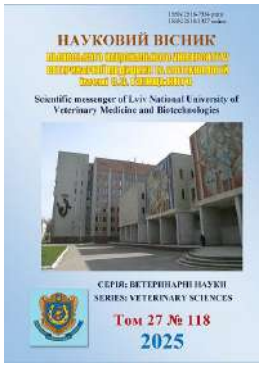

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Preclinical studies of the effects of a drug based on ethylmethylhydroxypyridine succinate and a phosphodiesterase-3 inhibitor on laboratory animals

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The study of a drug's toxicity is one of the key stages in pharmaceutical development. Preclinical studies form the evidence base for the therapeutic efficacy and safety of a medicinal product. The data obtained in such studies allow for the prediction of long-term effects associated with the use of a new drug. The aim of the present study was to experimentally determine the parameters of acute and chronic toxicity of the drug Bendamin in laboratory animals, as well as to assess its cumulative properties.The determination of the acute toxicity of Bendamin revealed that, when administered intragastrically, the drug belongs to toxicity class IV, with an LD_{50} value exceeding 5000 mg/kg. It does not exhibit cumulative effects, with a cumulation coefficient of 8.31 units. The study of chronic toxicity of Bendamin in white rats demonstrated that prolonged administration for 30 days, either in a therapeutic dose or in a fivefold dose, did not cause clinical signs of poisoning. This was confirmed by physiological fluctuations within normal limits in the studied hematological and biochemical parameters of the rats' blood. However, prolonged administration of Bendamin in a tenfold therapeutic dose resulted in slight suppression of the animals' physiological condition. This was evidenced by a 10.1 % decrease in total erythrocyte count and hemoglobin concentration, accompanied by a significant 59.8 % increase in leukocyte count ($P < 0.001$). Additionally, a reduction in liver function was observed, indicated by a statistically significant decrease in total protein (by 8.0 %) and urea (by 13.5 %), as well as an increase in the activity of AlAT, AsAT, and alkaline phosphatase by 31.6%, 7.4%, and 53.9 %, respectively. Significant changes in liver and spleen mass indices were also identified. Administration of a tenfold therapeutic dose of Bendamin in rats caused disturbances in the hemodynamics of various organs, accompanied by protein-related dystrophic changes with focal localization in the parenchyma of the liver, kidneys, and myocardium. These changes are interpreted as compensatory reactions of the organism to the administration of an elevated dose of the drug and are mostly reversible in nature.

Key words: *toxicology, pharmaceutical drug, toxicity, cumulation, phosphodiesterase-3 inhibitor, ethylmethylhydroxypyridine succinate.*

Introduction

In recent years, veterinary medicine has made significant progress, particularly in the field of diagnostics, which now allows the prevention of the development of severe pathologies in animals (Varkholiak, 2016; Vus & Kozenko, 2019; Oldach et al., 2019; Brett et al., 2020; Hotsulia et al., 2021; Smychok et al., 2023; Gutyj et al., 2024). Cardiology is one of the most rapidly advancing branches of veterinary medicine. In recent years, several major studies have been conducted in the treatment of heart failure, new diagnostic methods have been introduced, genetic charac-

teristics of certain pathologies have been identified, and new therapeutic agents have become available, including pimobendan, polyunsaturated fatty acids, sildenafil, levosimendan, torasemide, nebivolol, lisinopril, clopidogrel, among others (Varkholiak, 2016; Tjostheim et al., 2019; Yata et al., 2019; Varkholiak & Gutyj, 2020). However, data on the use of new therapeutic agents for cardiovascular diseases remain limited.

Therefore, there is a justified need to conduct comprehensive studies of the mechanisms of action of medicinal products, their interactions with other drugs, clinical efficacy, and safety of use.

One of the necessary stages in the development and implementation of any new drug is toxicological evaluation, including the determination of acute and chronic toxicity parameters in white rats under conditions of prolonged intragastric administration (Gutyj et al., 2017; Todoriuk et al., 2018; Kushnir et al., 2023).

Our previous studies have shown that the drug Bendamin, when administered intragastrically to white mice and rats in the maximum permissible dose, does not cause clinical signs of poisoning or behavioral abnormalities (Varkholiak & Gutyj, 2018; Varkholiak et al., 2021; 2022).

Aim of the Study

The study aimed to experimentally determine the parameters of acute and chronic toxicity of the drug Bendamin in laboratory animals.

Materials and Methods

The study was conducted on young, sexually mature male Wistar rats aged 2–3 months and weighing 170–185 g. The animals were maintained on a standard diet in the vivarium of the State Scientific Research Control Institute of Veterinary Medicinal Products and Feed Additives.

The experimental procedures were carried out in accordance with the principles of biomedical research involving animals, including the selection of analogs, establishment of control groups, and uniform feeding and housing conditions throughout the experiment (Kotsiumbas et al., 2006; Gutyj et al., 2022).

Acute Toxicity Assessment. The parameters of acute toxicity of the drug Bendamin were evaluated on white mice aged 2–3 months and weighing 19–22 g, and white rats aged 2–3 months and weighing 170–180 g. The drug was administered intragastrically as a single dose.

At the initial stage, doses of 50, 500, and 5000 mg/kg body weight were administered to both rats and mice. Three animals were used for each dose. At the final stage, animals received a dose of 5000 mg/kg body weight. High doses were administered in small portions at intervals of 2 to 4 hours. Six animals were used for each tested dose. The experiment was conducted in duplicate.

