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Original Article

General toxicity of a vitamin K_1 2,3-epoxide reductase (VKOR) inhibitor, 3-acetyl-5-methyltetronic acid, in rats

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ABSTRACT — We previously reported that 3-acetyl-5-methyltetronic acid (AMT) had inhibitory effects on rat renal vitamin K_1 2,3-epoxide reductase (VKOR), as well as anti-fibrotic effects on Thy-1 glomerulonephritis and cisplatin-induced renal fibrosis in rats. In the present study, we investigated the general toxicity of AMT in male Crl:CD (SD) rats following a single or 2-week oral administration. After a single oral dose up to 1,500 mg/kg, no death or bleeding tendency was observed in any animal. In the 2-week repeated toxicity study, we performed clinical observations, body weight measurements, a urinalysis, hematology, blood chemistry, gross autopsy, organ weight measurements, and histopathology. The result obtained showed significant decreases in the red blood cell count, hematocrit value, hemoglobin concentration, and urinary calcium. However, no bleeding tendency was observed, even at the highest dose of 400 mg/kg. We also confirmed that the oral bioavailability of AMT was 56.7% in a pharmacokinetic study, and the area under the blood concentration (AUC) at 400 mg/kg of the 2-week oral toxicity study in rats was markedly larger than that in renal fibrosis model rats at 30 mg/kg intravenously. We concluded that AMT does not cause systemic bleeding in rats at the dose levels which AMT showed anti-fibrotic effects.

Key words: 3-Acetyl-5-methyltetronic acid (AMT), Vitamin K_1 2,3-epoxide reductase (VKOR), Rats, Toxicity, Bleeding, Pharmacokinetics

INTRODUCTION

Vitamin K_1 2,3-epoxide reductase (VKOR) is a major enzyme of the vitamin K cycle that controls blood coagulation, and is also involved in mesangial cell proliferation via the vitamin K-dependent activation of Gas6 (Yanagita, 2004a). VKOR inhibitors, such as warfarin, effectively block mesangial cell proliferation and restore renal function (Yanagita, 2004b); however, they also inhibit the biosynthesis of blood coagulation factors in the liver and may cause bleeding (Limdi *et al*., 2009). The novel VKOR inhibitor, 3-acetyl-5-methyltetronic acid (AMT), is a small molecular organic compound that has demonstrated anti-fibrotic effects in two experimental rat models: anti-Thy-1 molecular antibody-induced glomerulonephritis (Uchida *et al*., 2012) and cisplatin-induced renal fibrosis (Uchida *et al*., 2017). In these experiments, we preliminary conducted blood coagulation tests and anti-fibrotic pharmacological assessments of AMT. In the present study, we assessed the toxicity of AMT including its anti-coagulation potency in normal rats, and conducted single and 2-week toxicity studies following oral administration. We also performed a pharmacokinetic study to compare the systemic exposure of AMT in rats between

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pharmacological studies on renal fibrotic models and the present study.

MATERIALS AND METHODS

Ethical considerations

Animal experiments were approved by the animal ethics committee of Toray industries, Inc., and conducted according to the Guidelines for Animal Experiments, Research and Development Division, Toray Industries, Inc.

Materials

AMT was synthesized at Pharmaceutical Research Laboratories, Toray Industries, Inc. (Tokyo, Japan). Carboxymethyl cellulose (CMC) was purchased from Wako Pure Chemical (Tokyo, Japan). Sterilized water for injections and normal saline (both Japanese Pharmacopoeia grade) were purchased from Otsuka Pharmaceutical Factory, Inc. (Tokushima, Japan).

Animal experiments

Male Crl:CD(SD) rats were obtained from Charles River Laboratories Japan, Inc. (Shiga, Japan) at 5 weeks of age. Animals were quarantined and acclimated for 1 week, and a pelleted diet (radiation sterilized CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water (tap water via an automatic water supply system) were supplied *ad libitum*. In the single and 2-week toxicity studies, animals were housed individually in stainless-steel wire mesh cages. In the pharmacokinetic study, five animals were housed in each stainless-steel wire mesh cage. Animal rooms were maintained at 19 to 25°C, relative humidity at 40 to 60%, air ventilation of 10 to 15 times per hour, and a 12-hr light dark cycle. After a 1-week quarantine/acclimation period, animals were randomized by the MiTOX system (Mitsui Zosen Systems Research Inc., Tokyo, Japan) into five groups consisting of 5 males in each toxicity study. AMT was dissolved in 0.5% CMC to prepare dosing solutions for oral administration and in saline to prepare dosing solutions for intravenous administration. Dosing solutions were prepared just before dosing. Dose levels in the three studies were set as follows: Exp.1 (single dose toxicity study), 0, 296, 444, 666, 1,000, and 1,500 mg/kg; Exp.2 (2-week repeated dose toxicity study), 0, 50, 100, 200, and 400 mg/kg/day; Exp.3 (pharmacokinetics study), 30, and 300 mg/kg.

