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Editorial address:
Palladin Institute of Biochemistry of the NAS of Ukraine, 9, Leontovich Street, Kyiv, 01054, Ukraine;
Tel.: +3 8 044–235–14–72; ***E-mail:*** biotech@biochem.kiev.ua; ***Web-site***: www.biotechnology.kiev.ua

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BIOMEDICAL APPLICATION OF K5 PLASMINOGEN FRAGMENT

L. G. KAPUSTIANENKO, A. O. TYKHOMYROV

Palladin Institute of Biochemistry
of the National Academy of Sciences of Ukraine, Kyiv

E-mail: kapustyanenko@biochem.kiev.ua

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Aim. Plasminogen kringle 5 is an endogenous angiogenic inhibitor. The purpose of the present review was to highlight the potential biomedical application of kringle 5 in the regulation of angiogenesis and tumor growth.

Methods. Angiogenesis is a complex process that involves endothelial cell proliferation, migration, basement membrane degradation, and neovessel organization. Since the uncontrolled growth of new blood vessels causes the progression of many common diseases, first of all, oncological diseases, autoimmune disorders, neovascular damage of the eye, the use of angiostatins can be a promising pharmacotherapeutic approach to the prevention and adjuvant therapy of these pathological conditions. The advantages of angiostatins application are their non-toxicity even at high doses, non-immunogenicity, lack of tolerance of target cells to their action. Angiostatins comprise a group of kringle-containing proteolytically-derived fragments of plasminogen/plasmin, which act as potent inhibitory mediators of endothelial proliferation and migration. Among all known angiostatin species, isolated K5 plasminogen fragment was shown to display the most potent inhibitory activity against proliferation of endothelial cells via triggering multiple signaling pathways, which lead to cell death and resulting angiogenesis suppression.

Results. Current literature data suggest that in addition to expressed and highly specific cytotoxicity in relation to endotheliocytes and some types of tumor cells, the kringle domain 5 of human plasminogen has other advantages as an antiangiogenic and antitumor regulator, including its specific inhibitory activity, which affects only activated, proliferating endothelial cells, and therefore is non-toxic to other types of normal cells. As an endogenous protein, which is formed in the human organism, K5 does not provoke an immune response. K5 as a small polypeptide molecule with a stable structure can be obtained as a recombinant protein in *E. coli* cells, and can also be used in pharmacokinetic systems of targeted delivery and sustained release.

Conclusions. The prospect of successful use of K5 as a therapeutic agent to manage pathological processes associated with dysregulation of angiogenesis makes it necessary to develop and improve methods of its production and to further test its plausible pleiotropic biological activities.

Key words: angiostatins; plasminogen fragment kringle 5; angiogenesis; endothelial cells; neovascular diseases; tumor growth; retinopathy.

Angiogenesis is the process of outgrowth of new blood vessels from pre-existing ones. It plays an important role in development, regeneration, and repair. However, pathological angiogenesis occurs not only in tumor formation, but also in a number of non-neoplastic diseases, which can be classified together as “angiogenesis-dependent diseases”. Viewing the process of angiogenesis as an “organizing principle” in biology can provide intriguing insights into the molecular mechanisms of seemingly unrelated

phenomena. This has important implications for the clinical use of angiogenesis inhibitors and drug discovery, not only to optimize cancer treatment, but perhaps also to develop therapeutic approaches for different, otherwise unrelated diseases [1].

Normally, in an adult organism, the formation of new blood vessels processes occur with low intensity due to the maintenance of a balance between pro- and antiangiogenic factors and are activated only during regenerative processes. Dysregulation of new vascular formation and associated pathological angiogenesis are indicated for age-related changes in tissues, oncological processes, atherosclerosis, diabetes, peptic ulcers, some autoimmune diseases, Alzheimer's disease, and a number of developmental pathologies. Neovascularization plays a significant role in tumor development and metastasis [1]. Among many physiological inhibitors of angiogenesis, fragments of various kringle-containing proteins, including plasminogen, urokinase, tissue type plasminogen activator, hepatocyte growth factor (HGF), play special roles. Proteolytic fragments of the glycoprotein protein — plasminogen, containing varying amounts of its kringle domains, are considered one of the most powerful suppressors of angiogenesis and are called angiostatins [1]. Angiostatins are most intensively generated by primary tumor cells due to dysregulation of the activity of a number of proteinases and can be markers of tumor growth. They effectively suppress the proliferative activity and migration of endothelial cells, trigger the processes of their apoptosis, preventing the formation of new blood vessels and thereby inhibiting the development of metastases. Angiostatins are involved not only in processes associated with oncogenesis, but also modulate angiogenesis in other disorders accompanied by activation of inflammatory reactions, for example, in diabetes mellitus. The role of angiostatins in the development of diabetes-associated angiopathy, as well as the its involvement in the development of diabetic complications such as coronary heart disease and retinopathy, is a complex and controversial issue that needs to be further explored.

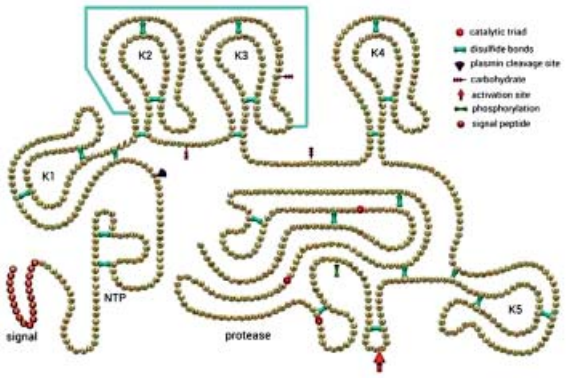

Fig. 1. **Domain structure of a native human plasminogen molecule [3]**

Plasminogen/plasmin system is involved in normal (physiological) and pathological angiogenesis. Physiological angiogenesis (from the development of a fetus and the birth to formation of normal vessels in a grown up organism) proceeds with moderate intensity and accelerates during a number of processes, including regeneration of injured tissues, recanalization of thrombi, and scarring. In contrast to normal vascular network, pathological angiogenesis (i.e., in the course of growth and metastasis of a tumor, myocardial infarction, wound healing, chronic inflammatory diseases, etc.) proceeds abnormally. In these conditions, vessels are heterogeneous, irregularly branched, have multiple fenestrations, and are hyperpermeable for plasma proteins [2].

Angiostatins: general information. Products of limited proteolysis of plasminogen, containing different amounts of its kringle domains (K1-3, K2-3, K1-4, K1-4.5, K1-5, K5), known under the general name "angiostatins", perform the functions of endogenous inhibitors in the body neovascularization and vessel growth (Fig. 1) [4, 5].

The biological effects of angiostatins are related to their ability to specifically inhibit the proliferation of activated endothelial cells, induce apoptosis, and inhibit cell migration. It has been established that various variants of angiostatins suppress tumor-associated angiogenesis, thus restraining the growth of the primary tumor and the progression of metastases [6]. They play an important role in the pathogenesis of neovascular eye diseases. Angiostatins maintain the angiogenic balance in the retinal tissue, preventing its excessive vascularization, in particular during vision correction using laser photocoagulation [7]. Suppression of the production of angiostatins in the retina can be one of the triggers for the development of diabetic retinopathy [8]. Angiostatins are among the proteins of the tear fluid proteome, forming on the surface of the cornea of the eye, thus preventing the formation of blood vessels in it, which is important for maintaining its optical transparency [9]. The anti-adhesive and anti-inflammatory effects of angiostatins have been

shown [10, 11]. Traditionally, angiostatin is considered as a structure corresponding to the kringle domain 1–3 fragment (K1–3) or kringle domain 1–4 (K1–4) fragment of plasminogen/plasmin molecule. Each kringle consists of 80 amino acids held together by three disulfide bonds and formed in loops. Later, by proteolysis of plasminogen or autolysis of plasmin, angiostatin K1–3, containing the first three kringles, and angiostatin K1–4.5, containing kringles 1–4 and 85% kringle 5 of plasminogen, were obtained. It has been shown that angiostatin K1–3 is a weaker inhibitor of endothelial cell proliferation than angiostatin K1–4 [12]. Angiostatin K1–4.5 inhibited angiogenesis and tumor growth at a dose 50 times less than K1–4 [13]. Comparative studies of plasminogen fragments (angiostatin, K1, K3, K2-3, etc.) have shown that kringle 5 (K5) exerts the greatest inhibitory activity [14].

Studies of the therapeutic effects of angiostatins is divided into two groups depending on the nature of the studied proteins, either native or recombinant. It has been suggested that one of the mechanisms of therapeutic action of laser retinal photocoagulation, which is aimed to avoid vision loss in retinopathy is the induction of the formation of endogenous pool of angiostatins [15]. Results of another study has indicated possibility of pharmacocorrection of diabetes-induced retinopathy by modulating angiostatin levels in the injured retina. It has been shown that inhibitors of proapoptotic enzyme PARP-1 are able to restore production of angiostatins in retinas of diabetic rats near to control levels [16]. The prospect of delivery of a genetically engineered construct containing an angiostatin-coding sequence (rAAV-AS K1–4) to retinal tissue in diabetic retinopathy has been declared [17].

Pearce et al. have shown that angiostatins under a normal physiological state are characterized by a wide localization in the structures of the eye. Using immunochemical methods, angiostatins were detected in the nerve fiber layer, ganglion cells, inner and outer plexiform layers, and photoreceptor layer of the retina of the eye of the cat, cow, dog, and rat, while in the retina of the eye of horses and pigs, additional immunostaining of angiostatins was shown in the matrix of the inner nuclear layer [18]. At present, the populations of astroglial and some other retinal cells responsible for the generation of angiostatin in this eye structure remain unknown, and their identification requires additional efforts.

Since the uncontrolled growth of new blood vessels causes the progression of many common diseases, first of all, oncological diseases, autoimmune disorders, neovascular damage of the eye, the use of angiostatins can be a promising pharmacotherapeutic approach to the prevention and adjuvant therapy of these pathological conditions. The advantages of using angiostatins are their non-toxicity even at high doses, non-immunogenicity, lack of tolerance of target cells to their action.

The results of a number of experimental works support the effectiveness of the use of exogenous angiostatins or the corresponding genetic engineering structures that encode them, with the aim of suppressing proangiogenic signaling in diabetic retinopathy. The authors of the paper [19] used a recombinant adenosine virus vector (rAAV) corresponding to the sequence of the K1–4 fragment of human plasminogen for the expression of angiostatin in the retina of rats with STZ-induced hyperglycemia. It was shown that expression of rAAV-AS significantly reduced capillary permeability in the retina of hyperglycemic rats. The use of the proposed gene delivery system has significant prospects for the therapy of eye diseases, since rAAV-AS is characterized by high stability, the ability for long-term expression, which allows achieving a significant therapeutic effect even after a single injection. It is assumed that native angiostatins are promising as agents that normalize vascularization in the retina. It was shown that a single injection of angiostatin K1–4 into the vitreous body at a dose of 7.5 μg per eye significantly reduced the degree of retinal capillary permeability with oxygen- and streptozotocin-induced diabetic retinopathy [20].

Although most of the work aimed at studying the effects of angiostatins in diabetic retinopathy has been conducted with the use of fragments of plasminogen consisting of the first three or four kringle domains, the special role of isolated K5 is increasingly becoming the subject of research. During the first trials, K5 proved itself as a promising potential therapeutic agent for the treatment of diabetic retinopathy. It was shown that the delivery of the gene encoding the K5 sequence to retinal cells reduced capillary permeability, inhibited VEGF overexpression, and inhibited retinal neovascularization under conditions of ischemia [21]. The results of the work [22] demonstrate that K5 is able to exert a direct effect on Müller glia cells, which are the main source of pro-inflammatory and pro-angiogenic

factors, including VEGF, in the retina and play a key role in maintaining neovascularization under conditions of hyperglycemia. Data were obtained that other angiostatins, including K1–4, do not interact with the K5 binding site on the surface of Müller cells, which indicates the specific nature of the association of K5 with cells. Moreover, it has been shown that K5 in the retina enhances the production of an endogenous inhibitor of angiogenesis — a pigment epithelial derived factor (PEDF) [23]. It is assumed that the physiological effects of this plasminogen fragment are realized due to its ability to bind specifically and with high affinity to the potential-dependent anion channel (VDAC1), as it occurs in endotheliocytes [24].

It is known that any disturbances in the trophism and functioning of the retinal pigment epithelium lead to indirect damage to photoreceptors, which leads to a malfunction of the entire visual apparatus. Dysfunction of retinal pigment epithelial (RPE) cells is the main reason for the development of such disorders as Stargardt's disease, Best's macular dystrophy, retinitis pigmentosa, rod-cone retinal dystrophy, age-related macular degeneration, etc. [25]. The new data from our laboratory indicate the absence of cytotoxic properties of angiostatins in relation to RPE cells. These data are of great practical importance in the context of the possibility of safe use of these angiogenesis inhibitors for the purpose of targeted inhibition of the activity of vascular endothelial cells in the treatment of various eye diseases associated with retinal neovascularization. Its antiproliferative effect is several times higher than that of angiostatin, as well as that of any single kringle domain. This may be due to the fact that the anti-endothelial effect of K5 and other kringle domains is realized by different mechanisms. For example, electro-dependent anion channel (VDAC1) may play a role of the K5 receptor on the surface of endothelial cells. K5 binding to endothelial cells induces a decrease in intracellular pH and hyperpolarization of the mitochondrial membrane [26]. ATP synthase associated with the cytoplasmic membrane of endothelial cells, and integrin $\alpha v\beta 3$ has been reported to be angiostatin receptors [27].

It is concluded from these *in vitro* studies [47] that the ranking order of endothelial cell inhibition is K5 > K1, K2, K3 > K1, K2, K4 > K1 > K3 > K2 > K4. However, these *in vitro* data have not been directly translated into antiangiogenic activity *in vivo*. For example, K5 has been found to be less active than angiostatin in suppression of angiogenesis in the chick chorioallantoic membrane assay and the mouse corneal angiogenesis model [28, 29]. Insufficient suppression of *in vivo* angiogenesis by K5 is mainly due to its relatively short half-life *in vivo*. Thus, the antiangiogenic effect of a given compound must be tested in *in vivo* angiogenesis models and not only in *in vitro* endothelial cell cultures [30].

Kringle 5: structure and biological properties. Among all known variants of angiostatins, K5 (Fig. 2) attracts a special attention. K5 has a molecular weight of ~15 kDa and exists as a compact structure with the distinct globular type of hydrophobic core, stabilized by three disulfide bonds. Amino acid analysis of the NH_2-terminal region of K5 revealed two elastase cleavage

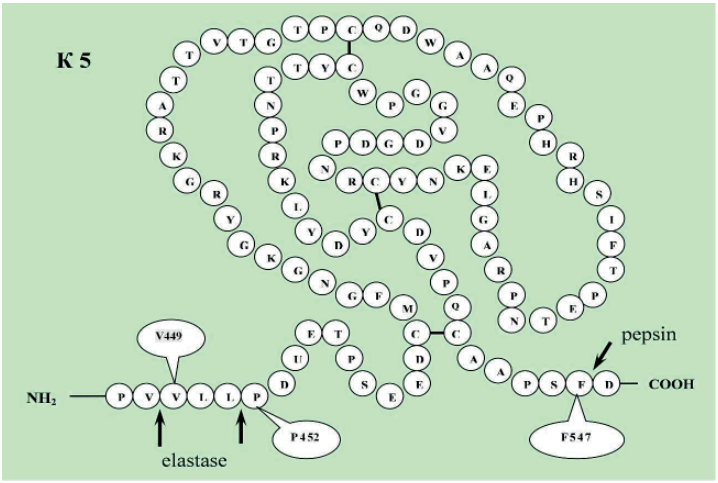

Fig. 2. **The structure of kringle 5 (K5) domain of plasminogen molecule [31]**

sites located both between Val448 and Val449, and between Leu451 and Pro452, and pepsin cleavage site at the position between Phe547 and Asp548. According to the result of the electrophoresis, K5 runs as two bands with molecular mass of 14.9 and 15.7 kDa, correspondingly K5Pro452-Phe547 and K5Val449-Phe547 (Fig. 3).

The antiproliferative and antimigratory effects of K5 in relation to endothelial cells are several times higher of those of other angiostatins, as well as of any individual kringle domain. The high angiostatic potential of K5 is realized due to its ability to specifically interact with a number of alternative receptor molecules exposed by endotheliocytes and some other types of cells, including oncotransformed ones. Today, special attention is paid to the mechanisms of antiangiogenic properties of the K5 fragment of plasminogen. It is believed that the main target of K5 is the protein VDAC1, which was first identified on the outer membrane of mitochondria, and later identified on the plasma membranes of some cells.

The primary function of this pore-forming protein is thought to be regulation of ATP release, transport of ions and metabolites, maintenance of mitochondrial volume, and regulation of redox balance. By interacting with pro- and anti-apoptotic factors, VDAC1 plays a role as a regulator of mitochondria-mediated signaling pathways, which may determine cell death or survival. Thus, VDAC1 is involved in carcinogenesis and the development of neurodegenerative conditions [32]. Amino acid homology between plasminogen activator streptokinase and VDAC1 is shown. It turned out that the binding site of K5 in the streptokinase molecule is located between residues Tyr252-Lys283, and is a homologous site in the primary structure of VDAC1 Tyr224-Lys255. Antibodies against these sequences interact with VDAC1 and recognize this protein on the plasma membrane of human endothelial cells. K5 binds with high affinity (K_d = 28 nM) to endothelial cells, and this interaction is blocked by specific antibodies. Purified VDAC1 binds to K5, but exclusively in the liposomal form. It is suggested that K5 disrupts the mechanisms that control intracellular Ca^{2+} levels precisely through interaction with VDAC1.

The binding of K5 to endothelial cells also induces a decrease in the intracellular pH value and the amount of hyperpolarization of the mitochondrial membrane. However, the exact role and underlying mechanisms of VDAC1 in K5-induced endothelial cell apoptosis remain to be elucidated. In the study [33], authors showed that K5 increased protein level of VDAC1, which initiated the mitochondrial apoptosis pathway of ECs. They also showed that K5 inhibited the ubiquitin-dependent degradation of VDAC1 by promoting the phosphorylation of VDAC1, possibly at Ser-12 and Thr-107. The phosphorylated VDAC1 was attenuated by the AKT agonist, glycogen synthase kinase (GSK) 3β inhibitor, and siRNA, suggesting that K5 increased VDAC1 phosphorylation via the AKT-GSK3β pathway. Furthermore, K5 promoted cell surface translocation of VDAC1, and binding between K5 and VDAC1 was observed on the plasma membrane. HKI protein blocked the impact of K5 on the AKT-GSK3 pathway by competitively inhibiting the interaction of K5 and cell surface VDAC1.

Moreover, K5-induced EC apoptosis was suppressed by VDAC1 antibody. Thus it was demonstrated that K5-induced EC apoptosis is mediated by the positive feedback loop of "VDAC1-AKT-GSK3β-VDAC1", which may provide new insights on the mechanisms of K5-induced apoptosis [33]. It is important to note that annexin II and ganglioside GM1 bind to the plasminogen molecule through the LBS located in K1, while VDAC1 interacts via a

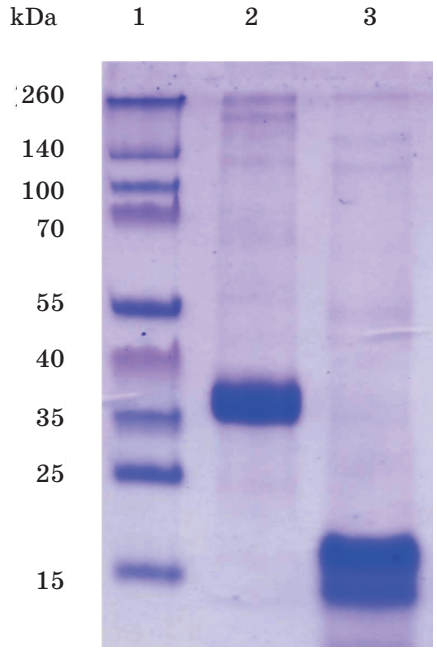

Fig. 3. **Electrophoregram of a K5 fragment obtained by limited proteolysis of plasminogen by pepsin:**
1 — molecular weight markers,
2 — mini-plasminogen, *3* — K5

site located in K5 [26, 33]. In addition, both the zymogen molecule via its K5 and t-PA via the finger domain bind to VDAC1 exposed on the cell surface. This mediated interaction of zymogen and its activator leads to a decrease in the value of K_m and an increase in the value of K_{max} for the reaction of converting plasminogen to plasmin [34].

K5 promoted an increase in the ratio of Bak/Bcl_{xl} on the mitochondrial membrane, which led to its depolarization, leakage of cytochrome c, and activation of caspases 7, 8, and 9 in endothelial cells, which led to their apoptosis [35]. It is possible that the pro-apoptotic properties of K5, which are realized through its binding to VDAC1, determine antitumor activity of this plasminogen kringle, as it was shown in some types of gastric cancer [36]. It was shown that K5 domain of the plasminogen molecule is a substrate for the NADH-dependent reductase activity of VDAC1. It turned out that such a ternary complex is an effective proteolytic machine that removes β-peptide deposits in the brain, as well as disposes of cellular debris from damaged tissue [34]. In particular, one of the receptors for K5 on the surface of endothelial cells is the potential-dependent anion channel (VDAC1). It is assumed that K5 disrupts the mechanisms that control intracellular Ca^{2+} homeostasis precisely through interaction with the VDAC1 molecule [26].

The therapeutic effect of K5 is due to its suppressive effect on the cell cycle of endothelial cells and the subsequent initiation of apoptosis [37]. Unlike other types of angiostatins, K5 has been shown to exert cytotoxic effects directly on cancer cells. The pro-apoptotic properties of K5, which are realized through its binding to VDAC1, determine the antitumor activity of this kringle domain of plasminogen, as shown in some types of gastric cancer [36]. The antiangiogenic and cytotoxic effects of K5 fused to galectin-3 (PK5-RL-Gal-3C) were described in a model of hepatocellular carcinoma [38]. We recently found that isolated K5, obtained by pepsinolysis of mini-plasminogen, halved the invasive/migratory potential of the highly invasive mouse mammary adenocarcinoma cell line 4T1 [unpublished data]. Suppression of the migratory activity of tumor cells by kringle domain 5 opens up prospects for its use as an antimetastatic agent.

Despite the fact that most of the studies aimed at studying the effects of angiostatins in diabetic retinopathy were conducted using fragments of plasminogen consisting of the first three or four kringle domains, the special role of isolated K5 has paid a peculiar attention [19]. During the first experiments, K5 proved to a promising therapeutic agent for the treatment of diabetic retinopathy. It was shown that the delivery of the gene encoding the K5 sequence to retinal cells contributed to a decrease in the degree of capillary permeability, inhibited the overexpression vascular endothelial growth factor (VEGF), and inhibited retinal neovascularization under ischemia conditions [24]. At the same time, the therapeutic effects of K5 in the retina were not accompanied by a cytotoxic effect on the cells of the pigment epithelium.

Inhibition of tumor angiogenesis has an important role in antitumor therapy. However, a recent study indicates that antiangiogenesis therapy may lead to glucose-related protein 78 (GRP78) associated antiapoptotic resistance [36]. Fang and co-authors discovered the dual effects of plasminogen kringle 5 (K5) on tumor angiogenesis and apoptosis induction by targeting hypoxia-inducible factor 1α (HIF-1α) and GRP78. K5 promoted the sumo/ubiquitin-mediated proteasomal degradation of HIF-1α by upregulating von Hippel-Lindau protein under hypoxia, resulting in the reduction of vascular endothelial growth factor and thus suppressing tumor angiogenesis. Furthermore, K5 decreased GRP78 expression via downregulation of phosphorylated extracellular-regulated protein kinase, leading to caspase-7 cleavage and tumor cell apoptosis.

Blocking voltage-dependent anion channel abrogated the effects of K5 on both HIF-1α and GRP78. K5 significantly inhibited the growth of gastric carcinoma xenografts by inhibiting both angiogenesis and apoptosis (Fig. 4) [36]. Gastric cancer is an aggressive malignancy that is frequently diagnosed at an advanced stage with poor prognosis. Although surgery and/or a combination of chemotherapy improve the survival rates, the 5-year relative survival rates of the patients receiving these treatments remains low at 30% and that of the patients with advanced disease is < 1 year [39, 40]. Therefore, it is necessary to develop more effective therapeutic strategies. The dual effects suggest that K5 might be a promising bio-therapeutic agent in the treatment of gastric cancer.

HIF-1α pathway has been proposed as a suitable target for future anticancer therapy [41–44]. Some previous research confirmed that K5 reduced the HIF-1α levels in the retina of retinopathy model and the retinal

capillary endothelial cells [23]. Another study of the same authors demonstrated that HIF-1α was expressed apparently both in nuclear and cytoplasmic compartments of LLC cells induced by hypoxic conditions, and K5 significantly down-regulated HIF-1α expression *in vivo* and *in vitro*. The protein level of intracellular HIF-1α is determined mainly by its rate of proteasomal degradation [45]. Briefly, HIF-1α is hydroxylated by prolyl hydroxylases (PHDs) under normoxic conditions. This modification allows the binding of the tumor-suppressor protein von Hippel-Lindau (VHL) to HIF-1α, and then promotes the formation of E3 ubiquitin ligase complex. The VHL protein mediates polyubiquitination of the HIF-1α subunit at three lysine residues, which results in its degradation by the proteasome. Thus, PHDs and VHL may be the candidate target molecules for the stabilization of HIF-1α during hypoxia. It has been confirmed earlier that K5 promoted the ubiquitin-proteasomal degradation of HIF-1α by inducing VHL, resulting in the decreased protein level of intracellular HIF-1α. Authors demonstrated that K5 treatment significantly reduced the amount of HIF-1α in cytoplasm and lead to a more marked reduction of HIF-1α in nucleus, suggesting that K5 not only down-regulated the protein level of HIF-1α, but also inhibited HIF-1α nuclear accumulation. The rapid nuclear translocation of HIF-1α represents an efficient way to escape from degradation and is the essential steps for HIF-1α in the transactivation of hypoxia-responsive genes [46]. The anti-metastasis effect of K5 is likely to be mediated by suppressing the protein stabilization and nuclear accumulation of HIF-1α, consequently inhibited the HIF-1α transcriptional activity that could be responsible for decreasing gene expression of VEGF and CXCR4, resulting in the inhibition of angiogenesis and tumor chemotaxis movement which are indispensable steps in the progression of metastasis (Fig. 5) [41].

K5 up-regulates VHL and consequently promotes ubiquitin-proteasome medicated protein degradation of HIF-1α. Moreover, K5 decreased HIF-1α protein stabilization, reduced nuclear HIF-1α accumulation and then inhibited transcriptional activation. Consequently, K5 down-regulated the gene expression of CXCR4 and VEGF, which were the downstream genes of HIF-1α pathway. VEGF and CXCR4 play key roles in angiogenesis and chemotaxis migration which both are requisites to metastasis promotion. This may be responsible for the dual inhibitory effects of K5 on tumor metastasis.