After administration of Bendamin, the animals were observed for 14 days. During the first day, animals were under continuous monitoring. Clinical observations included: general condition, appearance, behavioral characteristics, coordination of movements, locomotor activity (intensity and pattern), presence of convulsions, response to external stimuli (tactile, auditory, visual), condition of the hair coat, visible mucous membranes, food response, respiratory rate and rhythm, onset and nature of intoxication, severity, course, time of death or recovery.

Chronic Toxicity Assessment. The design of the chronic toxicity study was based on the results of the acute toxicity trial. Four experimental groups were formed, each consisting of six white rats. The first group served as the control. The remaining groups received Bendamin at therapeutic 5, and 10 therapeutic doses. The drug was administered orally, once daily, on an empty stomach via a gavage tube for 30 consecutive days. At the beginning and end of the experiment, rats from both control and experimental groups were weighed and monitored for clinical condition and behavior throughout the study.

Behavioral responses were assessed using the following criteria: horizontal locomotor activity, vertical activity, grooming, and emotional responsiveness.

On day 31, five rats from each group were used to assess the liver detoxification function using the thiopental test (. A 1 % solution of thiopental sodium was administered intraperitoneally at a dose of 45 mg/kg, and the average sleep duration (from lateral positioning until awakening) was recorded.

Simultaneously, the swimming endurance test was conducted on the other five rats from each group. A glass aquarium was used with a water column of 50 cm and water temperature maintained at 12–13 °C. A load (metal weight) equal to 5 % of the animal's body weight was attached to its tail. Each rat was weighed and then assigned an appropriate load. Control and experimental animals of similar body mass were placed in the tank simultaneously. The swimming time until the animal sank was recorded as a measure of physical endurance.

On the following day, under light ether anesthesia, the animals were decapitated. Hematological and biochemical analyses were performed using standard methods. Organ mass coefficients were determined in comparison to the control group.

Assessment of Cumulative Properties. The cumulative properties of Bendamin were studied on 12 white rats weighing 150–160 g, divided into two groups: control and experimental. The method of K.S. Lima et al. (1961) was used to assess the degree of cumulation.

Bendamin was administered starting from a dose of 0.1 LD_{50}, with the dose increasing by a factor of 1.5 every 4 days. Throughout the study, the general condition and mortality of the rats were monitored.

Based on the effects of the drug at each dose, the cumulation coefficient was calculated using the formula proposed by Yu.S. Kagan and V.V. Stankevych:

$$C_{cum} = DL_{50\,n} / DL_{50\,1},$$

Where: C_{cum} – The cumulation coefficient was calculated based on LD_{50n} and LD_{501} — the mean lethal doses under multiple and single administration, respectively.

To assess the effects of the administered doses of Bendamin on the organism, on the day following the final administration in the cumulation study, animals from each group were weighed and decapitated under light ether anesthesia. Blood samples were collected for hematological and biochemical analyses. Following necropsy, internal organs were excised, weighed, and their relative mass coefficients were calculated in comparison to the control group.

The results were analyzed using the Statistica 6.0 software package. The significance of differences between groups was assessed using Student's *t*-test. Differences were considered statistically significant at $P \leq 0.05$.

Results and discussion

The assessment of acute toxicity is a mandatory stage in the investigation of new pharmaceutical substances. It enables evaluation of the potential hazard posed by a compound under short-term exposure, determination of its toxicity class, and estimation of the therapeutic index. Therefore, in the first stage of this study, the acute toxicity of the cardiotropic drug Bendamin was assessed, including the determination of its tolerated, toxic, and median lethal doses in laboratory animals.

The acute toxicity of Bendamin was studied in rats and mice following intragastric administration. During dose selection, the maximum allowable dose for toxicity class IV – 5000 mg/kg – was used as the limiting value.

Under conditions of intragastric administration of Bendamin, no mortality was observed in white rats (Table 1).

Table 1
Toxicity Indicators of the Cardiovascular Drug Bendamin in White Rats

Number of animals in the group	Drug dose, mg/kg	Number of dead animals		
		all	in %	mean time of death
3	50	0	0	0
3	500	0	0	0
3	5000	0	0	0
12 (twice 6 animals)	5000	0	0	0

Following intragastric administration of the drug at the above-mentioned doses, no clinical signs of intoxication were observed in rats. The animals remained clean, active, and demonstrated good appetite; urination and defecation processes were within normal limits. The rats responded adequately to auditory and visual stimuli, and no signs of respiratory distress or seizures were detected. Reflex excitability was preserved in all animals.

Only transient suppression was observed in animals that received the highest dose of 5000 mg/kg, which is likely attributable to the large volume of the administered substance. By the following day, no changes in the clinical condition of the experimental animals were noted. Similar results were obtained upon repeated administration of Bendamin at a dose of 5000 mg/kg.

Thus, the LD_{50} of the drug following intragastric administration in white rats exceeds 5000 mg/kg body weight.

The results of the acute toxicity assessment of Bendamin administered intragastrically to white mice are presented in Table 2. No mortality was observed in the mice at any of the administered doses. Furthermore, no significant signs of intoxication were recorded in any of the animals during the course of the experiment.

Table 2
Toxicity indicators of the cardiopreparation Bendamine on white mice

Number of animals in the group	Drug dose, mg/kg	Number of dead animals		
		all	in %	mean time of death
3	50	0	0	0
3	500	0	0	0
3	5000	0	0	0
12 (twice 6 animals)	5000	0	0	0

The general condition of animals in the experimental groups did not differ from that of the intact (control) animals: motor coordination and skeletal muscle tone were preserved, and responses to pain, tactile, and auditory stimuli were adequate. In animals that received Bendamin at a dose of 5000 mg/kg body weight, slight suppression was observed, which is likely associated with the administration of a large volume of the drug.