Examinations and observations

In the single toxicity study, clinical observations were conducted twice every day from Day 1 (for 30 min after dosing; 4 hr after dosing) to Day 15. In the 2-week toxicity study, clinical observations were conducted once or twice every day from Day 1 (for 30 min after dosing) to Day 14 (for 30 min after dosing; 4 hr after dosing), and all animals were killed on Day 15. Body weights were measured on Days 1, 2, 4, 6, 8, 10, 13, and 15 in the single toxicity study, and on Days 1, 3, 7, 10, 14, and 15 in the 2-week toxicity study during the administration period. Food consumption was measured on Days 2 to 3, Days 6 to 7, and Days 13 to 14 in the 2-week toxicity study.

Urinalysis

A urinalysis was performed on Day 14 on 0 (vehicle), 200, and 400 mg/kg/day groups in the 2-week toxicity study. The following parameters were measured in 4-hr urine samples collected under non-fasting conditions with free access to water using metabolic cages for rats: pH, protein, ketone bodies, occult blood, bilirubin, nitrite, and urobilinogen by CLINITEK 200+ (Siemens Healthcare Diagnostics Inc., Illinois, USA), color, and urinary sediment. The following parameters were examined in 17-hr urine samples collected under non-fasting conditions with free access to water using metabolic cages for rats: sodium (Na), potassium (K), chloride (Cl), calcium (Ca), and creatinine (CRE) were measured by Hitachi 7070 (Hitachi Ltd., Tokyo, Japan), and urine volume.

Hematology

Hematology was examined on Day 15 in the 2-week toxicity study. Blood samples were collected from the abdominal aorta under pentobarbital anesthesia into blood collection tubes containing EDTA-2K for the blood cell count and 3.13% sodium citrate buffer for coagulation tests. The following parameters were examined: the red blood cell count (RBC), white blood cell count (WBC), platelet count (PLT), hemoglobin value (HGB), hematocrit concentration (HCT), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were measured by Sysmex K-4500 (Sysmex Corp., Hyogo, Japan), and the prothrombin time (PT), activated thromboplastin time (APTT), fibrinogen (FIB), thrombo test (TTO), and hepaplastin test (HPT) were measured by Sysmex CA-1000 (Sysmex Corp., Hyogo, Japan).

Blood chemistry

Blood chemistry was examined on Day 15 in the 2-week toxicity study. Blood samples were collected from the abdominal aorta under pentobarbital anesthesia into heparinized tubes. Plasma was obtained by centrifugation at 1,500 × *g* and 4°C for 15 min. The following parame-

ters were examined: alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), leucyl aminopeptidase (LAP), lactate dehydrogenase (LDH), total bile acid (TBA), glucose (GLC), triglycerides (TG), phospholipids (PL), total cholesterol (TC), non-esterified fatty acids (NEFA), total bilirubin (TB), total protein (TP), albumin (ALB), β-lipoprotein (BLP), blood urea nitrogen (BUN), CRE, uric acid (UA), Na, K, Cl, Ca, and phosphate (P) were measured by Hitachi 7070 (Hitachi High-Technologies Co., Tokyo, Japan).

Gross autopsy and organ weights

In the single toxicity study, gross autopsy was performed on the 1,000 and 2,000 mg/kg/day groups 14 days after administration. The autopsied animals were rats that were euthanized by cutting the abdominal aorta under anesthesia. In the 2-week toxicity study, after blood sample collection, all animals were euthanized by cutting of the abdominal aorta under anesthesia, and the following organs were removed and fixed in phosphate-buffered 10% formalin: the liver, kidneys, thymus, spleen, and thigh bone (including bone marrow). The weights of the brain, pituitary gland, thyroid gkands, lungs, heart, thymus, spleen, liver, kidneys, adrenal glands, prostate gland, epididymides, seminal vesicles, and testes were also measured.

Histopathology

In the 2-week toxicity study, the fixed liver, kidneys, thymus, spleen, and thigh bone (including bone marrow) were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE) and then examined histopathologically.