Advantages K5 application. In addition to pronounced and highly specific cytotoxicity in relation to endotheliocytes and some types of tumor cells, other advantages of kringle domain 5 of human plasminogen as an antiangiogenic and antitumor regulator are also obvious. Firstly, K5 shows its inhibitory activity specifically, affecting only activated, proliferating endothelial cells, and therefore is non-toxic to other types of normal cells, including the predecessors of endothelial cells and epitheliocytes [47]. Secondly, plasminogen is an endogenous protein present in the human body, and therefore K5 does not provoke an immune response. Thirdly, K5

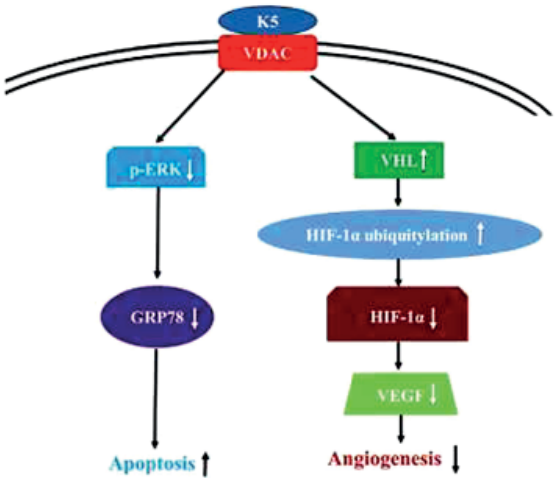

Fig. 4. **The schematic diagram of the signalling pathway** affected by K5 **in the regulation of tumor angiogenesis and tumor cell apoptosis (from [36])**

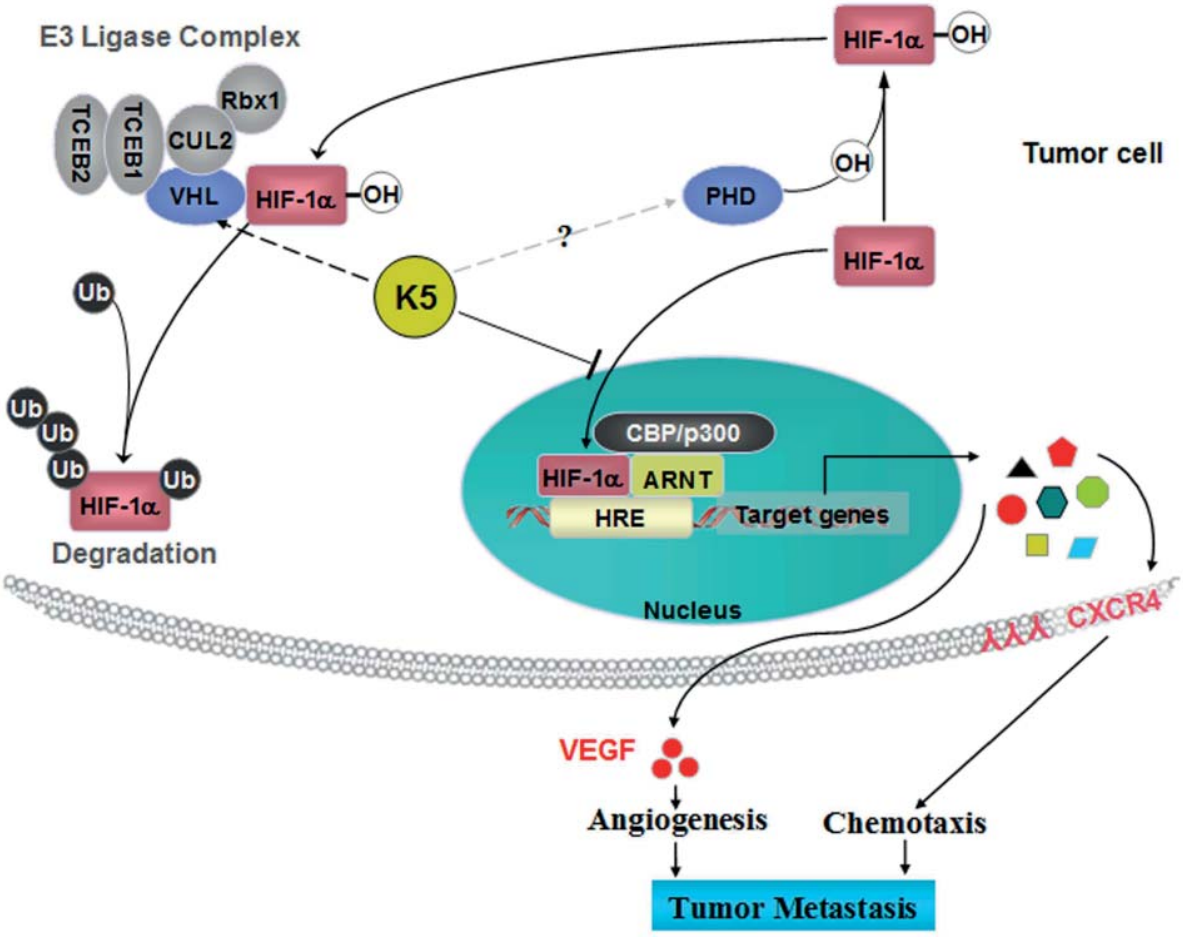

Fig. 5. **A schematic overview of the potential mechanism involved in K5-mediated inhibition of HIF-1α in tumor cells [41]**

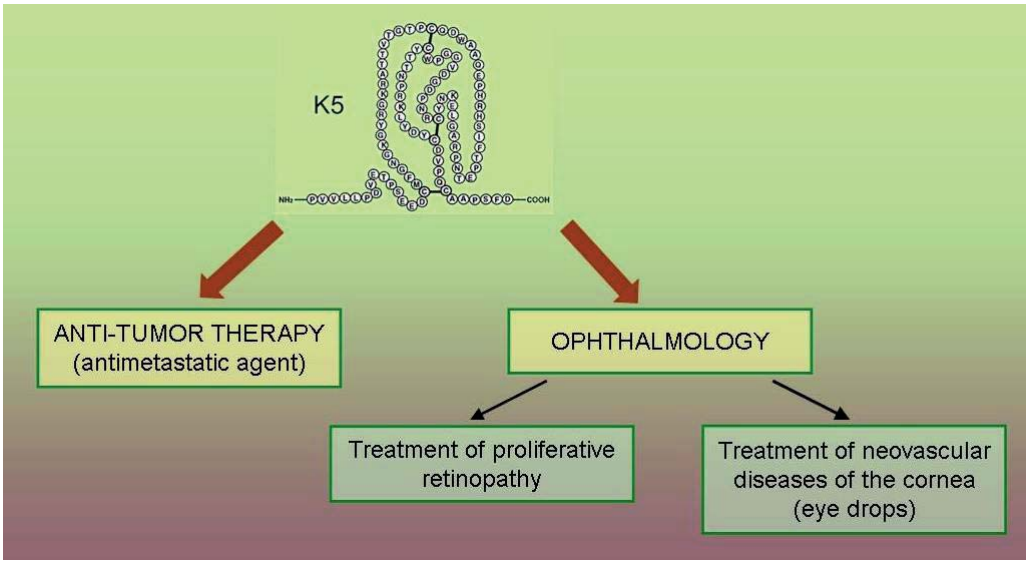

Fig. 6. **Directions of potential pharmacological application of rK5**

as a small polypeptide molecule with a stable structure can be obtained as a recombinant protein in *E.coli* cells, and can also be used in pharmacokinetic systems of targeted delivery and sustained release. In contrast to K5, it has proved extremely difficult to obtain soluble forms of higher molecular weight angiostatins with qualifications that meet the requirements for clinical application (Fig. 6) using expression systems based on *E.coli,* baculoviruses, yeast and mammalian cells.

Usually, the expression of such genetic constructs in *E. coli* resulted in the formation of insoluble protein aggregates of uncertain composition, unsuitable for further use, and in other expression systems the yield of the target protein was extremely low. Also, considering the high cost of native K5 preparations, which are obtained from blood plasma plasminogen, producing a genetically engineered form of this protein in order to create new drugs is expedient and profitable from an economic point of view.

Conclusions

Thus, the prospect of successful use of K5 as a therapeutic agent in pathological processes associated with dysregulation of angiogenesis makes it necessary to develop methods of obtaining it for the creation of new drugs with an antiangiogenic therapeutic effect.

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БІОМЕДИЧНЕ ЗАСТОСУВАННЯ ФРАГМЕНТА ПЛАЗМІНОГЕНУ К5

Л. Г. Капустяненко, А. О. Тихомиров

Інститут біохімії ім. *О.В.* Палладіна НАН України, *Київ,* Україна

E-mail: kapustyanenko@biochem.kiev.ua

~~Плазміноген кринґл 5 є ендогенним інгібітором ангіогенезу.~~

Мета. Висвітлити потенційне біомедичне застосування кринґла 5 у регуляції ангіогенезу та розвитку пухлини.

Методи. Ангіогенез є складним процесом, який включає проліферацію ендотеліальних клітин, міграцію, деградацію базальної мембрани та організацію нових судин. Оскільки неконтрольований ріст нових кровоносних судин є причиною прогресування багатьох поширених захворювань, насамперед, онкологічних та аутоімунних захворювань, неоваскулярних уражень ока, застосування ангіостатинів може бути перспективним фармакотерапевтичним підходом до профілактики та допоміжної терапії цих патологічних станів. Перевагами застосування ангіостатинів є їхня нетоксичність навіть у високих дозах, неімуногенність, відсутність толерантності клітин-мішеней до їхньої дії. Ангіостатини включають групу протеолітичних фрагментів плазміногену/плазміну, що містять кринґлові структури, які діють як потужні інгібувальні медіатори ендотелійної проліферації та міграції. Показано, що серед усіх відомих видів ангіостатинів ізольований фрагмент плазміногену К5 демонструє найпотужнішу інгібіторну активність проліферації ендотелійних клітин через запуск багатьох сигнальних шляхів, які призводять до загибелі клітин і, як наслідок, пригнічення ангіогенезу.

Результати. Сучасні наукові дані літератури свідчать про те, що, окрім вираженої та високоспецифічної цитотоксичності по відношенню до ендотеліоцитів та деяких типів пухлинних клітин, кринґловий домен 5 плазміногену людини має інші переваги як антиангіогенний та протипухлинний регулятор: він виявляє інгібіторну активність, зокрема, впливає лише на активовані ендотеліальні клітини, що проліферують, і тому не є токсичним для інших типів нормальних клітин; як ендогенний протеїн, присутній в організмі людини, К5 не провокує імунну відповідь; і К5 у вигляді невеликої поліпептидної молекули зі стабільною структурою може бути отриманий у вигляді рекомбінантного білка в клітинах *E. coli*, а також може бути використаний у фармакокінетичних системах спрямованого доставляння та пролонгованого вивільнення.

Висновки. Перспектива застосування К5 як ефективного засобу терапії при патологічних процесах, пов'язаних з порушеннями регуляції ангіогенезу, зумовлює як необхідність розроблення та вдосконалення методів його отримання, так і подальшого тестування його ймовірної плейотропної біологічної активності.

Ключові слова: ангіостатини; фрагмент плазміногену кринґл 5; ангіогенез; ендотелійні клітини; неоваскулярні захворювання; розвиток пухлини; ретинопатія.

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INFLUENCE OF BIOLOGICAL INDUCTORS ON THE SYNTHESIS AND BIOLOGICAL ACTIVITY OF MICROBIAL METABOLITES

T. P. PIROG[1, 2], *M. S. IVANOV*[1]

[1]National University of Food Technologies, Kyiv, Ukraine
[2]Institute of Microbiology and Virology of NASU, Kyiv, Ukraine

E-mail: tapirog@nuft.edu.ua

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The increasing antibiotic resistance is a severe concern for humanity. Co-cultivation of microorganisms is a promising method for obtaining new secondary antimicrobial metabolites. An effective strategy for co-cultivation of microorganisms involves the usage of certain biological inductors.

The *aim* of this review is to summarize existing scientific research in the literature related to the influence of physiologically different types of biological inductors on the synthesis and biological activity of microbial secondary metabolites.

An analysis of the literature has shown that in such studies, either live or inactivated cells of the inductor are added to the culture medium at significantly lower concentrations compared to the producer cells of the final metabolites, or the supernatant (filtrate) after cultivation of a competitive microorganism is used as an inductor.

According to the literature and our own experimental studies, the using inductors is an effective approach not only for intensifying the synthesis of bacteriocins, surfactants, and antibiotics, but also for increasing their biological activity. Additionally, it often leads to the production of novel antimicrobial compounds that are not typical for the producer.

However, the mechanisms of effect of inductors on the synthesis of biologically active secondary metabolites require further research, as the literature suggests that their introduction into the cultivation medium of producer does not always lead to an intensification of the synthesis of the final product. Moreover, the biological activity of secondary metabolites depends on the cultivation conditions of the producer, including the presence of biological inductors in the culture medium. Therefore, it is essential to conduct further research on the interaction between producers and competitive microorganisms to regulate the biological activity of the synthesised metabolites. In addition, there is a necessity to search for more cost-effective substrates for the biosynthesis of secondary metabolites, optimize the composition of the culture medium and expand the range of both pro- and eukaryotic inductors.

Key words: co-culture; inductor; physiological state of the inductor; antimicrobial metabolites.

In our previous publication [1], it was mentioned that the increase in antibiotic resistance of pathogenic microorganisms in recent decades has led the scientific community to research for novel environmentally friendly antimicrobial metabolites of natural origin with stable properties. One of the ways to solve this issue was the strategy of co-cultivation of microorganisms, in which the producer of practically valuable metabolites is cultivated together with competitive microorganisms. This is considered to be an effective strategy to induce producing by microorganisms secondary metabolites with increased antimicrobial activity or/and will stimulate the production of bioactive secondary metabolites, which cannot be obtained in the corresponding pure culture [1, 2].

Studies on the influence of competitive microorganisms on the synthesis of antimicrobial compounds can be divided into three categories:

1) both strains (the producer and the competitive microorganism) are introduced into the culture medium of antimicrobial metabolites producer in a 1:1 ratio, i.e. in almost the same concentration [2, 3];

2) live or inactivated inductor cells are added to the medium at a significantly lower concentration compared to the cells of the final metabolite producer [4–23];

3) the supernatant (filtrate) after cultivation of a competitive microorganism is used as an inductor [4, 21, 22, 24–29].

For each of these three categories of experiments, the corresponding terminology is used. The first category is the classical co-cultivation of two microorganisms, the cultivation of artificial microbial associations (consortia). Competitive microorganisms (or their supernatants) used in the second and third categories are called inductors or elicitors.

Related to the above stated, the aim of this review is to summarize current literature data on the effect of physiologically different types of biological inductors on the synthesis and biological activity of microbial metabolites.

Heat-inactivated inductors

There is information in the literature on the effect of heat-inactivated inductors on the synthesis and properties of bacteriocins [4], microbial surfactants [5–7], pigments [8–10], antibiotics [11], and other secondary metabolites [12–15].

Bacteriocins. It was found in [4] that the introduction of heat-inactivated *Staphylococcus aureus* ATCC 43090 cells (2 and 3% v/v) or *Bacillus* sp. cells (3% v/v) into the culture medium of the bacteriocin producer *Bacillus subtilis* NK16 was accompanied by a 2-4-fold increase in the synthesis of the final product compared to that established for the NK16 strain. The scientists suggested that one of the mechanisms for the increase in bacteriocin synthesis could be the recognition of certain proteins or receptors on the surface of inactivated inductor cell fragments and the implementation of a protective mechanism against a competitive microorganism. This was supported by the results of determining the antimicrobial activity of the synthesised bacteriocins against inductor cells (*S. aureus* ATCC 43090, *Bacillus* sp.): the growth inhibition zone was 25 and 23 mm, respectively. At the same time, without inductor cells, the growth inhibition zone was from 5 to 15 mm after treatment with the obtained bacteriocins.

Microbial surfactants. The researchers found [5] that the introduction of inactivated *Listeria monocytogenes* ATCC 7644 or *Aspergillus niger* IFL5 cells into the culture of *Bacillus* sp. P34 increased the synthesis of the antimicrobial lipopeptide iturin A by more than 2 times compared to that established for the P34 strain, but had virtually no effect on the synthesis of fengycins A and B. An interesting fact was that, despite the increased synthesis of lipopeptides, their antimicrobial activity was twice as low as that of those synthesized in the medium without inductor.

In the work [6], it is reported that the synthesis of iturin A by the *Bacillus amyloliquefaciens* P11 strain was increased by 0.5 and 3 times in the presence of heat-inactivated cells of *S. aureus* ATCC 25923 or *Aspergillus parasiticus* (strain number not provided), respectively. Furthermore, the introduction of inductors into the cultivation medium of *B. amyloliquefaciens* P11 was accompanied by the synthesis of new compounds not typical for the producer, such as subtilosin A and fengycin. However, the researchers did not specify their concentrations.

When inactivated *Candida albicans* SC 5314 yeast cells were added to *B. subtilis* RLID 12.1 culture medium, a 3–4-fold increase in the concentration of cyclic lipopeptides AF3 and AF5 was observed [7], which were characterized by high antifungal activity against *Candida auris* yeast, with minimum inhibitory concentrations (MIC) of 4–16 μg/ml.

Pigments. The effect of inactivated prokaryotic and yeast inductors on the synthesis of the antimicrobial pigment prodigiosin by actinobacteria of the genus *Streptomyces* and bacteria of the genus *Serratia* was studied by a few authors [8–10].

For example, researchers [8] found that the presence of inactivated *Escherichia coli*, *B. subtilis* or *Saccharomyces cerevisiae* cells (2–3%) in the culture medium of *Serratia marcescens* S23 was accompanied by a 7–9-fold increased production of this pigment compared to that of the S23 strain, moreover, the concentration of prodigiosin did not depend on the nature of the inductor (pro- or eukaryotic). The authors assumed that one of the mechanisms of induction is direct contact between the producer and the inductor cells, as they found no evidence of the participation of certain signal molecules in this process.

The authors in the study [9] demonstrated a 30–100% concentration increased of prodigiosin synthesis by *S. marcescens* (strain number not given) in the presence of the corresponding inductors (*E. coli, B. subtilis*, or *S. cerevisiae*), but compared to the studies described in work [8], the maximum induction of prodigiosin synthesis was observed in the presence of bacterial inductors.

The authors reported [10] that the introduction of heat-inactivated *Lactobacillus rhamnosus* LGG cells (0.5–1%) into the culture medium of actinobacteria *Streptomyces coelicolor* (strain number not given) was associated with an increased synthesis of prodigiosin up to 9.8 mg/l, which is 7 times higher than without the inductor. The authors hypothesised that the lactic acid bacteria lysis products could work as a precursor to the synthesis of the final product. It should be noted that the synthesis ability of *S. coelicolor* is significantly lower than that of *S. marcescens* [8, 9].

Antibiotics. The authors in the study [11] managed to significantly enhance the synthesis of the antibiotic phenazine by *Pseudomonas aeruginosa* (strain number not given) up to 43–300% by adding heat-inactivated *E. coli, B. subtilis*, and *S. cerevisiae* cells to the medium. *S. cerevisiae* yeast cells proved to be the most effective inductor, in the presence of which the phenazine concentration increased up to 30 mg/l. The determination of the antimicrobial activity of phenazine against the inductor cells showed that the growth inhibition zones of *E. coli, B. subtilis* and *S. cerevisiae* were 1.6, 2.9 and 4.3 mm, respectively.

Other secondary metabolites. During the cultivation of *Streptomyces* sp. RKND-216 in the presence of inactivated cells of *Alteromonas* sp. RKMC-009 or *Mycobacterium smegmatis* ATCC 120515, the production of two novel alkaloids was found: N-carbamoyl-2-hydroxy-3-methoxybenzamide and carbazoquinocin G [12]. The obtained metabolites did not show antimicrobial activity, but carbazoquinocin G was characterised by cytotoxic activity against breast cancer cell lines MCF7 and HTB26 (IC_{50} was 3.07 and 3.67 μM, respectively).

It was found in the work [13] that the introduction of heat-inactivated *S. aureus* cells into the culture of *Streptomyces* sp. MH-133 was accompanied by the synthesis of a complex of unidentified antibacterial metabolites that inhibited the growth of *S. aureus, E. coli, Klebsiella pneumonia*, *Enterobacter cloacae* (growth inhibition zones were 24, 22, 24 and 16 mm, respectively).

It is reported in studies [14, 15] about the effect of heat-inactivated inductors on the production of antimicrobial metabolites by micromycetes.

Six novel 16-residue peptaibols (acremopeptibodies A-F) were obtained from the culture of the micromycete *Acremonium* sp. IMB18-086 cultivated in the presence of autoclaved *Pseudomonas aeruginosa* cells [14]. Acremopeptabolites A and F showed antimicrobial activity against *S. aureus, B. subtilis, P. aeruginosa, Candida albicans*: the growth inhibition zones were 15, 16, 10 and 12 mm, respectively.

Other studies [15] showed that the introduction of autoclaved *P. aeruginosa* cells into the culture of *Chaetomium* sp. led to the production of four novel butenolide derivatives, as well as two analogues of shikimic acid (chetoisochorismine, shikimeran B), which were not observed in either the micromycete or inductor culture.

In Table 1, we summarised the data on the influence of heat-inactivated inductors on the production and the antimicrobial activity of secondary metabolites. These data indicate that the use of these inductors is a technologically simpler and effective method of not only intensifying the production of bacteriocins, surfactants, and antibiotics, but also increasing their biological activity, and is often accompanied by the synthesis of novel antimicrobial metabolites not typical for the producer.

Live inductors

There is scientific data showing that live cells of prokaryotic and eukaryotic inductors can affect the production and properties of bacteriocins [4, 16], surfactants [17, 18], pigments [8, 9, 19], antibiotics [11, 20–22] and other secondary metabolites [23].

Bacteriocins. It was shown in the work [4] that the introduction of live cells of *S. aureus* ATCC 43090 (0.5%), *E. coli* (0.2%) or *A. niger* (0.75%) into the culture medium of the bacteriocin producer *B. subtilis* NK16 caused an increase in the synthesis of the final product by 4–8 times compared to the rates without inductors. The produced bacteriocins exhibited high antimicrobial activity against *S. aureus* ATCC 4309, *E. coli* and *A. niger* cells: the growth inhibition zones were 27, 22 and 24 mm, respectively, and were 20–50% higher than when using bacteriocins synthesised without inductor.

Table 1

Effect of heat-inactivated inductors on the synthesis and antimicrobial activity of secondary metabolites

Producer	Carbon source	Biological inductor	Concentration (activity) of secondary metabolites		Test-cultures for determining antimicrobial activity	Antimicrobial activity	References
			without inductor	with an inductor			
1	2	3	4	5	6	7	8
Bacteriocins							
Bacillus subtilis NK16	Dextrose	*Staphylococcus aureus* ATCC 43090 / *Bacillus* sp. ATCC 6633	80 AU/ml	320 / 160 AU/ml	*Staphylococcus aureus* ATCC 43090	Growth inhibition zone of 25 mm (100 µg/disc)	4
					Bacillus sp. ATCC 6633	Growth inhibition zone of 23 mm (100 µg/disc)	
Microbial surfactants							
Bacillus sp. P34	Dextrose	*Listeria monocytogenes* ATCC 7644 / *Aspergillus niger* IFL5	Iturin A 100 mg/l	Iturin A 250 mg/l	*Aspergillus niger* IFL5	400 AU/ml*	5
					Listeria monocytogenes ATCC 7644	1600 AU/ml*	
Bacillus amyloliquefaciens P11	Dextrose	*Staphylococcus aureus* ATCC 25923 / *Listeria monocytogenes* ATCC 7644 / *Aspergillus parasiticus* **(strain number not given)**	Iturin A 100 mg/l	Iturin A 300 mg/l Surfactin** Subtilisin A** Fengycin**	*Staphylococcus aureus* ATCC 25923	1333 AU/ml*	6
					Listeria monocytogenes ATCC 7644	933 AU/ml*	
					Aspergillus parasiticus **(strain number not given)**	1600 AU/ml*	
Bacillus subtilis RLID 12.1	Glucose	*Candida albicans* SC 5314	AF3 441 mg/l	AF3 1280 mg/l	*Candida auris* **(strain number not given)**	MIC 4–10 µg/ml	7
			AF5 263 mg/l	AF5 960 mg/l	*Candida auris* **(strain number not given)**	MIC 4–16 µg/ml	
Pigment prodigiosin							
Serratia marcescens S23	Starch	*Escherichia coli* / *Bacillus subtilis* / *Saccharomyces cerevisiae* **(strain number not given)**	0.45 g/l	4.1 / 3.5 / 4.1 g/l	-----	N.d.	8

Table 1 (Continuend)

1	2	3	4	5	6	7	8
Serratia marcescens **(strain number not given)**	Trypton, yeast extract	*Bacillus subtilis* / *Escherichia coli* / *Saccharomyces c erevisiae* **(strain number not given)**	100 mg/ml	200 / 170 / 130 mg/ml	-----	N.d.	9
Streptomyces coelicolor **(strain number not given)**	Glucose	*Lactobacillus rhamnosus* LGG	1.4 mg/l	9.8 mg/l	-----	N.d.	10
Antibiotics							
Pseudomo-nas aeru-ginosa **(strain number not given)**	Trypton, yeast extract	*Escherichia coli* / *Bacillus subtilis* / *Saccharomyces cerevisiae* **(strain number not given)**	Phenazine 9.2 mg/l	Phenazine 13.4 / 19.4 / 30 mg/l	*Escherichia coli* **(strain number not given)**	Growth inhibition zone of 1.6 mm (200 µl/disc)	11
					Bacillus subtilis **(strain number not given)**	Growth inhibition zone of 2.9 mm (200 µl/disc)	
					Saccharomyces cerevisiae **(strain number not given)**	Growth inhibition zone of 4.6 mm (200 µl/disc)	
Other secondary metabolites							
Streptomyces sp. RKND-216	Dextrose	*Alteromonas* sp. RKMC-009 / *Mycobacterium smegmatis* ATCC 120515	N-carbamoyl-2-hydroxy-3-methoxybenzamide** carbazoquinocin G** (alkaloids)		-----	N.d.	12
Streptomyces sp. MH-133	Trypton, yeast extract	*Staphylococcus aureus* **(strain number not given)**	Complex of unidentified antibacterial metabolites		*Klebsiella pneumonia* **(strain number not given)**	Growth inhibition zone of 24 mm (100 µl/disc)	13
					Staphylococcus aureus **(strain number not given)**	Growth inhibition zone of 24 mm (100 µl/disc)	
					Escherichia coli ATCC 25922	Growth inhibition zone of 22 mm (100 µl/disc)	

Table 1 (End)

1	2	3	4	5	6	7	8
					Enterobacter cloacae **(strain number not given)**	Growth inhibition zone of 16 mm (100 µl/disc)	
Acremonium sp. IMB18-086	Rice	*Pseudomonas aeruginosa* ATCC 27853	Acremopeptaibols A-F** (16-residue peptaibols)		*Staphylococcus aureus* ATCC 33591	Growth inhibition zone of 15 mm	14
					Bacillus subtilis ATCC 6633	Growth inhibition zone of 16 mm	
					Candida albicans ATCC 10231	Growth inhibition zone of 12 mm	
					Pseudomonas aeruginosa ATCC 27853	Growth inhibition zone of 10 mm	
Chaetomium sp. **(strain number not given)**	Rice	*Pseudomonas aeruginosa* **(strain number not given)**	Hetobutenolide A, C** Methyl ester WF-3681** (butenolide derivatives) Hetoisochorismine** Shikimeran B** (shikimic acid analogues)		-----	N.d.	15

Notes. N.d. - not determined; * — activity was determined as the last dilution giving a growth inhibition zone and indicated in units of activity per millilitre; ** — synthesis of new compounds not typical for monoculture.

Other authors [16] found that paracin 1.7 production by *Lactobacillus paracasei* strain HD1-7 increased by almost 75% in the presence of live cells of *B. subtilis* ATCC 11774.

Microbial surfactants. There is information in the literature about the effect of live cells of prokaryotic [17] and eukaryotic [18] inductors on the synthesis of surfactants.

In work [17], the emulsifying activity of *S. marcescens* surfactants (strain number not given) was increased by 1.7–3.5 times when introduced into the culture medium of live *E. coli* or *S. aureus* cells. The surfactants synthesised in the presence of inductors showed antimicrobial activity against the test cultures of *S. aureus, K. pneumonia, E. coli, B. subtilis*: the growth inhibition zones were in the range of 14–16 mm.

The synthesis of iturin A by the producer *B. amyloliquefaciens* CX-20 increased by an average of 10% in the presence of live cells of the micromycetes *Aspergillus oryzae* 92011 or *Trametes* sp. 48424 [18].

Pigments. A number of studies reported the influence of live prokaryotic [8, 9, 19] and yeast [8, 9] inductors on the synthesis of pigments by bacteria of the genus *Pseudomonas* and *Serratia*.

The level of prodigiosin synthesised by *S. marcescens* S23 increased by 1.4–7 times by adding to the culture medium *E. coli, B. subtilis*, or *S. cerevisiae* cells [8], and the highest concentration of this particular metabolite (3.1 g/l) was achieved with the introduction of a yeast inductor.

In the study [9], it was found that the addition of live *S. cerevisiae* cells to the *S. marcescens* culture medium increased the synthesis of prodigiosin to 170 mg/ml, which is 70% above the level obtained when the producer was cultivated without an inductor. The use of prokaryotic inductors (*E. coli, B. subtilis*) resulted in an increasing of the final product synthesis by *S. marcescens* strain to 220–250 mg/ml, which is 50% higher than in case of using yeast inductors.

The authors in the work [19] found that the addition of live *S. aureus* (0.5, 0.75%), *K. pneumonia* (0.5, 0.75%), or *B. subtilis* (0.25, 0.5%) cells to the

P. aeruginosa culture medium increased the synthesis of the pyocyanin pigment in 2.3 times compared to the cultivation of the producer in the medium without inductors. Pyocyanin showed high antimicrobial activity against *S. aureus, K. pneumonia, B. subtilis*, and *E. coli* cells: growth inhibition zones were in the range of 26–38 mm.

Antibiotics. There is available data in literature about the influence of live prokaryotic cells [20, 11], yeast [20, 21, 11] and micromycetes [21, 22] on the synthesis of antibiotics by actinobacteria of the genus *Streptomyces*.

For instance, researchers [20] found that in the presence of live *Bacillus cereus* or *S. cerevisiae* cells, the production of valinomycin by *Streptomyces lavendulae* strain ACR-DA1 increased for 34–62% compared to the levels observed without inductor.

In the study [21], the concentration of rimocidin was increased by 42% by introducing the yeast inductor *S. cerevisiae* into the culture medium of the producer *Streptomyces rimosus* M527. When the micromycete *Fusarium oxysporum* f. sp. *cucumerinum* was used as an inductor, the antibiotic concentration almost twice increased compared to the values obtained without the inductor.

The authors reported [22] that the introduction of live cells of *A. niger* AS 3.6472 (0.2%) or *Penicillium chrysogenum* AS 3.5163 (0.4%) into the culture medium of *Streptomyces natalensis* HW-2 increased the production of the antibiotic natamycin by 25-36% compared to that of the HW-2 strain grown without micromycetes.

Other researchers [11] demonstrated that in the presence of live cells of the prokaryotes *B. subtilis, E. coli* and the yeast *S. cerevisiae*, the concentration of phenazine synthesised by *P. aeruginosa* was 80–145% higher than in the case of cultivation of the producer without inductors. The most effective inductor was *E. coli* cells, in the presence of which the concentration of phenazine increased to 18.8 mg/l, which is 2.5 times higher than without inductor. Phenazine showed antimicrobial activity against the inductor cells of *B. subtilis, E. coli, S. cerevisiae*: the growth inhibition zones of the tested cultures were 2.9, 1.6, 4.3 mm, respectively.

Other secondary metabolites. During the cultivation of *Saccharopolyspora erythraea* ATCC 31772 in the presence of cells of the micromycete *Fusarium pallidoroseum* ATCC 74289, three novel analogues of decalin-type tetraminic acids (N-demethylophiosetin, pallidorosetin A, pallidorosetin B) were identified [23].

In Table 2, we summarised the influence of live cell inductors on the synthesis and antimicrobial activity of secondary metabolites. These data indicated that the use of both prokaryotic and yeast and microbial inductors can increase the synthesis of bacteriocins, surfactants, antimicrobial pigments and antibiotics. The synthesis and the activity of the induced metabolites increased with the addition of live inductor cells, but almost did not depend on their nature (pro- or eukaryotic). In addition, the use of micromycetes as inductors proved to be an effective way to increase the synthesis of antibiotics.

Supernatant after cultivation of inductors

There is limited information in the literature regarding the impact of supernatant after cultivation of biological inductors on the synthesis and antimicrobial activity of secondary metabolites. Most of the research focuses on the induction of bacteriocin synthesis [4], pigments [24], surfactants [25], antibiotics [21, 22, 26–28], and other secondary metabolites [29].

Bacteriocins. In the study [4], it was found that the addition of supernatant after the cultivation of *S. aureus* ATCC 43090 (2 and 3%), *Bacillus* sp. ATCC 6633 (2 and 3%), *A. niger* (2 and 3%), or *S. cerevisiae* (3%) into the culture medium of bacteriocins producer *B. subtilis* NK16 was accompanied by a 2–4-fold increase in the synthesis of the final product compared to the controls without inductors. Bacteriocins exhibited high antimicrobial activity against cells of the inductor strains (*S. aureus* ATCC 43090, *Bacillus* sp. ATCC 6633, *A. niger*, and *S. cerevisiae)*: the growth inhibition zones were 27, 25, 25 and 21 mm, respectively.

Pigments. It was reported in the research [24] that the addition of supernatant after cultivation of the lactic acid bacteria *Leuconostoc mesenteroides* or *Lactobacillus plantarum* to the culture of *S. coelicolor* led to the production of the pigment prodigiosin, which is not typical for that microorganism.

