

Subacute toxicological evaluation of AT-533 and AT-533 gel in Sprague-Dawley rats

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Abstract. As a novel heat shock protein 90 inhibitor, AT-533 exhibits various biological activities *in vitro*, including anti-viral, anti-tumor and anti-inflammatory activities. Moreover, AT-533 gel, a gel dosage form of AT-533, has been suggested to have anti-keratitis and herpes simplex virus type-1 infection-induced effects on the skin lesions of animals. However, the safety evaluation of AT-533 and AT-533 gel has, to the best of our knowledge, not been examined in *in vivo* toxicological tests. Therefore, these toxicological tests were carried out in the present study. A 30-day subacute toxicity test for AT-533 was conducted at doses of 1, 2 and 4 mg/kg in Sprague-Dawley rats, while that for AT-533 gel was conducted using a single dose of 5 g/kg. The toxicological tests showed that a high-dose of AT-533 caused lethality and side effects in Sprague-Dawley rats. However, no mortality, loss of appetite and body weight, adverse reactions, or toxicologically relevant alterations in hematology, biochemistry and macroscopic findings (except for skin) occurred in rats exposed to low-dose AT-533 and single-dose AT-533 gel (5 g/kg) during a 30-day subacute

dermic toxicity study. The aforementioned results suggested that AT-533 gel is non-toxic for Sprague-Dawley rats, as shown by a dermic subacute toxicity test and that except for slight skin irritation, AT-533 gel had almost no side effects when administered percutaneously for 30 days.

Introduction

Heat shock protein 90 (Hsp90), a molecular chaperone, is present in the cytoplasm of human cells in the form of homodimers ($\alpha\alpha$, $\beta\beta$), which are essential for the regulation of stability and function of client proteins (1). Human Hsp90 has four subtypes: Cytoplasmic chaperone Hsp90 α (inducible/major), Hsp90 β (constitutive/minor), homologous endoplasmic reticulum partner glucose-associated protein 94 and mitochondrial homolog Hsp75/tumor necrosis factor receptor-associated protein 1 (2,3).

Hsp90 is composed of an N-terminal adenosine triphosphate (ATP) binding domain, an intermediate domain and a C-terminal dimerization domain containing a tetrachloropeptide repeat binding motif (4,5). There are numerous classical Hsp90 inhibitors, including 17-dimethylaminoethylamino-17-demethoxygeldanamycin (6), radicicol (7), PU24FCI (8), PH-H71 (9), CNF-2024/BIIB021 (10,11) and geldanamycin and its derivative 17-allylamino geldanamycin (12). These classical Hsp90 inhibitors prevent the maturation of the super-chaperone complex by interfering with ATP binding to the N-terminal pocket of Hsp90, which in turn inhibits the activation of client proteins and ultimately leads to their degradation through the ubiquitin-proteasome pathway (12,13).

Research indicates that the majority of these compounds are associated with poor water solubility, high toxicity, drug resistance and other disadvantages. Therefore, novel Hsp90 inhibitors would be beneficial alternatives. Representative candidate small molecule inhibitors of Hsp90 are artificially synthesized and include BJ-B11, AT-760 (SNX-2112) and AT-533 (SNX-25a). These structurally novel compounds are different derivatives with benzamide as the basic nucleus (14).

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X-ray diffraction has confirmed that these small molecule inhibitors can competitively bind to the ATP site on Hsp90 (15).

In our previous studies, the wide application of novel Hsp90 inhibitors for anti-tumor, anti-viral and anti-inflammatory purposes was demonstrated. AT-533 was revealed to exhibit high selectivity in inhibiting the tumorigenic properties of various cancer cell lines, including K562 (leukemia), Hep-2 (laryngeal cancer), A549 (lung cancer), Hep-G2 (liver cancer), SW620 (colon carcinoma), A375 (melanoma), MCF-7 (breast cancer) and HeLa (cervical cancer) cell lines (16). In addition, AT-533 exerted a potent inhibitory effect on human herpes simplex virus type 1 (HSV-1) by blocking HSV-1 nuclear egress and assembly *in vitro* (17,18). Additionally, AT-533 gel efficiently inhibited keratitis caused by HSV-1 infection in a rabbit keratitis model (19) and ameliorated HSV-1-induced skin lesions in C57BL/6 mouse zosteriform (18) and guinea pig skin herpes models (data not shown). AT-533 also suppressed HSV-1-induced inflammation through inhibition of the nuclear factor κ -light-chain-enhancer of activated B cells signaling pathway and the cleavage of pro-interleukin-1 β in an NLR family pyrin domain containing 3-independent manner *in vitro* (20). Furthermore, AT-533 has been demonstrated to attenuate angiogenesis in breast cancer through the hypoxia inducible factor-1 α /vascular endothelial growth factor/vascular endothelial growth factor receptor-2 signaling pathway *in vitro* and *in vivo* (21). Although the potential bioactivities of AT-533 and AT-533 gel have been extensively investigated, their possible side-effects and toxicities remain to be determined.

In the present study, subacute toxicological experiments were performed with AT-533 gel and its active pharmaceutical ingredient AT-533, in order to determine the non-toxic dosage and potential toxicity in rats, in accordance with the relevant guiding principles (22). The present study provides an important reference for further preclinical research into the use of AT-533 gel in animals and its clinical application as a skin medication for humans.

Materials and methods

Test substance. AT-533 was acquired as previously described (20). AT-533 (batch no. 20180520) was extracted and purified by our laboratory in conjunction with Kunming Jisheng Biotechnology Co., Ltd. (Kunming, China). The synthesis of AT-533 was as follows and as shown in Fig. 1A. First, 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (Compound 1) and 2-fluoro-4-hydrazino-benzonitrile (Compound 2) were synthesized in parallel, and then condensed to obtain 2-fluoro-4-(3,6,6-trimethyl-4-oxo-4,5,6,7-tetrahydro-1H-indazol-1-yl)benzonnitrile (Compound 3). Compound 3 further reacted with trans 4-hydroxycyclohexylamino to obtain 2-(4-hydroxycyclohexylamino)-4-(3,6,6-trimethyl-4-oxo-4,5,6,7-tetrahydro-1H-indazol-1-yl)benzonnitrile (Compound 4). Finally, compound 4 and hydrogen peroxide were reacted under alkaline conditions to get the product 2-[(4-hydroxycyclohexyl) amino]-4-(4,5,6,7-tetrahydro-3,6,6-trimethyl-4-oxo-1H-indazol-1-yl)-benzamide (AT-533).

Purity analysis was performed using high performance liquid chromatography (HPLC) on a Shimadzu LC-16 instrument (Shimadzu Corporation) with a GL WondaCract ODS-2 (5 μ m, 4.6x150 mm). The mobile phase was prepared by a 55/45 (v/v) mixture of methanol/water. The injection volume

was 20 μ l. The UV detector was set at a wavelength of 254 nm. The HPLC purity of AT-533 was determined to be >99.9%. The HPLC chromatogram and chemical structure of AT-533 are shown in Fig. 1B. AT-533 was dissolved in propylene glycol, PEG400 and sterile water for skin absorption. AT-533 gel (batch no. 20180913; drug content, 0.04%) was prepared by Guangzhou (Jinan) Biomedicine Research and Development Center. The preparation process was as follows: AT-533 was dissolved in propylene glycol and dispersed in carbomer 940. Following dispersing, sterile water was added to the mixture, which was stirring until the carbomer was completely swollen. Finally, triethanolamine was added and the mixture was stirred evenly to form a gel. All solvents met the requirements of the Chinese pharmacopoeia (23).

Animals. Specific pathogen-free male and female Sprague-Dawley rats (age, 6-8 weeks; weight, 180-220 g), were purchased from the Laboratory Animal Center of Southern Medical University (Guangzhou, China). All rats were housed separately in groups of 5 rats per cage with labels attached for identification. The animal code, test code, sex and date of the first test were marked on the label. The rats were kept in an animal room with a 12/12 h light/dark cycle and under standard conditions of temperature (22 \pm 2 $^{\circ}$ C) and humidity (70 \pm 10%). All rats had free access to food and water. After 7 days of acclimation, the fur (about 20 cm 2) of the rats from the back to the abdomen was shaved. Depilatory cream was then uniformly applied to ensure thorough fur removal. Rats were then returned to the cage overnight to recover from the stimulation of the depilatory cream. All animal experiments were performed using protocols approved by the Institutional Animal Care and Use Committee of Jinan University.

Grouping and administration. A total of 140 rats (70 male and 70 female) were randomly assigned to the control, vehicle, AT-533 (1, 2 and 4 mg/kg), blank gel or AT-533 gel (5 g/kg, maximum capacity) groups. A total of 10 female rats and 10 male rats were assigned to each group. All rats were transdermally treated with solvent or test substance once per day for 30 days. The test substance was applied directly to the skin and exposure was for 2 h per day. After 2 h the test substance was wiped off using warm water.

