Safety Evaluation of N-((3S,4S)-4-(3,4-Difluorophenyl)piperidin-3-yl)-2-fluoro-4-(1-methyl-1H-pyrazol-5-yl) benzamide (Hu7691) by a Repeated Dose 14-Day Oral Exposure Toxicity Study in Rats

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Article

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Abstract: Hu7691 represents a novel Pan-Akt kinase inhibitor, demonstrating excellent selectivity towards non-AGC kinase families and pronounced inhibitory effects on the proliferation of multiple tumor cell lines. However, there is currently a notable absence of in vivo toxicological research evidence concerning Hu7691. This study represents the first investigation into the 14-day repeated dose toxicity of Hu7691 in male and female Sprague-Dawley (SD) rats. Male rats were administered daily doses of 12.5, 50, 100, and 150 mg/kg, while female rats received doses of 12.5, 25, 50, and 75 mg/kg for 14 consecutive days. Hematological assessments, organ weights, and histopathological examinations revealed corresponding alterations, suggesting potential target organs for toxicity including the spleen, thymus, and gastrointestinal tract. It is worth noting that the test substance may also impact the liver, kidneys, heart, and ovaries. The No Observed Effect Level (NOAEL) was determined to be no greater than 12.5 mg/kg. Based on the observed gender-related toxicity differences in preliminary trials, it is recommended that the high dose reference dose for male animals in formal experiments should not be less than 100 mg/kg, while for female animals, it should be less than 50 mg/kg.

Keywords: Hu7691; 14-day exposure; oral toxicity study; SD rats; formulation; histopathology

1. Introduction

Cancer stands as one of the most formidable challenges in human medicine. Presently, the global landscape of oncological therapeutics is rapidly advancing towards the era of precision medicine, with the key components being genetic sequencing, tumor profiling, and personalized treatment. Currently, the most mature domain within precision medicine primarily revolves around targeted therapies, exemplifying the treatment of cancer with drugs tailored to specific molecular targets [1]. The phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway represents a critical signaling axis regulating various biological events in cancer, including proliferation, apoptosis, and angiogenesis. Aberrant activation of this pathway plays a pivotal role in the malignant progression of tumors. Consequently, it stands as a prominent focus in current research and development efforts for novel anti-cancer therapies [2–5]. Clinical data indicates that in over 30% of malignant tumors, the PI3K/Akt/mTOR signaling pathway is aberrantly activated. Substantial laboratory evidence further supports the notion that inhibiting this signaling pathway can effectively suppress tumor growth [6–15].

Akt is a serine/threonine protein kinase, and the Akt family primarily consists of three subtypes: Akt1, Akt2, and Akt3. These subtypes are closely related and play a pivotal role in regulating cell growth, proliferation, survival, and metabolism. They are central proteins within the

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PI3K/Akt/mTOR signaling pathway [16]. Following PI3K activation, the interaction between PIP3 and the PH domain of Akt occurs. This interaction leads to the translocation of Akt from the cytoplasm to the cell membrane, accompanied by a conformational change in Akt that exposes its serine and threonine residues. Subsequently, PIP3 binds to the PH domain of Akt, leading to the phosphorylation of serine residues by PDK1 and threonine residues by PDK2. Only when both serine and threonine residues are phosphorylated does Akt become activated. Activated Akt then shuttles between the cell membrane, cytoplasm, and nucleus. It continues to modulate downstream signaling molecules through phosphorylation, including mTOR, Bad, cyclin D1, and various other cellular proteins, promoting cell growth and inhibiting apoptosis [17].

Hu7691 is a novel Pan-Akt kinase inhibitor that exhibits excellent selectivity toward non-AGC kinase families. It demonstrates pronounced inhibitory effects on the proliferation of various tumor cell lines, particularly showing significant suppression in neuroblastoma, gastric cancer, osteosarcoma, and renal cancer xenograft models. In comparison to other Akt kinase inhibitors, Hu7691 holds promise as a prospective candidate for clinical drug development [18].

However, currently, there is a lack of information regarding the Investigational New Drug (IND) clinical studies of Hu7691, including animal toxicology studies. Therefore, investigating the in vivo toxicity profile of Hu7691 is of paramount significance for better understanding its clinical applicability. This study represents the first exploration of potential toxic effects of Hu7691, involving a 14-day toxicity experiment on SD rats exposed to varying doses of Hu7691. The results indicated that oral administration of Hu7691 resulted in significantly higher toxicity in vivo compared to the solvent control group of rats. Through clinical symptoms, changes in body weight, biochemical and hematological indicators, gross anatomical examination, organ weight ratios, and histopathological evaluations, the toxicity response of this test substance was assessed and determined. This information serves as a basis for dose selection in further long-term toxicity studies.

