# MULTI-VECTOR AND MULTI-LEVEL INTERACTIONS OF GA-40 PEPTIDES AND THEIR UNIQUE PROPERTIES: SELF-ASSEMBLING AND TENDING FOR HARMONIZATION

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ABSTRACT

The article reveals the main biochemical mechanisms of immune cascades activation using plant-derived peptides of the GA-40 preparation. In studies on volunteers, it was found that peptides GA-40 induce the synthesis of tumor necrosis factor alpha (TNF-α) in the presence of protein kinases, reduce the intracellular concentration of hydrogen peroxide and stimulate the activity of factor NF-kB. The results obtained allow us to reasonably assume that the peptides maintain the necessary balance of inducing and inhibitory reactions in the cell and, therefore, the activation of immunocompetent molecules, in particular NF-kB, proceeds along the most efficient pathway. In other words, in the case of insufficient cell activity in counteracting to damaging factors and pathogens, regulatory peptides increase the strength of the immune response, and in the case of hyper-response of cellular systems to damage and when high risks of excessive apoptosis or autoimmune processes occur, GA-40 reduces the intensity of interactions. This is due to the unique evolutionarily formed properties of peptides - self-assembling and a tending to harmonization. GA-40 provides multivector and multilevel interactions in immune cascades, which are aimed at improving the viability of normal cells and the destruction of cells dangerous to the macroorganism. This allows to significantly expand the spectrum of clinical use of the drug: from infectious-inflammatory and autoimmune processes to hormonal disorders and cancer.

**Key words:** self-assembling, GA-40, plant peptides, immune cascades, NF-kB, cancer, apoptosis.

The mechanisms of action of GA-40 have been studied in laboratories and clinics in different countries for more than 20 years. Of greatest interest are the biochemical properties of the preparation established during experiments: its ability to increase the production of the proinflammatory cytokine TNF-alpha (TNF-α) in the presence of protein kinase inhibitors and tyrosine protein kinases, its ability to influence the intracellular production of hydrogen peroxide, and also stimulate the detachment of the inhibiting of p50-p65 subunit from the complex NF-kB, that naturally leads to the implementation of immune cascades.

GA-40 is the first gene protector and immunocorrector based on regulatory plant peptides, which is used in clinical practice. It is known that peptides GA-40, due to their relatively small size (15-98 kDa), can act not only on membrane receptors, but also penetrate into cells through the pores of the membrane. This allows them to interact with intracellular structures: regulatory peptides, such as factor NF-kB, various ligands and enzymes.

The uniqueness of GA-40 lies in the fact that plant peptides under conditions of reduced antitumor immunity, due to aggressive tumor activity or after antitumor chemotherapy, restore immune surveillance and promote apoptosis of cancer cells [1]. Under opposite conditions, that is, under conditions of hyperproduction of immunocompetent molecules in inflammatory processes, in particular in the liver and kidneys (with hepatitis, cirrhosis, nephritis), plant peptides are able to reduce the intensity of peptide-peptide interactions and prevent unwanted damage to healthy cells [2,3] . This paradox can be explained by the evolutionarily formed properties of regulatory peptides - self-ordering and the desire for harmonization. The history of the discovery and study of the immunotropic and genoprotective properties of GA-40 is set forth in the monograph by Professor George Aleksidze “GA-40. New immunotherapeutic and anticancer drug ”, which was published in 2014 in the USA - / Lambert Academic Publishing 02/12/2014 /.

**Induction of TNF-a synthesis**

At a certain stage, the possible mechanisms of the induction of TNF-alpha by the GA-40 preparation were investigated, in particular, the dependence of this process on protein kinases and tyrosine kinases, the main participants in intracellular signal transmission.

Protein kinase C (PKC) is a family of Ca2 + / phospholipid-dependent serine-threonine kinases that play an important role in signal transmission and regulation of cell growth, differentiation and apoptosis. [4,5,7,10,11] At least 12 PKC isoforms that differ in structure, cell diversity and biological functions. [6,8,9]

Tyrosine protein kinases (TPK) are participants in signal transduction involving nuclear transcription factors at the final stage, resulting in a modification of the expression of specific genes, the effect of which is manifested in the regulation of cell growth and differentiation. [12-15]

There are 2 types of TPK: receptor and non-receptor. The first type includes 19 classes of protein kinases, which are most often receptors of growth factors (epidermal growth factor, platelet growth factor, insulin, insulin-like growth factor, etc.). Non-receptor TPKs include 9 families (Jak kinases, Srs kinases, Syk / Zap70 kinases, FAK kinases, etc.). [16,17]

For inhibitory analysis, staurosporin (antibiotic AM-2282), the main non-selective inhibitor of PKC and genistein (4 ', 5,7, trihydrodroxy-solflavone), a highly specific inhibitor of TPK, a competitive inhibitor in various protein kinase reactions, was used.

