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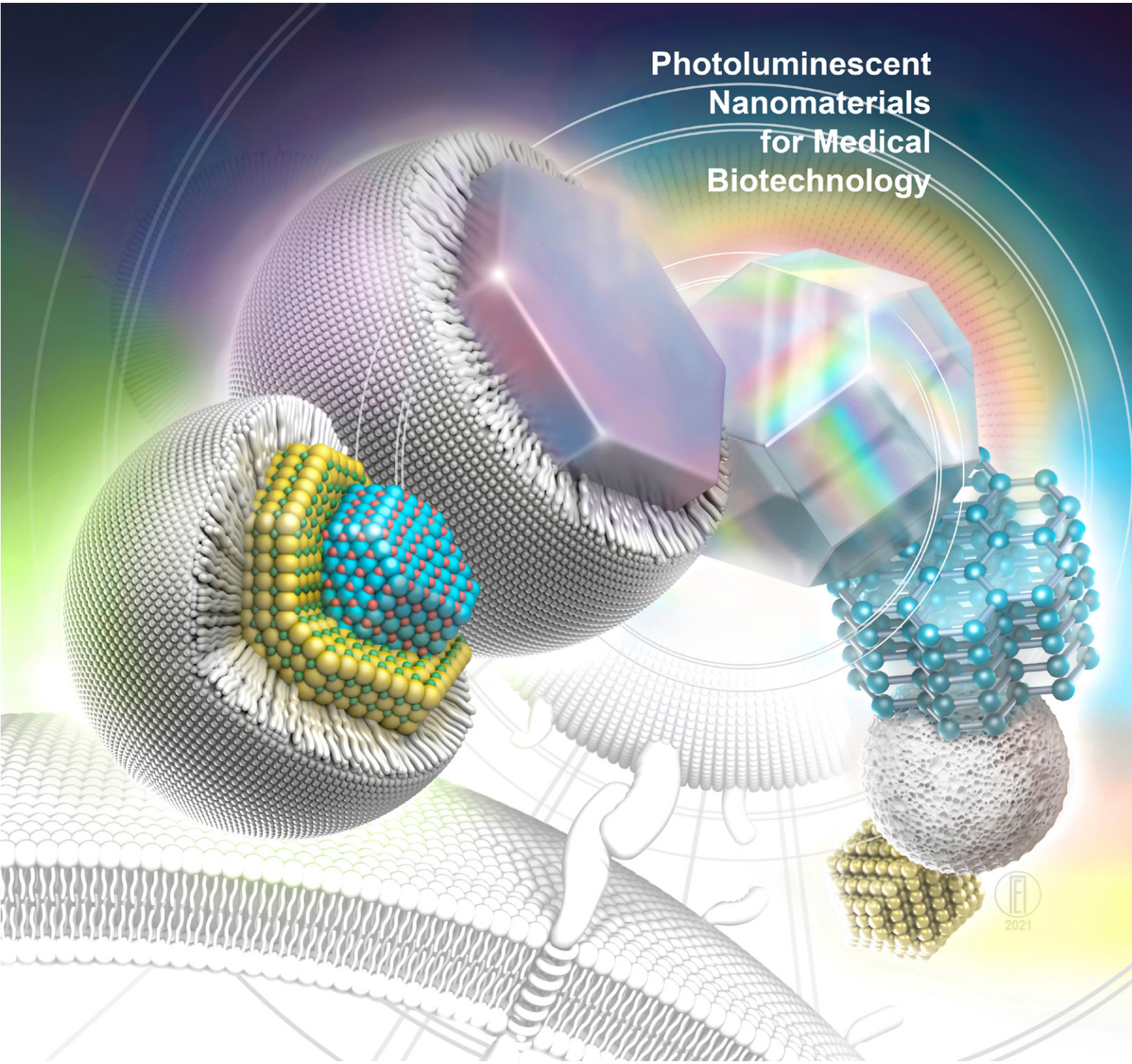
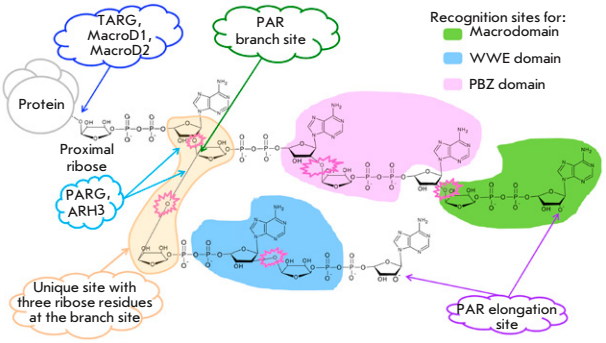


Poly(ADP-Ribosyl) Code Functions

N. V. Maluchenko, D. O. Koshkina, A. V. Feofanov, V. M. Studitsky, M. P. Kirpichnikov

Poly(ADP-ribosyl)ation plays a key role in cellular metabolism. Covalent poly(ADP-ribosyl)ation affects the activity of the proteins engaged in DNA repair, chromatin structure regulation, gene expression, RNA processing, ribosome biogenesis, and protein translation. The review discusses how structurally different poly(ADP-ribose) (PAR) molecules composed of identical monomers can differentially participate in various cellular processes acting as the so-called "PAR code." Impaired PAR metabolism is associated with the development of pathological processes causing oncological, cardiovascular, and neurodegenerative diseases.

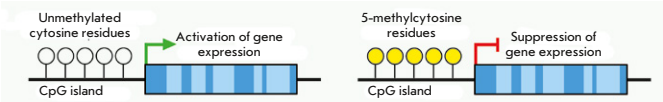


Structure of PAR on the protein globule surface and recognition sites for PAR readers and erasers

DNA Methylation As an Epigenetic Mechanism in the Development of Multiple Sclerosis

I. S. Kiselev, O. G. Kulakova, A. N. Boyko, O. O. Favorova

The epigenetic mechanisms of gene expression regulation are a group of the key cellular and molecular pathways that lead to inherited alterations in genes' activity without changing their coding sequence. This review summarizes the data on the molecular mechanisms underlying DNA methylation and the MS risk factors that can affect the DNA methylation profile and, thereby, modulate the expression of the genes involved in the disease's pathogenesis. The focus of our attention is centered on the analysis of the published data on the differential methylation of DNA from various biological samples of MS patients obtained using both the candidate gene approach and high-throughput methods.

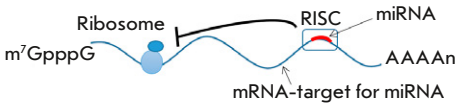


Methylation of cytosine residues in the CpG island located in the gene promoter region

MicroRNAs in the Myelodysplastic Syndrome

Y. A. Veryaskina, S. E. Titov, I. B. Kovynev, S. S. Fedorova, T. I. Pospelova, I. F. Zhimulev

Myelodysplastic syndromes (MDSs) are a group of hematological disorders characterized by ineffective differentiation of hematopoietic precursors, bone marrow dysplasia, genetic instability, and an increased risk of developing acute myeloid leukemia, which occupies a special place among blood cancers. The review discusses the role of microRNAs in the regulation of hematopoiesis and MDS pathogenesis. The relationship between miRNA expression levels and prognosis of overall survival and response to therapy is described.

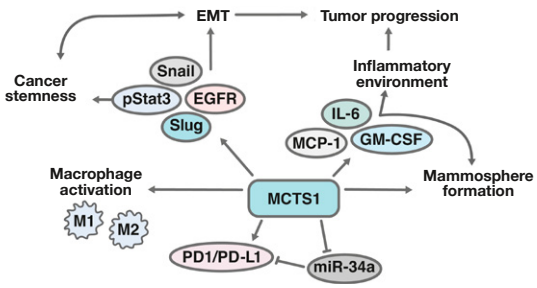


Mechanism of mRNA translational repression by the miRNAs incorporated into a RISC, which includes mature miRNAs

The Role of the MCTS1 and DENR Proteins in Regulating the Mechanisms Associated with Malignant Cell Transformation

E. Y. Shyrokova, V. S. Prassolov, P. V. Spirin

MCTS1 and DENR proteins are involved in translation reinitiation. Increased expression of MCTS1 and DENR is believed to be associated with the development of a number of malignancies. This review addresses the issue of using these proteins as potential prognostic and therapeutic targets in the fight against malignant diseases.



Schematic illustration of the contribution of MCTS1 to EMT, tumor escape from immune surveillance, and activation of pro-inflammatory factors by tumor cells

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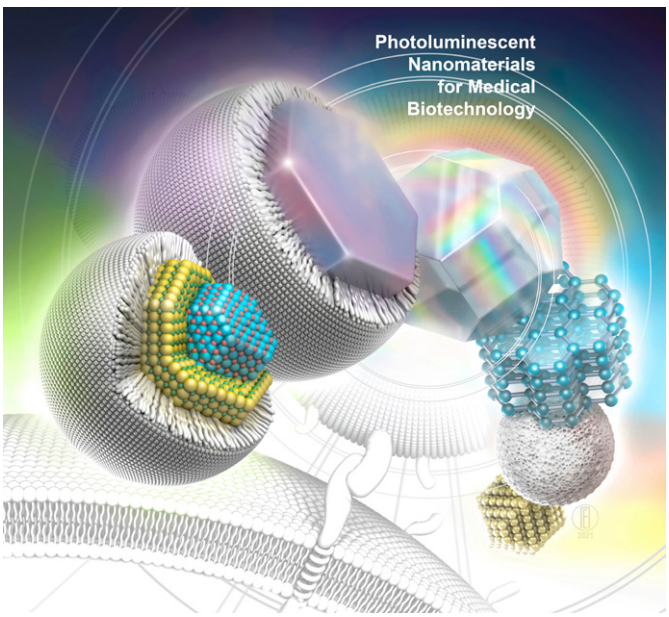

IMAGE ON THE COVER PAGE
(see the article by E. L. Guryev et al.)

MicroRNAs in the Myelodysplastic Syndrome

Y. A. Veryaskina[1,2*], S. E. Titov[2,3], I. B. Kovynev[4], S. S. Fedorova[4], T. I. Pospelova[4], I. F. Zhimulev[2]
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ABSTRACT **The myelodysplastic syndrome (MDS) holds a special place among blood cancers, as it represents a whole spectrum of hematological disorders with impaired differentiation of hematopoietic precursors, bone marrow dysplasia, genetic instability and is noted for an increased risk of acute myeloid leukemia. Both genetic and epigenetic factors, including microRNAs (miRNAs), are involved in MDS development. MicroRNAs are short non-coding RNAs that are important regulators of normal hematopoiesis, and abnormal changes in their expression levels can contribute to hematological tumor development. To assess the prognosis of the disease, an international assessment system taking into account a karyotype, the number of blast cells, and the degree of deficiency of different blood cell types is used. However, the overall survival and effectiveness of the therapy offered are not always consistent with predictions. The search for new biomarkers, followed by their integration into the existing prognostic system, will allow for personalized treatment to be performed with more precision. Additionally, this paper explains how miRNA expression levels correlate with the prognosis of overall survival and response to the therapy offered.**
KEYWORDS **myelodysplastic syndrome, miRNA, acute myeloid leukemia.**

INTRODUCTION

MicroRNAs (miRNAs) are short non-coding RNAs that regulate gene expression post-transcriptionally. To date, more than 2,600 human miRNAs have been identified, each with the potential to regulate hundreds of target genes [1]. MicroRNAs play key regulatory roles in all biological processes, including cell proliferation, cellular differentiation, cell cycle control, apoptosis, and angiogenesis [2–5]. In addition, miRNAs can act as either oncogenes or as suppressors of tumors of various origins, including hematological malignancies [6, 7]. MicroRNAs are important regulators of the differentiation and maintenance of hematopoietic stem cells (HSCs), and changes in their expression levels obviously promote the development of myeloid and lymphoid neoplasms [8].

Myelodysplastic syndromes (MDSs) are a heterogeneous group of HSC disorders characterized by bone marrow cell dysplasia and a deficiency of one or more blood cell types that have to do with inefficient hematopoiesis [9]. Although no epidemiological data on MDS have been gathered yet in Russia, the Surveillance, Epidemiology, and End Results (SEER) states that the incidence of the disease in the U.S. was above 28,032 in 2012–2017, with the majority of patients being above 70 years of age [10]. In addition, MDS presents an increased risk of transformation into acute myeloid leukemia (AML) [11]. The mechanisms of MDS initiation and development are not yet fully understood, and the current methods of treatment and diagnosis are not sufficiently efficient, since the disease comes in many facets [12].

Since information about the role of miRNAs in MDS development and prognosis is fragmentary, this overview considers the role miRNAs play in normal hematopoiesis and provides a comprehensive analysis of the variations in their expression levels in MDS patients with normal and aberrant karyotypes. Special attention is given to the examples portraying miRNAs as promising markers for predicting the development of MDS and the effectiveness of the therapy offered.

MicroRNA BIOGENESIS

The discovery of the small non-coding RNA *lin-4* in *Caenorhabditis elegans* in 1993 laid the foundation for a new line of research. The main finding of that discovery was the fact that *lin-4* downregulates the *lin-14* gene post-transcriptionally by complementarily

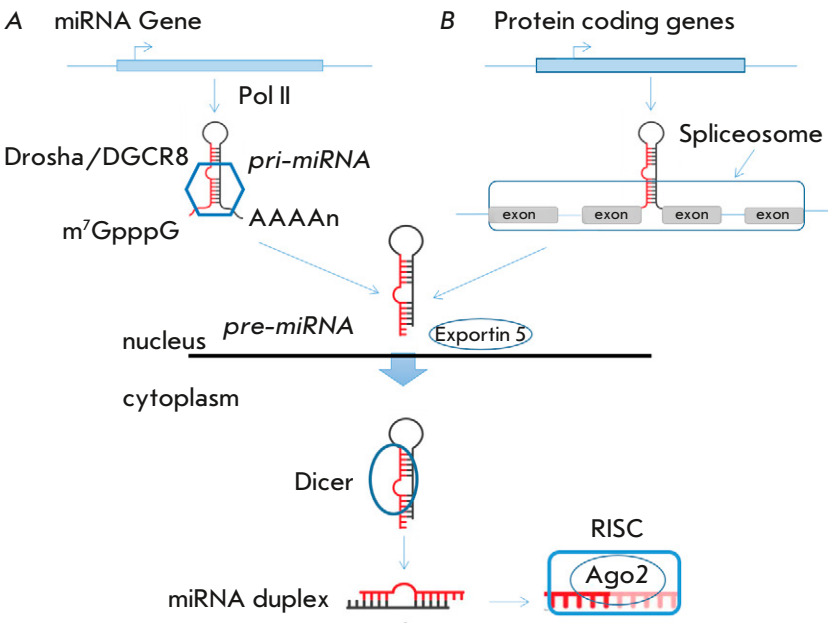

Fig. 1. MicroRNA processing. (*A*) – Canonical pathway. MicroRNAs are transcribed by RNA polymerase II to produce primary transcripts (pri-miRNAs). The pri-miRNAs are cleaved by a microprocessor that includes Drosha and DGCR8 to form precursor miRNAs (pre-miRNAs). The pre-miRNAs are exported from the nucleus to the cytoplasm by the protein Exportin 5. Dicer cleaves the loop, and one strand of the miRNA duplex binds to the Ago2 protein to form the RNA-induced silencing complex (RISC). (*B*) – One of the non-canonical pathways for miRNA processing (Drosha-independent). Splicing results in the formation of short intron hairpins to become a substrate for further miRNA processing

binding the unique short miRNA sequence of *lin-4* (the seed region) to the 3' untranslated region (3'-UTR) of the *lin-14* gene [13, 14]. According to PubMed, about 100,000 articles reporting on the results of miRNA studies have been published to date. Some miRNA genes are located within other genes, mostly in the introns and occasionally in the exons of protein-coding genes, but many more are located in intergenic regions and are regulated by their own promoters, which are similar to those of protein-coding genes. However, transcript processing in the former is more complex [15].

Mature miRNA is 18-24 nucleotides in length, and its formation is a multistep process involving a large number of enzymes: DNA → primary miRNA (pri-miRNA) → precursor miRNA (pre-miRNA) → mature miRNA. The miRNA biogenesis pathway can be either canonical or non-canonical (*Fig. 1*). The canonical pathway is more common, and if miRNA biosynthesis follows it, pri-miRNAs are transcribed by RNA polymerase II (Pol II) and converted into pre-miRNAs by a complex consisting of the RNA-binding protein DGCR8 and the enzyme Drosha. Next, a hairpin RNA ~70 nt in length (pre-miRNA) is exported to the cytoplasm by a complex consisting of the Exportin5/RanGTP proteins. Then, the endonuclease Dicer removes the terminal loop, resulting in a mature miRNA duplex. Because alternative strands can be differentially represented in different tissues, mature miRNAs are often suffixed "5p" or "3p" to denote the functional miRNA strand. Normally, the strand with the lower 5' stability or with the 5' uracil is called 'the guide strand' and eventually

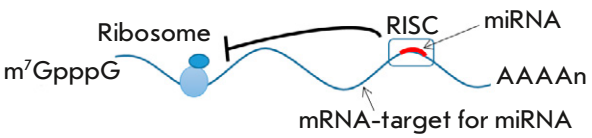

Fig. 2. Mechanism of mRNA translational repression by the miRNAs incorporated into a RISC, which includes mature miRNAs. The RISC interferes with the ribosome advancing along the mRNA and, thus, stops the translation

becomes incorporated into the RNA-induced silencing complex (RISC) directly involved in the regulation of target genes, while the other strand, called 'the passenger strand', is removed (*Fig. 2*). The target gene is silenced by mRNA cleavage at 10–11 nt upstream of the 5'-end of the guide strand. This cleavage is mediated by the activity of the Ago2 protein to be one of the main components of the RISC complex [16].

In addition, there are non-canonical pathways for miRNA biogenesis. These pathways use various combinations of the proteins involved in the canonical pathway and largely differ by whether they have Drosha and Dicer in them or not [17]. Drosha/DGCRS-independent pathways can generate pre-miRNA-like hairpins serving as Dicer substrates. This is how splicing would result in short intron hairpins called 'mirtrons'. Thus, splicing can act as an alternative to Drosha. In addition, this kind of hairpin can form as a byproduct of the processing of other RNAs: for example, tRNA. Dicer-independent pathways are rather rare special

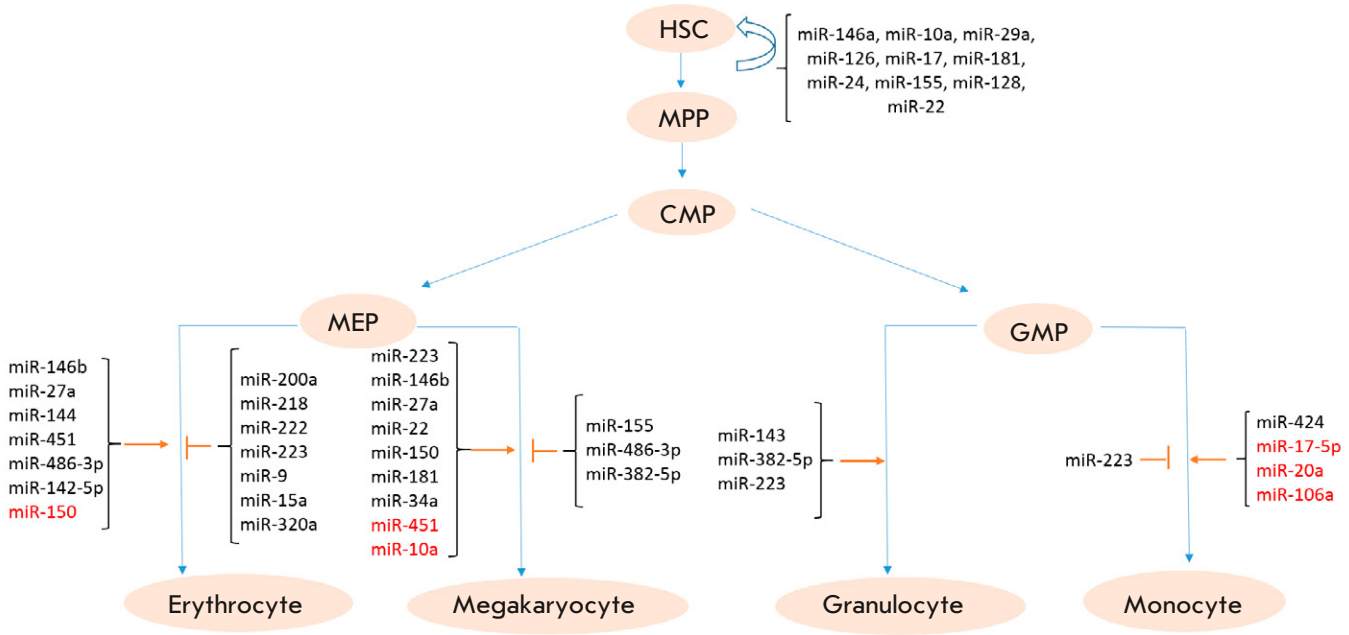

Fig. 3. A Schematic of myelopoiesis with a list of the miRNAs involved in the regulation of various stages of normal hematopoiesis. The names of miRNAs with increased expression levels are typed in black, and k; the names of those with decreased expression levels during hematopoiesis regulation – in red

cases of the processing of certain miRNAs. In particular, the processing of miRNA-451, which holds an important place in hematopoiesis, follows this non-canonical pathway. Primary miRNA-451 is processed by enzymes in the Drosha/DGCR8 complex, and the resulting pre-miRNA-451 directly binds to the Ago2 protein, the main component of the RISC complex [18]. Relatively rare non-canonical pathways are not considered in detail in this overview.

In most cases, miRNAs interact with the 3'-UTR of target mRNAs; however, interactions of miRNAs with other regions, including the 5'-UTR, the coding sequence, and gene promoters, have also been reported [16]. Mature miRNAs largely interact with target mRNAs due to complete or partial complementary binding of the seed region of the miRNAs to the 3'-UTR of the target mRNAs. It should be noted that binding with imperfect complementarity is possible, because a single miRNA can target multiple genes [19]. The degree of complementarity determines what will take place: Ago2-dependent cleavage of the target mRNA or translational suppression [16]. Currently, there are information resources (miRTarBase, TargetScan, mirDB, miRWalk, miRanda) that allow one to predict the genes targeted by miRNAs and, thus, identify the most specific miRNAs for the disease under study. However, to understand the role of miRNA in the mechanisms of initiation and development of blood diseases, comprehensive knowledge of miRNA functions in maintaining normal hematopoiesis is required.

MicroRNAs IN NORMAL HEMATOPOIESIS

Hematopoiesis is a process of blood cell formation that begins in early embryogenesis and appears as a cascade of divisions and differentiation of hematopoietic stem cells (HSCs) [20]. Stem cells undergo symmetric and asymmetric division, and, thus, their population is maintained and differentiated cells form. Symmetric division implies the formation of two identical cells, while asymmetric division results in the formation of one initial and one differentiated cell, the latter being capable of making it all the way from a multipotent precursor to a mature blood cell. Multipotent precursors (MPPs) produce a common lymphoid progenitor (CLP) and a common myeloid progenitor (CMP). MDSs are a group of diseases of hematopoietic stem cells and are characterized by multilineage dysplasia in immature myeloid cells and ineffective hematopoiesis. Impaired myeloid cell development is the primary cause of MDS [21].

Both genetic and epigenetic regulatory mechanisms, including miRNAs, are involved in the maintenance of normal hematopoiesis (*Fig. 3*). Chen *et al.* published one of the first works describing the role of miRNAs

in the differentiation control of hematopoietic lineages in mammals [22]. According to many studies, miRNAs are involved in the regulation of all branches of the hierarchical tree of blood cell development [23, 24]. Importantly, mutations of the *Dicer* gene, whose product is a key participant in miRNA processing, affect normal hematopoiesis, suggesting that miRNA regulation plays an important role in it [25, 26]. The balance between the self-renewal and differentiation of stem cells is also controlled by miRNAs. Georgantas *et al.* name 33 miRNAs specific to hematopoiesis; in particular, miRNA-17, -24, -146, -155, -128 and -181, which block HSC differentiation into more mature blood cells [27]. MicroRNA-22 is yet another player in the control of HSC self-renewal [28]. Gupta *et al.* emphasize the point that the expression levels of miRNA-146a, -10a, -29a, -126, -17, and miRNA-181 are increased in HSCs and that their function is to maintain the HSC phenotype and to regulate the transition of MPPs to CMPs or CLPs [8]. It was further established on mouse cells that miRNA-125a, -99, -130a, and miRNA-33 are involved in the control of HSC self-renewal [26, 29–31].