Observation and detection indicators. The food consumption of the rats was recorded daily, and their body weight was measured three times per week. Daily observation parameters included body weight, skin, hair color, eyes, legs, genitals, autonomic activity, diet, defecation, survival and overall appearance of mice. After 30 days of treatment, all rats were anesthetized with pentobarbital sodium (60 mg/kg interperitoneally) and dissected for blood sampling and exsanguination from the abdominal aorta. Routine and biochemical examinations were performed on the blood samples. Hematological tests were performed for white blood cell count (WBC) and differential, erythrocyte count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), red cell distribution width (RDW), platelet count (PLT), mean platelet volume (MPV), reticulocyte count (RET) and differential, prothrombin time (PT) and thrombin time (TT). Biochemical indexes included total

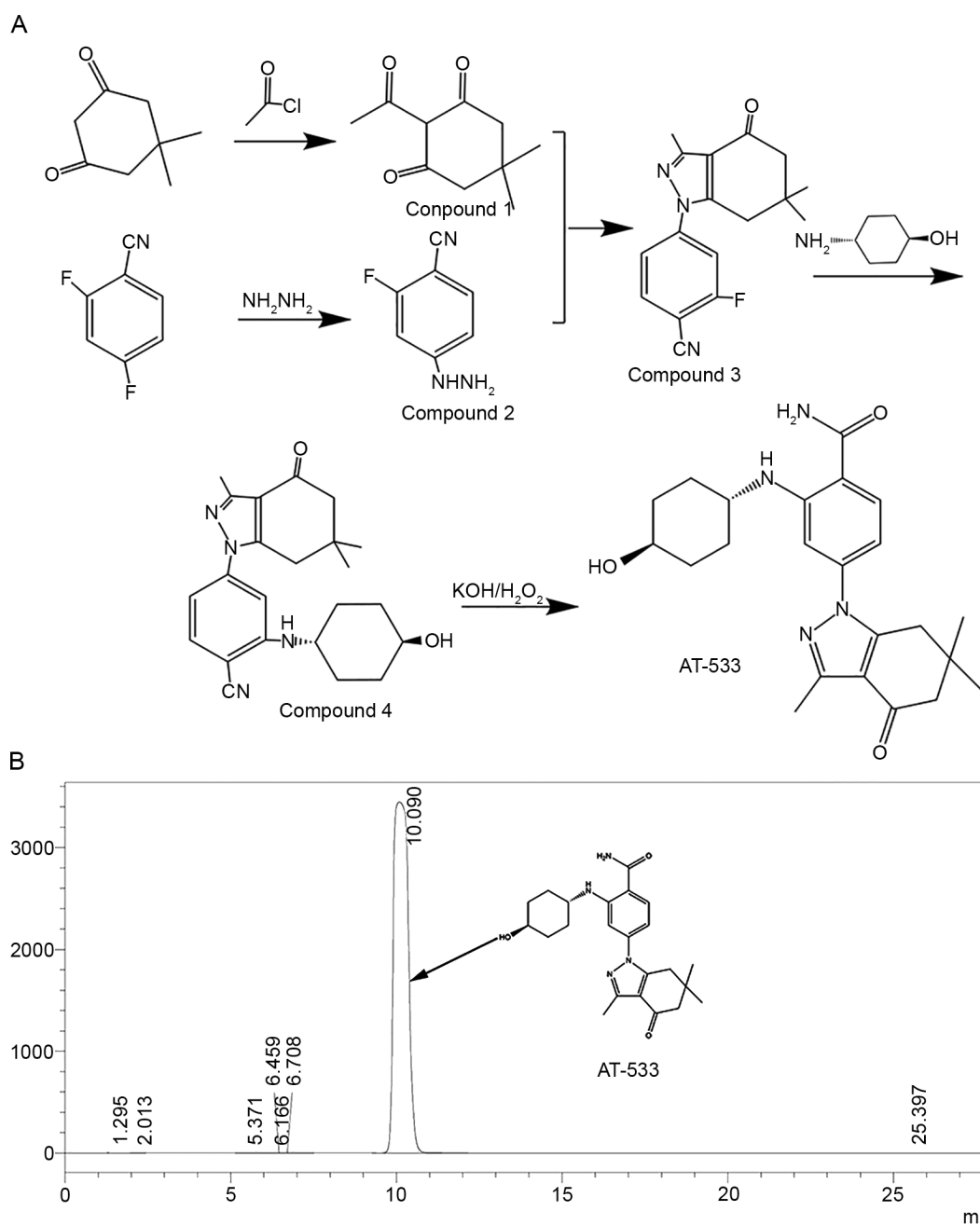


Figure 1. Synthesis of AT-533. (A) Mechanism of AT533 synthesis. (B) High performance liquid chromatography chromatogram and chemical structure of AT-533.

serum protein (TP; cat. no. K134), albumin (ALB; cat. no. K135), alanine-aminotransferase (ALT; cat. no. K114), serum aspartate aminotransferase (AST; cat. no. K118), total bilirubin (T-Bil; cat. no. S219), alkaline phosphatase (ALP; cat. no. K115), creatinine (CRE; cat. no. K131), urea nitrogen (BUN; cat. no. K132), uric acid (UA; cat. no. K122), triglycerides (TG; cat. no. K119), blood glucose (GLU; cat. no. K121), total cholesterol (CHOL; cat. no. K120), high-density lipoprotein-cholesterol (HDL; cat. no. K116), low density lipoprotein-cholesterol (LDL; cat. no. K117), creatine kinase (CK; cat. no. K119), creatine kinase isoenzyme (CK-MB; cat. no. K110), sodium (Na; cat. no. K170), potassium (K; cat. no. K169), and chloride (Cl; cat. no. K171). All the kits were purchased from Shanghai Kehua Bio-engineering Co., Ltd., and used in accordance with the manufacturer's protocol.

Hematological examination was performed using an automatic hematology analyzer (BC-3000plus; Shenzhen Mindray Bio-Medical Electronics Co., Ltd.). Blood biochemical indexes were detected by automatic biochemical analyzer (7020; Hitachi, Ltd.). Gross necropsy and anatomy analyses were performed. The tissues and organs requiring pathological examination included the heart, liver, pancreas, spleen, lungs, adrenal glands, kidneys, brain, esophagus, stomach, duodenum, mesenteric lymphoid node, colon, spinal cord (cervical, thoracic and lumbar), bone marrow, prostate, epididymis, testis, ovaries, uterus, mammary gland, sciatic nerve, bladder, pituitary gland, trachea, thyroids, thymus, salivary glands, optic nerve and dosing site (skin). All of these tissues and organs were fixed with 4% paraformaldehyde for 8 h at room temperature, dehydrated successively using 50, 75,

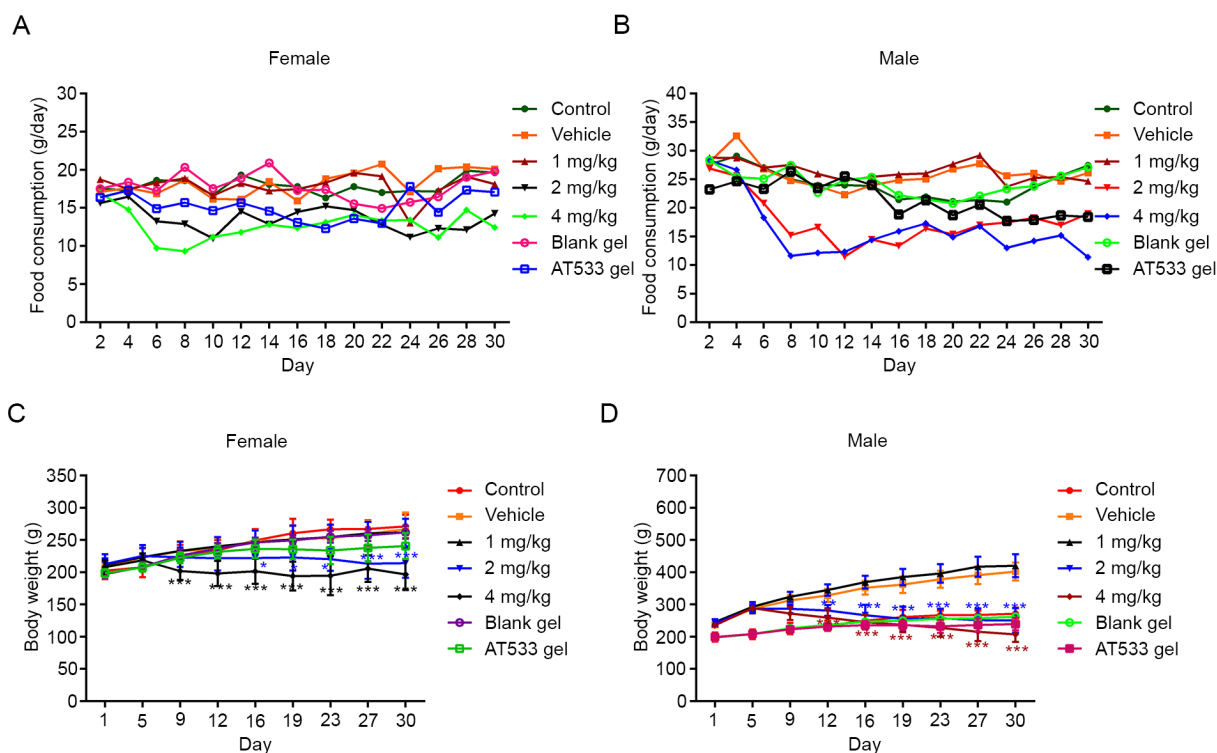


Figure 2. Food consumption and body weight of rats. Daily mean food consumption of (A) female and (B) male rats and mean body weight of (C) female and (D) male rats treated with different doses of AT-533 and single dose of AT-533 gel for 30 days (n=10). Data are presented as the mean \pm SD. A separate one-way ANOVA analysis was performed for each time point. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. vehicle group.