In the course of determining the acute toxicity of the cardiotropic drug Bendamin, it was not possible to establish the exact LD_{50} value, which indicates a low level of toxicity of the tested compound. Therefore, the LD_{50} for Bendamin after intragastric administration in white mice exceeds 5000 mg/kg body weight.

Consequently, in the extended trial, Bendamin was administered at a dose of 5000 mg/kg body weight to six white mice and six white rats. As the results of the study demonstrated (Table 3), no mortality was recorded in either species following a single intragastric administration of Bendamin at this dose.

It was also established that Bendamin at the given dose did not affect the body weight of the animals or the relative organ weight coefficients (Tables 3, 4).

It was found that the body weight of mice and rats in the control group increased by day 14 of the experiment compared to earlier time points, reaching 20.6 ± 0.41 g and 185.6 ± 0.96 g, respectively. In the experimental group, the body weight of the mice and rats was 20.5 ± 0.44 g and 186.6 ± 1.72 g, respectively.

Determination of the relative organ weight coefficients in laboratory animals revealed a slight decrease in the liver weight coefficient in both mice and rats.

Table 3
Effect of the drug Bendamine on the body weight of experimental animals (M ± m, n = 6)

Groups of animals	Kind of animals	Body weight, g		
		3 day	7 day	14 day
I (control)	Mice	19.1 ± 0.50	20.2 ± 0.35	20.6 ± 0.41
	Rats	168.1 ± 2.62	175.3 ± 1.41	185.6 ± 0.96
II (experimental)	Mice	19.0 ± 0.41	20.3 ± 0.24	20.5 ± 0.44
	Rats	171.5 ± 2.50	176.3 ± 2.11	186.6 ± 1.72

Table 4
Coefficients of mass of internal organs of laboratory animals (M ± m, n = 6)

Groups of animals	Kind of animals	Mass coefficients of internal organs					
		liver	spleen	heart	thymus	kidneys	
						right	left
I (control)	Mice	51.4 ± 0.3	9.8 ± 0.2	5.4 ± 0.1	5.1 ± 0.1	9.0 ± 0.2	9.9 ± 0.2
	Rats	35.6 ± 1.1	4.1 ± 0.1	3.5 ± 0,1	2.5 ± 0.2	3.1 ± 0.1	3.3 ± 0.2
II (experimental)	Mice	49.8 ± 0.5	9.6 ± 0.1	5.6 ± 0.2	5.0 ± 0.2	8.7 ± 0.3	9.6 ± 0.3
	Rats	34.2 ± 0.6	4.0 ± 0.1	3.4 ± 0.1	2.4 ± 0.2	3.3 ± 0.1	3.4 ± 0.1

In summary, the conducted studies indicate that the newly developed cardiotropic drug “Bendamin” belongs to the category of low-toxicity substances, classified as Class IV according to SOU 85.2-37-736:2011.

Assessment of Chronic Toxicity of the Drug “Bendamin” in Laboratory Animals. The chronic toxicity of the drug “Bendamin” was assessed in white rats—a well-studied species of laboratory animals—in accordance with the requirements for conducting preclinical studies of veterinary medicinal products, as outlined in the “Preclinical Study of Veterinary Drugs.” The administration route used in the experiment corresponds to the one intended for clinical veterinary practice. Four experimental groups of white rats aged 2–3 months and weighing 170–185 g were formed. The first group served as the control. The remaining groups received “Bendamin” at therapeutic, 5-fold, and 10-fold therapeutic doses, respectively. Throughout the experiment, clinical observation of the laboratory rats was performed.

The aim of the chronic toxicity assessment was to identify any toxic effects of the drug resulting from its prolonged administration to experimental animals.

According to the results of the study, administration of the newly developed cardiotropic drug “Bendamin” to white rats at therapeutic, 5-fold, and 10-fold therapeutic doses did not result in any visible signs of intoxication. No significant disturbances in appearance, general condition, or behavioral responses were observed. The animals retained a stable appetite throughout the entire 30-day experimental period.

During the chronic toxicity study of “Bendamin,” no statistically significant behavioral changes were detected (Table 5), and the characteristic behavioral responses indicated a normal functional state of the central nervous system. However, mild suppression of general condition was noted in rats that received the 10-fold therapeutic dose.

The assessment of emotional-behavioral responses after daily administration of “Bendamin” for 30 days at therapeutic and 5-fold doses revealed no significant impact on the nervous system. Under these conditions, a tendency toward behavioral suppression was observed in the group receiving the 10-fold dose. This was characterized by a reduction in horizontal (number of crossed squares) and vertical locomotor activity (number of rearings), as well as grooming behavior (number of grooming episodes). However, exploratory behavior (number of hole pokes) and emotional activity (number of defecations and urinations) did not differ significantly between experimental groups (E_1, E_2, and E_3) and the control group.