Materials and methods

The study involved 8 healthy volunteers aged 24 to 33 years.

Blood was taken in the morning on an empty stomach from the ulnar vein into a test tube with heparin at a concentration of 20 Units / ml.

Mononuclear cells (MNCs) were isolated using a single-stage density gradient of ficoll-verographin (d = 1.077 (PanEco) by the method of Boyum A., 1968. For this, heparinized blood was diluted twice with medium 199 (PanEco) and layered on ficoll. The tubes were centrifuged for 20 minutes 400g at t of 20-25 ° C. Cells at the interphase were sterilely harvested and washed twice in 199 medium. Cell viability after isolation was 98% -99% (by staining with 0.1% trypan blue (Serva) solution). diluted to a concentration of 2x106 cells / ml with complete culture medium (RPM I 1640 (Sigma), 10% inactivated fetal calf serum (Sigma) and 2 mM L-glutamine (Sigma)).

Staurosporin (Sigma) inhibitors (10, 100, 500 nM) and genistein (Sigma) (1, 10, 100 μM) for 1 h were added to 4x105 cells for 1 h, then activators of the name-α production (100 ng / ml LPS and 100 μg / ml of “GA-40”) and incubated for 48 hours in 5% CO2 at 37 ° C.

The production of TNF-α cytokine was determined in supernatants of cell cultures using enzyme-linked immunosorbent assay using a commercial test system of the company "Vector Best" (Russia).

Results.

Pretreatment of MNCs for 1 h with staurosporine in the studied doses caused a significant decrease in the production of TNF-α stimulated by GA-40, similarly to LPS-induced. Inhibition was dose-dependent in nature and at the maximum doses of staurosporine (200 and 500 nM) led to a decrease in cytokine synthesis to the background level during both GA-40 and LPS induction. Spontaneous cytokine production ranged from 0 to 48 pg / ml. With a minimum used dose of staurosporin of 10 nM, the least inhibition of

TNF-α production was observed (4.5 times for GA-40 and 9 times for LPS) (Table 1, Fig. 1).

Genistein, a tyrosine protein kinase inhibitor, also reduced the production of TNF-α in a dose-dependent manner in all tested doses under the action of the studied drug and LPS on mononuclear cells. So, with a minimum dose of genistein 1 μM, a similar decrease in the cytokine synthesis for GA-40 and LPS was 2 times, with a maximum dose of an inhibitor of 200 μM, almost complete suppression of TNF-α production to a spontaneous level was noted (a decrease of 16 and 21 times respectively) (Table 1, Fig. 2).

Table 1. Production of TNF-α (PCG / ml) by peripheral blood mononuclear cells of healthy volunteers. MNCs were pretreated with protein kinase or tyrosine kinase inhibitors (staurosporine or genistein) for 1 h, then incubated in the presence of GA-40 or LPS for 48 h (M ± m).

| Inhibitor \ Inductor | | GA-40, 100 mcg / ml | LPS 200ng / ml |
|---|---|---|---|
| Staurosporin, nM | 0 | 534±258,4 | 406±112,2 |
| | 10 | 118±31,5* | 43±11,4* |
| | 100 | 60±19,2* | 15±4,5* |
| | 200 | 38±19,9* | 12±3,1* |
| | 500 | 26±16,6* | 10±3,8* |
| Geninstein, microns | 0 | 534±258,4 | 406±112,2 |
| | 1 | 264±194,7* | 209±39,2* |
| | 10 | 121±79,3* | 88±39,7* |
| | 100 | 58±35,8* | 41±20,6* |
| | 200 | 34±9,2* | 19±8,6* |

* - significant differences compared with the values obtained in the absence of inhibitors, $p < 0.05$