90 and 100% ethanol and embedded in paraffin. The tissues were then sectioned into 3- μ m slices and stained with hematoxylin and eosin (H&E) for 1-3 min at room temperature. Prior to fixation, the heart, liver, spleen, lung, adrenal glands, kidneys, brain, thymus, epididymis, testis, ovaries and uterus were weighed and the organ coefficients (organ-to-final-body-weight and organ-to-brain-weight ratios) were calculated.

Statistical analysis. All data were analyzed using Graph Pad Prism 5.0 (GraphPad Software, Inc.) and Microsoft Excel 2013 (Microsoft Corporation) and are presented as the mean \pm SD. Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparison test. $P < 0.05$ was considered to be statistically significant.

Results

Laboratory observation, food consumption and body weight.

During continuous administration of treatment no mortality was observed in the control, vehicle, 1 mg/kg AT-533, blank gel and AT-533 gel group rats. In the 2 mg/kg AT-533 group two animal deaths occurred (1 male and 1 female) and in the 4 mg/kg AT-533 group 6 deaths (3 males and 3 females) were recorded during the drug exposure period. The symptoms presented following transdermal administration of AT-533 and AT-533 gel included a decreased appetite, skin irritation and hypersensitivity (manifested as scratching and bleeding), particularly in the rats from the 2 and 4 mg/kg AT-533 groups. As shown in Fig. 2A and B, food consumption in female and male rats from the 2 and 4 mg/kg AT-533 group decreased sharply from day 4, as compared with the vehicle group,

though this did not occur in the 1 mg/kg group female and male rats. There was no statistically significant difference in food consumption and body weight between the blank gel and AT-533 gel groups. There was no statistically significant difference in body weight between the normal control and vehicle/blank gel groups in both female and male rats, indicating that the vehicle/blank gel was safe for rats. As compared with the vehicle group, the body weight of female rats in the 4 mg/kg group decreased sharply from day 9 and in the 2 mg/kg AT-533 group from day 16 (Fig. 2C). Fig. 2D revealed that the body weight of male rats in the 2 and 4 mg/kg AT-533 groups decreased significantly from day 12. Compared with the vehicle/blank gel group, there was no statistically significant body weight change in the rats of the 1 mg/kg AT-533 and AT-533 gel groups during a continuous 30-day administration.

Hematology and biochemical examination.

Following consecutive administration of AT-533 for 30 days, hematology and serum biochemistry was performed in the rats. As shown in Table I, the WBC, MCHC and neutrophil (NE) counts and the NE, lymphocyte (LY) and monocyte (MO) percentages of female rats in the 2 and 4 mg/kg AT-533 groups were significantly increased as compared with the vehicle group, whereas the MCV and MCH levels and the HCT, eosinophil (EO) and basophil percentages were reduced. For the AT-533 gel experiment, NE % and LY % in female rats were increased and thrombin time (TT) was decreased at an AT-533 gel dose of 5 g/kg in comparison with vehicle while other hematological parameters were not obviously changed after 30 days of treatment. However, except for the counts of WBC and NE, other alterations were not considered to be toxicologically

Table I. Hematological values of female rats treated with AT-533 or AT-533 gel for 30 days.

Parameters	Control	Vehicle	1 mg/kg	2 mg/kg	4 mg/kg	Blank gel	AT-533 gel
WBC ($\times 10^9/l$)	4.69 \pm 2.49	5.59 \pm 0.85	8.63 \pm 3.83	9.96 \pm 5.64	17.32 \pm 4.40 ^b	5.53 \pm 1.73	4.74 \pm 2.42
RBC ($\times 10^{12}/l$)	7.59 \pm 0.62	7.71 \pm 0.36	7.92 \pm 0.57	7.48 \pm 0.42	7.98 \pm 0.79	7.94 \pm 0.50	7.50 \pm 0.46
HGB (g/l)	151.33 \pm 13.49	148.50 \pm 5.58	149.67 \pm 11.09	136.67 \pm 6.98	145.20 \pm 12.34	156.50 \pm 9.33	143.67 \pm 7.74
HCT (%)	42.53 \pm 3.59	43.63 \pm 1.44	43.32 \pm 2.76	39.53 \pm 1.24 ^a	41.28 \pm 2.49	44.42 \pm 2.48	40.70 \pm 1.77
MCV (fl)	56.03 \pm 1.59	56.68 \pm 2.31	54.75 \pm 1.63	52.97 \pm 1.77 ^b	51.88 \pm 2.10 ^b	55.97 \pm 0.66	54.33 \pm 1.46
MCH (pg)	19.92 \pm 0.45	19.27 \pm 0.73	18.90 \pm 0.60	18.30 \pm 0.48 ^a	18.24 \pm 0.3 ^a	19.73 \pm 0.35	19.17 \pm 0.35
MCHC (g/l)	355.83 \pm 5.23	340.33 \pm 4.72	345.33 \pm 6.31	345.50 \pm 8.71	351.20 \pm 8.50 ^a	352.33 \pm 5.39	353.00 \pm 9.30
PLT ($\times 10^9/l$)	1,201.33 \pm 157.57	1,105.50 \pm 132.27	1,117.33 \pm 175.16	1,316.67 \pm 155.22	1,337.25 \pm 174.21	1,258.60 \pm 139.33	1,212.00 \pm 154.72
RDW-SD (fl)	27.38 \pm 1.42	28.28 \pm 0.70	28.30 \pm 0.83	27.45 \pm 0.97	28.12 \pm 0.62	27.88 \pm 1.78	25.97 \pm 1.02
RDW-CV (%)	15.10 \pm 1.33	15.62 \pm 1.33	16.52 \pm 1.05	16.42 \pm 1.55	17.62 \pm 1.68	16.08 \pm 1.62	14.77 \pm 1.40
PDW (fl)	9.65 \pm 0.52	9.47 \pm 0.60	9.42 \pm 0.52	8.70 \pm 0.37	9.72 \pm 0.73	9.50 \pm 0.21	9.32 \pm 0.47
MPV (fl)	8.67 \pm 0.41	8.57 \pm 0.46	8.43 \pm 0.37	8.02 \pm 0.23 ^a	8.40 \pm 0.21	8.57 \pm 0.29	8.32 \pm 0.23
P-LCR (%)	15.57 \pm 3.06	14.78 \pm 3.32	14.42 \pm 2.62	10.92 \pm 1.77	14.32 \pm 2.17	14.73 \pm 1.94	13.32 \pm 1.95
PCT (%)	1.04 \pm 0.11	0.95 \pm 0.12	0.94 \pm 0.14	1.05 \pm 0.12	1.05 \pm 0.18	1.07 \pm 0.09	0.95 \pm 0.19
NE ($\times 10^9/l$)	0.64 \pm 0.24	0.73 \pm 0.23	1.06 \pm 0.50	3.53 \pm 2.22 ^a	5.85 \pm 1.54 ^c	0.74 \pm 0.38	1.22 \pm 0.55
LY ($\times 10^9/l$)	4.01 \pm 2.54	4.66 \pm 0.84	7.13 \pm 3.65	5.84 \pm 3.53	10.37 \pm 3.48	4.68 \pm 1.37	4.31 \pm 3.10
MO ($\times 10^9/l$)	0.13 \pm 0.03	0.20 \pm 0.05	0.16 \pm 0.07	0.47 \pm 0.25	0.98 \pm 0.33	0.18 \pm 0.09	0.25 \pm 0.15
EO ($\times 10^9/l$)	0.08 \pm 0.06	0.09 \pm 0.02	0.10 \pm 0.07	0.12 \pm 0.08	0.09 \pm 0.07	0.13 \pm 0.09	0.08 \pm 0.03
BA ($\times 10^9/l$)	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.01 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00
NE (%)	11.70 \pm 6.46	13.34 \pm 3.86	18.22 \pm 8.64	37.76 \pm 8.94 ^b	34.65 \pm 7.94	9.87 \pm 2.53	27.00 \pm 6.34 ^e
LY (%)	83.98 \pm 7.82	83.08 \pm 5.70	79.62 \pm 8.94	59.03 \pm 9.67 ^b	64.84 \pm 15.21 ^a	85.42 \pm 3.72	67.15 \pm 6.38 ^e
MO (%)	3.07 \pm 1.19	3.50 \pm 0.76	2.02 \pm 0.28	4.24 \pm 1.30	6.66 \pm 2.41 ^a	2.70 \pm 0.73	4.12 \pm 1.00
EO (%)	1.25 \pm 0.28	1.63 \pm 0.34	1.37 \pm 0.40	1.18 \pm 0.57	0.54 \pm 0.43 ^c	2.26 \pm 0.87	1.36 \pm 0.59
BA (%)	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.02 \pm 0.04	0.10 \pm 0.12 ^a	0.00 \pm 0.00	0.02 \pm 0.04
RET ($\times 10^9/l$)	233.42 \pm 43.40	243.38 \pm 30.01	257.95 \pm 41.78	197.48 \pm 50.31	227.48 \pm 75.54	282.12 \pm 37.05	270.95 \pm 78.70
RET (%)	3.09 \pm 0.59	3.16 \pm 0.42	3.29 \pm 0.70	3.02 \pm 1.12	2.86 \pm 0.95	3.55 \pm 0.40	3.62 \pm 1.01
PT (s)	9.43 \pm 0.34	9.02 \pm 0.30	8.68 \pm 0.25	8.60 \pm 0.33	9.36 \pm 2.00	9.62 \pm 1.40	9.13 \pm 0.52
TT (s)	45.47 \pm 4.15	40.77 \pm 6.06	49.33 \pm 5.12	37.75 \pm 4.21	44.74 \pm 6.95	47.28 \pm 4.19	42.05 \pm 4.19 ^d