Table 5
Indicators of the physiological state and activity of white rats after 30-day administration of the drug “Bendamin” (M ± m, n = 6)

Indicators	Animal group / drug dose			
	C (control)	E_1 (therapeutic)	E_2 (5-fold)	E_3 (10-fold)
Number of crossings	13.5 ± 1.57	13.2 ± 1.57	12.67 ± 1.33	11.3 ± 1.08
Number of stands	1.83 ± 0.60	2.0 ± 0.51	1.5 ± 0.43	1.0 ± 0.37
Number of burrow visits	4.17 ± 0.83	4.0 ± 0.93	3.83 ± 0.70	4.0 ± 0.68
Number of washings (grooming)	2.33 ± 0.42	2.5 ± 0.43	2.16 ± 0.31	1.66 ± 0.67
Number of defecations	2.33 ± 0.42	2.17 ± 0.31	2.0 ± 0.45	2.17 ± 0.40
Number of urine	1.5 ± 0.43	1.16 ± 0.31	1.33 ± 0.21	1.67 ± 0.42

One of the integral indicators reflecting the level of metabolic processes in animals under conditions of intoxication is the change in body weight and the mass of individual internal organs. Therefore, an important aspect in assessing the chronic toxicity of the drug "Bendamin" is the evaluation of body weight and the relative organ weight coefficients.

Table 6 presents the data on body weight and relative weights of selected internal organs in rats on day 31 of the study on chronic toxicity of "Bendamin." It was established that administration of the drug at a 10-fold therapeutic dose resulted in a 5 % decrease in body weight in white rats compared to the control group. Under these conditions, rats in groups E_1 and E_2 showed a tendency toward a slight increase in body weight.

Table 6

Body weight and weight coefficients of individual internal organs of rats on the 31st day of the experiment to study the chronic toxicity of the drug "Bendamine" (M ± m, n = 6)

Internal organs Body weight, g	Animal group			
	Control	Experimental		
		E_1	E_2	E_3
Liver	191.11 ± 1.25	194.9 ± 4.12	192.5 ± 5.15	180.9 ± 2.48**
Right kidney	36.2 ± 1.70	36.5 ± 2.32	37.0 ± 1.44	43.9 ± 2.10*
Left kidney	3.11 ± 0.23	3.29 ± 0.12	3.17 ± 0.33	3.01 ± 0.13
Spleen	3.14 ± 0.18	3.35 ± 0.06	3.24 ± 0.40	3.08 ± 0.13
Heart	3.85 ± 0.32	3.88 ± 0.14	3.94 ± 0.56	4.22 ± 0.47*
Lungs	4.52 ± 0.39	4.54 ± 0.35	4.31 ± 0.72	3.94 ± 0.21
Internal organs	10.02 ± 1.56	9.90 ± 1.05	9.27 ± 0.91	9.50 ± 0.75

Notes: in this and the following tables, the degree of probability compared to the data of the control group: – P < 0.05;** – P < 0.01; *** – P < 0.001

In the study of the relative weight coefficients of internal organs, it was found that administration of "Bendamin" at a 10-fold therapeutic dose resulted in a statistically significant increase in the relative weights of the liver and spleen (43.9 ± 2.10 and 4.22 ± 0.47, respectively) compared to the corresponding control values (36.2 ± 1.70 and 3.85 ± 0.32).

Administration of "Bendamin" at therapeutic and 5-fold doses led to a slight increase in the kidney weight coefficient, while administration of the drug at a substantially higher dose resulted in a reduction of the weight coefficients of the studied organs.

At the 10-fold dose relative to the therapeutic one, a 12.8 % decrease in the heart weight coefficient was observed in group E_3 compared to the control group. A reduction in lung mass was also recorded following administration of the drug at 5- and 10-fold therapeutic doses, amounting to 9.27 ± 0.91 and 9.50 ± 0.75, respectively.

It is known that the duration of thiopental-induced sleep in animals depends on the capacity of liver microsomal enzymes to neutralize thiopental through glucuronidation. In clinically healthy rats with normal liver function, sleep typically lasts 25–30 minutes. The sleep duration was measured from the moment the animal assumed a lateral position to the first attempts to change it, and was expressed in minutes.

It was established that in rats of the control group, the average sleep duration was 28.7 ± 1.66 minutes. In rats administered "Bendamin" at a 10-fold therapeutic dose, the average duration of thiopental-induced sleep increased by 23 %. The average swimming time for rats in this experimental group was 9.01 ± 1.32 minutes, whereas in the control group it was 12.58 ± 1.45 minutes (Table 7).

Table 7

Results of functional tests (M ± m, n = 5)

Animal group	Thiopental test	Swimming test
	Average sleep time, min.	Average swimming time, min.
control	28.7 ± 1.66	12.58 ± 1.45
E_1	29.4 ± 1.88	12.87 ± 1.57
E_2	30.7 ± 0.65	11.69 ± 1.72
E_3	32.3 ± 1.58*	9.01 ± 1.32*

"Bendamin" administered at therapeutic and 5-fold doses did not affect the results of functional tests, as indicated by the average sleep durations in rats from groups E_2 and E_3, which were 29.4 ± 1.88 and 30.7 ± 0.65 minutes, respectively, and the average swimming times, 12.87 ± 1.57 and 11.69 ± 1.72 minutes.

Therefore, administration of "Bendamin" at therapeutic and 5-fold doses for 30 days does not significantly affect the functional state of internal organs in the test animals.

Hematological studies revealed a tendency toward an increased level of hemoglobin and mean corpuscular hemoglobin (MCH) in all animals administered "Bendamin" compared to the control group. Specifically, in animals of group E_1, these parameters increased by 10.3 % and 4.7%, and in group E_2 by 5.1 % and 4.1 %, respectively (Table 8).

Following administration of the drug at a 10-fold

therapeutic dose, the hemoglobin level in the blood of rats in experimental group E_3 decreased to 110.4 ± 3.2 g/L, and the MCH decreased by 6.5 % relative to the control group. The red blood cell (RBC) count in groups E_1 and E_2 increased by 5.2 % and 1.0 %, respectively, while in group E_3 it decreased by 4.1 % compared to the control group.