2. Materials and Methods

2.1. Test article and chemicals

Hu7691 (97.98% purity) was obtained from Hangzhou University Institute of Innovation Medicine (Hangzhou, China). Dose formulations were prepared with cosolvents. All dosing solutions were currently prepared before administration. Cosolvents, Methyl cellulose M450 were purchased from Sinopharm Chemical Reagent Co., Ltd. (China).

2.2. Experimental animals

SD rats (six weeks old) were purchased from Hangzhou Vital River Laboratory Animal Technologies Co., Ltd. (Hangzhou, China). There were no significant abnormalities in animals during the acclimatization period. It was indicated that the batch of laboratory animals could be used in this study. Feed and water were supplied ad libitum, and alternated light and dark every 12-hour. The room temperature and humidity of animal rooms were set at 22 ± 3 ℃ and 50 ± 10% respectively. The animal use application (AUP) for this study has been approved by the Institutional Animal Care and Use Committee (IACUC), and the IACUC No. was IACUC-18-286.

2.3. Experimental design

According to the previous study, the maximum tolerated dose of Hu-7691 was 50 mg/kg in male and 25 mg/kg in female animals, respectively. The dose of Hu-7691 used in this study where 200 mg/kg or 600 mg/kg in male and 100 mg/kg or 150 mg/kg in female animals was found applicable to induce perianal filth, weight loss and food consumption reduction. The previous study showed that Hu-7691 could induce gastrointestinal toxicity, thymic toxicity and cardiotoxicity.

Thirty rats were randomly divided into 5 groups, each consisting of 6 rats (3/sex/group). The male rats in groups 2 to 5 orally received Hu-7691 12.5 mg/kg, 50 mg/kg, 100 mg/kg and 150 mg/kg for 14 days, respectively. The female rats in groups 2 to 5 orally received Hu-7691 12.5 mg/kg, 25 mg/kg, 50 mg/kg and 75 mg/kg for 14 days, respectively. Groups 5 were given 0.5% MC and set as

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control. Body weights were measured on D1, D4, D8, D11 and D14 before and post administration. Average food consumption was calculated weekly. Blood samples were collected on D14 before necropsy for analysis of hematology and biochemistry parameters. Organs were collected after necropsy for organ weight ratio calculation and histopathology observation.

2.4. Clinical observation

The animals in each group were observed on clinical symptoms, including appearance, skin, behavior, gland secretion, respiration, eyes, ears, nose, anus and fecal properties, limbs before administration once a day.

2.5. Body weights and food consumption

The animals were observed and recorded once a day, including morbidity and mortality. The body weights were examined before administration and on D1, D4, D8, D11, D14 post Hu7691 administration. Body weights were expressed as Mean ± SD according to sex of the animals. The total food consumption of each cage was examined and the average food consumption of each animal was calculated weekly.

2.6. Hematology and biochemistry Analysis

Blood samples were collected from abdominal aorta via vein with a vacuum blood collection needle and put into the vacuum negative pressure blood collection vessel with anticoagulant EDTA-K_2 (hematology examination), coagulant (biochemistry examination) or anticoagulant sodium citrate (coagulation examination) on D14. Before blood samples collection, all animals were fasted overnight. Then hematology parameters were examined by an automated hematology analyzer Sysmex XT-2000i (Sysmex Corporation, Japan) as follows: white blood cell count (WBC), red blood cell count (RBC), neutrophils (NEUT), lymphocytes (LYMPH), monocytes (MONO), eosinophils (EO), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hematoglobin (MCH), mean corpuscular hematoglobin concentration (MCHC), blood platelet count (PLT), reticulocyte count (RET#).

Biochemistry parameters were examinated on an automatic chemistry analyzer Cobas c311 (Roche Diagnostics, IN, USA) as follows: total protein (TP), albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TB), alkaline phosphatase (ALP), blood urea nitrogen (BUN), glucose (GLU), triglyceride (TG), total cholesterol (TC), creatine kinase (CK), sodium (Na^+), potassium (K^+) and chloride (CL^-) ions.

Coagulation was examinated on an automatic blood coagulation analyzer CA-1500 (Sysmex Diagnostics, IN, Japan) as follows: activated partial thromboplastin time (APTT), thrombin time (TT).

2.7. Necropsy, organ weight and histopathology

Before necropsy, animals in each group were fasted overnight and then profoundly anesthetized using pentobarbital sodium via intramuscular injection, and then enthanasia was performed by exsanguination after anesthesia, and subsequently proceed to a pathology examination for the necropsy. Tissues and organs were collected and weighted as follows: the liver, heart, kidneys, spleen, lungs, brain, cerebellum, thymus, adrenals, testicle, epididymis, ovaries and womb. The relative organ weights were calculated. The collected tissues were preserved in neutral buffered formalin solution. The tissues in the control and highest dose group, the abnormal tissues or organs in gross anatomy and the preserved tissues or organs in morbidity and mortality animals were dehydrated, embedded in paraffin, sliced and stained in hematoxylin and eosin for histopathological examination.