Values are presented as the mean \pm SD for 6 rats in each group (5 rats in the 4 mg/kg group). ^aP<0.05, ^bP<0.01, ^cP<0.001 vs. vehicle. ^dP<0.05, ^eP<0.001 vs. blank gel. BA, basophil; EO, eosinophil; HCT, hematocrit; HGB, hemoglobin concentration; LY, lymphocyte; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MO, monocyte; MPV, mean platelet volume; NE, neutrophil; P-LCR, PLT, platelet count; PT, prothrombin time; RBC, erythrocyte count; RDW, red cell distribution width; RET, reticulocyte count; TT, thrombin time; WBC, white blood cell count.

Table II. Hematological values of male rats treated with AT-533 or AT-533 gel for 30 days.

Parameters	Control	Vehicle	1 mg/kg	2 mg/kg	4 mg/kg	Blank gel	AT-533 gel
WBC ($\times 10^9/l$)	5.27±4.29	7.34±2.66	10.83±3.44	12.74±3.92	14.45±4.06 ^a	8.56±1.21	11.77±1.55
RBC ($\times 10^{12}/l$)	7.65±1.02	8.32±0.51	8.10±0.35	7.66±0.78	7.08±0.22	7.89±0.35	8.67±0.48
HGB (g/l)	154.00±6.38	160.33±8.12	156.83±5.91	141.00±13.10 ^a	130.33±1.53	154.43±9.50	166.00±7.21 ^a
HCT (%)	41.90±5.31	46.72±2.53	45.82±1.68	40.00±3.27 ^b	36.83±1.08 ^a	44.14±2.51	46.72±2.07
MCV (fl)	54.86±1.29	56.18±1.64	56.58±1.66	53.60±3.42	52.10±2.91 ^a	55.94±2.10	53.92±2.64
MCH (pg)	18.36±1.54	19.28±0.33	19.35±0.38	18.47±0.27	18.47±0.81	19.57±0.75	19.18±0.56
MCHC (g/l)	335.00±30.94	343.50±6.09	342.33±4.03	345.17±18.65	354.00±7.00	349.86±2.54	355.40±10.24
PLT ($\times 10^9/l$)	1,144.50±78.86	1,188.67±104.82	1,240.17±88.11	1,488.50±114.31 ^c	1,511.00±109.08 ^c	1,165.00±69.38	1,131.25±138.69
RDW-SD (fl)	29.26±1.90	29.32±1.23	30.17±0.83	29.30±1.22	28.80±1.61	29.39±1.32	28.48±1.54
RDW-CV (%)	17.00±1.98	17.30±0.87	17.35±0.87	17.73±1.44	16.97±2.60	17.19±0.63	17.92±1.10
PDW (fl)	10.42±1.64	9.30±0.20	9.43±0.27	9.55±0.68	8.43±0.15	9.80±0.77	9.86±0.59
MPV (fl)	8.96±0.57	8.33±0.16	8.42±0.27	8.57±0.39	7.73±0.06 ^a	8.63±0.48	8.64±0.42
P-LCR (%)	18.28±5.03	13.35±1.33	14.30±1.81	15.32±3.08	9.33±0.21	15.83±3.85	15.76±3.22
PCT (%)	1.00±0.08	0.99±0.07	1.05±0.06	1.28±0.07	1.17±0.09	0.97±0.06	0.91±0.15
NE ($\times 10^9/l$)	0.71±0.48	1.20±0.50	2.83±0.71	4.45±1.35 ^c	8.61±1.58 ^c	2.26±0.59	1.90±1.47
LY ($\times 10^9/l$)	3.70±2.80	6.23±2.31	6.53±1.76	8.19±1.96	4.85±2.34	6.48±0.61	7.99±2.59
MO ($\times 10^9/l$)	0.12±0.08	0.29±0.17	0.37±0.16	0.81±0.32 ^b	0.91±0.30 ^c	0.37±0.15	0.54±0.32
EO ($\times 10^9/l$)	0.08±0.05	0.14±0.06	0.13±0.07	0.08±0.05	0.07±0.05	0.13±0.03	0.09±0.08
BA ($\times 10^9/l$)	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.01	0.00±0.01	0.00±0.00	0.01±0.01 ^e
NE (%)	22.68±11.89	17.85±5.43	27.17±7.74	35.63±5.99 ^b	60.60±5.93 ^c	19.00±8.57	30.93±13.14
LY (%)	72.32±11.22	77.12±5.96	68.35±8.16	57.48±5.62 ^c	32.40±6.15 ^c	76.14±9.76	67.60±14.28
MO (%)	3.16±0.74	3.64±1.22	3.12±0.58	6.23±0.90 ^c	6.47±2.42 ^b	3.54±1.07	5.24±1.3 ^d
EO (%)	1.84±0.72	1.70±0.47	1.13±0.35	0.68±0.20 ^c	0.50±0.26 ^c	1.30±0.34	0.92±0.55
BA (%)	0.00±0.00	0.00±0.00	0.02±0.04	0.05±0.05	0.03±0.06	0.03±0.05	0.16±0.21f
RET ($\times 10^9/l$)	276.10±75.90	284.65±52.34	269.70±55.99	309.68±65.48	161.27±6.10 ^a	301.59±33.05	141.52±122.70 ^e
RET (%)	3.56±0.64	3.41±0.49	3.34±0.74	4.58±2.08	2.28±0.11	3.83±0.42	1.03±0.61 ^f
PT (s)	9.74±0.46	9.48±0.39	9.70±0.34	8.75±0.43 ^a	8.50±0.50 ^b	9.37±0.26	9.72±0.54
TT (s)	47.74±5.39	51.35±7.93	51.87±7.05	35.62±5.71 ^b	37.17±6.57 ^b	48.62±8.52	50.50±5.94

Values are presented as the mean \pm SD for 6 rats in each group (4 rats in the 4 mg/kg group). ^aP<0.05, ^bP<0.001 vs. vehicle. ^cP<0.05, ^dP<0.001 vs. blank gel. BA, basophil; EO, eosinophil; HCT, hematocrit; HGB, hemoglobin concentration; LY, lymphocyte; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MO, monocyte; MPV, mean platelet volume; NE, neutrophil; P-LCR, PLT, platelet count; PT, prothrombin time; RBC, erythrocyte count; RDW, red cell distribution width; RET, reticulocyte count; TT, thrombin time; WBC, white blood cell count.

Table III. Serum biochemical values of female rats treated with AT-533 or AT-533 gel for 30 days.

Parameters	Control	Vehicle	1 mg/kg	2 mg/kg	4 mg/kg	Blank gel	AT-533 gel
TP (g/l)	62.85±4.06	55.87±4.46	64.40±11.77	51.58±9.53	50.90±7.73	63.58±3.78	59.25±7.33
ALB (g/l)	29.82±1.38	27.40±2.34	29.90±5.81	22.15±5.22	16.84±3.74 ^c	30.70±1.32	27.72±2.82 ^a
ALT (U/l)	57.67±21.34	42.17±6.31	39.17±11.69	57.50±20.04	79.60±25.83	53.83±10.25	62.25±7.63
AST (U/l)	126.80±18.67	83.17±5.34	102.33±43.57	182.50±94.05	316.50±229.56 ^a	103.50±11.43	165.00±45.21 ^b
T-Bil (umol/l)	0.12±0.09	0.12±0.05	0.24±0.13	0.19±0.11	0.14±0.07	0.10±0.10	0.15±0.16
ALP (U/l)	145.83±31.57	135.40±29.05	167.80±63.15	158.80±4.55	307.20±245.60	175.50±1.73	189.00±39.17
BUN (mmol/l)	7.03±1.72	5.85±0.70	5.89±0.98	6.19±1.16	8.20±3.79	6.56±0.86	6.43±0.70
CRE (umol/l)	35.05±10.73	23.77±3.69	30.88±8.51	33.35±6.96	28.24±10.03	33.27±6.67	27.55±4.18
UA (umol/l)	110.55±34.80	93.38±8.14	121.86±11.47	104.70±34.12	85.53±46.07	79.25±13.48	64.92±18.43
GLU (mmol/l)	19.88±4.06	15.93±3.38	17.12±1.93	13.65±2.55	12.36±4.07	16.88±2.16	15.69±3.89
TG (mmol/l)	0.97±0.55	1.30±0.84	0.99±0.53	1.02±0.48	0.49±0.07	0.75±0.34	1.01±0.44
CHO (mmol/l)	1.95±0.22	1.63±0.28	2.07±0.45	1.73±0.43	1.20±0.62	1.85±0.22	1.73±0.32
HDL (mmol/l)	0.77±0.11	0.66±0.15	0.81±0.18	0.74±0.20	0.52±0.29	0.74±0.09	0.72±0.15
LDL (mmol/l)	0.24±0.03	0.19±0.04	0.21±0.05	0.25±0.07	0.22±0.04	0.20±0.04	0.25±0.04 ^d
CK (U/l)	1,326.50±190.12	579.60±338.26	1,544.50±391.89	899.00±301.61	2,246.60±1528.21	1,594.83±679.49	1,627.20±784.94
CK-MB (U/l)	391.50±140.6	303.20±54.87	505.80±190.8	271.50±39.26	341.33±38.53	415.20±43.64	434.40±106.4
K ⁺ (mmol/l)	5.50±0.66	5.22±0.90	5.81±1.35	6.18±1.80	5.34±1.04	5.05±0.83	5.87±1.42
Na ⁺ (mmol/l)	141.32±6.21	125.38±6.51	132.37±18.12	129.52±18.95	130.88±10.78	141.48±7.59	141.92±5.21
Cl ⁻ (mmol/l)	109.62±2.78	100.88±4.85	105.02±15.9	101.28±18.31	104.50±7.69	109.65±3.25	111.07±5.67