The mean corpuscular volume (MCV) of erythrocytes was the lowest in rats from experimental groups E_1 and E_3. In group E_2, the MCV was close to the control values.

Intragastric administration of "Bendamin" to animals in group E_1 for 30 days led to an increase in the hematocrit value to 33.10 ± 1.50 %, while in the control group this value was 32.72 ± 3.21 %. The lowest hematocrit value was observed in group E_3 and amounted to 29.71 ± 2.53 %.

Following administration of the cardiotropic drug "Bendamin" at therapeutic, 5-fold, and 10-fold doses, the number of leukocytes in the blood of rats from experimental groups E_1, E_2, and E_3 increased by 4.5 %, 26.9 %, and 59.7 %, respectively, compared to the control group. The platelet count increased only in experimental group E_1, reaching 553.0 ± 75.03 × 10^9/L, whereas in the control group it was 499.5 ± 67.4 × 10^9/L.

Table 8

Morphological blood parameters of white rats on the 31st day of the experiment to study the chronic toxicity of the drug "Bendamine" (M ± m, n = 6)

Indicators	Groups of animals			
	C (control)	E_1	E_2	E_3
Red blood cells, T/L	7.27 ± 0.58	7.65 ± 0.74	7.33 ± 0.95	6.97 ± 0.35
Hemoglobin, g/L	122.8 ± 3.4	135.5 ± 2.2**	129.1 ± 2.1	110.4 ± 3.2**
Hematocrit, %	32.72 ± 3.21	33.10 ± 1.50	32.84 ± 3.11	29.71 ± 2.53
Average hemoglobin content in erythrocytes, pg	16.9 ± 0.85	17.7 ± 0.33	17.6 ± 0.70	15.8 ± 0.62
Average volume of erythrocytes, μm^3	45.0 ± 0.54	43.3 ± 0.57*	44.8 ± 0.41	42.6 ± 0.80*
Average hemoglobin concentration in erythrocytes, g/100 ml	37.5 ± 0.41	40.9 ± 0.64	39.3 ± 0.68	37.2 ± 0.71

The lymphocyte count in the blood of the experimental groups increased proportionally to the administered dose. In group E_1, lymphocytes increased by 1.7 %; in group E_2 by 4.1 %; and in group E_3 by 10 % compared to the control group (Table 9).

The leukogram of rats' blood showed a decrease in the relative number of monocytes to 1.90 ± 0.49 % in group E_3, 2.12 ± 0.25 % in group E_2, and 2.50 ± 0.50 % in group E_1.

The leukogram also revealed a slight reduction in the number of band and segmented neutrophils in the blood of rats from groups E_1 and E_2. The lowest values for these parameters were recorded in group E_3, which received a dose 10 times higher than the therapeutic level, with reductions of 1.4 % and 2.9 %, respectively, compared to the control group.

Table 9

Leukogram in white rats on the 31st day of the experiment to study the chronic toxicity of the drug Bendamine (M ± m, n = 6)

Indicators	Groups of animals			
	C (control)	E_1	E_2	E_3
Leukocytes, G/L	7.28 ± 1.13	7.61 ± 1.65	9.24 ± 2.10	11.63 ± 1.10**
Platelets, G/L	499.5 ± 67.4	553.0 ± 75.0	489.8 ± 97.1	497.0 ± 62.4
Lymphocytes, %	61.46 ± 1.93	62.50 ± 2.22	64.00 ± 3.05	67.60 ± 1.33*
Monocytes, %	2.60 ± 0.41	2.50 ± 0.50	2.12 ± 0.25	1.90 ± 0.49
Eosinophils, %	2.50 ± 0.50	2.00 ± 0.57	1.67 ± 0.66	1.40 ± 0.25*
Basophils, %	1.97 ± 0.26	1.83 ± 0.16	1.93 ± 0.22	1.91 ± 0.24
Neutrophils (segmented), %	25.30 ± 2.67	25.51 ± 1.64	25.00 ± 2.00	22.40 ± 1.17
Neutrophils (rod-shaped), %	6.17 ± 1.74	5.66 ± 0.90	5.28 ± 1.35	4.79 ± 1.50

The percentage of eosinophils and basophils was lowest in experimental group E_3, amounting to 1.40 ± 0.25 % and 1.91 ± 0.24 %, respectively, whereas in the control group of rats, these values were significantly higher, ranging from 2.50 ± 0.50 % to 1.97 ± 0.26 %.

In addition, a slight increase in indicators of the non-specific immune response was observed in white rats, specifically in neutrophil phagocytic activity and the phagocytic index (Table 10).

The highest phagocytic activity of neutrophils was recorded in the blood of rats from experimental group E_3, where it increased by 2.43 % compared to the control group. Similar changes were observed in the phagocytic index, which was higher in groups E_1 and E_3 by 2.1 % and 21 %, respectively, while in group E_2, this parameter was 7.6 % lower than that of the control group.

Table 10
Indicators of nonspecific immunity in white rats on the 31st day of the experiment to study the chronic toxicity of the drug "Bendamine" (M ± m, n = 6)

Indicators	Groups of animals			
	C (control)	E_1	E_2	E_3
Phagocytic activity, %	20.20 ± 1.01	21.87 ± 0.57	23.35 ± 1.33*	22.63 ± 0.51*
Phagocytic index, m.t./neutrophil	10.28 ± 0.58	10.50 ± 1.10	9.5 ± 0.17	12.45 ± 1.44

The results of the biochemical analysis of rat blood serum on day 30 of Bendamin administration at various doses are presented in Table 11. It was established that administration of the drug at the therapeutic dose did not cause deviations in blood parameters indicative of liver function.