Pharmacokinetics

In the pharmacokinetic study, plasma concentrations of AMT after the single oral and intravenous administration at dose levels of 30 and 300 mg/kg to rats were measured by liquid chromatography tandem mass spectrometry (LC-MS) using the Agilent 1200 series RRLC system (Agilent Technologies, California, USA) combined with the tandem API-5000 MS/MS system (AB SCIEX, Ontario, Canada) to evaluate the systemic exposure of AMT. The LC system was run in the isocratic mode with a 80% acetonitrile aqueous solution containing 0.1% formic acid (v/v). The flow rate was 0.60 mL/min and the column temperature was 40°C. The analytical column was CAPCELLPAK C18 MGIII 2 mm × 50 mm, 3 μm (Shiseido, Tokyo, Japan). The MS system was run using the Q1 multiple ion scan with the positive ion mode ($[M + H]^+ = 157.12$). Plasma was obtained by centrifugation at 1,500 × *g* and 4°C for 15 min. Fifty microliters of each plasma sample was mixed with a three-fold volume of methanol, and centrifuged at 1,500 × *g* and 4°C for 2 min. The supernatant was filtered through a 96-well GF/F filter microplate (Whatman, New Jersey, USA), 100 μL of distilled water was added, and then the supernatant was injected (10 μL) into the LC-MS system. LC-MS data were analyzed using Analyst ver. 1.4.2 (AB SCEIX), and plasma concentrations of AMT were quantified by absolute quantitation using a linear regression equation of the calibration curve by the least-squares method. Pharmacokinetic parameters were calculated by a non-compartmental method using Phoenix WinNonlin ver 6.2 (Certara Inc., New Jersey, USA).

Statistical analysis

Values are expressed as means ± S.D. Data on body weight, food consumption, urinalysis, hematology, and blood chemistry in the 2-week toxicity study were statistically analyzed using the MiTOX system. The significance of differences in mean values between the control (vehicle) group and each dose group was assessed by the Bartlett test to assess the homogeneity of variance, followed by ANOVA when the homogeneity of variance was confirmed or a Kruskal-Wallis analysis of ranks when variance was not homogeneous. Dunnett's test was used for multiple comparisons, and differences among means were considered significant at P values of < 0.05 and 0.01. Regarding some graded categorical data in the urinalysis, e.g. pH, color, protein, ketone bodies, bilirubin, occult blood, and urobilinogen, these values were analyzed by an a × b chi-squared test, and differences from the control group were considered to be significant at P values of < 0.05 and 0.01.

RESULTS

Clinical observations, body weights, and food consumption

In the single and 2-week toxicity studies, none of the animals died throughout the observation period. At the lowest dose level of 296 mg/kg in the single toxicity study, no AMT-related changes were observed; however, the prone position and inanimation were noted at dose levels of 444 mg/kg or higher, a decrease in locomotor activity was additionally detected at dose levels of 666 mg/kg or higher, and emaciation and irregular respiration were observed at dose levels of 1,000 mg/kg or higher. These changes had mostly resolved by the next day of administration, Day 2. In the 2-week toxicity study, no clinically significant observations were not-

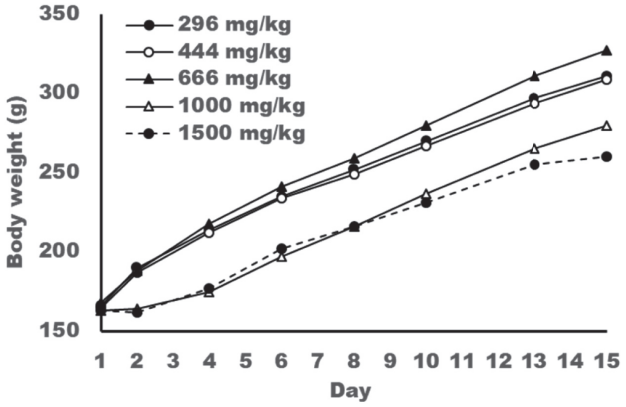

Fig. 1. Group mean body weight changes in a single oral toxicity study of AMT in rats. These data were represented as means (n = 5).

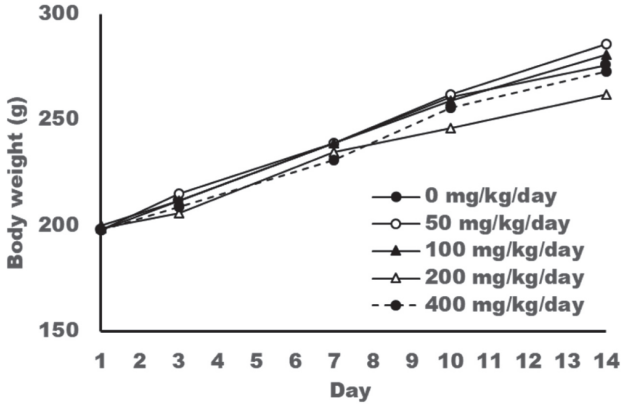

Fig. 2. Group mean body weight changes in a 2-week repeated oral toxicity study of AMT in rats. These data were represented as means (n = 5).

ed, except for an animal at the dose level of 200 mg/kg/day that exhibited emaciation, inanimation, and crepitation. In the single toxicity study, body weights during the observation period were lower in animals in the 1,000 and 1,500 mg/kg groups, but slightly increased after Day 2 to similar values as those in the other groups (Fig. 1). In the 2-week toxicity study, no AMT-related changes in body weights were observed, except for one animal in the 200 mg/kg/day group that had clinical findings (Fig. 2). Regarding food consumption following the 2-week toxicity study, significant differences were noted between 50 and 100 mg/kg/day groups at the sampling period of Day 14; however, this was clearly not dose dependent and was attributed to unintentional and non-

