EXPERIMENTAL STUDIES

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THE IMMUNOMODULATORY ACTIVITY OF THE BETULONIC ACID BASED COMPOUND

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The immunomodulatory activity of a betulonic acid-based compound with furocoumarin (BABCF; 2-azido, 9-N-methylpiperazinomethyl oreozelone) has been investigated. Male C57BL/6 mice (aged 3 months) treated with the cytostatic agent cyclophosphamide (CP) and intact individuals served as experimental models. The expression of genes was studied in bone marrow (*IL-12*, *IL-10*, *IL-1β*, *TNF-α*, *TGF-β*, *M-CSF*, *GM-CSF*) or in the suspension of peritoneal cells (*IL-12*, *IL-10*; as the injection site). The surface markers of T-lymphocytes (CD3, CD4, and CD8) in fractions of venous blood mononuclear cells (MNCs) were determined by means of flow cytometry using antibodies. Histological and morphometric studies were performed to assess the impact of CP and BABCF on the thymus. BABCF caused a pronounced (about 3-fold) increase in relative expression of the *GM-KSF* gene. BABCF caused a local increase in the expression of *IL-12* in the peritoneal cavity cells and restored the relative content of T-lymphocytes in the blood of CP-treated mice treated affecting mainly $CD3^+CD4^+$ lymphocytes. This substance reduced the tissue density of the thymic cortex and thymic medulla in CP-treated mice. Thus, results of this study suggest that BABCF exhibits a stimulating effect on the cellular link of immunity and promotes maintenance of the number of T-lymphocytes in the blood due to their migration from the central organs of the immune system.

Key words: betulonic acid; furocoumarins; cytokines; bone marrow; thymus; T-lymphocytes

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INTRODUCTION

About 20 years ago, potential immunotropic properties of betulinic acid were discovered [1]. Since then, this substance and its derivative betulonic acid (BA) attract much attention in the context of promising biologically active substances of natural origin. They exhibit anti-inflammatory [2], antioxidant [3], and antitumor [1, 3] activities.

Furocoumarins (psoralens) represent another class of compounds with pronounced immunotropic properties; they play a protective role in the plants. Psoralens exhibit photosensitizing activity and are used in clinical practice for the treatment of autoimmune and hyperproliferative skin diseases [4, 5]. Advances in organic chemistry resulted in the synthesis of compounds containing BA and furocoumarins [1, 6].

One of the important directions in modern pharmacology is the search for drugs influencing the cytokine system, which provides interaction between different cells of the immune system. More than 200 polypeptides belonging to the cytokine family are known; together they form an extensive network of interactions. Cytokines regulating the inflammation process attract much interest; these include IL-12, IL-10, IL-1β, and TNF-α [7]. Being polypotent biologically active compounds, many cytokines not only regulate protective reactions

Abbreviations used: AIDS – acquired immunodeficiency syndrome; a.u. – arbitrary units; BA – betulonic acid; BABCF – a betulonic acid based compound (2-azido, 9-N-methylpiperazinomethyl oreozelone); CD – cluster of differentiation; COX-2 – cyclooxygenase-2; CFU – colony forming unit; CP – cyclophosphamide; DMSO – dimethyl sulfoxide; EDTA – ethylenediaminetetraacetate; FITC – fluorescein isothiocyanate; *GAPDH* – glyceraldehyde-3-phosphate dehydrogenase gene; GM-CSF – granulocyte-macrophage colony-stimulating factor; HIV – human immunodeficiency virus; HKG – housekeeping gene; IL – interleukin; iNOS – inducible nitric oxide (NO) synthase; LPS – lipopolysaccharide; MNCs – mononuclear cells; M-CSF – monocytic stimulating factor; NF-κB – nuclear factor kappa-bi; NK – natural killer cells; PE – phycoerythrin; RNA – ribonucleic acid; TGF-β – transforming growth factor-β; TNF-α – tumor necrosis factor-α; *β-act* is the gene encoding the β-actin protein.

of the body to pathogens, but also maintain homeostasis. For example, colony-stimulating factors GM-CSF and M-CSF are stimulators of proliferation and differentiation of precursors of peripheral blood cells, as well as TGF-β.

Besides the cytokine regulation system, studies of new compounds also include analysis of shifts in the cellular link of immunity, particularly in populations of T-lymphocytes. In the case of exposure to various stressful factors, infections (especially viral ones), the most important role is attributed to the T-cell immune response [8]. It is also important to recognize processes occurring under the influence of new compounds in the central organs of the immune system: the bone marrow and the thymus gland.

Since BA and furocoumarins exhibit the immunotropic properties it was interesting to synthesize compounds combining BA and furocoumarins. The aim of this study was to investigate the effect of a BA-based compound with furocoumarin (BABCF; 2-azido, 9-N-methylpiperazinomethyl oreozelone) on the immune system.