A MDS diagnosis is based on the identification of dysplastic changes in at least one hematopoietic lineage. Morphological changes in the cells involved in erythro-, granulocyto- and megakaryocytopoiesis in the bone marrow and blood are very diverse, and the ratio of normal to dysplastic elements varies significantly from one patient to another. A cell line is considered to be modified if its dysplastic elements amount to more than 10%. Now, let us consider the role of miRNAs in the development of each hematopoietic lineage in more detail.

Erythropoiesis is a process of CMP differentiation into a common megakaryocyte-erythrocyte progenitor, followed by the formation of erythrocytes. Erythrocyte dysplasia appears as a change in the shape of red cells; in particular, due to cytoskeletal abnormalities. A common concomitant pathology in MDS is anemia, associated either with a decrease in the number of erythrocytes or with a decrease in their hemoglobin levels. Analysis of literature data has shown that miRNAs control every step of hematopoiesis. Some miRNAs promote – while others block – the differentiation of precursors into mature blood cells (*Fig. 4*). In particular, increased expression levels of miRNA-200a, -218, -221, -222, -223, 9, -15a, and -320 block, while miRNA-27a, -451, -144, -486-3p, and -146b promote erythropoiesis [32–46]. In addition, decreased expression levels of miRNA-150 promote erythropoiesis [39]. Jin *et al.* note that the expression levels of miRNA-142-3p, miRNA-142-5p, miRNA-146a, and miRNA-451 dynamically change during the differentiation of the erythroid lineage [40]. An interesting fact was noted by Sun *et al.*: a high-altitude hypoxic environment substantially increases the number of erythrocytes and influences the miRNA profiles of human erythrocytes. A substantial increase in expression levels was especially noted for miRNA-144-5p and miRNA-30b-5p [47].

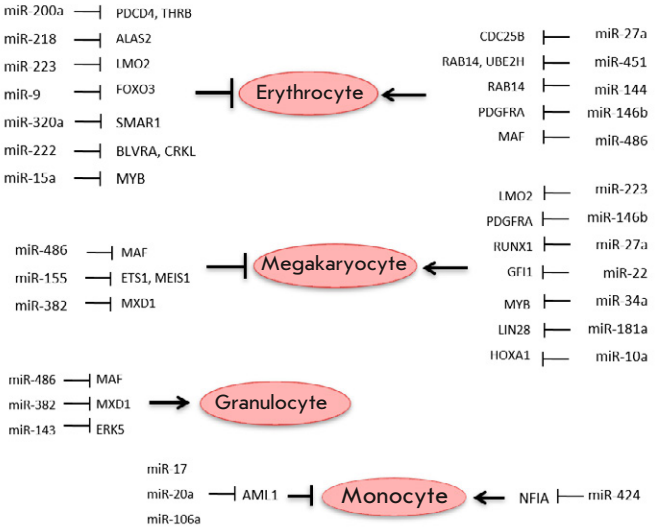

Fig. 4. MiRNAs and their target genes involved in the regulation of hematopoiesis

Megakaryocytopoiesis occurs in bone marrow (BM) and is a multi-stage process whose final stage is platelet formation [48]. As was noted above, miRNA-451 promotes erythropoiesis; however, the expression level of this miRNA is decreased during the differentiation of megakaryocytes, indicating the decisive role of this miRNA at the stage of megakaryocyte-erythrocyte progenitor differentiation [49]. MicroRNA-150 acts similarly: its increased expression levels promote megakaryocytopoiesis; and the decreased levels – erythropoiesis [50]. Analysis of literature data has shown that increased expression levels of miRNA-223, -27a, -22, -146b, -34a, and -181a promote – and increased expression levels of miRNA-155, -486-3p, and 382-5p inhibit – the differentiation of megakaryocytes [35, 46, 51–56]. In addition, the miRNA-10a expression is downregulated during megakaryocyte differentiation [57].

The formation of granulocytes and monocytes occurs because of the successive stages of differentiation, starting from CMPs. The morphological and functional abnormalities of granulocytes in part account for the bacterial infections in MDS. It is noted that miRNA-486-3p promotes granulocyte differentiation and suppresses macrophage differentiation [45]. Increased expression levels of miRNA-223 promote granulopoiesis and block monocyte-macrophage differentiation [37].

Table 1. Differential miRNA expression in MDS

Material sampled	miRs with increased expression	miRs with decreased expression	Ref.
BM	miR-21, miR-720	miR-671-5p, miR-BART13	[63]
BM/MNC	miR-17-3p, miR-17-5p, miR-21, miR-155, miR-18a, miR-126, miR-181a, miR-10a, miR-10b, miR-15a, miR-16, miR-222		[64]
PB	miR-17-3p, miR-17-5p, miR-21, miR-18a, miR-15a, miR-142-3p		[64]
BM	miR-299-3p, miR-299-5p, miR-323-3p, miR-329, miR-665, miR-370, miR-409-3p, miR-431, miR-432, miR-494, miR-654-5p	miR-196a, miR-423-5p, miR-525-5p, miR-507, miR-583, miR-940, miR-1284, miR-1305	[65]
BM	miR-194-5p, miR-320a		[66]
BM		miR-378	[67]
BM		miR-93-5p	[68]
BM/MNC		miR-124a, miR-155, miR-182, miR-200c, miR-342-5p, let-7a	[69]
BM	miR-99a-5p		[70]
BM	miR-4462	miR-30d-5p, miR-222-3p, miR-30a-3p	[71]
BM	miR-661		[72]
BM/MNC		miR-124	[73]
BM/MNC	miR-636	miR-103, miR-140, miR-150, miR-342, miR-378, miR-483, miR-632	[74]
BM	miR-21		[75]
BM/MNC	miR-222, miR-10a, miR-196a, miR-320, miR-100	miR-124, miR-206, miR-326, miR-197, miR-875-5p, miR-146a, miR-150, let-7e	[76]
BM	miRNA-550a-5p		[77]
BM	miRNA-210 and miRNA-155		[78]
BM	miRNA-10a and miRNA-10b		[79]
Plasma		miRNA-16 and let-7a	[80]
Plasma	miRNA-150-5p	miRNA-16-5p, miRNA-27a3p, miRNA-199a-5p, miRNA-451a	[81]
BM	miRNA-205-5p		[82]
Plasma/ vesicles	miRNA-10a-5p, miRNA-29a-3p, miRNA-34a-5p, miRNA-99b-5p, miRNA-125a-5p, miRNA-146b-5p and miRNA-150-3p/5p		[83]
Plasma	let-7a-3p, miRNA-21-3p, miRNA-221-3p, miRNA-221-3p/5p and miRNA-223-3p		[83]

BM – bone marrow, PB – peripheral blood, MNC – mononuclear cells.

In addition, increased expression levels of miRNA-143 and -382-5p are observed during granulocyte differentiation [56, 58]; increased miRNA-424 and decreased miRNA-17-5p, 20a, and 106a – during monocyte differentiation [59–61]. Rajasekhar *et al.* developed a miRNA profile of mature monocytes and granulocytes isolated from umbilical cord blood. These authors identified 46 miRNAs whose expression levels in both cell types were dissimilar to those of their CMPs. It is noteworthy that the miRNA-125b and miRNA-10a expression levels decreased 10- and 100-fold, respectively, in mature cells [62].

MicroRNAs IN MDS

MicroRNAs are among the regulators of normal hematopoiesis, and it is not surprising that changes in their expression levels contribute to hematologic neoplasm development. Over the past ten years, several large-scale studies of MDS-specific miRNA expression profiles (*Table 1*) have been published [63–83]. However, only part of the results obtained aligned, because different authors worked with samples that were prepared differently and of different quality, while they also used different methods of analysis and statistical data processing.

Table 2. Differential miRNA expression in karyotype-dependent MDS

Chromosomal aberration	miRs with increased expression	miRs with decreased expression	Ref.
del(5q)	miR-34a, miR-148a, miR-451, miR-486, miR-125a/b, miR-151, miR-199a, miR-10a/b, miR-29c, miR-130a, miR-24, miR-126, miR-335, miR-99b, miR-21, miR-17, miR-18a, miR-155	miR-128b, miR-95, miR-213, miR-520c, miR-146a, miR-449a, miR-300, miR-210, miR-193a-3p, miR-874, miR-589, miR-150, miRNA-143, miRNA-378, miR-145	[85]
monosomy 7/ del(7q)	miR-144, miR-451, miR-92a, miR-96, miR-340, miR-433, miR-105	miR-140-5p, miR-196b, miR-25, miR-590-3p, miR-511, miR-134	[85]
trisomy 8	miR-511, miR-146b, miR-134, miR-410, miR-153, miR-433, miR-105, miRNA-383	miR-10b, miR-452, miR-152, miR-181b, miR-28, miR-92, miR-10a, miR-324-3p, let-7a, miR-497, miR-24, miR-196b, miR-19a, miR-181c, miR-20a, miR-130b, miR-99a, miR-100, miR-515-3p, miR-199a	[85]
del(20q)	miR-206, miR-296-5p, miR-34b, miR-323-5p, miR-499-5p, miR-493, miR-503, miR-632, miR-98, miR-769-5p	miR-144, miR-451, miR-92a	[85]
monosomy 7/ del(7q)		miR-595	[86]
t(2;11)(p21;q23)	miRNA-125b-1		[87]
trisomy 1		miRNA-194-5p	[66]

Ozdogan *et al.* relate an interesting fact: in MDS, not only miRNA expression levels are changed, but also the expression of the *DICER1* gene, a key participant in the canonical miRNA processing pathway, is decreased [71]. In particular, Jang *et al.* conclude that increased expression levels of miRNA-205-5p promote MDS by suppressing *PTEN* and, thus, acting as an oncogene in hematopoietic cells. In addition, increased expression levels of miRNA-205-5p are not associated with a decrease in the overall survival rate or with a certain prognostic group of MDS patients. This indicates that miRNA-205-5p is involved in the initiation, but not in the progression, of MDS [82]. Li *et al.* suggest that increased expression levels of miRNA10a/b are associated with myeloblast population growth [79].

MicroRNAs detected in the blood are referred to as "circulating miRNAs." MicroRNAs are analyzed not only in the blood, but also in special structural elements named 'exosomes' that are nano-sized membrane vesicles that play an important role in the tumor microenvironment. It is noted that tumor cells release many more exosomes into the tumor microenvironment than normal cells do, leading to an increased level of exosomes in the circulatory system. The gene that promotes tumor growth can be transported by exosomes and promote metastasis. In particular, the miRNAs located in exosomes can contribute to oncogenesis [84]. Hrustincova *et al.* performed a unique comparative analysis of the expression levels of the miRNAs in total blood plasma and those of the miRNAs encapsulated in vesicles. They found that the populations of many hematopoiesis-associated miRNAs were substantially increased in MDS patients, mostly in both plasma and vesicles, although some miRNAs were unique to either plasma or vesicles. In addition, the expression levels of miRNA-103a-3p, -103b, -107, -221-3p, -221-5p, and miRNA-130b-5p were substantially decreased in the plasma of patients in a later stage of MDS compared to early-stage MDS patients. By contrast, the expression levels of miRNA-127-3p, -154-5p, -323b-3p, -382-3p, -409-5p, and miRNA-485-3p clustered in the chromosomal region 14q32 were increased at the early stage of MDS. The authors pointed out that certain profiles of miRNAs in plasma and vesicles appeared to represent two distinct biomarkers [83].

MicroRNAs AND GENETIC CHANGES IN MDS

It has been shown repeatedly that a karyotype change correlates with a unique profile of miRNA expression (*Table 2*).

Kuang *et al.* did their best to present as fully as possible the data obtained from the studies of correlations between miRNA expression levels and MDS-specific karyotypes [85]. Unbalanced chromosomal abnormalities are characteristic of MDS, and the most common are del(5q), monosomy 7 or del(7q), trisomy 8 and del(20q) [88]. Alkhatabi *et al.* showed that miRNA-595 expression levels are substantially decreased in MDS with -7/7q, as well as when a patient has a complex karyotype including chromosome 7 abnormalities [86]. Comparative analysis of miRNA expression levels in the presence of trisomy 1 demonstrated a decrease in the relative expression level of miRNA-194-5p in MDS patients with trisomy 1 compared to patients with the

normal karyotype [66]. Another work provides data on the role of miRNA-150 in the MDS developing in del(5q) individuals. It was pointed out that this miRNA targets a MYB transcription factor for suppression and that its suppression promotes proliferation inhibition [89]. Fang *et al.*, too, focus on the role of miRNAs in MDS developing in del(5q) individuals. It was demonstrated that MDS with this karyotype was characterized by an aberrant expression of more than 20 miRNAs, and most of them were located outside the deleted region 5q32 [90]. Analysis of the expression profile of 13 miRNAs located on 5q showed that the expression levels of miRNA-145 and miRNA-146a were substantially decreased in the BM cells of MDS patients with del(5q), as compared with the control group and patients with diploid karyotype [91]. However, Votavova *et al.* found that the expression levels of miRNA-378 and miRNA-146a were substantially decreased, and those of miRNA-34a were increased in del(5q) patients' BM cells, while the expression levels of miRNA-143 and miRNA-145 were somewhat increased [92].

Balanced chromosomal rearrangements in MDS patients are rare. One of the chromosomal translocations in MDS is t(2;11)(p21;q23). Increased expression levels of miRNA-125b-1 that is located close to the chromosome 11 breakpoint provide additional support to the idea that changes in miRNA expression profiles are associated with fragile sites [87].

Analysis of the expression levels of the miRNAs located on chromosome 8 showed that trisomy 8 results in an increase of miRNA-383 expression only. This result indicates that no increase in ploidy entails an increase in most of the miRNAs on this chromosome, confirming the complexity of the miRNAs-mediated regulatory mechanisms of MDS initiation [90].

Mutations are an integral part of the genetic changes leading to MDS; in particular, mutations to the *SF3B1*, *SRSF2,* and *U2AF1* genes involved in splicing are frequent in this disease [88]. It has been shown that the expression levels of let-7, miRNA-423, and miRNA-103a are decreased in MDS samples with mutations to these genes when compared with wild-type samples, suggesting the presence of complex molecular genetic cascades in MDS [93]. Analysis of the relationship between the presence of somatic mutations and the levels of circulating miRNAs in MDS demonstrated that the mutation to *Dnmt3a* was associated with changes in the expression levels of about 30 miRNAs in plasma and about 20 miRNAs in vesicles, and the presence of a mutation to *SF3B1*, with about 20 miRNAs expressing differentially in plasma and about 10 in vesicles, while only miRNA-100-5p and miRNA-450b-5 displayed unidirectional changes in expression levels, both in plasma and in vesicles [83].

MicroRNAs AND MDS THERAPY

The last decade has witnessed a breakthrough in MDS treatment. Three hypomethylating drugs have been approved therapeutically: azacitidine, decitabine, and lenalidomide. Nevertheless, it is still not always possible to achieve a proper response to the therapy [94]. A large number of works have been published seeking to analyze the correlations between miRNA expression levels and the response to the therapy offered in MDS. For example, analysis of miRNA-21 expression levels helps predict the response to hypomethylating agents and patients with low miRNA-21 expression levels in the serum had higher response rates [95].

Meng *et al.* noted that miRNA-124 expression levels are lower in MDS patients than in healthy donors, but that treatment with low doses of decitabine led to an increase in the expression in 7 out of 18 patients [73].

Analysis of miRNA expression levels in bone marrow before and during treatment with azacitidine showed that the response to the therapy was much better in patients with increased miRNA-17-3p and decreased miRNA-100-5p and miRNA-133b. Importantly, high expression levels of miRNA-100-5p at the beginning of the study were associated with a shorter overall survival rate. In addition, there was a decrease in the expression levels of miRNA-10b-5p, miRNA-15a-5p/b-5p, miRNA-24-3p, and miRNA-148b-3p in responders [100]. Another study noted that analysis of the expression profiles of miRNA-423-5p, -126-3p, -151a-3p, -125a-5p, and miRNA-199a-3p in MDS patients' plasma allowed one to predict their response to treatment with azacitidine [83].

Lenalidomide is an immunomodulatory and antiangiogenic drug used for treating del(5q) MDS. Interestingly, analysis of miRNA expression levels in bone marrow cells obtained from such patients showed that the miRNAs mapped to 14q32 were differentially expressing during treatment with lenalidomide [97]. It remains unknown whether the change in miRNA expression profiles is due to one of the actions of lenalidomide or simply a result of the abnormal clones' population decline. In another study, analysis of miRNA expression levels in peripheral blood monocytes demonstrated a decrease in miRNA-34a-3p and miRNA-34a-5p expression levels, and an increase in miRNA-378-3p and miRNA-378-5p following exposure to lenalidomide compared to the expression levels before therapy [98]. Venner *et al.* pointed out that there was an increase in miRNA-143 and miRNA-145 expression levels following exposure to lenalidomide, noting their role in the response to the therapy offered [99]. Naming lenalidomide's exact mechanism of action in MDS is important for oncohematology. However, there is not yet a diagnostic test that can predict the response to

Table 3. MicroRNAs associated with disease progression

miRNAs	Material sampled	Expression levels in MDS	Implications	Ref.
miRNA-422a, -617, -181a, -222, and miRNA-210	BM	increased	disease progression	[85]
miRNA-196b-5p	BM	increased	risk of transformation to AML	[101]
miRNA-29b	BM	decreased	risk of transformation to AML	[102]
miRNA-125a	BM	increased	overall survival decreased	[103]
let-7a	BM	increased	overall survival decreased	[85]
miRNA-194-5p	BM	decreased	overall survival decreased	[66]
miRNA-22	BM	increased	overall survival decreased	[28,104]
miRNA-661	BM	increased	overall survival decreased	[72]
miRNA-126 and miRNA-155	BM	increased	overall survival decreased or no disease progression	[69]
miRNA-124a	BM	increased	overall survival decreased	[69]
miRNA-223-3p	plasma	decreased	overall survival decreased	[81]
miRNA-451	plasma	decreased	overall survival decreased without disease progression	[81]

BM – bone marrow.

this drug. Thus, miRNAs are promising candidates to predict lenalidomide response.

MicroRNAs AND MDS PROGNOSIS

The prognosis of the disease is assessed using the Revised International Prognostic Scoring System (IPSS-R), which divides patients into five risk subgroups, taking into account the karyotype, the number of blast cells, and the degrees of deficiency of various blood cell types. In particular, this prognostic system allows one to assess the overall survival rate and the risk of transformation to AML in MDS patients [100]. However, this system does not fully reflect the genetic complexity of this disease. In addition to the recognized predictors, miRNAs are some of the promising markers for making predictions of the outcome in MDS. MDS is regarded as a disease preceding leukemia, and about 30% of MDS patients eventually develop AML [11]. Analysis of literature data has shown that miRNA expression profiles differ between early and advanced stages of MDS, confirming the involvement of miRNAs in the pathogenesis of MDS and, consequently, in MDS-to-AML transformation (*Table 3*).

Kuang *et al.* conducted an overview of data on the correlation of miRNA expression levels depending on the degree of disease progression. In particular, increased expression levels of miRNA-422a, -617, -181a, and miRNA-210 were associated with disease progression; increased expression levels of miRNA-17-5p, mRNA-20a, and miRNA-34a – with low-risk MDS [85]. In later studies, the understanding of the contribution of miRNAs to MDS was expanded. Wen *et al.* demonstrated that miRNA-196b-5p expression levels were increased in higher-risk MDS patients and in their peers who developed AML and proposed this miRNA as a biomarker associated with the risk of MDS transforming into leukemia [101]. In addition, Kirimura *et al.* emphasized that a decrease in miRNA-29b expression levels promoted the transformation of MDS into AML as well [102]. Choi *et al.* found that, according to IPSS, the expression levels of miRNA-21, miRNA-146b-5p, miRNA-126, and miRNA-155 were substantially higher in high-risk than in low-risk patients. Moreover, high expression levels of miRNA-126 and miRNA-155 correlated with a substantially lower overall survival rate and survival without transformation into leukemia, suggesting that these miRNAs could be associated with MDS progression and transformation to AML [69]. Alkhatab *et al.* determined that the expression level of miRNA-595 was lower in high-risk MDS; however, they emphasized that the data obtained required further research to involve a larger cohort of patients. In addition, this miRNA directly targeted the *RPL27A* gene and its downregulation disrupted erythropoiesis [88]. As was noted, miRNA-125a also contributed to impaired erythropoiesis, its expression was increased in MDS and negatively correlated with the overall survival rate of patients [103].

Some studies note correlations between miRNA expression levels and the chance of survival for MDS patients. In particular, decreased expression levels of miRNA-181 and miRNA-21 correlate with longer overall survival and increased expression levels of let-7a correlate with shorter patient survival [85]. In addition,

low expression levels of miRNA-194-5p correlate with a decrease in the overall conditions of MDS patients [66].

Not only MDS patients' BM cells, but also blood can be used as a source material, allowing one to identify miRNAs as readily available markers for the analysis of the development and prognosis of the disease. Analysis of circulating miRNAs in the plasma of MDS patients showed that the expression levels of miRNA-27a-3p, -150-5p, -199a-5p, -223-3p, and miRNA-451a were decreased in higher-risk MDS individuals. In addition, low expression levels of miRNA-451 were associated with decreased, progression-free survival rate; low expression levels of miRNA-223-3p, with a substantial decrease in the overall survival rate [81]. Zuo *et al.* presented a diagnostic panel including miRNA-144, -16, -25, -451, -651, -655, and let-7a, enabling one to select normal-karyotype patients based on the prognosis of survival [105].

Besides *de novo* MDS, cases of secondary MDS following treatment with cytostatic agents and characterized by a number of genetic changes identical to those in primary MDS are known in clinical practice. However, IPSS-R is focused only on primary MDSs. Secondary MDSs are heterogeneous as well and require the same careful classification into risk groups as primary MDSs; therefore, the search for prognostic markers in secondary MDSs, along with *de novo* MDSs, is an important line in hematology-oncology [88]. Very few analyses of miRNA expression levels in secondary MDS have been published. In particular, Le *et al.* showed that the expression levels of miRNA-99a-5p were higher in high-risk MDS patients and in patients with secondary MDS than in low-risk peers [70].

MUTATIONS IN miRNA GENES AND MDS

Changes in miRNA expression levels and, as a consequence, the development of neoplasms are associated not only with mutations in protein-coding genes but also directly with mutations in miRNA genes. Thus, a study identified seven MDS patients with mutations in miRNA genes. All mutations were heterozygous, and most of them were located in the seed region of the gene encoding miRNA-142-3p. Mutations in the seed region lead to a gain of new target genes as well as to a loss of the target genes specific to this miRNA. Therefore, a conclusion can be made that the mutations affecting the seed region reduce the target specificity and provoke leucosis [106].

Similar to protein-coding genes, miRNA-encoding genes are regulated post-transcriptionally and this represents yet another mechanism for regulating miRNA expression levels, alongside mutations. In particular, the transcription factor TWIST-1 promotes an increase in miRNA-10a/b expression by binding directly to the promoters of the genes encoding these miRNAs and, thus, promoting the initiation of MDS [79]. Another example is miRNA-34a regulation by a transcription factor encoded by the tumor suppressor gene *p53*; in addition, it is noted that the expression levels of this miRNA are increased in patients with early-stage MDS [85].

Hypermethylation of miRNA promoters is another factor of MDS progression. In particular, the miRNA-34b gene promoter was found hypermethylated in MDS patients and this status had probably been acquired during progression to AML [85]. In addition, hypomethylation of the let-7a-3 and miRNA-124-3 gene promoters correlates with a poor survival chance of MDS patients and a poor prognosis of the disease [107, 108].

CONCLUSION

Molecular genetic markers are gradually becoming more and more popular in describing MDS; in addition, they discriminate MDS from other BM conditions [109–113]. As described above, unique miRNA expression profiles are characteristic of different MDS subtypes. Nevertheless, further research is needed to understand the complex regulatory mechanisms operating between miRNAs and their target genes in MDS. Most of the works are based on the analysis of miRNA expression levels in BM or peripheral blood. Unlike mRNAs, miRNAs are highly stable, allowing the BM material embedded in paraffin or fixed on coverslips to be accessible to the analysis.

It should be admitted that a universal prognostic scoring system covering all important MDS parameters has yet to be developed. Therefore, an important task before clinical oncology is to search for additional molecular-genetic markers that can be integrated into the existing international prognostic systems, and some of the most promising candidates for that role are miRNAs. Current data on the roles of miRNAs in MDS suggest that these molecules have the potential to become tools for the diagnosis and prognosis of MDS and may be relevant to the response to treatment.

In addition to using miRNAs in diagnostic and prognostic tasks, one of the promising avenues in scientific research is trying them out as therapeutic targets. MicroRNA-mimics-34 encapsulated in lipid nanoparticles is the most studied potential therapeutic agent for the treatment of lung cancer. In addition, several preclinical studies have explored an antitumor strategy based on the suppression of oncomiRNAs with the use of antisense oligonucleotides (anti-miRNAs). In MDS, a chemically modified inhibitor of miRNA-21 promotes normal erythropoiesis and increases hematocrit [114].