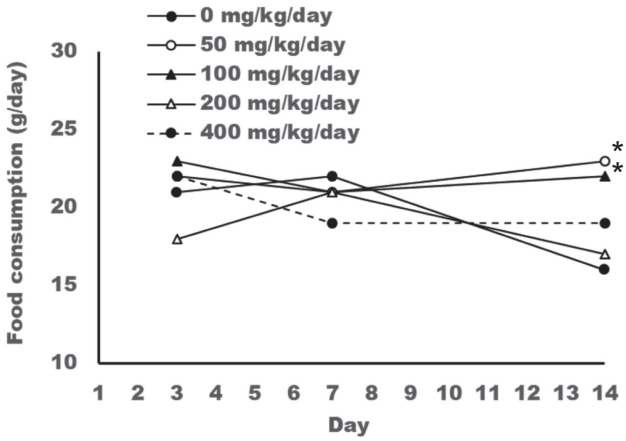

Fig. 3. Group mean food consumption in a 2-week repeated oral toxicity study of AMT in rats. These data were represented as means (n = 5). Comparison to control group, *: $P < 0.05$; **: $P < 0.01$

toxicological changes (Fig. 3).

Urinalysis

The daily excretion of calcium was significantly lower in the 200 and 400 mg/kg/day groups than in the control group, and the pH of urine slightly decreased in a dose-dependent manner (Table 1, Table 2). In the urinalysis test using CLINITEK 200+, one of the five individual data sets in the 200 mg/kg/day group was missing because of the failure to collect urine from the animal.

Hematology

RBC in the 200 mg/kg/day group or higher and HB and HCT in the 400 mg/kg/day group were significantly lower than those in the control group. PLT in the 400 mg/kg/day group was significantly higher than that in the control group. No AMT-related changes were observed in blood coagulation tests in any group (Table 3).

Blood chemistry

No significant AMT-related changes were observed in any group (Table 4).

Organ weights and gross autopsy

No significant AMT-related changes were observed in organ weights in any group (Table 5). The moderate and marked atrophy of the spleen and thymus, respectively, which may have been caused by decreased immune function and/or physiological stresses, marked gas including and dilatation of the gastrointestinal organs, possibly due

Table 1. Urinalysis in a 2-week repeated oral toxicity study of AMT in rats.

Dose (mg/kg/day)	AMT		
	0 (control)	200	400
Na (mEq/l)	166.8 ± 62.9	166.1 ± 19.5	156.2 ± 22.4
Na (mEq/day)	1.67 ± 0.28	1.39 ± 0.73	1.35 ± 0.29
K (mEq/l)	245.8 ± 121.7	255.4 ± 44.7	262.0 ± 37.9
K (mEq/day)	2.29 ± 0.64	2.04 ± 0.99	2.26 ± 0.50
Cl (mEq/l)	206.9 ± 92.7	203.1 ± 34.4	200.8 ± 26.8
Cl (mEq/day)	1.99 ± 0.28	1.70 ± 0.86	1.74 ± 0.42
Ca (mEq/l)	11.6 ± 6.9	10.1 ± 8.8	6.0 ± 2.8
Ca (mEq/day)	1.07 ± 0.26	0.54 ± 0.19**	0.50 ± 0.16**
Cre (mg/day)	8.3 ± 1.0	6.8 ± 2.8	7.6 ± 1.6

These data were represented as means ± S.D. (n = 5)
Comparison to control group, *: $P < 0.05$; **: $P < 0.01$

Table 2. Urinalysis (graded categorical data) in a 2-week repeated oral toxicity study of AMT in rats.

Dose (mg/kg/day)		AMT		
		0 (control)	200	400
Urine volume (mL)		12.3 ± 7.7	8.6 ± 5.1	8.9 ± 2.7
pH				
	5.0	0	0	1
	5.5	0	0	1
	6.0	0	0	1
	6.5	0	1	1
	7.0	0	1	1
	7.5	0	1	0
	8.0	0	1	0
	8.5	1	0	0
	> 9	4	0	0
Color				
	Y	3	4	4
	PY	2	0	1
Protein				
	-	0	0	1
	+/-	1	0	0
	1+	4	4	4
Glucose				
	-	5	4	5
Ketone body				
	-	1	0	1
	+/-	2	4	4
	1+	2	0	0
Bilirubin				
	-	5	4	5
Occult blood				
	-	5	4	5
Urobilinogen				
	0.1	4	2	3
	1	1	2	2

All groups were consisting of five animals except the group of 200 mg/kg/day which had four animals.

Table 3. Hematology in a 2-week repeated oral toxicity study of AMT in rats.