MATERIALS AND METHODS

The Studied Compound

BABCF (Fig. 1) was synthesized in the Laboratory of Medicinal Chemistry (Vorozhtsov Novosibirsk Institute of Organic Chemistry, SB RAS). It was obtained in the CuAAC reaction of azide-alkyne cycloaddition of betulonic acid propargyl ether with the corresponding 2-azidofurocoumarin in the presence of $CuSO_4 \cdot 5H_2O$ and sodium ascorbate in a solution of CH_2Cl_2/H_2O.

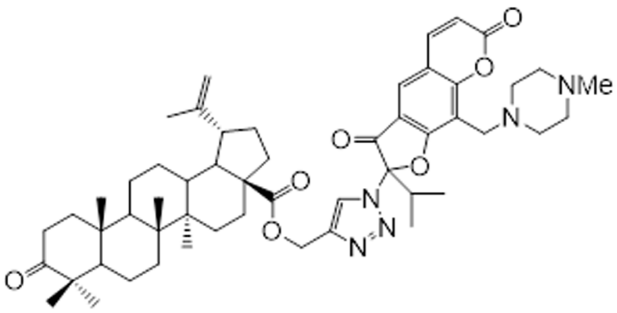

Figure 1. Structural formula of the tested compound of 2-azido-9 substituted oreoselone with BA (BABCF).

The Model of CP-Induced Immunosuppression

Experiments were carried out using 3 months-old male C57BL/6 mice.

An experimental cytostatic model was developed using a CP preparation (Endoxane, Baxter, Germany) dissolved in saline. The drug was administered once intraperitoneally (i.p.) at a dose of 200 mg/kg (sublethal dose) [9] one day after a single i.p. administration of the test compound (BABCF) at a dose of 30 mg/kg. BABCF was dissolved in DMSO, and then in saline; the final concentration of DMSO in the injected BABCF solution was 1%. On day 6 after BABCF administration, mice were killed by carbon dioxide inhalation. After that, peritoneal cells, bone marrow, thymus and venous blood samples were isolated for flow cytometry (Table 1).

Gene Expression Analysis

Isolation of RNA from the biological material was carried out using TRI-REAGENT (MRC, USA) according to the manufacturer's protocol. Reverse transcription was performed using a M-MuLV-RH RT kit (Biolabmix, Russia), 2–5 μg of total RNA, primers with random sequences, and oligo-dT primers. Quantitative PCR with real-time detection (RT-PCR) was performed using BioMaster HS-qPCR SYBR Blue(2×) (Biolabmix). In order to avoid errors that may occur during PCR on different plates, and thereby introduce distortions into the final result, universal conditions were selected for amplification of genes selected for the study: 95°C — 3 min; 45 cycles — (95°C — 12 s, 57°C — 15 s, 72°C — 20 s). *GAPDH* and *β-act* were used as the reference genes. The relative levels of gene expression were calculated by the $2^{-\Delta Ct}$ method. In all samples the efficiency of RT-PCR was close to the maximum or equal to it (E=2).

Expression of *IL-12* and *IL-10* was investigated in peritoneal cell suspensions and expression of *IL-12*, *IL-10*, *IL-1β*, *TNF-α*, *TGF-β*, *GM-CSF*, and *M-CSF* was investigated in bone marrow fragments (Table 2).

Flow Cytometry of Peripheral Blood Samples

Isolation of lymphoid cells from the whole blood samples was performed using a ficoll-verografin solution and sodium EDTA as an anticoagulant

Table 1. The scheme of *in vivo* experiments

	Day 1	Day 2	Day 6
Group 1 (Control)	0.9% NaCl + DMSO	—	Isolation of peritoneal cells (5 mice in the group), bone marrow (10 mice in the group), thymus samples (5 mice in the group) and venous blood samples (5 mice in the group)
Group 2	0.9% NaCl + DMSO	CP (200 mg/kg)	
Group 3	BABCF in DMSO + 0.9% NaCl	CP (200 mg/kg)	
Group 4	BABCF in DMSO + 0.9% NaCl	—	

Table 2. Target genes and oligonucleotide sequences used in the evaluation of gene expression