2.8. Statistical analysis

All collected data were organized by each dose group by sex. Data were expressed as Mean ± SD for the quantitative results. One-way analysis of variance (ANOVA) was performed to assess

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differences in these continuous variables, followed by Kruskal Wallis test when it was non-parametric. The results listed the statistically significant differences between each dose group and control group for *p*-value below 0.05. All statistical analysis was conducted by SPSS v26.0 (SPSS Inc. USA).

3. Results

3.1. Clinical observations

One animal (5M03) at 150 mg/kg dose group died on Day 8(D8), one animal (5M01) at 150 mg/kg dose group died on D9, one animal (4F01) at 50 mg/kg dose group died, one animal (5F01) at 75 mg/kg dose group died. On D5~D8, red staining around the nose, bristling hair, emaciation, and arched back were observed in the dead animals. Then, large gastrointestinal volumes, dilatation of the jejunum and duodenum, and fluid filling in the stomach, jejunum, and duodenum were following observed.

Red-stained nose, arched back and emaciation were observed in some female animals at the dose group (Hu-7691 ≥ 25 mg/kg,

The female animals At the dosage of ≥25 mg/kg, the female animals showed red-stained nose, arched back and emaciation. The animals at the dosage of more than 50 mg/kg showed the symptom of vertical hair and matte hair, and the animals at the dosage of 75 mg/kg also showed the symptom of anal filth. At the dose of ≥ 100 mg/kg, the results showed that showed the symptoms of vertical hair, dull hair, red nose, arched back, and emaciation of male animals. No other significant clinical changes were observed.

The clinical symptoms were shown in Table 1.

Table 1. Effect of Hu7691 on clinical symptoms after repeated administration for 14 days.

Dosage (mg/kg)	Sex	Clinical observations (a/b)					
		Bristles/matte hair	Red around the nose	Perianal filth	Hunched	Emaciated	Dead
0	♂	0/3	0/3	0/3	0/3	0/3	0/3
12.5	♂	0/3	0/3	0/3	0/3	0/3	0/3
50	♂	0/3	0/3	0/3	0/3	0/3	0/3
100	♂	3/3	2/3	0/3	3/3	0/3	0/3
150	♂	3/3	1/3	0/3	3/3	3/3	2/3
0	♀	0/3	0/3	0/3	0/3	0/3	0/3
12.5	♀	0/3	0/3	0/3	0/3	0/3	0/3
25	♀	0/3	1/3	0/3	1/3	1/3	0/3
50	♀	1/3	2/3	0/3	2/3	2/3	1/3
75	♀	3/3	3/3	1/3	3/3	3/3	1/3

Notes: a/b: the proportion animals of the symptoms in the total.

3.2. Body weight

The mean body weight of male and female rats was calculated and shown in Figure 1A,B. Compared with the vehicle control group, the dosage of ≥ 50 mg/kg in female animals was significantly decreased on D4~D14, and the dosage of 25mg/kg in female animals was significantly decreased on D11~ D14 ($p < 0.05$~0.01) The number of male animals at the dose of 150 mg/kg was significantly decreased on D4~D8 (the number of the animals at the dose 150 mg/kg group was 1 after D8, which did not meet the statistical crietria), and the number of the animals at the dose 100 mg/kg group significantly decreased on D4~ D14 ($p < 0.05$~0.001) (Figure 1A,B).

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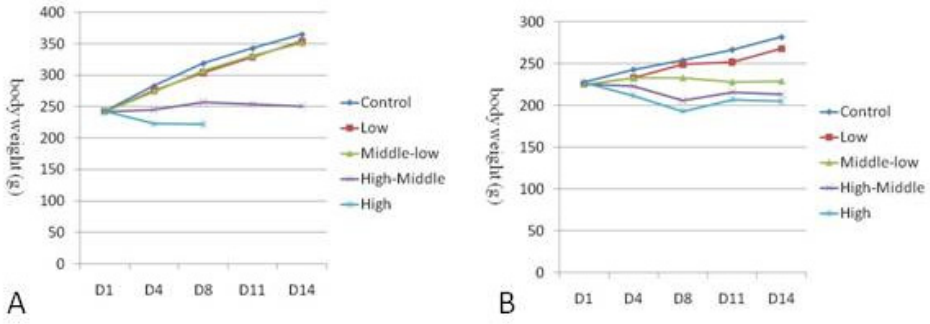

Figure 1. Mean body weights (g) of male (**A**) and female (**B**) rats orally exposed to Hu7691 for 14 days.