Dose (mg/kg/day)	AMT				
	0 (control)	50	100	200	400
RBC ($\times 10^4/\mu L$)	644 ± 34	619 ± 26	612 ± 10	597 ± 96	564 ± 22**
HGB (g/dl)	14.1 ± 0.8	13.9 ± 0.5	13.5 ± 0.5	12.9 ± 1.7	12.1 ± 0.6**
HCT (%)	40.2 ± 2.5	38.8 ± 1.6	38.4 ± 1.3	37.5 ± 5.6	35.1 ± 1.8**
MCV (fl)	62.3 ± 2.5	62.7 ± 1.6	62.8 ± 1.5	62.9 ± 1.7	62.2 ± 1.1
MCH (pg)	21.8 ± 0.7	22.0 ± 0.5	22.1 ± 0.6	21.7 ± 0.7	21.5 ± 0.5
MCHC (g/dl)	35.0 ± 0.3	35.0 ± 0.3	35.2 ± 0.5	34.4 ± 0.6	34.6 ± 0.5
PLT ($\times 10^4/\mu L$)	97.9 ± 15.2	100.5 ± 10.0	98.4 ± 17.1	101.2 ± 21.5	123.7 ± 11.2
PT (sec.)	14.3 ± 0.5	14.0 ± 0.4	13.6 ± 0.2	13.8 ± 0.6	13.5 ± 0.3
APTT (sec.)	20.3 ± 4.2	17.0 ± 0.6	16.6 ± 0.8	17.7 ± 0.7	16.6 ± 1.1
FIB (mg/dl)	224.6 ± 15.9	223.0 ± 7.2	231.6 ± 16.6	227.8 ± 8.9	224.8 ± 17.0
WBC ($\times 10^2\mu L$)	58 ± 25	49 ± 14	48 ± 11	44 ± 13	58 ± 5
TTO (sec.)	23.5 ± 0.6	23.8 ± 1.1	22.6 ± 1.2	25.1 ± 3.2	22.5 ± 0.4
HPT (sec.)	29.7 ± 2.1	31.0 ± 0.8	29.0 ± 2.0	27.7 ± 3.8	29.1 ± 0.6

These data were represented as means ± S.D. (n = 5)
Comparison to control group, *: $P < 0.05$; **: $P < 0.01$

Table 4. Blood chemistry in a 2-week repeated oral toxicity study of AMT in rats.

Dose (mg/kg/day)	AMT				
	0 (control)	50	100	200	400
ALP (U/l)	751 ± 157	734 ± 87	699 ± 127	632 ± 78	683 ± 169
AST (U/l)	63 ± 12	59 ± 4	135 ± 168	72 ± 31	72 ± 18
ALT (U/l)	31 ± 10	31 ± 3	81 ± 114	49 ± 47	38 ± 10
LDH (U/l)	348 ± 370	192 ± 50	639 ± 989	210 ± 62	269 ± 129
LAP (U/l)	72 ± 5	70 ± 4	70 ± 6	69 ± 6	64 ± 10
GLC (mg/dl)	169 ± 13	171 ± 8	180 ± 16	182 ± 22	164 ± 12
TC (mg/dl)	64 ± 14	64 ± 7	70 ± 7	74 ± 10	79 ± 9
TG (mg/dl)	76 ± 47	97 ± 29	92 ± 10	85 ± 29	96 ± 51
PL (mg/dl)	129 ± 27	135 ± 10	143 ± 12	147 ± 7	157 ± 13
NEFA (mcEq/l)	390 ± 107	397 ± 107	455 ± 177	377 ± 88	356 ± 142
TB (mg/dl)	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
BA (mg/dl)	11.6 ± 6.6	8.8 ± 4.3	10.5 ± 7.4	15.5 ± 7.4	15.3 ± 8.3
BUN (mg/dl)	15.8 ± 1.3	14.8 ± 2.1	16.5 ± 1.8	16.7 ± 4.5	14.7 ± 1.8
CRE (mg/dl)	0.39 ± 0.03	0.36 ± 0.05	0.39 ± 0.03	0.39 ± 0.04	0.35 ± 0.05
UA (mg/dl)	1.11 ± 1.05	0.82 ± 0.71	1.01 ± 0.44	1.53 ± 1.50	0.95 ± 0.61
TP (g/dl)	5.15 ± 0.24	5.03 ± 0.05	5.13 ± 0.13	4.87 ± 0.31	4.95 ± 0.19
ALB (g/dl)	2.07 ± 0.10	2.05 ± 0.05	2.07 ± 0.11	2.04 ± 0.06	1.97 ± 0.07
BLP (mg/dl)	119 ± 56	148 ± 38	144 ± 13	140 ± 28	154 ± 61
Ca (mg/dl)	10.2 ± 0.4	10.0 ± 0.2	10.2 ± 0.2	10.2 ± 0.3	10.0 ± 0.4
P (mg/dl)	7.10 ± 0.43	7.41 ± 0.90	7.55 ± 0.74	8.56 ± 1.46	8.15 ± 0.68
Na (mEq/l)	144.1 ± 2.7	143.6 ± 2.5	143.6 ± 1.6	145.0 ± 3.0	142.2 ± 3.3
K (mEq/l)	4.28 ± 0.62	4.11 ± 0.43	4.09 ± 0.36	4.48 ± 0.61	4.01 ± 0.30
Cl (mEq/l)	103.8 ± 0.9	103.4 ± 1.3	103.0 ± 1.7	102.9 ± 1.5	101.8 ± 2.1
CRE (mg/dl)	0.12 ± 0.02	0.10 ± 0.02	0.10 ± 0.02	0.11 ± 0.02	0.10 ± 0.02