Gene name	Oligonucleotide sequences
GAPDH	F: 5′-TAGACAAAATGGTGAAGGTCGG-3′ R: 5′-CCTGGAAGATGGTGATGGG-3′
β-act	F: 5′-CCTGAGGAGCACCCTGTG-3′ R: 5′-GGAGAGCATAGCCCTCGTAG-3′
IL-12	F: 5′-AGTGTGGCACTGATGCTGATG-3′ R: 5′-GTAGCCAGGCAACTCTCGTT-3′
IL-10	F: 5′-TGGGTTGCCAAGCCTTATCG-3′ R: 5′-CTCTTCACCTGCTCCACTGC-3′
IL-1β	F: 5′-TGCCACCTTTTGACAGTGATGA-3′ R: 5′-ATCAGGACAGCCCAGGTCAA-3′
TNF-α	F: 5′-TCAGTTCTATGGCCCAGACC-3′ R: 5′-ACCACTAGTTGGTTGTCTTTGAG-3′
TGF-β	F: 5′-TGATACGCCTGAGTGGCTGTCT-3′ R: 5′-CACAAGAGCAGTGAGCGCTGAA-3′
GM-CSF	F: 5′-GTTGAATGAAGAGGTAGAAGTC-3′ R: 5′-ATCCGCATAGGTGGTAAC-3′
M-CSF	F: 5′-TGAGTCTGTCTTCCACCT-3′ R: 5′-GTGTGCCCAGCATAGAAT-3′

(1.8 mg EDTA per 1 ml of blood). The upper layer of cells (lymphocytes) was collected, diluted with RPMI medium, and washed to remove ficoll contaminations by centrifugation (1500 rpm) for 20 min. The resulting cells were used for immunophenotyping of T-lymphocytes using antibodies to CD3FITC (BioLegend Inc., USA), CD4PE, CD8APC (Abcam, UK). Briefly, 0.5 μl of monoclonal antibodies was added in the presence of 1% albumin to 50 μl of each blood cell sample. Stained lymphocytes were analyzed using a CytoFlexS-100 flow cytometer (Beckman Coulter, USA).

Histological Examination

The object of the study was the mouse thymus. For light microscopy, thymus fragments were fixed in a 4% paraformaldehyde solution, dehydrated in a series of increasing concentration of alcohols, cleared in xylene, and embedded into paraffin according to the standard histological method. The resulting sections with a thickness of 4-6 μm were stained with hematoxylin and eosin. Light-optical and morphometric studies of the thymus were performed in transmitted light using an Axio Imager.M2 universal microscope (Carl Zeiss, Germany) at magnifications of ×50 and ×100.

Micrographs were taken using a Zeiss Axiocam 208 color digital camera (Carl Zeiss) and ZEN Image Analysis Software. One thymus fragment was analyzed from each animal in the group (n=5). Morphometry was performed using a semi-automated image processing software tool ImageJ. In view of the accidental involution of the thymus in CP-treated animals, thymus preparations in the groups "CP" and "CP+BABCF" were analyzed at a magnification of ×100, and in the groups "Control" and "BABCF" at a magnification of ×50.

The relative volume of the thymus cortex and medulla was calculated and the cortex-medulla index was determined. The tissue density of the thymus cortex and medulla was estimated by measuring the average value of the light field illumination intensity (in a.u.) in 3 sectors of the medulla and 5 sectors of the cortex.

For assessment of the regenerative effect of the test compound on the structure of the thymus, the thymus was weighed and the ratio of the weight of the organ to the body weight of the animal ("organ index") ((m thymus, g)/(m animal, g)×100) was determined.

Statistical Analysis

The significance of differences between studied groups was determined using the STATISTICA 12 software package.

Normally distributed data are expressed as the mean of several independent experiments ± standard deviation (M±SD). Non-normally distributed data are presented as median (Me), upper and lower quartiles [Q1–Q3]. The statistical significance of differences was assessed using the non-parametric Wilcoxon-Mann-Whitney test with a Bonferroni correction for the two tests. The results were considered as statistically at $p<0.025$.

RESULTS AND DISCUSSION

Relative Gene Expression

Treatment of animals with CP was accompanied by increased expression of gene, encoding anti-inflammatory TGF-β, stimulating cell differentiation and exhibiting an antitumor effect [7], and GM-CSF, stimulating the regenerative activity of the bone marrow in response to CP administration.

Administration of BABCF to animals resulted in a 3-fold increase in the level of *GM-CSF* expression as compared to intact animals. However, in mice treated with CP after BABCF, *GM-CSF* expression in the bone marrow was lower than in animals treated with CP only. GM-CSF is a cytokine that stimulates granulocytic and monocytic hematopoietic lineages. Data of Table 3 show that CP and BABCF have opposite effects on the expression of *M-CSF* and *GM-CSF*. However, it should be noted, that changes in the *M-CSF* expression are at the level of a statistical trend. The influence of BABCF on the expression of *IL-12*, *IL-10*, *IL-1β*, *TGF-β,* and *TNF-α* in the bone marrow was not found.

