 ± 0.13**
Hb	g/dl	15.8 ± 0.4	15.9 ± 0.4	15.4 ± 0.4	15.7 ± 0.4	16.3 ± 0.3	13.7 ± 0.2**
HCT	%	47.1 ± 0.4	46.7 ± 1.6	45.8 ± 1.6	44.0 ± 1.5**	48.0 ± 0.6	39.8 ± 0.9**
PLT	$x10^{12}/l$	0.81 ± 0.08	0.84 ± 0.09	0.79 ± 0.18	0.63 ± 0.05**	0.84 ± 0.13	1.06 ± 0.08*
WBC	$x10^{9}/l$	7.36 ± 0.83	6.66 ± 0.36	6.58 ± 0.35	6.46 ± 0.83	8.22 ± 0.68	6.30 ± 0.40**
PT	Sec.	14.6 ± 0.6	14.3 ± 0.8	14.8 ± 0.8	14.9 ± 0.9	16.3 ± 0.5	15.2 ± 0.3**
APTT	Sec.	24.7 ± 2.1	24.2 ± 4.1	25.6 ± 2.7	35.6 ± 0.8*	30.0 ± 6.7	25.2 ± 2.6
Female							
RBC	$x10^{12}/l$	9.01 ± 0.14	9.02 ±0.12	8.77 ± 0.58	9.00 ±0.51	8.58 ± 0.20	6.64 ± 0.08**
Hb	g/dl	16.2 ± 0.2	16.2 ± 0.3	15.9 ± 1.0	16.3 ± 0.7	15.6 ± 0.3	12.7 ± 0.5**
HCT	%	46.2 ± 1.0	45.8 ± 1.0	45.1 ± 3.1	45.1 ± 2.6	44.6 ± 0.9	34.8 ± 0.6**
PLT	$x10^{12}/l$	0.72 ±0.08	0.71 ± 0.21	0.78 ± 0.16	0.67 ± 0.15	1.04 ± 0.06	1.22 ± 0.08**
WBC	$x10^{9}/l$	8.78 ± 1.05	7.62 ± 1.33	5.98 ± 1.06**	5.24 ±0.31**	5.84 ± 0.65	6.24 ± 1.2
PT	Sec.	13.9 ± 0.6	14.0 ± 0.8	14.1 ± 0.4	14.9 ± 0.9	14.7 ± 05	14.4 ± 0.4
APTT	Sec.	26.5 ± 0.9	26.3 ± 4.9	27.5 ± 7.3	44.3 ± 9.0 **	24.1 ± 3.1	19.8 ± 1.3*

Data are mean ± S.D. values
*,**: Significantly different from the relevant control at $p<0.05$, $p<0.01$, respectively

Table 2. Biochemical findings for male rats after 28 days of treatment with 2-mercaptobenzimidazole and a 2-week recovery period.

Groups(Dose)		Treatment				Recovery	
No. of animals		Control 5	2 mg/kg 5	10 mg/kg 5	50 mg/kg 5	Control 5	50 mg/kg 5
TP	g/dl	5.98 ± 0.16	6.01 ± 0.15	6.20 ± 0.22	6.99 ±0.08**	6.32 ± 0.16	5.79 ±0.10**
ALB	g/dl	4.25 ± 0.07	4.24 ± 0.12	4.33 ± 0.10	4.86 ± 0.06**	4.38 ± 0.05	3.94 ± 0.09**
A/G		2.47 ± 0.14	2.39 ± 0.09	2.32 ± 0.19	2.29 ± 0.18	2.26 ± 0.15	2.14 ± 0.12
BUN	mg/dl	8.89 ± 1.26	8.09 ± 0.89	6.65 ± 0.31**	12.77 ± 0.49**	12.1 ± 1.40	11.1 ± 0.4
CRN	mg/dl	0.28 ± 0.03	0.27 ± 0.04	0.24 ± 0.02	0.28 ± 0.02	0.34 ± 0.07	0.24 ± 0.02*
GLC	mg/dl	118 ± 4	120 ± 13	124 ± 6	124 ± 6	130 ± 7	108 ± 8**
NEFA	mEq/l	0.78 ± 0.13	0.84 ± 0.15	0.83 ± 0.12	0.82 ± 0.07	0.82 ± 0.05	0.95 ± 0.12
PL	mg/dl	103 ± 3	108 ± 9	107 ± 9	226 ± 16**	121 ± 5	160 ± 12**
TG	mg/dl	76 ± 15	82 ± 14	74 ± 23	65 ± 10	157 ± 23	85 ± 12**
T-CHO	mg/dl	52 ± 4	54 ± 6	65 ± 7*	180 ± 11**	59 ± 2	105 ± 11**
F-CHO	mg/dl	7.6 ± 1.6	7.1 ± 2.3	10.0 ± 1.7	46.4 ± 3.5**	11.4 ± 1.6	23.1 ± 3.7**
ALP	mU/ml	323 ± 29	312 ± 9	206 ± 24**	187 ± 26**	198 ± 11	262 ± 28**
ALT	mU/ml	38 ± 9	40 ± 5	32 ± 7	29 ± 5	33 ± 7	33 ± 7
AST	mU/ml	82 ± 8	81 ± 4	69 ± 8*	54 ± 3**	62 ± 16	64 ± 6
CHE	mU/ml	174 ± 15	186 ± 21	536 ± 53	2013 ± 289**	173 ± 32	480 ± 42**
γ-GTP	mU/ml	1.29 ±0.40	1.33 ± 0.22	1.42 ± 0.37	1.98 ± 0.09**	0.01 ± 0	0.01 ± 0
LAP	mU/ml	47 ± 2	45 ± 1	47 ± 2	65 ± 4	42 ± 2	48 ± 2**
LDH	mU/ml	539 ± 104	503 ± 118	483 ± 128	374 ± 47	340 ± 47	334 ± 129
Ca	mg/dl	9.9 ± 0.1	9.9 ± 0.2	9.9 ± 0.2	9.8 ± 0.3	10.5 ± 0.1	10.2 ± 0.2*
Mg	mg/dl	2.1 ± 0.1	2.2 ± 0.1	2.0 ± 0.2	2.0 ± 0.1	2.0 ± 0.1	2.0 ± 0.1
Pi	mg/dl	7.9 ± 0.3	7.7 ± 0.3	7.1 ± 0.3**	5.4 ± 0.2**	7.3 ± 0.3	7.6 ± 0.3
Na	mEq/l	137 ± 1	137 ± 1	138 ± 1	142 ± 1**	136 ± 1	136 ± 1
K	mEq/l	5.2 ± 0.2	4.9 ± 0.3	4.7 ± 0.2**	3.5 ± 0.1**	4.3 ± 0.3	5.0 ± 0.2**
Cl	mEq/l	100 ± 0	99 ± 1*	98 ± 2	98 ± 1*	99 ± 1	103 ± 1**