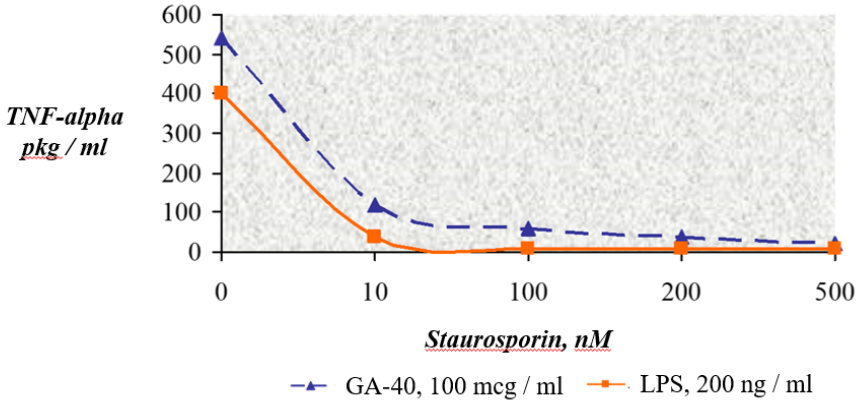


Figure 1. Production of TNF-α (PCG / ml) by peripheral blood mononuclear cells of healthy volunteers in the presence of staurosporin.

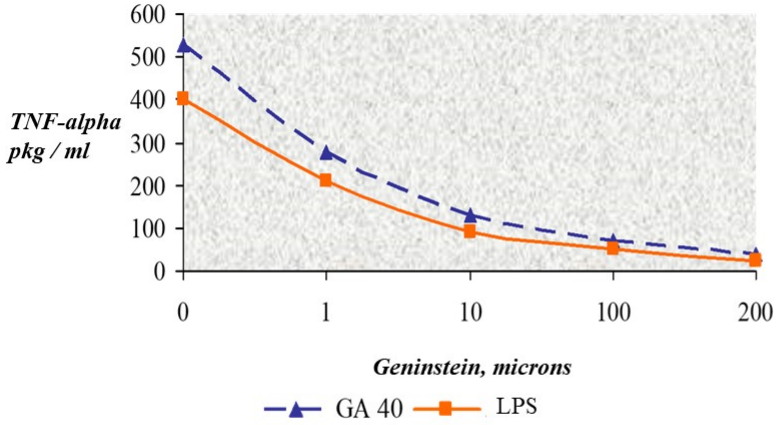


Figure 2. Production of TNF-α (pkg / ml) by mononuclear cells of the peripheral blood of healthy volunteers in the presence of genistein.

**Hydrogen peroxide production**

The effect of GA-40 on the intracellular production of hydrogen peroxide by peripheral blood cells was also studied. It is important to note that the intracellular accumulation of oxygen radicals indicates an increase in the bactericidal potential of the cell, activation of a number of signaling molecules that trigger the protective mechanisms of the immune system. An intracellular increase in the level of hydrogen peroxide is key for

the activation of a number of signaling molecules [Junn et al., 2000; kim et., 2001; Koh et al., 2001]. The participation of hydrogen peroxide in the activation of an important transcription factor NF-kB is known [Kamata et al., 2002]. Also, it should be noted that hydrogen peroxide (H2O2) is a non-radical active form of oxygen that is formed in many pathological and physiological conditions. At present, hydrogen peroxide is universally recognized as an important mediator of redox processes. Depending on its spatiotemporal accumulation profile, this molecule can act as a signal messenger or cause oxidative damage. The ability of a cell to resist oxidative influences determines its resistance to carcinogenic and other damaging factors [18,19].

An important aspect is a comprehensive assessment of the data that peroxisomes, organelles, best known for their role in cellular lipid metabolism, also serve as centers in the H2O2 signaling network. A deeper understanding of how peroxisomes integrate into the H2O2 cell signaling network is key to determining the exact the role of production and removal of peroxisomal H2O2 in normal and pathological conditions. [20,21,24] The primary messenger effect of H2O2 depends on its ability to oxidize various target proteins with a high degree of specificity, mainly through a reaction with nucleophilic thiolate cysteine groups (Cys-S-), which can be found in specific protein microenvironments. Oxidation of such deprotonated cysteine residues can lead to the formation of unstable sulfenic acid intermediates (-SOH), which can again be reduced and react with other proximal thiol groups to form intra- or intermolecular disulfide bonds. [22,23,25]. The formation of a disulfide bond can cause conformational changes, leading to changes in macromolecular interactions, protein localization, their functional needs, activity and stability. [26,27]