Table 3. Relative expression of cytokine genes ($2^{-\Delta Ct}$) in the bone marrow of animals treated with CP and BABCF (n=10)

	Control	CP	CP+BABCF	BABCF	*p* (Control/BABCF); *p* (CP/CP+BABCF)
IL-12	0.035 [0.028; 0.092]	0.054 [0.031; 0.145]	0.051 [0.024; 0.093]	0.042 [0.029; 0.054]	0.970; 0.734
IL-10	0.043 [0.037; 0.054]	0.033 [0.02; 0.043]	0.036 [0.022; 0.050]	0.032 [0.023; 0.065]	0.713; 0.734
IL-1β	0.0004 [0.0001; 0.0020]	0.0001 [0.00001; 0.0015]	0.0004 [0.0002; 0.0010]	0.00076 [0.00039; 0.0016]	0.473; 0.653
TNF-α	0.020 [0.019; 0.044]	0.027 [0.019; 0.029]	0.024 [0.012; 0.029]	0.029 [0.023; 0.032]	0.990; 0.521
GM-CSF	0.00028 [0.00016; 0.00033]	0.0013 [0.00039; 0.00230]	0.00058 [0.00038; 0.00072]	0.0015 [0.00085; 0.00190]	0.021; 0.162
M-CSF	0.00017 [0.000018; 0.000500]	0.00005 [0.000022; 0.000330]	0.000066 [0.000023; 0.000410]	0.000019 [0.000014; 0.000270]	0.273; 0.850
TGF-β	0.00056 [0.00034; 0.00100]	0.00096 [0.00066; 0.00190]	0.0014 [0.00043; 0.00170]	0.00049 [0.00044; 0.00076]	0.910; 0.713

Control is a group of animals treated with 0.9% NaCl+DMSO; CP is a group a animals treated with 0.9% NaCl+DMSO and then with CP. CP+DMSO is a group of animals initially treated with BABCF and then with CP. BABCF is a group of animals treated with BABCF only (see Table 1 for details).

Table 4. Relative expression of cytokine genes ($2^{-\Delta Ct}$) in suspension of peritoneal cells of animals treated with CP and BABCF (n=5)

	Control	CP	CP+BABCF	BABCF	*p* (Control/BABCF); *p* (CP/CP+BABCF)
IL-12	0.020 [0.011; 0.046]	0.015 [0.005; 0.037]	0.073 [0.073; 0.084]	0.094 [0.064; 0.096]	0.022; 0.144
IL-10	0.035 [0.0250; 0.0470]	0.024 [0.0052; 0.0490]	0.038 [0.0300; 0.0570]	0.045 [0.0390; 0.0950]	0.296; 0.296

Table 5. Results of flow cytometry of blood samples of experimental animals (n=5). The values of the relative content of $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ lymphocytes in serum (%), and the ratio of $CD4^+/CD8^+$ lymphocytes are presented

	Control	CP	CP+BABCF	BABCF	*p* (Control/BABCF); *p* (CP/CP+BABCF)
$CD3^+$	42.20 [33.8; 51.8]	26.85 [26.6; 27.4]	40.15 [33.3; 47.0]	43.60 [36.1; 45.9]	0.936; 0.005
$CD3^+CD4^+$	22.85 [20.0; 30.2]	6.85 [6.1; 7.7]	26.15 [21.2; 33.0]	35.60 [33.0; 44.1]	0.689; 0.005
$CD3^+CD8^+$	30.90 [26.5; 44.4]	14.90 [11.7; 17.8]	41.05 [36.0; 53.0]	48.70 [45.7; 51.0]	0.173; 0.005
$CD4^+/CD8^+$	0.73 [0.68; 0.74]	0.48 [0.43; 0.52]	0.58 [0.53; 0.68]	0.72 [0.67; 0.96]	0.109; 0.019

Administration of BABCF caused a 4-fold increase in the relative expression of *IL-12* by the peritoneal cavity cells as compared with control (Table 4). This may indirectly indicate its immunoactivating effect, as well as its ability to attract cells producing IL-12 to the injection site.

Flow Cytometry of Peripheral Blood Samples

The flow cytometry analysis of a suspension of MNC cells, provided information about the relative content of T-lymphocyte fractions ($CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$) (Table 5), and the $CD4^+/CD8^+$ ratio was calculated.

The number of cells in blood samples of CP-treated animals was significantly lower than in intact mice. CP reduces the absolute number of blood leukocytes, acting mainly on the lymphocyte lineage of hematopoiesis, namely T-lymphocytes [9].

It has been found that BABCF was able to restore the relative content of $CD3^+$ lymphocytes (T-lymphocytes), which was reduced after CP administration. The content of T-lymphocytes in the blood of animals of the group "CP+BABCF" was about 1.6 times higher than in CP-treated animals. At the same time, BABCF mainly stimulated growth of the $CD3^+CD4^+$ cell subpopulation.