3.3. Hematology

Hematology parameters were evaluated after Hu7691 administration in rats and provided in Table 2. Compared with the vehicle control group, the WBC in female animals at the dose ≥25mg/kg significantly increased ($p < 0.05$). The levels of NEUT in male animals at the dose ≥ 50mg/kg and in female animals at the dose ≥ 50mg/kg significantly increased and the levels of LYMPH in male animals at the dose ≥ 50mg/kg and in female animals at the dose ≥ 50mg/kg significantly decreased ($p < 0.05$~0.001). There were significant Hu-7691 related changes on hematology during the administration period.

Additionally, the change of Hb, MCV, MCH, MCHC and PLT levels were sporadic, not related to Hu7691 concentration, and within the normal range for the strain, which was not considered to be of biological relevance [19].

Table 2. List of hematology examination (Values are mean ± SD for 3 rats/sex/group).

Dosage (mg/kg)	Sex	WBC (10^9/L)	%NEUT (%)	%LYMPH (%)	%MONO (%)	%EOS (%)	RBC (10^{12}/L)
0	♂	10.93±1.82	7.3±1.2	90.8±1.3	1.4±0.3	0.5±0.2	7.12±0.44
12.5	♂	6.91±1.16	7.4±2.6	90.4±3.3	1.7±1.0	0.5±0.2	6.84±0.42
50	♂	8.73±1.70	11.0±2.6**	85.5±3.7*	3.0±1.1	0.6±0.3	7.54±0.12
100	♂	11.83±5.38	68.9±6.1***	25.3±5.2***	5.0±2.9	0.7±0.4	6.51±1.33
0	♀	3.44±2.23	7.6±4.2	89.6±5.1	1.7±0.9	1.0±0.1	7.22±0.21
12.5	♀	3.78±1.33	12.3±8.8	85.2±9.6	1.5±0.2	1.0±0.7	7.29±0.53
25	♀	9.92±2.65*	23.2±19.0	73.9±20.1	1.9±1.2	1.0±0.3	6.67±0.16*
50	♀	10.01±0.91*	49.5±1.6**	46.3±1.8**	3.3±0.0	0.9±0.3	6.18±0.66
75	♀	7.17±3.15*	53.7±25.2***	42.9±23.8***	2.0±0.4	1.5±1.1	5.68±1.79
Dosage (mg/kg)	**Sex**	**Hb (g/dL)**	**HCT (%)**	**MCV (fL)**	**MCH (Pg)**	**MCHC (g/dL)**	**PLT (10^9/L)**
0	♂	14.5±0.3	42.5±0.6	59.8±2.7	20.4±0.9	34.1±0.2	1,083±31
12.5	♂	14.1±0.6	40.7±1.3	59.6±2.1	20.6±0.5	34.6±0.4*	1,036±67
50	♂	15.2±0.3	43.7±1.2	58.0±0.7	20.2±0.1	34.9±0.3**	981±100*
100	♂	13.0±2.7	36.0±6.6	55.5±1.2	19.9±0.1	35.9±1.0*	1,300±304
0	♀	13.9±0.7	39.4±1.5	54.6±0.7	19.2±0.4	35.1±0.4	975±65
12.5	♀	14.7±0.7	41.0±1.6	56.4±2.1	20.1±0.8	35.8±0.4	925±51
25	♀	13.7±0.5*	38.2±1.4	57.3±0.8*	20.5±0.2*	35.7±0.2*	1,085±354
50	♀	12.1±1.7	34.7±3.2	56.1±0.8	19.6±0.6	34.8±1.7**	1,733±296*
75	♀	11.1±3.7	31.7±10.5	55.6±1.0	19.5±0.3	35.1±0.1*	1,566±274

Note: The significance levels observed are *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ in comparison to control group values.

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3.4. Serum chemistry

Clinical blood biochemistry was examined after Hu7691 administration and summarized in Table 3. Most of these parameters did not alter after repeated administration of Hu7691. The BUN level was significantly decreased in all female rats exposed to Hu7691 ($p < 0.05$, $p < 0.01$, and $p < 0.01$, respectively), and the GLU level showed a significant decrease in all male rats exposed to Hu7691 compared with the control group ($p < 0.01$). Moreover, a significant increase of TB, Na^+ ions, Cl^- ions levels occurred in all male rats receiving Hu7691 administration ($p < 0.01$, $p < 0.01$, and $p < 0.05$, respectively). Similarly, an obvious increase of TB, Na^+ ions levels was observed in female rats receiving 1000 mg/kg dose ($p < 0.05$), in comparison to the control group.

Table 3. List of serum chemistry examination (Values are mean ± SD for 3 rats/sex/group).