In contrast, administration of the investigational drug at 5-fold and 10-fold doses led to increased enzymatic activity in rats from experimental groups E_2 and E_3. Specifically, alanine aminotransferase (AlAT) activity increased by 12.7 % and 31.6 %, respectively, compared to the control group. Similar changes were observed in aspartate aminotransferase (AsAT) activity, which increased by 3.9 % in group E_2 and by 7.4 % in group E_3 relative to control animals.

Furthermore, blood serum analysis showed an increase in alkaline phosphatase (ALP) activity by 11 % in group E_1, 25 % in group E_2, and 54 % in group E_3, respectively.

Table 11
Biochemical blood parameters of white rats on day 31 of the study of chronic toxicity of the drug Bendamin (M ± m, n = 6)

Indicators	Groups of animals			
	C (control)	E_1	E_2	E_3
AlAT, U/L	53.4 ± 2.45	54.2 ± 3.16	60.2 ± 4.10	70.3 ± 3.34**
AsAT, U/L	173.4 ± 3.85	178.2 ± 5.01	180.1 ± 3.50	186.2 ± 4.24*
LF, μkat/L	1.80 ± 0.30	2.00 ± 0.42	2.25 ± 0.25*	2.77 ± 0.42*
Creatin, μmol/L	74.07 ± 2.66	67.4 ± 2.11*	70.16 ± 2.85	86.6 ± 3.14*
Urea, mmol/L	5.65 ± 0.20	5.70 ± 0.32	5.76 ± 0.26	4.89 ± 0.47
Total protein, g/L	66.65 ± 2.70	69,36 ± 3.55	66.91 ± 4.10	61.32 ± 4.02
Albumin, g/L	26.30 ± 1.82	31,00 ± 3.64	25.04 ± 3.36	18.95 ± 3.11*
Globulins, g/L	40.35 ± 3.61	38.36 ± 4.85	41.87 ± 5.20	42.37 ± 5.31

The protein-synthesizing and detoxification functions of the liver in the experimental groups of animals did not undergo significant changes; the corresponding indicators showed a slight upward trend. For instance, the total protein level in the blood serum of experimental group E_1 increased by 4 %, while the concentration of urea rose by 1 % compared to the control group. Only in experimental group E_3 was suppression of the protein-synthesizing and detoxification functions of the liver observed, as evidenced by a decrease in total serum protein to 61.32 ± 4.02 g/L compared to 66.65 ± 2.70 g/L in the control group, and a reduction in urea concentration to 4.89 ± 0.47 mmol/L compared to 5.65 ± 0.20 mmol/L in the control.

Analysis of protein fractions in the blood serum of rats in the experimental groups revealed that the albumin level in group E_1 increased by 17.9 %, whereas in group E_3 it decreased by 17.9 %. The globulin level in the blood of rats in group E_1, which received Bendamin at the therapeutic dose, decreased by 5 %, while in groups E_2 and E_3, the globulin level increased by 4 % and 5 %, respectively, compared to the control group.

A statistically significant ($P < 0.001$) increase in serum creatinine levels to 86.6 ± 3.14 μmol/L, along with a 20.5 % reduction in urea concentration to 4.89 ± 0.47 mmol/L in rats of group E_3, indicates systemic impairments involving not only the liver but also the kidneys.

Thus, summarizing the results of clinical, morphological, biochemical, and nonspecific immunity parameters, it can be concluded that administration of the drug Bendamin to experimental animals at therapeutic and 5-fold doses over a 30-day period does not cause evident clinical signs of intoxication, and the studied hematological and biochemical parameters remain within the reference range of the control group.

Postmortem examination of rats in the control and experimental groups revealed no external injuries. The fur coat was uniform, smooth, shiny, and firmly held in the hair follicles. Natural orifices were closed, with no discharges.

Internal examination showed anatomically correct positioning of thoracic and abdominal organs. The peritoneum was smooth, shiny, and moist, without any deposits. The content of the thoracic and abdominal cavities was minimal, transparent, and watery in consistency. The lungs were pale pink, loose in consistency, divided into lobes; the pulmonary and costal pleurae were smooth and shiny, with no deposits. The heart was conical in shape, with a transparent pericardium free of deposits; the myocardium was firm and uniformly red in color. The stomach and small intestines were moderately filled with feed mass; the large intestines contained formed fecal matter. The mucous membrane was pale pink, shiny, moist, and free of deposits.

The liver was dark red in color, with a smooth capsule and sharp edges, displaying a characteristic structure on section and a firm consistency. In rats that received a 10-fold therapeutic dose of Bendamin, isolated light brown foci with a smoothed parenchymal pattern were observed on the cut surface.

The kidneys were bean-shaped, dark red in color, with converging cut edges and a well-defined boundary between the cortical and medullary zones. The capsule was easily removable. In some rats that received a 10-fold therapeutic dose of Bendamin, a slight enlargement of the organ with uneven coloration of the subcapsular surface was observed.

The spleen was dark cherry-red, with sharp edges; its structure was preserved on section. Scraping ranged from minimal to moderate in different individuals.

The urinary bladder was moderately filled with urine; the wall was not thickened, and the mucous membrane was pale pink, without deposits.