Surfactants. The authors in the study [25] reported a 107.4% increase in

Table 2

Effect of live inductor cells on the synthesis and antimicrobial activity of secondary metabolites

Producer	Carbon source	Biological inductor	Concentration (activity) of secondary metabolites		Test-cultures for determining antimicrobial activity	Antimicrobial activity	References
			without inductor	with an inductor			
1	2	3	4	5	6	7	8
Bacteriocins							
Bacillus subtilis NK16	Dextrose	*Staphylococus aureus* ATCC 43090 / *Escherichia coli* / *Aspergillus niger* **(strain number not given)**	Bacteriocins 80 AU/ml	Bacteriocins 640 / 320 / 320 AU/ml	*Staphylococcus aureus* ATCC 43090	Growth inhibition zone of 27 mm (100 µl/disc)	4
					Escherichia coli **(strain number not given)**	Growth inhibition zone of 21 mm (100 µl/disc)	
					Aspergillus niger **(strain number not given)**	Growth inhibition zone of 21 mm (100 µl/disc)	
Lactobacillus paracasei HD1-7	Glucose	*Bacillus subtilis* ATCC 11774	Paracin 1.7 60 AU/ml	Paracin 1.7 105 AU/ml	-----	N.d.	16
Microbial surfactants							
Bacillus amyloliquefaciens CX-20	Glucose	*Aspergillus oryzae* 92011/ *Trametes* sp. 48424	Iturin A 1.74 g/l	Iturin A 1.88 / 1.95 g/l	-----	N.d.	18
Serratia marcescens **(strain number not given)**	Trypton, yeast extract	*Escherichia coli* / *Staphylococcus aureus* **(strain number not given)**	0.27 (units of emulsifying activity)	0.46 / 0.94 (units of emulsifying activity)	*Staphylococcus aureus* **(strain number not given)**	Growth inhibition zone of 14 mm (40 µg/disc)	17
					Klebsiella pneumonia **(strain number not given)**	Growth inhibition zone of 16 mm (40 µg/disc)	
					Escherichia coli **(strain number not given)**	Growth inhibition zone of 14 mm (40 µg/disc)	
					Bacillus subtilis **(strain number not given)**	Growth inhibition zone of 16 mm (40 µg/disc)	

Table 2 (Continued)

1	2	3	4	5	6	7	8
Antimicrobial pigments							
Pseudomonas aeruginosa **(strain number not given)**	Trypton, yeast extract	*Staphylococcus aureus* / *Klebsiella pneumonia* / *Bacillus subtilis* **(strain number not given)**	Pyocyanin 17 mg/ml	Pyocyanin 38 / 40 / 33 mg/ml	*Staphylococcus aureus* **(strain number not given)** *Bacillus subtilis* **(strain number not given)**	Growth inhibition zone of 34 mm (40 μg/disc) Growth inhibition zone of 28 mm (40 μg/disc)	19
					Escherichia coli **(strain number not given)**	Growth inhibition zone of 26 mm (40 μg/disc)	
					Klebsiella pneumonia **(strain number not given)**	Growth inhibition zone of 38 mm (40 μg/disc)	
Serratia marcescens S23	Starch	*Escherichia coli* / *Saccharomyces cerevisiae* / *Bacillus subtilis* **(strain number not given)**	Prodigiosin 0.45 g/l	Prodigiosin 2.5 / 3.1 / 0.65 g/l	-----	N.d.	8
Serratia marcescens **(strain number not given)**	Trypton, yeast extract	*Bacillus subtilis* / *Escherichia coli* / *Saccharomyces cerevisiae* **(strain number not given)**	Prodigiosin 100 mg/ml	Prodigiosin 250 / 220 / 170 mg/ml	-----	N.d.	9
Antibiotics							
Streptomyces lavendulae ACR-DA1	Dextrine	*Bacillus cereus* / *Saccharomyces cerevisiae* **(strain number not given)**	Valinomycin 50 mg/ml	Valinomycin 81 / 67 mg/ml	-----	N.d.	20
Streptomyces rimosus M527	Soybean flour, mannitol	*Saccharomyces cerevisiae* / *Fusarium oxysporum* f. sp. *cucumerinum*	Rimocidin 0.21 g/l	Rimocidin 0.3 / 0.39 g/l	-----	N.d.	21
Streptomyces natalensis HW-2	Glucose, maltose	*Aspergillus niger* AS 3.6472 / *Penicillium chrysogenum* AS3.5163	Natamycin 0.639 g/l	Natamycin 0.799 / 0.875 g/l	-----	N.d.	22

Table 2 (End)

1	2	3	4	5	6	7	8
Pseudomonas aeruginosa **(strain number not given)**	Trypton, yeast extract	*Escherichia coli*, або *Bacillus subtilis* / *Saccharomyces cerevisiae* **(strain number not given)**	Phenazine 7.6 mg/ml	Phenazine 18.8 / 13.8 / 14.5 mg/ml	*Escherichia coli* **(strain number not given)**	Growth inhibition zone of 1.6 mm (200 µl/disc)	11
					Bacillus subtilis **(strain number not given)**	Growth inhibition zone of 2.9 mm (200 µl/disc)	
					Saccharomyces cerevisiae **(strain number not given)**	Growth inhibition zone of 4.3 mm (200 µl/disc)	
Other secondary metabolites							
Saccharopolyspora erythraea ATCC 31772	Dextrose	*Fusarium pallidoroseum* ATCC 74289	N-Demethylophiosetin* Palidorosetin A* Palidorosetin B* (tetraminic acid analogues)	-----	-----	N.d.	23

Notes. N.d. — not determined; * — synthesis of new compounds not typical for monoculture.

were synthesized, minimum inhibitory concentrations of which against *B. subtilis* BT-2 and *S. aureus* BMC-1 were 5.6-11 times lower compared to those obtained for biosurfactants synthesized in a medium without the inductor.

In the study [32], it was found that the introduction of live *B. subtilis* BT-2 cells into the *R. erythropolis* culture medium with ethanol (2%, v/v) was accompanied by the synthesis of surfactants that were characterized by higher antimicrobial activity than surfactants synthesized in the medium without inductor. The minimum inhibitory concentrations against test-cultures of these biosurfactants were 6 µg/ml, which were 8 times lower than the values established for the preparations obtained without the inductor. In further research [33], heat-inactivated cells of *B. subtilis* BT-2 were used instead of live cells of the inductor. It was established that the antimicrobial activity of surfactants synthesized under such cultivation conditions was 16-32 times higher than that of preparations formed during the cultivation of IMV As-5017 strain in medium without an inductor.

The summarized information on the effect of live and inactivated *B. subtilis* BT-2 cells on the antimicrobial activity of *N. vaccinii* IMV B-7405, *A. calcoaceticus* IMV B-7241 and *R. erythropolis* IMV AS-5017 biosurfactants is shown in Table 4.

These data showed that the antimicrobial activity of surfactants (*R. erythropolis* IMV Ac-5017 [32, 33], *N. vaccinii* IMV B-7405 [30] and *A. calcoaceticus* IMV B-7241 [31]) was almost unaffected by the physiological state of the inductors (live or inactivated cells).

It should be noted that there are only few reports on the influence of inductors in various physiological states on the biological activity of microbial surfactants (see Tables 1–3). In contrast to those described in the available literature, the synthesis of the surfactants we have studied is based on low-cost substrates, including industrial waste (crude glycerol, used sunflower oil). Additionally, synthesized in the presence of biological inductors biosurfactants by *R. erythropolis* IMV Ac-5017, *N. vaccinii* IMV B-7405, and *A. calcoaceticus* IMV B-7241 showed exceptionally high antimicrobial activity (MIC values ranging from 0.85 to 20 µg/ml, see Table 4).

lipopeptide synthesis (name not given) by *Streptomyces bikiniensis* strain HD-087 in the presence of supernatant after cultivation of *Magnaporthe oryzae* Guy11. The authors suggested that one of the mechanisms of induction is the presence of fungal intermediates in the supernatant, which are precursors to the biosynthesis of fatty acids, the components of lipopeptides.

Antibiotics. The influence of the supernatant after inductor cultivation on the synthesis of antibiotics by actinobacteria of the genus *Streptomyces* was studied in [21, 22, 26–28]. Most of the articles are devoted to the synthesis of natamycin, and micromycete supernatant was used as an inductor in these studies.

For instance, the authors of [21] showed that the introduction of *F. oxysporum* f. sp. *cucumerinum* or *S. cerevisiae* supernatant into the *S. rimosus* M527 culture medium was accompanied by an increase in rimocidin synthesis by 42 and 72%, respectively, compared to the values without inductors.

In the work [22], it was found that the synthesis of natamycin by strain *S. natalensis* HW-2 increased by 1.3 to 3 times with the addition of supernatant of micromycetes *A. niger* AS 3.6472 (1.5 and 2%) or *P. chrysogenum* AS 3.5163 (1.5 and 2%). At the same time, the presence of *S. cerevisiae* AS 2.2081 supernatant (2.5%) in the culture medium of the producer had practically no effect on the synthesis of the antibiotic.

In the study [26], the synthesis of natamycin by *S. natalensis* HW-2 strain was increased by 32% when *P. chrysogenum* AS 3.5163 supernatant was added to the culture medium, which was caused by overexpression of the ilvH gene in the antibiotic producer under such culture conditions. In addition, the method of RNA sequencing showed changes in the transcriptome of *S. natalensis* HW-2 under the influence of the inductor supernatant. In further studies [27], it was found that when the concentration of *P. chrysogenum* AS 3.5163 supernatant was increased to 6%, and introduced into the *S. natalensis* HW-2 cultivation medium after 24 h from the start of the process, the concentration of natamycin doubled compared to cultivation without inductor.

In the presence of the supernatant after the cultivation of *A. niger* or *P. chrysogenum* (5%) in the cultivation medium of *Streptomyces natalus* N5, there was a 1.7- to 2-fold increase in the concentration of natamycin was observed compared to the values without inductors [28].

Other secondary metabolites. In 2020 [29], researchers isolated, but did not characterise, a group of antibacterial metabolites from the culture of *Promicromonospora kermanensis* DSM 45485 in the presence of *P. aeruginosa* UTMC 1404 supernatant. The obtained compounds exhibited antimicrobial activity against *S. aureus* UTMC 1401: the growth inhibition zone was 23 mm.

In Table 3, we summarised the effect of the supernatant after cultivation of biological inductors on the production and antimicrobial activity of secondary metabolites. As well as in the presence of live (see Table 1) or inactivated (see Table 2) inductor cells, the use of the supernatant led to an intensification of the synthesis of bacteriocins, surface-active lipopeptides, and antibiotics.

The effect of prokaryotic inductors on the antimicrobial activity of microbial surfactants of *Nocardia vaccinii* IMV B-7405, *Acinetobacter calcoaceticus* IMV B-7241 and *Rhodococcus erythropolis* IMV AS-5017

Our own experimental studies [30–33] have shown the possibility of regulating the antimicrobial activity of surfactants of *N. vaccinii* IMV B-7405, *A. calcoaceticus IMV* B-7241 and *R. erythropolis* IMV AS-5017 by introducing live and inactivated cells of *B. subtilis* BT-2 and *E. coli* IEM-1 into the medium with ethanol and industrial waste (waste sunflower oil, crude glycerol).

In the study [30], it was observed that the presence in medium of *N. vaccinii* IMV B-7405 cultivation with sunflower oil (2% v/v), both live and inactivated cells of *B. subtilis* BT-2 led to the production of biosurfactants, antimicrobial activity of which against test cultures (*B. subtilis* BT-2, *S. aureus* BMC-1) was 3-6 times higher compared to the levels established for biosurfactants synthesized under cultivation of strain IMV B-7405 without the inductors.

Similar patterns were demonstrated in studies with another biosurfactant producer — *A. calcoaceticus* IMV B-7241 [31]. The results showed that live cells of *B. subtilis* BT-2 were more effective inductors: in their presence in a cultivation medium containing crude glycerol (5% v/v), biosurfactants

Table 3

Effect of supernatant after cultivation of inductors on the synthesis and antimicrobial activity of secondary metabolites

Producer	Carbon source	Biological inductor	Concentration (activity) of secondary metabolites		Test-cultures for determining antimicrobial activity	Antimicrobial activity	References
			without inductor	with an inductor			
1	2	3	4	5	6	7	8
Bacteriocins							
Bacillus subtilis NK16	Dextrose	*Staphylococus aureus* ATCC 43090 / *Bacillus* sp. ATCC 6633 / *Saccharomyces cerevisiae* / *Aspergillus niger* **(strain number not given)**	Bacteriocins 80 AU/ml	Bacteriocins 160 / 160 / 320 / 160 AU/ml	*Staphylococcus aureus* ATCC 43090	Growth inhibition zone of 27 mm (100 µl/disc)	4
					Bacillus sp. ATCC 6633	Growth inhibition zone of 25 mm (100 µl/disc)	
					Saccharomyces cerevisiae	Growth inhibition zone of 25 mm (100 µl/disc)	
					Aspergillus niger	Growth inhibition zone of 21 mm (100 µl/disc)	
Antimicrobia pigments l							
Streptomyces coelicolor **(strain number not given)**	Dextrin, peptone	*Leuconostoc mesenteroides* / *Lactobacillus plantarum* **(strain number not given)**	Prodigiosin* (concentration not given)		-----	N.d.	24
Microbial surfactants							
Streptomyces bikiniensis HD-087	Starch, glucose	*Magnaporthe oryzae* Guy11	Lipopeptides 285.6 mg/l	Lipopeptides 531.3 mg/l	-----	N.d.	25
Antibiotics							
Streptomyces rimosus M527	Soybean flour, mannitol	*Saccharomyces cerevisiae* / *Fusarium oxysporum* f. sp. *cucumerinum*	Rimocidin 0.21 g/l	Rimocidin 0.36 / 0.3 g/l (polyene macrolide)	-----	N.d.	21

Table 3 (Continued)

1	2	3	4	5	6	7	8
Streptomyces natalensis HW-2	Glucose, maltose	*Saccharomyces cerevisiae* AS 2.2081 / *Aspergillus niger* AS 3.6472 / *Penicillium chrysogenum* AS 3.5163	Natamycin 0.639 g/l	Natamycin 0.7 / 1.62 / 1.84 g/l (polyethylene antibiotic)	----	N.d.	22
Streptomyces natalensis HW-2	Oat	*Penicillium chrysogenum* AS 3.5163	Natamycin 0.85 g/l	Natamycin 1.25 g/l	----	N.d.	26
Streptomyces natalus N5	Glucose	*Aspergillus niger* / *Penicillium chrysogenum* **(strain number not given)**	Natamycin 0.85 g/l	Natamycin 1.44 / 1.69 g/l	----	N.d.	28
Streptomyces natalensis HW-2	Glucose	*Penicillium chrysogenum* AS 3.5163	Natamycin 1.2 g/l	Natamycin 2.49 g/l	----	N.d.	27
Other secondary metabolites							
Promicromonospora kermanensis DSM 45485	Dextrose	*Pseudomonas aeruginosa* UTMC 1404	The name is not given (group of antibacterial metabolites)		*Staphylococcus aureus* UTMC 1401	Growth inhibition zone of 23 mm (50 µg/disc)	29

Notes. N.d. — not determined; * — synthesis of new compounds not typical for monoculture.

***

Analysis of the published literature on the effect of biological inductors of various physiological states on the synthesis of antimicrobial secondary metabolites (see Tables 1–3) showed that the mechanisms responsible for increasing the synthesising ability of antimicrobial compound producers are currently unclear. The researchers identified some of them:

1) an increase in the synthesis of antimicrobial compounds as a protective mechanism against a competitive organism; the producer recognised particular proteins or receptors of inactivated inductor cells [4];

2) the presence of heat-inactivated inductor cells affected the expression of genes related to the synthesis of antimicrobial peptides [6];

3) the mechanism of interaction may be due to direct contacts between the cells [8];

4) inactivated inductor cells contain lysed compounds that can perform the role of precursors for metabolite production [10];

5) production of specific metabolites by fungi that stimulated transcriptional activation of a silent cluster of biosynthetic genes for the biosynthesis of antimicrobial compounds [27].

The available literature on the effect of biological inductors on the synthesis of antimicrobial compounds is much less than the literature concerning co-cultivation of microorganisms [1–3]. At the same time, the using biological inductors to obtain novel antimicrobial metabolites [12-15, 23, 29], increase the synthesis or activity of already known ones [4–11, 16–22, 25–28] is more technologically advanced, as such processes are easier to scale and implement (in particular, using inactivated inductor cells).

At the same time, it is necessary to pay attention to the costly growth substrates used for the biosynthesis of secondary metabolites. Obviously, the next stage of research should be devoted to finding lower-cost substrates for biosynthesis and optimising the composition of culture media. In addition, different scientists have often used the same biological inductors for different producers of various secondary metabolites. Expanding the range of both pro- and eukaryotic inductors should also be taken into account in further studies.

Table 4

Antimicrobial activity of microbial surfactants synthesized by *N. vaccinii* IMV B-7405, *A. calcoaceticus* IMV B-7241 and *R. erythropolis* IMV AS-5017 in the presence of live and inactivated *Bacillus subtilis* BT-2 cells

Producer	Carbon source	Physiological state of inductor cells	Test culture	Minimum inhibitory concentrations (μg/ml) of surfactants synthesized		References
				without inductor	with an inductor	
Nocardia vaccinii IMV B-7405	Used sunflower oil	Live	*Bacillus subtilis* BT-2	120	20	30
			Staphylococus aureus BMC-1	80	20	
		Heat-inactivated	*Bacillus subtilis* BT-2	120	40	
			Staphylococus aureus BMC-1	80	20	
Acinetobacter calcoaceticus IMV B-7241	Crude glycerol	Live	*Bacillus subtilis* BT-2	9.8	0.85	31
			Staphylococus aureus BMC-1	4.9	0.85	
		Heat-inactivated	*Bacillus subtilis* BT-2	9.8	2.2	
			Staphylococus aureus BMC-1	4.9	2.2	
Rhodococcus erythropolis IMV Ac-5017	Ethanol	Live	*Bacillus subtilis* BT-2	48	6	32
			Staphylococus aureus BMC-1	48	6	
		Heat-inactivated	*Bacillus subtilis* BT-2	96	3	33
			Staphylococus aureus BMC-1	48	3	

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ВПЛИВ БІОЛОГІЧНИХ ІНДУКТОРІВ НА СИНТЕЗ ТА БІОЛОГІЧНУ АКТИВНІСТЬ МІКРОБНИХ МЕТАБОЛІТІВ

Т. П. Пирог [1, 2], *М. С. Іванов*[1]

[1]*Національний університет харчових технологій*
[2]*Інститут мікробіології і вірусології ім. Д. К. Заболотного НАН України*

E-mail: tapirog@nuft.edu.ua

Зростаюча антибіотикорезистентність є серйозною проблемою для людства. Спільне (комбіноване) культивування мікроорганізмів є перспективним методом для отримання нових антимікробних метаболітів. Перспективним варіантом спільного культивування мікроорганізмів є використання так званих біологічних індукторів.

Мета огляду — узагальнення наявних у літературі наукових досліджень, що стосуються впливу фізіологічно різних типів біологічних індукторів на синтез та біологічну активність мікробних вторинних метаболітів.

Аналіз даних літератури показав, що у таких дослідженнях живі або інактивовані клітини індуктора вносять у середовище у значно нижчій концентрації порівняно з клітинами продуцента цільових метаболітів, або як індуктор використовують супернатант (фільтрат) після вирощування конкурентного мікроорганізму.

Згідно даних літератури і власних експериментальних досліджень використання індукторів є ефективним способом не тільки інтенсифікації синтезу бактеріоцинів, поверхнево-активних речовин, антибіотиків, а й підвищення їх біологічної активності, а також часто супроводжується утворенням нових антимікробних сполук, не характерних для продуцента.

Разом з тим потребують подальших досліджень механізми дії індукторів на синтез біологічно активних сполук, оскільки за даними літератури їх внесення у культуру продуцента не завжди супроводжувалося інтенсифікацією синтезу цільових продуктів. Крім того, біологічна активність вторинних метаболітів залежить від умов культивування продуцента, у тому числі від наявності біологічних індукторів у середовищі. Тому важливим є проведення подальших досліджень щодо взаємодії продуцентів з конкурентними мікроорганізмами для контролю біологічної активності синтезованих метаболітів. Необхідним є також пошук дешевших субстратів для біосинтезу вторинних метаболітів, оптимізація складу поживних середовищ і розширення спектру як про-, так і еукаріотичних індукторів.

Ключові слова: спільне культивування; індуктор; фізіологічний стан індуктора; антимікробні метаболіти.

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MECHANISMS OF ANTIVIRAL ACTIVITY OF FLAVONOIDS

O. I. GOLEMBIOVSKA, O. Ya. BESPALOVA, A. B. PROSVETOVA, S. M. SAMSONENKO, N. L. POYEDYNOK

National Technical University of Ukraine "Igor Sikorsky Kyiv Polytechnic Institute", Ukraine

E-mail: e.bespalova25.05@gmail.com

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The article examines the multifaceted mechanisms underlying the antiviral activity of flavonoids, compounds widely distributed in the plant kingdom.

The *aim* of the work was to review literature data on mechanisms of antiviral activity of flavonoids.

Methods. Publications were selected based on the PubMed (https://pubmed.ncbi.nlm.nih.gov/) databases published in 2015–2023. They include information on mechanisms of antiviral activity of flavonoids.

Results. Beginning with an overview of flavonoid structures, the document navigates through the intricate interactions between flavonoids and various stages of the viral life cycle. Drawing upon a comprehensive analysis of *in vitro* and *in vivo* studies, the review highlights the diverse ways in which flavonoids inhibit viral entry, replication, and release. Depending on their antiviral mechanisms, flavonoids can serve as preventive inhibitors, therapeutic inhibitors, or indirect inhibitors by influencing the immune system.

Conclusion. The synthesized information not only contributes to the advancement of antiviral research but also lays the foundation for the development of novel therapeutic interventions against a spectrum of viral infections.

Key words: flavonoids; antiviral activity; viral infection; bioactive compounds; host-pathogen interaction.

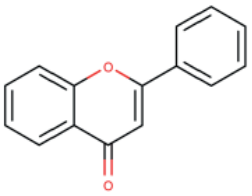
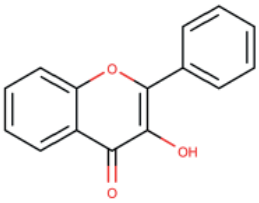
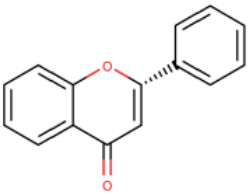
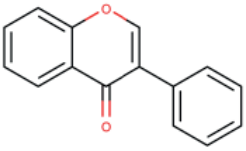
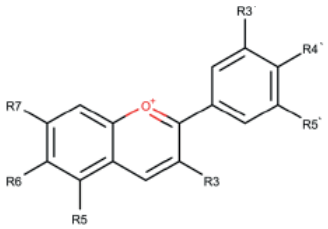
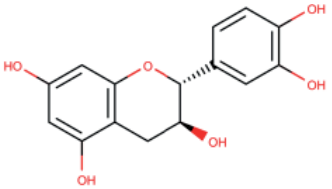
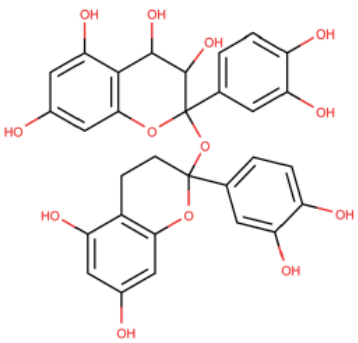
In the past few years, there has been an increased focus on exploring natural reservoirs of antioxidants. Flavonoids are a diverse group of polyphenolic compounds found in various plants and are known for their wide range of biological activities and health benefits [1, 2]. The flavonoids act at different stages of viral infection, such as viral entrance, replication and translation of proteins. They play important roles in plant biology, including pigmentation, UV protection, and defense against pathogens and herbivores [3].

Flavonoids are characterized by their chemical structure, which consists of two aromatic rings (A and B) connected by a three-carbon bridge forming a heterocyclic ring (C-ring). Depending on the substitution of this basic structure, flavonoids can be further categorized into different classes, including flavones, flavonols, flavanones, isoflavones, anthocyanins, and flavanols (catechins and proanthocyanidins) [4] (Table 1).

Flavonoids are crucial natural compounds with the capacity to demonstrate antiviral properties. Their importance in this context lies in their capability to engage with viruses at multiple stages of the viral life cycle, thereby making them promising candidates for the development of antiviral therapies [5].

Flavonoids have been demonstrated to disrupt viral replication by specifically targeting critical enzymes and essential proteins involved in the replication process [6]. One notable example is their ability to inhibit the function of viral polymerases, which play a vital role in synthesizing viral genetic material. This interference results in the disruption of

Table 1. **Common chemical structures of different flavonoid classes**

Class	Examples of compounds	The common structure of some classes of flavonoids
Flavones	Luteolin, tangeretin, apigenin	
Flavonols	Quercetin, kaempferol	
Flavanones	Eriodictyol, blumeatin, naringenin	
Isoflavones	Genistein, daidzein	
Anthocyanins	Cyanidin, delphinidin, malvidin, peonidin	
Flavanols (catechins and proanthocyanidins)	Catechin, epicatechin, epigallocatechin	 Structural formula of Catechin  Structural formula of Proanthocyanidins

new virus particle production, consequently restricting the infection's spread.

Numerous flavonoids are renowned for their potent antioxidant and anti-inflammatory characteristics [7]. Viral infections frequently induce oxidative stress and inflammation within the host, which can worsen the disease's impact. Flavonoids have the capacity to alleviate these effects, thereby diminishing tissue damage and alleviating the severity of symptoms linked to viral infections.

Flavonoids possess the ability to regulate the immune response, bolstering the body's capacity to protect against viral infections [5]. They have the potential to trigger the generation of immune cells and cytokines, crucial components of the antiviral immune response. This immunomodulatory impact can empower the host to mount a more efficient defense against the virus.

Certain flavonoids have been identified as effective blockers of viral attachment and entry into host cells [8]. They can disrupt viral attachment proteins or receptors on the surface of host cells, thus impeding the virus from entering and commencing the infection.

Flavonoids derived from food sources also display significant anti-viral effects by inhibiting the reverse transcriptase of various retroviruses, including HIV. Research has confirmed that extracts derived from hyssop leaves, which include tannic acids and flavonoids, effectively blocked the activity of reverse transcriptase, prevented syncytium formation, and reduced the expression of P17 and p24 HIV antigens in HIV-infected cells. Furthermore, recent preliminary studies have suggested that flavonoids and polyphenolic compounds like ferulic, gallic, and caffeic acids, ethyl gallate, curcumin, and a-tocopheryl-succinyl-O-ethyl ferulate can inhibit HIV replication by as much as 80% and also safeguard against the depletion of cellular glutathione [9].

- Flavonoids can interfere with viral entry by disrupting the integrity of the viral envelope.
- They can inhibit viral membrane fusion by affecting the conformational changes required for fusion.
- Flavonoids can disrupt the stability of the viral capsid, rendering the virus non-infectious.
- They can inhibit the activity of viral proteases, which are essential for viral replication and entry.
- Flavonoids can inhibit endocytosis, a process by which viruses enter host cells through vesicular uptake.

This mechanism plays a pivotal role in thwarting the initial phases of viral infection.

Flavonoids have the capacity to diminish the viral load in individuals who are infected, a critical factor in managing the virus's transmission and enhancing clinical outcomes. Reduced viral loads can result in milder symptoms and a shorter duration of illness.

Many flavonoids demonstrate a broad-spectrum antiviral capability, implying that they may have the potential to be effective against a diverse array of viruses, encompassing both RNA and DNA viruses [10]. This adaptability renders them valuable in the pursuit of antiviral treatment development.

Flavonoids are typically regarded as safe for consumption and exhibit low toxicity, particularly when contrasted with certain synthetic antiviral medications. This characteristic renders them appealing candidates for incorporation into antiviral therapies, as they are less prone to induce adverse side effects.

Owing to their various modes of action, flavonoids have the potential to reduce the emergence of antiviral resistance. When treatments target multiple phases of a virus's life cycle simultaneously, viruses are less inclined to develop resistance against them.

To highlight and understand all possible mechanisms of flavonoid's antiviral activity this systematic review was made.

Materials and Methods

The systematic review methodology for this article involves a structured approach to identifying, selecting, and analyzing relevant studies.

Search criteria was a research question and relevant keywords and phrases related to flavonoids, antiviral activity, synergies, and combinations such as "flavonoids," "antiviral," "mechanism," and specific flavonoid names (e.g., "quercetin," "rutin," "epigallocatechin gallate") etc.

Results and Discussion

Flavonoids have been extensively researched for their effectiveness against a diverse array of DNA and RNA viruses. Broadly, flavonoids employ multiple mechanisms of action. Mechanisms by which flavonoids can interfere with virus attachment and entry into host cell are direct interaction with viral receptors, inhibition of viral fusion, disruption of viral envelope integrity,

modulation of host cell signaling, stimulation of innate immune response, through anti-inflammatory, immunomodulatory effects and antioxidant activity, interference with viral replication (Fig. 1).

Therefore, flavonoids can impede the viruses by preventing their attachment and entry into host cells, disrupt various stages of viral replication processes, hinder translation and polyprotein processing, thereby curtailing the release of viruses for infecting other cells. Various flavonoids have been discovered to inhibit viruses through diverse mechanisms. Depending on their antiviral mechanisms, flavonoids can serve as preventive inhibitors, therapeutic inhibitors, or indirect inhibitors by influencing the immune system [11].

I. Direct Interaction with Viral Receptors

Flavonoids can bind directly to viral receptors, blocking the attachment of the virus to host cells. They have the capacity to bind specifically to viral receptors on the surface of viruses. These receptors are often proteins or glycoproteins that viruses use to recognize and attach to host cells. Flavonoids can form non-covalent interactions, such as hydrogen bonds or van der Waals forces, with these receptors, effectively blocking their active sites.

1. *Direct Binding to Viral Envelope Proteins and Altering Envelope Protein Conformation*: Flavonoids can directly bind to viral envelope proteins, such as glycoproteins, spike proteins, or hemagglutinins, which are responsible for recognizing and attaching to host cell receptors. Epigallocatechin-3-gallate inhibit hepatitis C virus E2 envelope glycoprotein *in silico* [12–14]. This interaction can interfere with the ability of the viral protein to bind to its cellular receptor, effectively blocking the initial attachment step.