In conclusion, cases of secondary MDS associated with morphological BM cell abnormalities and imbalance between different blood cell types, similar to those in primary MDS, are known in practice. It is possible that secondary MDS develops after cytostatic therapy or in patients with autoimmune diseases, solid tumor neoplasms, some infections, and other pathologies accompanied by secondary hematopoietic conditions. Of special interest are MDS in untreated patients with malignant lymphomas. An important discovery is the fact that NHML patients with signs of erythroid lineage dysplasia develop anemia about twice as often as their peers with morphologically normal BM cells, and collectively these facts are associated with rapid tumor progression, a low three-year survival rate, and resistance to the treatment offered [115].

Thus, the search for additional prognostic markers for the diagnosis of both *de novo* MDS and secondary lesions to BM will allow us to develop personalized treatment that is as precise as possible. ●

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Photoluminescent Nanomaterials for Medical Biotechnology

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ABSTRACT **Creation of various photoluminescent nanomaterials has significantly expanded the arsenal of approaches used in modern biomedicine. Their unique photophysical properties can significantly improve the sensitivity and specificity of diagnostic methods, increase therapy effectiveness, and make a theranostic approach to treatment possible through the application of nanoparticle conjugates with functional macromolecules. The most widely used nanomaterials to date are semiconductor quantum dots; gold nanoclusters; carbon dots; nanodiamonds; semiconductor porous silicon; and up-conversion nanoparticles. This paper considers the promising groups of photoluminescent nanomaterials that can be used in medical biotechnology: in particular, for devising agents for optical diagnostic methods, sensorics, and various types of therapy.**
KEYWORDS **photoluminescent nanomaterials, biotechnological application, optical diagnostics and therapy, chemical sensors, quantum dots, gold clusters, carbon dots, nanodiamonds, porous silicon, up-conversion nanoparticles**

INTRODUCTION

In recent decades, there has been a qualitative shift in medicine towards more precise and personalized treatment through a combination of early diagnosis, therapy, and subsequent monitoring of the course of a disease. This approach is called theranostics. Nanotechnology – in combination with optical, acoustical and other methods of non-invasive application – occupies a dominant niche in this area. Nanoparticles are able to successfully combine several functions thanks to their unique properties, such as the programmability of physical and chemical characteristics, presence of reactive functional groups, a large surface to volume ratio, and optimal size. These features allow nanoparticles to act not only as independent therapeutic and/or contrast agents and delivery vehicles, but also as a platform for creating multifunctional complexes. In this context, optically active nanoparticles open up wide possibilities for the visualization of target cells or subcellular structures, in combination with a simultaneous targeted therapeutic effect.

One of the groups of nanomaterials used in optical diagnostics methods, sensorics, and therapy is the plasmon-resonance particles of gold, silver, and other metals. They have been proposed as a basis for a number of sensors for the qualitative and quantitative determination of various chemical compounds and biological macromolecules, as well as agents for visualizing and affecting target cells [1–3]. However, the overwhelming majority of the solutions rely upon the use of photoluminescent nanomaterials (PLNMs). Depending on their chemical structure, shape and size, the properties of such materials differ significantly, making them a suitable means to solving a wide range of practical problems. Nowadays, the most widely used in biomedical research are quantum dots, small gold clusters, carbon dots, nanodiamonds, semiconductor porous silicon, and up-conversion nanoparticles.

In this paper, we considered the PLNM groups that are of interest as a basis for devising agents for medical biotechnology: in particular, for optical diagnostic methods, sensorics, and various types of therapy.

Quantum dots

Quantum dots (QDs) are the most thoroughly studied PLNMs [4–6]. They are inorganic nanocrystals usual-

ly consisting of elements of the II and VI or III and V groups and measuring in size between 2 and 10 nm. Most often, QDs are synthesized from such compounds as CdSe, CdS, CdTe, InAs, and GaAs with semiconductor properties in their bulk state. QDs possess photoluminescence (PL), with a quantum yield greater than 50% and a narrow symmetrical peak emission, whose position is determined by the particle size and composition (*Fig. 1*) [7, 8].

The PL properties of QDs are determined by the discrete energy levels that occur due to the restricted free motion of charge carriers (electrons and holes). Upon absorption of a quantum of exciting radiation, an electron enters a conduction zone with an excited state lasting from a few to tens of nanoseconds. A photon is emitted as a result of the radiative recombination of an electron-hole pair, and the photon energy corresponds to the difference between the highest hole and the lowest electron levels. Smaller particles have a larger energy difference between the corresponding levels, resulting in a higher energy of emitted photons and shorter wavelength.

In biomedicine, as a rule, QDs of improved structure are used, most often of improved core /shell structure, where the last forms from compounds with a similar crystal structure featuring the properties of wider-gap semiconductors [13–15]. CdSe/ZnS QDs are used more often than others: they exhibit PL in the entire visible region of the spectrum depending on their particle size. The shell provides an increased PL quantum yield, contributes to QD surface stabilization, and prevents heavy metal ions from entering the environment, thereby reducing the toxic effect of such QDs relative to QDs without a shell [16, 17].

During synthesis, the surface of semiconductor QDs is covered with hydrophobic compounds, making them practically insoluble in water. To achieve colloidal stability and biocompatibility, the surface can be modified in various ways. One approach involves substituting hydrophobic surface ligands for hydrophilic ones or coating with amphiphilic compounds [18]. An alternative solution is to create an additional outer shell of either organic polymers [19, 20] or inorganic compounds (silicon oxide) [21]. The obtained QDs lack colloidal stability, which limits the scope of their poential use in biomedical applications. Various approaches to solving this problem have been described: however, a reliable and reproducible protocol has not yet been developed [22].

QDs have several useful photophysical properties, such as their high PL quantum yield and extinction coefficient, which enable one to visualize single nanoparticles; a wide absorption range and narrow symmetric PL emission peaks, making them useful in multiplex analysis [23]; long-term photostability, which allows for long-term tracking of individual molecules; and their wide multiphoton excitation range, which favourably distinguishes QDs from organic fluorophores [24]. In addition to the above-listed properties, many approaches to the surface functionalization of QDs and the attachment of various targeting/toxic modules specific to target molecules have been developed to date, enabling one to produce multifunctional complexes with the desired set of properties [25–27].

Fluorescence imaging of cells, tissues, and organs is the main area of QD application (*Fig. 1*). With the more than 20 years it has been in use, QD imaging of cellular

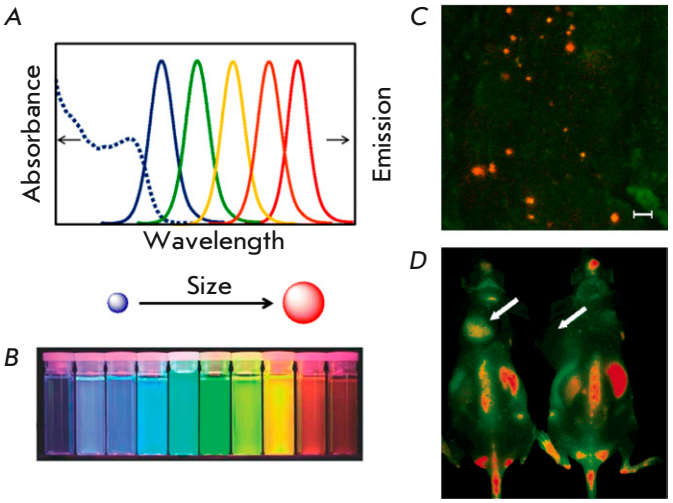


Fig. 1. (*A*) – Size-dependence of the CdSe/ZnS QD fluorescence emission spectrum. Adapted from [9] with permission from the copyright holder: © 2017 by the authors. Licensee MDPI, Basel. (*B*) – Fluorescence photograph of QD suspensions irradiated with ultraviolet light (emission maxima at 443, 473, 481, 500, 518, 543, 565, 587, 610, and 655 nm). Adapted from [10] with permission from the copyright holder: John Wiley and Sons. © 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (*C*) – Visualization of targeted QD conjugates (QD-4D5scFv) in a xenograft SK-BR-3 tumor. The image was obtained by confocal fluorescence microscopy. Scale bar 10 μm. Adapted from [11] with permission from the copyright holder. © 2019 by the authors. Licensee MDPI, Basel. (*D*) – Intravital visualization of the distribution of targeted QD conjugates (QD705-RGD) in the body of a mouse carrying the U87MG xenograft tumor (indicated by an arrow). Mouse tissue autofluorescence is shown in green; QD fluorescent signal – in red. Adapted from [12] with permission from the copyright holder. © 2006 American Chemical Society

structures has become the standard approach. Specificity in staining certain cell components is achieved by using such targeting molecules as antibodies, peptides, nucleic acid fragments, and others. External modules are attached to particles by either chemical conjugation [28, 29], or by self-assembly, when illumina-specific adapters are added to streptavidin-biotin beads [30, 31], or by barnase-barstar [32–35]. Such targeted complexes are actively used in optical microscopy, cell flow cytometry [36, 37], and immunohistochemical [38, 39] and enzyme immunoassay [40, 41].

A number of photophysical properties make QDs indispensable in the cases where organic fluorophores are of little use. In particular, QDs photostability enables one to study molecular dynamics: several studies have been performed to track receptors [42–44], integrins [45, 46], transport proteins [47], and membrane lipids [48].

One of the downsides of QDs is the intermittent nature of their PL (blinking) that occurs when one or both components of an exciton (electron and hole) hit the surface of the particle, which leads to the appearance of a charge on the particle and quenching of the PL as a result of nonradiative recombination [49]. In order to overcome this drawback, several methods have been designed that provide complete or partial blinking suppression [50, 51].

QDs are used to create sensors capable of assessing the quantitative content of various compounds in a medium. For this purpose, the changes in the emission characteristics (peak positions, intensity, polarization, kinetic parameters) associated with the attachment of target molecules to a QD surface are exploited [52–56]. Many sensors using QDs as one of the participants in the Förster resonance energy transfer (FRET) pair have already been developed. Such systems are being successfully used to study the interaction between a ligand and a receptor, the specific detection of DNA sequences, and the detection of changes in protein molecule conformation [57– 59].

Sensors are being actively developed that combine these approaches and are designed for a wide range of tasks such as detecting viruses and bacteria, determining the activity of enzymes and the presence of small organic molecules and various ions, and pH measuring [60–62].

The wide choice of synthesized QD components makes it possible to obtain particles with a PL emission in the near-IR region falling into the transparency window of biological tissue and to minimize light absorption and scattering [63]. The emission peaks of such particles remain narrow and symmetrical; and the QD size – within a few nanometers. Such characteristics enable one to actively use QD-based agents for noninvasive *in vivo* imaging of cells, tissues, and organs. Several studies have reported on the successful delivery of QD-based agents to tumor cells of various origins and to endotheliocytes of tumor vessels [12, 35, 64–66]. QDs emitting in the near-infrared region can effectively mark primary tumors and can be used to search for metastases [67–69], map lymph nodes [70, 71], study the vasculature [72], and track target tumor cells [73, 74].

QD complexes have an obvious therapeutic potential, particularly, in photodynamic therapy. When energy is transferred from QDs through organic dyes (FRET technology) or directly to an oxygen molecule, a pronounced photosensitizing effect can be observed [75, 76]. Finally, QDs can be used to monitor the efficiency of drug [77, 78] and nucleic acids delivery [79, 80]. Their use in clinical practice is constrained by their undesirable toxic effects associated with the presence of heavy metal ions and other hazardous substances (Cd, Pb, As, Te, Se) in their composition. The dynamics of their release into the surrounding environment mainly depends on their polymer coating. For instance, a biodegradable coating results in a significant release of components and obvious toxic effects [81–83]. A stronger polymer shell minimizes the side effects but greatly increases their retention time in the kidneys and spleen [84–86]. These features can significantly increase the risk of toxicity in clinical use. The way to overcome the described limitations, apparently, lies in the search for and design of PL agents that have a different chemical composition.

Small gold clusters

Small gold clusters consisting of 2–100 atoms differ significantly in their properties from larger gold nanoparticles with a size of several nanometers or more. Gold clusters have intense fluorescence with a significant Stokes shift; a long-excited state lifetime; a high quantum yield; as well as photostability and biocompatibility. Their PL is determined by the transition of electrons between discrete molecular energy levels. The size and composition of the clusters determine the position of the PL emission peaks in a range from UV to IR (*Fig. 2A,B*) [87, 88].

Small gold clusters are used both as imaging agents – in particular for tracking cell differentiation and movement – and as highly sensitive fluorescent probes (*Fig. 2C,D*) [89–91]. The attachment of targeting molecules of various kinds (proteins, peptides, polymers, or small molecules) makes it possible to obtain conjugates of small gold clusters with targeted properties [92–95].

As imaging agents, gold clusters allow one to achieve high image clarity and localization accuracy [96, 97].

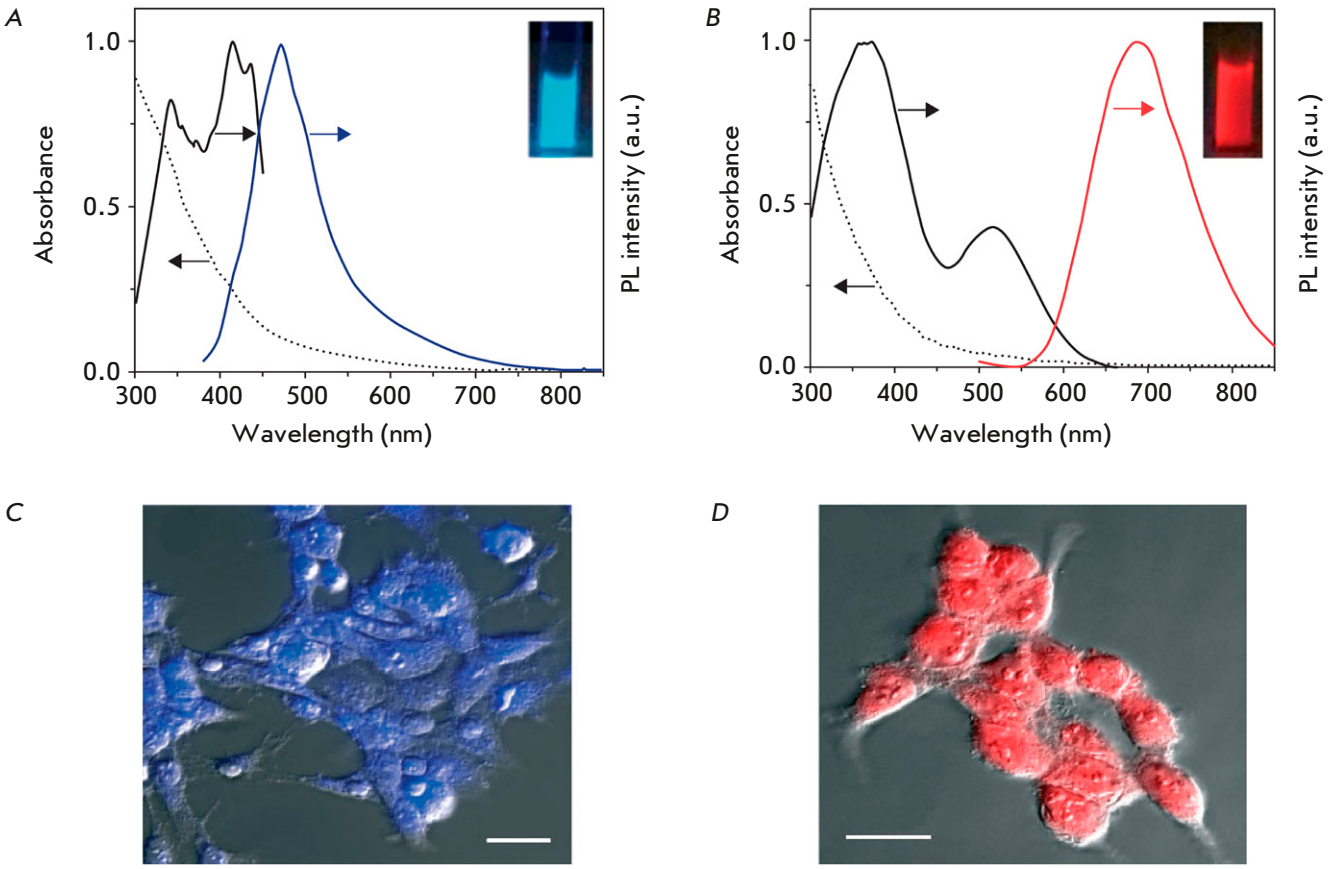


Fig. 2. (*A*), (*B*) – Absorbance (dotted line), excitation (black), and PL emission (color) spectrum of gold nanoclusters. Insets – fluorescent photographs of gold nanocluster suspensions. (*C*), (*D*) – Visualization of HEK293 cells using gold nanoclusters emitting in the blue region of the spectrum (*C*) and gold nanoclusters coated with bovine serum albumin, emitting in the red region of the spectrum (*D*). Superposition of the transmitted light image and the PL signal of gold nanoclusters. Scale bar 50 μm. Adapted from [88] with permission from the copyright holder: John Wiley and Sons. © 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Gold clusters coated with bovine serum albumin enable one to quickly and efficiently visualize tumor cells and whole tumors [98]. After entering the cells, small gold clusters are capable of emitting fluorescence for a long time (up to 28 days *in vitro*). Compared to QDs, they have lower cytotoxicity and insignificantly affect cell viability at comparable doses [99]. Their optical properties have made them an effective tool for such analytical methods as biomacromolecules detection [100] and tracking of drug distribution, as well as accumulation *in vivo* and *in vitro* [101].

Another interesting feature of small gold clusters is electroluminescence. So, they are widely used in the development of sensors [102]; in particular, for DNA and microRNA detection. One such development is a proposed biosensor for the detection of peroxidase genes using fluorescent gold clusters as a label [103].

Small gold clusters can also be used for a targeted delivery of the drugs attached to their surface. Effective delivery and controlled release of anticancer drugs (doxorubicin, cisplatin, captopril, and 6-mercaptopurine) using gold clusters encapsulated in dendrimers has been demonstrated in [104]. Gold clusters can also be used in gene therapy, providing systemic gene delivery and the visualizing of intracellular transport. As vectors, they favourably distinguish themselves by their low cytotoxicity, good photostability, and lack of an immune response [105].

Another interesting property of gold clusters is their radiosensitizing ability thanks to a high ionizing radiation absorption coefficient that is significantly higher

than that of organic molecules [106, 107]. Being able to increase the radiosensitivity of tumor cells *in vivo* enables the clusters to increase the therapeutic efficacy of radiation therapy by locally increasing the Au concentration in the tumor [108].

The use of fluorescent gold clusters as contrast agents is "hindered" by a broad peak of PL emission, which makes it difficult to use several agents simultaneously [88]. Also, the problem related to the safety of nanomaterials made of gold and other noble metals remains unresolved. There is evidence that small gold clusters cause oxidative stress; disruption of the mitochondrial function; have a negative effect on nucleic acids, as well as on the level of proinflammatory cytokines; induce liver destruction, etc. [3, 109, 110]. On the other hand, the variety of structures and compositions of the agents based on small gold clusters used in these studies prevent us from drawing any definitive conclusion regarding the specific reasons behind these negative consequences.

Carbon dots

Carbon dots (C-dots) are clusters of carbon atoms 2–8 nm in size with photoluminescent properties. They contain a significant amount of hydrogen and oxygen atoms, as well as traceable amounts of nitrogen, and can be of either amorphous (carbon in sp2- and sp3-hybridization) or graphene structure (sp2-hybridized atoms) [111, 112]. The advantages of C-dots are their photostability, wide surface modification capabilities, and low production cost, since they can be obtained using chemical treatment from the soot of many carbon-containing materials, including those of plant origin. [113–115].

C-dots are characterized by a bright PL in a range of 300–500 nm determined by the defects in the particle surface, exciton recombination, and quantum-size effects (*Fig. 3A,B*). The absence of toxicity allows us to count on the widespread use of carbon dots in biomedicine, as has been indicated in many studies [114, 116, 117].

Carbon dots are effectively used as fluorophores in the development of sensors, in particular, to determine the metal ion content. Adding selective ligands makes it possible to create sensors for the Ag^{+}, Al^{3+}, Zn^{2+}, Hg^{2+}, and Cu^{2+} ions [120–124]. Connecting carbon dots (PL in the blue region) with quantum dots (PL in the red region) and coating with bovine serum albumin has given us a ratiometric sensor for the supersensitive determination of copper ions [125]. C-dots are successfully used to create highly sensitive systems for the immunofluorescence and enzyme immunoassay of various antigens [126, 127]. Thanks to the FRET technology, a pH-sensitive probe based on C-dots and a pH-sensitive dye (FITC) acting as an acceptor has been developed [128, 129]. The possibility to use C-dots in ratiometric complexes for assessing intracellular temperature has been demonstrated. Complexes of two types of carbon clusters differing in their PL emission spectrum that are thermosensitive in a range from 15 to 90°C and stable at pH values ranging from 4 to 9 and can be used for cell temperature mapping [130].

Apart from sensoring, C-dots are also used as drug carriers. Particularly, conjugates with the antitumor drug oxaliplatin have been obtained by covalent attachment to their modified surface [131]. An alternative drug delivery system is conjugates of C-dots and gold nanorods having pH-sensitive bonds. Such conjugates demonstrate an active release of bound doxorubicin upon changes in pH and exposure to radiation. The functionalization of such conjugates with folic acid has made it possible to create a theranostic complex suitable both for efficient visualization of tumor cells and targeted drug delivery with controlled release [132]. The targeting action of folic acid makes it possible to detect even single tumor cells [133].

Another theranostic application of C-dots has been complexes including organosilica nanospheres. These spheres are mesoporous, and as so they can include anticancer drugs in their composition, making the complexes capable of pH-dependent drug release (doxorubicin) and photothermal activity upon irradiation in the near-IR range [134].

Being non-toxic and biocompatible, C-dots open up prospects for use as an alternative to semiconductor quantum dots: however, their photophysical properties need to be modified to shift the PL emission maxima to the near-IR range [135, 136].

Nanodiamonds

Another type of carbon nanomaterials, nanodiamonds (NDs), has similar photoluminescent properties [137, 138]. NDs are composed of carbon sp3 hybridization atoms assembled into a crystal lattice of cubic syngony. The defects in the lattice structure that form localized excited states upon absorption of light quanta in the visible range cause NDs to be photoluminescent [139] (*Fig. 4A*). For this purpose, nanodiamond particles are doped with nitrogen atoms that form local defects of various types during synthesis [140] and the position of the emission maxima and their intensity are determined by the types of defects and the total amount of nitrogen doped. In particular, negatively charged nitrogen vacancies (NV–) cause a PL that is located in the 650–700 nm region, which is most preferable for bioimaging [141–144].

