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CELL IMMUNITY OF LABORATORY ANIMALS UNDER THE INFLUENCE OF 5-INDOLYLMETHYLENE RHODANINE-3-CARBOXYLIC/SULPHONIC ACID DERIVATIVE

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The aim. *To study the cell immunity status under influence of 3-[5-(1H-indol-3-ylmethylene)-4-oxo-2-thioxo-thiazolidin-3-yl]-propionic acid, as a prominent 4-thiazolidinone derivative and a class of biologically active compounds with polypharmacological properties.*

Materials and methods. *Experimental method on the model of laboratory animals (guinea pigs); intradermal allergy tests; relative and absolute content in the peripheral blood of T- and B-lymphocytes subpopulations; hematological indexies: index of the ratio of lymphocytes and monocytes, index of the ratio of neutrophils and monocytes, index of the ratio of neutrophils and eosinophils, phagocytic index, phagocytic number; ELISA; organic synthesis; pharmacological screening.*

Results. *The effect of 3-[5-(1H-indol-3-ylmethylene)-4-oxo-2-thioxo-thiazolidin-3-yl]-propionic acid has antifungal properties and affect cellular component of immunity in vivo in the guinea pigs model. There are no changes in the skin of guinea pigs during and after chemical applications of the skin and after intradermal tests. The compound stimulate the immune cells, in particular the lymphocyte (increase in the absolute number of CD3 T-lymphocytes by 21.46 % and the absolute number of CD8 T-suppressors by 27.15 %), but with a selective inhibitory effect on certain units (decrease the relative number of NK cells CD16 by 11.57 % and B-lymphocytes CD22 by 23.08 %). There was an increase in the activity of the macrophage phagocytic system (increase in PN by 439.87 % and PI by 62.73 % at 120 minutes), which indicates the reliability of the absorbing function of phagocytes, but with a decrease in their ability to endocytosis (PCI decreased significantly by 78,72 %).*

Conclusions. *Synthesized 3-[5-(1H-indol-3-ylmethylene)-4-oxo-2-thioxo-thiazolidin-3-yl]-propionic acid has a selective activating effect on certain parts of cellular immunity and on phagocytic activity. Derivate influence on the phagocytic activity of neutrophils is ambiguous, and the effect of the compound directed to the cellular part of the immune system does not cause cellular immunodeficiency. The studied derivative is promising for further study of the drug-like molecule with antifungal and antitumor effects*

Keywords: *2-thioxo-4-thiazolidinones, indolecarbaldehydes, synthesis, cellular immunity, phagocytosis, immunotropic activity, leukocytes, guinea pigs*

1. Introduction

Cancer is the cause of every sixth death on the planet and ranks second among the leading causes of death [1]. However, according to the WHO, mortality from infectious diseases that cannot be treated due to antibiotic resistance of pathogens by 2050 will be among the top three causes of death in the world. Therefore, it is important to search for new molecules with polypharmacological properties capable of polytarget activity, in particular with respect to antibiotic-resistant microorganisms and chemotherapeutic-resistant cancer cells [2].

4-thiazolidinone derivatives are an important class of "drug-like" molecules with anticancer [3, 4], antiprypanosomal [5], antiviral [6], antimicrobial [7], antifungal [8], anti-inflammatory [9], antioxidant [10] activity. In addition, the thiazole heterocycle is present in drugs with antimicrobial activity, such as penicillin, monobactam, sulfathiazole, thiabendazole, nizatidine, as well as drugs for the treatment of cancer, such as sunitinib, which makes thiazole/thiazolidinone derivatives a potential source of new agents with polytargetative action.

We have synthesized a number of derivatives of rhodanine-3-carboxylic/sulfonic acid and studied their antibacterial and antifungal activity. The prospects for the use in medicine of the isolated compound-leader (Fig. 1) became the basis for the study of its effect on the cellular immune system and allergenic action in vivo.