Flavonoids can induce structural changes in viral envelope proteins.

Such compounds as flavonols (quercetin, kaempferol), flavones (apigenin, nobiletin), isoflavones (genistein), flavanones (naringenin), gingerols (6-gingerol, 8-gingerol), polyphenols (resveratrol), and catechins (epigallocatechin gallate, EGCG) were studied against E protein of the SARS CoV-1 with patch-clamp electrophysiology and a cell viability assay [15]. EGCG showing the highest inhibitory activity.

In another study [16] among different studied flavonoids (baicalein, fisetin, hesperetin, naringenin/ naringin, quercetin and rutin) that possess anti dengue activity only quercetin can interrupt the fusion process of virus by inhibiting the hinge region movement and by blocking the conformational rearrangement in envelope protein *in silico*.

This structural disruption can hinder the proper conformation of viral proteins required for attachment to host cells. As a result, the

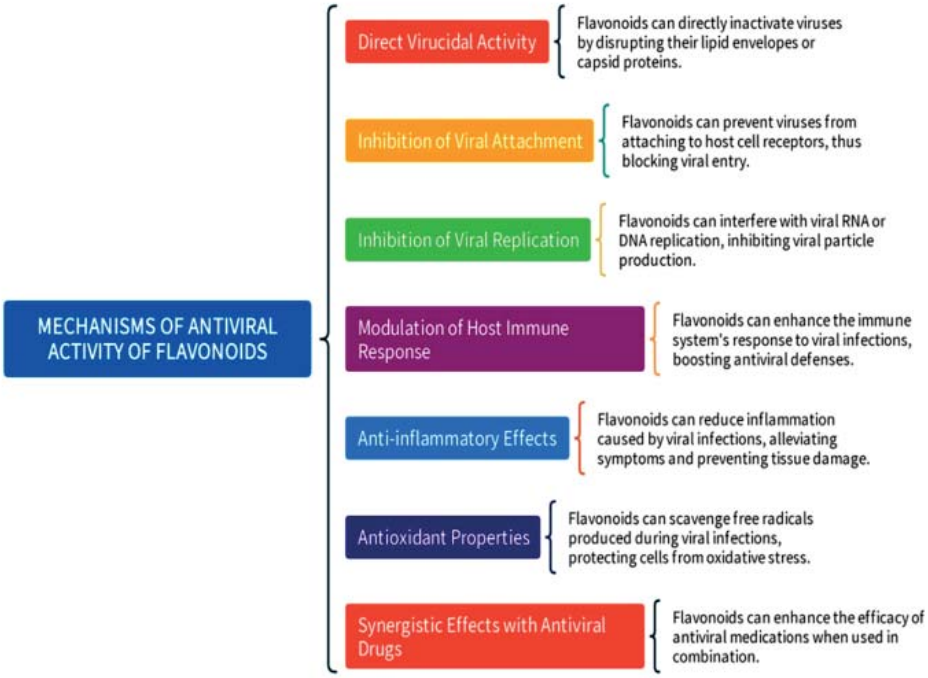


Fig. 1. **Common schema for mechanisms of antiviral activity of flavonoids**

virus may lose its ability to recognize and bind to cellular receptors effectively.

2. *Inhibition of Fusion Peptides*: Some flavonoids can interact with fusion peptides present in viral envelope proteins [17]. These peptides play a crucial role in facilitating the fusion of the viral envelope with the host cell membrane [18]. Flavonoids can interfere with this fusion process by binding to or blocking the fusion peptides. This physical interaction hinders the initial step of viral entry, preventing infection.

Summarized aspects of mechanisms of interaction between flavonoids and virus are described in [13] and represented in Fig. 2.

II. Inhibition of Viral Fusion

Several flavonoids have shown promising effects in inhibiting viral entry by interfering with the attachment and fusion processes [10, 19]. While the effectiveness of specific flavonoids may vary depending on the virus and host cell type, here are some examples of flavonoids that have demonstrated antiviral properties in inhibiting viral entry:

Quercetin inhibits the attachment of influenza A virus to host cells by interfering with the interaction between viral envelope proteins and host cell receptors [20, 21]. It can also inhibit viral entry by preventing the fusion of the viral envelope with the host cell membrane [22].

Epigallocatechin-3-*O*-gallate has been shown to block the binding of various types of enveloped DNA, (+)-RNA, and (–)-RNA viral attachment proteins to host cell receptors [14, 23–25]. It can also inhibit viral entry by interfering with the fusion process between the viral envelope and the host cell membrane.

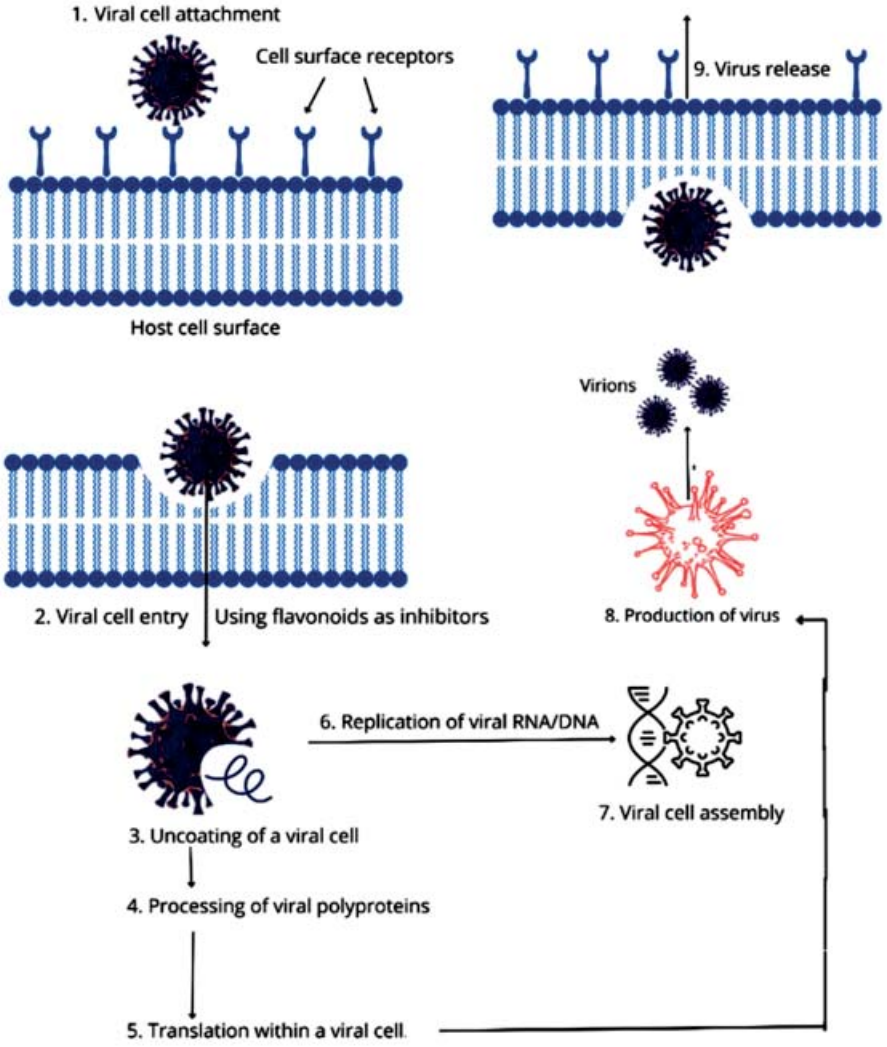


Fig. 2. **The mechanism of interaction between flavonoids and virus**

Baicalin interferes with dengue virus attachment by inhibiting the binding of viral glycoproteins to host cell receptors [24, 26].

Rutin, apigenin and luteolin can block viral attachment by interfering with the interaction between HIV-1 viral glycoproteins and host cell receptors [27–31]. It can also inhibit viral entry by disrupting the fusion process between the viral envelope and the host cell membrane.

It's important to note that while these flavonoids have shown promise in laboratory studies for their antiviral effects, their effectiveness can vary depending on the specific virus, the concentration of flavonoids used, and the experimental conditions.

III. Disruption of Viral Envelope Integrity

Disruption of viral envelope integrity is yet another mechanism through which flavonoids can exert their antiviral effects as it was described for rotavirus for catechin isomers and proanthocyanidins in [32]. Many viruses rely on their outer lipid envelope for protection. This interference can lead to changes in the fluidity, organization, or structural integrity of the lipid membrane, making it more vulnerable to damage.

IV. Modulation of Host Cell Signaling

1. *Competitive Binding*: Flavonoids can compete with viral particles for binding to host cell receptors. By occupying these receptors, flavonoids effectively block the attachment of the virus to host cells. This competitive binding is particularly relevant for viruses that require specific receptors to enter host cells.

2. *Modulation of Receptor Expression*: Some flavonoids can modulate the expression of host cell receptors involved in viral attachment and entry. For example, they may downregulate the expression of these receptors, making it more challenging for viruses to find and attach to host cells.

3. *Alteration of Receptor Properties*: Flavonoids may affect the physical properties of host cell receptors, such as changes in receptor conformation or charge. These alterations can make it more difficult for viral attachment proteins to bind to the receptors effectively.

4. *Inhibition of Signaling Pathways*: Flavonoids can interfere with host cell signaling pathways involved in the regulation of receptor expression and viral entry. By modulating these pathways, flavonoids can reduce the susceptibility of host cells to viral attachment and entry. through the activation of specific kinases or transcription factors that are required for viral replication. By disrupting these signaling pathways, flavonoids can impede viral multiplication. For example, luteolin can interfere with various cell signaling pathways – it may inhibit the PI3K/Akt/mTOR pathway, which is involved in cell survival and proliferation [21].

Flavonoids can modulate signaling pathways that control the balance between pro-apoptotic and anti-apoptotic factors. For instance, they may activate stress-activated protein kinases, such as JNK (c-Jun N-terminal kinase) or p38 MAPK (mitogen-activated protein kinase), which can promote pro-apoptotic signals.

5. *Strengthening the Host Immune Response*: Flavonoids with immunomodulatory properties can enhance the host immune response. A robust immune response can reduce the viral load and the likelihood of successful viral attachment and entry into host cells [33].

It's important to note that the specific interactions between flavonoids, viral envelope proteins, and host cell receptors can vary depending on the flavonoid compound and the virus in question. Additionally, the efficacy of flavonoids as antiviral agents may be influenced by factors such as the concentration of flavonoids, the timing of treatment, and the viral strain's characteristics.

V. Stimulation of Innate Immune Response

Flavonoids can influence the host's immune system to combat viral infections through various mechanisms [34]. Their immunomodulatory properties make them valuable in enhancing the body's ability to defend against viruses.

This mechanism is carried out through the stimulation of cytokine production (flavonoids can promote the release of interferons (IFNs), which have antiviral properties and help the immune system combat viral infections); enhancement of antigen presentation (flavonoids can improve antigen presentation by antigen-presenting cells (APCs) such as dendritic cells. This facilitates the recognition of viral antigens by immune cells like T cells, leading to a more robust immune response).

Other aspects of this action are activation of natural killer (NK) cells; modulation of T cell responses (flavonoids can influence T cell responses, including the activation and proliferation of cytotoxic T cells (CTLs) that directly target infected cells [35]. This

helps eliminate virus-infected cells from the body); regulation of inflammatory responses; protection of immune cells from damage caused by oxidative stress, which is often elevated during viral infections; enhancement of humoral immunity; modulation of inflammatory signaling pathways (flavonoids can interfere with pro-inflammatory signaling pathways, such as nuclear factor-kappa B (NF-κB), which are often activated during viral infections), that contribute to a balanced immune response; reduction of immunosuppression and enhancement of mucosal immunity.

Found in green tea, EGCG exhibits immunomodulatory properties by enhancing the activity of NK cells and promoting the production of interferons [36]. It can also suppress the production of pro-inflammatory cytokines, helping to control excessive inflammation during viral infections.

Baicalin, derived from *Scutellaria baicalensis* (Chinese skullcap), has demonstrated immunomodulatory effects by enhancing the phagocytic activity of macrophages and increasing the production of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), which are important for antiviral responses [37, 38, 39].

VI. Anti-Inflammatory Effects

Flavonoids possess anti-inflammatory properties that can help regulate excessive inflammation during viral infections. By reducing inflammation, flavonoids can alleviate symptoms and limit tissue damage [40].

Flavonoids can inhibit the production and release of pro-inflammatory mediators, such as cytokines (e.g., interleukin-1 beta, interleukin-6, tumor necrosis factor-alpha), chemokines, and prostaglandins. By reducing the levels of these inflammatory molecules, flavonoids dampen the overall inflammatory response triggered by viral infections [41, 42].

Flavonoids can interfere with signaling pathways involved in inflammation. For example, they may inhibit the activation of nuclear factor-kappa B (NF-κB), a key transcription factor that promotes the expression of pro-inflammatory genes. By blocking NF-κB activation, flavonoids reduce the production of inflammatory cytokines [34].

Quercetin is a widely studied flavonoid found in various foods such as apples, onions, and citrus fruits. It possesses strong antioxidant properties and has been shown to inhibit the production of pro-inflammatory cytokines, making it a potent anti-inflammatory agent in [43–45].

VII. Interference with Viral Replication

Flavonoids can impact viral replication and multiplication within host cells through various mechanisms. Their ability to interfere with different stages of the viral life cycle makes them valuable candidates for controlling viral infections. Here's an exploration of how flavonoids can affect viral replication and multiplication:

1. *Inhibition of Viral Enzymes*: Many flavonoids have been shown to inhibit key viral enzymes involved in replication. For example, flavonoids can inhibit viral RNA polymerases or reverse transcriptases, essential for the replication of RNA and retroviruses, respectively. By blocking these enzymes, flavonoids can significantly reduce viral genome replication, hindering the production of new virus particles. In [46, 47] quercetin potently inhibits Enterovirus 71 and porcine epidemic diarrhea virus activity 3C protease activity, thereby blocking its replication. Proanthocyanidins from blueberry has strong antiviral activity against hepatitis C virus (HCV) and human T-lymphocytic leukemia virus type 1 (HTLV-1) via inhibition of ACE2 and viral 3CLpro (3-chymotrypsin-like) enzymes [48]. Herbacetin, rhoifolin and pectolinarin in the study [49] demonstrated inhibitory activity against SARS-CoV 3C-like protease.

2. *Interference with Viral Protein Synthesis*: Flavonoids may also interfere with the synthesis of viral proteins, which are crucial for the assembly of new virus particles. By inhibiting viral protein synthesis, flavonoids can disrupt the virus's ability to replicate and multiply within host cells. Myricetin demonstrated both in vitro and in vivo blocking HSV infection through direct interaction with virus gD protein [50, 51].

Disruption of RNA/DNA replication - flavonoid-mediated inhibition of viral enzymes and RNA/DNA replication represents a multifaceted approach to disrupting the viral life cycle.

• Interference with Nucleotide Incorporation: Flavonoids can interfere with the incorporation of nucleotides into the growing viral RNA or DNA strand [52]. By competing with nucleotides for binding to the viral polymerase or by modifying the structure of nucleotides, flavonoids can disrupt the elongation of the viral genome, preventing the

formation of complete viral genetic material.

• Template Strand Destabilization: Flavonoids can destabilize the template RNA or DNA strand that serves as a blueprint for viral genome replication, for example against SARS-CoV-2 targets [53, 54]. This destabilization can make it more challenging for viral polymerases to use the template for accurate replication, leading to errors in the newly synthesized genetic material.

• RNA/DNA Cleavage: Some flavonoids possess the ability to cleave RNA or DNA molecules [55, 56]. By inducing breaks in the viral genome, flavonoids can introduce mutations and inhibit proper replication. This can lead to the production of nonfunctional viral genetic material.

• Inhibition of Helicases: Helicase enzymes are essential for unwinding the viral genome during replication. Flavonoids can inhibit helicase activity, preventing the separation of the DNA or RNA strands required for replication. Authors [57] report for the first time myricetin, quercetin, kaempferol and licoflavone C as selective inhibitors of SARS-CoV-2 nps13 helicase with low micromolar activity in both *in silico* and *in vitro*.

• Impairment of Nucleotide Synthesis: Flavonoids can affect the host cell's ability to synthesize nucleotides, which are essential building blocks for viral RNA and DNA replication as it is described for human T cell leukemia virus by the plant flavonoid baicalin [58]. By disrupting nucleotide biosynthesis, flavonoids limit the availability of raw materials required for viral genome replication.

These mechanisms not only hinder the synthesis of viral genetic material but can also introduce errors and mutations into the viral genome, further compromising the virus's ability to replicate effectively.

4. *Induction of Host Antiviral Responses and Reduction of Oxidative Stress*: Some flavonoids can stimulate the host's innate antiviral responses. They can promote the production of antiviral cytokines, such as interferons, and activate immune cells like natural killer (NK) cells. These responses can limit viral replication and the spread of infection.

Viral replication often generates oxidative stress in host cells. Flavonoids, known for their antioxidant properties, can help mitigate this stress by scavenging reactive oxygen species (ROS). Lowering oxidative stress can indirectly hinder viral replication, as viruses may exploit ROS for their own replication.

5. *Preventing Viral Assembly and Budding*: Some flavonoids can interfere with the assembly and budding of new virus particles from host cells. By inhibiting the interaction between viral structural proteins and host cell membranes, flavonoids can block the release of virions, reducing viral replication and spread.

6. *Modulation of Cellular Microenvironment*: Flavonoids can modify the cellular microenvironment, making it less conducive to viral replication. For example, they may alter the pH within endosomes or lysosomes, which can hinder the release and processing of viral components.

7. *Impairment of Viral Protein Transport and Decreased Viral Entry and Attachment*: Flavonoids can interfere with the transport of viral proteins within host cells. This disruption can prevent the proper assembly of new virus particles and reduce viral multiplication.

Flavonoids can also impact viral replication by reducing viral entry and attachment, as discussed in previous responses. By blocking these early stages of infection, they limit the number of cells that become infected and reduce the potential for viral multiplication.

VIII. Antioxidant Activity

Almost all polyphenolic compounds and flavonoids among them posseses antioxidant activity because of the presence of phenolic hydroxyl radicals.

Flavonoids can act as scavengers of reactive oxygen species, such as superoxide radicals ($O_2 \cdot -$), hydroxyl radicals ($\cdot OH$), and hydrogen peroxide (H_2O_2). They neutralize these harmful molecules, preventing oxidative damage to cellular components. Flavonoids can chelate metal ions like iron and copper, which can participate in the generation of ROS through Fenton reactions. By binding to these ions, flavonoids reduce their ability to catalyze oxidative reactions [59]. Flavonoids can upregulate the activity of endogenous antioxidant enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase. These enzymes help detoxify ROS and maintain cellular redox balance. Some flavonoids have the capacity to regenerate other antioxidants like vitamin C and vitamin E, which further enhances the cellular defense against oxidative stress [60].

But it is not only antioxidant properties of flavonoids are responsible for this mechanism. Rutin is known for its antioxidant properties, which help reduce oxidative stress and inflammation [61]. During viral infections, the virus can induce oxidative stress within

host cells. Viral replication processes and host immune responses can generate ROS, leading to oxidative damage to cellular structures. And rutin's antioxidant activity may protect cells from damage caused by free radicals.

Viral-induced oxidative stress can result in DNA damage, protein oxidation, and lipid peroxidation. These effects can impair cell function and promote viral replication. Flavonoids, by acting as antioxidants, can protect cellular components from oxidative damage. They scavenge ROS and reduce the oxidative burden on DNA, proteins, and lipids. Flavonoids' anti-inflammatory properties can help mitigate the oxidative stress associated with viral infections. By suppressing inflammation, they indirectly reduce ROS production. This group of biologically active substances can boost the activity of endogenous antioxidant enzymes, reinforcing the cell's ability to neutralize ROS generated during viral infections.

Some studies [55, 62] suggest that flavonoids may directly inhibit viral replication by disrupting the redox balance required for efficient

IX. Induction of Cell Death

Flavonoid-induced apoptosis is a mechanism by which certain flavonoid compounds can trigger programmed cell death in virus-infected cells. Apoptosis is a tightly regulated and controlled process that plays a critical role in the body's defense against viral infections [63].

Flavonoid-induced apoptosis works to eliminate virus-infected cells through recognition of virus-infected cells, activation of apoptotic pathways, inhibition of anti-apoptotic proteins such as Bcl-2 and Bcl-xL, release of pro-apoptotic factors, such as cytochrome c, from the mitochondria into the cytoplasm, activation of caspases (they cleave and activate downstream effector proteins), DNA fragmentation and cell shrinkage, formation of apoptotic bodies with its further phagocytosis and as a result limiting viral spread (Fig. 3).

Further research is needed to understand the precise mechanisms of flavonoid-induced apoptosis and its potential applications in antiviral therapies.

X. Synergistic Effects with Antiviral Drugs

The combination of flavonoids with conventional antiviral agents can offer several potential benefits in the management of viral infections [64]. While flavonoids alone may not replace conventional antiviral drugs, they can complement these agents in several ways:

– *Enhanced Antiviral Activity*: Combining flavonoids with conventional antiviral agents may enhance their overall antiviral activity. Flavonoids can target different stages of the viral life cycle, potentially inhibiting viral replication through mechanisms that differ from those of conventional antivirals.

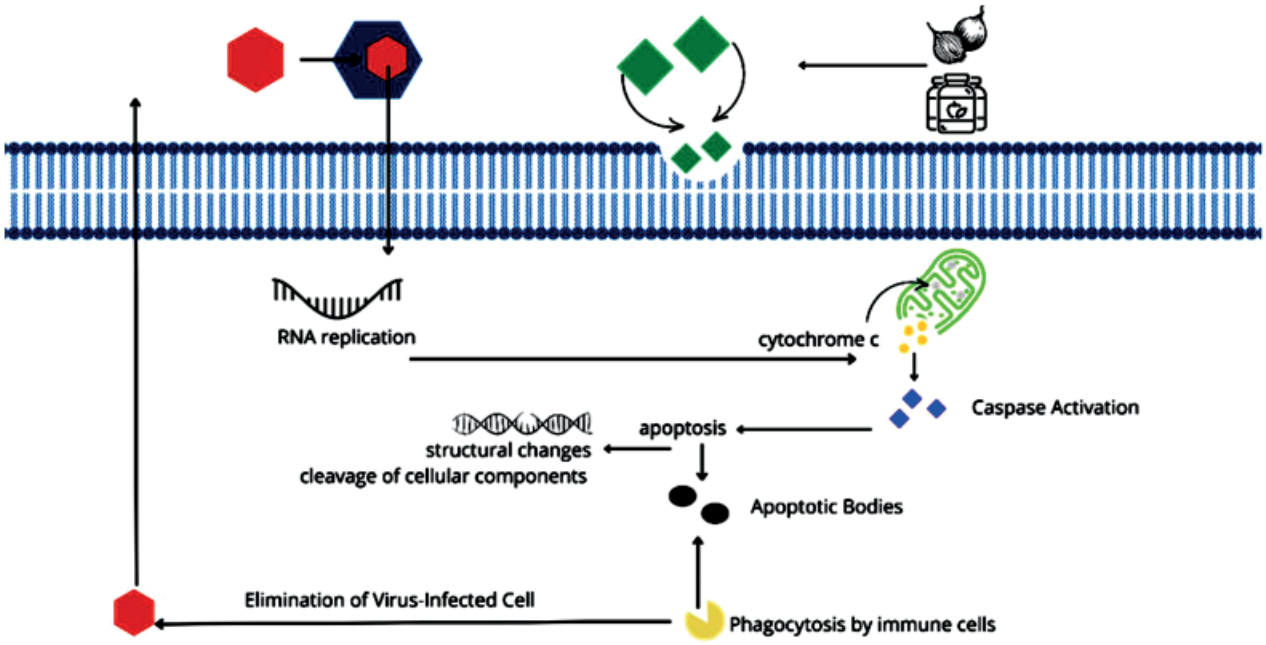


Fig. 3. **Flavonoids' potential in reducing viral spread through programmed cell death**

– *Reduced Antiviral Resistance*: The use of combination therapy with flavonoids and conventional antiviral drugs can reduce the likelihood of antiviral resistance. Viruses that develop resistance to one type of drug may still be vulnerable to inhibition by flavonoids, providing an alternative means of control.

– *Broad-Spectrum Antiviral Activity*: Flavonoids often exhibit broad-spectrum antiviral activity, meaning they can be effective against multiple types of viruses. This versatility is valuable when dealing with viral infections for which specific antiviral drugs may not be available.

– *Immunomodulation*: Flavonoids can modulate the immune response, helping to strike a balance between viral suppression and preventing excessive inflammation [65]. This can be especially important in cases where the immune response itself contributes to tissue damage and disease severity.

– *Reduction of Drug Toxicity*: Some conventional antiviral drugs can have side effects or toxicity concerns. Flavonoids, being natural compounds found in many foods, are generally considered safe and may help reduce the overall toxicity of antiviral treatments when used in combination.

– *Antioxidant and Anti-Inflammatory Effects*: Viral infections often induce oxidative stress and inflammation. Flavonoids' antioxidant and anti-inflammatory properties can help mitigate these effects, improving the overall health of the host and potentially reducing disease severity [66].

– *Support for the Immune System*: Flavonoids can support the immune system, enhancing its ability to combat viral infections. This can include the activation of immune cells, the regulation of cytokine production, and the reduction of immunosuppression induced by some viruses.

– *Potential Synergistic Effects*: In some cases, flavonoids and conventional antiviral agents may have synergistic effects, meaning their combined action is more effective than the sum of their individual effects. This synergy can lead to improved viral control.

– *Reduced Reliance on High Drug Doses*: Using flavonoids in combination with antiviral agents may allow for the use of lower doses of the conventional drugs. This can help reduce the risk of side effects associated with high drug doses.

The effectiveness of combination therapy with flavonoids and conventional antiviral agents may vary depending on the specific flavonoid compounds, the viral strain, and the stage of infection. Clinical studies and trials are necessary to determine the optimal combinations and dosages for specific viral infections. Additionally, healthcare professionals should be consulted when considering the use of such combinations to ensure safety and efficacy.

Conclusions

The antiviral activity of flavonoids involves multiple mechanisms:

Inhibition of Viral Entry: Flavonoids may interfere with viral attachment to host cells, preventing successful entry.

Disruption of Viral Envelope: Some flavonoids can destabilize viral envelopes, compromising the integrity of the viral structure.

Interference with Viral Replication: Flavonoids might target various stages of the viral replication cycle, inhibiting synthesis of viral components.

Modulation of Host Immune Response: Flavonoids may enhance the host immune system, aiding in the recognition and elimination of infected cells.

Antioxidant Effects: The antioxidant properties of flavonoids could contribute to their antiviral activity by reducing oxidative stress associated with viral infections.

Flavonoids such as quercetin, kaemferol, myricetin, catechin, and epigallocatechin gallate have been found to block the attachment of viruses [14, 45, 67, 68]. On the other hand, flavonoids including luteolin, apigenin, naringenin [69], hesperidin [70], and chrysoriol have been identified as inhibitors of viral replication. Certain combinations of these flavonoids, such as quercetin with luteolin or kaemferol with apigenin, have shown potential synergistic effects. However, the most effective combination of flavonoids will vary depending on the type of virus, the host cell, and other factors. Flavonoids also offer additional health benefits, including antioxidant, anti-inflammatory, and anti-cancer properties. Due to these properties, flavonoids hold promise as natural antiviral agents and may be considered for the development of antiviral drugs.

In conclusion, the diverse mechanisms by which flavonoids act against viruses make them a promising class of antiviral agents. Their ability to target different stages of the viral life cycle, coupled with potential immunomodulatory effects, highlights the potential of flavonoids in the development of novel antiviral therapies.

However, it's essential to note that further research and clinical studies are needed to fully understand their efficacy and safety in specific viral infections.

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МЕХАНІЗМИ ПРОТИВІРУСНОЇ ДІЇ ФЛАВОНІДІВ

Голембіовська О. І., Беспалова О. Я., Просвєтова А. Б., Самсоненко С. М., Поєдинок Н. Л.

Національний технічний університет України
«Київський політехнічний інститут імені Ігоря Сікорського», Україна

E-mail: e.bespalova25.05@gmail.com

У статті розглядаються багатогранні механізми, що лежать в основі противірусної активності флавоноїдів — сполук, широко поширених у царині рослин.

Мета. Огляд даних літератури щодо механізму противірусної дії флавоноїдів.

Методи. Публікації відбиралися на основі баз даних PubMed (https://pubmed.ncbi.nlm.nih.gov/), опублікованих у 2015–2023 роках. Вони містять інформацію про механізми противірусної дії флавоноїдів.

Результати. Починаючи з огляду структур флавоноїдів, у документі обговорюється складна взаємодія між флавоноїдами та різними стадіями життєвого циклу вірусу. Спираючись на комплексний аналіз досліджень *in vitro* та *in vivo*, висвітлюються різноманітні способи, якими флавоноїди пригнічують проникнення, розмноження та вивільнення вірусу. Залежно від їхніх антивірусних механізмів, флавоноїди можуть слугувати профілактичними інгібіторами, терапевтичними інгібіторами або непрямими інгібіторами, впливаючи на імунну систему.

Висновок. Синтезована інформація не тільки сприяє розвитку антивірусних досліджень, але й закладає основу для розроблення нових терапевтичних методів подолання вірусних інфекцій.

Ключові слова: флавоноїди; противірусна активність; вірусна інфекція; біоактивні сполуки; взаємодія хазяїн-збудник.

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RADIATION AND HYPOXIA STUDIES: EFFECTS OF HIGH-ENERGY ATMOSPHERIC PARTICLES ON BIOLOGICAL ORGANISMS AND POSSIBILITIES OF THEIR REHABILITATION

O. M. KLYUCHKO[1], *G. V. LIZUNOV*[2], *P. V. BELOSHITSKY*[3]

[1]National Aviation University, Educational and Research Institute of Air Navigation, Kyiv, Ukraine
[2]Space Research Institute of the National Academy of Sciences of Ukraine — State Space Agency of Ukraine, Kyiv
[3]P. Tychyny Uman State Pedagogical University, Ukraine

E-mail: kelenaXX@nau.edu.ua

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The influences of cosmic radiation on atoms and molecules in the Earth's atmosphere were observed with subsequent transformation of atoms, molecules of gases, as well as development of states of oxygen deficiency (hypoxic) in biological organisms, some recommended ways of such disorders correction.