The method for assessing the accumulation of intracellular hydrogen peroxide is based on the ability of fluorochrome to glow after its oxidation by various forms of oxygen products. The dye, dichlorofluorescein diacetate (DCF-DA), which is initially non-fluorescent, passively penetrating into the cell, is treated with esterases - the acetate group is cleaved and it passes into the polar compound, which is unable to diffuse back from the cell. When interacting with hydrogen peroxide generated during an oxygen “explosion”, DHF-DA turns into a fluorescent compound (green spectrum region), which allows you to analyze cells by the intensity of the dye glow using flow cytofluorimetry.

Materials and methods

The study involved 8 healthy volunteers. Blood was taken

in the morning on an empty stomach from the cubital vein into a test tube with heparin at a concentration of 10 U / ml.

To 4 ml of heparinized blood was added 2 ml of 3% gelatin in phosphate-buffered saline (PBS) (0.15 M NaCl, lM K2HPO4, lM KH2PO4, pH 7.4), stirred and incubated for 10-15 minutes at 37 ° C for erythrocyte sedimentation. White blood cells from the supernatant were collected and washed twice in phosphate-buffered saline (PBS). The leukocyte concentration was adjusted to 2x106 / ml for segmented neutrophils in the FSB.

Assessment of the accumulation of intracellular peroxide

A study of the effect of the GA-40 preparation on the production of healthy donors by the leukocytes of intracellular peroxides was carried out according to the method described in Bass et al., 1983. Leukocytes were pre-incubated with DHP-DA in the presence of sodium azide for 20 min., At 37 ° C 96- well plate (Nunc). Then, GA-40 was added at a concentration of 100, 10, 1 μg / ml, phosphate-saline buffer and phorbol-12-myristate-13-acetate (FMA) as a positive control. After a 30-minute incubation at 37 ° C, the cells were pelleted by centrifugation. The red blood cells were destroyed by lysis buffer (0.15 M NH4 Cl, 0.01 M NaHCO 3, 0.1 mM Na 2 EDTA, pH 7.4) and the cells were washed with PBS. Samples were analyzed using a FACSCalibur flow cytometer in the CellQuest program on channel FL1. As a result of the analysis of the samples, indicators of the average fluorescence intensity were evaluated.

Results

The results are presented in table 2. It can be seen from the table that the GA-40 preparation of 10 and 100 μg / ml causes a decrease in the level of intracellular hydrogen peroxide in the neutrophils of the peripheral blood of healthy donors. When using "GA-40" at a concentration of 100 μg / ml, a significant decrease in this indicator of 27% was observed, and 1 μg / ml had no effect. Figure 4 presents an example of a cytofluorogram demonstrating the accumulation of hydrogen peroxide in the neutrophils of a healthy donor under the influence of FMA and GA-40.

Table 2. The effect of the “GA-40” preparation on the accumulation of intracellular hydrogen peroxide by neutrophils and peripheral blood monocytes of healthy donors. The average fluorescence values in the green spectrum are indicated, M ± m.

| Test cells | control | FMA | «GA-40», µg / ml | | |
|---|---|---|---|---|---|
| | | | 100 | 10 | 1 |
| neutrophils | 70,5±7,13 | 377,6±162,55* | 51,0±11,07* | 58,4±12,86 | 69,3±25,06 |
| monocytes | 53,5±4,84 | 101,8±9,24* | 38,4±5,95 | 36,0±6,98 | 43,9±11,52 |

* - significant differences compared with control, p <0.5

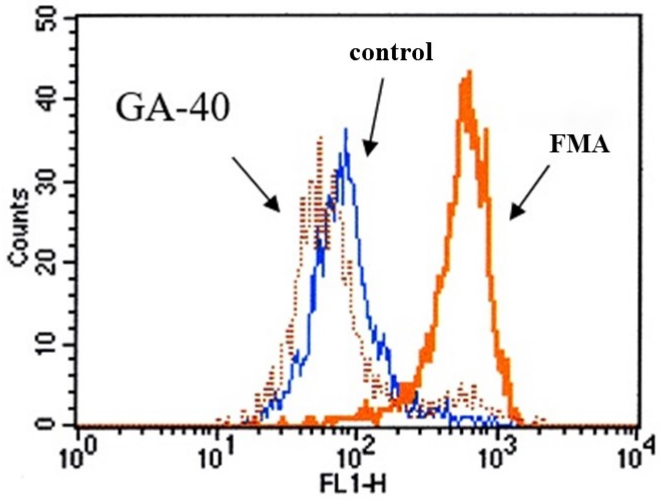


Figure 4. The effect of the GA-40 preparation at a concentration of 100 µg / ml on the content of intracellular hydrogen peroxide in the neutrophils of the peripheral blood of a healthy donor, the abscissa axis shows the fluorescence intensity in the green spectrum, and the ordinate axis shows the number of cells.