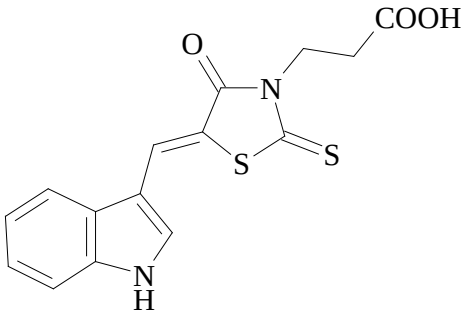

Fig. 1. Structural formula of 3- [5- (1*H*-indol-3-ylmethylene) -4-oxo-2-thioxo-thiazolidin-3-yl]-propionic acid

The aim. To study the state of the cellular immune system of laboratory animals under the influence of the newly synthesized derivative of 3-[5-(1*H*-indol-3-ylmethylene)-4-oxo-2-thioxo-thiazolidin-3-yl]-propionic acid.

2. Planning (methodology) of research

The plan of the experiment is graphically depicted in Fig. 2.

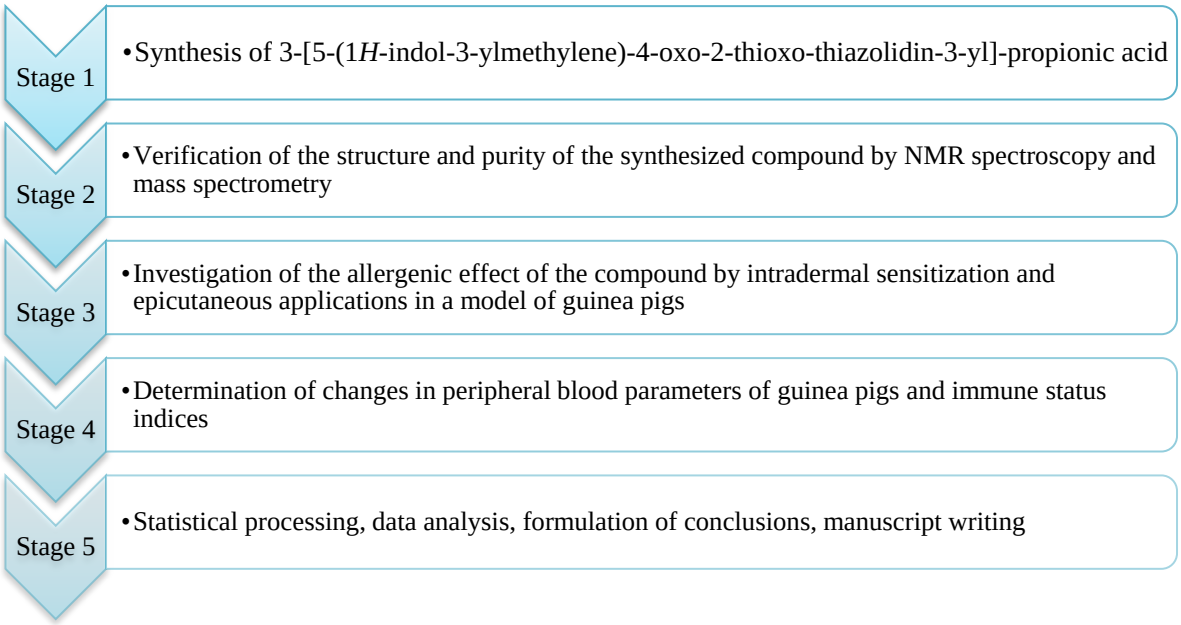

Fig. 2. The plan of the study

3. Materials and methods of the research

Synthesis of 3-[5-(1*H*-indol-3-ylmethylene)-4-oxo-2-thioxo-thiazolidin-3-yl]-propionic acid. Place 10 mmol of rhodanine-3-propionic acid, 15 mmol of indole-2-carbaldehyde, 10 mmol of anhydrous sodium acetate and 10 ml of glacial acetic acid in a round bottom flask under reflux. The reaction mixture is boiled for three hours and cooled. The reaction product is filtered off and recrystallized from acetic acid [8]. The structure and purity of the synthesized compound were confirmed by ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry.

Determination of lipophilicity index (Log P) of the test compound. One of the most common principles for determining the medicinal properties of compounds is the application of the Lipinski (or five) rules [11]. According to these rules, the value of LogP lipophilicity for potential "drug-like" molecules should be in the range from -2 to 5. The study of the lipophilicity parameter of the test compound was performed using the software package ACD/Labs 6.00 and was 2.43±0.80. In addition, the ability to be a donor and proton acceptor was evaluated using this software package and was 2 and 5, respectively. This ability is an important feature for possible binding by intermolecular hydrogen bonding to biotarget and also fully meets the criteria for drugs compounds.