These data were represented as means ± S.D. (n = 5)

to a gastrointestinal disturbance and irregular gas intake, and slightly or moderately small livers, kidneys, prostate glands, seminal vesicles, epididymides, and testes, which may have been related to a decrease in body weight, were observed in one animal with clinical findings in the 200 mg/kg/day group. The slight congestion of the lungs was noted in one animal in the control group and two animals in the 100 mg/kg/day group. Moderate atrophy and focal scarring of the liver was found in one animal in the 50 mg/kg/day group, which were not AMT-related chang-

Table 5. Absolute and relative organ weights in a 2-week repeated oral toxicity study of AMT in rats.

Dose (mg/kg/day)		AMT				
		0 (control)	50	100	200	400
Body weight		287 ± 19	293 ± 21	287 ± 13	270 ± 69	283 ± 20
Brain	g	1.88 ± 0.05	1.85 ± 0.07	1.90 ± 0.09	1.92 ± 0.08	1.92 ± 0.07
	g/100 g	0.657 ± 0.032	0.635 ± 0.038	0.662 ± 0.025	0.760 ± 0.248	0.680 ± 0.045
Pituitary	mg	9.8 ± 0.6	10.6 ± 1.6	9.6 ± 1.2	10.2 ± 3.1	9.8 ± 0.9
gland	mg/100 g	3.42 ± 0.18	3.64 ± 0.58	3.32 ± 0.35	3.81 ± 0.70	3.46 ± 0.29
Thyroid	mg	17.4 ± 2.4	19.9 ± 4.9	20.4 ± 0.9	18.7 ± 2.6	20.4 ± 2.7
glands	mg/100 g	6.05 ± 0.68	6.83 ± 1.75	7.11 ± 0.53	7.24 ± 1.61	7.19 ± 0.67
Thymus	mg	591 ± 89	594 ± 136	539 ± 96	482 ± 248	517 ± 115
	mg/100 g	206 ± 27	203 ± 43	188 ± 37	165 ± 69	181 ± 29
Heart	g	0.98 ± 0.11	1.01 ± 0.08	0.99 ± 0.08	0.96 ± 0.23	1.00 ± 0.08
	g/100 g	0.340 ± 0.022	0.344 ± 0.027	0.343 ± 0.020	0.359 ± 0.029	0.355 ± 0.019
Lungs	g	1.17 ± 0.13	1.21 ± 0.12	1.15 ± 0.08	1.16 ± 0.15	1.18 ± 0.11
	g/100 g	0.407 ± 0.045	0.414 ± 0.030	0.400 ± 0.0230	0.466 ± 0.094	0.417 ± 0.017
Liver	g	12.17 ± 1.98	12.11 ± 1.10	12.46 ± 0.79	11.64 ± 2.96	12.95 ± 1.03
	g/100 g	4.22 ± 0.44	4.13 ± 0.10	4.33 ± 0.19	4.31 ± 0.02	4.58 ± 0.29
Kidneys	g	2.17 ± 0.13	2.39 ± 0.23	2.25 ± 0.16	2.30 ± 0.34	2.28 ± 0.06
	g/100 g	0.757 ± 0.021	0.819 ± 0.074	0.784 ± 0.041	0.881 ± 0.160	0.808 ± 0.050
Spleen	g	0.67 ± 0.08	0.62 ± 0.08	0.63 ± 0.08	0.59 ± 0.23	0.74 ± 0.07
	g/100 g	0.234 ± 0.020	0.210 ± 0.019	0.221 ± 0.033	0.209 ± 0.045	0.262 ± 0.032
Adrenal	mg	49.5 ± 2.9	50.0 ± 5.1	53.9 ± 11.7	49.3 ± 6.3	46.2 ± 4.7
glands	mg/100 g	17.30 ± 1.56	17.07 ± 1.21	18.77 ± 4.16	19.16 ± 4.89	16.34 ± 1.64
Testes	g	2.68 ± 0.07	2.62 ± 0.19	2.77 ± 0.18	2.63 ± 0.32	2.62 ± 0.27
	g/100 g	0.937 ± 0.075	0.898 ± 0.091	0.963 ± 0.036	1.023 ± 0.264	0.928 ± 0.095
Prostate	mg	604 ± 64	667 ± 92	624 ± 191	573 ± 206	649 ± 97
gland	mg/100 g	211 ± 30	230 ± 43	217 ±66	207 ± 37	230 ± 36
Epididymides	mg	609 ± 59	617 ± 89	629 ± 33	539 ± 102	601 ± 89
	mg/100 g	213 ± 31	210 ± 23	219 ± 19	203 ± 25	213 ± 36

These data were represented as means ± S.D. (n = 5)

es because there was no similar observations or findings in other animals.