Purposes of this work were to study radiation effects in ionosphere with subsequent high-energy transformations of atoms, molecules of gases at different heights above the Earth surface; interaction of some high-energy atmospheric particles with biological objects at near Earth's heights up to 5.500 m above sea level, and oxygen roles in consequences of biological organisms' irradiation.

Methods. Analysis of results of satellite and rocket observations of the Earth atmosphere gases exploring at different altitudes above sea level. The investigations were done in mountain conditions at EMBS research station of the National Academy of Sciences of Ukraine. The comparative analysis of results of long-term observation of patients using standard laboratory methods, complex of methodological techniques such as clinical, physiological studies of respiratory, and cardiovascular systems. The research has been carried out concerning hematological, immunological states; functional state of higher nervous activity, mental and neurotic state; antihypoxants use, histochemical, biophysical methods, math modelling, others.

Results. The data obtained during the satellites atmosphere exploring were presented: studies of influences on the structure of atoms, molecules in atmosphere, concentrations of gases from ionosphere to the Earth surface, such phenomena as photochemical processes, photoionization. The notion "information" was discussed basing on the phenomena, described in the article. Described studies of gases particles modification, oxygen deficiency in organisms (hypoxic states) were supplemented with the results of irradiated Chernobyl patients' examinations, rehabilitation by Ukrainian doctors, scientists in mountain conditions.

Conclusions. Phenomena of solar radiation influence on atoms, molecules and molecular complexes in the Earth's atmosphere was observed. The main attention was concentrated on the studies of gases concentrations at different heights with linked effects of oxygen roles in consequences of organisms' irradiation and rehabilitation. Practical recommendations for patients' medical care and rehabilitation were done.

Key words: radiation damage of organisms; hypoxia; high altitudes; high-energy particles; free radicals.

Humanity solves a number of contemporary practical problems high above the Earth's surface. These are high-altitude aviation flights, as well as space flights, in which crewmembers and passengers are exposed to significant doses of radiation as well as molecules of oxygen or other gases deficiency in case of contact of organism with the surrounding atmosphere. Some publications in this item were prepared in Ukraine [1–4] and abroad [5–7]. Our predecessors in science suggested the concept of "environment conditionally suitable for life" (approximately above 3 thousand m above sea level (a.s.l.)) and "environment unsuitable for life" (approximately above 5 thousand m a.s.l.) of human and other higher living organisms in mountains [3]. In our previous works, we observed a number of physical factors in the Earth atmosphere, like ones, reveled during earthquakes studies that make environment unsuitable for life [8, 9]. In present publication, we consider briefly the impact of cosmic radiation factors on atoms, molecules and molecular complexes in the Earth's atmosphere [1, 10–15]. Consequently, the first group of methods that had given the data to present article included the results of satellite observations and rocket observations [1–4, 16–21]. Because of this influence, the stay of humans and higher living organisms above the indicated limits becomes impossible. Today's task is to diagnose the full range of gas and electrodynamic parameters that characterize the ionosphere. Such diagnostics is possible only *in situ*, on spacecraft launched into the ionosphere [1, 3, 22–28]. For this purpose, special low-orbit satellites and high-apogee sounding rockets are used. Satellites are good because they allow ones to place solid instrumentation systems on board and take measurements on a planetary scale [1–4, 29–35]. During the space age, about 20 ionospheric satellites were launched, the last of which, the Chinese Seismo-Electromagnetic Satellite Mission (CSES), was realized in 2018 [1, 3, 36, 37]. The heights of satellite orbits are strictly limited from below by the deceleration factor against the atmosphere — at least 250–300 km. Even then, if the orbit height is maintained with the help of corrective engines [38–41] this open the possibilities of novel equipment usage for discoveries [42–45], as well as new methods development [46]. In most cases, ionosphere satellites are launched to altitudes of 500–700 km, well developed for the needs of remote sensing, into the outer part of the ionosphere (Fig. 1) [47–53]. Thus, the D and E regions and the lower part of the F region, which are so important for understanding the ionosphere and space weather, are beyond the capabilities of satellite sensing [47–52]. Satellite and rocket experiments not only complement each other, they must be combined with each other [53–57]. This gives the possibility for mathematical and program modelling [58–61], as well as for theoretical studies and conclusions [60]. Finally, all such data basing today in science and technique are the results of satellite observations as well as obtained in process of rocket observations [1–5, 7–62]. The second group of methods we used for our studied was linked with investigations in high-mountain conditions at research station of the National Academy of Sciences of Ukraine (EMBS). There are the comparative analysis of the results of long-term observation of patients in hospital conditions using many standard laboratory methods of their states examinations. The conducted scientific research consisted of a complex of methodological techniques and approaches such as clinical and physiological studies of respiratory and cardiovascular systems, hematological and immunological states, and functional state of higher nervous activity, mental and neurotic state. Administration of antihypoxants, histochemical, biophysical and other methods were used to evaluate oxybiotic processes. Mathematical processing of the results, as well as methods of mathematical modeling were applied. In addition, the next question that we raised in the process of these studies was: what measures should be taken in order to secure the stay of people above such limits. Our well-grounded suggestions to use some specific pharmacological preparation for the prevention of some biological system damage were done previously [3, 62–65].

So, the sequence of material in present article is the following: 1) to observe briefly the impact of cosmic radiation on atoms and molecules in the Earth's atmosphere. Further, to examine subsequently a chain of interconnected natural phenomena: 2) transformation of atoms and molecules of gases in high-altitude atmospheric conditions, 3) as a result, damages of respiration effects in biological organisms and development of oxygen deficiency (hypoxic) states in them. Finally, 4) giving some recommendations of the ways of possible correction of developed hypoxic states and some other pathological states linked with cosmic radiation influences. In parallel, some information aspects of the organization of substances at different altitudes will be considered.

The purposes of this observation were to study deeply radiation effects in the ionosphere of the Earth with subsequent high-energy transformations of gases molecules at different altitude levels above the Earth surface, interaction of some high-energy atmospheric particles with biological objects at near Earth's altitudes (up to 5.500 m a.s.l.), and the impact on information processes in such complex systems. In addition, the manifestation of hypoxia phenomena was of our interest as well as its study in living systems by scientific groups of the National Academy of Sciences of Ukraine with possibility of organisms' further rehabilitation.

Atoms and molecules in the upper layers of the Earth's atmosphere: their transformation under the space radiation and satellite methods of their research. Research of the ionosphere with spacecrafts. In this chapter we observe briefly some main types of substances transformation in the Earth atmosphere under the influence of solar and galactic radiation [1, 3, 7–62]. Such results were obtained in process of many-years investigations of great groups of ionosphere researchers [1–5] and others. So, under the influence of the space factors (galactic and solar radiation, some others), first, a change in the chemical composition of the atmosphere happens. Adapted such materials from [1, 57] are on Fig. 1 (Chamberlain graph, 1981) and Fig. 2 (Kelley graph, 1989). The main means of ionosphere sounding have been and remain remote radio physical means — networks of ionosondes, incoherent scatter radars, systems for radio translucence of the ionosphere with GPS signals, etc. These tools make it possible to control the electron density distribution in the ionosphere with high accuracy. From the standpoint of the science today, this knowledge is not enough!

The ionosphere, as a material medium, is a weakly ionized gas located in the Earth's magnetic field, in which variations in the parameters of neutral and charged components and the electromagnetic field are closely related. In many cases, exactly these connections are interesting. Moreover, the study of a single ionosphere parameter (f. e. electron concentration), can disorient the researcher, for which the history of ionosphere research knows a number of examples.

Today's task is to diagnose the full range of gas and electrodynamic parameters that characterize the ionosphere. Such diagnostics is possible only *in situ*, on spacecraft launched into the ionosphere. For this purpose, special low-orbit satellites and high-apogee sounding rockets were used.

Satellites are good because they enable to place solid instrumentation systems on the board and do the measurements on a planetary scale. During the space age, about 20 ionospheres satellites were launched, the last of which, the Chinese Seismo-Electromagnetic Satellite Mission (CSES), was commissioned in 2018. Up to the last years, another similar satellite, Mikrosat-M, was being prepared for launching in Ukraine.

The ionosphere is formed because of solar radiation absorption by the atmosphere at altitudes of 100–200 km. In the region of wavelengths less than 1000 Å (extreme ultraviolet and X-rays), the energy of photons exceeds the

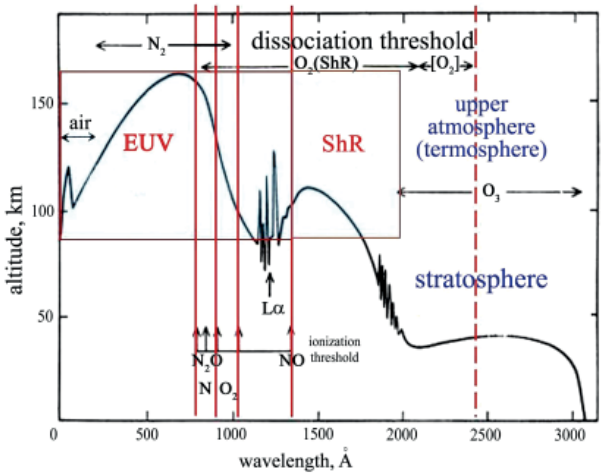

Fig. 1. **Some wave phenomena in the Earth atmosphere**
In the Schumann-Runge continuum (ShR), the flow of energy is: $F \sim 15$ erg/cm^{2}s. In extreme ultraviolet (EUF): $F \sim 2$ erg/cm^{2}s. For comparison: solar constant F* = 1.38 kW/m^2 = 1.38×10^6 erg/cm^{2}s. That is, F / F* ~ 0.001% (adapted from [25])

thresholds for dissociation and ionization of atmospheric gases, which causes the phenomenon of so-called "boiler of photochemical reactions" in the atmosphere, and this radically changes all properties of the atmosphere.

First, these processes cause a change in the chemical composition of the atmosphere (Fig. 2). If below 80 km the atmosphere consists of nitrogen molecules N_2 (78%), oxygen O_2 (21%), as well as small components — gases Ar, He, etc. (1%), then photodissociation of molecules occurs at high altitudes. Reactive atomic oxygen becomes the main one. The ionosphere being penetrated by solar radiation turns out to be an aggressive environment, a space factor that affects space-based systems.

Second, atmospheric gases are ionized, but at ionospheric heights, the degree of ionization is low. For example, in the region of ionospheric maximum at a height of ~300 km, the ratio of the concentrations of charged and neutral particles is less than 0.1%. Only in the magnetosphere does this ratio change in the opposite direction.

Thirdly, the ionosphere is colossally heated by the Sun up to temperatures of about 1000 °K. Since the brightness of the Sun in the short-wavelength part of the spectrum is a variable value, depending on the level of solar activity and under the influence of individual flares, the parameters of the ionosphere demonstrate significant variations (Figs. 2, 3).

We see that the ionosphere is not a static object, but a stationary process of circulation of neutral and charged particles. Arising under the action of solar ionizing radiation, charged particles can recombine partially with each other, returning to the mother's neutral atmosphere. Other charged particles partially flow along the lines of force of the Earth's magnetic field upwards into the magnetosphere. At night, the plasma stored in the magnetosphere descends to the heights of its birth and recombines. Thus, the ionosphere, like a candle flame, retains its shape, despite the fact that a new one continuously replaces the substance that forms it (Fig. 3).

Consequently, we can subdivide the groups of factors that influence on the content of atmosphere at different levels above the Earth. Being summarized, this information evidences about the changes in chemical composition of the air along the vertical line from the ionosphere to the surface of the Earth. Consequently,

1) Below 80 km: N_2 — 78%, O_2 — 21% (plus 1% — small components). Above 80 km: separation of components is according to individual barometric laws plus photo dissociation of O_2. At the heights of the F region, atomic oxygen O becomes the main gas component.

2) Plasma is formed, but it is a small chemical admixture to the neutral gas (in the maximum density of the ionosphere at an altitude of 250–300 km, the degree of ionization is < 0.1%).

3) The absorption of solar ultraviolet causes a colossal heating of the atmosphere (around 1000 K). Since the brightness of the Sun in this part of the spectrum is variable, the temperature and density of the upper atmosphere undergo enormous variations.

Photoionization. Photoionization phenomenon was described enough completely in [60]. Photoionization of the neutral

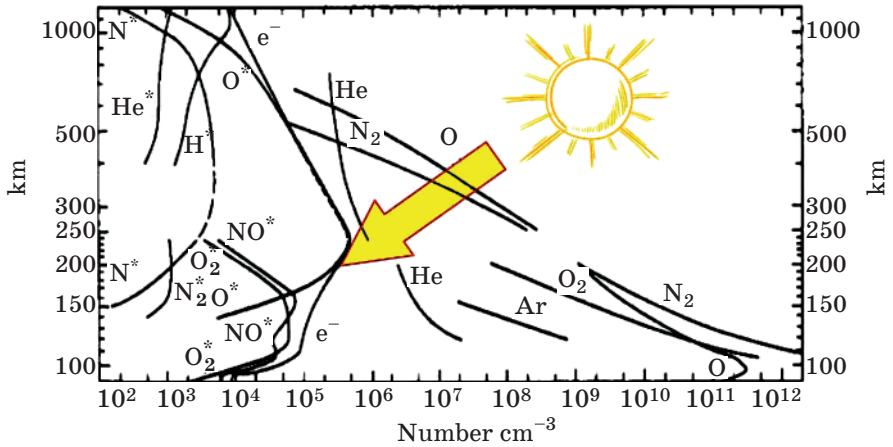

Fig. 2. **The concentration of neutral and charged components of the atmosphere (horizontal axis) as a function of height (vertical axis)**
The upper atmosphere is a chemically active environment under the aggressive influence of solar radiation. The upper atmosphere is a photochemical boiler. Plasma is born, drifts up and down and recombines. In this way, the ionosphere is formed (adapted from [1, 57])

components of the atmosphere by the extreme ultraviolet and X-ray radiation of the Sun is the primary reason for the ionosphere existence and it is the main factor that define gas content of it and atmosphere in general [60]. The data about the ionization potentials and the corresponding wavelengths for a number of atmospheric components are known for today. Respectively, it is known that for the Earth's atmosphere ionizing radiation is with , and for the main components with [60].

The most significant photoionization processes for the ionosphere are the following [60]:

$$N_2 + h\upsilon \rightarrow N_2^+ + e^-, \quad (1)$$

$$O_2 + h\upsilon \rightarrow O_2^+ + e^-, \quad (2)$$

$$O + h\upsilon \rightarrow O^+ + e^-, \quad (3)$$

In the D region:

$$NO + h\upsilon \rightarrow NO^+ + e^-, \quad (4)$$

$$O^2(^1D_a) + h\upsilon \rightarrow O_2^+ + e^-, \quad (5)$$

Dissociative ionization is also possible:

$$N + h\upsilon \rightarrow N^+ + N + e^-, \quad (6)$$

$$O_2 + h\upsilon \rightarrow O^+ + O + e^-, \quad (7)$$

whose contribution to the resulting photoopization rate is small ($q(6) \cong 0.02q(1)$; $q(7) \cong 0.15q(2)$, where q(6) — is the rate of photoopization in the process (6)).

Less important, but noticeable processes are [60]:

$$N + h\upsilon \rightarrow N^+ + e^-, \quad (8)$$

$$He + h\upsilon \rightarrow He^+ + e^-, \quad (9)$$

$$H + h\upsilon \rightarrow H^+ + e^- \quad (10)$$

The rate of photoionization of the $n^{\text{-th}}$ component of a neutral gas is q_n (z) i.e., the number of photoionization events per unit of volume is determined by the following expression:

$$q_n(z) = n_n(z) \sum_{\lambda \le \lambda_m} I_\lambda(z)\sigma_{n\lambda} = n_n j_n, \quad (11)$$

where n_n — is the concentration of gas component of sort n; $I_\lambda(z)$ — *i*s a stream of photons with a wavelength X at a height z; $\sigma_{n\lambda}$— is the cross-section of photoionization of gas component of sort n. Photoionization coefficient is for radiation with wave length $\lambda \le \lambda_m$. For $I_x\{z\}$ we have, as in the case of dissociating radiation:

$$I_x\{z\} = I_2\infty exp(-\tau_\lambda) = I_2\infty exp\,|-sec\chi > \sigma_{n\lambda}{}^a|\, n_n dz|,$$

where τ_λ — is the optical depth for radiation n; wavelength X; $\sigma_{n\lambda}{}^a$ — is the cross section of a photon absorption with a wavelength X of a gas component of type n; x — is the zenith angle of the Sun. For $x > 80^e$, the function *sec* χ should be replaced by the Chapman function Ch(y).

So, these equations describe the effects of solar and galactic radiation on particles in atmosphere: atoms of the lightest elements and the simplest gas molecules. When particles in the atmosphere (especially in upper atmosphere – ionosphere) are exposed to solar or galactic radiation, energy excited them. Being in excited states, the simplest molecules and atoms of elements of atmospheric gases start their transformation. Such forms of the matter as free radicals, ions, another types of charged particles with high energies that are able to damage biological organisms are formed in these conditions. For example, at 600 km a.s.l., the concentration of particles is ~10^6 cm^{-3}, and in interplanetary space is ~10 cm^{-3}. In our previous publication [1–3] it was grounded, that for the processes

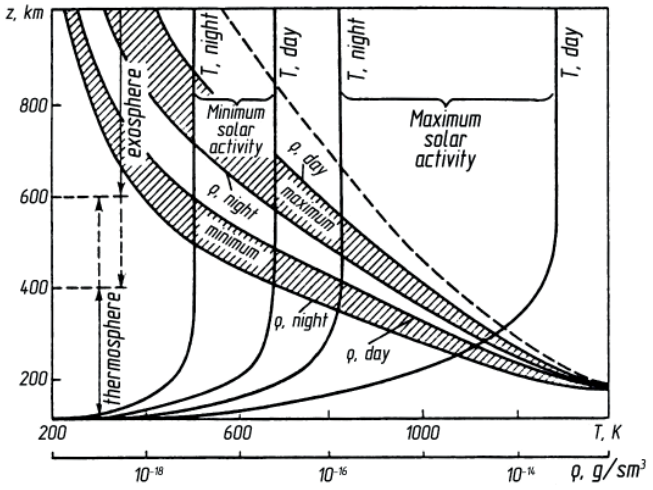

Fig. 3. **Variations in temperature and density of the upper atmosphere (adapted from [29])**

understanding in atmosphere and radiation influences on the matter not only such characteristic, as density of particles in atmosphere (or particles concentration) is important, but also the length (distance) of the free path between the particles. The closer to the Earth's surface, the shorter the free distances (paths) between the particles in atmosphere. On the other side, the higher above the Earth's surface — the greater the free path between these particles. These dependencies in characteristics changes we had demonstrated below in this chapter. It is necessary to mention too, that above we had described the state and processes in the upper atmosphere. But according to Figs. 1–3 we can see "tail effects" moving to the Earth's surface: some processes and particles characteristics became stronger revealed, other — weaker revealed. In addition, such regulations we had shown on Fig.4 with the further explanations.

We tried to summarize natural effects linked with two types of phenomena — 1) space radiation (solar, galactic, other) by itself, and 2) excited atmospheric particles which can be radioactive – can be the reasons of radioactive transformations of the matter close to the Earth's surface and damages of biological objects. The results of such theoretical generalization for space electromagnetic radiation (solar, galactic, others) we had already published in the first article on this item [62]. Doing this for our today continuation of investigations – excited atmospheric particles which can be radioactive — we had subdivided also four groups of effects, and they are listed below in similar manner. They are given on Fig. 4, compare them with [62].

Dependencies in molecular particles characteristics under the influences of space radiation at different altitudes above the Earth surface up to the ionosphere:

The vertical "ionosphere – Earth surface" (1, *a, b*).

1, *a*. Increasing the effects along the vertical

1) The densities of matter particles at different altitudes above the Earth surface are increased (gases, microscopic dust particles others — up to biomolecules and solid matters). 2) Number of neutral particles in atmosphere is increased. 3) Protective properties of the atmosphere are increased.

1, *b*. Decreasing the effects along the vertical

1) Various effects associated with high-energy, radiative effects on substances decrease. 2) The closer to the Earth's surface, the shorter the free distances (paths) between atmospheric particles. 3) Radiation doses, obtained by persons during the flight. 4) Radiation influences on the surfaces of the aircrafts. 5) Number of charged particles and free radicals in atmosphere decreases.

The vertical "Earth surface – ionosphere" (2, *a, b*)

2, *a*. Increasing the effects along the vertical

Numerous effects described in the point 1, *b* demonstrate increasing: 1) Various effects associated with high-energy, radiative effects on substances are increased; 2) The further from the Earth's surface, the longer the free distances (paths) between atmospheric particles; 3) Radiation doses, obtained by persons during the flight;

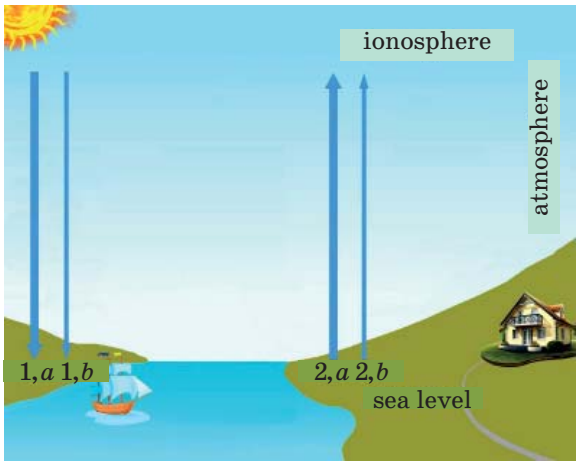

Fig. 4. **Changes in characteristics of matter particles (gases, microscopic dust particles, free radicals, ions, and other types of charged and neutral particles) at different levels above the Earth surface.**
Adapted from [62]

4) Radiation influences on the surfaces of the aircrafts. 5) Number of charged particles and free radicals in atmosphere is increased.

2, *b*. Decreasing the effects along the vertical

Various effects listed in the point 1, *a* demonstrate decreasing: 1) The densities of matter particles at different altitudes above the Earth surface are decreased (from biomolecules and solid matters — to gases, microscopic dust particles others); 2) Number of neutral particles in atmosphere is decreased; 3) Protective properties of the atmosphere is decreased.

Important general regularity was registered in process of these investigations. With shortening the free distances (paths) between atmospheric particles, the densities of matter particles are growing near the surface of the Earth and on its surface. Respectively at such distances, where the particles begin to "feel" each other (i.e., forces of attraction-repulsion arise between them), one can speak of the origination of the concept of information as a measure of the ordering of these particles.

So, we can see, that atmospheric gases particles in excited state (ions, free radicals, radioactive isotopes of H^+, O^-, N^+, NO^+, others) can be registered and more close to the Earth's surface — up to a few kilometers above and even at the a.s.l. In our previous publications [3, 62] we had examined electromagnetic radiation (solar, galactic), which also demonstrate its "tail effects" close to the Earth's surface. These two types of phenomena — 1) radiation (solar, galactic) by itself, and 2) excited atmospheric particles, which can be radioactive — can be the reasons of radioactive transformations of the matter close to the Earth's surface and damages of biological objects. These natural phenomena cause different effects associated with high-energy, radiative effects on substances in the atmosphere and at the surface of the Earth's, as well as on living organisms at different heights above the Earth's surface (Figs. 5, *a, b*; 6). The atoms of elements in atmosphere, which we see as the most damaged by radiation (C, O, N with their ions, free radicals etc.) are involved as well into the chains of biochemical reactions of organism. Oxygen plays the leading role among all other elements in subsequent scenarios. F.e. changes in oxygen transportation and/or utilization leads to hypoxic states development, and so on). Therefore, modification of these elements in atmosphere under the radiation influence with further involving them in such reactions causes notable effects on the organism state (Fig. 6). General image of Krebs cycle give us possibility to imagine great damages of it functions in case of attacks by atmospheric "hot particles" with high energies O, O^*, O_2, O_2^*, N^*, N_2^*, NO^*, others (compare with Figs. 1, 2). Such phenomena — radiation influence on atoms and molecules in living organisms will be explained and described in details further in this publication.

Effects of the particles with high energies that appeared in atmosphere as result of the space radiation on condensed matter close to the Earth's surface and biological objects. Condensed ordered matter and notion of "information". We have already described above the effect of production of particles with high energies in atmosphere as result of space radiation, and the most usual among them were H^+, O^-, N^+, NO^+, "hot particles" with high energies O, O^*, O_2, O_2^*, N^*, N_2^*, NO^* and some others. Being spread as the "tails" to the Earth's surface they interact with the matter there, where matter densities is increased more and more (sure, in such concentrated (condensed) media free distances (paths)

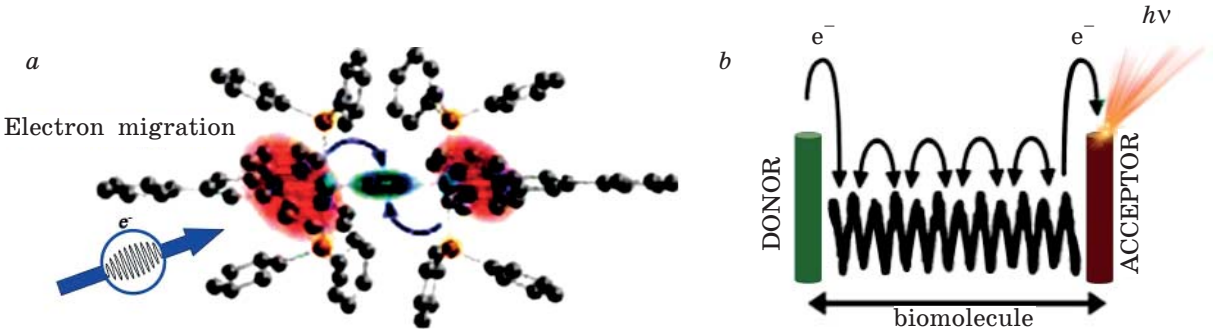

Fig. 5. **Action of electron with high energy on biological macromolecule with migration of its additional energy along the molecule:**
a — the initial moment of attack of biological macromolecule by a high-energy quantum; *b* — hypothetic scheme of capture of quantum of radiation energy by acceptor part of biomolecule and schematic representation of transfer of this energy along a helix of this biomolecule

between "close–to–surface" matters particles are shortening). In such conditions, the crystals were formed — inorganic as well as organic nature. Consequently, such condensed forms of matter demonstrate an order in their structures — the notion "information" appears: "information is a property of the orderliness of condensed systems (including living systems)". Such "crystals in organic Nature" we usually call "biological objects" — biological macromolecules, their complexes, DNA, viruses, membranes... and further more highly organized structures — living systems.

Well known, that listed above H^+, O^-, N^+, NO^+, and some other elements and simple fragments of molecules with high energies from the atmosphere [60] can be captured easily and incorporated into the structures of such condensed media and more highly organized structures. But they are "not normal" — their inner energies are higher than in structures in normal conditions. Such great energies cause different effects in such "inorganic" and "organic" crystals. Below we observe some effects of such "high energy particles" on biological objects, which captured such particles. Main information below was presented in [65] — excellent review, a book with results and their analysis. So, phenomena of interaction of simple fragments of molecules with high energies from the atmosphere with biological objects and linked problems [66–85] will be observed below.

Effects of oxygen on the matter at the Earth's surface and biological objects. In the experimental studies, it was registered that oxygen influences greatly on the effect of irradiation of dry enzymes, nucleic acids, dry seeds, spores, etc. This demonstrated convincingly that the oxygen effect extends to the direct action of radiation and, therefore, is realized in other ways than in aqueous solutions [65, 79, 84]. Further, it was shown that oxygen can enhance the effect of radiation even being added to biological object after irradiation, i.e. in period, when the primary products of irradiation, due to short time of their lives, have already disappeared [60, 65, 78, 84].

General image of Krebs cycle give us possibility to imagine great complex damages of its functions in case of attacks by electrons with high energies and atmospheric "hot particles" with high energies O, O^*, O_2, O_2^*, N*, N_2^*, NO* others, see formulas (1)–(10) and Fig. 6. Numerous links and elements of the Krebs cycle can be changed due to such influences. The arrows at the figure indicate only some possible points of attack of elements of the Krebs cycle and some involved substances by such electrons or/and high-energy particles, the possibility of some

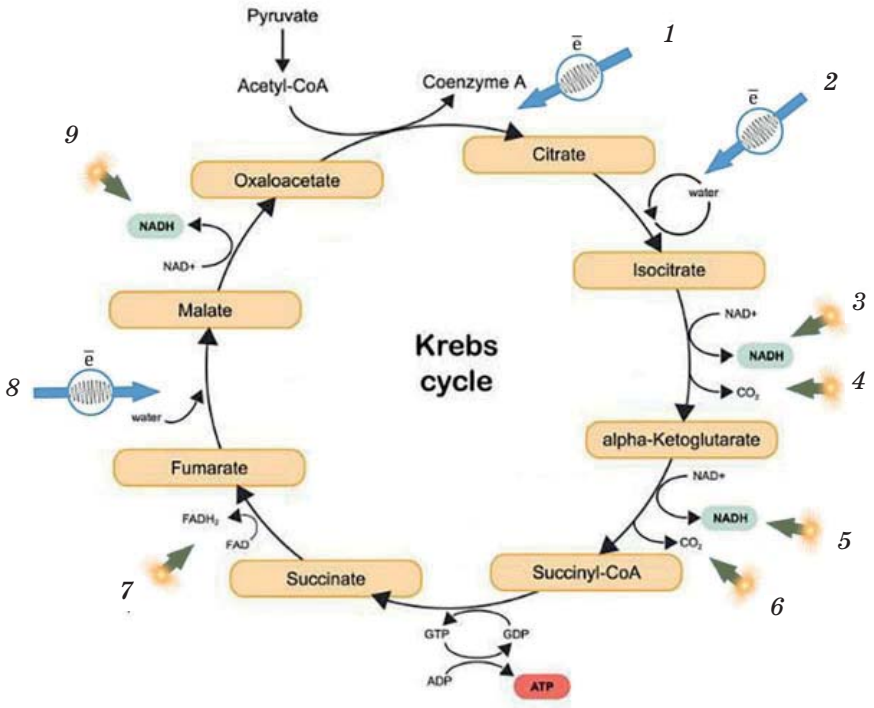

Fig. 6. **One of the various natural phenomena that cause numerous effects associated with high-energy, radiative effects on substances in the atmosphere and at the surface of the Earth**

atoms in compounds replacement (H, O, C, N, others) with subsequent damage of the nearest bonds, etc. Naturally, there are much more such vulnerable points in the cycle, taking into account equations (1–10, 13–16); atoms, compounds of almost the entire cycle are vulnerable. The numbers near the arrows are linked with the numbers of photoionization reactions above:

Reactions with high energy electrons participation (3, 8, 10) — arrows 1, 2, 8.