The experiment was performed on 16 white guinea pigs (8 animals in each group) aged 3–3.5 months and weighing 280–350 g. The animals were kept in a vivarium of Danylo Halytsky Lviv National Medical University at a temperature of 19.0–20.5 °C in the conditions of a natural light cycle. The diet is standard with free access to water and food [12]. Animal research has followed the principles of bioethics, legislation and requirements in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Research and Scientific Purposes (Strasbourg, 1985). Council of Europe Directive № 2010/63/EU and the Law of Ukraine № 3447-IV "On the protection of animals against cruel treatment" as amended by № 440-IX of 14.01.2020.

Studies of the allergenic effect of the compound were performed according to the guidelines [13, 14]. Sensitization was performed by the method of O. G. Alekseeva, A. I. Petkevich [15] by intradermal injection of 200 μg of the derivative diluted in 0.02 ml of solvent (saline for the control group of pure and 20 % solution of ethyl alcohol) in the skin of the outer surface of the ear animals. Control animals were injected with 0.02 ml of solvent. After 10 days, an additional 20 applications were applied to the pre-depilated area of the lateral surface of the body. The degree of sensitization was established after intradermal testing in dilutions: 1:10, 1:100. The severity of the allergenic effect of the compound was established in comparison with the control, the intensity of local hyperemia, edema, the presence of erosions and tissue necrosis. The body's response was assessed by visual inspection of the skin surface (erythema diameter, infiltrate or edema at the inoculation site or generalized) at the injection site after 20–30 minutes, 4–5 hours and 24 hours after administration and according to the results of clinical and allergic tests.

Changes in peripheral blood parameters (leukocytes and white blood cell differential) were determined [16, 17]. On the basis of the leukocyte formula, the ratio of individual lymphocyte populations (CD-3, CD-4, CD-8, CD-16, CD-22) was calculated, which can be used

as a general characteristic of cellular reactions of non-specific and specific defense of the organism. Hematological indices were taken into account: index of the ratio of lymphocytes and monocytes (IRLM), which indicates the presence of interaction of effector and affective parts of the immune response; index of the ratio of neutrophils and monocytes (IRNM), index of the ratio of neutrophils and eosinophils (IRNE), which characterize the role of each component of the microphage and macrophage defense system of the body [18, 19].

The state of non-specific resistance of the organism was studied by phagocytic index (PI) – the percentage of neutrophils involved in the process of phagocytosis; phagocytic number (PN) – the number of particles of polystyrene latex absorbed by one phagocyte [20]. To determine the phagocytic activity of neutrophils in the hole of the plastic plate was made 0.5 ml of blood, 0.5 ml of suspension of polystyrene latex and mixed. After a 30-minute incubation at 37 °C the tubes were centrifuged. Smears were prepared from the precipitate, air-dried for at least 1 hour, fixed and stained. 200 neutrophils were counted under a light microscope, and the percentage of cells containing latex granules (phagocytic index) and the average number of latex granules in 1 phagocyte (phagocytic number) were determined.

The phagocytosis completion index was determined.

The state of immunological reactivity of the organism was evaluated by the relative and absolute content in the peripheral blood of subpopulations of T- and B-lymphocytes by the reaction of rosette formation with erythrocytes, which adsorbed monoclonal antibodies against receptors CD_3 (T-lymphocytes), CD_4 (T - helpers), CD_8 (T-cytotoxic), CD_{16} (natural killers), CD_{22} (B-lymphocytes). The studies were performed using erythrocyte diagnosticums (manufactured by TOV NVL "Granum", Ukraine, Kharkiv). Immunoregulatory index was also calculated (the CD_4/ CD_8 ratio).

The obtained data were expressed as a percentage and in absolute units per 1 liter of blood (10*9/l), (G/l). The reliability of the obtained changes was evaluated using Student's t-test.

The permission to conduct experiments on animals was approved by the protocol No. 6 of the commission on ethics of scientific research, experimental development and scientific works of Danylo Halytsky LNMU from June 25, 2018.

Statistical data processing was performed using Microsoft Excel software. Data were presented as arithmetic mean (M) and standard deviation (m). The reliability of the obtained data was evaluated using Student's t-test (probable changes were taken at $p<0.05$).