Histopathology

Moderate and marked decreases in lymphocytes in the cortex and medulla of the thymus, a moderate increase in foamy cells in the cortex of the thymus, a slight increase in adipose tissue in bone marrow, and a moderate decrease in hematopoiesis in the spleen were detected in one animal with clinical findings in the 200 mg/kg/day group. There was slight focal necrosis in the liver of one animal in each of the 50 and 100 mg/kg/day groups, slight focal single cell necrosis in the liver of two animals in the 100 mg/kg/day group, moderate focal fibrosis, slight diffuse multinucleated giant cells, and a moderate increase in bile ducts in the portal area in one animal in the 50 mg/kg/day group, moderate focal hemorrhage in the liver of one animal in the 100 mg/kg/day group, and slight mononuclear cell infiltration in the liver of one or two animals in each of the groups examined; however, these results were not dose dependent and were often detected spontaneously in rats. Therefore, these changes were considered to be unintentional and non-toxicological (Table 6).

Pharmacokinetics

The mean areas under the concentration-time curve to the extrapolated time point ($AUC_{0-\infty}$) were 40.8 and 231 μg·hr/mL after the intravenous and oral administration to rats at dose levels of 30 and 300 mg/kg, respectively. Therefore, relative oral bioavailability was calculated to be 56.7%. The plasma concentration at the earliest sampling point, which was 5 min post-dose (C_{5min}) after intravenous dosing, was 113 μg/mL, whereas the maximum concentration (C_{max}) after oral dosing was 59.7 μg/mL (Table 7).

DISCUSSION

We previously reported that AMT had inhibitory effects

Table 6. Histopathological findings in a 2-week repeated oral toxicity study of AMT in rats.

Organ	Findings	Grade	AMT (mg/kg/day)				
			Dose	0 (control)	50	100	200	400
			Number of animals	5	5	5	5	5
Spleen	Decrease hematopoiesis	0		5	5	5	4	5
		2		0	0	0	1	0
Thymus	Cyst, Cortex, Focal	0		4	5	5	5	5
		2		1	0	0	0	0
	Decrease in lymphocytes, Cortex	0		5	5	5	4	5
		3		0	0	0	1	0
	Decrease in lymphocytes, Medulla	0		5	5	5	4	5
		2		0	0	0	1	0
	Increase in foamy cells, Cortex	0		5	5	5	4	5
		2		0	0	0	1	0
Bone marrow	Increase in adipose tissue	0		5	5	5	4	5
		1		0	0	0	1	0
Liver	Necrosis, Lobule, Focal	0		5	4	4	5	5
		1		0	1	1	0	0
	Single cell necrosis, Lobule, Focal	0		5	5	3	5	5
		1		0	0	2	0	0
	Fibrosis, Focal	0		5	4	5	5	5
		2		0	1	0	0	0
	Hemorrhage, Lobule, Focal	0		5	5	4	5	5
		2		0	0	1	0	0
	Multinucleated giant cell, Lobule, Diffuse	0		5	4	5	5	5
		1		0	1	0	0	0
	Mononuclear cell infiltration, Lobule, Focal	0		4	5	5	5	5
		1		1	0	0	0	0
	Mononuclear cell infiltration, Portal area, Focal	0		5	5	3	4	5
		1		0	0	2	1	0
	Increase in bile ducts, Portal area, Focal	0		5	4	5	5	5
		2		0	1	0	0	0
Kidney	Cyst, Cortex, Focal	0		4	5	5	5	5
		1		1	0	0	0	0

Grade, 0: negative; 1: slight; 2: moderate; 3: marked.

on renal VKOR in rats, as well as anti-fibrotic effects in renal fibrotic models (Uchida *et al*., 2012; Uchida *et al*., 2017). In pharmacological experiments, we preliminary assessed the anti-coagulant potency of AMT using fibrotic model rats. The findings obtained showed no AMT-related changes in blood coagulation. In the present study, we assessed the toxicity of AMT including anti-coagulant potency in single and 2-week repeated oral toxicity studies using normal rats. In the single toxicity study, rats had a temporarily prone position, inanimation, decreased locomotor activity, and irregular respiration just after dosing, which may have been due to the effects of AMT on the circulatory system in rats. In the 2-week toxicity study, a significant increase in PLT was observed at the highest dose of 400 mg/kg/day. We conducted a single intravenous dose toxicity study in mice and noted an increase in PLT at 7 days post-dose (data not shown), which was similar to the same drug-related response observed in rats. In addition, RBC in the 200 and 400 mg/kg/day groups, and HB and HCT in the 400 mg/kg/day group were significantly lower than those in the control group. The results suggest that AMT exacts effects on the hematopoietic system in rats. However, in these studies, there was no death or no significant anti-coagulation effects in any rats at all dose levels. The strong VKOR inhibitor, warfarin does not have any direct effects on the circulatory or hematopoietic system. These findings on AMT may not be related to inhibitory potency against VKOR.