Values are the mean ± SD for 6 rats in each group. ^aP<0.05, ^bP<0.01, ^cP<0.001 vs. vehicle. ^dP<0.05, ^eP<0.01 vs. blank gel. ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, urea nitrogen; CHOL, cholesterol; Cl⁻, chloride; CRE, creatinine; GLU, glucose; HDL, high-density lipoprotein cholesterol; K⁺, potassium; LDL, low density lipoprotein-cholesterol; Na⁺, sodium; T-Bil, total bilirubin; TG, triglycerides; TP, total serum protein; UA, uric acid.

Table IV. Serum biochemical values of male rats treated with AT-533 or AT-533 gel for 30 days.

Parameters	Control	Vehicle	1 mg/kg	2 mg/kg	4 mg/kg	Blank gel	AT-533 gel
TP (g/l)	61.30±6.22	59.12±8.13	57.15±9.59	50.88±9.44	48.78±4.55	60.78±3.86	62.98±3.86
ALB (g/l)	27.52±1.74	27.93±3.65	25.53±4.26	17.73±2.64 ^c	14.22±2.28 ^c	28.23±1.65	23.44±4.70 ^d
ALT (U/l)	53.00±5.35	52.80±13.65	42.50±9.65	38.80±6.26	133.83±143.50	55.00±16.49	54.50±14.84
AST (U/l)	124.50±16.8	138.80±30.7	100.17±21.4	94.33±32.6	272.50±156.2	116.00±18.6	120.50±46.3
T-Bil (umol/l)	0.11±0.05	0.44±0.14	0.23±0.18	0.06±0.07	0.24±0.20	0.13±0.06	0.07±0.06
ALP (U/l)	268.2±33.5	252.2±49.2	195.2±43.5	272.5±119.7	289.8±131.2	249.2±22.02	259.5±25.38
BUN (mmol/l)	6.60±0.96	5.86±1.30	5.30±0.73	5.43±2.08	15.46±9.61 ^a	6.78±1.28	6.42±0.45
CRE (umol/l)	27.34±5.90	25.90±5.68	23.92±7.13	20.10±8.60	23.03±10.80	29.47±3.91	40.88±8.23 ^e
UA (umol/l)	112.8±19.8	111.4±28.0	114.4±37.7	81.2±27.9	75.8±24.1	90.1±20.8	93.5±16.8
GLU (mmol/l)	17.24±1.84	16.69±3.03	14.28±2.23	13.06±3.09	9.84±2.36 ^c	20.56±2.89	17.60±5.92
TG (mmol/l)	1.84±0.35	2.16±0.67	1.52±0.42 ^a	1.03±0.08 ^c	0.35±0.18 ^c	1.71±0.22	0.96±0.56 ^d
CHO (mmol/l)	1.45±0.14	1.62±0.36	1.52±0.35	1.22±0.46	0.92±0.23 ^b	1.82±0.22	1.47±0.52
HDL (mmol/l)	0.60±0.04	0.68±0.16	0.61±0.14	0.55±0.20	0.37±0.12 ^b	0.74±0.11	0.58±0.21
LDL (mmol/l)	0.26±0.02	0.23±0.04	0.22±0.03	0.24±0.07	0.25±0.08	0.26±0.03	0.32±0.01 ^f
CK (U/l)	2,059.0±584.5	2,541.6±811.2	1,816.8±575.2	966.3±617.5 ^b	815.7±689.7 ^b	1,929.3±1224.6	1,681.0±787.1
CK-MB (U/l)	486.0±114.4	470.8±111.9	642.40±216.13	271.33±161.47	358.20±350.24	452.50±68.25	370.80±52.08
K ⁺ (mmol/l)	6.61±0.99	5.68±1.46	5.59±1.69	6.10±2.82	4.86±2.10	5.72±0.53	5.18±0.94
Na ⁺ (mmol/l)	140.64±3.74	132.28±11.46	127.92±16.86	119.87±23.25	120.10±22.52	143.08±4.79	149.24±3.35 ^d
Cl ⁻ (mmol/l)	109.88±4.43	105.68±8.89	101.83±13.63	98.44±16.09	93.42±23.34	111.67±4.55	117.60±3.10 ^d

Values are the mean ± SD for 6 rats in each group. ^aP<0.05, ^bP<0.01, ^cP<0.001 vs. vehicle. ^dP<0.05, ^eP<0.01, ^fP<0.001 vs. blank gel. ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, urea nitrogen; CHOL, cholesterol; Cl, chloride; CRE, creatinine; GLU, glucose; HDL, high-density lipoprotein cholesterol; K, potassium; LDL, low density lipoprotein-cholesterol; Na, sodium; T-Bil, total bilirubin; TG, triglycerides; TP, total serum protein; UA, uric acid.

Table V. Absolute and relative weights of organs of female treated with AT-533 or AT-533 gel for 30 days.

A, Absolute weights									
Parameters	Control	Vehicle	1 mg/kg	2 mg/kg	4 mg/kg	Blank gel	AT-533 gel		
Body weight (g)	275.2±22.0	269.4±30.8	261.2±24.8	219.7±18.3 ^a	185.2±35.1 ^c	261.9±12.1	250.2±15.5		
Brain	1.93±0.09	1.97±0.07	1.92±0.12	1.89±0.09	1.83±0.04	2.08±0.35	1.96±0.13		
Heart	0.94±0.10	0.97±0.11	0.98±0.13	0.89±0.11	0.82±0.14 ^b	0.97±0.06	0.98±0.10		
Liver	9.34±1.39	9.35±1.25	8.62±1.04	8.50±0.40	6.86±1.24	9.69±0.40	8.80±0.59		
Spleen	0.60±0.07	0.54±0.08	0.56±0.06	0.46±0.04	0.44±0.08	0.62±0.09	0.50±0.06 ^d		
Lung	1.20±0.16	1.12±0.06	1.20±0.09	1.06±0.13	1.08±0.10	1.18±0.04	1.24±0.18		
Kidneys	1.83±0.40	1.71±0.19	1.72±0.24	1.51±0.18	1.57±0.21	1.96±0.10	1.71±0.10		
Adrenals	0.068±0.015	0.080±0.013	0.081±0.006	0.066±0.010	0.073±0.008	0.090±0.012	0.066±0.012		
Thymus	0.43±0.14	0.40±0.08	0.45±0.12	0.24±0.06 ^a	0.20±0.10 ^a	0.45±0.02	0.38±0.13		
Ovaries	0.16±0.02	0.16±0.03	0.16±0.03	0.14±0.02	0.11±0.01 ^b	0.17±0.02	0.15±0.02		
Uterus	0.52±0.11	0.57±0.12	0.64±0.12	0.48±0.16	0.40±0.24	0.61±0.16	0.58±0.22		

B, Organ-to-body weight ratio (%)									
Parameters	Control	Vehicle	1 mg/kg	2 mg/kg	4 mg/kg	Blank gel	AT-533 gel		
Brain	0.71±0.07	0.74±0.08	0.74±0.09	0.87±0.10	1.02±0.21 ^b	0.79±0.15	0.79±0.07		
Heart	0.34±0.03	0.36±0.01	0.37±0.03	0.41±0.02 ^a	0.44±0.03 ^c	0.37±0.02	0.39±0.04		
Liver	3.39±0.42	3.48±0.03	3.31±0.04	3.88±0.27	3.71±0.27	3.70±0.12	3.53±0.28		
Spleen	0.22±0.02	0.20±0.01	0.22±0.04	0.21±0.01	0.24±0.01 ^b	0.24±0.04	0.20±0.02		
Lung	0.43±0.03	0.42±0.03	0.46±0.05	0.48±0.06	0.60±0.11 ^c	0.45±0.03	0.50±0.07		
Kidneys	0.66±0.12	0.64±0.02	0.66±0.04	0.69±0.08	0.86±0.12 ^c	0.75±0.02	0.69±0.04		
Adrenals	0.025±0.006	0.030±0.003	0.031±0.004	0.030±0.003	0.041±0.012 ^a	0.035±0.006	0.027±0.005		
Thymus	0.16±0.06	0.15±0.02	0.17±0.03	0.11±0.02 ^a	0.10±0.04 ^a	0.17±0.01	0.15±0.05		
Ovaries	0.060±0.009	0.061±0.009	0.062±0.012	0.062±0.009	0.061±0.014	0.062±0.009	0.058±0.009		
Uterus	0.19±0.04	0.22±0.06	0.25±0.04	0.22±0.08	0.21±0.10	0.24±0.07	0.23±0.08		