Data are mean ± S.D. values
*,**: Significantly different from the relevant control at $p<0.05$, $p<0.01$, respectively

Table 3. Biochemical findings for female rats after 28 days of treatment with 2-mercaptobenzimidazole and a 2-week recovery period.

Groups(Dose)		Treatment				Recovery	
		Control	2 mg/kg	10 mg/kg	50 mg/kg	Control	50 mg/kg
No. of animals		5	5	5	5	5	5
TP	g/dl	5.92 ± 0.16	5.81 ± 0.15	5.88 ± 0.06	6.70 ± 0.23**	6.29 ± 0.25	6.31 ± 1.05
ALB	g/dl	4.30 ± 0.12	4.29 ± 0.10	4.29 ± 0.05	4.60 ± 0.14**	4.44 ± 0.19	3.78 ± 0.08**
A/G		2.67 ± 0.18	2.83 ± 0.11	2.71 ± 0.10	2.19 ± 0.13**	2.41 ± 0.15	1.66 ± 0.5*
BUN	mg/dl	10.17 ± 1.09	9.95 ± 0.91	7.35 ± 0.92	18.08 ± 5.23	11.4 ± 1.4	10.7 ± 1.0
CRN	mg/dl	0.29 ± 0.04	0.29 ± 0.04	0.27 ± 0.03	0.41 ± 0.07**	0.32 ± 0.01	0.30 ± 0.02
GLC	mg/dl	116 ± 5	115 ± 6	116 ± 7	120 ± 5	117 ± 10	110 ± 10
NEFA	mEq/l	0.69 ± 0.07	0.64 ± 0.08	0.60 ± 0.05	0.94 ± 0.20	0.82 ± 0.14	0.86 ± 0.16
PL	mg/dl	154 ± 8	139 ± 9	123 ± 7**	265 ± 22**	173 ± 14	187 ± 14
TG	mg/dl	52 ± 2	49 ± 12	41 ± 4	65 ± 12**	55 ± 11	64 ± 15
T-CHO	mg/dl	85 ± 7	74 ± 5	73 ± 6	208 ± 22**	95 ± 4	121 ± 13**
F-CHO	mg/dl	19.0 ± 2.0	15.8 ± 1.4	15.2 ± 1.7	59.1 ± 4.0**	23.3 ± 1.5	30.0 ± 3.1**
ALP	mU/ml	200 ± 29	200 ± 28	138 ± 7*	183 ± 51	131 ± 12	153 ± 18
ALT	mU/ml	33 ± 5	32 ± 5	28 ± 4	29 ± 5	29 ± 7	28 ± 7
AST	mU/ml	74 ± 7	75 ± 6	68 ± 4	62 ± 4**	63 ± 4	61 ± 5
CHE	mU/ml	1230 ± 235	1350 ± 146	1380 ± 152	2096 ± 41**	1566 ± 293	1421 ± 147
γ-GTP	mU/ml	0.70 ± 0.18	0.57 ± 0.21	0.69 ± 0.24	1.25 ± 0.31**	0.03 ± 0.02	0.10 ± 0.21
LAP	mU/ml	46 ± 3	44 ± 1	45 ± 1	68 ± 3**	43 ± 3	48 ± 3*
LDH	mU/ml	338 ± 25	362 ± 74	334 ± 33	328 ± 76	323 ± 82	268 ± 83
Ca	mg/dl	9.8 ± 0.1	9.6 ± 0.3	9.5 ± 0.1	9.6 ± 0.5	9.9 ± 0.4	10.0 ± 0.2
Mg	mg/dl	2.0 ± 0.1	2.1 ± 0.1	2.1 ± 0.1	2.1 ± 0.1	2.05 ± 0.06	2.07 ± 0.09
Pi	mg/dl	6.4 ± 0.3	6.1 ± 0.2	6.1 ± 0.3	5.5 ± 0.2**	5.1 ± 0.3	6.8 ± 0.3**
Na	mEq/l	138 ± 0	138 ± 1	140 ± 1	143 ± 2**	137 ± 0	135 ± 1**
K	mEq/l	4.5 ± 0.2	4.4 ± 0.2	3.9 ± 0.1**	3.1 ± 0.1**	4.4 ± 0.2	4.6 ± 0.1
Cl	mEq/l	101 ± 1	102 ± 1	96 ± 0	97 ± 3	104 ± 1	104 ± 1