Histological examination of the liver in all study groups of rats revealed preserved lobular structure with hepatic cords arranged radially around the central vein. The portal triad region exhibited no structural abnormalities. The organ's blood vessels were either empty or moderately filled. Hepatocytes were polygonal in shape, with homogeneous, well-stained cytoplasm, and nuclei that were round and clearly delineated.

In rats of the second experimental group, which were administered a 5-fold dose of the drug, moderate dilation of intralobular capillaries and venous lumens was observed, along with granular dystrophy of hepatocytes in the centrilobular zone of the hepatic lobules.

In rats from the Group III experimental group, which received a 10-fold therapeutic dose of the drug Bendamin, disorganization of the lamellar structure of hepatic lobules was more frequently observed. Hepatocytes were arranged in a disordered manner, and sinusoidal capillaries were dilated. Throughout the lobular area, hepatocytes with uneven cytoplasmic staining and granularity were noted. Hepatocytes with pale (rarefied) cytoplasm were observed more frequently. Among them, cells with lysed and pyknotic nuclei and indistinct cell borders were present. In the portal triad region, isolated clusters of round-cell elements were detected. These findings indicate the development of protein dystrophy and necrobiotic processes.

The histological structure of the kidneys in rats from all studied groups was preserved. No changes were detected in rats from experimental groups I and II.

In the kidneys of rats administered a 10-fold therapeutic dose of the drug "Bendamin" over a period of 30 days, focal granular protein dystrophy of the epithelium of the convoluted renal tubules was observed, accompanied by narrowing of their lumens. The nuclei of some nephroepithelial cells were enlarged, with individual cells showing signs of karyopyknosis and karyorrhexis. The capillary network of certain renal glomeruli appeared condensed, and the space between the capsule of Shumlyansky–Bowman was expanded, with no contents present.

The myocardium of rats in the control, Group I, and Group II experimental groups consisted of bundles of muscle fibers with preserved transverse striation; the nuclei of cardiomyocytes were distinct and centrally located. Moderate accumulation of transudate was observed in the intermuscular space. In the myocardium of rats from the Group III experimental group, foci of fiber disintegration, thickening, fragmentation of muscle fibers, and loss of both transverse and longitudinal striation were noted.

The sarcoplasm in some areas contained acidophilic granularity; the nuclei were mostly preserved, although some appeared swollen with low chromatin content. The intermuscular spaces were moderately expanded and infiltrated with weakly oxyphilic transudate, indicating the development of moderate edema and dystrophic changes in the organ.

Thus, following oral administration of "Bendamin" to rats for 30 days, the macroscopic and microscopic structure of the examined internal organs remained preserved in all animal groups. In the second experimental group, moderate histostructural changes were detected in the liver and kidneys, which were reversible in nature. In rats that received a 10-fold therapeutic dose of Bendamin, histological analysis revealed disturbances in hemodynamics and dystrophic changes, primarily of protein origin, with focal localization in the parenchyma of the liver, kidneys, and myocardium. These alterations were largely reversible and are considered to be the result of compensatory responses of the organism to the administration of an elevated dose of the test compound.

Degree of accumulation of the drug "Bendamin" in the organism of white rats. To evaluate the cumulative properties of the drug "Bendamin," it was administered to laboratory animals starting at a dose of 0.1 DL_{50}, with subsequent 1.5-fold dose increases every 4 days. During the experiment, the general condition of the animals and any mortality were monitored. Under the conditions of this cumulative study, no mortality was observed among the experimental animals receiving Bendamin at increasing doses. The total average administered dose (DL_{50n}) per rat throughout the entire experiment was:

$$DL_{50n} = (500 \cdot 4) + (750 \cdot 4) + (1125 \cdot 4) + (1687{,}5 \cdot 4) + (2531{,}25 \cdot 4) + (3796{,}875 \cdot 4) = 41562{,}5 \text{ мг/кг}.$$

$$DL_{50n} = 2000 + 3000 + 4500 + 6750 + 10125 + 15187{,}5 = 41562{,}5 \text{ мг/кг}.$$

According to the formula, the cumulation coefficient (Kcum) is:

$$C_{cum} = 41562{,}5:5000 = 8{,}31 \text{ units}$$

Thus, the cumulation coefficient of the drug "Bendamin" was 8.31 units, indicating that the drug does not exhibit cumulative properties.

To assess the ability of "Bendamin" to accumulate in the organs and tissues of white rats, the mass coefficients of internal organs were determined. As shown in Table 12, the determination of organ mass coefficients in the experimental group of animals revealed a tendency toward an increase in the liver and spleen mass coefficients by 5.2 % and 5.1 %, respectively. The heart mass coefficient in the experimental group increased by 5.7 %, and that of the thymus by 7.6 % compared to the control group.

Table 12
Coefficients of mass of internal organs of white rats from the study of cumulative properties of the drug "Bendamin" (M ± m, n = 6)

Internal organs	Control	Experimental
Liver	34.6 ± 1.10	36.4 ± 2.15
Heart	3.5 ± 0.16	3.7 ± 0.29
Spleen	3.9 ± 0.20	4.1 ± 0.13
Lungs	8.3 ± 0.31	8.7 ± 0.45
Right kidney	3.3 ± 0.13	3.1 ± 0.14
Left kidney	3.7 ± 0.12	3.5 ± 0.11
Thymus	2.25 ± 0.23	2.42 ± 0.27

It was found that in the experimental group of rats, the relative kidney mass coefficient slightly decreased compared to the control group, where the relative mass of the right kidney was 3.1 ± 0.14 and the left kidney – 3.5 ± 0.11, while in the control group, these values were 3.3 ± 0.13 and 3.7 ± 0.12, respectively.