Reaction with hydrogen with high energy participation (10) — arrows 3, 5, 7, 9.

Reaction with oxygen with high energy participation (3, 7) — arrows 4, 6.

Since tissues of human organism consist on 65–70% of water, the primary radiation chemical reactions develop primarily in the aqueous phases. These reactions we had described already in [62]; so, arrows 2, 8 point also to locations in a cycle that can be damaged due to the water radiolysis effects. Reactions of free radical oxidation will be observed below in the next sub-chapter.

Due to the basic investigations, there was formed an idea about sub lethal and potentially lethal radiation damages of the cells. Such damages were possible to eliminate more or less successfully by the work of enzymatic systems of intracellular repair. With the development of these ideas, it became clear that oxygen also participates in the processes of realization and repair of radiation damage. It was found that these last processes are not only energy-dependent, but they are also oxygen-dependent. Thus, the main effect associated with the presence of oxygen in the irradiated biological environment. Due to its involvement in the reactions of radiation the consequences was the fixation of potentially lethal radiation damages in cells, and these damages were transformed into irreversible damages [65, 79, 83, 84].

Numerous studies have shown that a decrease in the oxygen concentration in the irradiated tissue volume reduces the radiosensitivity of this tissue and reduces the severity of its damage. Tissue hypoxia can be achieved by reducing the oxygen concentration in the inhaled air to 10–7–5% immediately before and during the irradiation [65], limiting oxygen transport by blood, and other methods discussed below.

The role of free radical oxidation in the pathogenesis of radiation damages. It was shown above that free radicals were chemical structures with specific properties formed under the influence of high energies in the Earth's ionosphere in great quantities. Their number decreased noticeably with approaching to the Earth's surface. These rare remained radicals from atmosphere can be captured by biological structures with fatal consequences for them (biostructures damages, destroying, etc.)

At the same time, free radicals can be formed in living systems by themselves [65, 83, 84]. Free radicals in biological systems, first of all, act as initiators of peroxidation process. When free radical interacts with a molecule of organic compound — a new molecule and new radical are formed, and the latter continue the chain of interactions. Thus, peroxide oxidation proceeds as a chain process. B.N.Tarusov and M.N. Emanuel demonstrated that the kinetics of peroxidation of organic compounds corresponded to the mechanism of branched and degenerate-branched radical reactions [60, 65].

Radicals–initiators of reactions of peroxidation (PO) can appear under the influence of radiation quanta — ionizing, ultraviolet and even visible [84]. These quanta, falling from outside or being produced inside of organic substrate (due to the content of natural radionuclides in it) predetermine PO. The role of PO initiators can play radicals formed during the electron transport chains functioning during the interaction of iron ions with oxygen and so on. Practically in the cells of any organism at every moment of its life, there are radicals of different structures that can play the role of PO initiators [84–103].

The next stage of the process – continuation of the chain — is a sequence of radical-molecule reactions [65]:

$$RH + {\bullet}OH \rightarrow R{\bullet} + H_2O$$

$$\text{or} \quad RH + HO{\bullet} \rightarrow R{\bullet} + H_2O \qquad (13)$$

$$R{\bullet} + O_2 \rightarrow ROO{\bullet} \qquad (14)$$

$$ROO{\bullet} + RH \rightarrow ROOH + R{\bullet} \qquad (15)$$

$$R{\bullet} + O_2 \rightarrow ROO{\bullet} \text{ and so on} \qquad (16)$$

At this stage, a gradual increase in numbers of active radicals continue to form chain. Finally, in reality, the events of opposite direction inevitably take place – chain termination as a result of interaction (recombination) of radicals with each other [65, 84]:

$$R{\bullet} + R{\bullet} \rightarrow R - R^{*} \rightarrow R \rightarrow R + h\nu \qquad (17)$$

$$ROO{\bullet} + ROO{\bullet} \rightarrow ROOR^{*} + O_2 \rightarrow \rightarrow ROOR + O_2 + h\nu \qquad (18)$$

The excited products formed during such reactions give off excess of energy of electronic excitation in the form of radiated quanta. This phenomenon initiates the effect of chemiluminescence.

Another variant of chain termination occurs when the radical interacts with the molecule of inhibitor substance [65, 83, 84]:

$$R\bullet + Hin \rightarrow RH + In\bullet \qquad (19)$$

Outwardly, this reaction does not differ from usual reactions of chain propagation [65]. However, the fundamental difference is that the radical formed in result of reaction is relatively stable and does not continue the chain.

If the frequency of circuits' breaks prevails over the frequency of branching, PO process is terminated. With the reverse ratio of these reactions, the rate of PO gradually increases with the increasing of amount of active products and increasing the substrate molecules number involved in this process. Hence, one of the most important features of PO is the process develops even in the absence of specific catalysts (enzymes), self-accelerating, autocatalytically under favorable conditions: temperature, free access of molecular oxygen and sufficient amount of radical initiators [65, 85–103].

The honor of free radical reactions of PO discovering in tissues and liquid media of organism belongs to B.N. Tarusov [65]. He had discovered that PO reactions develop most effectively in lipid-containing structures, primarily in biological membranes, when these objects were exposed to ionizing radiation.

Reactive oxygen species were found in great variety of cellular organelles, although in very low concentrations they were approximately in 10^{-11} mol/L.

The superoxide anion radical has been found in membranes (nuclear, plasma, microsomal, and mitochondrial). Ability of anion radical to penetrate easily through biological membranes anion channels without specific carriers in chloroplasts was registered [65].

Experimental evidences of superoxide anion radical ability to activate directly the processes of lipid peroxidation were obtained in 1982. The process of formation of lipid peroxides is chain free radical process. Peroxidation is initiated under the condition when free radicals appear in lipid phase, and they can interact with easily oxidized lipid molecule (LH). For example, LH can be unsaturated fatty acids of phospholipids in biological membranes. In this case, a free radical of lipid L• is formed. In presence of oxygen, reaction between the L• radical and O_2 molecule is going [65].

$$L\bullet + O_2 \rightarrow LOO\bullet \qquad (20)$$

Lipid peroxide radicals appear in result of this reaction. The rate constant of this reaction is 10^7–10^8 mol/L/s, activation energy is close to zero. This means that at oxygen concentrations above 10^{-6} M, all L radicals are converted into $LOO^{\bullet}$ radicals.

The peroxide radical can interact with new molecules of unsaturated fatty acids with the formation of hydroperoxide LOOH and the new radical L• [65].

$$LOO\bullet + LH \rightarrow LOOH + L\bullet \qquad (21)$$

This reaction also has low activation energy and high rate constant, which value depends on the type of compound being oxidized.

More and more new LH lipid molecules and molecules of oxygen are involved in the process. As a result, LOOH hydroperoxides are accumulated, but the number of L• and LOO• radicals does not change (the principle of indestructibility of free valence). Although structurally radicals L•, L_1•, L_2•, etc., as well as LO•, LO_1•, LO_2• etc. can differ from each other.

In the presence of metals with variable valence, the process described above acquires a branched type due to the reaction [65]:

$$LOOH + Fe^{2+} \rightarrow LO\bullet + \bullet OH + Fe^{3+} \qquad (22)$$

So, new free radicals and ions were produced, and hence new products of peroxidation appeared too. Further course of branched chain processes leads to the formation of new products of lipid oxidation: peroxides, epoxides, acidic compounds, aldehydes and ketones, unsaturated fatty acids, which in excess concentrations cause toxic effect. Oxygenase systems, including cytochrome P-450 play an important role in the inactivation of lipid toxic substances in animals.

According to E.B. Burlakova and co-authors [65], the intensity of free radical lipid peroxidation processes (LPO) is linked with the composition and physical state of phospholipids (their fluidity), with the structure and functions of biological membranes, with their sensitivity to signals and extreme influences. So, POL is extremely important for the regulatory and informational role of membranes in cellular metabolism (in case if it is normal).

The participants of LPO reaction are following [65]. Lipids (unsaturated fatty

acids) of biomembranes, biological fluids and molecular oxygen, the resulting lipid peroxidation products (primary, secondary, final). LPO catalysts (stimulators) are active forms of oxygen (free radicals, peroxides) that are formed in living systems as intermediates in number of enzymatic reactions, products of photo- and radiochemical reactions, and free metal ions with variable valence and their molecular complexes. Finally, there are variety of antioxidant mechanisms that provide structural-spatial and biochemical obstacles on the way of lipid peroxidation and breakage of chains of free radical oxidation.

The main indicators of the intensity and dynamics of lipid peroxidation in living systems are the products of lipid peroxidation by themselves. They act also (at least, their primary products) as catalysts for the process, ensuring its self-accelerating autocatalytic process. The second source of information is the state of antioxidant systems — the amount of antioxidants (AO) of different types, the activity of antioxidant enzyme systems [65, 83–103].

LPO products. The primary products of LPO are free oxidative radicals: superoxide, hydroperoxide, and hydroxyl •OH, hydroperoxides, lipid peroxides, epoxides, and diene conjugates. The secondary products of lipid peroxidation are aldehydes, in particular, malondialdehyde (MDA), determined in the reaction with 2-thiobarbituric acid (TBA), as well as gaseous products of oxidative degradation of fatty acids (ethane, pentane); they are formed when double bonds in the carbon chain are broken. The final products of lipid peroxidation are fluorescent products of oxidative co-polymerization of lipids and proteins — Schiff bases (lipofluorescent, lipofuscin pigments), determined by the methods of fluorescence analysis [65].

As a result of observation of complex of all problems associated with the development of LPO in living systems, following provisions were stated [65]:

a) Objective prerequisites for the development of non-enzymatic reactions of free radical oxidation (LPO) exist in all living systems, without exception. They are due to the presence of easily oxidizable organic compounds in their structure (primarily in biomembranes). These compounds can accumulate potential energy in their molecules. Among organic molecules, the most vulnerable to peroxidation reactions are polyene molecules of fatty acids (linoleic, linolenic, and especially arachidonic), which are part of the phospholipids of biological membranes and blood lipoproteins.

b) The presence of free oxygen in the biosphere, its use in the life of plant and especially animal organisms, its presence in biological fluids and extracellular space makes constant contact of oxygen with membrane lipids unpreventable. Therefore, the spheres of LPO reactions are the areas of these contacts.

c) The use of oxygen in such important intracellular processes as biological oxidation and oxidative phosphorylation (the inner membrane of mitochondria), oxidative macrosomal destruction of xenobiotics, presence and functioning of specialized electron transport chains in these organelles, formation of free radical intermediates in process of enzymatic catalysis and due to the existence of natural radiation background are accompanied by the appearance of reactive oxygen species such as radicals and peroxides, which play the role of catalysts and products of non-enzymatic lipid peroxidation. Their presence even in the most negligible quantities ensures that the activation barrier is overcome. This creates conditions for processes of free radical lipid peroxidation reactions, for realizing potential possibilities listed above (a, b).

d) Ions of metals with variable valence (Fe, Cu, Co, Mo, Mn, etc.) can act as branching factors for free radical oxidation chains and, consequently, for general increase of lipid peroxidation.

e) The combination of listed prooxidant factors determines the universal nature, the ubiquitous distribution of LPO processes in all living and actively metabolizing systems. Moreover, the dual role of LPO intermediates, their ability to act also as autooxidation catalysts cause real danger of progress of free radical chain reactions and, as a result, complete destruction of membrane structures, cells and organisms with oxygen access. Only the presence of factors with opposite action, antioxidant systems, keeps the lipid peroxidation process at a stationary basal level, which does not change normal life activity. The resulting prooxidant-antioxidant balance is the most important mechanism of homeostasis.

f) Any significant stress in living system functioning, caused by unusual external agents (in their strength, duration, quality) is accompanied by the increase of oxidative metabolism, an increase of production of reactive oxygen species and activation of lipid peroxidation process, which is able to overcome the AO-protection barrier. Thus, external stress

impacts, together with internal prerequisites, act as components of causal complex that determines the development of a wave of LPO activation ("explosion") in living systems.

Exposure to ionizing radiation is the most impressive example of stress effect that causes the activation of free radical lipid peroxidation in the tissues of irradiated organism [65, 103].

Physiological antioxidant system of biological organism under the influence of radiation. All currently existing living organisms have a number of inherited, genetically determined adaptive means of protection against toxic destructive action of free molecular oxygen — this the most universal poison. Means that help to overcome the danger of oxidative destruction of complex organic compounds and biological structures [65, 79, 83–105].

From one side, the oxidative capacity of oxygen is used in animal and human organisms to provide energy and use it for new biosynthesis, to maintain organism temperature, muscle work, oxidative destruction of xenobiotics, harmful microorganisms, etc. The material expression of this way of solution of oxygen problem was the creation of complex membrane-bound enzyme ensembles — the systems of electron transport of mitochondria, the endoplasmic reticulum, the enzyme system of "oxidative explosion" in the membranes of phagocytes, etc.

On the other hand, protection of biological structures from oxygen excess, and, especially, the most vulnerable membrane formations, was solved in Nature, at least partially, by creating specialized enzyme systems — antioxidant enzymes (AO-enzymes), capable of maintaining prooxidant-antioxidant balance in intracellular and intercellular fluids and in lipid structures of membranes. In such a way appear the "order" in organization of molecular consequences and biochemical pathways in living Nature; further it was logically linked with the notion of "information".

It should be emphasized that both problems — biologically necessary utilization of free oxygen and AO–protection from it – are solved in the most closely interconnected way. The first line of cell defense from O_2 toxic effects is to prevent the producing of its active forms. The cytochrome C-oxidase enzyme carries out a four-electron reduction of O_2 to H_2O without formation of active intermediates. The second line of defense is formed by AO-enzyme systems, localized in the cell primarily in the most vulnerable loci — mitochondria and microsomes — organelles that implement the function of electron transport systems. The stationary level of O_2 and H_2O_2 in intact mitochondria changes from 10^{-11} to 10^{-9} mol/L, respectively. AO–enzymes prevent the "leakage" of reactive oxygen species (radicals, $HO_2^{\bullet}$, and hydrogen peroxide) from actively functioning systems of biological oxidation, preventing the danger of uncontrolled oxidative destruction of biological structures of cells.

AO-enzymes include superoxide dismutase (SOD), which inactivates superoxide radical anion; catalase, which decomposes hydrogen peroxide H_2O_2, as well as enzymes of glutathione system (GSH); glutathione peroxidase (GPO), which decomposes organic (lipid) peroxides (along with H_2O_2); glutathione reductase (GR), which reduces glutathione oxidized during enzymatic (GPO) and non-enzymatic reactions, as well as family of glutathione transferases (GT), which alkylate by glutathione various toxic metabolites and xenobiotics. Finally, to AO-enzymes the ceruloplasmin belongs (main AO-enzyme of the blood), as well as transferrin (with some restrictions).

Superoxide dismutase (SOD) catalyzes the reaction:

$$O_{2^-} + O_{2^-} + 2H^+ \rightarrow O_2 + H_2O_2 \qquad (23)$$

As result of the reaction, hydrogen peroxide is formed, which is capable to inactivate SOD. Therefore, SOD is localized and usually functions in collaboration with catalase, which quickly and efficiently decomposes H_2O_2. The rate of superoxide dismutase reaction is very high; the second-order rate constant reaches 2×10^9 $mol^{-1}s^{-1}$ [65]. The active center of the enzyme contains metal atoms with variable valence.

Mn-SOD of mitochondria and Cu, Zn-SOD of the cytosol are the most important AO-enzymes that inactivate the superoxide radical and, accordingly, reduce the overall toxic effect of oxygen and its active forms.

Catalase is a hemeprotein that catalyzes the reaction:

$$H_2O_2 + H_2O_2 \rightarrow O_2 + 2H_2O \qquad (24)$$

AO-enzymes SOD and catalase, functioning together, in the most cases timely inactivate reactive oxygen species (ROS), O_{2^-}, H_2O_2, which are formed both during normal cell activity and under conditions of significant LPO activation, including pathologically conditioned. However, LPO activation

develops most effectively in the lipid (phospholipid) structures of biomembranes and is accompanied by the formation of lipid peroxides, which only slightly can be eliminated by SOD-catalase system.

Glutathione peroxidase (GPO) — is a selenoprotein. It is possible that, at least in human serum, GPO is present as selenoglycoprotein. The GPO molecule has a molecular weight of about 74 kDa and consists of four identical subunits. GPO neutralizes not only H_2O_2, but also organic peroxides (including lipid peroxides), formed in organism during the activation of lipid peroxidation [65].

Glutathione transferase (GT) is a whole family of enzymes with polyfunctional activity that mainly detoxifies various xenobiotics, including peroxides. E- and S-GT destroy organic (lipid) peroxides. GT unites at least 11 isoforms (A, B, C, etc.) [65].

The origin of antioxidant enzymes was probably the oldest protective system. The archaic origin of this mechanism can be confirmed due to the discovery of AO-enzymes or their simpler analogues in all living organisms in contemporary World. Each of them is aimed specifically at eliminating one of the dangerous initiators of LPO or its products [65].

Methods of medical treatment and rehabilitation of patients irradiated in Chernobyl zone basing on the results of hypoxia studies at EMBS NASU. The pathological states we observed in this article, associated with the action of various types of radiation effects on organism and the role of oxygen in these phenomena, just were within the competence of scientists and doctors who worked at the Elbrus Medical And Biological Station (EMBS NASU) [3, 63–65]. A great contribution to these works was made by Prof. Komisarenko S.V. [104, 105].

These groups of professionals specialized in the development of new methods of treatment and rehabilitation of persons who received various doses of radiation during the accident at the Chernobyl nuclear power station in 1986. Among them, there were representatives of the civil population "chernobyltsy", as well as people who worked for the liquidation of the accident consequences "liquidators".

At EMBS the concept of gradual adaptation to hypoxybaria, oxygen regimes of organism and their regulation, and functional respiratory system were proposed and substantiated; consequently, a number of mathematical models were elaborated. This made it possible to characterize different types of hypoxic states not only qualitatively, but also quantitatively, to estimate their degrees, to predict changes in the state of organism under the influence of extreme factors, to analyze the role of certain physiological reactions in compensation of oxygen deficiency, and to transform the science of hypoxia from experimental-descriptive sphere to an exact one. Research conducted at EMBS revealed the destructive (pathogenic) and constructive (cyanogenic) mechanisms of development of hypoxic conditions in organism, allowed for the first time in world practice to justify and develop new highly effective methods of treatment, prevention, rehabilitation, increasing the organism's stability and performance — hypoxytherapy. Hypoxytherapy can be implemented in mountain conditions, pressure chambers or using various hypoxicators. Hypoxytherapy methods are widely used today in spa medicine, cardiology, pulmonology, neurology, psychiatry, pediatrics, gynecology, aviation and space medicine, and training of athletes.

On the basis of many years of research, the "Elbrus" classification of hypoxic conditions was created [65], the terminology in this field is formulated, which is widely used by contemporary researchers.

New highly effective methods for medical treatment and rehabilitation of patients irradiated during nuclear accident in Chernobyl. If to speak about the new highly effective methods for medical treatment and rehabilitation of patients irradiated during nuclear accident in Chernobyl (1986), that were developed at scientific base EMBS in Caucasus it is necessary to remember following data. Among such methods there were the following: gradual adaptation to high-altitude conditions, training in pressure chambers, inhalation of gas mixtures with low oxygen content, and the effect of intermittent hypoxia. These methods are successfully used in numerous medical institutions, hospitals, and sports centers in different countries.

For the first time, the method of gradual adaptation to low pO_2 in inhaled air was used in a pressure chamber for the treatment of patients with bronchial asthma, and later – children with whooping cough. In mountain conditions, the method of gradual adaptation was initially used to treat patients with some mental illnesses (catatonic form of

schizophrenia), bronchial asthma, and chronic non-specific lung diseases [65]. Subsequently, for the treatment of patients by methods of adaptation to hypoxybaria at EMBS at altitude of 2100 m a.s.l. special inpatient department was organized for the recovery of patients from areas with ecologically unfavorable conditions — town Shevchenko (Kazakhstan) and town Chornobyl (Ukraine).

The methods of medical treatment using adaptation to hypoxic environment in Elbrus were successful for many patients with various diseases. There are: respiratory allergies, anemia, hypertension, diabetes, coronary heart disease, arrhythmias, neurodystonic and "post-Chernobyl" syndromes, for girls with juvenile dysfunctional disorders etc. [65]. In the process of medical treatment, doctors deeply studied the peculiarities of the genesis of hypoxic conditions, the mechanisms of sanogenesis. Important work for the treatment of people injured during the Chernobyl accident and the liquidators of the consequences of this accident began immediately after the accident in May 1986. As a result, the symptoms of the liquidators' diseases were determined, as well as the characteristics of radiation-induced diseases of children from Chernobyl zone.

At EMBS, it was shown that in the genesis of the "Chernobyl syndrome" polyfunctional disorders in the systems of oxygen transport and utilization, which led to the development of hypoxic conditions, are of primary importance. The clinical picture of vegetative-vascular dystonias, anemias, respiratory allergies, dyscirculatory encephalopathies etc manifested these conditions.

In the process of adaptation to the mountain conditions, in process of usage of developed methods of medical treatment, people irradiated in Chernobyl nuclear accident demonstrated following positive results [65]:

– the psycho-emotional state and regulation of vegetative functions, indicators of functional mobility and dynamism of nervous processes were improved;

– indicators of breathing, hemodynamics, immune status of blood, heart rate and its electrical activity were normalized;

– degenerative changes in blood cells decreased;

– regeneration processes were activated;

– aerobic and anaerobic enzymes in tissues;

– oxygen content in arterial blood increased;

– activities of succinate dehydrogenase and creatine phosphatase were changed in positive for organism directions;

– increased lysosomal activity of white blood cells;

– increased DNA synthesis;

– the economization of oxygen transport systems took place.

So, in patients with listed disorders, "mountain-treatment" or "mountain-therapy" caused general condition and well-being improvements, increase in the adaptation reserve, transition to a new level of regulation, so on.

Conclusions

In present article the impacts of cosmic radiation on atoms and molecules in the Earth's atmosphere were analyzed.

1. The results of exploring of various characteristics of the Earth atmosphere gases content at different altitudes above the Earth were suggested. The data obtained during the atmosphere exploring by satellite were presented. Concentrations of the gases starting from the ionosphere to the Earth surface were revealed and described.

2. Further, the chains of interconnected natural phenomena were examined subsequently. Phenomena of solar radiation influence on atoms, molecules and molecular complexes in the Earth's atmosphere were observed (including photochemical processes, photoionization). Transformation of atoms and molecules of gases in high-altitude atmospheric conditions were described. Some other natural phenomena that effect on the structure of atoms, molecules and molecular complexes in the Earth atmosphere were observed.

3. Along with this, some information aspects of the organization of substances at the height of the ionosphere and near the Earth's surface were considered.

4. The most attention was concentrated on the studies of oxygen concentrations at different altitudes, transformations of oxygen molecules in ionosphere, lower levels of atmosphere and linked with these effects developments of hypoxic states in human organisms.

5. As a result, some damages of respiration effects in biological organisms and development of oxygen deficiency (hypoxic) states in them were examined.

6. Some recommendations of the ways of possible correction of developed hypoxic states and some other pathological states linked with cosmic radiation influences were suggested. Described studies of oxygen deficiency in

organisms (hypoxic states) were demonstrated on the results of the groups of Ukrainian scientists who worked in high mountain conditions at EMBS — scientific base of the National Academy of Sciences of Ukraine.

Some data of hypoxic pathological states studies were presented as well as some ways of their corrections. On the base of these studies practical recommendations for patients' medical treatment and rehabilitation were done. Some conclusions made on the basis of works on the rehabilitation of the people that were exposed to the consequences of the Chernobyl accident "chernobyltsy" as well as ones who liquidated consequences of accident "liquidators" since 1986. The obtained results can be spread on the treatment and rehabilitation of the people from other contingents of radiation risk.

General complete conclusions of such studies with recommendations were published. There are only a few in brief list below.

1. The symptoms of liquidators disorders were studied and described at EMBS. Radiation-caused morbidity of the children from the "4th radiation zone" was determined concerning different disorders: gastrointestinal — 78.6%, respiratory — 58.9%, thyroid gland — 57.1%, vegetative-vascular dystonia — 19%.

2. Disorders in oxygen transport system and oxygen utilization system caused hypoxic states development in irradiated people; and these disorders were primary for further development of "Chernobyl syndrome". Consequently, hypoxic states were developed in irradiated people in the result of anemias, vegetative-vascular dystonias, dyscirculatory encephalopathies, respiratory allergies etc.

3. Ten criteria of organism state were defined at EMBS: efficiency of processes of oxygen transport and utilization, organism's oxygen balance, degree of progressive action of hypoxia, physical and mental capacity, adaptability and level of adaptation, and etc. These criteria were defined as the most informative criteria for the estimation of "mountain-therapy" or "mountain-treatment" [62].

4. At EMBS there were registered that persons, which chronically obtained small doses of radiation, the organism's reserve capacities were reduced. There are: indicators of oxygen consumption, efficiency of oxygen transport systems, and activity of respiratory enzymes responsible for urgent adaptation.

The methods that were called at EMBS "mountain-therapy" or "mountain-treatment" of rehabilitation/treatment of the persons from radiation risk contingents were found as very effective. Their effectiveness was based on the adaptation to the mountains natural conditions (in Ukraine they can be Carpathians now, or mountains in other countries). Treatments, rehabilitation using hypoxia simulation were also effective; there were methods of hypoxytherapy (normobaric, hypobaric, hypercapnic, pulsed, periodic hypoxia, interval), as well as hypoxic states, simulated in the conditions of hypoxicator, barochamber, inhalation of hypoxic mixtures, and etc.). These methods, as well as hypobaric or normobaric interval hypoxia were effective in the replacement of the stepwise mountain adaptation. The Ukrainian sciantists suggested the most effective rehabilitation complex in which, together with "mountaintherapy", were united diet, phytotherapy, thermobarotherapy, developed complexes of breathing and physical exercises, intake of silicon waters, bromine-iodine waters, sulfate and dolomite natural waters, and etc.

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ДОСЛІДЖЕННЯ РАДІАЦІЇ ТА ГІПОКСІЇ: ВПЛИВ ВИСОКОЕНЕРГЕТИЧНИХ ЧАСТИНОК АТМОСФЕРИ НА БІОЛОГІЧНІ ОРГАНІЗМИ ТА МОЖЛИВОСТІ ЇХ РЕАБІЛІТАЦІЇ

О. М. Ключко[1], *Г. В. Лізунов*[2], *П. В. Білошицький*[3]

[1]Національний авіаційний університет, Київ, Україна,
[2]Інститут космічних досліджень НАНУ-ДКАУ, Київ, Україна,
[3]Уманський державний педагогічний університет імені Тичини, Україна

E-mail: kelenaXX@nau.edu.ua

Розглянуто вплив космічного випромінювання на атоми та молекули газів земної атмосфери з подальшим ланцюгами їхніх перетворень, а також розвиток відповідних станів дефіциту кисню (гіпоксії) у біологічних організмах, рекомендовано шляхи корекції таких порушень.

Метою роботи було дослідити радіаційні ефекти в іоносфері з подальшим перетворенням атомів, молекул газів на різних висотах над поверхнею Землі; взаємодії деяких високоенергетичних частинок атмосфери з біологічними об'єктами на висотах від поверхні Землі до 5500 м над рівнем моря (н.р.м.), а також роль кисню в наслідок опромінення біологічних організмів.

Методи. Аналіз результатів супутникових і ракетних спостережень газів земної атмосфери на різних висотах над рівнем моря. Дослідження в гірських умовах на науково-дослідницькій станції ЕМБС НАН України: порівняльний аналіз результатів багаторічного спостереження за хворими з використанням стандартних лабораторних методів, комплекс методичних прийомів: клінічні, фізіологічні дослідження дихальної, серцево-судинної систем; гематологічних, імунологічних станів; функціональний стан вищої нервової діяльності, психічний і невротичний стан; застосування антигіпоксантів, гістохімічні, біофізичні методи, математичне моделювання та інші.

Результати. Представлено дані, отримані під час дослідження атмосфери супутниками: вплив на структуру атомів, молекул в атмосфері, концентрації газів від іоносфери до поверхні Землі, описані такі явища, як фотохімічні процеси, фотоіонізація. Обговорюється поняття «інформація» на основі феноменів, описаних у статті. Описано вплив модифікації частинок газів та виникнення у зв'язку з цим кисневої недостатності в організмах (гіпоксичний стан). Проаналізовано результати реабілітації осіб, опромінених унаслідок Чорнобильської аварії (із отриманням низьких доз радіації) українськими лікарями та науковцями в гірських умовах і подано відповідні рекомендації.

Висновки. Розглянуто вплив сонячної радіації на атоми, молекули та молекулярні комплекси в атмосфері Землі. Досліджено вплив концентрації газів на різних висотах та роль кисню при опроміненні організмів. Надано практичні рекомендації щодо лікування та реабілітації опромінених пацієнтів.

Ключові слова: радіаційне ураження організмів; гіпоксія; великі висоти; високо-енергетичні частинки.

EXPERIMENTAL ARTICLES

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POLARIZED ACTIVATION OF HUMAN PERIPHERAL BLOOD PHAGOCYTES BY BACTERIOPHAGE–DERIVED DOUBLE-STRANDED RNA (LARIFAN) *in vitro*

R. Dovhyi[1]
M. Rudyk[1]
Ye. Hurmach[2]
T. Serhiichuk[1]
Yu. Yumyna[1]
A. Dvukhriadkina[1]
K. Ostrovska[1]
D. Pjanova[3]
L. Skivka[1]

[1]Taras Shevchenko National University of Kyiv, Ukraine

[2]Bogomolets National Medical University, Kyiv, Ukraine

[3]Latvian Biomedical Research and Study Centre, Riga, Latvia

E-mail: roman_dovhyi@knu.ua

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Aim. This study aimed to examine the effect of Larifan on metabolic characteristics of human blood monocytes and granulocytes *in vitro*.

Methods. Four healthy adult men aged 21–26 years were recruited to participate in the study as blood donors. The metabolic profile of human blood monocytes and granulocytes was evaluated by phagocytic activity, reactive oxygen species production, nitric oxide generation, and arginase activity. Phagocytosis of FITC-labeled inactivated Staphylococcus aureus and reactive oxygen species generation were estimated by flow cytometry. Arginase activity was assessed in cell lysates, and nitric oxide generation in supernatants was examined using the Griess reaction.