Data are mean ± S.D. values
*,**: Significantly different from the relevant control at $p<0.05$, $p<0.01$, respectively

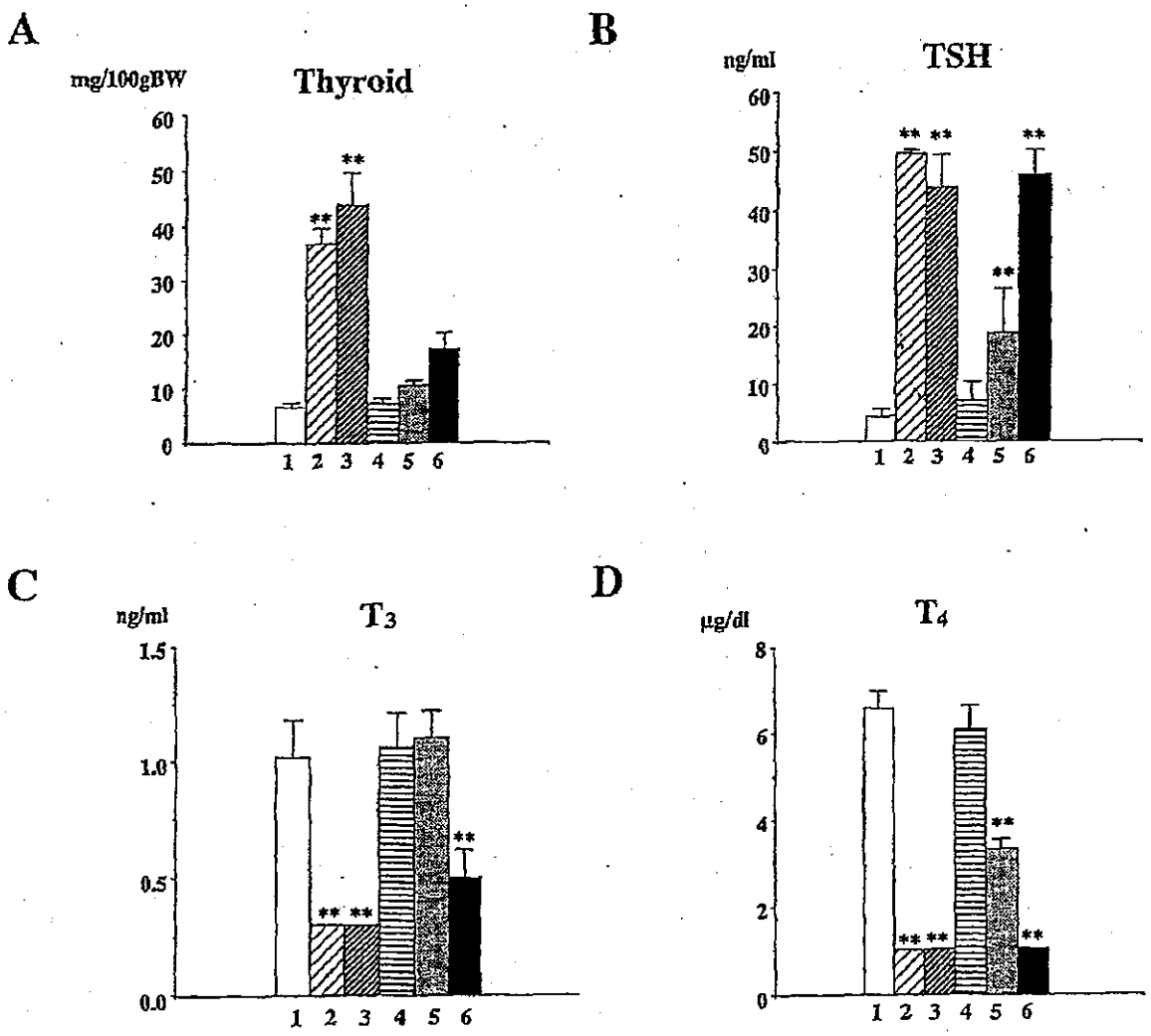

Fig. 4. Thyroid weights and serum thyroid-related hormone levels in male rats after gavage treatment with antithyroid thioureylene compounds 2-MBI, TU and ETU for 14 days.

A: Relative thyroid weights (mg/100g body weight)
B: Serum levels of TSH C: Serum levels of T3
D: Serum levels of T4
1 : Control (Corn oil alone) 2 : 0.15mmole 2-MBI/kg
3 : 0.3mmole 2-MBI/kg 4 : 0.3mmole TU/kg
5 : 0.15mmole ETU/kg 6 : 0.3mmole ETU/kg

Table 4. Organ weights for male rats after 28 days of treatment with 2-mercaptobenzimidazole and a 2-week recovery period.