In the "CP+BABCF" group, the content of $CD3^+CD4^+$ cells (T-helpers) was about 5.6 times higher than in the group of CP-treated animals. At the same time, the content of $CD3^+CD8^+$ cells in the same group showed only a 3.2-fold increase after the administration of BABCF (Fig. 2).

The direction of action to T-helpers is also demonstrated by the increase in the ratio of $CD4^+/CD8^+$ after the introduction of the test substance. Interestingly, the animals treated with only BABCF had approximately the same $CD4^+/CD8^+$ ratio and blood $CD3^+$ levels as the control mice (Table 5).

The Histological Examination of the Thymus

Treatment of mice with CP caused atrophy of the thymus cortex, blurring of its contours, and a decrease in the cell density both in the thymus cortex and in the thymus medulla (Fig. 3). Analysis of the cortex-medulla ratio of the thymus showed that CP reduced the volumetric density of the thymus cortex. At the same time, it was found that the test compound did not promote the restoration of the volume fraction of the thymus cortex after CP treatment, however, in the mono mode it reduced it (p=0.017) (Table 6).

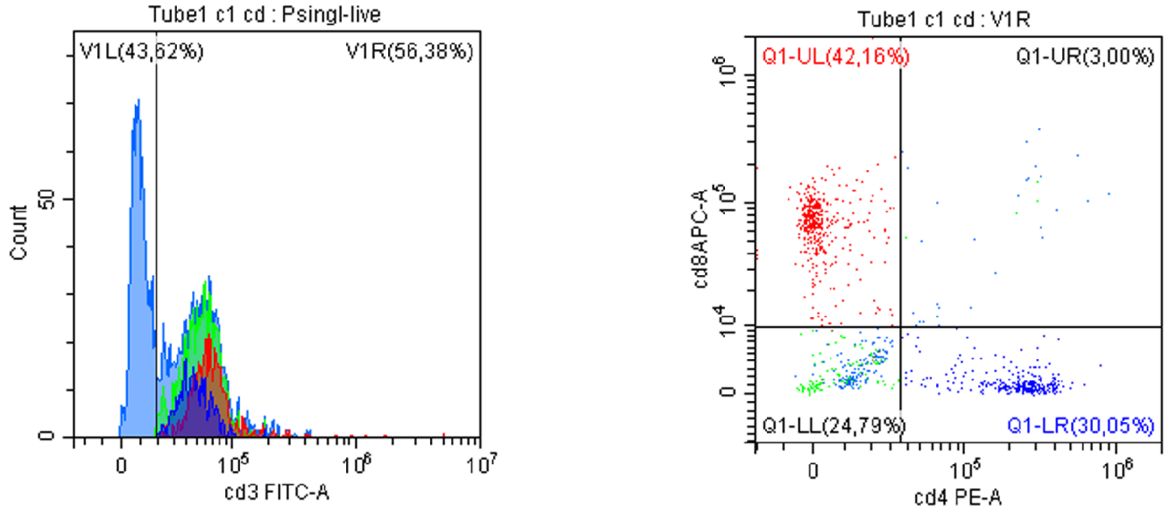

Figure 2. Fractionation of mononuclear cells with FITC-labeled anti-CD3, PE anti-CD4, APC anti-CD8 antibodies. On the left, the total yield of $CD3^+$ cells is shown (56.38% live cells, 43.62% debris). On the right, the subpopulation of $CD3^+CD8^+$ cells (cytotoxic T-lymphocytes, T-killers) is displayed in red, the subpopulation of $CD3^+CD4^+$ cells (T-helpers) is displayed in blue. An example of flow cytometric analysis of an animal blood sample after administration of BABCF is given. The color version of this figure is available in the electronic version of this article.