NDs are currently being considered as a promising system for targeted drug delivery characterized by

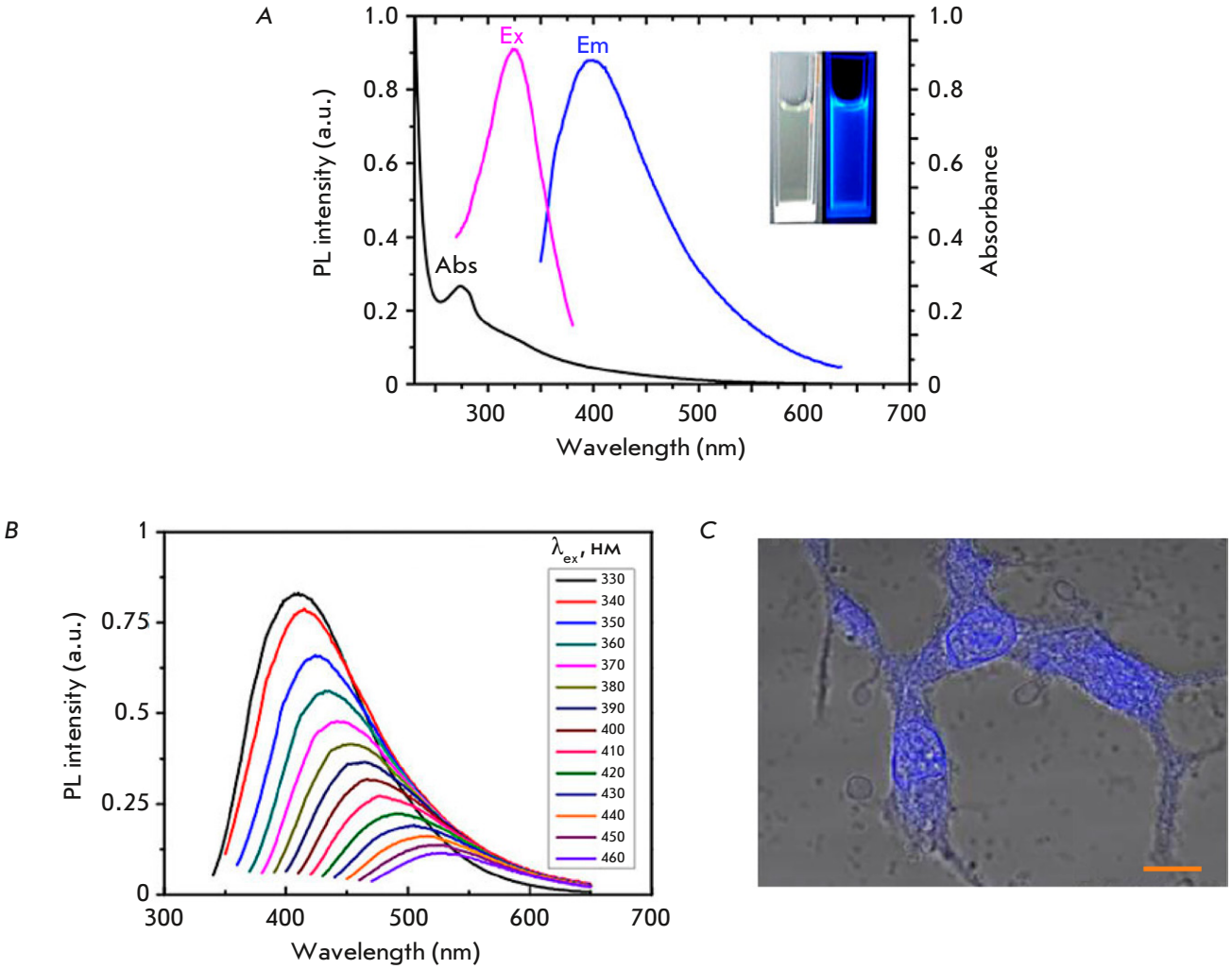


Fig. 3. (*A*) – Spectrum of absorbance, excitation and PL emission of C-dots. Inset – brightfield and fluorescent photographs of suspensions of carbon dots. (*B*) – PL emission spectrum of C-dots upon excitation by light with different wavelengths. Adapted from [118] with permission from the copyright holder: © 2019 The Authors, Royal Society Publishing. (*C*) – Visualization of 293T cells using nitrogen-doped C-dots. Superimposed image in transmitted light and PL signal of carbon dots. Scale bar 10 μm. Adapted from [119] with permission from the copyright holder: Dove Medical Press Ltd. © 2016 Informa PLC, London

high delivery efficiency and low toxicity [148–150]. There are many potential biological and medical ND applications, including use in biocompatible composites and implants, targeted drug delivery, biosensor components, and as stable solid carriers for peptide synthesis (*Fig. 4B,C*). ND-based imaging and therapy helps in early diagnosis, treatment, and effective prevention of several diseases. The imaging methods make it possible to effectively determine the stage of a disease, carry out non-invasive monitoring of the effectiveness of treatment, and, as emphasized, predict the duration and degree of remission [151].

Zurbuchen *et al.* have demonstrated the subcellular multimodal imaging technique (using optical and electron microscopy) to facilitate the localization of NDs having fluorescent NV-centers. Thanks to their PL properties, the possibility of their use as agents for diagnosing nervous system diseases has been shown [152].

Having a large surface area, NDs are well suited for drug loading and functionalization. For instance, Huang *et al.* have demonstrated effective attachment of doxorubicin to nanodiamonds, with its subsequent release. It has been found that this compound is less toxic to

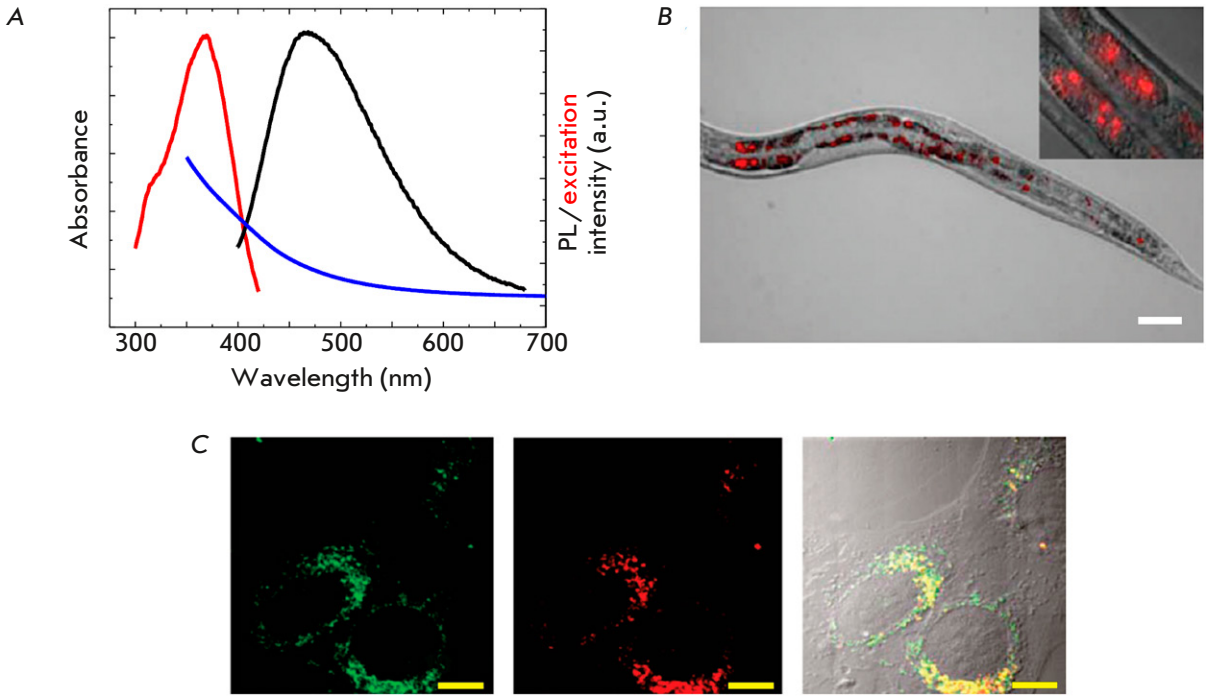


Fig. 4. (*A*) – Absorbance (blue), excitation (emission at 490 nm), and PL emission spectrum of nanodiamonds. Adapted from [145] with permission from the copyright holder: IOP Publishing. © Copyright 2020 IOP Publishing, Bristol. (*B*) – Visualization of the intestines of the free-living worms *Caenorhabditis elegans* using nanodiamonds coated with bovine serum albumin. Inset – an enlarged view of intestinal cells containing nanodiamonds. The overlay images were obtained by the method of differential interference contrast and epiluminescent images in a range above 600 nm, with excitation in a range of 510–560 nm. Scale bar 50 μm. Adapted from [146] with permission from the copyright holder: American Chemical Society. © Copyright (2010) American Chemical Society. (*C*) – Visualization of HeLa cells using nanodiamonds. From left to right: confocal fluorescence image of LysoTracker-stained liposomes obtained in the range of 500–530 nm; confocal fluorescent image of nanodiamonds obtained in the range of 600–750 nm; overlay images. Scale bar 10 μm. Adapted from [147] with permission from the copyright holder: American Chemical Society. © Copyright (2009) American Chemical Society

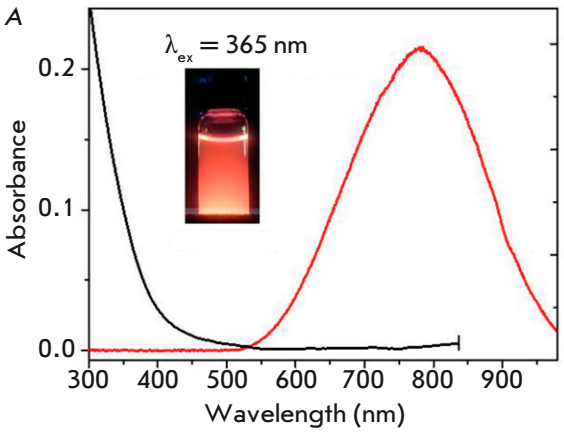
normal cells and exhibits a higher activity against human colorectal cancer cells than free doxorubicin. The prolonged release ensures the required drug concentration at a lower administered dose [153]. It has been shown that clusters of nanodiamonds are able to enclose the drugs being delivered to isolate the delivered agent from healthy cells, allowing for most of the administered dose to reach the target area, increasing the healing effect [154].

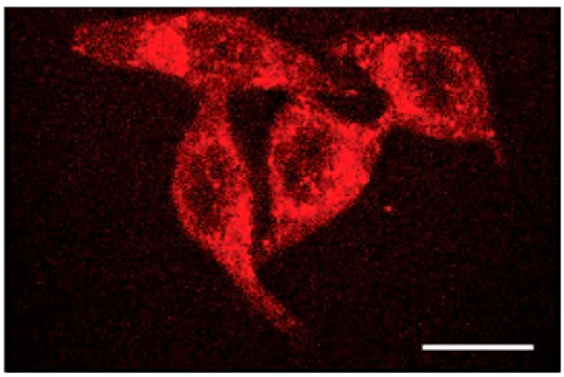
Nanodiamonds are also considered as a promising tool for gene delivery in order to significantly increase gene therapy effectiveness. For instance, efficient delivery and subsequent expression of the green fluorescent protein gene has been demonstrated with spiky NDs as a carrier [155]. Another interesting direction in this respect is regenerative tissue engineering. Yang *et al.* have developed a polymer-based nanocomposite frame containing NDs to support the growth and differentiation of osteoblasts, as well as their enhanced biomineralization to stimulate bone formation *in vitro* [156].

Despite the obvious advantages of nanodiamonds, their practical application is limited by the laboriousness associated to their synthesis. They also require a solution to the aggregation problem and correction of their PL properties.

Semiconductor porous silicon nanoparticles

The fluorescent properties of semiconductor porous silicon nanoparticles (PSiNPs), like those of QDs, depend on quantum-size effects. These particles are biocompatible, biodegradable, and have low toxicity [157, 158]. The position of their PL emission maxima in the visible or near-IR region (*Fig. 5*) depends on the particle size





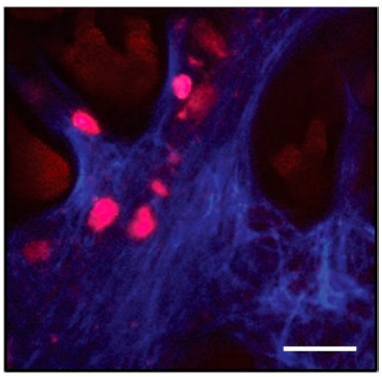


Fig. 5. (*A*) – Spectrum of absorbance and PL emission of porous silicon nanoparticles upon excitation by 365-nm light. Inset – photograph of a colloidal solution of porous silicon nanoparticles irradiated with 365-nm light. (*B*) – HeLa cells labeled with targeted conjugates based on porous silicon nanoparticles. The image was obtained by two-photon microscopy at an exciting radiation power of 10 mW. Scale bar 15 μm. (*C*) – *In vivo* image of a xenograft HeLa tumor after injection of targeted conjugates based on porous silicon nanoparticles, obtained by two-photon microscopy. Scale bar 75 μm. Adapted from [162] with permission from the copyright holder: John Wiley and Sons. © 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

and modification of their surface [138, 159, 160]. Large silicon particles that are not direct-gap semiconductors have a very low PL yield. On the contrary, particles up to 5 nm in diameter exhibit the properties of direct-gap semiconductors and bright PL, which, nonetheless, does not reach that of QDs [161].

The large content of silicon in the Earth's crust significantly reduces the cost of synthesizing silicon nanoparticles, in comparison to other inorganic nanomaterials. PSiNPs have been used to create effective sensors to measure the pH level; the concentration of heavy metals, carbohydrates, pesticides, antibiotics, and other compounds [163–165]. Long-term monitoring of PSiNPs biodistribution in living organisms is possible thanks to their PL emission in the near-IR range [158]. The attachment of a protein or other targeting modules to PSiNPs makes it possible to obtain nanocomplexes both for specific visualization of cells and subcellular structures, as well as for whole-body imaging (*Fig. 5*) [162, 166–168].

PSiNPs have been successfully used for the delivery and controlled pH-dependent release of drugs; in particular, doxorubicin [169]. They are capable of inducing a photothermal effect; in particular, heating a tumor tissue to 60°C when irradiated with a 1064 -nm laser beam to induce apoptosis and angiogenesis suppression *in vivo* [170]. Their porous structure makes them easy for drug-loading, e.g., by the capillary method when it is enough to immerse a particle in a concentrated solution of a drug [171, 172]. The PSiNPs surface in most cases has a negative surface charge, enabling absorption of positively charged molecules, such as immunoglobulin-binding protein A [173]. It is the absorption principle that makes controlled delivery of small protein molecules possible. However, due to weak drug/particle interactions, PSiNPs provide for only rapid unloading, as opposed to long unloading periods when a drug is covalently bound to the carrier [174]. On the other hand, the hydroxylated pore surface makes it possible to covalently load drugs, particularly, doxorubicin, with its subsequent release [175]. Binding drugs to porous silicon particles improves their solubility [176–178], increases their biostability [179], as well as the ability of drugs to penetrate the body's biological barriers.

Among the limitations associated with the use of PSiNPs, it is worth noting the problem of achieving bright PL in the transparency window of a biological

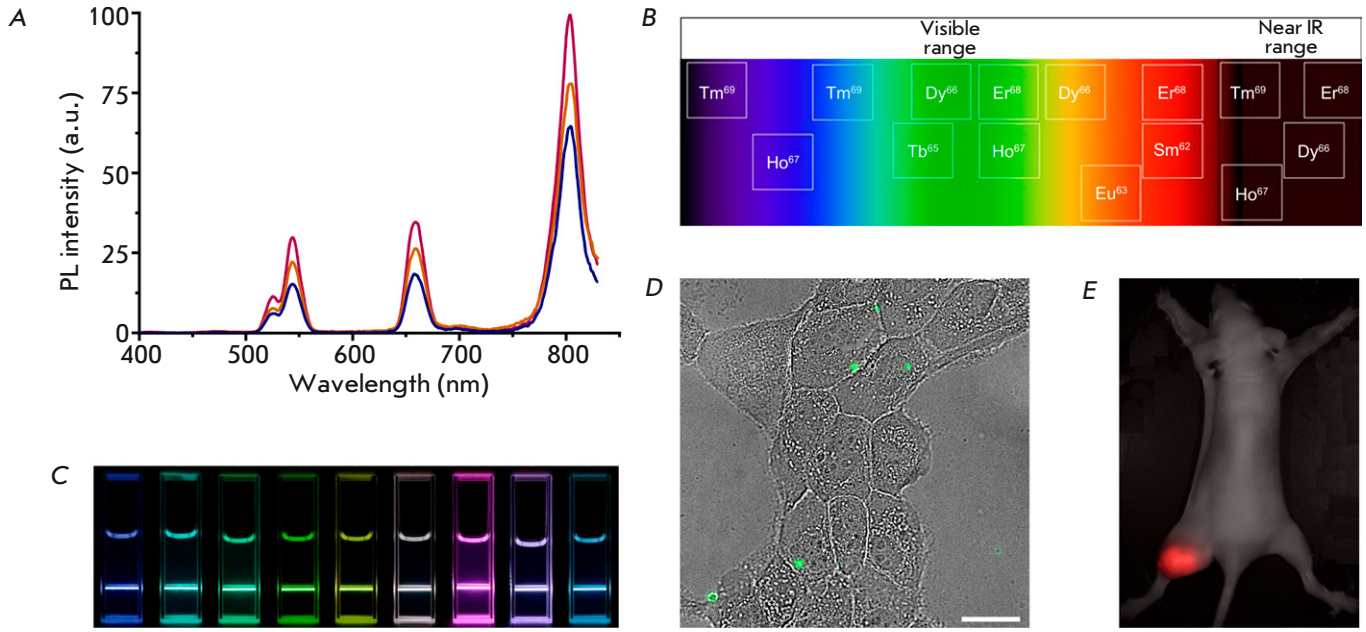


Fig. 6. (*A*) – PL emission spectrum of $NaYF_4$:Yb,Er,Tm UCNP upon excitation with 980-nm light of varying power. (*B*) – Dependence between dopant ion type and UCNP radiation wavelength. Adapted from [187] with permission from the copyright holder: Dove Medical Press Ltd. © 2019 Informa PLC, London. (*C*) – Photographs of various colloidal UCNP solutions irradiated with 980-nm light. Adapted from [188] with permission from the copyright holder: John Wiley and Sons. © 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (*D*) – Visualization of SK-BR-3 cells with targeted $NaYF_4$:Yb,Er UCNP complexes. The overlay of the translucent image and PL signal in the range 420–840 nm was obtained using a wide-field fluorescence microscopy system. Scale bar 20 μm. (*E*) – Visualization of a xenograft SK-BR-3 tumor with theranostic complexes based on UCNP with the composition of $NaYF_4$:Yb,Tm. Superposition of a brightfield image and PL signal in the range 485–831 nm, obtained using a laboratory imaging system

tissue. It is possible to shift the PL emission maximum to the near-IR region by increasing the particle size, but this will lead to a simultaneous significant decrease in the PL yield. In addition, the problem of obtaining stable colloidal aqueous PSiNPs solutions resistant to oxygen has not been completely solved [138].

Up-conversion nanoparticles

The significant autofluorescence of biological tissues complicates the registration of a target PL signal from different labels and probes [180, 181]. This is especially important in intravital imaging of individual cells or tissues of the body, where the level of autofluorescence is the main limitation to imaging sensitivity. The solution to this problem has been facilitated by studying up-conversion nanoparticles (UCNPs) that are inorganic nanocrystals consisting of an optically inert host matrix ($NaYF_4$, Y_2O_3, $NaPrF_4$, La_2O_3, Lu_2O_3, $LuPO_4$, $GdVO_4$, $NaGdF_4$) and optically active lanthanide ions acting as luminescence centers [182, 183]. The best-studied among them are the $NaYF_4$:Yb^{3+}:Er^{3+}/Tm^{3+} UCNPs actively used in biomedical applications [137, 184, 185].

The unique UCNP optical properties result from the up-conversion phenomenon, a nonlinear optical process where a nanoparticle sequentially absorbs two or more low-energy photons and emits a high-energy photon of a shorter wavelength. The energy of exciting IR light is absorbed by the ions of the sensitizer (Yb^{3+}) and is transmitted non-radiatively to the surrounding ions-sensitizers Yb^{3+} and the ions-activators Er^{3+} and/or Tm^{3+}. The excited states of lanthanide ions are long-lived, making it possible to absorb more than one quantum of light with a subsequent energy transfer to the same activator ion. The energy accumulated on these ions causes them to transition to high energy levels. The return to their initial state is accompanied either by non-radiative energy transfer or by photon emission, with the energies exceeding the energy of the exciting light. The Er^{3+} and Tm^{3+}

ions have several energy levels to provide several narrow emission peaks in the visible and IR spectral regions (*Fig. 6*) [186].

Thanks to their photophysical properties, UCNPs hold several advantages over other fluorophores used in biomedicine. The pronounced emission maxima make it possible to record the PL signal, clearly distinguishing it from tissue autofluorescence and the scattered excitation radiation. PL excitation by near-infrared light falling into the biological tissue transparency window makes it possible to achieve a greater visualization depth. When using thulium-doped UCNPs, the PL emission maximum is also in the near-IR region. The long PL lifetime (up to milliseconds) makes it possible to implement delayed detection optical schemes, increasing the SNR [189]. Finally, UCNPs have high chemical photostability and low toxicity [190, 191].

UCNP limitations include a lower radiation conversion coefficient (within 1–2%) if compared to linear fluorescent materials. As in the case of other nanomaterials, reliable and stable procedures for UCNP preparation, modification, and functionalization are required, as well as a study of the possible negative consequences of their application [183, 185, 192, 193]. Despite this, a lot of evidence has been accumulated showing that UCNPs can be successfully applied in the development of agents for optical and multimodal imaging [194, 195], sensors [196, 197], as well as for photodynamic and photothermal therapy [198, 199].

Nowadays, UCNPs are proving themselves to be not only excellent imaging agents for fluorescence diagnostics, but also a highly efficient platform for assembling multifunctional theranostic complexes [200, 201, 202]. Modifying their surface with immunoglobulin- and non-immunoglobulin targeting modules enables one to use UCNPs in high-precision optical diagnostics of oncological diseases. The possibility of using UCNPs for specific visualization of tumor cells and experimental tumors has been demonstrated in [190, 203–206]. Attachment of the bifunctional targeted toxins specific to the tumor cells of a certain molecular profile to biocompatible UCNPs makes it possible to open the therapeutic potential of the designed complexes [207, 208] and use the advantages of combined therapy. It has been shown that the efficacy of therapeutic modules (β-emitter and targeted toxin) increases by more than two orders of magnitude when they are used as parts of a theranostic nanocomplex to attack tumor cells [209].

UCNPs allow one to use deeply penetrating IR radiation to excite PL with its subsequent transfer to an organic molecule-effector (in the case of photodynamic therapy) or to gold/silver nanoparticles (in the case of photothermal therapy). Several studies have demonstrated the significant photodynamic effect UCNP complexes combining small molecules (rose Bengal, riboflavin) [210] and phototoxic proteins (KillerRed, mCherry) [106, 211] have on tumor cells.

Conclusion

The development of various nanomaterials with photoluminescent properties has significantly expanded the arsenal of approaches used in modern biomedicine. The unique photophysical properties of these new materials make it possible to significantly improve the sensitivity and specificity of diagnostic methods and also enable one to apply the theranostic approach to treatment using PL conjugates of nanoparticles and functional macromolecules. The size and surface properties of PL nanoparticles ensure efficient delivery of low-molecular-weight therapeutic agents of various natures, as well as biologically active macromolecules. Despite the positive features inherent in each type of the PL nanomaterials described above, it must be admitted that they also have a common downside that prevents their active introduction into widespread clinical practice and concerns the response of the immune system to the nanomaterials injected into the bloodstream for systemic delivery. The immune system cells that protect the body against foreign agents attack nanomaterials, and the latter fail to reach their target pathogenic cells and instead are quickly inactivated and accumulate in healthy tissues, primarily in the liver. This short circulation challenge has traditionally been solved by coating the nanomaterials with inert polymers to mask them from the immune system. These so-called stealth nanoagents, primarily liposomes, have been used recurrently over the past decades but have not become a cardinal solution to the problem. Recently, a fundamentally new approach has been proposed that makes it possible to significantly extend the circulation time of nanoagents and, as a consequence, to increase their therapeutic effect. The approach, called “cytoblockade of the mononuclear phagocytic system,” does not require any modification of the nanoparticles and consists in introducing a relatively small amount of antibodies against the body’s own red blood cells. As a result, the immune system “focuses” on attacking its own erythrocytes and for some time “ceases to see” the injected nanomaterials. During this time, the materials manage to locate target pathogenic objects and provide a therapeutic effect. An important characteristic feature of this approach is its versatility: i.e., independence of the nature, size, and other properties of the nanoparticles [212]. Ideologically close to this approach is the method in which “inert” nanoagents are first introduced into the body, triggering an attack by the immune

system, and only after that are drug-loaded nanoparticles introduced [213]. Thus, it can be concluded that studies of the practical application of theranostic PL drugs should focus on a combination of highly effective, targeted nanoagents capable of detecting a pathogenic centre with high accuracy [214] and technologies that ensure their sufficiently long circulation time in the bloodstream. ●

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Quality Control Mechanisms in Bacterial Translation

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ABSTRACT **Ribosome stalling during translation significantly reduces cell viability, because cells have to spend resources on the synthesis of new ribosomes. Therefore, all bacteria have developed various mechanisms of ribosome rescue. Usually, the release of ribosomes is preceded by hydrolysis of the tRNA–peptide bond, but, in some cases, the ribosome can continue translation thanks to the activity of certain factors. This review describes the mechanisms of ribosome rescue thanks to *trans*-translation and the activity of the ArfA, ArfB, BrfA, ArfT, HflX, and RqcP/H factors, as well as continuation of translation via the action of EF-P, EF-4, and EttA. Despite the ability of some systems to duplicate each other, most of them have their unique functional role, related to the quality control of bacterial translation in certain abnormalities caused by mutations, stress cultivation conditions, or antibiotics.**
KEYWORDS **translation, bacteria, quality control, termination, *trans*-translation.**

INTRODUCTION

In a bacterial cell, protein synthesis involves the 70S ribosome that consists of the small 30S and large 50S subunits (*Fig. 1*) [1–3]. Translation initiation begins with an interaction between the 30S subunit associated with the IF3 factor and the mRNA internal ribosome binding site. Then, the initiation factor IF2 associated with GTP delivers the initiator fMet-tRNA to the P site and IF1 binds to the A site. Initiation is completed by the binding of the 50S subunit, GTP hydrolysis, and the dissociation of initiation factors. During elongation, the ternary complex aa-tRNA (aminoacyl-tRNA)–EF-Tu–GTP binds to the A site of the ribosome. After correct recognition of a codon by the tRNA anticodon, GTP undergoes hydrolysis. The acylated end of the tRNA moves to the peptidyl transferase center (PTC), and EF-Tu is released. Through the transpeptidase reaction catalyzed by the large ribosomal subunit, the peptide chain is transferred to the aminoacyl-tRNA occupying the A site. The EF-G factor catalyzes the movement of the ribosome forward along the mRNA by one codon, after which the deacylated tRNA moves to the E site, and the peptidyl-tRNA enters the P site, thereby freeing the A site for the next aa-tRNA. After dissociation of EF-G, the elongation cycle is repeated. When a stop codon enters the A site, it is recognized by the class I release factors RF1 or RF2, which triggers termination of the protein synthesis. Both factors contain the conserved GGQ motif that catalyzes the hydrolysis of the peptidyl–tRNA bond, thus releasing the newly synthesized peptide. The class II release factor RF3, which also exhibits GTPase activity, promotes the dissociation of RF1 or RF2 from the ribosome. Further, the RRF and EF-G proteins facilitate the disassembly of the 30S and 50S ribosomal subunits and the subsequent binding of IF3 to the small subunit removes the tRNA and mRNA. The translation cycle is complete.