The mass coefficients of the lungs and thymus during the study period ranged from 8.7 ± 0.45 and 2.42 ± 0.7 in the experimental group to 8.3 ± 0.31 and 2.25 ± 0.23 in the control group, respectively.

Following the examination of hematological parameters in rats on day 24 of the study evaluating the cumulative properties of the drug "Bendamin," an increase in red blood cell count to 6.13 ± 0.27 T/L, white blood cell count to 8.42 ± 0.54 G/L, and hemoglobin concentration to 132.4 ± 2.44 g/L was observed, compared to the corresponding control values: 5.81 ± 0.25 T/L, 8.34 ± 2.76 G/L, and 126.1 ± 2.11 g/L (Table 13).

Table 13
Morphological parameters of rat blood on the 24th day of the experiment to study the cumulative properties of the drug "Bendamin" (M ± m, n = 6)

Indicators	Animal groups	
	Control	Experimental
Hemoglobin, g/L	126.1 ± 2.11	132.4 ± 2.44*
Erythrocytes, T/L	5.81 ± 0.25	6.13 ± 0.27
Leukocytes, G/L	8.34 ± 2.76	8.42 ± 0.54
Eosinophils, %	2.14 ± 0.14	1.04 ± 0.31**
Neutrophils, %	25.2 ± 1.61	26.1 ± 1.40
Lymphocytes, %	69.6 ± 1.95	71.1 ± 1.48
Monocytes, %	3.06 ± 0.32	1.76 ± 0.23**

Following the analysis of the leukocyte profile in rats on day 24 of the study investigating the cumulative properties of the drug "Bendamin," a decrease in the number of eosinophils and monocytes by 1.1 % and 1.3 %, respectively, was observed compared to the control group. The number of lymphocytes in the blood of the experimental group of rats increased by 1.5 %, while neutrophils increased by 0.9 % relative to the control group.

It was established that prolonged administration of "Bendamin" in increasing doses affected certain biochemical parameters of the blood serum of the experimental animals (Table 14). Examination of total protein levels revealed a slight increase in the blood of the experimental group of rats on day 24 of the study, by 5.2 % compared to the control group. The activity of aminotransferases in the blood serum of the experimental rats also increased by 13 % and 8 %, respectively, compared to the control. The activity of alkaline phosphatase in the serum of experimental rats rose to 162.6 ± 31.2 U/L, whereas in the control group it was slightly lower, amounting to 158.5 ± 25.4 U/L.

Table 14
Biochemical parameters of blood in rats on the 24th day of the experiment to study the cumulative properties of the drug "Bendamine" (M ± m, n = 6)

Indicators	Animal groups	
	Control	Experimental
Total protein, g/L	68.45 ± 1.77	72.05 ± 2.32
AlAT, U/L	63.2 ± 2.46	71.4 ± 2.37*
AsAT, U/L	161.1 ± 3.66	174.3 ± 4.58*
LF, U/L	158.5 ± 25.4	162.6 ± 31.2
Creatinine, μmol/L	77.2 ± 1.19	74.8 ± 1.83
Urea, mmol/L	6.58 ± 0.41	6.64 ± 0.25
Total bilirubin, μmol/L	3.76 ± 0.52	3.43 ± 0.40

Along with the increase in enzyme activity in the blood serum of rats from the experimental group, a decrease in creatinine and total bilirubin levels was also observed, by 3.1 % and 8.8 %, respectively.

The concentration of urea in the blood of rats on day 24 of the study on the cumulative properties of the drug "Bendamin" ranged within 6.64 ± 0.25 mmol/L, compared to the control value of 6.58 ± 0.41 mmol/L.

Thus, summarizing the obtained results, it can be concluded that the drug "Bendamin" does not exhibit cumulative activity, with a cumulation coefficient of 8.31 units. Under these conditions, administration of the drug stimulated hematopoiesis and showed a tendency to enhance the protein-synthesizing function of the liver.

Conclusions

The determination of the acute toxicity of the drug "Bendamin" demonstrated that, upon intragastric administration, it belongs to toxicity class 4, with an LD_{50} of over 5000 mg/kg. The drug does not exhibit cumulative effects, with a cumulation coefficient of 8.31 units.

The study of the chronic toxicity of "Bendamin" in white rats indicated that prolonged administration over 30 days in a therapeutic or 5-fold dose does not cause clinical signs of intoxication, as evidenced by physiological ranges of the examined morphological and biochemical blood parameters in the rats.

Prolonged administration of a 10-fold therapeutic dose of "Bendamin" in rats was accompanied by mild suppression of the physiological condition of the organism, as indicated by a decrease in total erythrocyte count and hemoglobin content by 10.1 %, alongside a 59.8 % increase in leukocyte count ($P < 0.001$). Additionally, a reduction in liver function was observed, evidenced by a significant decrease in total protein content by 8.0% and urea by 13.5 %, along with elevated activity of AlAT, AsAT, and alkaline phosphatase by 31.6 %, 7.4 %, and 53.9 %, respectively. Significant changes in liver and spleen mass coefficients were also established.

Administration of a 10-fold therapeutic dose of Bendamin in rats resulted in impaired hemodynamics in various organs, accompanied by dystrophic protein-origin changes with focal localization in the parenchyma of the liver, kidneys, and myocardium. These changes are interpreted as a compensatory response of the organism to the administration of an increased dose of the investigated drug and are largely reversible.

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Conflict of Interest

The authors declare no conflict of interest.

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