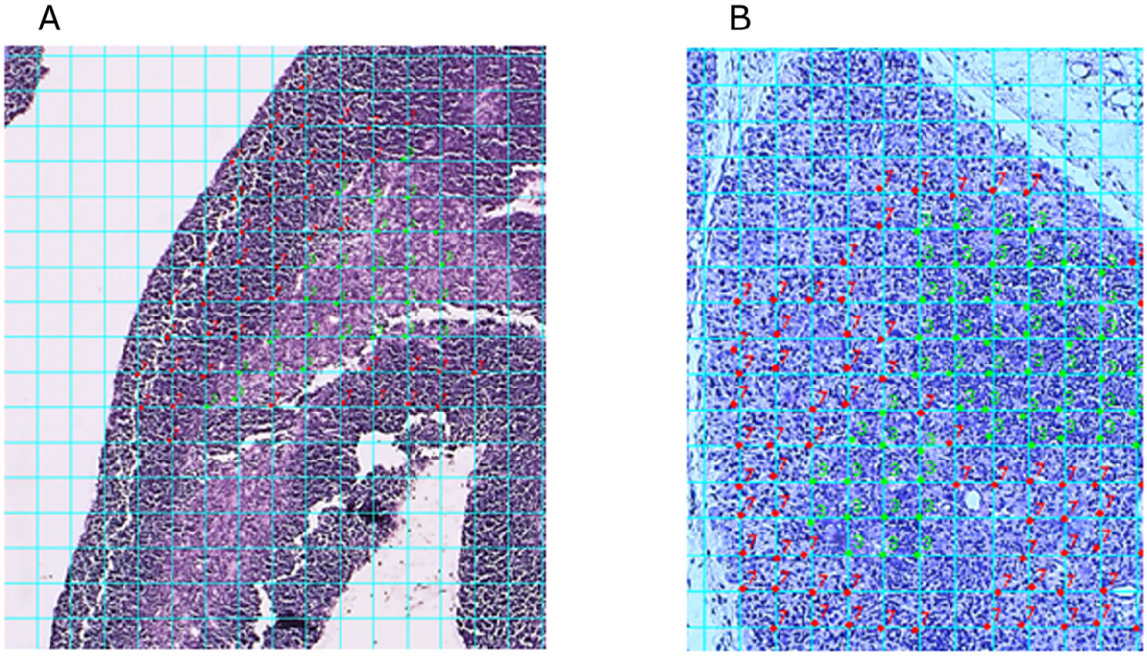

Figure 3. The structure of the thymus of control C57BL/6 mice (**A**; magnification ×50) and CP-treated C57BL/6 mice (**B**; magnification ×100). An example of the analysis of the of the cortex-medulla ratio. Staining with hematoxylin and eosin.

Table 6. The ratio of the volume fractions of the cortex and medulla of the thymus of animals treated with CP and BABCF. Analysis of the density ("cellularity") of the cortex and medulla of the thymus in groups of experimental animals (n=5)

	Control	CP	CP+BABCF	BABCF	*p* (Control/BABCF); *p* (CP/CP+BABCF)
Cortex-medulla index	3.28 [2.73; 7.65]	1.50 [1.19; 2.08]	1.50 [1.09; 1.86]	2.93 [2.32; 4.28]	0.017; 0.169
Cortex density, a.u.	1.73 [1.62; 1.83]	1.84 [1.72; 1.99]	2.00 [1.81; 2.17]	1.62 [1.48; 1.80]	0,026; 0.011
Medulla density, a.u.	2.10 [2.00; 2.27]	1.66 [1.60; 1.80]	1.81 [1.66; 1.99]	2.06 [1.90; 2.29]	0.361; 0.003

Table 6 shows the values of the illumination intensity (in a.u.) of the light field in the cortex and medulla of the thymus (in 10 fields of view for each preparation). The tissue density was inversely proportional to the intensity of its illumination, so the higher the intensity, the lower the density was. Apparently, due to a decrease in the cell density, the illumination of the cortex and medulla of the thymus in the group of CP-treated animals was higher (i.e. the tissue density was lower) than in animals of the control group. However, in animals treated with CP and BABCF there was a significant increase in the average intensity of illumination of the cortex and medulla as compared to the CP-treated animal. Consequently, the tissue density of the cortex and medulla of the thymus in the in animals treated with CP and BABCF was even lower than in the CP-treated mice.

According to the literature data, treatment with CP was accompanied by the thymus "exhaustion", a decrease in the number of proliferating lymphocytes of the thymus cortex, and inclusion of connective tissue in its structures [9]. In our experiment, this was confirmed by a decrease in the cortex-medulla index in CP-treated animals, and a decrease in the tissue density of the thymus cortex and medulla.

The calculation of the thymus organ index has shown that administration of CP caused about 3-fold decrease in the thymus weight as compared with the control animals. No effect of BABCF on thymus weight after CP injection was found (Fig. 4). Administration of BABCF also did not have any significant effect on the thymus weight in CP-untreated mice.

The density of thymus tissues in animals treated with CP+BABCF was lower than in CP-treated animals. Taking into account the flow cytometry data, it can be assumed that the effect of BABCF is aimed at maintaining the number of T-lymphocytes circulating in the blood, including their increased output from the thymus (mainly from the cortical zone of the thymus, due to a decrease in the cortex-medulla ratio induced by BABCF). In the mouse bone marrow, BABCF increased the content of GM-CSF mRNA. GM-KSF is produced by various cells of the body:

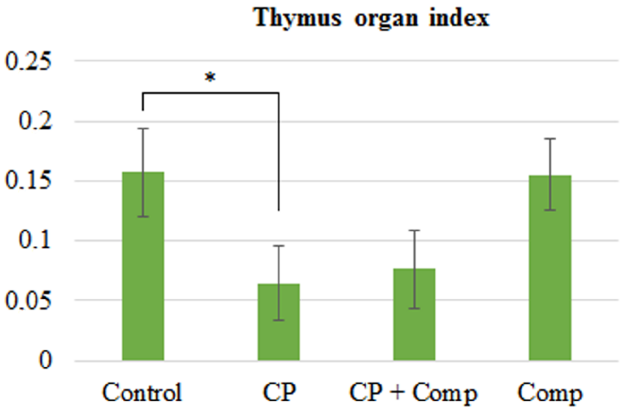

Figure 4. The thymus organ index in groups of experimental animals ((thymus weight, g)/(animal weight, g)×100). The asterisk * shows statistically significant differences between groups (p<0.025)

neutrophils, macrophages, eosinophils, stem cells, etc. The expression of this factor is stimulated by inflammatory mediators (IL-1, IL-6, and TNF-α). Receptors for GM-KSF are expressed on the surface of hematopoietic cells (CFU of granulocytes) [7]. The biological role of GM-KSF consists in stimulation of the growth and differentiation of hematopoietic cells, mainly of the myeloid lineage and to a lesser extent the monocytic lineage. This cytokine increases the functional activity (oxidative metabolism, cytotoxicity) of neutrophils, monocytes, and macrophages; it increases their number in peripheral blood, and prevents apoptosis of their precursors [7]. During the experiment, the effects of CP and BABCF on the *GM-KSF* gene expression were similar. Some studies have shown that after exposure to CP, bone marrow cells, in response to a sharp decrease in the number of peripheral blood leukocytes, increased *GM-KSF* expression [10]. It is reasonable to suggest that *GM-KSF* expression is inhibited after treatment with CP and BABCF due to a decrease in the number of cells producing this cytokine. Administration of BABCF had no effect on the expression of *IL-1β*, *IL-12*, *TNF-α*, and *IL-10*. The first three genes encode pro-inflammatory cytokines, which are produced mainly by macrophages in response to components of viruses and bacterial LPS;

the *IL-10* gene encodes a cytokine that suppresses the inflammatory response. There is little information in the literature about the production of these cytokines in the bone marrow exposed to cytostatics, but a compensatory increase in their production has been reported [10].

BABCF increased the content of T-lymphocytes in the blood and the $CD4^+/CD8^+$ ratio in mice treated with CP and BABCF. Therefore, the BABCF effect is aimed at maintaining the number of circulating $CD3^+CD4^+$ T-lymphocytes. This is also supported by the fact that this substance promoted release of T-lymphocytes from the thymus gland. In animals treated with CP and BABCF, the density of the tissues of the cortical and medullar zones was even lower than in CP-treated mice. The thymus cortex contains mainly immature and proliferating T-lymphocytes that have migrated from the bone marrow. They pass through sequential stages of selection, during which cells, containing receptors for self-antigens, are eliminated by apoptosis. The medulla contains mature cells ready to leave the thymus and populate the organs of the peripheral immune system (i.e., the lymph nodes). Thus, BABCF replenishes the pool of circulating T-lymphocytes by both young forms and mature T-lymphocytes. $CD3^+CD4^+$ lymphocytes (or T-helpers) play a major role in the immune response to extracellular antigens. They are able to be activated upon contact of their own T-cell receptors with an antigen in combination with MHC-I present on the membrane of the antigen-presenting cell. After activation, T-helpers (Th2 subpopulation), interacting with B-lymphocytes via CD4 and CD40L, trigger B-lymphocyte transformation into the antibody-producing plasma cells. A full humoral response requires cooperation of $CD3^+CD4^+$ and B-lymphocyte. In addition, T-helpers (Th1-subpopulation) through the production of IL-2, IFN-γ, and TNF-α contribute to the activation of $CD3^+CD8^+$ (T-killers)-cytotoxic cells. Thus, without a sufficient content of $CD3^+CD4^+$ cells in the body, neither humoral nor cellular immunity is possible. HIV infection is one of the most formidable conditions accompanied by a deficiency of T-helpers is [8]. It is possible that BABCF can be considered as a potential corrector of the immunodeficiency states, including the cases of profound immunodeficiency.

Intraperitoneal administration of BABCF caused a 4-fold increase in the relative expression of *IL-12* by the peritoneal cavity cells as compared with the control mice. This indicates an immunoactivating effect of the tested compound, since this cytokine is a stimulator of the cellular link of acquired immunity [7]. It is possible that this effect is due to attraction of macrophages, the main producers of this cytokine, into the abdominal cavity. These results are consistent with our earlier data on the increase in *IL-12* expression by U-937 cell culture in response to the action of BABCF [11]. In addition, BABCF was shown to reduce the expression of the anti-inflammatory cytokine *IL-10* gene in U-937 cells. This effect was not noted in the animal peritoneal cell model, apparently due to the heterogeneous cellular composition of the peritoneal lavage.