Dosage (mg/kg)	Sex	TP (g/L)	Alb (g/L)	ALT (U/L)	AST (U/L)	TBIL (umol/L)	ALP (mmol/L)	BUN (umol/L)
0	♂	56.6±1.7	41.7±0.6	45.0±5.9	147.9±10.3	0.8±0.3	230±9	5.3±1.0
12.5	♂	56.3±1.7	41.9±2.4	40.2±5.9	106.4±15.2*	0.8±0.5	249±57	5.1±1.6
50	♂	56.9±0.1	39.9±1.3	43.6±10.2	137.3±17.8	0.8±0.4	162±37*	4.0±0.5
100	♂	52.2±1.6*	29.5±3.3**	23.4±8.9	99.4±32.1	0.2±0.3	106±22***	10.3±1.3**
0	♀	60.1±2.3	46.7±2.2	32.9±4.6	110.8±10.8	1.4±0.4	103±21	6.7±0.6
12.5	♀	62.2±2.1	47.9±2.8	33.9±6.7	114.6±16.0	1.7±0.5	100±3	6.8±0.8
25	♀	61.2±6.5	46.6±5.7	34.7±9.7	100.0±11.2	1.1±0.7	69±34	9.2±1.6
50	♀	46.3±0.0*	26.3±0.6**	37.0±5.5	86.3±11.2	0.8±0.3	50±2*	10.4±2.1
75	♀	56.3±6.6	35.7±9.1	38.7±26.0	117.7±22.7	1.4±0.4	79±21	12.1±6.1
Dosage (mg/kg)	**Sex**	**Cr (g/L)**	**Glu (g/L)**	**K (mmol/L)**	**NA (mmol/L)**	**CL (umol/L)**	**TG (mmol/L)**	**TC (mmol/L)**
0	♂	26±1	5.50±0.71	4.90±0.13	142±1	100.5±1.6	0.51±0.20	1.25±1.05
12.5	♂	26±2	6.23±0.40	4.64±0.26	144±1	102.4±0.9	0.55±0.13	0.55±0.81
50	♂	25±4	5.97±0.10	4.59±0.09*	143±1	100.5±0.2	1.36±0.51	2.09±0.32
100	♂	29±2	4.81±0.59	4.74±0.23	143±2	103.2±1.1	1.22±0.54	2.83±0.59
0	♀	38±6	5.64±0.26	4.15±0.60	142±2	99.6±2.3	0.40±0.12	1.38±0.23
12.5	♀	39±2	6.32±0.60	4.10±0.07	143±0	101.0±1.1	0.38±0.04	2.25±0.29*
25	♀	40±5	5.87±0.59	3.88±0.12	142±2	98.9±3.7	0.46±0.08	1.96±0.63
50	♀	34±4	4.90±0.67	4.92±0.05	142±1	103.2±1.6	2.55±1.68	2.26±0.24*
75	♀	36±4	5.84±1.07	4.76±0.49	144±1	102.0±0.9	0.73±0.08	2.52±0.01**

Note: Compared with the control group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.5. Blood Coagulation Detection Index

Clinical blood biochemistry was examined after Hu7691 exposure and the data were summarized in Table 4. Blood Coagulation Detection Indexs did not alter after repeated exposure to Hu7691.

Table 4. List of blood Coagulation Detection Index examination (Values are mean ± SD for 3 rats/sex/group).

Dosage (mg/kg)	Sex	APTT(s)	PT(s)
0	♂	8.0±1.5	7.8±0.3
12.5	♂	10.5±0.4	7.8±0.1
50	♂	9.0±2.2	7.7±0.1
100	♂	8.9±0.5	7.7±0.0
0	♀	9.8±0.7	8.6±0.6
12.5	♀	10.6±3.1	9.1±1.0
25	♀	9.4±1.8	8.5±0.2
50	♀	10.7±10.1	8.1±7.8
75	♀	11.4±0.1	8.0±0.4

Note: The significance levels observed are $p > 0.05$ in comparison to control group values.

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3.6. Necropsy, organ weights and histopathology

The gross anatomy of 3/3 male animals in the Hu7691 100 mg/kg dose group showed that the spleen and thymus were slightly smaller, bleeding points were observed in the glandular stomach, and duodenum and jejunum were dilated; The gross anatomy of the spleen and thymus in 2/3 of the 75 mg/kg dose group female animals is relatively small, while in 1/3 of the 50 mg/kg dose group female animals, the gross anatomy of the spleen and thymus is relatively small, and in 1/3 of the 25 mg/kg dose group female animals, the gross anatomy of the spleen and thymus is relatively small.

Compared with the solvent control group, the absolute weight, relative body weight coefficient, and relative brain weight coefficient of the spleen and thymus of male animals in the Hu7691 ≥ 50 mg/kg group were significantly reduced (p <0.05-0.001), while the absolute weight and relative brain weight coefficient of the spleen and thymus of female animals in the 12.5 mg/kg and ≥ 50 mg/kg groups were significantly reduced (p <0.05-0.001).