Unlike eukaryotic cells, where translation is preceded by mRNA processing, bacteria are unable to control the quality of the template before protein biosynthesis. Translation in a bacterial cell occurs simultaneously with transcription. This coupling of the two most important processes in time and space, on the one hand, is an advantage: it not only enables the cell to produce proteins at a higher rate, but also underlies the regulatory mechanism of attenuation. On the other hand, the

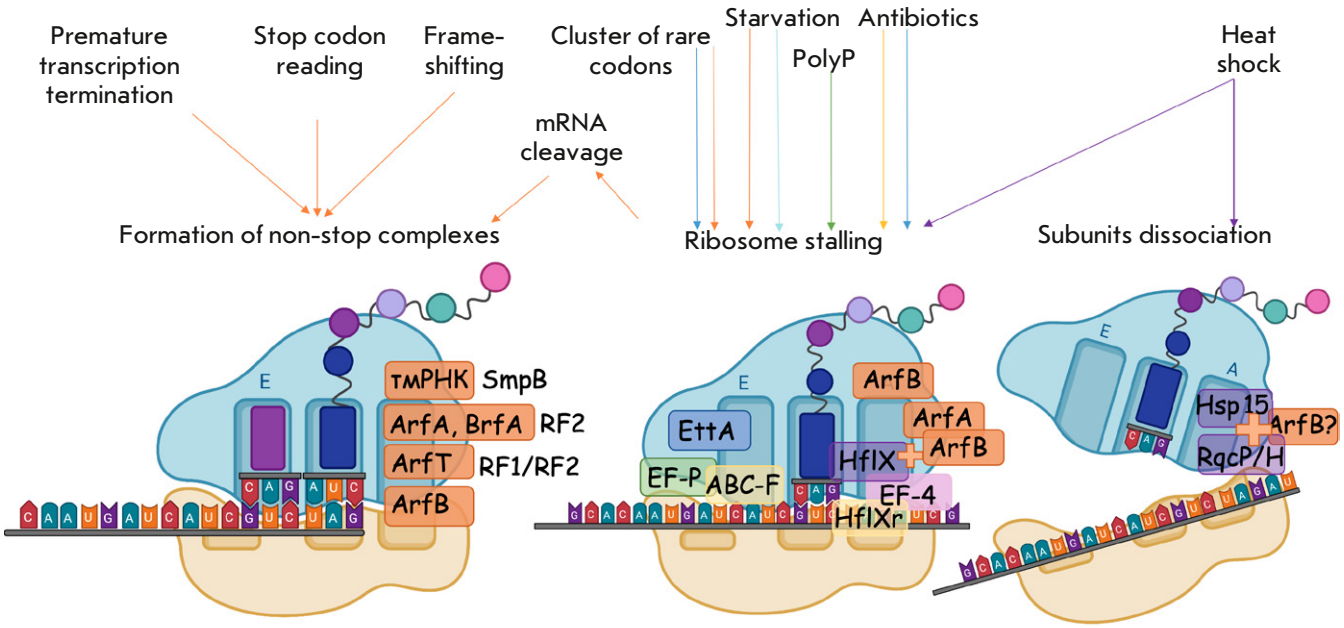

Fig. 1. Main causes of translational stalling in a bacterial cell and ways of solving these problems. The figure shows possible causes of translational stalling in a bacterial cell and the tools used by the cell to solve the problems. Left: a non-stop complex formed during translation. This type of substrate is recognized by the factors causing emergency translational termination, followed by the hydrolysis of the peptidyl-tRNA (tmRNA, ArfA, BrfA, ArfB, ArfT). Middle: a ribosome stalled on an intact template. In the case of starvation, this ribosome is stabilized in a hibernation state by Etta; during the passage of a polyproline sequence, EF-P promotes the resumption of translation. Resumption of translation is also provided by EF-4. If this complex is formed under the action of an antibiotic, it can be a substrate for a number of ABC-F proteins, HflX, and, possibly, HflXr. If stalling is caused by a cluster of rare mRNA codons, then the ribosome is likely rescued by ArfB. Right: spontaneous dissociation of ribosomal subunits. The RqcP/H and Hsp15 factors can promote the release of the 50S subunit. (All illustrations are created on BioRender.com)

absence of any control over the mRNA before translation inevitably leads to ribosome stalling during the protein synthesis on a template damaged by various factors. The most common cause of these occurrences is ribosome stalling on a damaged mRNA and the formation of the so-called non-stop complex [3]. The list of problems that may arise during translation is not limited only to the lack of a stop codon in the mRNA (*Fig. 1*). Movement of the ribosome can also stop on an intact template; e.g., during translation of "rare" codons and polyproline sequences [4] or under amino acid starvation conditions. Ribosome stalling in the cell also occurs in the presence of antibacterial agents that disrupt protein biosynthesis [5]. Of course, this wide range of potential problems has led to the development of various mechanisms aimed at solving them. In some cases, translation stalling is used to regulate gene expression, so it should not be perceived by the cell as a problem requiring a particular solution [6]. This review discusses the main causes of the problems arising during protein biosynthesis in a bacterial cell and the means used by bacteria to rescue stalled ribosomes. Investigation of some of them is of great practical importance, because the activity of some rescue systems underlies the mechanisms of antibiotic resistance.

The factors that solve the problem of stalled translation may be divided into two types:

1. Factors causing emergency termination of translation, first and foremost, with subsequent hydrolysis of the peptidyl-tRNA and release of the ribosome' and

2. Factors causing the reactivation of translation in emergency conditions.

Let us consider in more detail the causes behind translation stalling and the rescue systems operating in each specific case.

FACTORS CAUSING EMERGENCY TRANSLATION TERMINATION WITH SUBSEQUENT PEPTIDYL-tRNA HYDROLYSIS AND RIBOSOME RESCUE

One of the most common problems that the ribosome may encounter during mRNA translation is the absence of a stop codon [3]. This error can occur

for a variety of causes. These include premature transcription termination, frameshifting, endo- and exonuclease activity, and stop codon readthrough [3]. Non-stop complexes can also form under the action of some of the endoribonuclease toxins that are necessary for translation arrest under stress conditions [7]. The formation and accumulation of non-stop complexes is toxic to the cell, and the lack of special mechanisms for the elimination of these complexes leads to a rapid decrease in the cell's ability to synthesize proteins [3, 8, 9]. In this case, the cell viability is affected not only by the deficiency in proteins, the synthesis of which is suddenly interrupted, but also, to a greater extent, by the lack of ribosomes for the translation of other mRNAs. Usually, ribosomes cannot easily dissociate, as they are part of a non-stop complex, since interactions among the peptidyl-tRNA, ribosome, and mRNA firmly hold the complex together [1, 10]. Therefore, bacteria are faced with the primary problem of rescuing stalled ribosomes. Its complexity is related to the need for selective hydrolysis of the desired peptidyl-tRNA. In other words, the mechanism should quite accurately distinguish non-stop complexes from the ribosomes involved in normal elongation.

Trans-translation

The most common mechanism for the rescue of ribosome complexes is the *trans*-translation performed by transport-messenger RNA (tmRNA), which is encoded by the *ssrA* gene, and the SmpB protein. The tmRNA structure and the *trans*-translation mechanism are described in detail in a number of papers [3, 11–14]. tmRNA derived its name from its ability to combine the functions of both transfer and messenger RNA. The 5'- and 3'-ends of tmRNA form a structure resembling that of Ala-tRNA, which is recognized by alanyl-tRNA synthetase. In addition to a tRNA-like domain, tmRNA contains two to four pseudoknots and a specialized reading frame that encodes a short peptide (8–35 amino acids long, depending on the species). It lacks a start codon, which excludes its normal translation [3].

To perform its function, tmRNA requires the SmpB protein [15]. SmpB stabilizes tmRNA, promotes its recognition by alanyl-tRNA synthetase, and provides binding of the EF-Tu necessary for the delivery of tmRNA to the ribosome. The interaction between tmRNA and EF-Tu is similar to the binding of EF-Tu and aa-tRNA, which is confirmed by the stabilization of this complex on the ribosome in the presence of kirromycin [16].

At the first step of *trans*-translation, the tmRNA–SmpB–EF-Tu–GTP complex binds to the A site of the ribosome. Unlike a ternary complex that interacts with mRNA at the A site, the tmRNA–SmpB–EF-Tu–GTP complex interacts with an empty A site. In this case, the codon–anticodon interaction is replaced by the interaction between SmpB and a ribosome site that binds mRNA on the 3'-side of the P site during normal translation. In this case, the tmRNA–SmpB–EF-Tu complex triggers GTP hydrolysis. If the mRNA channel is empty, then the tmRNA remains in the A site to continue the translation of the tmRNA coding part. If mRNA is present in the channel, the interaction is prevented because of steric overlap. Thus, the *trans*-translation mechanism does not affect translating ribosomes [17].

Entry of the tmRNA–SmpB complex into the A site leads to the transfer of a polypeptide chain to Ala-tmRNA and is accompanied by subsequent translocation of deacylated tRNA from the P site to the E site, and the peptidyl-tmRNA–SmpB from the A site to the P site. During translocation, the tmRNA reading frame enters the mRNA channel, such that its first codon, known as the "resume codon," displaces the C-terminal tail of SmpB from the decoding center. *Trans*-translation continues until a stop codon of the tmRNA is reached, which is recognized by the canonical release factor RF1 or RF2 that terminates translation and releases the polypeptide with a tmRNA-encoded tag. Further, the polypeptide is recognized by several proteases, including ClpXP, ClpAP, HflB, and Tsp13, which leads to its rapid degradation (*Fig. 2*) [3, 18].

The interaction between the protease and the ssrA tag is provided for by the SspB adaptor protein. The original mRNA involved in the non-stop complex is also degraded to avoid repeated translation and a recurrence of emergency situations [3]. In *Escherichia coli* cells, this process is carried out by the RNase R that is recruited by tmRNA–SmpB [19]. Thus, tmRNA plays three important roles in the life of the cell: it is involved in ribosome rescue and in the quality control of the protein and mRNA [13].

Reserve pathways of ribosome rescue involving ArfA and BrfA

In the case of limited *trans*-translation activity, the ribosome is rescued through an alternative pathway using the Arfa (alternative ribosome rescue factor A) protein. ArfA recruits RF2 to the ribosome, which in turn hydrolyzes the peptidyl-tRNA in non-stop complexes (*Fig. 3*) [20, 21].

ArfA compensates for the absence of a stop codon at the A site and promotes peptidyl-tRNA hydrolysis by RF2 [22]. Therefore, the RF2 GGQ motif hydrolyzing peptidyl-tRNA plays the central role in ribosome rescue by ArfA, while the SPF motif recognizing a stop codon is not that important [23]. In contrast to *trans*-translation, ArfA activity leads only to the release of ribosomes but is not accompanied by a subse-

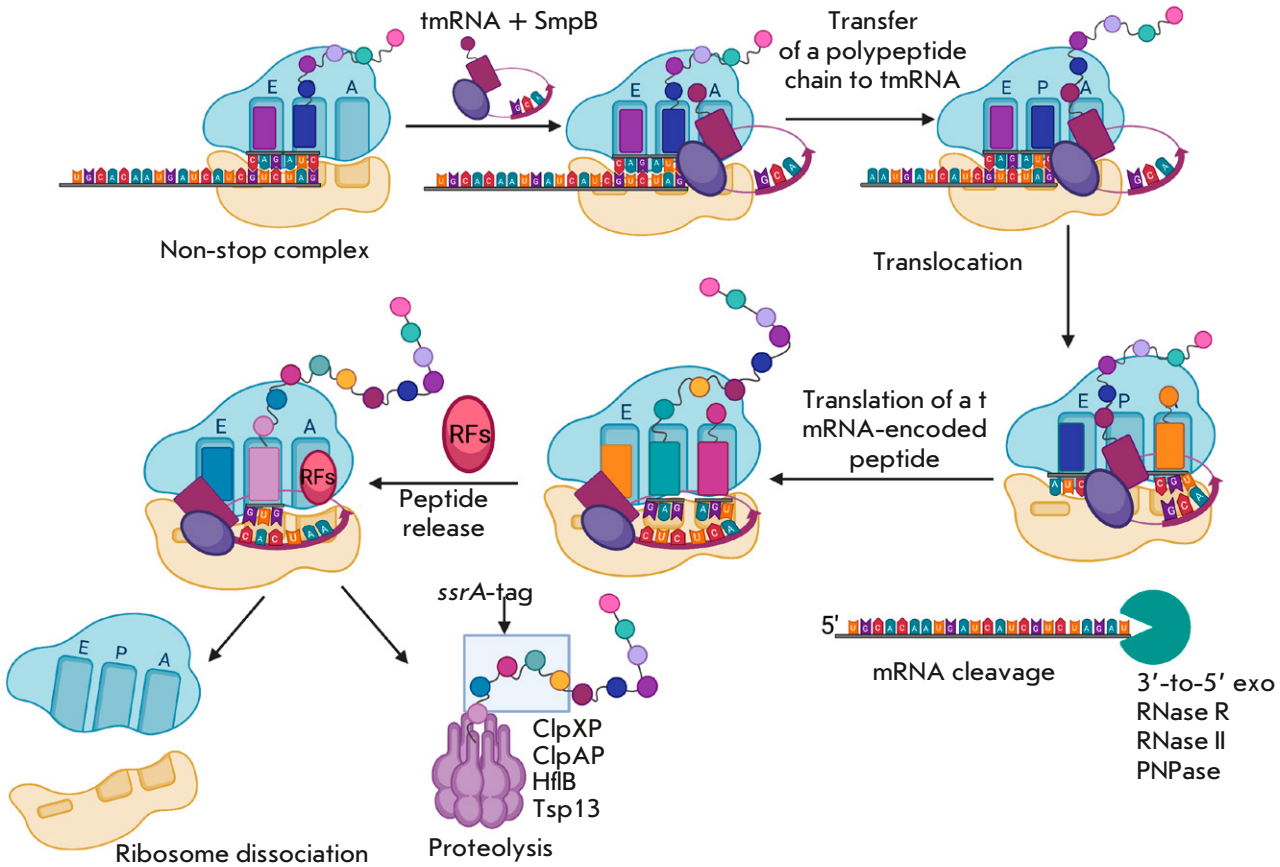

Fig. 2. Ribosome rescue by *trans*-translation. The tmRNA–SmpB complex recognizes the ribosome within a non-stop complex and binds in a free A site. Binding of the tmRNA–SmpB complex in the A site leads to the transfer of a polypeptide chain to the Ala-tmRNA and is accompanied by subsequent translocation of the deacylated tRNA from the P site to the E site and the peptidyl-tmRNA–SmpB from the A site to the P site. *Trans*-translation continues until s tmRNA stop codon is reached, which is recognized by the canonical termination factor RF1 or RF2, which stops translation and releases the polypeptide with a tmRNA-encoded tag. Further, the polypeptide is recognized by several proteases, including ClpXP, ClpAP, HflB, and Tsp13, which leads to its rapid degradation [3, 11–14]

quent degradation of nascent polypeptides or mRNA [20–24]. Interestingly, ArfA recruits only RF2, but not RF1. RF2 is capable of releasing arrested ribosomes with rather low activity, while ArfA enhances this activity [25] through direct interaction with RF2 [26].

It should be noted that ArfA is synthesized from non-stop mRNA, and its expression is directly regulated by the *trans*-translation system [27]. In *E. coli*, the ArfA mRNA adopts a hairpin structure and contains an RNase III cleavage site; RNase III removes the stop codon and the final 18 codons of the open reading frame. The *arfA* gene of *Neisseria gonorrhoeae* lacks an RNase III cleavage site; however, the hairpin facilitates transcription termination before the stop codon, thereby providing inhibition of ArfA synthesis [28]. Ribosomes stalled on ArfA mRNA are released during *trans*-translation, and the protein undergoes rapid proteolysis [29]. In some cases, ArfA mRNA can retain a stop codon; then, the classical variant of translation termination with the formation of a full-length product occurs but the C-terminal region of the full-length ArfA contains a hydrophobic area that promotes protein aggregation, with the protein being cleaved by intracellular proteases. If the activity of *trans*-translation is limited or impaired, then a truncated ArfA lacking the ssrA degradation tag is formed. This truncated product replaces the tmRNA–SmpB system. This regulation mechanism makes ArfA a true reserve ribosome rescue system that operates only when *trans*-translation activity is low or absent [27].

The ribosome rescue mechanism involving the ArfA protein is used by only gram-negative bacteria. In

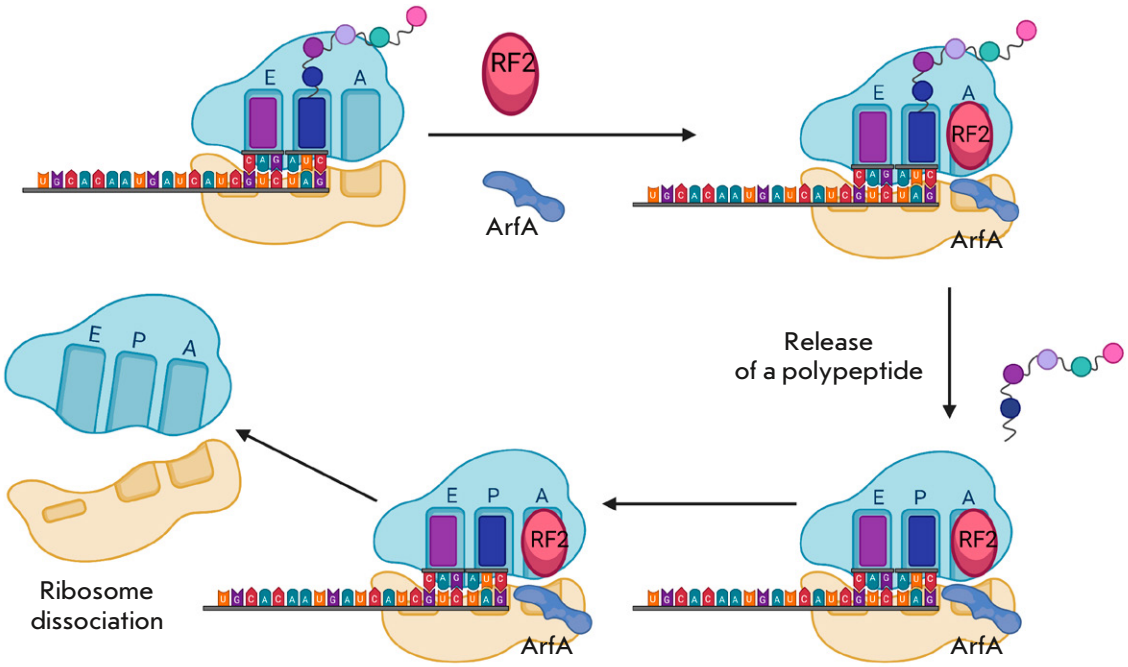

Fig. 3. Ribosome rescue by ArfA. ArfA binds at the 3′-end of the mRNA [22] and promotes hydrolysis of the peptidyl-tRNA by RF2

gram-positive bacteria, other mechanisms are present, and, for a long time, the canonical release factors were believed not to be involved in them. However, a mechanism of ribosome rescue similar to the action of ArfA was recently described in *Bacillus subtilis* cells [30]. The protein BrfA (Bacillus ribosome rescue factor A) plays a central role in this mechanism. Like ArfA, it recognizes non-stop complexes and recruits the RF2 release factor to a stalled ribosome. The C-terminal region of the protein also binds to the mRNA channel only if the channel is not occupied by part of the mRNA on the 3'-end of the P site. The similarity with ArfA is also observed at the regulation level: BrfA is synthesized from a non-stop mRNA, and its expression depends on the activity of *trans*-translation. However, the ArfA and BrfA proteins lack structural similarity and are evolutionarily distant from each other. In addition, despite the fact that both proteins recruit RF2, the interaction of each of these proteins with RF2 is different [30]. Probably, gram-positive and gram-negative bacteria developed in parallel reserve ribosome rescue mechanisms to secure the *trans*-translation system.

ArfB: an alternative rescue system

An alternative way to rescue stalled ribosomes is provided by the protein ArfB (alternative ribosome rescue factor B). The *arfB* gene was first identified as a lethality suppressor in an *E. coli* mutant lacking both *trans*-translation and the ArfA protein [24]. Homologues of the *arfB* gene were found in 34% of the sequenced genomes of both gram-positive and gram-negative bacteria [31]. Unlike ArfA, ArfB homologues are also present in eukaryotic cells [32].

The ArfB N-terminal domain is homologous to the catalytic domains of RF1 and RF2. This domain contains the GGQ motif that plays a crucial role in ArfB-mediated peptidyl-tRNA hydrolysis. In this case, several important amino acid residues necessary for the recognition of the retained complex and binding of the stalled ribosome are located not in the N-, but in the C-terminal domain of the protein. ArfB lacks a domain capable of interacting with a stop codon [33]. Purified ArfB from *E. coli* and *C. crescentus* is able to hydrolyze the peptidyl-tRNA in non-stop complexes *in vitro* in the absence of the RF1 and RF2 release factors (*Fig. 4A*) [24, 31].

The ribosome with a free A site serves as a substrate for tmRNA and ArfA; a similar arrangement was suggested for ArfB, but ArfB was found to interact with ribosomes even when a small mRNA segment extends from the P site [34]. In this situation, the nucleotides of the decoding center are re-arranged, which leads to the expansion of the mRNA tunnel. This plasticity prevents steric overlap of the ArfB C-terminal domain and a short mRNA fragment, thereby facilitating ribosome rescue. The C-terminal domain serves as a sensor that recognizes ribosomes with a free A site or a re-arranged decoding center. After its binding in the mRNA tunnel, a flexible linker region of the protein promotes entry of the N-terminal domain into the PTC to release the peptide. Then, rotation of the ribosome subunits relative to each other leads to the transfer of

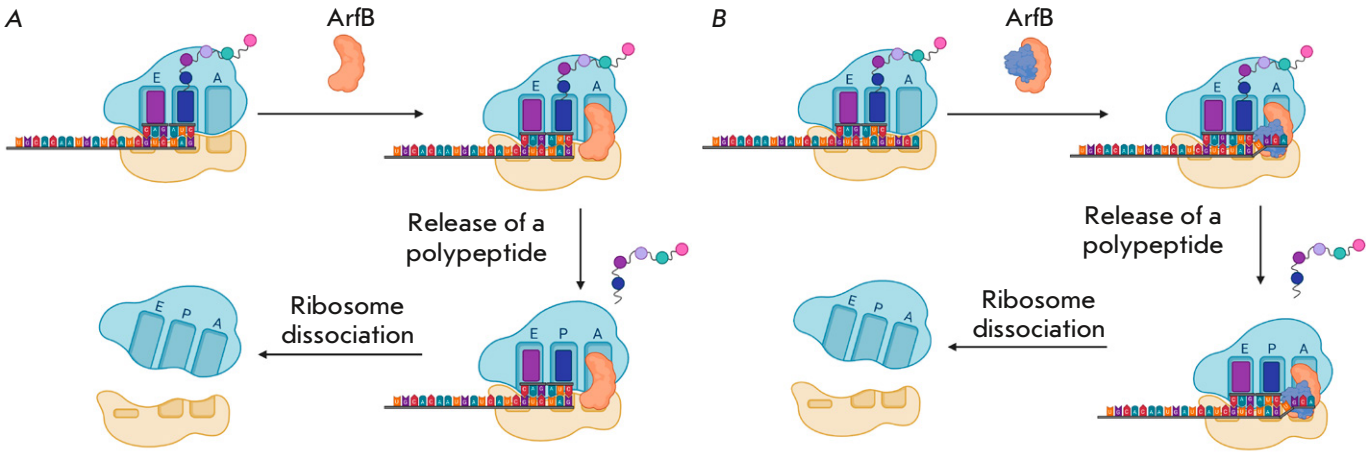

Fig. 4. (*A*) – Model of ribosome rescue by ArfB. ArfB binds to the mRNA tunnel of a stalled ribosome. Once bound, the flexible linker region of the protein allows the N-terminal domain to enter the PTC to release a peptide. Then, the ArfB–ribosome complex dissociates [24]. (*B*) – Scenario of ribosome rescue by ArfB when the A site is occupied. If an extended mRNA fragment protrudes from the P site, this fragment moves outside the mRNA tunnel into the intersubunit space and is stabilized there by an additional copy of the ArfB protein [35]. In this case, catalytic ArfB hydrolyzes the peptidyl-tRNA. Then, the ArfB–ribosome complex dissociates

the deacylated CCA-end of the tRNA to the E site. The ArfB–ribosome complex dissociates, and its subsequent disassembly is facilitated by the ribosome recycling factor RRF [35]. Like ArfA, ArfB releases the ribosome without degradation of a synthesized peptide.

