OPINION
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of a Dissertation submitted for defense under the procedure for awarding the scientific and educational degree "PhD" in the Professional field 5.11. Biotechnology, Scientific specialty "Technology of biologically active substances"
Topic: "Isolation of natural molecules of plant origin modulating the function of transcription factor Nrf2"
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There have long been traditions around the world that use plants to treat a variety of illnesses. Real opportunities for the identification and characterization of natural products with anti-inflammatory, antibacterial, antifungal, antitumor, etc. activities and their application in pharmaceutical and therapeutic practice are created by the modern methodological level and scientific and technological analysis algorithms.

The effects of pharmacologically influencing the transcription factor Nrf2 in a number of hard-to-treat, chronic diseases are based on the presence of many different genes affected by it. The use of natural plant molecules, with a highly selective action and a suitable pharmacokinetic profile, to modulate the functionality of Nrf2 is an innovative approach for the study and therapy of such diseases and their accompanying pathologies.

Neutrophils are key cells for innate immunity. Neutrophils are among the blood cells that play a significant role in some of the most common inflammatory and degenerative joint diseases. The study of the influence of Nrf2 on their function is of important scientific and scientific-applied importance.

In the dissertation's literature review, within 52 pages, which is about 1/3 of the presented material, detailed information is included on all biologically active substances of plant origin and pharmacological potential described in the literature. Among them are substances of plant origin approved for therapeutic use and undergoing clinical trials. A detailed reference has been made to the methods for obtaining, characterizing, and isolating biologically active substances. The plant objects *Balota nigra L, Clinopodium vulgare L, Leonurus cardiaca L and Haberlea rhodopensis Friv.* as well as the biologically active substances identified in them and their biological activity, are described in detail.

Considerable attention has been devoted to the structure, function, and mechanisms for modulating the activity of the transcription factor Nrf2. The concluding part of the review describes the role of Nrf2 expression in degenerative and inflammatory joint diseases.

To achieve the main goal clearly formulated in the dissertation, eight main tasks follow, which outline the highlights of the dissertation, namely to identify low molecular weight compounds from plant extracts of *B. nigra* L, *C. vulgare* L, *L. cardiaca* L and *H. rhodopensis* Friv. that affect the expression and activation of the transcription factor Nrf2 in neutrophils and to investigate their therapeutic effect in models of joint inflammatory and degenerative diseases.

The Materials and Methods chapter includes and combines more standard cultivation, chemical, and biochemical methods with some of the most advanced analytical methods in the field of modern biotechnology, such as cell isolation, cultivation, and differentiation; high performance liquid chromatography (HPLC); flow cytometric analysis; immunofluorescence; RT - qPCR; NMR; methods to identify Nrf2 expression at the transcriptional and translational levels; statistical analyses; etc. An important methodological element in the development is the introduction of a method for in vitro cultivation of H. rhodopensis Friv in order to preserve this endemic species in nature. The use of all described methods for the development of the present dissertation characterizes the doctoral student as a well-established specialist with a wide and specialized arsenal for research activity in the field of the technology of biologically active substances.

The "Results" chapter begins with the results of the metabolomic analysis of extracts and pure substances isolated from *H. rhodopensis*, *B. nigra*, *C. vulgare*, and *L. cardiaca*. It is important to point out the rationale for determining the type of extracts and the biologically active molecules contained in them that will be used in the development, as well as the objects of their impact.

The original results obtained were due to the optimization of the methods for measuring the protein and gene expression of Nrf2 in neutrophils. For this purpose, the triterpenoid CDDO-Me, an activator of Nrf2, was used. The effect of CDDO-Me on apoptosis was evaluated in mouse bone marrow neutrophils and in human granulocytes. An activator of Nrf2 in unstimulated neutrophils improves cell survival. CDDO-Me has been shown to inhibit the senescence susceptibility phenotype in Ly6G+CD11b+ neutrophils and may act as a potent regulator of neutrophil senescence during knee joint injury progression.

The effect of local application of CDDO-Me on an osteoarthritis induction model and on the phenotype of neutrophils in this model was also investigated. Improvement of histological parameters was found, along with inhibition of local secretion of metalloproteinase MMP-9 and tumor necrosis factor TNF-α. Mathematical analysis has shown a good correlation between disease activity and the expression of the chemokine receptor CXCR4 in neutrophils.

It has been proven that the NFE2L2 gene expression level changes under the influence of various plant extracts and pure substances. Application of miconoside, forcytoside, verbascoside, and balonigrin led to higher expression compared to *H. rhodopensis* plant extract, and leonurine and stachydrin decreased its level compared to *L.cardiaca* extract.