Groups(Dose)		Treatment				Recovery	
No of animals		Control 5	2 mg/kg 5	10 mg/kg 5	50 mg/kg 5	Control 5	50 mg/kg 5
Body weight	g	224 ± 8	223 ± 7	218 ± 14	153 ± 3**	285 ± 11	186 ± 8**
Brain	g	1.73 ± 0.04	1.79 ± 0.04	1.73 ± 0.07	1.65 ±0.02*	1.83 ± 0.04	1.67 ± 0.04**
Heart	g	0.72 ± 0.02	0.70 ± 0.02	0.60 ± 0.03**	0.39 ±0.02**	0.80 ± 0.06	0.56 ± 0.02**
Lung	g	0.83 ± 0.04	0.87 ± 0.03	0.86 ± 0.05	0.63 ±0.02**	0.92 ± 0.04	0.77 ± 0.07**
Liver	g	6.39 ± 0.30	6.72 ± 0.32	8.09 ± 0.79**	6.50 ± 0.24	8.36 ± 0.52	5.29 ± 0.30**
Kidney	g	1.47 ± 0.09	1.56 ± 0.05	1.57 ± 0.11	1.04 ±0.07**	1.82 ± 0.11	1.15 ± 0.03**
Spleen	g	0.44 ± 0.04	0.46 ± 0.02	0.43 ± 0.02	0.22 ± 0.01**	0.57 ± 0.03	0.42 ± 0.02**
Testis	g	2.55 ± 0.10	2.59 ± 0.07	2.48 ± 0.10	2.40 ± 0.09*	2.80 ± 0.05	2.49 ± 0.11**
Pituitary	mg	7.3 ± 1.5	6.9 ± 1.2	8.3 ± 0.9	7.6 ± 1.0	8.3 ± 1.0	6.7 ± 2.0
Thyroid	mg	9.4 ± 1.3	11.6 ± 1.5	39.0 ± 5.2*	81.2 ± 12.4**	11.8 ± 1.5	47.4 ± 8.2**
Adrenal	mg	32.0 ± 3.0	32.7 ± 3.1	31.3 ± 3.0	31.6 ± 3.1	32.8 ± 1.9	28.1 ± 3.4*
Submaxillary G.	g	0.36 ± 0.04	0.38 ± 0.04	0.33 ± 0.05*	0.20 ± 0.04**	0.47 ± 0.05	0.27 ± 0.02**
Thymus	g	0.34 ± 0.05	0.26 ±0.02**	0.23 ± 0.04**	0.13 ± 0.02**	0.33 ± 0.02	0.18 ± 0.01**
Brain	g%	0.78 ± 0.04	0.80 ± 0.03	0.80 ± 0.03	1.08 ± 0.03**	0.64 ± 0.02	0.90 ± 0.04**
Heart	g%	0.31 ± 0.01	0.31 ± 0.01	0.27 ± 0.02**	0.26 ± 0.01**	0.28 ± 0.01	0.30 ± 0.02*
Lung	g%	0.37 ± 0.01	0.39 ± 0.01	0.40 ± 0.02**	0.41 ± 0.01**	0.33 ± 0.01	0.41 ± 0.03**
Liver	g%	2.85 ± 0.08	3.01 ± 0.10	3.71 ± 0.17**	4.25 ± 0.14**	2.93 ± 0.09	2.85 ± 0.07
Kidney	g%	0.65 ± 0.02	0.70 ± 0.02*	0.72 ± 0.02**	0.68 ± 0.05	0.64 ± 0.02	0.62 ± 0.02
Spleen	g%	0.19 ± 0.01	0.21 ± 0.01	0.19 ± 0.01	0.15 ± 0.01**	0.20 ± 0.02	0.23 ± 0.01**
Testis	g%	1.13 ± 0.01	1.16 ± 0.05	1.14 ± 0.04	1.57 ± 0.03**	0.99 ± 0.03	1.34 ± 0.07**
Pituitary	mg%	3.3 ± 0.6	3.1 ± 0.6	3.8 ± 0.2	5.0 ± 0.7**	2.9 ± 0.4	3.7 ± 1.2
Thyroid	mg%	4.2 ± 0.5	5.2 ± 0.6	17.9 ± 2.2*	53.0 ± 7.6**	4.2 ± 0.6	25.7 ± 5.1**
Adrenal	mg%	14.2 ± 1.0	14.7 ± 1.6	14.4 ± 1.3	20.7 ± 1.8**	11.5 ± 1.0	15.2 ± 2.0**
Submaxillary G.	g%	0.16 ± 0.01	0.17 ± 0.02	0.15 ± 0.02	0.13 ± 0.03*	0.17 ± 0.02	0.15 ± 0.02*
Thymus	g%	0.15 ± 0.02	0.12 ± 0.01*	0.11 ± 0.02**	0.09 ± 0.01**	0.12 ± 0.01	0.10 ± 0.01**

Data are mean ± S.D. values

*,**: Significantly different from the relevant control at $p<0.05$, $p<0.01$, respectively

Table 5. Organ weights for female rats after 28 days of treatment with 2-mercaptobenzimidazole and a 2-week recovery period.