Substrates of ArfB also include ribosomes with a rather extended mRNA fragment (*Fig. 4B*) [35]. In this case, the nucleotides of the decoding center do not change their position and a completely different mechanism operates. The extending mRNA is transferred outside the mRNA tunnel into the intersubunit space and is stabilized there by an additional copy of the ArfB protein, while the catalytic ArfB performs hydrolysis. Therefore, ArfB can act in both monomeric and multimeric forms, which enables the enzyme to efficiently recognize two groups of substrates. Therefore, the protein is able to release stalled ribosomes not only upon template breakage, but also in the case of rare codons or polyproline sequences. This demonstrates the similarity of ArfB to its eukaryotic homologue, the ICT1 protein that, according to some data, releases mitochondrial ribosomes stalled during translation of a cluster of rare codons in [32].

Deletion of *arfB* in *C. crescentus* does not affect viability, but it is lethal in combination with deletion of *ssrA* [31]. However, ArfB cannot fully compensate for the loss of *trans*-translation, because the *ΔssrA C. crescentus* strain has a pronounced growth defect [3]. In addition, unlike ArfA, the synthesis of ArfB is not associated with *trans*-translation activity and it most probably does not act exclusively as a reserve system for *trans*-translation [24, 31]. The action of ArfB, like ArfA, releases the ribosome but does not lead to subsequent targeted degradation of a synthesized peptide or mRNA. Perhaps, ArfB is necessary for the recognition of other possible translation abnormalities: e.g., the release of the ribosome from the non-stop complexes formed due to heat shock [3, 35].

ArfT releases ribosomes through a different mechanism

An unusual mechanism of ribosome rescue was found in the causative agent of tularemia, *Francisella tularensis*. *Francisella tularensis* lacks ArfA and ArfB, but inactivation of the ssrA/SmpB system is not a lethal mutation for this bacterium. Transposon mutagenesis followed by deep sequencing revealed a new alternative ribosome rescue factor called ArfT [36].

Deletion of the *arfT* gene was found to lead to a loss of viability only in *F. tularensis* mutants incapable of *trans*-translation. Overexpression of ArfT, on the contrary, promotes the intensive growth of these cells [36]. ArfT is, to some extent, similar to ArfA, and these two factors probably recognize non-stop complexes in a similar way. The C-terminal tail of ArfA binds in an empty mRNA channel of stalled ribosomes using several lysine and arginine residues, including the conserved KGKGS motif. None of these residues by itself is important for the activity of ArfA; however, replacement of individual residues reduces the activity of ribosome rescue *in vitro*. The KKGGSTNKK sequence near the C-terminus of ArfT contains, like ArfA, a number of

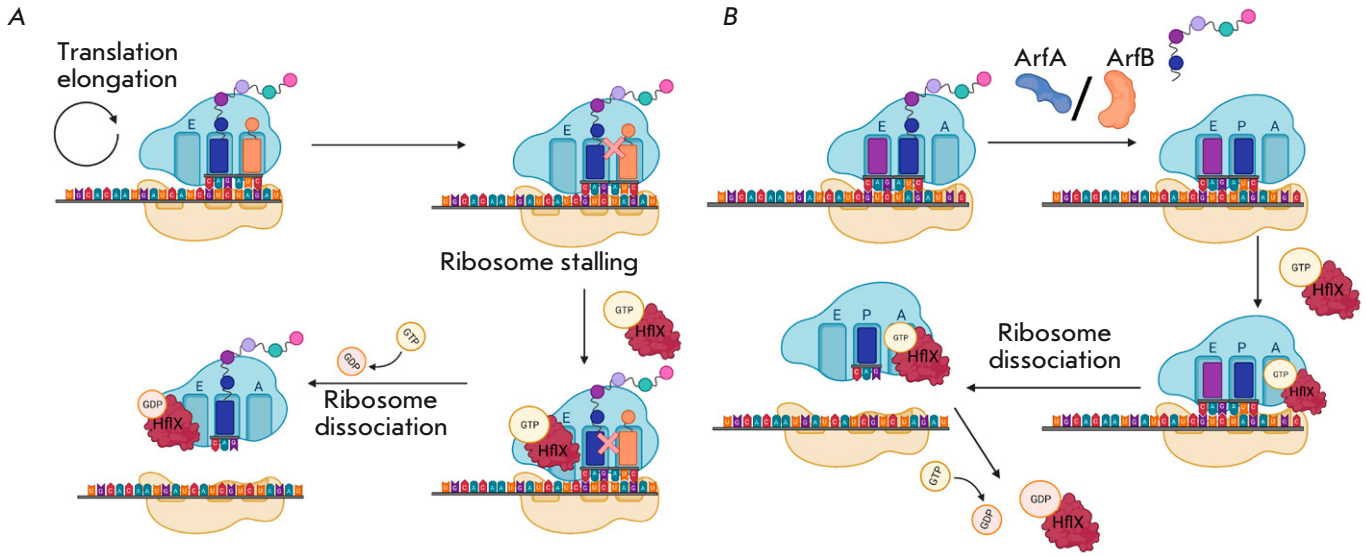

Fig. 5. Possible mechanisms of HflX activity. (*A*) – HflX binds to a free E site [38]. The stalled peptide in the PTC is a signal for HflX to hydrolyze GTP. Then, HflX cleaves the 70S subunit into the 50S and 30S ribosomal subunits that can later be used in another round of translation. (*B*) – HflX binds to the A site of a stalled ribosome [39]. The peptide is released by the rescue factor ArfA or ArfB. Then, HflX–GTP binds to the A site and causes dissociation of ribosomal subunits

positively charged residues; therefore, ArfT can probably use this sequence to bind the ribosome [37]. ArfT causes hydrolysis of the peptidyl-tRNA by acting together with termination factors; however, unlike ArfA recruiting only RF2, ArfT interacts with both RF2 and RF1. For example, in the course of *in vitro* modeling of abnormal translation, the addition of ArfT and RF1 from *F. tularensis* to the non-stop complex led to the hydrolysis of the peptidyl-tRNA with an efficiency of 95%, and the addition of ArfT and RF2 from *F. tularensis* led to the same hydrolysis with an efficiency of 84% [36].

Despite the similarity of the C-terminal sequence of ArfT and ArfA, the ability of ArfT to activate both RF1 and RF2 may mean that ArfT interacts with release factors differently than ArfA does. In addition, it is worth noting that ArfT formation is not regulated by translation termination.

FACTORS CAUSING EMERGENCY TRANSLATION TERMINATION NOT ASSOCIATED WITH PEPTIDYL-tRNA HYDROLYSIS

HflX

Heat shock is another cause of translation stalling. In this case, rescue systems interact with the 70S ribosome containing a peptidyl-tRNA in the P site and intact mRNA in the A site. One of the factors that can recognize this substrate is the *E. coli* HflX protein.

There are several potential mechanisms of HflX activity. According to one of them, Hf1X can bind to a free E site (*Fig. 5A*) [38]. A peptide stalled in the PTC serves as a signal for the hydrolysis of GTP by HflX. Then, HflX splits the 70S ribosome into 50S and 30S subunits, which can then be used in another round of translation. After splitting of the ribosome, HflX can bind to the A site to prevent re-binding of the 50S and 30S subunits and block binding of other GTPases [38]. HflX was shown to bind to the A site of a stalled ribosome (*Fig. 5B*) [39]. In this model, a peptide is released by the rescue factor ArfA or ArfB. Then, HflX–GTP binds to the A site and causes ribosomal subunits dissociation.

HflXr

The action mechanism of numerous antibacterial agents is based on translation suppression. Many of them bind to the PTC, thereby inhibiting the peptidyl transferase reaction [40]. Resistance to these antibiotics is usually associated with the action of efflux pumps or the mechanisms that modify or inactivate an antibiotic molecule [41]. In addition, deletion of the *hflX* gene in the pathogenic bacterium *Mycobacterium abscessus* was recently found to increase sensitivity to macrolide antibacterial agents. The product of this gene is capable of disassembling the ribosomes blocked by macrolides and, as thus, plays an important role in the development of antibiotic resistance in some pathogens [42].

A nontrivial mechanism of resistance, which is probably related to the activity of the HflXr protein, was described in *Listeria monocytogenes* [5, 43]. This protein is a homologue of *E. coli* HflX whose function is to disassemble a stalled ribosome [5]. Although HflXr is also capable of disassembling ribosomal subunits, it cannot be argued that its action is directly related to the displacement of an antibiotic. For example, despite the fact that deletion of the *hflXr* gene renders bacteria more sensitive to erythromycin and lincomycin, the sensitivity phenotype manifests itself only upon simultaneous deletion of another gene, *lmo0919* [5].

Release of the ribosome by RqcH and RqcP

Among the causes behind the abrupt arrest of protein biosynthesis, there is a rather unusual one–premature dissociation of ribosomal subunits. The release of the 50S subunit from a complex with the peptidyl-tRNA occurs using several mechanisms. One of them involves the RqcH and RqcP proteins (*Fig. 6*) [44]. The action of these proteins partially duplicates ssrA/tmRNA activity, because it also produces a polypeptide with a tag recognized by intracellular proteases.

The Rqc2 homolog (RqcH) found in *B. subtilis* is a homologue of the eukaryotic translation quality control factor Rqc2. In a model shown in *Fig. 6*, the RqcP protein binds to the 50S ribosomal subunit and stabilizes tRNA on the P site [44, 45]. RqcH delivers charged alanine tRNA to the 50S, which occupies a free A site. RqcH specifically binds Ala-tRNA due to the fact that the nucleotides G35 and C36 of the tRNA anticodon and the amino acid residues of the RqcH NFACT-N domain form Watson–Crick-like interactions [46]. Further, a polypeptide chain is transferred. Then, RqcP loses its affinity for the ribosome, which facilitates a translocation-like movement of the ribosome: in this case, the deacylated tRNA moves to the E site and the peptidyl-tRNA moves to the P site. Later, to stabilize the peptidyl-tRNA at the P site, RqcP binds again. RqcH either dissociates or, being bound to the ribosome, recruits Ala-tRNA. The cycle of this "elongation" can repeat itself until the RqcH factor dissociates, and

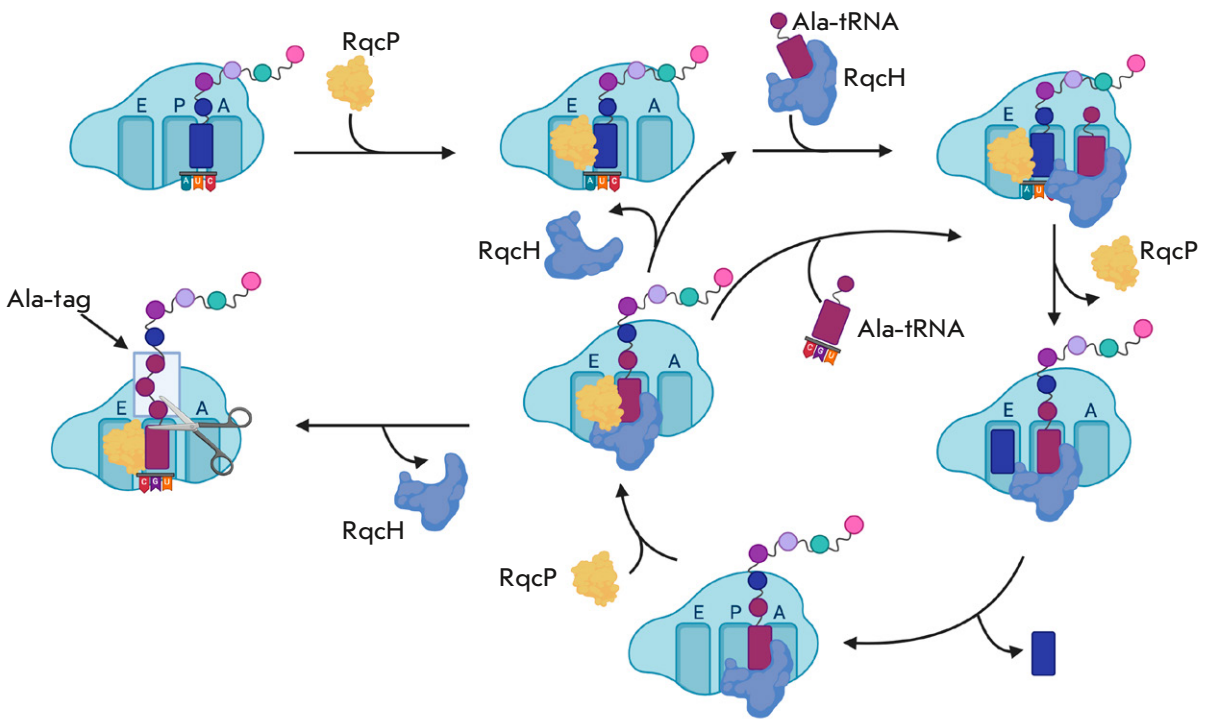

Fig. 6. Mechanism of action of the RqcP and RqcH (YabO) proteins. RqcP binds to the 50S subunit and stabilizes tRNA at the P site [44, 45]. RqcH delivers the charged alanine tRNA to the 50S, which occupies a free A site. Further, a polypeptide chain is transferred. Then, RqcP loses its affinity to the ribosome and undergoes a translocation-like movement: in this case, the deacylated tRNA moves to the E site and the peptidyl-tRNA moves to the P site. Later, RqcP rebinds to stabilize the peptidyl-tRNA at the P site. The ribosome-bound RqcH recruits Ala-tRNA. Further, the cycle of this "elongation" can be repeated until the RqcH factor dissociates, and the polypeptide is released. The factor hydrolyzing the peptidyl-tRNA is not exactly known. Probably, it is ArfB

the polypeptide is released. The factor that hydrolyzes the peptidyl-tRNA is not clearly known. ArfB is supposed to act in a similar way [44].

Hsp15

Actinobacteria and gamma-proteobacteria lack the RqcH and RqcP proteins. However, it should be noted that the RqcP protein is a homologue of the *E. coli* Hsp15 protein [44]. Like RqcH/RqcP, Hsp15 binds to the 50S subunit blocked after sudden disassembly of the ribosome. Hsp15 does not interact with 70S ribosomes, because the small subunit prevents its binding. In the case of unplanned ribosome disassembly, the large subunit becomes accessible to Hsp15. In this case, the peptidyl-tRNA can occupy the A site because of the absence of the 30S subunit. However, this is an unfavorable situation, because the release factor is unable to bind to the 50S subunit in the case of an occupied A site. The Hsp15 protein was found to promote movement of peptidyl-tRNA from the A site to the P site. Then, ArfB presumably performs the release of a polypeptide chain. A significant difference between this mechanism and the action of the RqcH and RqcP proteins is that the synthesized polypeptide chain is not targeted for degradation [47].

PrfH

In 1992, the *E. coli* K-12 gene encoding an amino acid sequence with high similarity to the RF1 and RF2 sequences was identified [48]. The element was called PrfH (protein release factor homologue). Later, a significant number of bacterial genomes, even evolutionarily distant from each other, were shown to contain orthologs of this gene. The PrfH protein is similar to the translation termination factors RF1 and RF2 and is regarded as their paralog [49].

There are several suggestions regarding the function of PrfH and which ribosome complex may constitute its substrate. The most plausible hypothesis is that PrfH is a ribosome rescue factor [49].

For example, *prfH* overexpression was found to increase the resistance of *Pseudomonas aeruginosa* bacteria to azithromycin [50]. In addition, by using a reporter system, *prfH* overexpression was shown to decrease the number of stalled ribosome–model mRNA complexes formed in the presence of azithromycin.

However, the role of PrfH is unknown and requires further investigation.

FACTORS INDUCING TRANSLATION REACTIVATION

Elongation factor P

It should be noted that template damage is not the only reason behind ribosome stalling during translation. Ribosomes are often stalled on intact mRNAs. This situation can develop in two scenarios: either elongation resumes, or mRNA is cleaved to form a non-stop complex. Ribosome profiling studies have demonstrated that this ribosome pausing is short-term, because it does not block the movement of other ribosomes translating the same template and does not disrupt gene expression [51, 52]. Many of these cases are caused by elongation delay due to a lack of the necessary aminoacyl-tRNA. In addition, the delay can be caused by pseudoknots and some elements of the mRNA sequence [52].

Stalled ribosomes are capable of spontaneous elongation resumption or translation termination, but specialized translation factors often help in these processes. One of them, EF-P, is a highly conserved protein, a eukaryotic eIF5A homologue, that promotes the synthesis of polyproline sequences [4, 53, 54]. EF-P orthologs in different groups of organisms contain modified amino acid residues whose identity may differ in different taxa [53]. For example, the *E. coli* EF-P contains a lysinyl-hydroxylysine moiety generated by the YfcM [55], YjeK, and YjeA [56–58] enzymes. EF-P from *P. aeruginosa* contains a rhamnose moiety [59, 60], and the appropriate residue in EF-P from *B. subtilis* is 5-aminopentanol [61]. In eukaryotic cells, eIF5A, the EF-P ortholog, contains a hypusine residue [62].

The formation of a peptide bond between proline residues is complicated and often leads to protein synthesis arrest [63]. Similar difficulties were shown to arise when the ribosome passes three or more consecutive prolines [64]. This motif is found, in particular, in the highly conserved valine-tRNA synthetase [63].

Structural studies of EF-P on the ribosome have shown that EF-P binds between the E site and the P site on the 50S subunit in close proximity to peptidyl-tRNA. Binding of EF-P stimulates elongation *in vivo* and *in vitro* when ribosomes are stalled on polyproline sequences (*Fig.* 7). EF-P is believed to promote the stabilization of the PTC substrate conformation productive for the peptidyl transferase reaction. Despite the fact that EF-P eliminates a small number of abnormalities, it is important enough to the physiology of a bacterial cell. For example, *E. coli* and *S. enterica* strains lacking EF-P have membrane integrity defects and exhibit increased sensitivity to some antibacterial agents [64].

EF-4 (LepA)

The well-known conserved translation factor EF-4, also known as LepA, was suggested as a promoter of elongation by catalysis of reverse translocation of stalled ribosomes [3]. However, ribosome profiling data show that EF-4 is involved mainly in the initiation stage and it is not yet known whether this protein plays a role

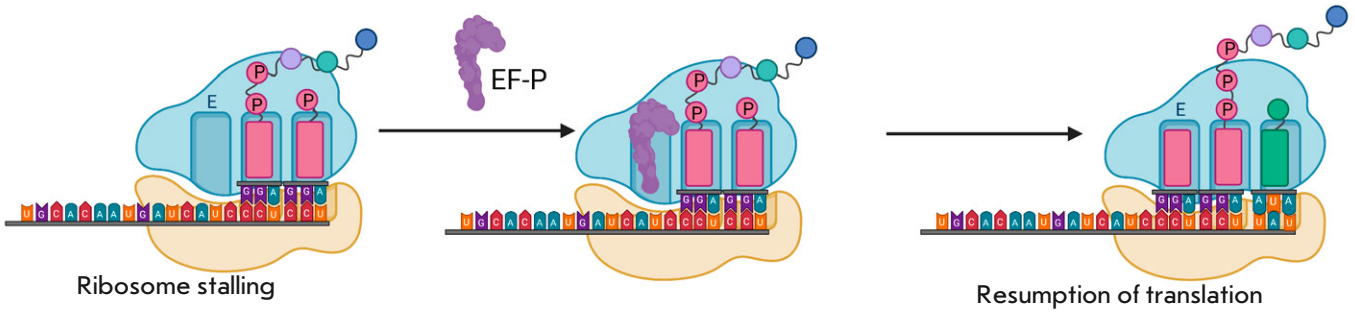

Fig. 7. Mechanism of action of the EF-P factor. Binding of EF-P stimulates elongation *in vivo* and *in vitro* when ribosomes are stalled on polyproline sequences. EF-P binds between the E site and the P site on the 50S subunit in close proximity to the peptidyl-tRNA. EF-P is believed to stabilize a PTC substrate conformation productive for the peptidyl transferase reaction [4]

in the rescue of ribosomes [65]. In addition, EF-4 was shown to remodel the A site tRNA, causing a displacement of the tRNA acceptor stem from the PTC. Further research is required to understand the functional significance of A/L distortion of A site tRNA [66].

EttA

ATP-binding cassette (ABC) type F proteins that bind to ribosomes and promote dissociation of the ribosome–antibiotic complex are capable of protecting the ribosome against antibiotics [43, 67, 68]. Of particular interest is EttA, an ABC-F protein found in *E. coli* [69]. EttA does not promote antibiotic resistance, but it acts as a translation factor limiting the activity of ribosomes in response to a low ATP level [70, 71]. At high ADP concentrations, EttA binds to the 70S ribosome at the P site, stabilizing it in the so-called hibernation state. This binding interferes with protein synthesis and enables tolerance of adverse conditions by limiting translation.

Also, some ABC-F proteins underlie the mechanisms of antibiotic resistance. A detailed review of the ABC-F proteins that protect the ribosome from antibiotics is presented in [40]. These ABC-F proteins bind on the E site of the ribosome. Binding causes a slight counterclockwise rotation of the 30S subunit relative to the 50S, which leads to a shift in the tRNA and allows the ARD domain of the protein to enter the PTC, resulting in a dissociation of the antibiotic. This is presumably associated with the fact that binding of the protein induces allosteric conformational changes in PTC nucleotides containing the antibiotic binding site. The ABC-F proteins found in many bacteria, e.g., *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *B. subtilis*, make these organisms resistant to a wide range of antibiotics [40].

CONCLUSION

The ability to release the ribosomes stalled on mRNA during translation markedly increases the chance of survival and, therefore, has been retained during natural selection (*Table*). Most bacteria need at least one ribosome rescue mechanism to survive. In this case, *trans*-translation has become the most widespread system: the *ssrA* and *smpB* genes are found in more than 99% of bacterial species [3]. Because components of the *trans*-translation system are present in almost all bacterial genomes, and mutations in the genes encoding these proteins reduce cell viability, the proteins involved in this system are considered as attractive targets for new antibacterial agents. These considerations have also been confirmed by the fact that *trans*-translation is specific to bacterial cells, which reduces the risk of possible side effects. Several compounds, potential inhibitors of the release of non-stop complexes through *trans*-translation, have been selected by high-throughput screening [8]. The mechanism of one of them is based on the prevention of polypeptide tagging, while others inhibit proteolysis of tag-containing proteins. One of the compounds inhibits both tag attachment and subsequent proteolysis of the protein.