C, Organ-to-body weight ratio (g/g)									
Parameters	Control	Vehicle	1 mg/kg	2 mg/kg	4 mg/kg	Blank gel	AT-533 gel		
Heart	0.49±0.05	0.49±0.05	0.51±0.09	0.48±0.08	0.45±0.08	0.48±0.08	0.50±0.05		
Liver	4.85±0.76	4.75±0.64	4.54±0.87	4.51±0.28	3.74±0.66	4.76±0.73	4.51±0.53		
Spleen	0.31±0.04	0.27±0.04	0.30±0.04	0.25±0.03	0.24±0.04	0.31±0.07	0.26±0.04		

Table V. Continued.

C, Organ-to-body weight ratio (g/g)						
Parameters	Control	Vehicle	1 mg/kg	2 mg/kg	4 mg/kg	AT-533 gel
Lung	0.62±0.10	0.57±0.02	0.63±0.08	0.56±0.08	0.59±0.05	0.63±0.08
Kidneys	0.95±0.21	0.87±0.08	0.90±0.15	0.80±0.10	0.85±0.11	0.88±0.04
Adrenals	0.035±0.006	0.041±0.006	0.042±0.005	0.035±0.006	0.040±0.004	0.034±0.007
Thymus	0.22±0.08	0.20±0.04	0.23±0.06	0.13±0.03	0.11±0.06 ^a	0.19±0.07
Ovaries	0.085±0.012	0.083±0.014	0.085±0.020	0.072±0.011	0.059±0.05 ^a	0.074±0.008
Uterus	0.27±0.05	0.29±0.06	1.92±0.12	1.89±0.09	1.83±0.04	0.29±0.11

Values are the mean ± SD for 6 rats in each group (5 rats in 4 mg/kg group). ^aP<0.05, ^bP<0.01, ^cP<0.001 vs. vehicle. ^dP<0.05 vs. blank gel.

significant, as the markers in question stayed within the normal reference ranges (24,25).

In male rats, the WBC, PLT, NE and MO counts, and NE % and MO % of the 2 and 4 mg/kg AT-533 groups increased, as compared with the vehicle group, while the HGB, MCV, MPV, RET, PT and TT levels and HCT, LY and EO percentages decreased. The HGB level and MO percentage increased and the number and percentage of RET decreased following treatment with 5 g/kg AT-533 gel in comparison with vehicle, while other hematological parameters were not obviously changed after 30 days of treatment (Table II). Except for the PLT, NE and MO counts, other alterations were not considered to be toxicologically significant, as the markers in question stayed within the normal reference ranges (24,25).

Serum biochemistry of female rats showed that the level of ALB was lower and the level of AST higher in rats treated with 4 mg/kg AT-533, as compared with the vehicle group, while other biochemical indicators were not obviously changed following 30 days of treatment. The levels of AST and LDL increased and the level of ALB decreased in female rats following treatment with 5 g/kg AT-533 gel in comparison with vehicle, while other biochemical indicators were not obviously changed following 30 days of treatment (Table III). However, the changes of female rats in the 5 g/kg AT-533 gel group were not considered to be toxicologically significant, as the markers in question stayed within the normal reference ranges (26,27).

Table IV revealed that the levels of ALB, GLU, TG, CHO, HDL and CK of male rats in the 4 mg/kg AT-533 group decreased, while the level of BUN was markedly increased in comparison with vehicle. Male rats treated with 1 mg/kg AT-533 exhibited a slight reduction of TG. The levels of ALB and TG decreased and the levels of CRE, LDL, Na⁺, and Cl⁻ increased in male rats at an AT-533 gel dose of 5 g/kg in comparison with vehicle, while other biochemical indicators were not obviously changed after 30 days of treatment. Except for the levels of ALB, CHO, HDL and BUN in the 4 mg/kg AT-533 group male rats, other alterations that stayed within the normal reference ranges were not considered to be toxicological (26,27). It may be hypothesized that the slightly low levels of GLU, TG and CK could be partly relevant to the decrease in food consumption.

Absolute and relative weight of organs. As shown in Table V, in female rats treated with 4 mg/kg AT-533, the absolute heart, thymus and ovary weight was decreased in comparison with that in the vehicle group. The absolute spleen weight decreased in female rats at an AT-533 gel dose of 5 g/kg; however, this change was not significant, as the value stayed within the normal reference ranges (28,29). The brain-to-body, heart-to-body, spleen-to-body, lung-to-body, kidney-to-body and adrenals-to-body weight ratios were elevated, while the thymus-to-body weight ratio was slightly decreased in female rats from the 4 mg/kg treatment group in comparison with vehicle. As compared with the vehicle group, the thymus-to-brain and ovary-to-brain weight ratios of female rats in the 4 mg/kg treatment were slightly decreased.

The male rats exhibited a sharp drop in food consumption and body weight in the 2 and 4 mg/kg AT-533 groups compared with the vehicle group and the absolute weight of the main organs, except for adrenal weight decreased; however, such differences were not significant, as values stayed within

Table VI. Absolute and relative weights of organs of male treated with AT-533 or AT-533 gel for 30 days.

A, Absolute weights									
Parameters	Control	Vehicle	1 mg/kg	2 mg/kg	4 mg/kg	Blank gel	AT-533 gel		
Body weight (g)	379.5±36.1	400.5±24.0	421.2±32.0	250.4±47.7 ^c	198.7±24.0 ^c	388.3±32.1	317.5±68.9 ^d		
Brain	1.97±0.17	2.05±0.10	2.12±0.10	1.99±0.05	1.87±0.13	2.07±0.11	1.93±0.16		
Heart	1.26±0.15	1.41±0.22	1.41±0.13	1.10±0.11 ^b	0.91±0.15 ^c	1.29±0.12	1.20±0.11		
Liver	13.70±1.26	14.29±1.71	14.95±2.23	9.17±0.73 ^c	7.20±1.83 ^c	14.87±1.90	11.01±2.66 ^c		
Spleen	0.72±0.18	0.68±0.03	0.72±0.10	0.61±0.07	0.38±0.16 ^c	0.75±0.12	0.56±0.09		
Lung	1.44±0.15	1.52±0.16	1.62±0.16	1.27±0.31	1.15±0.11 ^b	1.43±0.17	1.45±0.31		
Kidneys	2.65±0.20	2.59±0.34	2.85±0.24	2.09±0.20 ^a	2.02±0.40 ^b	2.67±0.27	2.42±0.34		
Adrenals	0.056±0.013	0.059±0.008	0.059±0.012	0.193±0.324	0.074±0.011	0.063±0.025	0.065±0.011		
Thymus	0.47±0.06	0.45±0.06	0.55±0.10	0.11±0.09 ^c	0.10±0.06 ^c	0.41±0.11	0.29±0.13		
Testis	2.93±0.35	3.09±0.31	3.21±0.43	2.57±0.43	2.21±0.64 ^b	3.15±0.38	3.14±0.18		
Epididymis	1.35±0.23	1.10±0.20	1.12±0.19	1.10±0.76	0.64±0.27	1.49±0.78	1.31±0.26		
B, Organ-to-body weight ratio (%)									
Parameters	Control	Vehicle	1 mg/kg	2 mg/kg	4 mg/kg	Blank gel	AT-533 gel		
Brain	0.52±0.03	0.51±0.05	0.51±0.05	0.82±0.14 ^c	0.95±0.10 ^c	0.53±0.04	0.63±0.12		
Heart	0.33±0.02	0.35±0.04	0.34±0.03	0.45±0.07 ^a	0.46±0.08 ^b	0.33±0.02	0.39±0.05 ^d		
Liver	3.62±0.23	3.56±0.29	3.54±0.35	3.73±0.48	3.59±0.62	3.82±0.31	3.46±0.28		
Spleen	0.19±0.04	0.17±0.01	0.17±0.02	0.25±0.05 ^b	0.19±0.06	0.19±0.03	0.18±0.02		
Lung	0.38±0.05	0.38±0.04	0.39±0.05	0.52±0.12 ^a	0.59±0.09 ^c	0.37±0.03	0.46±0.05 ^d		
Kidneys	0.70±0.06	0.65±0.05	0.68±0.07	0.85±0.13	1.03±0.28 ^c	0.69±0.06	0.78±0.12		
Adrenals	0.015±0.004	0.015±0.002	0.014±0.003	0.090±0.160	0.038±0.010	0.016±0.006	0.022±0.007		
Thymus	0.12±0.02	0.11±0.02	0.13±0.02	0.04±0.03 ^c	0.03±0.03 ^c	0.11±0.03	0.09±0.02		
Testis	0.77±0.03	0.77±0.09	0.76±0.07	1.03±0.10	1.13±0.39 ^a	0.83±0.09	1.02±0.18		
Epididymis	0.36±0.05	0.28±0.05	0.26±0.04	0.42±0.21	0.32±0.10	0.39±0.28	0.42±0.10		
C, Organ-to-body weight ratio (g/g)									
Parameters	Control	Vehicle	1 mg/kg	2 mg/kg	4 mg/kg	Blank gel	AT-533 gel		
Heart	0.64±0.06	0.69±0.12	0.67±0.07	0.55±0.05 ^a	0.48±0.06 ^c	0.62±0.04	0.63±0.08		
Liver	6.96±0.60	6.99±0.90	7.07±1.10	4.61±0.31 ^c	3.83±0.87 ^c	7.17±0.74	5.71±1.40 ^d		
Spleen	0.36±0.07	0.33±0.02	0.34±0.05	0.31±0.04	0.20±0.08 ^b	0.36±0.05	0.29±0.05 ^a		

Table VI. Continued.