In the past few years, the anti-inflammatory activity of BA has been recognized. This was confirmed by studies on cell cultures, where BA inhibited the production of inflammatory mediators, such as IL-1β, IL-6, iNOS, prostaglandins (by suppressing COX-2 activity). It was found that most of these effects were due to inhibition of the nuclear transcription factor NF-κB [2].

In general, the hybrid compound of BA and furocoumarin retains the anti-inflammatory properties of BA. However, it was shown in [6] that the introduction of an additional substituent at the C9 position of oreoselon furocoumarin in combination with BA (BABCF) led to the loss of its anti-inflammatory activity. This is consistent with our data on the absence of a decrease in the expression of pro-inflammatory cytokines after treatment with this substance [6].

CONCLUSIONS

The following conclusions can be drawn from the results obtained in this study:

1. The hybrid compound of betulonic acid and furocoumarin stimulates the expression of *GM-KSF* in the bone marrow; this indicates its stimulating effect on myeloid progenitor cells (CFU).

2. This compound has a stimulating effect on T-lymphocytes; this effect is especially pronounced on $CD3^+$,$CD4^+$-lymphocytes (T-helpers). The tested compound increased the $CD4^+/CD8^+$ ratio and restored the relative content of T-lymphocytes in peripheral blood after treatment of mice with CP.

3. The tested compound increased the expression of *IL-12* in peritoneal lavage cells. This suggests stimulation of the cellular component of acquired immunity. It is possible that the tested compound attracts macrophages to the injection site.

The direction of the action of the test compound in relation to the cellular link of acquired immunity is confirmed by an increase in the expression of the leukocyte hematopoietic factor (*GM-KSF*) gene in the bone marrow, an increase in the content of circulating blood T-lymphocytes under conditions of experimental artificial immunosuppression and a local increase in the expression of the *IL-12* gene at the injection site. This compound should be considered as a candidate for the role of a potential immunostimulant in primary and secondary immunodeficiency states (AIDS, post-chemotherapy, long-term glucocorticoid therapy, etc.).

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COMPLIANCE WITH ETHICAL STANDARDS

The animals were kept in accordance with the interstate standard GOST 33216-2014 and the European Convention for the Protection of Vertebrate Animals Used in Experiments and for Other Scientific Purposes. The publication of the material was approved by the Biomedical Ethics Committee at the Federal Research Center for Fundamental and Translational Medicine (No. 20 at the meeting dated June 28, 2023). During the experiment, the animals were kept under standard vivarium conditions under artificial illumination (12:12 light/dark cycle, in plastic cages with fine wood shavings, and received standard granulated chow (PK 120-3) and water *ad libitum*.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ИММУНОМОДУЛИРУЮЩАЯ АКТИВНОСТЬ СОЕДИНЕНИЯ БЕТУЛОНОВОЙ КИСЛОТЫ

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Изучена иммуномодулирующая активность одного из соединений бетулоновой кислоты с фурокумарином (2-азидо, 9-N-метилпиперазинометил ореозелоном) (далее Соед). Экспериментальной моделью служили самцы мышей линии C57BL/6 (возраст 3 месяца), подверженные воздействию цитостатического средства — циклофосфамида (ЦФ), контролем — интактные особи. Исследовали экспрессию *IL-12*, *IL-10*, *IL-1β*, *TNF-α*, *TGF-β*, *M-CSF*, *GM-CSF* в костном мозге, *IL-12*, *IL-10* во взвеси перитонеальных клеток (место введения Соед). Поверхностные маркеры Т-лимфоцитов (CD3, CD4 и CD8) во фракциях мононуклеарных клеток (МНК) венозной крови определяли с помощью проточной цитометрии с использованием антител. Для оценки воздействия ЦФ и Соед на тимус были выполнены его гистологическое и морфометрическое исследования. Выявлено стимулирующее влияние Соед на экспрессию гена *GM-KSF* (повышение относительной экспрессии в среднем в 3 раза). Соед вызывает локальное повышение экспрессии *IL-12* в клетках брюшной полости и восстанавливает относительное содержание Т-лимфоцитов в крови мышей, обработанных ЦФ, воздействуя главным образом на $CD3^+CD4^+$ лимфоциты. Показано, что исследуемое вещество снижает плотность ткани коркового и мозгового вещества тимуса у группы мышей, которым введён ЦФ. Полученные результаты свидетельствуют о том, что Соед оказывает стимулирующее влияние на клеточное звено иммунитета и способствует поддержанию числа Т-лимфоцитов в крови за счёт их миграции из центральных органов иммунной системы.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: бетулоновая кислота; фурокумарины; цитокины; костный мозг; тимус; Т-лимфоциты

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