4. Results

There were observed no changes in the skin of the guinea pig during the chemical application of skin applications and after intradermal tests in doses of 1:10 and 1:100.

Indicators of white blood cell differential of peripheral blood of the studied animals (Table 1) significantly changed in the experimental group compared with the control. In particular, the total number of leukocytes increased by 21.76 %, an increase in the absolute number of eosinophils. Absolute monocytosis (increase in the number of monocytes by 52.09 %), relative and absolute increase in the number of lymphocytes (by 5.02 % and 26.87 %, respectively) against the background of a decrease in the percentage of neutrophilic granulocytes were also recorded. The index of the ratio of neutrophils and monocytes (IRNM) decreased significantly by 35.43 % (Table 3). Other indicators have not changed significantly.

Table 1

Indicators of the white blood cell differential of guinea pig blood after epicutaneous sensitization by the test substance, M±m

Indicator	Control	Test substance	Change relative to control, %	Certainty*
Leukocytes, g/l	13.79±0.49	16.79±1.16	21.76	p<0.05
White blood cell differential:				
Basophils, %	0.25±0.16	0.25±0.16	0.00	
Basophils, g/l	0.03±0.02	0.04±0.03	35.69	
Eosinophils, %	2.63±0.18	2.88±0.23	9.52	
Eosinophils, g/l	0.36±0.02	0.49±0.05	35.38	p<0.05
Neutrophils, %	24.63±1.34	20.13±0.79	–18.27	p<0.05
Neutrophils, g/l	3.39±0.23	3.43±0.35	1.11	
Monocytes, %	2.75±0.25	3.50±0.42	27.27	
Monocytes, g/l	0.38±0.04	0.58±0.07	52.09	p<0.05
Lymphocytes, %	69.75±1.22	73.25±1.03	5.02	p<0.05
Lymphocytes, g/l	9.68±0.40	12.28±0.77	26.87	p<0.05

Note: significance is indicated only for p <0.05 and p <0.001, all other indicators have p≥0.05

Analysis of quantitative parameters of cellular adaptive immunity (Table 2) in the control group showed a significant increase in the absolute number of CD_3 T-lymphocytes by 21.46 % and the absolute number of CD_8 T-suppressors by 27.15 %, as well as a decrease in the relative number of CD_{16} NK cells by 11.57 % and B-lymphocytes CD_{22} by 23.08 %. Other indicators have not changed significantly.

Table 2

Subpopulation composition of peripheral blood lymphocytes guinea pig after epicutaneous sensitization by the test substance, M±m

Indicator	Control	Test substance	Change relative to control, %	Certainty*
Indicators of cellular immunity				
T-lymphocytes CD_3, %	49.75±1.10	47.50±0.93	–4.52	
T-lymphocytes CD_3, g/l	4.81±0.20	5.84±0.38	21.46	p<0.05
T-helpers CD_4, %	30.25±0.96	28.00±0.80	–7.44	
T-helpers CD_4, g/l	2.93±0.15	3.44±0.23	17.52	
T-suppressors CD_8, %	19.50±0.63	19.50±0.38	0.00	
T-suppressors CD_8, g/l	1.89±0.09	2.40±0.15	27.15	p<0.05
NK-cells CD_{16}, %	15.13±0.79	13.38±0.38	–11.57	p<0.05
NK-cells CD_{16}, g/l	1.48±0.11	1.65±0.11	11.77	
B-lymphocytes CD_{22}, %	22.75±1.08	17.50±0.5	–23.08	p<0.001
B-lymphocytes CD_{22}, g/l	2.21±0.17	2.16±0.17	–1.98	
Immunoregulatory index, (CD_4/CD_8)	1.56±0.08	1.43±0.05	–8.47	

Note: significance is indicated only for p<0.05 and p<0.001, all other indicators have p≥0.05

Among the indexes of immune status significantly increased PN (120 min, by 439.87 %), PI12 (120 min, by 62.73 %); significantly decreased PN12 (30 min, by 28.3 %), PI12 (30 min, 27.21 %), PCI (78.72 %) and IRNM (35.43 %) (Table 3), IRNE and IRLM have not changed significantly.