Compared with the solvent control group, the absolute weight and relative brain weight coefficients of the liver and kidneys of male animals in the Hu7691 100 mg/kg group decreased significantly (P<0.01-0.001). The absolute weight and relative brain weight ratio of the heart in male animals at the dose of 100 mg/kg and female animals at a dose of 75 mg/kg decreased, while the absolute weight and relative brain weight coefficients of the ovaries in female animals at a dose of ≥ 25 mg/kg decreased, but no dose correlation was observed, The changes in these indicators cannot be ruled out to be related to the administration of the test substance.

The absolute organ weight, organ body weight coefficient, and organ brain weight coefficient data are shown in Tables 5–7.

Table 5. List of absolute organ weight (Values are mean ± SD for 3 rats/sex/group).

Dosage (mg/kg)	Sex	SPLEEN	LIVER	KIDNEY	ADRENALS	THYMUS
0	♂	0.7249±0.0476	9.8091±0.9568	2.5069±0.0579	0.0428±0.0100	0.7038±0.1141
12.5	♂	0.6560±0.0804	9.6923±1.2075	2.4692±0.1912	0.0395±0.0030	0.5977±0.1305
50	♂	0.5780±0.0685*	9.9034±0.7158	2.3161±0.1122	0.0403±0.0056	0.3358±0.1020*
100	♂	0.2030±0.0202***	6.6913±0.4170**	1.5625±0.0597***	0.0300±0.0052	0.0761±0.0257***
0	♀	0.6360±0.0877	7.7315±0.6535	1.9792±0.2029	0.0631±0.0111	0.5214±0.1420
12.5	♀	0.4255±0.0225*	6.8255±0.1239	1.7739±0.0321	0.0557±0.0036	0.4555±0.0892
25	♀	0.3973±0.1707	6.3344±0.7277	1.5645±0.1521*	0.0542±0.0107	0.2082±0.0900*
50	♀	0.3414±0.0619*	6.6504±0.3591	1.3779±0.1180*	0.0488±0.0005	0.1233±0.1055*
75	♀	0.2458±0.0670*	6.4443±0.0599	1.4232±0.3043	0.0353±0.0006*	0.0970±0.0851*
Dosage (mg/kg)	**Sex**	**HEART**	**BRAIN**	**TESTICLE**	**EPIDIDIMS**	**OVARIES**
0	♂	1.2545±0.0688	1.8932±0.1410	2.6549±0.0202	0.6507±0.0917	
12.5	♂	1.2089±0.0494	1.8676±0.0664	2.8478±0.1281	0.6631±0.0919	
50	♂	1.1532±0.0469	1.9459±0.0988	2.8200±0.4947	0.6440±0.0882	
100	♂	0.8343±0.0399***	1.9045±0.0321	3.0220±0.3458	0.6166±0.0506	
0	♀	1.0607±0.0609	1.9342±0.0258			0.1396±0.0049
12.5	♀	0.9025±0.0885	1.8307±0.0090**			0.1147±0.0185
25	♀	0.8658±0.1433	1.7825±0.1327			0.1126±0.0099*
50	♀	0.8210±0.1025*	1.8012±0.0477*			0.0872±0.0306
75	♀	0.8776±0.0246*	1.9177±0.0360			0.0864±0.0081**

The significance levels observed are * p < 0.05, ** p < 0.01 and *** p < 0.001 in comparison to control group values. T-test.

Table 6. List of relative organ weight/body weight (Values are mean ± SD for 3 rats/sex/group).

Dosage (mg/kg)	Sex	SPLEEN	LIVER	KIDNEY	ADRENALS	THYMUS
0	♂	0.2109±0.0115	2.8544±0.2808	0.7295±0.0186	0.0125±0.0030	0.2047±0.0320
12.5	♂	0.1968±0.0135	2.9350±0.5525	0.7418±0.0045	0.0119±0.0006	0.1798±0.0393
50	♂	0.1773±0.0230*	3.0327±0.1533	0.7097±0.0303	0.0124±0.0020	0.1034±0.0334*
100	♂	0.0882±0.0107***	2.9074±0.2773	0.6774±0.0030**	0.0130±0.0026	0.0328±0.0097***
0	♀	0.2417±0.0379	2.9308±0.1816	0.7522±0.0913	0.0240±0.0043	0.1994±0.0620
12.5	♀	0.1684±0.0155*	2.6983±0.1569	0.7006±0.0150	0.0220±0.0023	0.1802±0.0366