Groups(Dose)		Treatment				Recovery	
		Control	2 mg/kg	10 mg/kg	50 mg/kg	Control	50 mg/kg
No of animals		5	5	5	5	5	5
Body weight	g	155 ± 7	150 ± 10	155 ± 7	115 ± 2**	168 ± 11	129 ± 5**
Brain	g	1.69 ± 0.04	1.71 ± 0.04	1.66 ± 0.02	1.54 ±0.02*	1.72 ± 0.06	1.63 ± 0.07*
Heart	g	0.50 ± 0.03	0.48 ± 0.02	0.49 ± 0.02	0.33 ± 0.01**	0.50 ± 0.04	0.42 ± 0.02**
Lung	g	0.71 ± 0.06	0.69 ± 0.03	0.71 ± 0.05	0.57 ± 0.02**	0.65 ± 0.04	0.63 ± 0.04
Liver	g	4.52 ± 0.18	4.26 ± 0.39	5.06 ± 0.31*	5.01 ± 0.26**	4.24 ± 0.42	3.94 ± 0.22
Kidney	g	1.08 ± 0.04	1.05 ± 0.06	1.18 ± 0.06**	0.91 ± 0.03**	1.05 ± 0.09	0.92 ± 0.06*
Spleen	g	0.35 ± 0.03	0.33 ± 0.02	0.35 ± 0.01	0.18 ± 0.01**	0.34 ± 0.04	0.35 ± 0.04
Ovary	mg	57.1 ± 5.5	55.8 ± 8.8	61.2 ± 4.7	23.8 ± 1.6*	51.7 ± 7.2	37.7 ± 0.9**
Pituitary	mg	9.6 ± 1.3	9.6 ± 1.1	8.1 ± 0.6	7.5 ± 1.5*	10.3 ± 2.1	9.4 ± 1.4
Thyroid	mg	8.8 ± 1.1	8.8 ± 1.6	29.2 ± 5.7**	73.6 ± 3.6**	10.6 ± 1.1	48.2 ± 13.4**
Adrenal	mg	42.2 ± 2.3	39.5 ± 1.8	38.3 ± 2.4*	35.3 ± 3.1**	39.3 ± 5.7	31.4 ± 2.2*
Submaxillary G.	g	0.30 ± 0.03	0.28 ± 0.02	0.30 ± 0.02	0.19 ± 0.01**	0.31 ± 0.02	0.25 ± 0.02**
Thymus	g	0.31 ± 0.04	0.25 ± 0.02**	0.22 ± 0.01**	0.12 ± 0.02**	0.27 ± 0.03	0.19 ± 0.02**
Brain	g%	1.10 ± 0.04	1.15 ± 0.08	1.08 ± 0.05	1.34 ± 0.03**	1.02 ± 0.04	1.26 ± 0.04**
Heart	g%	0.32 ± 0.01	0.32 ± 0.02	0.32 ± 0.02	0.28 ± 0.01**	0.30 ± 0.02	0.33 ± 0.01**
Lung	g%	0.46 ± 0.02	0.46 ± 0.03	0.46 ± 0.02	0.50 ± 0.03*	0.39 ± 0.02	0.49 ± 0.03**
Liver	g%	2.92 ± 0.05	2.84 ± 0.20	3.27 ± 0.14**	4.35 ± 0.18**	2.51 ± 0.10	3.04 ± 0.11**
Kidney	g%	0.70 ± 0.04	0.70 ± 0.02	0.76 ± 0.03**	0.79 ± 0.02**	0.62 ± 0.03	0.71 ± 0.03**
Spleen	g%	0.23 ± 0.02	0.22 ± 0.01	0.23 ± 0.01	0.16 ± 0.01**	0.21 ± 0.01	0.27 ± 0.03**
Ovary	mg%	36.9 ± 2.7	37.4 ± 6.3	39.7 ± 3.2	20.6 ± 1.2*	30.3 ± 3.0	29.1 ± 1.0
Pituitary	mg%	6.2 ± 1.0	6.4 ± 0.7	5.3 ± 0.6	6.5 ± 1.2	6.1 ± 1.0	7.2 ± 1.1
Thyroid	mg%	5.7 ± 0.8	5.9 ± 1.1	18.9 ± 3.6*	63.9 ± 2.4**	6.3 ± 0.9	37.3 ± 10.2*
Adrenal	mg%	27.3 ± 0.6	26.5 ± 1.4	24.8 ± 0.7*	30.7 ± 2.6	23.2 ± 1.9	24.8 ± 1.8
Submaxillary G.	g%	0.19 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	0.16 ± 0.1**	0.18 ± 0.01	0.19 ± 0.01
Thymus	g%	0.20 ± 0.02	0.16 ± 0.01**	0.14 ± 0.01**	0.10 ± 0.02**	0.16 ± 0.01	0.15 ± 0.01

Data are mean ± S.D. values
*,**: Significantly different from the relevant control at $p<0.05$, $p<0.01$, respectively