Results. Phagocytic index and reactive oxygen species generation were found to be lower in both human blood monocytes and granulocytes treated with Larifan. The drug caused a dose-dependent increase in nitric oxide production, as well as a decrease in the arginase activity of blood monocytes.

Conclusions. Our results indicate the ability of Larifan to reinforce the antiviral properties of resting phagocytes along with containment of oxidative stress development.

Key words: monocytes; granulocytes; phagocytosis; reactive oxygen species; nitric oxide; arginase; metabolic polarization.

Type I and III interferons (IFNs) are innate cytokines that are broadly expressed across many cells, and therefore they are important in the first line of defense against viruses. However, many viruses, particularly SARS-CoV-2, are known to inhibit these IFN responses at various points, from cytokine production to receptor signaling, leading to an increase in viral load [1].

Pathogens, including viruses, are known to express some conserved motifs which are not found in host organism, known as pathogen-associated molecular patterns (PAMPs). Those molecular patterns can be recognized by the corresponding pattern recognition receptors of the phagocytic cells, leading to the proinflammatory activation of those cells [2]. Due to the SARS-COV-2 viral load increase resulting from IFN inhibition, multiple PAMPs stimulate phagocytes, leading to the overactivation of a body's immune system that is manifested as the cytokine storm. And

cytokine storm is considered to be one of the main causes of acute respiratory distress syndrome and multi-organ failure [3].

Since COVID-19 is accompanied by delayed type I IFN response [1], regulation of interferon production can be considered a promising therapeutic option [4]. One such drug candidate is Larifan, comprising a heterogeneous population of dsRNA obtained biotechnologically from *E. coli* cells infected with f2sus11 amber mutant bacteriophage. It is already approved and registered for human use at the State Agency of Medicines of the Republic of Latvia as a treatment option for herpes virus infections and secondary immunodeficiency (Reg. No.04-0230). Moreover, it was demonstrated that pre- and post-infection administration of Larifan inhibited SARS-CoV-2 replication both *in vitro* and *in vivo* in golden Syrian hamsters. Also, Larifan decreased the severity of the infection-induced pathological lesions in the lungs of those animals, and is characterized by interferonogenic activity [5].

We have previously studied the effects of Larifan on the metabolic profile of macrophages of different localization. It was found that intranasally delivered Larifan is capable of re-educating glioma-associated microglia, thus abolishing the creation of pro-tumoral microglia infiltrates [6]. Also, in another experiment, Larifan increased nitric oxide (NO) synthesis and reduced arginase activity and reactive oxygen species (ROS) generation in rat peritoneal macrophages under normoxic conditions [7].

Given the literature data about the remarkable metabolic plasticity of monocytes and macrophages [8], as well as our results showing the ability of Larifan to reprogram the metabolic profile of tissue-resident macrophages, it can be assumed that this drug may prime blood phagocytes for a fight against SARS-COV-2 infection.

The purpose of this study was to examine the effect of Larifan (bacteriophage-derived DSRNA) on metabolic characteristics of human peripheral blood monocytes and granulocytes *in vitro*.

Materials and Methods

Study participants included four healthy adult men aged 21–26 years. The exclusion criteria were a history of somatic disease. Approval was obtained from the local ethical committee, and informed consent was obtained from all subjects before the commencement of the study.

Monocyte isolation. Monocytes were isolated from the buffy coat by double-density gradient centrifugation as described by Menck et al. [9] with slight modifications. Briefly, the buffy coat was subjected to a Ficoll-Hypaque gradient centrifugation (400 g, 30 min) to harvest peripheral blood mononuclear cells (PBMCs). Isolated PBMCs were washed twice by centrifugation in PBS-EDTA (1 mM), and were then layered on a slight hyperosmolar Percoll gradient (density = 1.064 g/m) followed by centrifugation (500 g, 30 min). Cell viability was determined by the Trypan blue exclusion test. The percentage of monocytes after the Percoll gradient was higher than 90% as confirmed by morphology and FACS analysis using anti-CD14 antibodies (BD).

Study design. To estimate the effect of Larifan on ROS generation and phagocytic activity of monocytes and granulocytes whole blood samples collected with EDTA were treated with the drug at growing concentrations (50, 100, and 150 μg/ml) for 30 min and then were analyzed by flow cytometry. To estimate the effect of Larifan on arginase activity and NO generation, isolated monocytes were treated with the drug at growing concentrations (50, 100, and 150 μg/ml) for 18h. Conditioned media and cells were harvested after the treatment with Larifan. Aliquots of media were sampled immediately and analyzed for nitrites. Arginase activity was analyzed in harvested cells.

Intracellular ROS assay. ROS levels were measured using 2′7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA, Invitrogen), which is converted into a non–fluorescent derivative (carboxy-H2DCF) by intracellular esterases as described earlier [10]. Carboxy-H2DCF is membrane impermeable oxidized to fluorescent derivative carboxy-DCF by intracellular ROS. 200 μl of EDTA-anticoagulated whole blood was incubated with 4.3 μl of PBS containing 10 μM carboxy–H2DCFDA for 30 min at 37 °C. A short recovery time was allowed for the cellular esterases to hydrolyze the acetoxymethyl ester or acetate groups and render the dye responsive to oxidation. Erythrocytes were lysed with lysis buffer. The cells were then transferred to polystyrene tubes with cell–strainer caps (Falcon, Becton Dickinson) and analyzed with flow cytometry (excitation: 488 nm, emission: 525 nm). Only living cells, gated according to scatter parameters, were used for the analysis. Neutrophils or monocytes were gated according to forward and side scatter.

Phagocytosis assay. The flow cytometry phagocytosis assay was performed as described above [10]. *Staphylococcus aureus* Cowan I cells (collection of the Department of Microbiology and Immunology of Taras Shevchenko National University of Kyiv) were grown on beef–extract agar and subsequently were heat-inactivated and fluorescein isothiocyanate (FITC) labeled.

The stock of FITC-labeled *S. aureus* at a concentration of 1×10^7 cells/mL in a volume of 5 µL was added to 200 µl of heparinized whole blood. A tube with whole blood only served as a negative control. All samples were incubated at 37 °C for 30 min. At the end of the assay, phagocytosis was arrested by the addition of a cold stop solution (PBS with 0.02% EDTA and 0.04% paraformaldehyde). Erythrocytes were lysed with lysis buffer. The fluorescence of phagocytes with ingested bacteria was determined by flow cytometry. Neutrophils or monocytes were gated according to forward and side scatter. Phagocytosis index (PhI) was calculated with the following formula:

$$[Gmean_{pos} / P_{pos}] - [Gmean_{neg} / P_{neg}],$$

where P_{pos} — percent of positive cells, $Gmean_{pos}$ — mean channel fluorescence, P_{neg} — percent of positive cells in the negative control, $Gmean_{neg}$ — mean channel fluorescence of the negative control.

Measurement of NO production. Griess reaction was used to measure the quantity of NO in each supernatant [11]. The amount of NO per 10^6 cells was calculated by dividing the NO metabolite readings by the total number of viable cells. The mean and standard error were computed as normalized values.

Determination of arginase activity. Arginase activity in cell lysates was determined using a conventional procedure [11]. In summary, cells were treated for 15 minutes at room temperature in a shaker with 100 µl/well of 0.1% Triton X-100 lysis solution. Subsequently, 10 µl of 10 mM $MnCl_2$ and 100 µl of 50 mM Tris-HCl, pH 7.5, were added to each lysate. The plates were heated to 56 °C for 7 minutes to activate the enzyme. The lysates were incubated for two hours with 100 µl of 0.5 M L-arginine (pH 9.7) at 37 °C to facilitate substrate hydrolysis. Using 800 µl of H_3PO_4 (85%), H_2SO (96%), and H_2O (1/3/7, v/v/v), the reaction was stopped. α-isonitrosopropiophenone (40 µl, 9% solution in ethanol) was added and the mixture was incubated for 30 minutes at 95 °C and then for 30 minutes at 4 °C to determine urea using colorimetric analysis. Spectrophotometric analysis was used to quantify the urea content. Each measurement was expressed as the urea level/h per 10^6 cells by dividing it by the total number of viable cells. The means and standard errors were normalized.

Statistical analysis. All experimental results are reported as mean ± standard error. Data statistical significance was determined by Student's t-test. The values of $P < 0.05$ were considered as significant.

Results and Discussion

A slight decrease of the phagocytic index was detected in human blood monocytes and granulocytes treated with all studied concentrations of Larifan compared to untreated cells (Fig. 1). Phagocytosis,

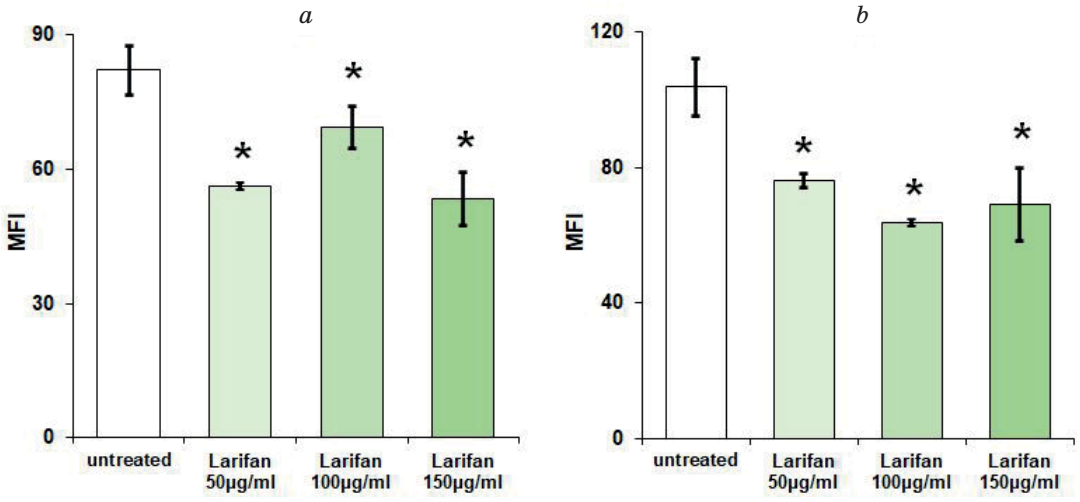

Fig. 1. **Phagocytic index in human blood monocytes:**
a — and granulocytes, *b* — treated with Larifan. MFI — mean fluorescence intensity.
The data are presented as mean ± standard error of the mean. *$P < 0.05$ as compared to untreated cells

specifically antibody-dependent, can be a double-edged sword for antiviral defense: there are reports demonstrating its protective [12], as well as deleterious role, due to phenomenon known as antibody-dependent enhancement (ADE) occurring through enhanced antibody-mediated virus uptake by Fc gamma receptor-expressing phagocytic cells [13]. There is no compelling evidence for ADE in COVID-19 patients so far [14]. Nonetheless, this phagocytosis-inhibiting effect of Larifan may be speculated to prevent ADE to some extent.

Similarly, ROS generation declined significantly ($P < 0.05$) in human blood monocytes after applying Larifan in 3 different concentrations (Fig. 2, *a*). Two higher doses of the drug also significantly lowered ROS production ($P < 0.05$) by human blood granulocytes as compared to untreated cells (Fig. 2, *b*). High ROS production is generally associated with pro-inflammatory metabolic profile of phagocytic cells, with ROS being responsible for direct antimicrobial effects mediated through interaction with bacterial DNA, RNA and proteins [15]. Similar properties of ROS were also described in the context of parasitic infections. However, it is important to note that in contrast to bacteria and parasites, viruses often benefit from increased ROS production [15–17]. To et al. (2017) have shown that Nox2-derived ROS, generated in response to infection with single-stranded RNA and DNA viruses, suppressed antiviral response by modifying highly conserved cysteine residue (Cys98) on Toll-like receptor-7, and targeted inhibition of those endosomal ROS molecules abrogated influenza A virus pathogenicity [17]. Also, according to the studies, ROS play an important role in the pathogenesis of COVID-19 [18]. We have also shown the ability of Larifan to attenuate ROS production in tissue-resident macrophages of different origins, such as rat peritoneal macrophages and microglial cells of C6 glioma-bearing rats [6, 7]. Therefore, the ROS suppressive effect of Larifan may be beneficial in the context of viral diseases, including COVID-19.

Adding 100 μg/ml and 150 μg/ml of Larifan caused a dose-dependent increase in NO production ($P < 0.05$ and $P < 0.01$, respectively) by human blood monocytes (Fig. 3, *a*). Apparently, it happens as a result of dsRNA stimulating RNA-dependent protein kinase, which in its turn activates inducible nitric oxide synthase (iNOS) through the NF-κB pathway [19]. An increase in NO production is usually regarded as a sign of proinflammatory activation of classically activated M1 macrophages [20], with NO acting as a broad-spectrum antimicrobial and antiviral agent effective, in particular, against SARS-COV-2. There are a multitude of specific and non-specific anti-viral mechanisms exerted by NO [21]. Moreover, in contrast to the widely recognized concept of NO being a pro-inflammatory molecule, there are recent reports demonstrating the ability of NO to downregulate leukocyte migration in the course of an acute inflammatory reaction, as well as to inhibit the production of pro-inflammatory cytokines and chemokines [22, 23].

The same higher doses of Larifan (100 μg/ml and 150 μg/ml) reduced the

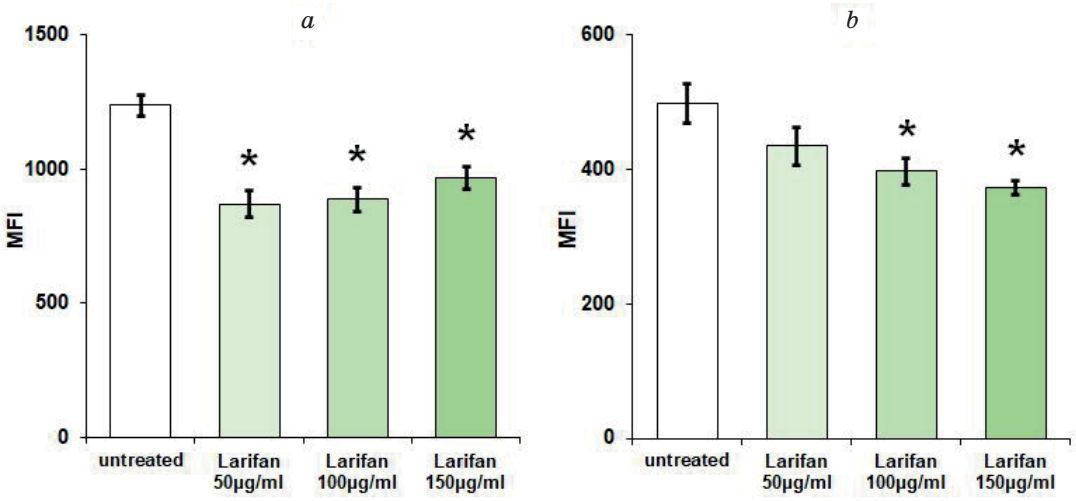

Fig. 2. **Reactive oxygen species generation in human blood monocytes:**
a — and granulocytes, ***b*** — treated with Larifan. MFI — mean fluorescence intensity.
The data are presented as mean ± standard error of the mean. * $P < 0.05$ as compared to untreated cells.

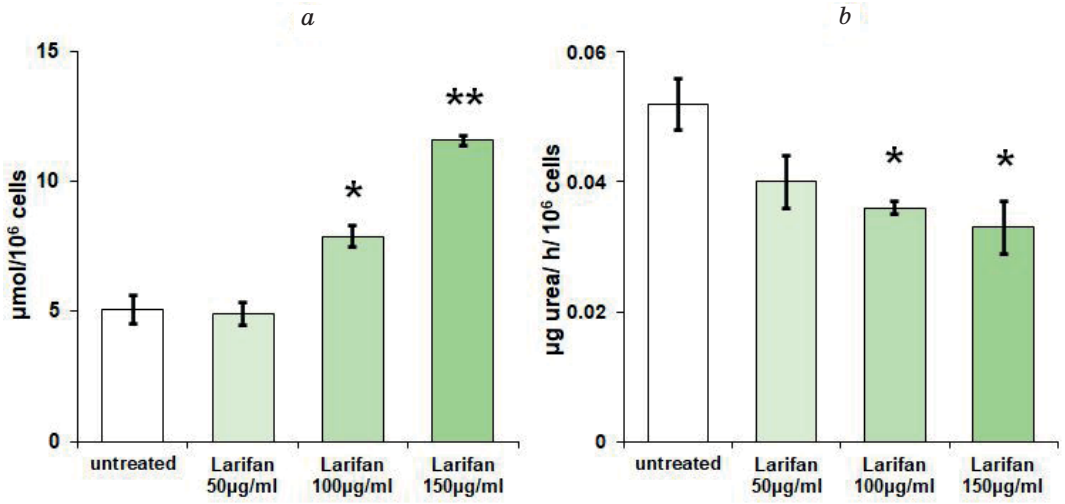

Fig. 3. **Nitrite production (*a*) and arginase activity (*b*) in human blood monocytes treated with Larifan**
The data are presented as mean ± standard error of the mean. * $P < 0.05$; ** $P < 0.01$ as compared to untreated cells.

arginase activity of human blood monocytes ($P < 0.05$) in comparison to untreated cells (Fig. 3, *b*). This decrease may be explained by the reduction of the amount of available L-arginine, which is a common substrate of iNOS and arginase, due to aforementioned significant increase in activity of iNOS (Fig. 3, *a*). Elevated arginase activity is associated with alternatively activated M2 repairing response of macrophages, which is appropriate when a viral pathogen has been eradicated and there is a need to restore tissues damaged by inflammation and viral replication [20]. Therefore, it is not surprising that arginase was inhibited by Larifan since the purpose of the latter is to enhance antiviral response when the infection is in full swing.

Conclusions

Treatment with Larifan causes a slight decrease in phagocytic activity, potent inhibition of reactive oxygen species generation along with an increase of NO release, and a moderate decrease of arginase activity. Taken together, our results indicate the ability of Larifan to reinforce the antiviral properties of resting phagocytes along with containment of oxidative stress development.

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Conflicts of Interest
Authors declare no conflict of interest.

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ПОЛЯРИЗОВАНА АКТИВАЦІЯ ФАГОЦИТІВ ПЕРИФЕРИЧНОЇ КРОВІ ЛЮДИНИ ДВОЛАНЦЮГОВОЮ РНК (ЛАРИФАН) ФАГОВОГО ПОХОДЖЕННЯ *in vitro*

Р. Довгий[1], *М. Рудик*[1], *Є. Гурмач*[2], *Т. Сергійчук*[1], *Ю. Юмина*[1], *А. Двухрядкіна*[1], *К. Островська*[1], *Д. П'янова*[3], *Л. Сківка*[1]

[1]ННЦ «Інститут біології та медицини», Київський національний університет імені Тараса Шевченка, Україна
[2]Національний медичний університет імені О. О. Богомольця, Київ, Україна
[3]Латвійський біомедичний дослідно-навчальний центр, Рига, Латвія

E-mail: roman_dovhyi@knu.ua

Метою роботи було вивчити вплив Ларифану на метаболічні характеристики моноцитів і гранулоцитів крові людини *in vitro*.

Методи. Донорами крові для цього дослідження були четверо здорових дорослих чоловіків віком 21–26 років. Метаболічний профіль моноцитів і гранулоцитів крові людини оцінювали за фагоцитарною активністю, утворенням реактивних форм кисню, продукуванням оксиду азоту та активністю аргінази. Фагоцитоз інактивованого *Staphylococcus aureus*, міченого FITC, і утворення реактивних форм кисню оцінювали за допомогою проточної цитометрії. Активність аргінази вимірювали в клітинних лізатах, а утворення оксиду азоту в супернатантах досліджували за допомогою реакції Грісса.

Результати. Фагоцитарний індекс і утворення активних форм кисню були нижчими як у моноцитів, так і у гранулоцитів крові людини, які отримували Ларифан. Препарат викликав дозозалежне підвищення продукції оксиду азоту, а також зниження аргіназної активності у моноцитів крові.

Висновки. Наші результати вказують на здатність Ларифану посилювати противірусні властивості фагоцитів та попереджати розвиток оксидативного стресу.

Ключові слова: моноцити; гранулоцити; фагоцитоз; реактивні форми кисню; оксид азоту; аргіназа; метаболічна поляризація.

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COMPLEXATION OF CURCUMIN WITH BOVINE SERUM ALBUMIN AND DIPHTHERIA TOXOID CRM197

D. A. Zhukova
O. O. Hrabovskyi

Palladin Institute of Biochemistry
of the National Academy of Sciences of Ukraine,
Kyiv

E-mail: dariya.a.zhukova@gmail.com

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Aim. The goal of the study is to demonstrate the binding sites for curcumin on the protein carriers — bovine serum albumin and diphtheria toxoid CRM197. BSA was chosen as a potential non-specific protein carrier because of its widely used in medicine as a drug carrier.

Methods. In the investigation, both spectrophotometric and molecular docking methods were used.

Results. Two stable binding sites were demonstrated for BSA to bind curcumin. CRM197 was taken as a well-studied carrier protein with its own antitumor activity and has been investigated as a specific carrier with a high affinity for cancer cells with overexpression of epidermal growth factor receptor.

Our results showed one possible curcumin binding site, making CRM197 an ideal specific curcumin delivery platform that provides at least an additive effect in anticancer therapies.

Conclusions. In conclusion, both studied proteins form stable complexes with curcumin that can lay in base of the commercial drug application.

Key words: curcumin; blood proteins; BSA; toxoid; CRM197; complex formation; macromolecular complexes; nanocomplex; protein structure; molecular docking.

Cancer is still a major factor threatening human life around the world, and anticancer drugs remain a huge unmet clinical need [1]. New approaches, use of synthetic molecules [2], cell therapy techniques [3], application of peptides [4] or nanocomplexes [5] are still under consideration.

The object of our study was CRM197 — a recombinant non-toxic diphtheria toxin derivative that differs from the native toxin by only one substitution in the amino acid sequence of the catalytic domain. CRM197 is obtained by replacing glycine in position 52 of the DT gene with glutamic acid [6]. This replacement leads to the loss of catalytic activity by the C-domain and the loss of cytotoxicity by the entire CRM 197 molecule [7]. Despite the loss of toxicity, the CRM 197 molecule retains all the structural components characteristic of the DT molecule, including the structures responsible for binding the R domain to the EGFR demonstrated an overexpression on the surface of the row of the cancer cells [8].

A non-toxic recombinant derivative of diphtheria toxin has antitumor effects in several types of tumor cells, in particular, CRM197 has been shown to block proliferation and angiogenesis and induce apoptosis in human SW-13 and H295R adenocarcinoma cells in culture and in mouse xenografts [9]. In addition, CRM197 has been shown to be a promising carrier for many drugs, such as paclitaxel [10], doxyrubicin [11], cisplatin [12].

All this makes CRM197 a promising possible carrier for curcumin, which can not only increase the bioavailability of anticancer drugs, but also have a synergistic effect on tumor cells.

We tested BSA as a vector for nonspecific delivery of curcumin to evaluate the difference between specific and nonspecific protein carriers. BSA was chosen because of its ability to bind a variety of chemicals such as paclitaxel, metal ions etc [4, 5].

In present study, we focused of the possible complexation of CRM197 with curcumin that is known low-molecular weight compound with prominent anticancer activity [13]. The aim of a present study was to explore the mechanism of complex formation between CRM197 and curcumin and to compare it to the complex formed by BSA and curcumin.

Materials and Methods

BSA (bovine serum albumin), tablets for buffer preparation, PVDF membrane, SDS acrylamide and Curcumin (5 mg/ml stock in 96% ethanol) were purchased from Sigma-Aldrich, USA.

The recombinant protein CRM197 was expressed in *E.coli* BL21(DE3) Rosetta (Sigma-Aldrich, USA) (0.5 mM IPTG (Sigma, USA), 5 hours at 30 °C) with a His-tag at the C-terminal. It was purified using His-Trap affinity column as per the manufacturer's protocol, and the residual imidazole was removed by dialysis against phosphate-buffered saline (PBS, pH 7.4).

SDS-PAGE (sodium dodecyl sulfate — polyacrylamide gel electrophoresis) was used for the characterization of obtained protein. SDS-PAGE was performed at 60 V, 20 min and 120 V, 60 min. Coomassie Brilliant Blue 40 was used as the protein dye. Obtained gels were scanned and protein concentrations were calculated by was purchased from Sigma-Aldrich, USA. PBS.

Formation of Curcumin-Protein Complexes. The curcumin stock solution (5 mg/mL) was prepared in 96% ethanol. A required amount of curcumin was added to the calculated amount of protein (CRM197 or BSA) in PBS solution (pH 7.4) to achieve desired molar ratios. Curcumin in PBS at the required concentration and solvent ratio was used as the control. Formed complexes were dialysated in PBS using PVDF dialysis tubing membrane (Sigma-Aldrich, USA)

Spectrophotometric characteristics. The absorption spectrum of complexes was obtained by spectrophotometry using Optizen-POP (Optizen, Korea). The measurement was provided against PBS solution (pH 7.4). Curcumin dissolved in PBS was used as a control when we examined the spectral properties of curcumin complexes both with BSA or CRM197.

Molecular docking. The protein structures of CRM197 (5I82) and BSA (2VUE) were acquired from Protein Data Bank (http://www.rcsb.org/) and prepared in Chimera software. Ligand structures were built by Marvin Sketch software. Ligands were then protonated and generated in the low-energy conformations (MarvinSketch version 21.16.0, ChemAxon (https://www.chemaxon.com)). A molecular docking simulation of protein and ligand was performed using SwissDock web server, which uses the protein–ligand docking program EADock DSS v3 [14]. A search space of 20×20×20 was used with a grid box centered on the binding sites. Additionally, an accurate docking type was selected with default parameters.

Results and Discussion

Expression of recombinant protein CRM197. Recombinant CRM197 molecule is presented on the Fig. 1. It was obtained from *E. coli* cell lysate by refolding of protein from inclusion bodies on affinity column (Ni-NTA-agarose). The purified protein was further characterized by SDS-PAGE (Fig. 2). The molecular weight of matured protein CRM197, purified from *E. coli* cell lysate, was approximately 60 kDa that coincides with literature data [15].

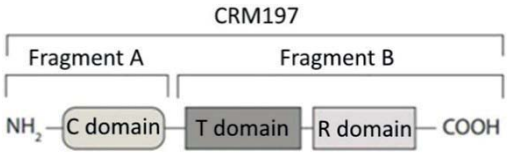

Fig. 1. **Schematic structure of nontoxic derivative od diphtheria toxin CRM197**

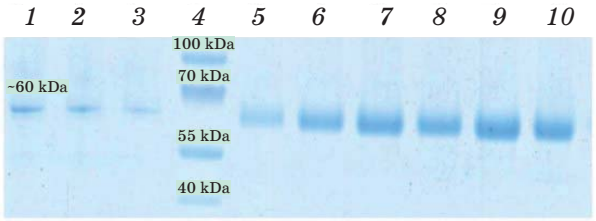

Fig. 2. **SDS-PAGE analysis of purified protein CRM197**
1–3 samples of CRM197 fractions; *4* — Molecular Mass Marker (PageRuler™ Prestained Protein Ladder, 10 to 180 kDa, ThermoFisher); *5–10* — gradient of BSA concentration: *5* — 0.3 mkg; *6* — 0.84 mkg; *7* — 1.38 mkg; *8* — 1.92 mkg; *9* — 2.46 mkg; *10* — 3.0 mkg

Spectrometric characteristic of curcumin complexes with BSA or CRM. CRM197 is a well-known protein with a high affinity to HB-EGFR. Row of cancer cell lines demonstrate of overexpression of these receptors that makes CRM197 is a possible specific curcumin deliver agent. We also used BSA as a widely used protein carrier of chemicals in medicine [16]. Both BSA and CRM197 were considered as the perspective non-specific carrier of curcumin in our investigation.

The absorption spectrum of curcumin was a broad band with maximum absorbance peak at a wavelength ~ 425 nm. However, complexes of curcumin with proteins demonstrated different spectrums with three peaks in the case of CRM197 (Fig. 3) and two peaks in the presence of BSA (Fig. 4).

Both tested proteins showed increasing of absorption rate with increasing of curcumin concentration. When ratio of saturation of proteins by curcumin was achieved there is no further increasing of absorption rate that is represented as a plato on the graph. Based on the data the calculated molar ratio was 1:2,5 and 1:3 for BSA and curcumin and CRM and curcumin respectively.

This allowed us to conclude that the spectral behavior of complexes can be evidence of the stoichiometry of complexes of curcumin with BSA or CRM197. We can speculate that more than one molecule of curcumin can interact with one protein. To prove this hypothesis, we used the molecular docking of BSA or CRM197 with curcumin using SwissDock.

Molecular modeling. The next stage of our work was research *in silico* of the possible binding sites of the curcumin molecule with the CRM197 or BSA molecules. The molecular docking method using the SwissDock software allowed predicting the possible binding modes for the formation of stable complexes.

Study of complexation of BSA and curcumin allowed to obtain two possible binding sites for such interactions (Fig. 5, *A, B*). According to the first binding site curcumin forms hydrogen bonds with Tyr138, Tyr161 and His146 of BSA molecule (Fig. 5, *A*).

Second binding mode of curcumin was characterized by the hydrogen bonds between Ser202, Ile290 and Pi-Pi interactions with Trp214 (Fig. 5, *B*).

Complex of CRM197 with curcumin were stabilized by formation of hydrogen bonds with Lys20, Gly18 and Pi-Pi interactions between aromatic moieties of curcumin and His17 and Tyr61 of CRM197 (Fig. 6).