C, Organ-to-body weight ratio (g/g)						
Parameters	Control	Vehicle	1 mg/kg	2 mg/kg	4 mg/kg	AT-533 gel
Lung	0.73±0.08	0.74±0.07	0.77±0.08	0.64±0.14	0.62±0.09	0.75±0.16
Kidneys	1.35±0.10	1.27±0.18	1.35±0.13	1.05±0.08	1.09±0.28	1.26±0.22
Adrenals	0.029±0.008	0.029±0.003	0.028±0.006	0.097±0.163	0.040±0.008	0.034±0.006
Thymus	0.24±0.03	0.22±0.02	0.26±0.05	0.06±0.05 ^c	0.03±0.03 ^c	0.15±0.07
Testis	1.48±0.11	1.51±0.15	1.52±0.20	1.29±0.19	1.20±0.41	1.63±0.10
Epididymis	0.69±0.11	0.54±0.10	0.53±0.08	0.55±0.37	0.35±0.16	0.68±0.12
						Blank gel
						0.69±0.05
						1.29±0.15
						0.030±0.012
						0.17±0.09
						1.52±0.15
						0.73±0.42

Values are the mean ± SD for 6 rats in each group (5 rats in 4 mg/kg group). ^aP<0.05, ^bP<0.01, ^cP<0.001 vs. vehicle. ^dP<0.05, ^eP<0.01 vs. blank gel.

the normal reference ranges (28,29). The absolute liver weight decreased in male rats at an AT-533 gel dose of 5 g/kg in comparison with the vehicle while the weight of other organs was not obviously changed after 30 days of treatment.

For male rats treated with 2 mg/kg AT-533, the brain-to-body, heart-to-body, spleen-to-body, and lung-to-body weight ratios increased, while the thymus-to-body-weight ratio decreased, as compared with the vehicle group. In comparison with male rats in the vehicle group, the brain-to-body, heart-to-body, lung-to-body, kidney-to-body, and testis-to-body weight ratios were elevated, while the thymus-to-body weight ratio was decreased in male rats treated with 4 mg/kg AT-533. Heart-to-body and lung-to-body weight ratios were increased in male rats at an AT-533 gel dose of 5 g/kg in comparison with the vehicle. As compared with their corresponding control groups, heart-to-brain (for male rats treated with 2 and 4 mg/kg AT-533), liver-to-brain (for male rats treated with 2, 4 mg/kg AT-533 and 5 g/kg AT-533 gel), spleen-to-brain (for male rats treated with 4 mg/kg AT-533 and 5 g/kg AT-533 gel) and thymus-to-brain (for male rats treated with 2 and 4 mg/kg AT-533) weight ratios decreased (Table VI).

Macroscopic and histopathological examination. Prior to gross necropsy and anatomy analyses, the appearance and morphology of rats were observed. In addition to the symptoms described above, gaseous distention was observed in the treatment groups (2, 4 mg/kg AT-533 and 5 g/kg AT-533 gel) following anatomy analysis. Histopathological observations were conducted for organs and tissues of all 30-day subacute toxicity portion rats. Although some of the absolute and relative weights of organs in rats from the treatment groups showed significant differences, no obvious pathological lesions were found in the organs described above, except for the liver. H&E staining results indicated that no evident pathological lesions were observed in the skin tissue (dosing site) of female and male rats treated with saline, blank gel, vehicle or 1 mg/kg AT-533 (Fig. 3A, B, D, E, H, I, K and L). However, male and female rats exhibited skin irritation and infiltration of a few inflammatory cells following continuous 2 mg/kg AT-533 and 5 g/kg AT-533 gel administration, respectively. No twisting and breaking of dermal elastic fibers was identified (Fig. 3C, F, J and M). In addition, the skin tissue of female and male rats from the 4 mg/kg AT-533 group was characterized by epidermal keratinization, thickening, twisting and breaking of dermal elastic fibers, as well as infiltration of a large number of inflammatory cells (Fig. 3G and N). Furthermore, liver tissue section results indicated that a few hepatocytes exhibited signs of vacuolar degeneration, while no hepatic edema, hyperemia or necrotic cells were observed in the liver of female and male rats treated with saline, vehicle, blank gel, 5 g/kg AT-533 gel or 1 mg/kg AT-533 (Fig. 4A-E and H-L). In the 2 and 4 mg/kg AT-533 groups, liver edema, cytoplasmic vacuolar degeneration and slight dilatation of blood vessels was observed, but no necrotic or inflammatory cell infiltration was identified in the rat liver (Fig. 4F, G, M and N). Apart from these, there were no pathological lesions in the heart, pancreas, spleen, lungs, adrenal glands, kidneys, brain, esophagus, stomach, duodenum, mesenteric lymphoid node, colon, spinal cord (cervical, thoracic and lumbar), bone marrow, prostate, epididymis, testis, ovaries, uterus, mammary gland, sciatic nerve, bladder, pituitary gland, trachea, thyroids, thymus, salivary glands and optic nerve of rats

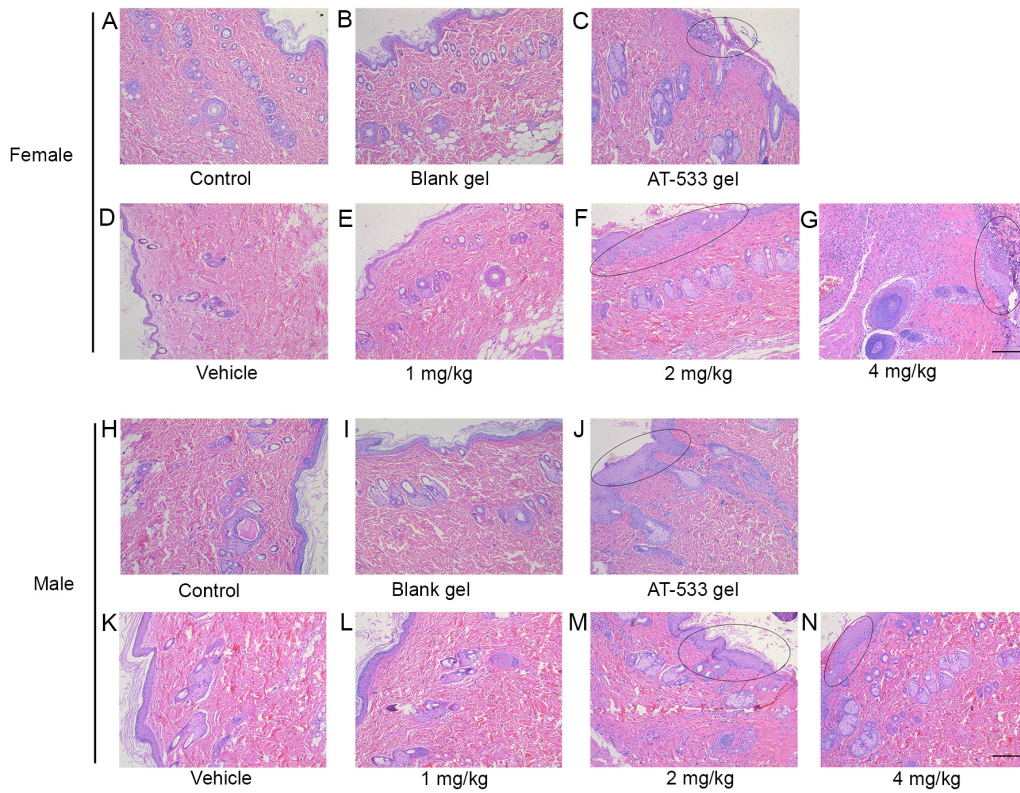


Figure 3. Histological analysis of rat skin treated with AT-533 or AT-533 gel in the 30-day subacute toxicity. Representative micrographs of skin sections from female rats under the conditions of (A) control, (B) blank, (C) AT-533 gel, (D) vehicle, (E) 1 mg/kg AT-533, (F) 2 mg/kg AT-533 and (G) 4 mg/kg AT-533 stained with hematoxylin and eosin. Representative micrographs of skin sections from male rats under the conditions of (H) control, (I) blank, (J) AT-533 gel, (K) vehicle, (L) 1 mg/kg AT-533, (M) 2 mg/kg AT-533 and (N) 4 mg/kg AT-533 hematoxylin and eosin. The elliptical circle represents epidermal keratinization, thickening and inflammatory cell infiltration. Magnification, x10.