A considerable amount of work has been done on the part reflecting the effect of *H. rhodopensis* extract, fractions, and pure substances on the expression of Nrf2 at the protein and transcription levels in human granulocytes. Fractions of *H. rhodopensis* extract differing in the ratio of myconoside and calceolarioside E in them were studied (D - 1:0.6, E - 0.25:1, and B - contains mainly myconoside). By RT-qPCR, the PMA-induced mRNA expression of the NFE2L2 gene and the Nrf2 target genes PRDX1 and SOD-1 and the effect of fraction D 200 μg/mL, myconoside 32 μg/mL and calciolarioside E 20 μg/mL on the expression of the NFE2L2 gene were determined.

An approximately 1.5-fold decrease in PRDX1 expression was found after treatment with CDDO-Me compared to undifferentiated (pre) cells. A 1.6-fold decrease in SOD-1 transcription in differentiated granulocytes compared to differentiated but untreated cells was also found. The data presented show that calciolarioside E induces PMA-induced expression of the NFE2L2 gene by also increasing the protein level of Nrf2. The miconoside shows an opposite effect similar to that of CDDO-Me, decreasing the transcripts for Nrf2 in PMA-stimulated cells, but the level of Nrf2 protein remains stable, and PI3K expression is increased. Myconoside positively affected PMA-induced expression of PRX1 and SOD-1 genes, while calceolarioside E had the opposite effect. In conclusion, it could be said that CDDO-Me, fraction D, miconoside, and calciolarioside E cause modification of Nrf2 expression in neutrophils, change in their migratory ability, induction of senescence, and inhibition of cytokine TNF-α production, with an important role in chronic inflammation.

The "Discussion" section is distinguished by an in-depth knowledge of the previous data on the investigated processes, a serious analysis of the obtained results, substantiated hypotheses, and revealing the huge potential of a large part of the studied plant extracts as a

source of metabolites with anti-inflammatory activity in activated neutrophils and the corresponding therapeutic significance.

On the basis of the obtained results, 11 conclusions and 9 main contributions of an original nature (6 with a scientific-fundamental and 3 with a scientific-applied nature) were formulated, as well as 3 with confirmatory ones. Both conclusions and contributions objectively reflect the results obtained.

In the present thesis, for the first time, a study was made on the influence of Nrf2 in neutrophils, and the relationship between senescence and CXCR4 was investigated. The Nrf2 activator CDDO-Me was found to downregulate CXCR4 expression on the surface of blood- and BM-derived neutrophils after LPS stimulation in vitro and during the progression of joint damage pathology.

The obtained data show that the synthetic methyl ester of 2-cyano-3,12-dioxo-oleana-1,9(11)-dien-28-oic acid (CDDO-Me) can act as a potent regulator of neutrophil senescence by time of progression of knee joint damage.

The results demonstrate that the intracellular expression of Nrf2 is stimulated by the use of either miconoside or calciolaroside E, either alone or in combination. The continuation of these studies will confirm the therapeutic application of the isolated compounds or fractions of H. rhodopensis in pathologies associated with failure/dysfunction in the activity or expression of Nrf2.

It should also be pointed out that in in vitro cultured plants of H. rhodopensis, the content of myconoside and calciolaroside E increased more than 12 and 30 times compared to the amounts in the wild species, with a more favorable ratio of these molecules.

CONCLUSION

In summary, it can be concluded that the dissertation that has been presented has the characteristics of a significant fundamental work. A great deal of experimental and analytical work has been carried out at a very high scientific level. The role of doctoral student Kristiana Amirova is undeniable, as is the high professional qualification she achieved. It can be categorically said that the dissertation work contains both scientific and scientifically applied original and significant results.

Based on the obtained results, PhD student Christiana Amirova participated as the first author in writing three articles, which were published in refereed journals with high scientometric indicators. The dissertation work submitted for official defense on the topic "Isolation of natural molecules of plant origin, modulating the function of the transcription

factor Nrf2" by PhD student Kristiana Amirova fully corresponds to all the main and additional criteria reflected in the ZRASB and the Regulations for its application in the BAS and in the Institute in Microbiology at the BAS.

On the basis of everything highlighted so far, I confidently suggest to the colleagues of the Scientific Jury to vote for awarding the scientific and educational degree "PhD" to Christiana Miroslavova Amirova in the professional field of 5.11 Biotechnologies.

15/09/2023

Signature:

/Assoc. Dr. Zlatka Alexieva/