The cells of almost all studied bacterial species capable of surviving in the absence of *trans*-translation contain an alternative release factor [72]. For example, the viability of cells with a *ssrA* deletion is maintained by *arfA* in *E. coli*, *brfA* in *B. subtilis*, *arfT* in *F. tularensis*, and *arfB* in *C. crescentus*. *Shigella flexneri* and *N. gonorrhoeae* cannot survive without *trans*-translation [27]. This may be explained by the fact that these pathogens lack an *E. coli* ArfA homologue capable of replacing the tmRNA–SmpB system [27, 73]. Note that

Factors and mechanisms of stalled ribosome rescue

Cause of stalling	Rescue factor	Mechanism of ribosome rescue	Occurrence
Formation of stalled complexes	*Trans*-translation (tmRNA/SmpB)	Resumption of translation using tmRNA. Tagging of a polypeptide and mRNA	99% of bacterial genomes
	ArfA	RF2 factor recruitment	Gram-negative
	BrfA	RF2 factor recruitment	*Bacullus subtilis*
	ArfT	Recruitment of RF or RF2	*Francisella tularensis*
	ArfB	Independent hydrolysis of peptidyl-tRNA	Gram-negative and gram-positive
	HflX	Disassembly of ribosomal subunits	Gram-negative and gram-positive
Abrupt dissociation of subunits	RqcH/RqcP + ArfB (?)	Mimicking of translation elongation for attaching an Ala tag to a polypeptide. Hydrolysis	Except for gamma-proteobacteria and actinobacteria
	Hsp15 +ArfB(?)	Transfer of peptidyl-tRNA to the P-site. Hydrolysis	Gram-negative and gram-positive
Rare codon cluster, polyproline sequence, secondary structure	EF-P	Assistance in peptide bond formation in passing a difficult segment	Gram-negative and gram-positive
	EF-4	Assistance in passing a difficult segment	Gram-negative and gram-positive
	ArfB	Hydrolysis of peptidyl-tRNA	Gram-negative and gram-positive
Action of antibiotics	HflXr	Disassembly of ribosome	*Listeria monocytogenes*
	ABC-F-proteins	Antibiotic dissociation	Gram-positive
	PrfH-?	Unknown	Gram-negative and gram-positive

ArfT interacts with *F. tularensis* RF1/2 but is unable to bind *E. coli* RF1/2. The BrfA factor interacts exclusively with RF2 of *B. subtilis*. Thus, the described ribosome rescue systems are not interchangeable in different species [26]. In this case, all the alternative rescue systems fail to provide sufficient activity in the absence of *trans*-translation. Deletion of *ssrA* or *smpB* results in many different phenotypes. For example, mutants lacking ssrA may exhibit increased sensitivity to antibiotics and temperature fluctuations and should have virulence defects [27, 74]. *Trans*-translation is preserved in all bacteria, but no species has adapted to the exclusive use of the ArfA, ArfB, or other system. Activity of tmRNA/smpB not only releases stalled ribosomes, but also promotes the removal of nascent polypeptides and damaged mRNAs, which also provides a significant advantage to the system over reserve rescue systems. A partial analogue of *trans*-translation is the RqcH–RqcP system, whose activity leads to the degradation of an incorrect polypeptide.

The additional ribosome rescue systems, both reserve and independent, may hardly be called quality control mechanisms in protein biosynthesis. These systems do not target damaged mRNAs, or the polypeptides synthesized on their basis, for degradation. Despite a variety of reserve mechanisms, none of them duplicates *trans*-translation; there is a suggestion that ribosome rescue is the primary mechanism in translation stalling. Of course, *trans*-translation is the most beneficial of the mechanisms, because it relieves the cell of unwanted and potentially toxic molecules. However, when it is limited or absent, the central need is still implemented – the rescue of stalled ribosomal subunits for subsequent rounds of protein synthesis. Thus, bacteria have acquired a variety of translation rescue systems aimed mainly not at controlling the quality of mRNA but at releasing ribosomal subunits. ●

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DNA Methylation As an Epigenetic Mechanism in the Development of Multiple Sclerosis

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ABSTRACT **The epigenetic mechanisms of gene expression regulation are a group of the key cellular and molecular pathways that lead to inherited alterations in genes' activity without changing their coding sequence. DNA methylation at the C5 position of cytosine in CpG dinucleotides is amongst the central epigenetic mechanisms. Currently, the number of studies that are devoted to the identification of methylation patterns specific to multiple sclerosis (MS), a severe chronic autoimmune disease of the central nervous system, is on a rapid rise. However, the issue of the contribution of DNA methylation to the development of the different clinical phenotypes of this highly heterogeneous disease has only begun to attract the attention of researchers. This review summarizes the data on the molecular mechanisms underlying DNA methylation and the MS risk factors that can affect the DNA methylation profile and, thereby, modulate the expression of the genes involved in the disease's pathogenesis. The focus of our attention is centered on the analysis of the published data on the differential methylation of DNA from various biological samples of MS patients obtained using both the candidate gene approach and high-throughput methods.**
KEYWORS **DNA methylation, epigenetics, multiple sclerosis.**
ABBREVIATIONS **BER – base excision repair; CIS – clinically isolated syndrome; CNS – central nervous system; DMS – differentially methylated CpG-site; DNMT – DNA methyltransferase; EDSS – expanded disability status scale; GWAS – genome-wide association study; HDAC – histone deacetylase; MBD – methyl-binding domain protein; MS – multiple sclerosis; NGS – next generation sequencing; PBMCs – peripheral blood mononuclear cells; PPMS – primary progressive multiple sclerosis; RRMS – relapsing-remitting multiple sclerosis; SAM – S-adenosyl methionine; SPMS – secondary progressive multiple sclerosis; TET – TET methylcytosine dioxygenase; Rep – repressor protein.**

INTRODUCTION

Epigenetic processes include inherited (at least during mitosis) changes in gene expression that do not affect the DNA nucleotide sequence [1]. However, this classical definition is today often extended to include stable, long-term variations in the cellular transcriptional profile that are not necessarily inherited in the number of epigenetic events [2].

The central mechanisms of epigenetic regulation of gene expression are presented in *Fig. 1*. They include DNA methylation (*A*); histone modification, i.e. functionally significant biochemical changes in chromatin that affect the accessibility of certain genomic loci to transcription enzymes (*B*); and the regulation of gene expression at different levels of genetic information implementation with the involvement of regulatory non-coding RNAs, among which the microRNAs regulating expression at the post-transcriptional level are the best studied (*C*) [3].

These mechanisms act synergistically and form a system that regulates the key cellular processes; therefore, they are crucial for a normal development and differentiation of all body cell types [4]. By now, the effect of numerous environmental factors has been proven to be mediated by various epigenetic mechanisms [5]. In some cases, this interaction leads to stable pathological changes that underlie many chronic diseases [6].

Although the investigation of the role of epigenetic mechanisms in the development of common human diseases first focused for the most part on oncological diseases [7], more and more of researchers' attention is currently focused on different pathologies, in particular autoimmune and neurodegenerative ones [8, 9].

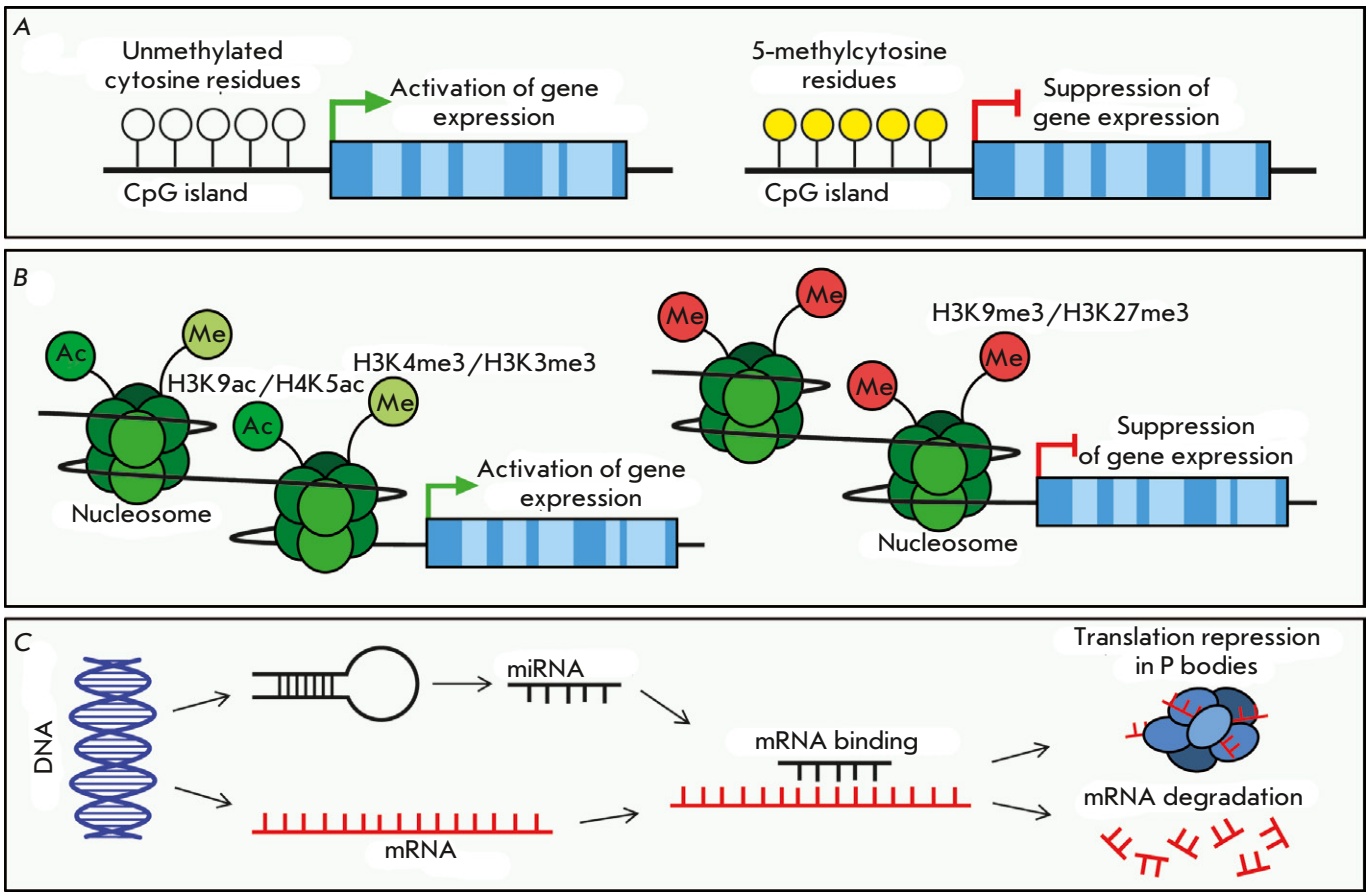

Fig. 1. The major epigenetic mechanisms regulating gene expression. The exon-intron structure of a gene is shown as dark blue and light blue rectangles, respectively. (*A*) – Methylation of cytosine residues in the CpG island located in the gene promoter region. (*B*) – The most common modifications of the histone proteins involved in gene expression activation (acetylation of either histone H3 lysine 9 or histone H4 lysine 5 (H3K9ac/H4K5ac) and trimethylation of either histone H3 lysine 3 or histone H3 lysine 4 (H3K4me3/H3K3me3)) and suppression (trimethylation of either histone H3 lysine 9 or histone H3 lysine 27 (H3K9me3/H3K27me3)). (*C*) – MicroRNA-mediated repression of mRNA translation and degradation

Identification of the features of the epigenetic regulation characteristic of these pathologies can help in our understanding of the mechanisms of their development and contribute to the creation of new effective therapeutic drugs.

In this review, we will focus on one of the key mechanisms of the epigenetic regulation of gene expression, namely DNA methylation, and its role in the development of multiple sclerosis (MS), a socially potent, severe disease of the central nervous system (CNS) characterized by chronic autoimmune inflammation and neurodegeneration.

MOLECULAR MECHANISMS OF THE EPIGENETIC REGULATION OF GENE EXPRESSION

DNA methylation is a universal epigenetic mechanism that suppresses gene expression in various ways and is involved in the regulation of the activity of the other two mechanisms mentioned above: histone modification and gene expression regulation by non-coding RNAs. In the overwhelming majority of cases, DNA is methylated at the C5 position of cytosine in CpG dinucleotides (CpG sites). The CpG sites that undergo methylation are unevenly distributed throughout the genome; they can form clusters called CpG islands. CpG islands are DNA regions at least 500 bp long with > 55% content of G and C nucleotides and a > 65% ratio of the actual number of CpG sites to the expected one with uniform distribution throughout the genome [10]. CpG islands and neighboring areas (shore) within 2 kb are of the greatest functional significance, since their methylation/demethylation effectively changes the expression level of nearby genes (see *Fig. 1A*). There are also distant areas (shelf) located within 2 kb from

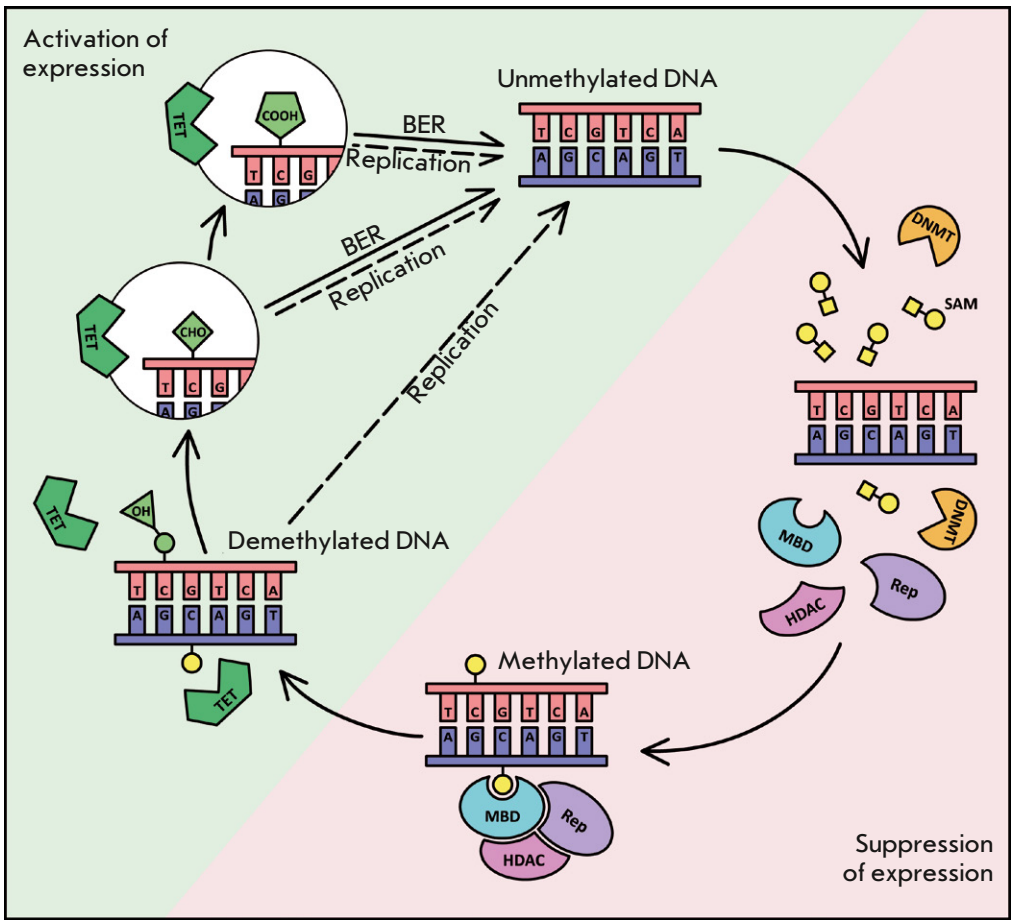

Fig. 2. DNA methylation as an epigenetic mechanism of gene expression regulation (see the text for details). BER – base excision repair; DNMT – DNA methyltransferase; HDAC – histone deacetylase; MBD – methyl-binding domain protein; SAM – S-adenosyl methionine; TET – TET methylcytosine dioxygenase; Rep – repressor protein

the neighboring regions and the rest of the genome (sea), where CpG sites are rare and distributed relatively evenly. About 70% of the gene promoters contain CpG islands [11], which determines the participation of the latter in gene expression regulation.

An overall scheme summarizing our current understanding of the molecular mechanisms of methylation and demethylation of CpG sites in the genome and their involvement in gene expression regulation is shown in *Fig. 2*.

DNA methylation is performed by DNA methyltransferases (DNMTs), enzymes that can transfer a methyl group to the fifth carbon atom of the cytosine residue to form 5-methylcytosine (5mC), using S-adenosyl methionine (SAM) as a donor [12]. The DNMT family includes DNMT1, DNMT2, and the DNMT3 subfamily consisting of DNMT3a, DNMT3b, and DNMT3L. DNMT1 is responsible for DNA methylation after replication and able to rapidly methylate the newly synthesized DNA strand complementary to the template strand. The DNMT3 subfamily is involved in *de novo* DNA methylation [13]. DNMT2/TRDMT1, tRNA (cytosine-5-)-methyltransferase, is technically not a DNA methyltransferase; it is involved in cytosine methylation at the 38 residue of the tRNA anticodon loop.

Methylation of CpG sites in the gene promoter region utilizes methyl-binding domain (MBD) proteins that are capable of suppressing gene expression through two different mechanisms. The first response to promoter methylation is the assembly of MBD-based protein complexes, including corepressor proteins (Rep) that provide rapid suppression of expression by preventing the binding of transcription factors [14]. For long-term stable gene suppression, MBD proteins can recruit histone deacetylases (HDACs) and, thus, initiate another mechanism of epigenetic regulation of gene expression: histone modification leading to chromatin condensation in the gene region [15, 16].

Demethylation of 5-methylcytosine involves TET methylcytosine dioxygenases 1, 2, and 3, which belong to the same family. They can catalyze the oxidation of 5-methylcytosine to 5-hydroxymethylcytosine, then to 5-formylcytosine, and finally to 5-carboxylcytosine

[17]. The resulting modified bases are not recognized as methylated ones by the cell molecular machinery and can remain relatively stable, being gradually lost during the synthesis of new DNA molecules in replication. This passive demethylation process is called replication-dependent dilution. In addition, 5-formylcytosine and 5-carboxylcytosine can be actively eliminated independently of replication through their cleavage from the sugar-phosphate backbone of DNA with the participation of thymine DNA glycosylase, with subsequent repair of the break by base excision repair [17].

As mentioned above, DNA methylation is closely related to the epigenetic mechanism of histone modification [16]. The most significant histone modifications include acetylation and methylation (see *Fig. 1B*). Histones are acetylated at lysine residues by histone acetyltransferases; the reverse process is carried out by histone deacetylases. High levels of histone acetylation contribute to less dense chromatin regions and, thus, increased DNA accessibility to chromatin-binding proteins and transcription enzymes, while a low acetylation level has the opposite effect. Methylation of histones at either lysine or arginine residues is catalyzed by histone methyltransferases, and the effect of methylation on chromatin density and, therefore, gene expression depends on the location of the amino acid residue and the number of methyl groups it possesses [18].

MicroRNA-mediated regulation of gene expression (see *Fig. 1C*) also largely depends on the level of DNA methylation, since it is performed at the post-transcriptional level, and the cellular microRNA level depends on the methylation status of their genes [19]. MicroRNAs are small (18–25 nt long) single-stranded non-coding RNA molecules that can complementarily bind to the target gene mRNA. Binding occurs mainly in the 3'-untranslated region of the target gene and triggers a cascade of reactions resulting in suppressed synthesis of its protein product. Full complementarity between a microRNA and its target mRNA upon their binding activates the enzymes of the endonuclease complex and a subsequent degradation of the target mRNA, while incomplete complementarity suppresses translation at either the initiation or elongation stage, cleavage of the mRNA poly-A sequence and translocation of the mRNA to P bodies for subsequent storage or degradation [20].

DNA methylation is the most studied process among the three described mechanisms of epigenetic regulation of gene expression. Considerable evidence indicating the key role of this process in the development of numerous autoimmune and neurodegenerative diseases in humans has been accumulated to date [8, 9]. These pathologies include MS. We will further consider a set of data that analyzes the contribution of DNA methylation to the development of this severe CNS disease.

EPIDEMIOLOGICAL, CLINICAL, AND ETIOLOGICAL FEATURES OF MULTIPLE SCLEROSIS

MS is a chronic autoimmune disease; its pathogenesis includes demyelination of CNS axons and neurodegeneration and is accompanied by progressive neurological dysfunction [3]. A steady increase in neurological deficit leads to irreversible disability in young, working age patients, which points to the high social and economic toll of the disease. MS is present almost all over the world, but its prevalence varies greatly in different populations. In the Russian Federation, the disease incidence is about 80 cases per 100,000 [21]. The disease's prevalence is on the increase, which is associated not only with growth in life expectancy and increasing success in the diagnosis of MS, but also with a real increase in its incidence [22].

MS is characterized by a pronounced clinical heterogeneity. Most patients have relapsing-remitting MS (RRMS) that is characterized by alternating periods of neurological deficit worsening (relapses) and reduction/disappearance of neurological symptoms (remissions). In the absence of effective drug therapy, about half of RRMS patients develop secondary progressive MS (SPMS) within 10 years from the onset of the disease, which is characterized by a steady increase in the degree of neurological deficit [23]. A similar clinical picture is observed from the very onset of the disease in 10–15% of patients, and this severe disease course is called primary progressive MS (PPMS) [24]. Different MS courses are characterized by different severities of the autoimmune, inflammatory, and neurodegenerative processes involved in its pathogenesis [25]. The changes in the degree of neurological deficit observed in different MS courses (RRMS, SPMS, and PPMS) are presented in *Fig. 3*.

Like other common autoimmune diseases with a pronounced inflammatory component, MS is generally considered a multifactorial disease; it develops in genetically predisposed individuals upon exposure to environmental factors. The effect of hereditary and external factors can be mediated by epigenetic mechanisms of gene expression regulation, mainly DNA methylation [3].

A fairly large number of environmental factors that can act as disease triggers have been identified so far, and many of them can affect the epigenetic mechanisms of gene expression regulation. These external factors include viral infections. For instance, there is a clear relationship between a high risk of MS and previous infectious mononucleosis, a disease caused by the

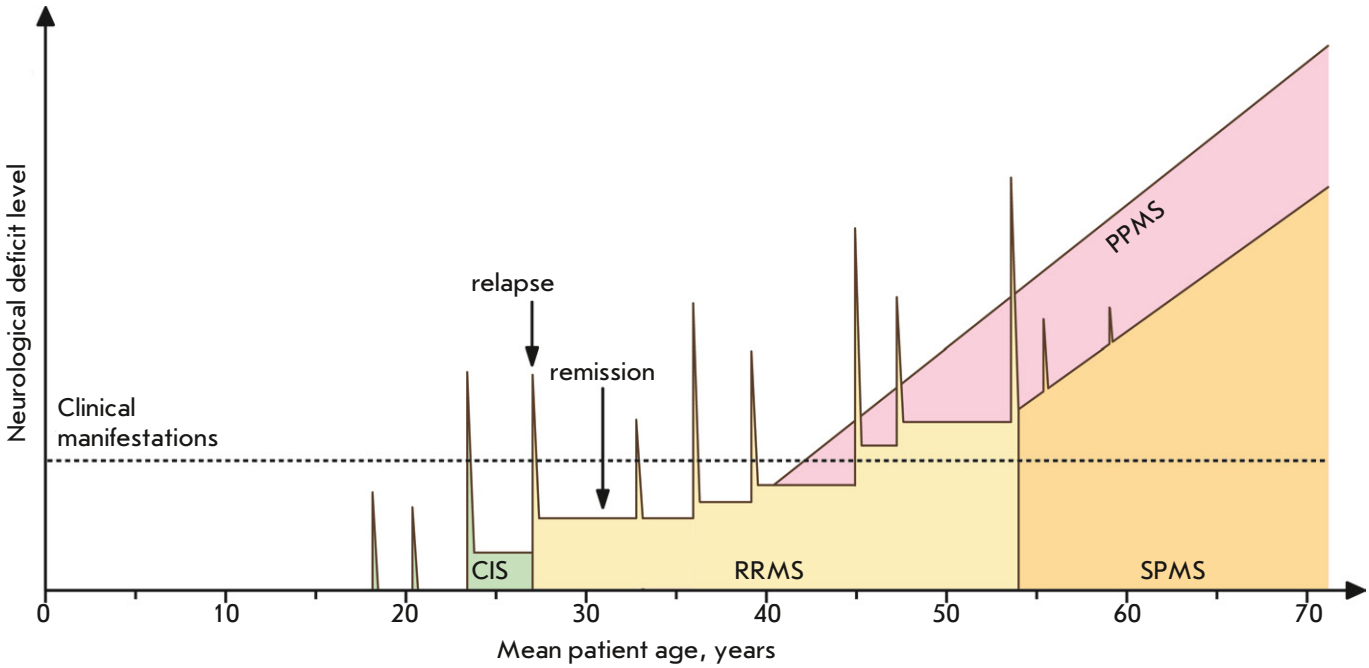

Fig. 3. Schematic representation of neurological changes during different clinical courses of MS. The dashed line indicates the neurological deficit level accompanied by clinical manifestations of the disease. The clinically isolated syndrome (CIS) is the first episode of clinical relapse typical of relapsing-remitting MS (RRMS). It can be followed by several years of clinical remission. Repeated relapses followed by periods of remission allow for diagnosing RRMS. Some patients with a prolonged medical history of RRMS develop secondary progressive MS (SPMS) characterized by a steady neurological worsening. Primary progressive MS (PPMS), which is characterized by neurological worsening without remissions from the onset of the disease, is also presented; PPMS manifests itself later than RRMS. The time scale is built based on the mean patient age at the onset of different clinical courses of MS [23, 24, 26]. The number and duration of relapses and remissions are shown schematically

Epstein–Barr virus [27]. Latent membrane protein 1 (LMP1) of the Epstein-Barr virus is homologous to the surface protein of CD40 B cells and is involved in the stimulation of B-cell-mediated immune and inflammatory responses, thereby increasing the risk of MS and other autoimmune diseases [28]. In addition to the direct stimulation of CD40-dependent signaling pathways, LMP1 can activate the epigenetic mechanism of DNA methylation in cells, which increases the overall methylation level of the host cell genome, resulting in a modulation of the expression of various genes [29].