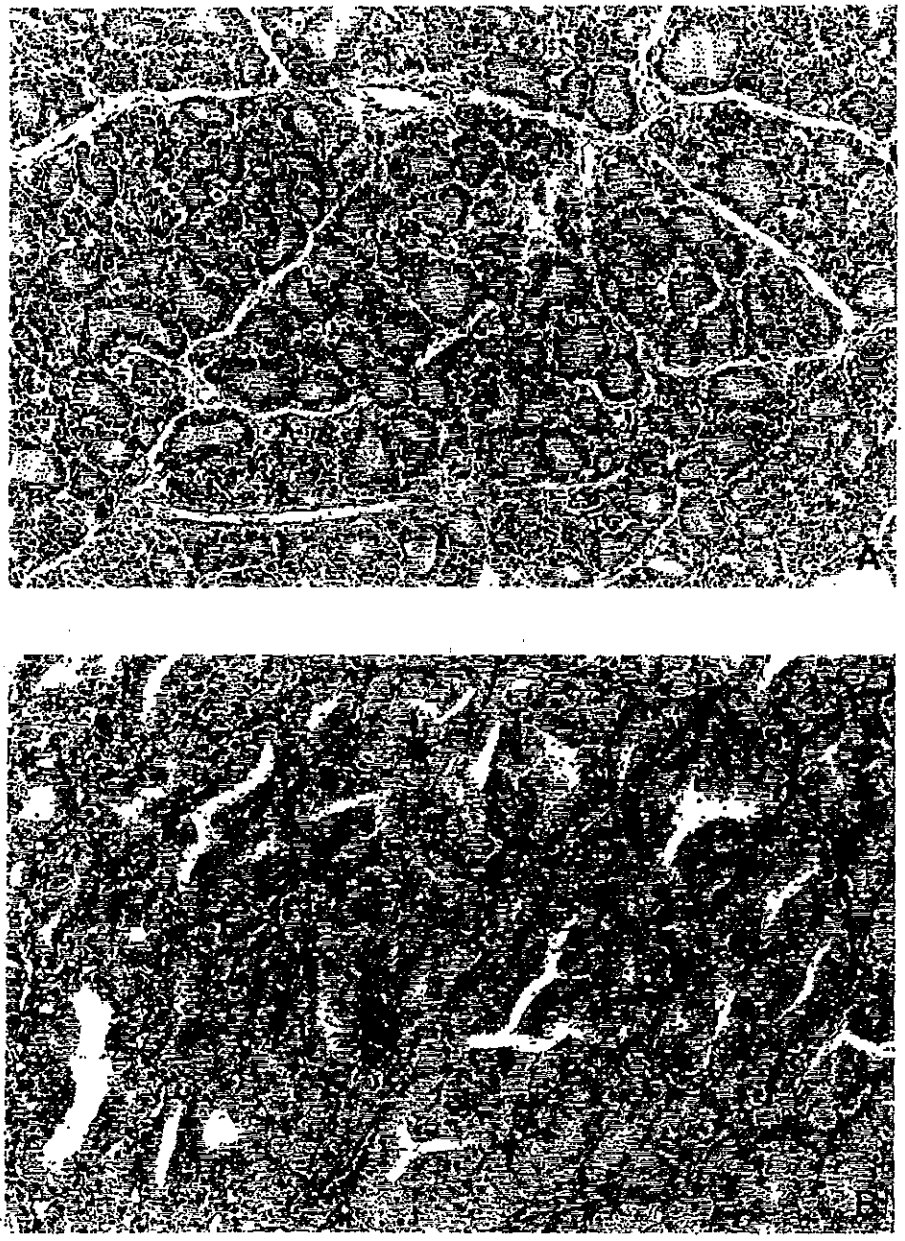

photo 1. A: Thyroid gland from a control rat. Note normal glands with follicles containing abundant colloid and lined by low cuboidal cells. HE ×48
B: Thyroid gland from a rat treated for 28 days with repeated doses of 2-MBI (50 mg/kg). Note diffuse follicular hyperplasia characterized by tall columnar epithelial cells and markedly decreased colloid. HE ×48

Table 6. Histopathological findings for rats after 28 days of treatment with 2-mercaptobenzimidazole and a 2-week recovery period.

		Treatment								Recovery			
Sex		Male				Female				Male		Female	
Groups(Dose mg/kg)		0	2	10	50	0	2	10	50	0	50	0	50
No. of animals examined		5	5	5	5	5	5	5	5	5	5	5	5
Heart													
Intramyocardial cell infiltration	±	0	0	2	0	0	0	1	0	0	1	1	0
Fibrosis	±	1	0	1	1	1	0	0	0	0	1	1	0
Myocardial calcification	±	0	0	0	0	0	0	1	0	0	0	0	0
Lung													
Alveolar wall thickening	±	0	2	1	0	1	0	1	0	1	1	0	1
Perivascular cell infiltration	±	0	1	0	0	0	0	0	0	0	0	0	0
Perivascular edema	±	0	1	1	0	1	1	0	2	0	1	2	1
Interstitial cell infiltration	±	1	0	0	0	1	0	0	0	1	0	0	0
Liver													
Microgranuloma formation	±	0	1	0	1	3	0	2	1	1	1	1	0
Interstitial cell infiltration	±	0	0	0	0	1	1	1	0	0	1	1	0
Kidney													
Eosinophilic bodies	±	5	5	5	5	0	0	0	0	5	5	0	0
Calcification of collecting tubules	±	0	0	0	1	0	0	0	1	0	0	0	0
Testis													
Interstitial edema	±	1	0	2	2	-	-	-	-	1	0	-	-
Spleen													
Hemosiderin deposition	±	0	0	0	0	0	0	0	0	1	0	1	0
Pancreas													
Acinar cell vacuolation	±	0	0	0	0	0	0	1	0	1	0	0	0
Acinar cell necrotic degeneration	±	0	0	0	0	0	0	0	0	1	0	0	1
Thyroid gl.													
Diffuse hyperplasia of follicles	±	0	0	1	0	0	0	1	0	0	0	0	0
	+	0	0	4	0	0	0	4	0	0	0	0	0
	++	0	0	0	5	0	0	0	5	0	5	0	5
Decrease of colloid	±	0	0	2	0	0	0	3	0	0	0	0	0
	+	0	0	3	0	0	0	1	0	0	0	0	0
	++	0	0	0	5	0	0	0	5	0	0	0	0
Fibrous capsule thickening	±	0	0	0	0	0	0	0	0	0	0	0	0
	+	0	0	0	0	0	0	0	0	0	0	0	1
	++	0	0	0	5	0	0	0	5	0	0	0	0
Pituitary gl.													
Increase of thyroidectomized cells	±	0	0	3	0	0	0	0	2	0	0	0	0
	+	0	0	1	0	0	0	0	0	0	3	0	0
	++	0	0	0	5	0	0	0	0	0	2	0	0
Adrenal cortex													
Hyperplasia of lipid-laden cells	±	0	0	3	0	0	0	0	0	0	0	0	0
	+	0	0	0	1	0	0	0	2	0	0	0	0
	++	0	0	0	4	0	0	0	3	0	0	0	0

RESULTS

Acute oral toxicity study

First stage treatment (n=3): Treatment with 10 mg/kg of 2-MBI did not induce any acute toxic signs. At 100 mg/kg, decreases in spontaneous movement, ataxic gait, eyelid closure, and lacrimation were observed within 1 hr of treatment. The rats then recovered within 24 hr and all survived. Treatment with 1000 mg/kg of 2-MBI immediately caused paralytic gait, prone position with coma, lacrimation and hypothermia, and 2 male rats and all 3 female rats died within 32 hr. The remaining one male rat died within 72 hr.