In the 2-week toxicity study, an animal exhibited clinical findings: a decrease in body weight, and histopatho-

Table 7. Pharmacokinetics parameters of AMT following a single oral and intravenous administration in rats.

Dose	(mg/kg)	Oral	Intravenous
		300	30
C_{max}	(μg/mL)	59.7 ± 42.7	-
T_{max}	(hr)	0.25 ± 0.00	-
$AUC_{0\text{-}24hr}$	(μg·hr/mL)	188 ± 61	39.9 ± 7.0
$AUC_{0\text{-}\infty}$	(μg·hr/mL)	231 ± 109	40.8 ± 7.0
CL_{total}	(mL/hr/kg)	-	750 ± 120
$t_{1/2}$	(hr)	6.98 ± 5.2	0.227 ± 0.077
BA	(%)	56.7 ± 26.8	

These data were represented as means ± S.D. (n = 3)
BA= (individual $AUC_{0\text{-}\infty}$, oral / Dose, oral) / (mean $AUC_{0\text{-}\infty}$, intravenous / Dose, intravenous) × 100
-: not applicable

logical changes during the treatment period. In clinical observations, no animal had the same symptoms, and this animal also had fine crackles such as a soft, high-pitched, and very brief noise in its breathing. Therefore, this animal may have injured its oral cavity or esophagus when the treatment was being administered orally.

We separately conducted a pharmacokinetic study to evaluate toxicokinetics and compare the systemic exposure of AMT in rats between previous pharmacological studies on renal fibrotic models and the present study. The results obtained showed that the oral bioavailability of AMT was as high as 56.7%, while the AUC of AMT at 400 mg/kg in the 2-week oral toxicity study in rats may have been markedly higher than that after its intravenous administration at a dose of 30 mg/kg, which was the highest dose level in pharmacological studies. After the oral administrations of AMT at 400 and 1,500 mg/kg to rats, extrapolated and estimated maximum plasma concentrations based on the toxicokinetics study were 79.6 and 299 μg/mL, which were 0.510 and 1.91 μmol/mL of AMT, respectively. Since the IC_{50} of AMT against rat liver VKOR is 3.20 μmol/mL, the results of the present study are very reasonable because there was no drug-related response or change in coagulation tests at a dose of 1,500 mg/kg in the single toxicity study or 400 mg/kg/day in the 2-week toxicity study. Yanagita *et al*. reported that low-dose warfarin exacted anti-fibrotic effects, but did not cause bleeding tendency in a rat experimental glomerulonephritis model, and these findings were attributed to serum concentrations of warfarin in rats (Yanagita *et al*., 2001). In the present study, we concluded that AMT has no bleeding tendency at the dose levels which AMT showed anti-fibrotic effects in rats, and these results are well explained by its inhibitory activity on VKORs and the systemic exposure of AMT in rats.

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Limdi, N.A., Beasley, T.M., Baird, M.F., Goldstein, J.A., McGwin, G., Arnett, D.K., Acton, R.T. and Allon, M. (2009): Kidney function influences warfarin responsiveness and hemorrhagic complications. J. Am. Soc. Nephrol., **20**, 912-921.

Uchida, M., Sakaguchi, Y. and Miyamoto, Y. (2012): A novel vitamin K1 2,3-epoxide reductase (VKOR) inhibitor, 3-acetyl-5-methyltetronic acid, reduces experimental glomerulonephritis. J. Vet. Med. Sci., **74**, 863-869.

Uchida, M., Miyoshi, T. and Miyamoto, Y. (2017): Pharmacological effects of a vitamin K1 2,3-epoxide reductase (VKOR) inhibitor, 3-acetyl-5-methyltetronic acid, on cisplatin-induced fibrosis in rats. J. Vet. Med. Sci., **79**, 1507-1515.

Yanagita, M., Arai, H., Ishii, K., Nakano, T., Ohashi, K., Mizuno, K., Varnum, B., Fukatsu, A., Doi, T. and Kita, T. (2001): Gas6 regulates mesangial cell proliferation through Axl in experimental glomerulonephritis. Am. J. Pathol., **158**, 1423-1432.

Yanagita, M. (2004a): The role of the vitamin K-dependent growth factor Gas6 in glomerular pathophysiology. Curr. Opin. Nephrol. Hypertens, **13**, 465-470.

Yanagita, M. (2004b): Gas6, warfarin, and kidney diseases. Clin. Exp. Nephrol., **8**, 304-309.