**NF-Kb expression**

Interesting are the results of a study of the expression of Nuclear Factor kB (NF-KB) under the influence of GA-40. It's known that the five components of NF-kB families are prominent mediators of inflammation, acting as key regulators of the transcription of hundreds of genes. Several signaling pathways activated by various stimuli converge upon activation of NF-kB, which leads to systemic and complex regulation of immune cascades. [28.30]. It is increasingly recognized that the number of components that affect the

phenotypic results of signal transmission paths may be higher than those that are taken into account to date. [29,31,36]. In vertebrates, NF-kB is activated by more than 150 different stimuli - such as stress, cytokines, free radicals, ultraviolet radiation, oxidized LDL, bacterial or viral antigens. In turn, there is evidence that active NF-kB is involved in transcriptional control of more than 400 genes. These genes include cytokines, chemokines and their modulators, immunoreceptors, proteins involved in antigen presentation, cell adhesion molecules, acute phase proteins, stress response proteins, cell receptor peptides, apoptosis regulators, growth factors, ligands and their modulators, early proteins response, transcription factors, their regulators and enzymes that control several phenomena, such as inflammation, as well as an innate and adaptive immune response. [32-34]

Given the central role of NF-kB in maintaining cellular homeostasis, it is not surprising that due to the violation of its finely tuned modulation, different pathologies often develop: cancer, autoimmune diseases and chronic infectious and inflammatory diseases. [35,37].

## Materials and methods

Stimulation of cells and preparation of a nuclear extract: the study was performed on cells of human monocytic lymphoma U937. Cells were diluted in PCB and incubated with or without stimulants (LPS E. coli (Sigma) 1 μg / ml, GA-40 100 μg / ml) in microtubes (Eppendor) in the amount of 1x106 cells per sample in an atmosphere of 5% CO2 at 37 ° C 10 min. Then the cells were pelleted by centrifugation, the supernatant was taken, and the cell pellet was lysed in 150 μl of hypotonic buffer (10 mM HEPES (Sigma), 10 mM KCl, 0.2 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, tablet protease inhibitors (Sigma), 0, 05% Triton-X100, 1mM dithiothritol) 5 min. in the cold. To precipitate cell nuclei, the samples were centrifuged at 3000 rpm for 15 min, the supernatant was collected, and 50 μl of lysis buffer (20 mM HEPES (Sigma), 0.4 M NaCl, 1 mM EDTA (PanEco), 20% glycerol, 1 mM phenylmethyl sulfonyl fluoride (Sigma) were added to the precipitate. , tablet protease inhibitors (Sigm), 0.1% Triton-X100, 1mM dithiothritol (LOBA)). Lysis was carried out for 1 h at + 4 ° C with constant stirring. Then the samples were mixed with sample buffer (0.5 M Tris-HCl, pH 6.8, DDS-Na 2%, 0.1% bromphenol blue, 20% glycerol, 0.05% mercaptomethanol) in a 1: 2 ratio and warmed up 5 min at + 95 ° C.

Western blotting

DDS electrophoresis was performed on a 12% polyacrylamide gel in an Mini-PROTEAN II electrophoresis cell (BioRad), after which proteins were transferred onto a nitrocellulose membrane (BioRad) in a Trans-Blot® SD dry transfer system (BioRad). The

membrane was blocked in a 2% bovine serum albumin solution overnight at + 4 ° C. The membrane was then washed with washing buffer (10 mM Tris-HC1, pH 7.4, 0.05% Tween-20) and incubated in a solution of anti-NF-kB antibodies (mouse antiNF-kV p65, Pharmingen) for 2 hours at room temperature with shaking . At the end of the incubation, the membrane was washed and horseradish peroxidase conjugate solution (antimouse Ig-HRP, Pharmingen) was added for 1 h at room temperature with shaking. After washing, the membrane was stained with a substrate mixture with chloronaphthol.