Second stage treatment (140-600 mg/kg, n=2): Depending on the dose administered, loss of spontaneous activity, ataxic gait, eyelid closure, lacrimation, paralytic gait, prone position and coma were observed. Rats treated with doses less than 225 mg/kg recovered within 24 hr but all rats treated with 370 and 600 mg/kg died within 2 days.

LD_{50} (Oral): According to the calculation method proposed by Lorke (1983) using the numbers of rats that died during the 14 days, the LD_{50} values for 2-MBI in this experiment were determined to be 300 mg/kg for both male and female rats.

Autopsy findings in the acute toxicity study: Slight congestion in lung, congestion in glandular stomach mucosa, and retention of administered oil in the stomach through to the colon were found in animals which died within 3 days of dosing. Rats that survived for 14 days exhibited no abnormalities.

Twenty eight-day repeated dose oral toxicity study

Clinical signs

No mortality due to 2-MBI treatment occurred for either male or female rats. No clinical signs related to 2-MBI administration were observed except for decrease in body weight gain and food consumption. The rats of both sexes receiving 50 mg/kg showed emaciation and severe suppression in body weight gain along with decreased food consumption one week after treatment started, as shown in Figs 2 & 3. No significant differences in both body weight gain or food consumption were observed in the groups that received 10 mg/kg 2-MBI or less.

Hematology

At the termination of treatment, significant decreases in WBC in the 10 and 50 mg/kg female rats and in PLT in 50 mg/kg male rats were observed. Significant WBC decrease in male rats was still noted as observed 2-weeks after termination of 2-MBI treatment. At the end of the recovery period, RBC, Hb and HCT were significantly decreased in both the male and female 50 mg/kg groups showing delayed onset of anemia (Table 1).

Increased active partial thromboplastin time (APTT, blood clotting time) was observed for both the males and females receiving 50 mg/kg.

Clinical biochemistry

At the termination of the 2-MBI administration, serum levels of TP, BUN, PL, T-CHO, F-CHO, CHE and γ-GTP were significantly increased in both males and females given 50 mg/kg. ALP, K^+ and Pi were decreased significantly in male rats receiving more than 10 mg /kg, and Na^+ was increased significantly in the 50 mg/kg treated animals. CHO and PL levels remained significantly high 2 weeks after termination of 2-MBI administration (Table 2 & 3). Serum levels of T_3, T_4 and TSH measured 2 weeks after repeated dose treatment with 2-MBI or the antithyroid agents, TU and ETU, are shown in Fig.4.

Organ Weight

At the termination of 2-MBI administration, dose related increases in absolute and relative weights of thyroid, liver and kidney were observed. At 10 mg/kg, the mean absolute and relative thyroid weights in both sexes were approximately 3 times those of the respective control rats, and in the 50 mg/kg dose group the increase in relative thyroid weight was more than 10-fold. Even after the 2-week recovery period, relative thyroid weights were approximately 6 times the control values. Dose-related decreases in absolute and relative thymus weights in all treatment groups of male and female rats and a decrease in relative spleen weight in both males and females receiving 50 mg/kg were also observed. Significant increases in brain, lung, adrenal and pituitary gland weights and decreases in heart and submaxillary glands of relative organ weights were also observed with 50 mg/kg. Some of these organ weight changes may have been due to the severe reduction in body weight gain as shown in Fig 2 & 3 and Tables 4 & 5.

Histopathology

As gross findings, marked enlargement of thyroid glands and thymus involution were evident in treated rats. Diffuse hyperplasia of tall and columnar epithelial cells of follicles, and decrease in colloid and thickening of fibrous capsule appeared dose-dependently(Photo. 1). In association with the thyroid changes, hypertrophic cells in the anterior pituitary glands were found, the so-called thyroidectomy cells. Calcification of the collecting tubules in kidney and fatty changes in adren-

al cortex were also observed. These histopathological changes mostly disappeared after the 2-week recovery period, as shown in Table 6. Despite the thymus weight being decreased dose-dependently, there were no obvious changes in its architecture.

DISCUSSION

The present subacute toxicity study of 2-MBI by gavage administration for 28 consecutive days followed by 2-week recovery period in Wistar rats demonstrated marked thyroid enlargement associated with characteristic diffuse hyperplasia in both male and female rats receiving a dose of 10 mg/kg 2-MBI or above. This thyroid toxicity was accompanied by significant decreases in circulating thyroid hormone (T_3&T_4) levels and a marked increase in the serum TSH level in the ancillary 14-day gavage administration study, the changes being more pronouced than with TU and ETU (Paynter *et al.*, 1988).The antithyroid effects in the present gavage study are consistent with the results observed in the inhalation study of Gaworski *et al.* (1991).The possibility of tumor promotion potential therefore arises (Onodera *et al.*, 1994). Hypertrophic cells increased in the anterior pituitary glands of male rats treated with more than 10 mg/kg 2-MBI, suggesting stimulated TSH generation due to the decrease in thyroid hormone levels by negative feedback regulation (Haynes, 1990). The pituitary and thyroid lesions induced by 2-MBI treatment were identical to those reported after thyroidectomy (Norford *et al.*, 1993).