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25	♀	0.1857±0.0878	2.9489±0.6734	0.7286±0.1627	0.0248±0.0037	0.0983±0.0507
50	♀	0.1701±0.0105	3.3385±0.2218	0.6904±0.0240	0.0246±0.0032	0.0589±0.0457
75	♀	0.1268±0.0143*	3.3985±0.5195	0.7381±0.0389	0.0186±0.0027	0.0475±0.0366
Dosage (mg/kg)	**Sex**	**HEART**	**BRAIN**	**TESTICLE**	**EPIDIDIMS**	**OVARIES**
0	♂	0.3651±0.0219	0.2047±0.0320	0.7725±0.0052	0.1896±0.0286	
12.5	♂	0.3645±0.0302	0.1798±0.0393	0.8575±0.0476	0.1999±0.0315	
50	♂	0.3536±0.0197	0.1034±0.0334*	0.8643±0.1515	0.1975±0.0284	
100	♂	0.3616±0.0054	0.0328±0.0097***	1.3080±0.1040***	0.2676±0.0247*	
0	♀	0.4027±0.0274	0.1994±0.0620			0.0530±0.0040
12.5	♀	0.3563±0.0329	0.1802±0.0366			0.0452±0.0059
25	♀	0.4017±0.0932	0.0983±0.0507			0.0518±0.0047
50	♀	0.4166±0.1013	0.0589±0.0457			0.0430±0.0101
75	♀	0.4642±0.0880	0.0475±0.0366			0.0452±0.0031

The significance levels observed are * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ in comparison to control group values. T-test.

Table 7. List of relative organ weight/brain weight (Values are mean ± SD for 3 rats/sex/group).

Dosage (mg/kg)	**Sex**	**SPLEEN**	**LIVER**	**KIDNEY**	**ADRENALS**	**THYMUS**
0	♂	38.3281±1.4699	518.1590±34.5187	132.8023±7.9521	2.2943±0.7151	37.0121±3.5065
12.5	♂	35.2493±5.5822	518.7932±58.5405	132.5686±15.1094	2.1220±0.2353	32.0979±7.5437
50	♂	29.7780±4.2084*	508.6051±11.9040	119.1164±5.4313	2.0837±0.3797	17.4630±6.0305**
100	♂	10.6696±1.2342***	351.6505±28.0513**	82.0323±2.3705***	1.5767±0.2993	3.9878±1.2975***
0	♀	32.8470±4.1430	399.7410±33.6162	102.3844±11.2781	3.2684±0.6109	26.9221±7.0784
12.5	♀	23.2407±1.1581*	372.8430±6.4049	96.9035±2.0308	3.0424±0.1934	24.8936±4.9750
25	♀	22.0046±8.6217	355.1793±29.4804	87.8005±6.6001	3.0611±0.7300	11.5635±4.7535*
50	♀	18.9152±2.9385*	369.0860±10.1691	76.4363±4.5296	2.7078±0.0991	6.7703±5.6781*
75	♀	12.8502±3.7331*	336.1378±9.4321	74.3752±17.2626	1.8414±0.0641	5.0982±4.5316*
Dosage (mg/kg)	**Sex**	**HEART**	**TESTICLE**	**EPIDIDIMS**	**OVARIES**	**WOMB**
0	♂	66.6880±8.6860	140.7071±9.5896	34.6570±6.6415		
12.5	♂	64.8128±4.2740	152.7603±12.1614	35.5677±5.4317		
50	♂	59.3902±4.4840	144.9807±25.1509	33.1594±4.9988		
100	♂	43.7945±1.4480*	158.6207±17.0399	32.4007±3.0500		
0	♀	54.8691±3.7782			7.2153±0.2069	19.0245±3.2897
12.5	♀	49.2874±4.6447			6.2701±1.0382	21.6684±2.6783
25	♀	48.4052±5.3772			6.3180±0.3267*	19.8641±6.5851
50	♀	45.6720±6.9008			4.8176±1.5724	16.2426±6.2479
75	♀	45.7604±0.4243*			4.5077±0.5086**	11.8367±1.0334

The significance levels observed are * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ in comparison to control group values. T-test.

The spleen and thymus of male animals in HU7691≥100 mg kg group and female animals in HU7691≥50 mg kg group showed decreased lymphocyte cellularity. Gastric edema and jejunal ulcer were found in HU7691 150 mg/kg group. No significant damage was observed in other animals (Figure 2).

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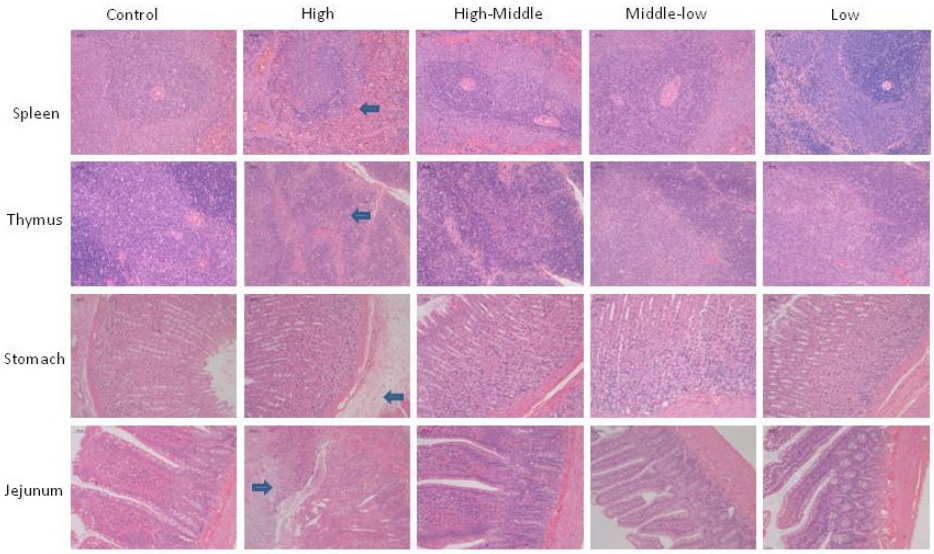