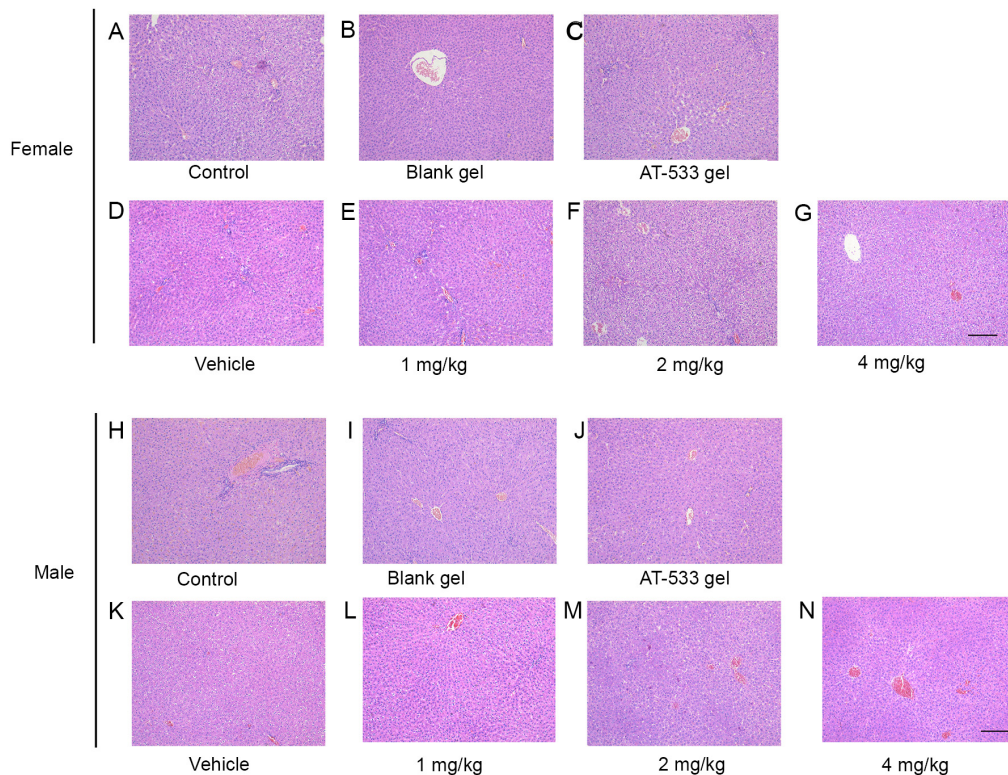


Figure 4. Histological analysis of rat livers treated with AT-533 or AT-533 gel in the 30-day subacute toxicity test. Representative micrographs from female rats under the conditions of (A) control, (B) blank, (C) AT-533 gel, (D) vehicle, (E) 1 mg/kg AT-533, (F) 2 mg/kg AT-533 and (G) 4 mg/kg AT-533 stained with hematoxylin and eosin. Representative micrographs from male rats under the conditions of (H) control, (I) blank, (J) AT-533 gel, (K) vehicle, (L) 1 mg/kg AT-533, (M) 2 mg/kg AT-533 and (N) 4 mg/kg AT-533 hematoxylin and eosin. Magnification, x10.

(data not shown). In combination, these results suggested that the AT-533 and AT-533 gel used in this test did little damage to organs and tissues.

Discussion

Drug safety evaluation is an essential part of the development of new drugs (22,30). In the present study, subacute toxicity tests were conducted in order to examine whether AT-533 or AT-533 gel produced adverse reactions. To the best of our knowledge, this is the first report demonstrating the safety of AT-533 and AT-533 gel through *in vivo* toxicological evaluation. In the present study, the most common symptoms resulting from the dermal administration of AT-533 and AT-533 gel were loss of appetite, skin irritation and hypersensitivity, particularly in rats from the 2 and 4 mg/kg AT-533 group rats. The weight loss in these rats may have been induced by nutrient absorption inhibition. Unexpectedly, dermal exposure to 5 g/kg AT-533 gel over a period of 30 days did not influence food consumption. Although the body weight of rats from the 5 g/kg AT-533 gel group was slightly lighter than that in rats from the blank gel group, there were no significant differences between them. It is known that blood parameters are key indicators of physiological and pathological status, and therefore alterations in them predict toxic effects of the test substances (31). The hematopoietic system is considered to be the most sensitive target for toxicities, and its index of physiological and pathological status is direct evidence for toxicology studies (32). Hematological analysis data suggested that, except for the WBC, NE and PLT counts, other hematological parameters showed no toxicologically significant differences. In rats from the 4 mg/kg AT-533 group, the reason for the abnormal increase in the WBC and NE counts was likely skin irritation caused by the dermal administration of AT-533. During the drug exposure period, some rats may have been uncomfortable, due to the stimulation of AT-533, and used their front paws to scratch the skin continuously in order to feel comfortable. As a result, continuous scratching caused skin damage and bleeding, leading to inflammation, as characterized by the abnormal increased numbers of WBC and NE (33). This phenomenon was concentration-dependent and rats in the 4 mg/kg AT-533 group were more severely affected than other medication-administration groups. Similarly, the abnormal increase in the number of PLT was related to the skin bleeding in male rats at an AT-533 dose of 2 and 4 mg/kg.

To evaluate the influence of AT-533 and AT-533 gel on organ (heart, liver and kidney) functions, blood biochemical examinations were carried out on day 30 of the drug exposure and recovery periods (data not shown). The level of ALB was abnormally decreased and the liver enzyme AST was relatively increased when female rats were administered 4 mg/kg AT-533. Furthermore, the levels of ALB, CHO and HDL were abnormally decreased when male rats were administered 4 mg/kg AT-533. The reason for the abnormal increase of the BUN level may be either an excessive production of BUN (due to high protein absorption from diet, diabetes, severe liver disease or high fever) or excretion disorder of BUN (such as mild renal dysfunction, hypertension, gout, multiple myeloma, urinary tract occlusion or postoperative oliguria Wait) (34). In addition to BUN, CRE is another biomarker of renal function (35) and the abnormal increase of the CRE level indicates excretory dysfunction associated with renal failure. However, there was no abnormal

change in the level of CRE. Furthermore, no abnormalities were identified in the kidney, heart and spleen tissue sections by H&E staining (Figs. S1-S3). These conflicting results between the blood biochemical indexes and histopathological examinations may have been due to several reasons; a main one may be a decrease in food consumption. Following the administration of 2 or 4 mg/kg AT-533 to rats, they showed loss of appetite and flatulence. Long-term malnutrition would lead to reduced levels of ALB and CHO to some extent.

Organ weight and organ coefficient are important indicators for evaluating the toxic effects of test substances in preclinical toxicology studies of new drugs, and have a high reference value for target organ confirming drug toxicity (22,30). The absolute weight of the major organs was altered in rats from the high-dose (2 or 4 mg/kg) AT-533 groups, but stayed within the normal reference ranges (28,29). The relative weight of most organs (brain, heart, spleen, lung, kidney, and thymus) in the 4 mg/kg group was changed, but no abnormalities were observed by pathological examination. These conditions may have been due to the weight loss of the rats at an AT-533 dose of 4 mg/kg.

In conclusion, the lowest observed adverse effect level was induced by 1 mg/kg daily exposure to AT-533 during a 30-day subacute toxicity test; thus, the no-observed-adverse-effect-level (NOAEL) was <1 mg/kg. The results of the 30-day subacute toxicity test of AT-533 gel indicated that 5 g/kg AT-533 gel did not cause lethality in either male or female rats; it only caused a slight body weight decrease and skin irritation. Therefore, the NOAEL of AT-533 gel was <5 g/kg. The effective dose of AT-533 gel is <5 g/kg and AT-533 gel at a therapeutic dose was not toxic to the mice (18,19). In the future work, whether AT-533 gel at a therapeutic dose will have toxic effects on rats will be confirmed. The aforementioned results provide a reference for subsequent subchronic and chronic toxicity tests of AT-533 and AT-533 gel. In our next subchronic and chronic toxicological study, considering the differences hematological values and organ weights between male and female rat, the sex of animals will be taken into consideration.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YH and YiW designed the study. YaW, XS, SQ, PC, LH, QW, TS, FL, XL and QL performed the experiments. YaW and XS performed the statistical analysis. YH, YiW, YaW and XS confirmed the legitimacy and authenticity of all raw data. YaW drafted the manuscript. SQ, PC and LH made significant conceptual contributions to the manuscript. YH and YiW reviewed the final version of the paper. All the authors provided intellectual content and approved the final version of the manuscript.

Ethics approval and consent to participate

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed and the study protocols were approved by The Institutional Animal Care and Use Committee of Jinan University (approval no. IACUC-2020-000038).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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