BSA and CRM197, a non-toxic derivative of diphtheria toxin, are promising carriers due to their binding capabilities and potential anticancer properties. The present study was dedicated to investigation of protein carriers for curcumin as drug-delivery platforms. Two promising protein carriers were evaluated such as BSA and CRM197, non-toxic derivatives of diphtheria toxin. BSA was chosen as a non-specific protein carrier because of its ability to bind different substances and deliver them to the cells [12, 18, 19]. Two stable binding sites were demonstrated for BSA to bind curcumin, which allow it serving as a promising carrier for anticancer therapy. CRM197 is a well-studied carrier protein [10, 20,] with own intrinsic anticancer activity [10] was investigated as a specific carrier with high

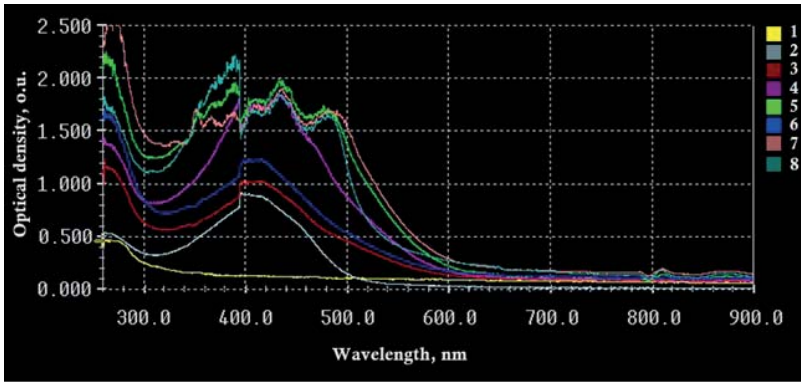

Fig. 3. **The absorption spectrum of curcumin and its complexes with CRM197**
1 — Solution of CRM197 (0.05%); *2* — solution of curcumin in PBS (50:1); *3* — 0.05% solution of CRM197 and curcumin (50:1); *4* — 0.05% solution of CRM197 and curcumin (25:1); *5* — 0.05% solution of CRM197 and curcumin (17:1); *6* — 0.05% solution of CRM197 and curcumin (33:1); *7* — 0.05% solution of CRM197 and curcumin (12:1); *8* — 0.05% solution of CRM197 and curcumin (8:1). All curcumin-containing solutions were prepared in the presence of 0.5% ethanol

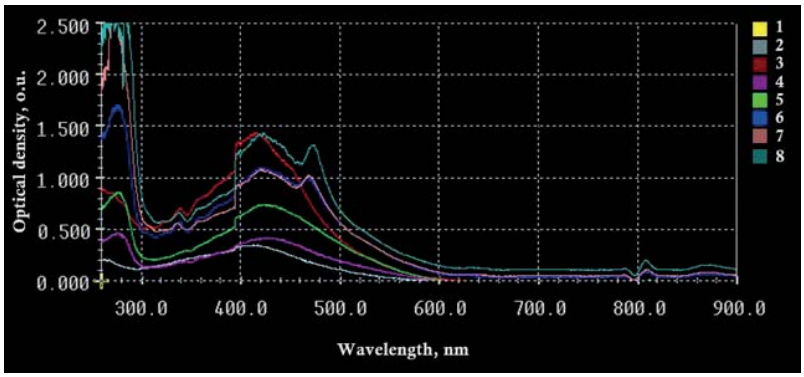

Fig. 4. **The absorption spectrum of curcumin and its complexes with BSA**
1 — PBS; *2* — solution of curcumin in PBS (50:1); *3* — 0.05% solution of BSA and curcumin (50:1); *4* — 0.05% solution of BSA and curcumin (25:1); *5* — 0.05% solution of BSA and curcumin (17:1); *6* — 0.05% solution of BSA and curcumin (33:1); *7* — 0.05% solution of BSA and curcumin (12:1); *8* — 0.05% solution of BSA and curcumin (8:1). All curcumin-containing solutions were prepared in the presence of 0.5% ethanol

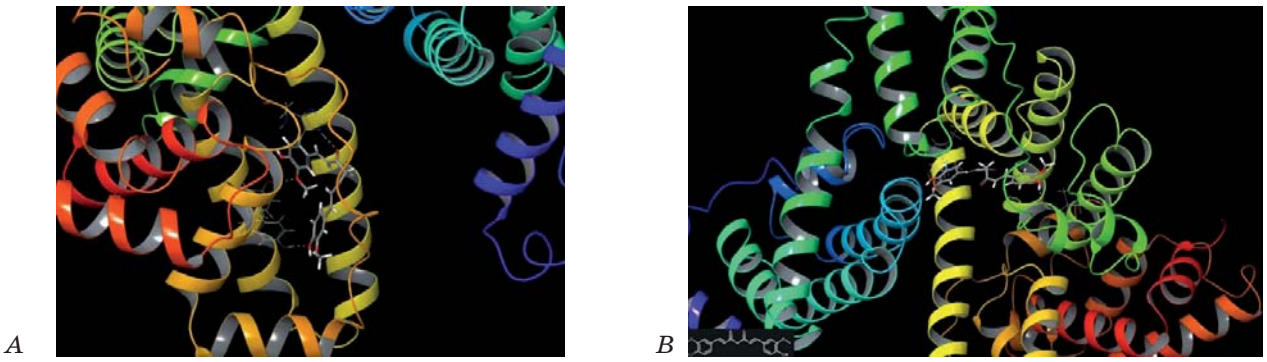

Fig. 5. **Binding modes of BSA and curcumin**
Yellow dashed lines — hydrogen bonds; blue dashed lines — Pi-Pi interactions

affinity to cancer cells with overexpression of marker proHB-EGF like A431 (human adenocarcinoma) or MDA-MB (human breast cancer) cell lines. These results showed one possible binding site for curcumin for CRM197 that makes it perfect specific drug-delivery platforms.

Conclusions

Two sites of curcumin binding with BSA were detected. This was confirmed by the spectral analysis and also by the docking in SwissDock. As for CRM197 *in silico* studies allowed us to detect only one binding site that contradict the spectrometry data. The domains of CRM197 are highly flexible and the availability of only one crystallographic structure did not allow us to take into consideration all conformational changes that can result in the formation of curcumin binding sites.

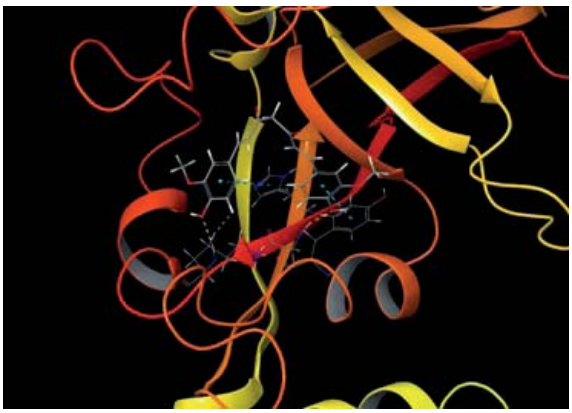

Fig. 6. **Binding modes of CRM197 and curcumin**
Yellow dashed lines — hydrogen bonds; blue dashed lines — Pi-Pi interactions

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КОМПЛЕКСОУТВОРЕННЯ КУРКУМІНУ З BSA ТА CRM197

Жукова Д. А., Грабовський О. О.

Інститут біохімії ім. О. В.Палладіна НАН України,
Київ

E-mail: *dariya.a.zhukova@gmail.com*

Мета. Метою дослідження було довести наявність сайтів зв'язування куркуміну з протеїновими носіями — сироватковим альбуміном великої рогатої худоби та дифтерійним токсоїдом CRM197. БСА був обраний як потенційний неспецифічний протеїновий носій через його широке застосування в медицині як носія багатьох лікарських засобів.

Методи. У дослідженні було використано як спектрофотометричний метод, так і метод молекулярного докінгу.

Результати. Було продемонстровано два стабільних сайта зв'язування БСА з куркуміном. CRM197 був обраний як добре вивчений протеїн з власною протипухлинною активністю, що широко застосовують у медичній практиці щодо терапії пухлинних клітин з підвищеним рівнем експресії EGFR. Наші результати показали один можливий сайт зв'язування для молекули куркуміну, що робить CRM197 ідеальною платформою специфічної доставки куркуміну, яка забезпечує принаймні адитивний ефект в протипухлинній терапії.

Висновки. Підсумовуючи, обидва досліджені протеїни утворюють стабільні комплекси з куркуміном, що може лягти в основу комерційного застосування ліків.

Ключові слова: кумин; протеїни крові; BSA; анатоксин; CRM197; комплексоутворення; високомолекулярні комплекси; нанокомплекс; структура протеїну; молекулярний докінг.

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DYNAMICS OF THE PHENOLIC CONSTITUENTS AND ANTIOXIDANT ACTIVITY IN SUBMERGED CULTURES OF *Xylaria* SPECIES

A. R. Atamanchuk
N. A. Bisko

M.G. Kholodny Institute of Botany
of the National Academy of Sciences of Ukraine,
Kyiv

E-mail: atamalyssa@gmail.com

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Purpose. This study was conducted to enhance comprehension of the dynamic process of synthesis of phenolic compounds by representatives of the genus *Xylaria*, and the correlation between phenol content and antioxidant properties found in biomass and culture liquid during submerged cultivation.

Methods. Cultivation of *Xylaria polymorpha* and *Xylaria longipes* fungal strains from the IBK Mushroom Culture Collection was carried out on a glucose-yeast-peptone nutrient medium under submerged conditions. Harvesting of both biomass and culture liquid was done on the 3rd, 5th, 7th, and 9th day of cultivation, followed by extraction with ethyl acetate. The total phenol content of extracts was determined using the Folin–Ciocalteu method and the antioxidant potential was evaluated through the DPPH assay.

Results. Findings revealed that the accumulation of phenolic compounds by fungal species of the *Xylaria* genus was specified on a strain level. Notably, *X. longipes* strains exhibited higher production of phenolic constituents compared to *X. polymorpha* and demonstrated superior antioxidant activity at a specific time of cultivation. Furthermore, a strong correlation was established between the dynamics of polyphenol accumulation and antioxidant activity in both mycelial biomass and culture liquid.

Conclusions. Natural phenolic compounds with antioxidant properties were extracted from the biomass and culture liquid of the studied strains. Significantly higher concentrations of phenolic compounds and values of antioxidant activity were found in the biomass compared to the culture liquid. The results indicate that a later day of cultivation is not necessarily equivalent to the production of more phenols, emphasizing the need for a comprehensive assessment of the accumulation of these compounds and the dynamic study of related parameters.

Key words: *Xylaria*; phenolic compounds; antioxidants; dynamics; biomass; culture liquid.

According to many scientists, fungi of the genus *Xylaria* Hill ex Schrank can be attributed to promising producers of a variety of biologically active substances, serving both the final and a side product during cultivation. In previous studies regarding metabolites of *Xylaria* species different groups of natural products such as diterpenoids, sesquiterpenoids, diterpene and triterpene glycosides, steroids, alkaloids and phenolics were discovered [1–3]. Since these compounds proved to possess antibacterial [4], antifungal [5, 6], cytotoxic [7], and other pharmacological activities, the interest in xylariaceous fungi as producers of biologically active substances has been growing. Among them, phenolic constituents are of particular interest because of their remarkable potential as free radical scavengers [8]. However, it is worth noting that most of these compounds were obtained from the fruiting bodies of these fungi, while there are rather limited studies on their cultivation. Nevertheless, the practical use of fungi is closely related to the production of mycelium through different methods of cultivation, which is why many modern studies are focused on the optimization of culture conditions [9, 10]. The method of submerged cultivation applied in this study presents a promising approach for obtaining both mycelium and culture liquid

containing bioactive compounds, facilitating their subsequent analysis.

As a result of a preliminary screening of species of the genus *Xylaria* from the IBK Mushroom Culture Collection for biological activity, *Xylaria polymorpha* (Pers.) Grev. and *Xylaria longipes* Nitschke were selected for the study. Cultural and morphological data on growth rates on different nutrient media were used to select two strains of both species [11]. This study aimed to analyze the growth-associated dynamics of phenol production and antioxidant capacity during submerged cultivation of these fungi. Since phenolic compounds have been registered among the main contributors to the antioxidant activity of fungi [12] a correlation between the accumulation of phenolic compounds and antioxidant activity in both biomass and culture liquid extracts was of particular interest.

Materials and Methods

The basal glucose-yeast-peptone nutrient medium (GYP) composed of (g/l): glucose, 25; peptone, 3; yeast extract, 3; $MgSO_4$, 0.25; KH_2PO_4, 1; K_2HPO_4, was used for the cultivation of the mycelium. Fungal strains IBK 2720, 2736 of *X. polymorpha* and IBK 2718, 2726 of *X. longipes* used in this study are from the IBK Mushroom Culture Collection of the M.G. Kholodny Institute of Botany. Strains were initially grown in Petri dishes for 7 days at 25±1 °C on a basal medium with an additional 20 g/l of agar-agar.

The obtained inoculum was homogenized and sterilely inoculated (10% v/v, in 6 duplicates) in 250 ml Erlenmeyer flasks containing 100 ml of GYP medium. Cultivation was carried out for 9 days in darkness on a laboratory shaker under the following conditions: temperature 25±1 °C, agitation speed 120 rpm. The mycelial biomass was harvested by filtration on 3, 5, 7, and 9th day of cultivation and dried at 60 °C until constant weight.

Biomass extraction was conducted with ethyl acetate in a ratio of 1:5 (w/v) for 24 h at room temperature (20±1 °C). Then, the extracts were centrifuged for 15 min at 3000 rpm, after which the supernatant was separated and concentrated using a vacuum rotary evaporator at 40 °C. The culture liquid was initially concentrated using a vacuum rotary evaporator at 40±1 °C and extracted with ethyl acetate in a ratio of 1:2, for 24 h at room temperature (20±1 °C). The upper ethyl acetate fraction was separated using a separatory funnel and then concentrated using a vacuum evaporator at 40±1 °C.

The antioxidant activity of the prepared extracts was determined by 2,2–Diphenyl–1–picrylhydrazyl (DPPH) scavenging assay following Liu et al. [13].

Total phenol content was estimated using a Folin–Ciocalteu (FC) reagent-based assay following Elfahri et al. [14]. The total phenolic value of the samples was obtained from the regression equation $y = 0.0033x+0.0462$ with $R^2 = 0.9904$. The content of total phenolics was estimated as gallic acid equivalents (GAE) and converted into mg/g of dry weight (for biomass extracts) and mg/ml of cultural media (for culture liquid extracts).

Experimental data are indicated as the mean value of at least three independent experiments ± SD (standard deviation). The Student's t-test was applied to express the significance; values at $P < 0.05$ were considered significant. Correlations were obtained by Pearson correlation coefficient in bivariate correlations. Results were analyzed in standard statistical packages Microsoft Excel and Statistics 6.

Results and Discussion

Diverse patterns were noted in the accumulation of phenolics for each strain on different days. In the case of *X. polymorpha* IBK 2720 and 2736 strains, the peak total phenolic content (TPC) was observed on days 3 and 7, with recorded amounts of 0.92±0.05 and 1.53±0.09, respectively (Figs. 1, 2). Strain *X. longipes* IBK 2726 demonstrated the highest TPC value of 2.53±0.10 on the 5th day of cultivation, surpassing all other strains studied. Conversely, the strain *X. polymorpha* IBK 2720 exhibited the lowest TPC value among all biomass extracts, also on the 5th day of cultivation (Fig. 1).

The distinct variations in phenolic accumulation across different strains may be attributed to various factors associated with the cultural and morphological characteristics of the strains, as well as their diverse origins. Contrary to the assumption that the decline in phenolic levels is a result of their extraction into the culture medium, the data did not support this claim.

The quantity of phenolics in the biomass exhibited similar fluctuations to those in the culture liquid, as illustrated in Fig. 1–4, but with higher rates. Notably, for the strain *X. longipes* IBK 2726 the phenolic content initially increased on day 5, decreased nearly threefold on day 7, and halved by the 9th day of cultivation. Similarly, but not so pronounced,

the content of phenols in the culture liquid of this strain changed (Fig. 4). A similar trend was observed when comparing the phenolic content in the biomass and culture liquid of *X. polymorpha* IBK 2720. However, in this case, the amount of phenolics first decreased, then increased sharply and fell again on the 9th of cultivation (Fig.1).

In general, the culture liquid extracts contained notably lower quantities of phenols compared to the biomass, with the peak value of 0.45±0.03 recorded on day 9 for strain IBK 2726. Although the method applied here allows estimation of the total phenolic content, it is susceptible to various factors that may affect the values. In fungal cultivation, factors such as extraction methods, solvent choices, and culture media composition can influence bioactive compound concentrations.

Regarding the antioxidant activity, the biomass extracts of all strains studied showed high rates of DPPH scavenging activity. The maximum value was recorded for *X. longipes* IBK 2718 – 87.82±0.19% on the seventh day of cultivation, and a close value was obtained for *X. polymorpha* IBK 2720 on the third day of cultivation at 87.37±0.75%. As compared to other antioxidant assays, the obtained values are high. For instance, in the already mentioned study conducted by Liu et al. [13], the ethyl acetate extracts of the endophytic *Xylaria* sp. had a substantially higher phenol content, than obtained by us. Nevertheless, their antioxidant activity was notably lower compared to the results obtained in our research and amounted to 29.66±0.97%.

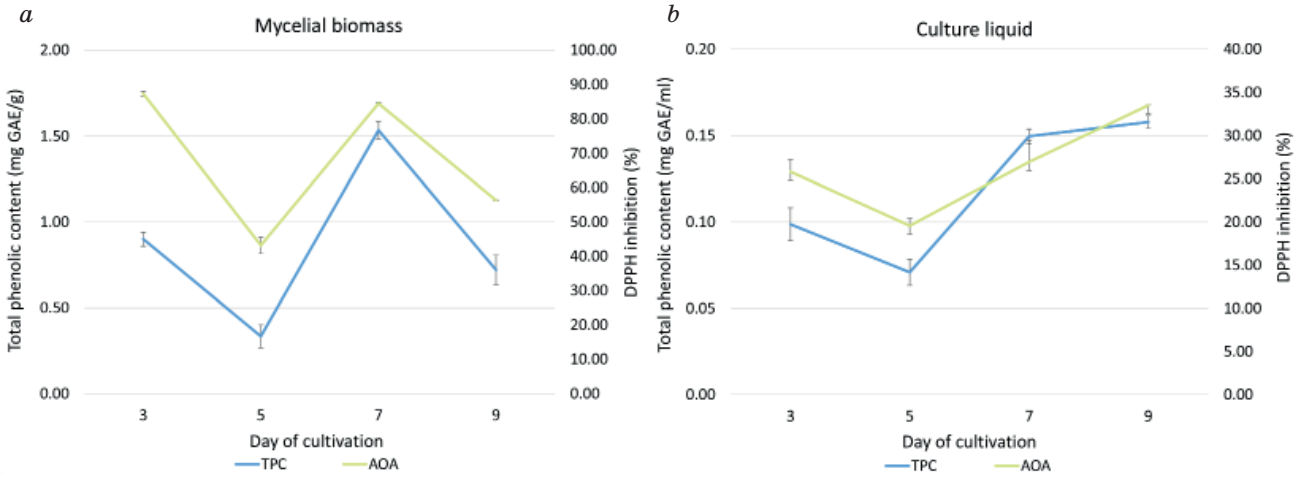

Fig. 1. **Dynamics of the total phenolic content and antioxidant activity in mycelial biomass (*a*) and culture liquid (*b*) of *X. polymorpha* IBK 2720**

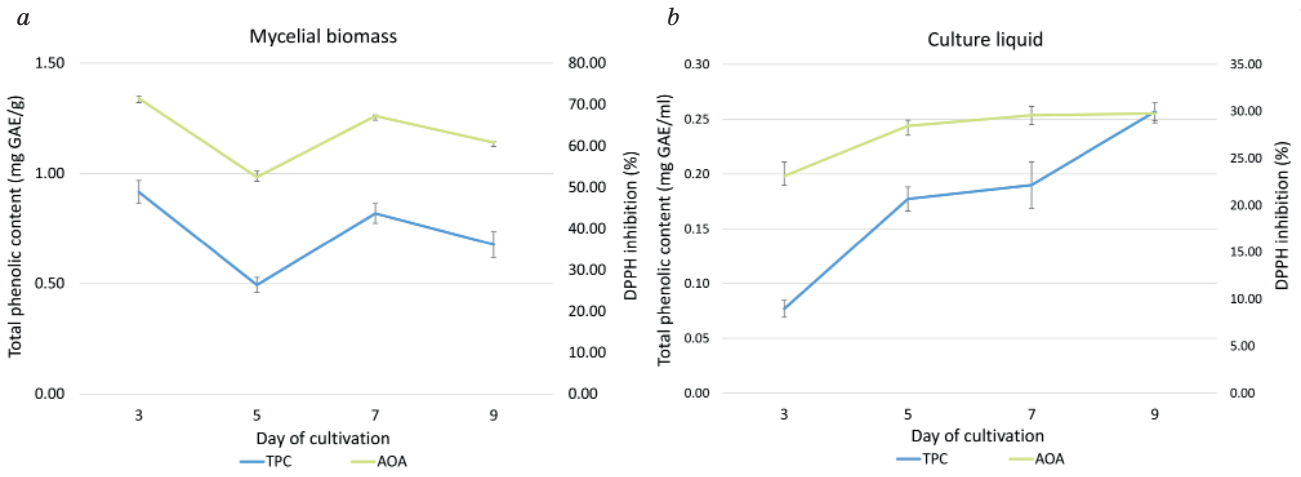

Fig. 2. **Dynamics of the total phenolic content and antioxidant activity in mycelial biomass (*a*) and culture liquid (*b*) of *X. polymorpha* IBK 2736**

Moreover, the strains we studied produced phenols in amounts comparable to other fungi, not only within their taxonomic group but also among fungi that are actively cultivated on the industrial scale. For example, according to Cheung et al. (2003) for the edible mushrooms *Lentinula edodes* (Berk.) Pegler (shiitake) and *Volvariella volvacea* (Bull.) Singer (straw mushroom) amounts of extracted ethyl acetate phenols were 0.03±0.01 and 0.21±0.08 mg of GAE/g of dry weight of fruiting bodies, respectively [15]. These amounts are comparable to our data for mycelium and culture liquid, even though researchers emphasize that fruiting bodies exhibit significantly higher concentrations of phenolic compounds compared to cultivated mycelium.

It has been observed by numerous authors that phenolic content correlates with antioxidant activity. This is attributed to the structural chemistry of phenolics, which facilitates hydrogen or electron donation from hydroxyl groups located along the aromatic ring. Such mechanisms contribute to effective free radical scavenging activities and demonstrate metal-chelating potential [16]. The correlation of total phenolic content with DPPH scavenging activities of extracts in our study is shown in Figs. 1–4 and calculated correlation coefficients are presented in Table 1. Notably, the biomass of *X. polymorpha* IBK 2736 displayed the strongest correlation between the total phenolic content and DPPH activity, with a Pearson's coefficient of 1.00.

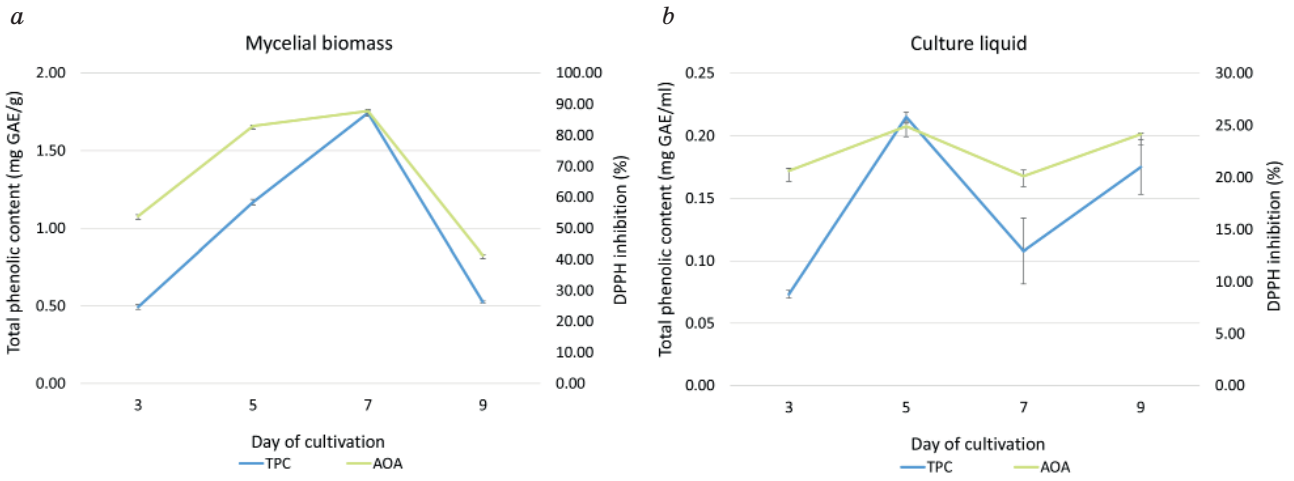

Fig. 3. **Dynamics of the total phenolic content and antioxidant activity in mycelial biomass (*a*) and culture liquid (*b*) of *X. longipes* IBK 2718**

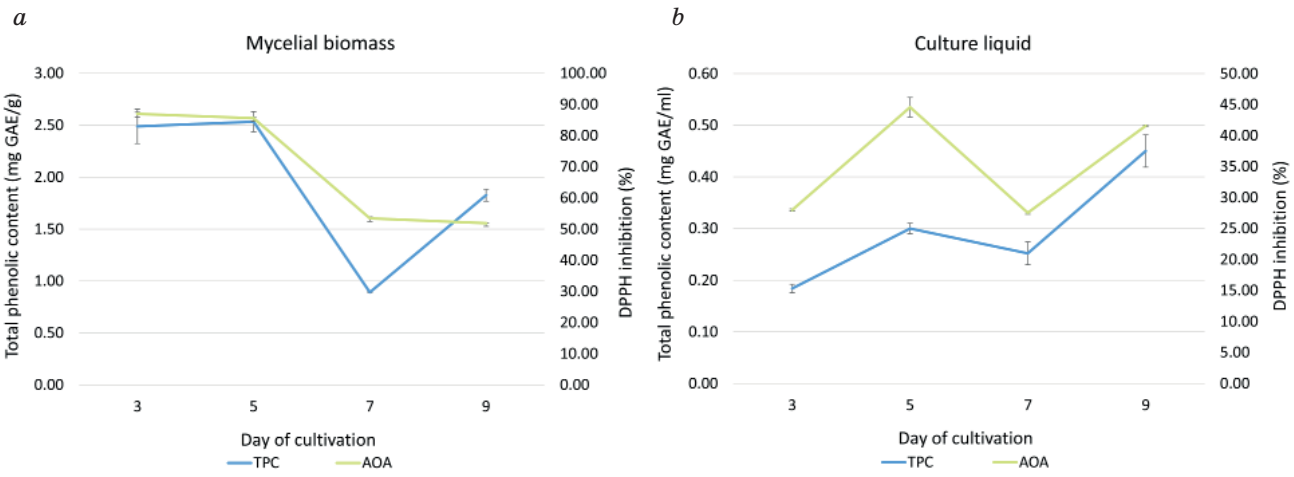

Fig. 4. **Dynamics of the total phenolic content and antioxidant activity in mycelial biomass (*a*) and culture liquid (*b*) of *X. longipes* IBK 2726**

Table 1

Pearson's correlation coefficients of antioxidant activity and phenolic contents

Species	IBK strain	Mycelial biomass	Culture liquid
X. polymorpha	2736	1.00	0.93
	2720	0.82	0.89
X. longipes	2718	0.92	0.94
	2726	0.85	0.71

These correlations do not account for distinguishing characteristics of phenolic profiles, which can vary both qualitatively and quantitatively, depending on the types of phenolics present in the samples. It is important to note that phenolic compounds possess different donor-proton capacities, which determine their antioxidant activity. Therefore, phenolic compounds and their characteristics remain to be investigated in representatives of the genus *Xylaria*.

Conclusions

The results demonstrate that *X. polymorpha* and *X. longipes* accumulate phenolic compounds strain-specifically, which should be considered when selecting strains that produce biologically active substances. Most noticeable concentrations of phenolic compounds were accumulated in biomass compared to culture liquid. Studied trains of *X. longipes* accumulated more phenolic compounds, reaching the maximum of 2.53±0.10 mg GAE/g for the strain IBK 2726 on the 5th day of cultivation. In comparison, the highest amount of TPC was 1.53±0.09 mg GAE/g *for X. polymorpha* IBK 2720 on the 7th day of cultivation. These results support the belief that extended cultivation time does not result in increased metabolite accumulation, and thus it is effective to study the variation of phenols through dynamico17s. A strong correlation between the total phenol content and DPPH radical scavenging activity was observed for all biomass and culture liquid extracts.

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ДИНАМІКА ВМІСТУ ФЕНОЛІВ ТА АНТИОКСИДАНТНОЇ АКТИВНОСТІ ПРИ ГЛИБИННОМУ КУЛЬТИВУВАННІ ВИДІВ РОДУ *Xylaria*

Атаманчук А. Р., Бісько Н. А.

Інститут ботаніки ім. М.Г. Холодного Національної академії наук України, Київ

E-mail: atamalyssa@gmail.com

Мета. Дослідження динаміки накопичення фенольних сполук представниками роду *Xylaria*, а також кореляції між вмістом фенолів та антиоксидантними властивостями, виявленими у біомасі та культуральній рідині при їх глибинному культивуванні.

Методи. Штами грибів *Xylaria polymorpha* та *Xylaria longipes* з Колекції культур шапинкових грибів Інституту ботаніки імені М.Г. Холодного НАН України (ІВК) вирощували на глюкозо-дріжджово-пептонному живильному середовищі за умов глибинного культивування. Біомасу та культуральну рідину відбирали на 3, 5, 7 та 9-ту добу культивування із подальшою екстракцією етилацетатом. Загальний вміст фенолів у всіх екстрактах визначали за допомогою методу Фоліна-Чокалтеу. Антиоксидантний потенціал оцінювали за допомогою спектрофотометричного аналізу поглинання вільних радикалів 2,2–дифеніл–1–пікрилгідразилу.

Результати. Встановлено, що накопичення фенольних сполук було штамоспецифічною характеристикою. Зокрема, штами *X. longipes* продукували більше фенольних сполук упродовж усього часу культивування, порівняно зі штамами *X. polymorpha*, та проявляли вищу антиоксидантну активність на певну добу. Крім того, було встановлено високу кореляцію між динамікою накопичення фенольних сполук та антиоксидантною активністю як у біомасі, так і в культуральній рідині.

Висновки. Фенольні сполуки з антиоксидантними властивостями було екстраговано із біомаси та культуральної рідини досліджених штамів. Значно вищі концентрації фенольних сполук та значення антиоксидантної активності було виявлено у біомасі порівняно з культуральною рідиною. Показано, що продовження процесу культивування не завжди призводить до збільшення концентрації фенольних сполук, що підкреслює необхідність подальшого комплексного вивчення накопичення цих речовин та взаємозв'язків із супутніми параметрами.

Ключові слова: *Xylaria*; фенольні сполуки; антиоксиданти; динаміка; біомаса, культуральна рідина.