It is well documented that thyroid hormones stimulate metabolism of cholesterol to bile acid and that hypercholesterolemia is characteristic of hypothyroid states (Haynes, 1990). In the present study, a 2-3 fold increase in serum cholesterol was observed in the 50 mg/kg treated rats and full return to the control level was not evident after the 2-week recovery period. Serum levels of phospholipid were also increased significantly upon 2-MBI administration, although the free fatty acids which were significantly decreased in the inhalation study (Gaworski *et al.*, 1991) did not appear to be affected by gavage administration.

Thyroid hormones are also known to participate regulation of the balance of intracellular and intercellular Na^+ and K^+ levels by active transport (Asano, 1982). The serum Na^+/K^+ ratios in this study were markedly altered from 26.4(control) to 40.6(50 mg/kg) for males and from 30.7 to 46.1 for females, respectively. This may be due to the severe hypothyroid state induced by 2-MBI administration. It is also well known clinically that serum levels of Ca^{2+}, Pi and ALP are altered by a parathyroid hormone imbalance which affects bone metabolism (Fujita,1987). In the present rat study, serum levels of ALP and Pi were significantly decreased , but Ca^{2+} was not changed by 2-MBI treatment. In contrast, Gaworski *et al.*(1991) observed an increased serum level of ALP, so this alteration may not be directly related to a hypothyroid state.

Anemia has been associated with hypothyroidism (Haynes and Murad, 1985) and was observed at termination in the 13-week inhalation study conducted by Gaworski *et al.* (1991). Clinical symptoms of anemia (decreases in RBC, Hb and HCT) were also detected 2 weeks after the termination of high dose 2-MBI administration in the present study. This delayed change may be partially due to the long retention of 2-MBI by hemoglobin (El Dareer *et al.*, 1984) and the long half-life of RBC.

2-MBI is known to be a metabolite of the immunomodulator, 3-(p-chlorophenyl)-2,3-dihydro-3-hydroxythiazolo[3,2a]benzimidazole-2-acetic acid (Janssen *et al.*, 1981). In the present 28-day gavage study in rats, a clear dose-related involution of the thymus was observed in all treatment groups. Severe decrease of body weight gain affects the thymus weight (Levin *et al.*, 1993) but the thymus change was observed even at doses which had no effects on body weight. Although manifest morphological alterations were not found in either the thymus or the spleen, 2-MBI might thus have potential immunotoxicity. A significant decrease in WBCs occurred in female rats at doses higher than 10 mg/kg and a decrease in the relative spleen weight in 50 mg/kg dose rats was also demonstrated. In the 13-week inhalation study (Gaworski *et al.*, 1991), a similar significant decrease in thymus weight associated with atrophy and decreased WBC counts due to lower numbers of circulating lymphocytes in male rats were also observed. Malmfors (1976) reported thymic involution even after a single dose of 2-MBI in rats but not in mice, guinea pigs or rabbits. Such thymic involution in rats was not observed after thyroidectomy (Malmfors, 1976). In our 14-day ancillary study (data not shown), TU and ETU also induced significant thymus involution without notable decrease in body weight gain. These facts suggest antithyroid function-linked thymus involution in rats. Further immunotoxicological investigation is required to clarify this prominent effect of 2-MBI and other antithyroid agents on the thymus.

A significant increase in relative liver weight was observed in the 10 and 50 mg/kg dose groups of both sexes, which is in agreement with the results of the

inhalation study (Gaworski,1991) in which liver injury associated with hepatic hypertrophy and granulomatous inflammation was found. Increased blood clotting time (APTT) also suggests an adverse effect on liver functions. However, in the present gavage study, no overt hepatocyte injury was apparent, which is consistent with no increases in serum AST and ALT levels. In addition, the relative liver weight increase in 50 mg/kg dose rats returned to the control level within the 2-week recovery period. Therefore, the observed liver changes appeared to be an adaptive response.

Slight, but statistically significant increases of BUN level in males, and of CRN level in females associated with a slight increase in relative kidney weight were observed in the higher 2-MBI dose groups. In consideration of the previous report demonstrating renal toxicity (Gaworski,1991), the present finding of calcification in the collecting tubules in one animal of each sex may be significant.

The non-observed-effect level (NOEL) of 2-MBI in rats in the present gavage study was evaluated to be less than 2 mg/kg based on the significant decreases in absolute and relative thymus weights of the lowest treatment dose group. Obviously, prudence must be taken in extrapolating animal data to the human situation. In safety evaluation of chemicals, an uncertainty factor of "100x" is usually adopted. If the available NOEL is based on a subacute study like that conducted here, an additional uncertainty factor of "5x " should be considered (to give 500x). Based on this safety evaluation, 4, μg/kg/day might be acceptable for the human exposure level of 2-MBI. However, the contamination level of 2-MBI in drugs must be reduced as far as possible or should be replaced by a less toxic rubber antioxidant. This might be particularly important in the case of drugs administered over long periods such as insulin. Furthermore, 2-MBI is a fatty soluble compound, and thus its accumulation in the body may occur with repeated administration. Toxicokinetic studies of 2-MBI and related compounds such as methylated derivatives are now under way.

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