Results

As can be seen from Figure 6, in the samples where the GA-40 stimulation was carried out, bands corresponding to a molecular mass of 65 kDa and similar to the positive control applied to antibodies to NF-kB were detected. Moreover, in spontaneous samples, such bands are of much lower intensity. Based on these results, it can be concluded that the addition of the GA-40 preparation to the U937 cell line causes the inhibitory subunit to detach from the NF-kB p50-p65 complex, as a result of which the p65 subunit migrates to the nucleus, where it was detected as a result of the experiment.

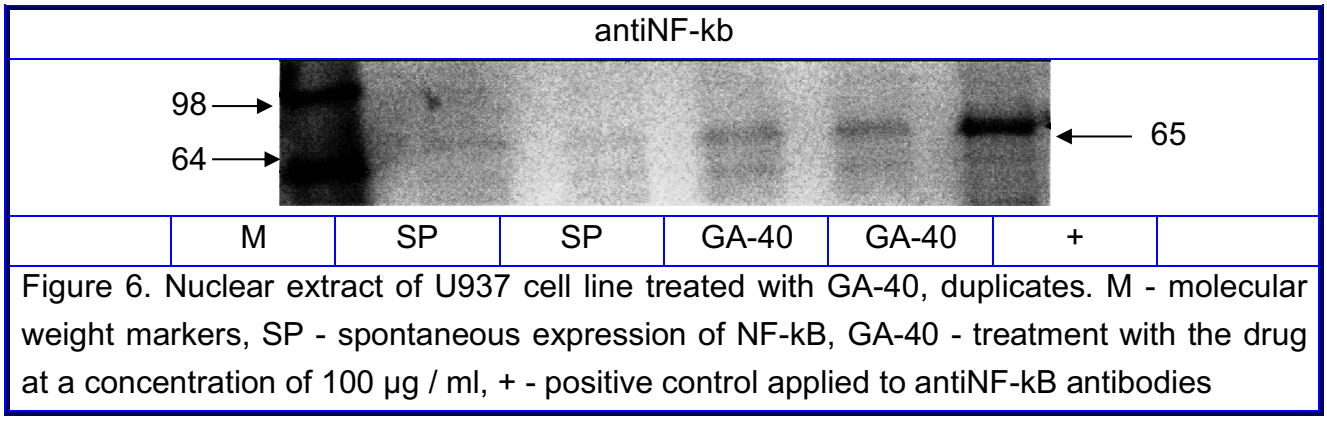


Figure 6. Nuclear extract of U937 cell line treated with GA-40, duplicates. M - molecular weight markers, SP - spontaneous expression of NF-kB, GA-40 - treatment with the drug at a concentration of 100 μg / ml, + - positive control applied to antiNF-kB antibodies

**Discussion**

The aim of this work was to study the possible mechanisms of action of the drug "GA-40". In particular, it was found that the average and high dose of the drug cause a significant increase in the production of TNF-alpha. Many studies describe the involvement of kinases of the PKC and TPK family as monocyte / macrophage signal-transmitting molecules necessary for the production of cytokines in response to LPS. We hypothesized that PKC and TRK could also participate in GA-40-mediated cell signaling when this drug induces TNF-a synthesis. To determine the signal components involved in GA-40-mediated production of TNF-a by human blood mononuclear cells, we investigated the

contribution of PKC and TPK using pharmacological inhibitors staurosporin and genistein, respectively.

Staurosporin and Genistein inhibit the production of TNF-a activated by the GA-40 test drug. This may indicate that PKC and TPK are involved in the regulation of cytokine production induced by GA-40.

Among PKC, both Ca2 + -dependent and Ca2 + -independent kinases are distinguished. The first type includes traditional PKC - α, β1, β2 and γ, which require activation of Ca2 + in the presence of phosphatidylserine - phospholipid, which is part of the cell membrane. This class of kinases is activated by diacylglycerol (DAG) and phorbol esters. The second type includes new RKS (δ, ε, η, θ), which are activated by DAG, phorbol esters or phosphatidylserine, but are Ca2 + -dependent. In addition, atypical PKCs (ζ, λ, μ, τ) - depend on phosphatidylserine, but are not activated by Ca2 +, DAG, or phorbol ethers.