Table 3

Index indicators of the immune status of laboratory animals, M±m

Indicator	Control	Test substance	Change relative to control, %	Certainty*
PN12 (phagocytic number), 30th minute, c.u.	60.20±2.63	43.16±3.81	–28.30	p<0.001
PN12 (phagocytic number), 120th minute, c.u.	13.00±1.39	70.18±2.91	439.87	p<0.001
PI12 (phagocytic index), 30th minute, %	5.89±0.17	4.29±0.30	–27.21	p<0.001
PI12 (phagocytic index), 120th minute, %	3.07±0.12	4.99±0.41	62.73	p<0.001
PCI (phagocytosis completion index)	3.47±0.32	0.74±0.03	–78.72	p<0.001
Index of the ratio of neutrophils and monocytes (IRNM)	9.21±1.15	5.95±0.76	–35.43	p<0.05
Index of the ratio of neutrophils and eosinophils (IRNE)	9.66±1.32	7.11±0.76	–26.33	
Index of the ratio of lymphocytes and monocytes (IRLM)	25.86±2.67	21.44±3.18	–17.09	

** Significance is indicated only for p<0.05 and p<0.001, all other indicators have p≥0.05*

5. Discussion

Neutrophilic granulocytes, which are cellular non-specific defense factors, are the first to take on the action of xenobiotics and are able to leave the bloodstream, migrating to the foci of inflammation, as evidenced by an increase in total white blood cells by 21.76 %. The studied derivative has a corresponding effect on the immune system, as evidenced by an increase in the absolute number of eosinophils (by 35.38 %) and monocytes (by 52.09 %), absolute and relative lymphocytosis (an increase of 26.87 % and 5.02 %, respectively).

It should be noted that if we compare the indicative leukograms with the reference values of the leukogram for guinea pig [13], all indicative leukograms of the study group remained within normal limits, but the reference values differ from one author to another [21].

An increase in the activity of the macrophage phagocytic system was noted. A significant increase in PN by 439.87 % at 120 minutes indicates an increase in the absorptive properties of neutrophils, but the inhibition of the digestive ability of phagosomes. A decrease in PI of 27.21 % at 30 minutes followed by an increase of 62.73 % at 120 minutes indicates an initial decrease followed by a significant increase in the number of active neutrophils [22]. The decrease in the digestive activity of neutrophils (PCI) was significantly reduced by 78.72 % and amounted to <1 is due to excessive accumulation of latex antigens and is due to incomplete phagocytosis [23–25].

Study limitations. A number of leukocyte formulas of guinea pigs are difficult to interpret as absolute changes in clinical and laboratory parameters, as the values of some indicators of the experimental group are within the reference values of white blood cell differential for guinea pigs, despite a significant change in control. For example, the detected absolute and relative increase in the number of eosinophils in the blood of experimental animals does not exceed the reference values and can not be considered eosinophilia in the classical sense.

Prospects for further research. It is planned to investigate the effect of the newly synthesized derivative on the humoral part of immunity and the effect on the level of pro-/anti-inflammatory cytokines.

6. Conclusions

1. The effect of the newly synthesized 3-[5-(1*H*-indol-3-ylmethylene)-4-oxo-2-thioxo-thiazolidin-3-yl]-

propionic acid derivative on the immune system *in vivo* was studied.

2. The effect of the substance on the immune system is manifested in an increase of the quantitative level of lymphocytes and monocytes against the background of maintaining the number of neutrophilic leukocytes at the same level. Activation of eosinophils is probably a response to the production of chemotactic factors by basophils, the number of which has also increased.

3. The effect of the substance on the phagocytic activity of neutrophils is ambiguous: the increase in the absorbing activity of phagocytes is accompanied by a decrease in the level of completeness of phagocytosis. The mechanism of reduction of phagocyte digestive activity may be polyetiological in nature (lysosomal enzyme deficiency, decreased cationic protein levels and inhibition of NO synthetase activity, impaired Fcγ-receptor receptor function), which needs to be clarified in further studies.

4. It was found that the effect of the test compound is directed to the cellular immune system: the number of CD3 and CD8 T lymphocytes increases against the background of a decrease in the relative number of natural killers and CD22 B lymphocytes. The decrease in the immunoregulatory index is insignificant (unreliable indicator) and, accordingly, indicates the absence of cell immunodeficiency. It is a question of activation of mechanisms of adaptive resistance.

5. The identified effects that accompany antitumor and antifungal activity are promising for further study of the drug-like molecule by polytarget action.

Conflict of interests

The authors declare that they have no conflicts of interest.

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