Figure 2. H&E stained sections of spleen, thymus, stomach and jejunum from SD rats treated with control and Hu7691 for 14 days (bars = 50 μm).

4. Discussion

Akt serves as the central protein in the PI3K/Akt/mTOR signaling pathway. Hu7691, as a novel Akt kinase inhibitor, has shown excellent performance in our center's research on anti-tumor efficacy. In our in vitro studies of its inhibitory effects on tumor cell proliferation, we have observed significant inhibitory effects on 21 different human tumor cell lines originating from various tissues, including glioblastoma, lung cancer, gastric cancer, and osteosarcoma (previous research conducted at our center, pending publication). Notably, these effects surpassed those of the positive reference. Moreover, in a 20-day oral administration study of Hu7691, it exhibited dose-dependent inhibition of tumor growth in nude mice with human gastric cancer HGC27, human osteosarcoma KHOS, and human renal cancer 786-O xenografts. Simultaneously, it showed a noticeable reduction in skin toxicity [20].

In this study, Hu7691 led to observable symptoms in rats, including piloerection, dull fur, redness around the nose, and kyphosis. Additionally, it caused a significant decrease in the body weight of the treated rats. Blood biochemical analysis results indicated that Hu7691 primarily induced an increase in WBC levels in female animals at doses ≥25 mg/kg, a significant increase in NEUT, and a marked decrease in LYMPH in both male and female animals at doses ≥50 mg/kg. These changes exhibited statistical significance and showed a dose-related trend, suggesting that the administration of the test substance may influence WBC and its subcategories, NEUT and LYMPH.

Macroscopic examination revealed reduced spleen and thymus volume in female animals at doses ≥25 mg/kg. Histopathological examination further showed a decrease in lymphocytes in the spleen and thymus of female animals at doses ≥25 mg/kg, consistent with the changes in organ weight and coefficients. These findings suggest that the administration of the test substance may have an impact on the immune system.

In the group receiving a dose of 100 mg/kg of Hu7691, all 3 male animals exhibited macroscopic findings of gastric glandular bleeding, as well as dilatation of the duodenum and jejunum. Histopathological examination revealed submucosal edema in the stomach and ulceration in the jejunum. These observations suggest that the administration of the test substance may have an impact on the digestive system.

Compared to the solvent control group, male animals in the Hu7691 100 mg/kg group exhibited reduced absolute liver and kidney weights and relative brain weight coefficients. Male animals at 100 mg/kg and female animals at 75 mg/kg also showed decreased absolute heart weights and relative brain weight coefficients. In female animals at doses ≥25 mg/kg, there was a reduction in absolute

ovarian weights and relative brain weight coefficients, although without a clear dose-response relationship. These changes in these indicators cannot exclude a potential association with the administration of the test substance. Subsequent long-term experiments should include histopathological examinations of the liver, kidneys, heart, and ovaries.

In summary, male Sprague-Dawley (SD) rats were administered doses of 12.5, 50, 100, and 150 mg/kg, while female rats received doses of 12.5, 25, 50, and 75 mg/kg once daily for 14 consecutive days. Animal fatalities were observed in the male group at the 150 mg/kg dose and in the female group at the 50 mg/kg dose. The potential target organs for toxicity appear to be the spleen, thymus, and gastrointestinal tract, with the possibility of the test substance affecting the liver, kidneys, heart, and ovaries not excluded.

5. Conclusion

In conclusion, male Sprague-Dawley (SD) rats were administered doses of 12.5, 50, 100, and 150 mg/kg, while female rats received doses of 12.5, 25, 50, and 75 mg/kg once daily for 14 consecutive days. Animal fatalities were observed in the male group at the 150 mg/kg dose and in the female group at the 50 mg/kg dose. The potential target organs for toxicity appear to be the spleen, thymus, and gastrointestinal tract, with the possibility of the test substance affecting the liver, kidneys, heart, and ovaries not excluded. The NOAEL was determined to be no greater than 12.5 mg/kg. Based on the observed gender-related toxicity differences in the study, it is recommended that the high-dose reference dose for male animals in long-term experiments should not be less than 100 mg/kg, while for female animals, it should be less than 50 mg/kg.

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