From the literature data it is known that intracellular signaling pathways associated with PKC activation lead to activation of the nuclear factor NF-kB (Yarilin A.A., 1999). NF-kB is a nuclear transcription factor that plays a key role in the functioning of the immune system. Cytokine signals (TNF-alpha, IL1), activation of the T-cell and B-cell receptors, CD40 receptor lead to activation of NF-kB, in addition, some non-receptor signals can also trigger activation of NF-kB (oxygen burst, UV irradiation (Saatapo J., Huter S.A., 2002)). Considering the data obtained regarding the participation of CSWs and the induction of the cytokine response with the GA-40 drug, NF-kB seems to be the most likely target of the GA-40 action. Experimental data confirmed this assumption. First, GA-40, by reducing the intracellular concentration of hydrogen peroxide, increases the ability of cells to withstand damaging factors and creates the necessary conditions for an adequate immune response without excessive release of cells into apoptosis and escalation of inflammation. Peptides maintain the necessary balance of activating and inhibitory reactions in the cell, and therefore the activation of immunocompetent molecules, in particular NF-kB, goes along the most efficient pathway. In other words, in the case of insufficient cell activity in opposition to damaging factors and pathogens, regulatory peptides increase the strength of the immune response, and in the case of hyper-response of cellular systems to damage and when high risks of excessive apoptosis or autoimmune processes occur, GA-40 reduces the intensity of interactions. It is important to note that NF-kB consists of two subunits with molecular weights of 50 and 65 kDa linked by the inhibitory IkB subunit. This complex is constantly located in the cytoplasm of the cell. When the activation signal arrives, IkB detaches and the released subunits migrate to the nucleus, where it binds to specific sequences of the promoter zones of the target genes (Hayden M.S., 2004). N65-subunit NF-kB was found in nuclear extracts of cells of the human monocytic line U937

incubated in the presence of 100 μg / ml of the drug. This fact suggests with a high degree of probability that the final stage of GA-40-induced signaling is the activation of the transcription factor NF-kB.

NF-kB regulates the expression of cytokines, growth factors, and enzymes involved in the formation of reactive oxygen species. Activation of the “oxygen explosion” in the cell indicates an increase in the bactericidal potential of the cell, activation of a number of signaling molecules that trigger the protective mechanisms of the immune system. An intracellular increase in the level of hydrogen peroxide is, in turn, key to the activation of a number of signaling molecules [Junn et al., 2000; Kim et al., 2001; Koh et al., 2001]. The addition of the drug to leukocytes in the maximum of the tested concentrations caused a significant decrease in intracellular hydrogen peroxide. Perhaps this effect is associated with the antioxidant effect of the drug and confirms the results obtained in previous studies (effect on leukocyte chemiluminescence). Or, the drug includes other signaling pathways that suppress excessive cell activation.

NF-kB activation is also expressed in the activation of macrophages and dendritic cells, their ability to absorb, destroy pathogens and present foreign antigenic material on their surface. For the effective presentation of a foreign peptide and the activation of T cells, the presentation of antigenic material as part of the main histocompatibility complex of the second class (HLA) and the expression of costimulatory molecules (CD40, CD80 / 86) are necessary. The preparation GA-40 at a concentration of 100 μg / ml during daily incubation had practically no effect on the expression of these molecules by human macrophages. However, this does not mean the absence of macrophage activation, perhaps other unexplored factors are activated, or structures interacting with GA-40 are not represented on the surface of macrophages.

**Conclusions**

Thus, it is possible to identify a phased pathway for cell activation under the action of the preparation "GA-40". The preparation “GA-40” interacts with both membrane structures - lectin receptors, T-cell receptors, phospholipids, glycoproteins, and with intracellular systems. As a result, changes in the structure of phosphatidylserine and activation of the Ca2 + -independent protein kinase C occur. The protein kinase pathway activates the transcription factor NF-kB and its translocation into the nucleus. Activation of NF-kB is expressed in increasing the production of TNF-alpha and the subsequent launch of the immune cascade, which is implemented as a powerful anti-infection and anti-tumor scenario.

Due to its unique properties - self-ordering and the desire for harmonization - GA-40 peptides provide multivector and multilevel interactions in the regulatory cascades of the

cell, which allows to significantly expand the range of clinical use of the drug: from infectious-inflammatory and autoimmune processes to hormonal disorders and cancer.

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