Another important risk factor for MS is tobacco smoking [30]. Recent studies have shown that, like infection with the Epstein–Barr virus, smoking also stimulates DNA methylation in MS patients [31, 32]. In addition, smoking promotes histone modification and changes in the miRNA expression profiles in a number of cell lines: i.e., it affects all three key mechanisms of the epigenetic regulation of gene expression [33, 34]. The effect of other risk factors for MS, such as the levels of vitamin D [35] and female reproductive hormones [36, 37], on gene expression can also be mediated significantly by epigenetic processes [38–40].

Significant attention has been historically focused on the investigation of the genetic characteristics of MS. The first genomic region that was shown to be associated with the disease was the HLA major histocompatibility complex genes' locus. To date, the *1501 allele of the highly polymorphic HLA class II *DRB1* gene is considered the main MS risk marker. Besides this allele, other *DRB1* variants are associated with the disease in European populations: *0301, *0405, *0801, *1303, etc., as well as a number of alleles of HLA class I genes (HLA-A*0301, HLA-B*3701, *3801, as well as *4402, HLA-C*05, and *07) [41]. Genome-wide association studies (GWAS) proved most efficient in detecting new MS susceptibility markers outside the HLA locus. They have revealed more than 200 disease-associated polymorphic variants to date. At the same time, according to various estimates, the overall contribution of all those identified genetic variants can explain ≤ 48% of heritability [42]. The epigenetic mechanisms affect-

Table 1. Data on DNA methylation in MS patients obtained using the candidate gene approach

DNA source	Study group	Main result	Year [ref.]
T lymphocytes	RRMS patients, control group	Hypermethylation in the alternative *VDR* promoter in RRMS patients	2017 [43]
	RRMS patients, control group	Hypermethylation in the *IL2RA* promoter in RRMS patients	2017 [44]
PBMCs	RRMS patients, control group	An association between hypermethylation of LINE-1 retrotransposons and a high risk of RRMS with a low effectiveness of IFN-beta therapy was found	2017 [45]
	RRMS patients, control group	*IL2RA* gene analysis revealed no differences in its methylation status between study groups	2017 [44]
	RRMS patients, control group	Hypermethylation of *TET2* and *DNMT1* gene promoters was detected in RRMS patients; there were no significant differences in global methylation	2014 [46]
	RRMS patients, control group	Hypermethylation of the *PTPN6* promoter in RRMS patients	2012 [47]
	RRMS patients, control group	Analysis of *PADI2* and *PADI4* showed hypomethylation of the *PADI2* promoter in RRMS patients	2012 [48]
	Monozygotic twins discordant for MS	Analysis of *CIITA* revealed no differences in the methylation status between groups	2008 [49]
Whole blood	RRMS patients	Hypomethylation of *BDNF* in patients with higher disease progression rates	2018 [50]
	RRMS patients during relapse and remission, control group	Analysis of *RUNX3*, *MLH1*, *IGF2*, *CDKN2A*, *SOCS1*, *NEUROG1*, *CACNA1G*, and *CRABP1* showed differential methylation of *RUNX3*, *CDKN2A*, *SOCS1*, and *NEUROG1* in RRMS patients compared to controls; there were no differences between relapse and remission patients	2018 [51]
	RRMS patients, control group	Analysis of *TMEM39A* revealed no differences in the methylation status between the study groups	2017 [52]
	RRMS patients, control group	Hypermethylation of LINE-1 retrotransposons was detected in patients; methylation level correlated with the average disability score according to the EDSS	2016 [53]
	RRMS and PPMS patients	Analysis of *HLA-DRB1**1501 and *HLA-DRB5* found no association between their methylation status and clinical MS course	2010 [54]
Blood serum	RRMS patients, control group	Hypermethylation of some L1PA2 members of LINE-1 retrotransposons in RRMS patients	2018 [55]
	RRMS patients during relapse and remission, control group	Hypermethylation of *MOG* in RRMS patients during relapse compared to remission patients and the control group	2016 [56]
	RRMS patients during relapse and remission, control group	Analysis of a panel of 56 genes revealed significant differences in their methylation levels between all three groups	2010 [57]
Brain tissues	RRMS patients, control group	Analysis of *IL2RA* showed no relationship between its methylation status in different study groups	2017 [44]
	RRMS patients, control group	Hypermethylation of *PADI2* in normal white matter of RRMS patients	2007 [58]

ing gene expression in various cells and tissues and unrelated to changes in the DNA nucleotide sequence may be key in solving the problem of missing MS heritability.

STUDY OF DNA METHYLATION IN MULTIPLE SCLEROSIS

Studies of DNA methylation in MS started more than 10 years ago with the use of various approaches, the most common of which were the analysis of the differential methylation of individual candidate genes and genome-wide methylation analysis using high-density DNA microarrays or next generation sequencing (NGS). The DNA methylation analysis of promising candidate genes became the first approach to be used, since it was the most accessible. In the majority of those studies, the analysis was performed using either pyrosequencing or MALDI-TOF mass spectrometry of DNA amplification products after DNA bisulfite conversion, as well as methylation-specific PCR, followed by a comparison of average CpG methylation levels in the studied fragments. Generally, RRMS patients were studied and the control groups consisted of healthy individuals. These studies were few (only 16 articles have been found) and were carried out using DNA obtained from whole blood, its fractions, and brain tissue (*Table 1*).

As can be seen from *Table 1*, differential methylation of the genes involved in the regulation of autoimmune responses (*IL2RA*, *PTPN6*, and *SOCS1*) [44, 47, 51] and CNS function (*PADI2*, *CDKN2A*, *RUNX3*, *NEUROG1*, and *BDNF*) [48, 50, 51] was detected in the whole blood and various leukocyte populations of RRMS patients. The observed differences in DNA methylation levels turn out to be divergent, indicating the involvement of this epigenetic process in both the activation [47, 51] and suppression of inflammatory responses in the CNS [44, 48, 50]. Hypermethylation of the *VDR* gene, which codes for the vitamin D receptor whose deficiency is considered one of the key non-hereditary triggers of MS, as well as the *DNMT1* and *TET2* genes involved in DNA methylation and demethylation, respectively, was noted in the blood cells [43, 46].

A study of a set of 56 genes in serum-circulating DNA revealed differences in these genes' methylation levels, which allow for distinguishing RRMS patients during relapses from patients in remission and healthy individuals of the control group with > 70% sensitivity and specificity [57]. Another study showed hypermethylation of *MOG*, which encodes one of the myelin sheath proteins, in the serum of RRMS patients [56]. According to the authors, this may indicate impaired expression of *MOG* in oligodendrocytes, whose DNA enters the bloodstream after their destruction by demyelination. An analysis of brain tissues demonstrated hypomethylation of the peptidyl arginine deiminase type 2 (*PADI2*) gene that is involved in the post-translational modification of the key myelin sheath protein in neurons; namely, the myelin basic protein (MBP) [58]. The fact that this gene is also hypomethylated in the peripheral blood mononuclear cells (PBMCs) of RRMS patients may be an indication of the involvement of the regulatory mechanisms, which are similar among different tissues, in gene expression modulation [48].

The only study comparing the methylation levels of HLA-*DRB1* and *HLA-DRB5* in the whole blood of RRMS and PPMS patients [54] found no significant differences between these groups.

Studies of the methylation level of LINE retrotransposons should be mentioned separately. Under normal conditions, these repeated sequences contain many methylated CpG sites, which prevents the transcription of their genes [59]. Therefore, analysis of their differential methylation is a simple way to assess the global level of genome methylation in various tumors and some autoimmune diseases [55]. The methylation level of LINE-1 family retrotransposons was analyzed in RRMS patients in PBMCs, whole blood, and blood serum: LINE-1 hypermethylation was observed in all cases [45, 53, 55]. In addition, an association was found between a greater methylation level of LINE-1 and both severe disability according to the EDSS score and a low efficacy of IFN-beta therapy for RRMS [45, 53]. A good reproducibility of the data on the hypermethylation of LINE-1 elements in MS patients, as well as an association between their methylation levels, disease severity, and drug therapy effectiveness, is an indication that LINE-1 retrotransposons could become promising diagnostic and prognostic markers of MS.

In general, the data obtained using the candidate gene approach have shown that DNA methylation is involved in MS pathogenesis and they paved the way for the investigation of this epigenetic mechanism of gene expression regulation in MS patients using less sensitive, but much more efficient, genome-wide methods. The use of these methods, which primarily include high-density DNA microarrays and NGS, allows for the detection of differentially methylated sites (DMSs), individual CpG sites whose methylation levels change in MS, throughout the genome. *Table 2* summarizes the results of genome-wide studies of DNA methylation in MS patients using different groups for comparison.

It is important to note that the threshold of statistical significance (p) for DMS detection at the genome-wide level greatly varies between different studies. In five out of 18 works presented in *Table 2*, corrections for multiple comparisons were applied and the differences were considered significant at $p_{FDR} < 0.05$ [32, 60–63]. Other studies used a less stringent threshold of statistical significance: a nominal p value in a range of 0.05–0.0005. In addition to the p value, the minimum difference in the mean CpG methylation level between the compared groups (β), which most often varies within 5–10%, is also used as a selection criterion for DMS [64, 65]. Since DMSs not meeting the criteria selected by the authors are often omitted in publications, we will further rely on the p and β values the authors used for DMS detection.

Various blood cells and fractions (whole blood, serum, PBMCs, CD4+ and CD8+ T cells, CD19+ B cells, and CD14+ monocytes) were mostly used as a source of DNA in the published papers; brain tissue has been studied in only a few works. In some papers, twins discordant for MS were studied. However, in most cases, RRMS patients were compared to unrelated healthy individuals. A few studies analyzed a change in DNA methylation in RRMS patients during therapy using various drugs, during relapse and remission, as well as when comparing RRMS individuals with SPMS and/or PPMS groups.

In *Table 2*, the data obtained when analyzing DNA methylation profiles in pairs of monozygous twins discordant for MS should be discussed separately from

Table 2. Data on DNA methylation in MS patients obtained using high-throughput methods

DNA source	Study group	Main result	Year [ref.]
CD4+ T lymphocytes	Monozygotic twins discordant for MS (combined group of RRMS, SPMS, and PPMS patients)	Differential methylation of *FIRRE*	2019 [61]
	RRMS patients, control group	Differential methylation in *MOG/ZFP57*, *HLA-DRB1*, *NINJ2/LOC100049716*, and *SLFN12* genes	2019 [66]
	RRMS and SPMS patients, control group	Hypermethylation of the last exons of *VMP1/MIR21* in RRMS patients compared to the control group and SPMS patients	2018 [67]
	RRMS patients before and after treatment with dimethyl fumarate	A total of 945 DMSs, 97% of which were hypermethylated after treatment, were found; DMSs of *SNORD1A*, *SHTN1*, *MZB1*, and *TNF* were located in the promoter region	2018 [64]
	RRMS patients, control group	Differential methylation of the HLA locus in the region of *HLA-DRB1*, *HLA-DRB5*, and *RNF39*; DMSs were also found in the region of *HCG4B*, *PM20D1*, and *ERICH1*	2017 [65]
	RRMS patients, control group	There were no significant differences in DNA methylation between RRMS patients and healthy controls	2015 [60]
	RRMS patients, control group	Differential methylation of the HLA locus (19 DMSs in the region of HLA-*DRB1* and 55 DMSs beyond it); many of them are located within genes whose association with MS had been previously shown	2014 [68]
	Monozygotic twins discordant for MS	There were no significant differences in DNA methylation between twins	2010 [69]
CD8+ T lymphocytes	RRMS patients, control group	Differential methylation of *HLA-DRB1* and *SLFN12* in RRMS patients; global DNA hypermethylation	2019 [66]
	RRMS patients, control group	A total of 79 DMSs, none of which was located within *HLA-DRB1*	2015 [70]
	RRMS patients, control group	DNA hypermethylation was found in RRMS patients compared to the control; no differences in methylation levels of individual DMSs were noted	2015 [60]
CD19+ B lymphocytes	RRMS patients during treatment, control group	Multiple DMSs were found within *LTA* and in the region of PC-associated genes *SLC44A2*, *LTBR*, *CARD11*, and *CXCR5*	2018 [71]
CD14+ monocytes	RRMS patients, control group	Two DMSs in *HLA-DRB1*	2018 [72]
CD4+, CD8+, CD19+, and CD14+ leucocytes	RRMS and SPMS patients, control group	DNA methylation levels were assessed separately in CD4+, CD8+, CD19+, and CD14+ cells, followed by selection of DMSs that are universal for different cell types. RRMS- and SPMS-specific methylation patterns were identified	2019 [73]
PBMCs	Monozygotic twins discordant for MS (combined group of RRMS, SPMS, and PPMS patients)	Differential methylation of *TMEM232* and *ZBTB16* was observed in MS patients and then replicated in an independent sample. IFN-beta therapy induces hypomethylation of *RSAD2*, *MX1*, and *PLSCR1*	2019 [61]
	RRMS and PPMS patients, control group	DNA hypermethylation was found in PPMS patients compared to both RRMS and control groups; 30 and 67 DMSs were detected in RRMS and PPMS compared to the control, respectively; 51 DMSs were found when comparing two MS forms with each other	2016 [74]
Whole blood	RRMS patients, control group	The relationship between smoking and DNA methylation level was found in RRMS patients. The differences were more significant for women and carriers of MS risk haplotypes in the HLA locus	2017 [32]
	RRMS patients, control group	There were no significant differences in DNA methylation between RRMS and control patients	2015 [60]
Brain tissues	RRMS patients, control group	Global DNA hypermethylation and 2,811 individual DMSs were detected in RRMS patients	2019 [62]
	Demyelinated and healthy brain tissue of RRMS patients	Differential methylation of 16 genes, whose expression is characteristic of astrocytes and neurons, was found in the demyelinated hippocampal tissue	2017 [75]
	RRMS patients, control group	Hypermethylation of genes involved in maintaining the vital activity of oligodendrocytes and hypomethylation of genes involved in proteolytic processes were detected in MS patients	2014 [63]

the other results. Comparison of DNA methylation levels in the CD4+ T cells of twins discordant for MS revealed no significant differences in any of the three pairs studied: the number of DMSs observed when comparing twins from each pair was lower than that obtained when comparing unrelated healthy individuals [69]. A study of the same lymphocyte population revealed differential methylation of the *FIRRE* gene between twins, while the analysis of DNA methylation in PBMCs showed the presence of DMSs in the regions of *TMEM232* and *ZBTB16* [61]. However, since the study group included patients with RRMS, SPMS, and PPMS, the detected DMSs can be considered only epigenetic markers characteristic of MS in general. It is safe to state that the studies carried out using the twin methods have not led to any unambiguous conclusions so far.

As seen from *Table 2*, most of the published works compared DNA methylation levels in T cells (primarily CD4+) between RRMS patients and the control group; however, contradictory data were obtained in most of the cases. In particular, the results of six studies performed using CD4+ T cells can be compared to each other. For instance, a study by S.D. Bos *et al.* showed no significant differences in DNA methylation between RRMS individuals and healthy donors [60]. In a study by B. Rhead *et al.*, DMSs were found in RRMS patients in the *MOG/ZFP57*, *HLA-DRB1*, *NINJ2/LOC100049716*, and *SLFN12* genes [66]. S. Ruhrmann *et al.* detected DMSs clusters in the last two exons of the *VMP1/MIR21* gene [67]. Another two studies carried out by the same research group also revealed significant differences in methylation profiles between RRMS patients and healthy individuals [65, 68]. The only differentially methylated region identified in both works was the HLA locus, which turned out to be hypermethylated in patients, mainly in the *HLA-DRB1* region, while the markers of differential methylation outside the HLA locus found in [65] and [68] differed between each other and were not identified in other studies.

The results obtained in three works on DNA methylation in CD8+ T cells are also difficult to compare. S.D. Bos *et al.* noted global DNA hypermethylation in RRMS individuals; however, no significant differences in the methylation of individual CpG sites were found [60]. The data on global DNA hypermethylation were confirmed by B. Rhead *et al.*, who detected DMSs in the region of *HLA-DRB1* and *SLFN12* in RRMS patients [66]. No trend towards global hypermethylation was observed in a study by V.E. Maltby *et al.*; however, 79 separate DMSs were detected throughout the genome, none of which were located within either *HLA-DRB1* or *SLFN12* [70].

An analysis of CD19+ B cells revealed a DMS cluster in the *LTA* gene, and a number of DMSs in the *SLC44A2*, *LTBR*, *CARD11*, and *CXCR5* genes, which, according to GWAS, are associated with MS [71]. It should be noted that the RRMS group was heterogeneous in that study: it included both patients without drug therapy and patients taking various immunomodulatory drugs. Reduced methylation of *HLA-DRB1* was observed in the CD14+ monocytes of RRMS patients, mainly in *DRB1**1501 allele carriers [72].

Special attention should be paid to a recent comprehensive study that evaluated the levels of DNA methylation in all mentioned populations of blood leukocytes (CD4+ and CD8+ T cells, CD19+ B cells, and CD14+ monocytes) in RRMS and SPMS patients, as well as in healthy individuals in the control group, followed by a selection of DMSs common to different cell types [73]. This significantly increased the power of the statistical analysis and allowed for the identification of methylation patterns specific to RRMS and SPMS patients, which were then validated in DNA samples from CD14+ monocytes and the whole blood of independent groups of patients and healthy individuals. Although the use of these integrated approach does not allow for a detailed analysis of the role of DNA methylation in the functioning of individual populations of blood leukocytes during disease development, it helps to identify the features of DNA methylation that characterize different clinical forms of MS. This may be useful for their differential diagnosis at early disease stages; in addition, it also provides clues as to the development of new drugs that are highly effective in the therapy of MS forms poorly responsive to treatment.

PBMCs, a fraction of blood cells mostly consisting of all the previously mentioned subpopulations of leukocytes, can be used as a more accessible object for the search for DNA methylation markers characteristic of different MS forms. We carried out research using the case-control design and analyzed DNA methylation levels in the PBMCs of RRMS and PPMS patients. This analysis showed preferential hypermethylation of PBMC DNA in PPMS patients compared to both RRMS individuals and the control group, and it also revealed a set of individual DMSs specific to each of the studied MS forms [74]. This is the only genome-wide study performed in PPMS patients so far, and its data undoubtedly require validation in independent samples.

Very sparse studies on DNA methylation before and after a course of therapy with immunomodulatory drugs should be mentioned also. DNA isolated from the CD4+ T cells of the same RRMS patients was shown to have many DMSs throughout the genome associated with treatment, 97% of which were hypermethylated after treatment [64]. N. Souren *et al.* showed that intake

Table 3. Genes differentially methylated in MS in different populations of blood leukocytes according to the data of at least two independent studies and the biological functions of their protein products according to the UniProt [76] and NCBI Gene [77] databases

Gene	Biological function of the protein product	Reference
AHRR	Aryl hydrocarbon receptor repressor; it is involved in metabolism of xenobiotics and regulation of cell growth and differentiation	[68, 73]
ATP11A	The catalytic component of the P4-ATPase flippase complex, which ensures the maintenance of asymmetric distribution of phospholipids in membranes	[73, 74]
DLGAP2	Protein product can participate in molecular organization of synapses and nerve cell signaling	[70, 73]
DYDC2	Unknown	[70, 73]
ERICH1	Unknown	[65, 73]
GNG7	The gamma subunit of the G protein; it is involved in signaling in adenylate cyclase-dependent pathways in certain brain regions	[68, 73]
HLA-DQB1	Protein product is involved in presentation of antigenic peptides to CD4+ T lymphocytes as part of MHC class II molecules	[68, 73]
HLA-DRB1	Protein product is involved in presentation of antigenic peptides to CD4+ T lymphocytes as part of MHC class II molecules	[65, 66, 68, 72]
HLA-DRB5	Protein product is involved in presentation of antigenic peptides to CD4+ T lymphocytes as part of MHC class II molecules	[65, 68, 73]
HOXC4	Transcription factor involved in cell positioning along the anteroposterior body axis during ontogenesis	[73, 74]
TNXB	Protein product mediates the interaction between the cells and the extracellular matrix	[70, 73]
USP35	Protein product is involved in suppression of NF-kB and inhibition of PARK2-mediated degradation of mitochondria	[68, 73]
ZFYVE28	Negative regulator of epidermal growth factor receptor signaling	[73, 74]

of IFN-beta by patients induces hypomethylation of *RSAD2*, *MX1*, and *PLSCR1* in PBMCs [61]. Although these data require independent confirmation, they are indicative of an important role for the epigenetic mechanism of DNA methylation in the development and suppression of a MS pathology. In addition, they show the importance of taking into account not only the type of MS course [74], but also the intake of immunomodulatory drugs [64] when selecting homogeneous groups of MS patients for a DNA methylation analysis.

Only a few works [32, 60] used serum and whole blood as a biological source to search for differential methylation profiles characterizing MS, since a wide range of different body cells can be the source of DNA entering the bloodstream, and the observed changes in DNA methylation levels are difficult to interpret. S.D. Bos *et al.* revealed no significant differences in DNA methylation profiles in whole blood samples [60]. Another work showed an association between smoking and the DNA methylation level in the whole blood of RRMS patients, with the most significant differences being found in women and carriers of MS risk haplotypes of the HLA locus [32]. To date, there are only three studies that have analyzed DNA methylation in various brain tissues in MS. Individual DMSs were identified when comparing demyelinated and normal hippocampus tissue in MS patients [75], as well as the white matter [62] and the frontal cortex [63] of RRMS patients and the control group. Differences in the design of studies and in the biological source of DNA do not allow us to reliably compare the results of these works.

In general, despite a rather extensive amount of accumulated data, the HLA gene locus is the only genomic region whose differential methylation in the same biological source has been confirmed in independent studies [65, 66]. Meanwhile, the results obtained in [73] show that universal patterns of differential DNA methylation (at least in different populations of blood leukocytes) can exist in MS. Based on this data, we searched for DMSs identified in more than one study using both the candidate gene approach (*Table 1*) and the high-throughput DNA analysis (*Table 2*), regardless of which leukocyte populations were used as a DNA source. The identified genes and the main functions of their protein products are presented in *Table 3*.

The genes presented in *Table 3* are involved in the immune response (*HLA-DQB1*, *HLA-DRB1*,

HLA-DRB5, and *USP35*), signal transduction (*AHRR*, *ATP11A*, *GNG7*, *HOXC4*, and *ZFYVE28*), and the interaction with the matrix (*DLGAP2*, *TNXB*). The role of the *DYDC2* and *ERICH1* genes remains unknown. Most of the listed genes were identified in [73] as MS markers universal for different leukocyte populations, which is indicative of their contribution to MS pathogenesis at the level of the integral systems regulating a cell's vital activity, which are common among different cell types. Differential methylation of *HLA-DRB1* in MS was observed in four studies in CD4+ and CD8+ T-lyphocytes, as well as CD14+ monocytes [65, 66, 68, 72]. Although the authors of [73] did not consider DMSs in *HLA-DRB1* as MS-associated ones, other HLA genes were included in this category: *HLA-A*, *HLA-H*, *HLA-J*, *HLA-DRA*, *HLA-DQB1*, and *HLA-DRB5*. In addition, *HLA-DRB1* was found among the markers of differential methylation characteristic of SPMS [73].

HLA genes are believed to play a leading role in genetic predisposition to MS, and the level of significance of the association between the *HLA-DRB1**15 allele and MS development in GWAS studies exceeds $p < 5 \times 10^{-1000}$ [42]. Thus, the fact that, of the more than 200 GWAS-identified MS risk genes differences in methylation levels in at least two independent studies were shown for only HLA genes seems quite indicative. In most cases, during disease development, the effects of DNA methylation and genetic variability apparently manifest themselves through different gene sets, a fact that determines the relative independence of these processes from each other. In addition, DNA methylation almost never affects the master genes but exerts a small effect on the expression levels of many other genes.

In conclusion, the data obtained to date indicate the involvement of the epigenetic mechanism of DNA methylation in MS, which takes place in various blood cells and brain tissues. Further expansion of the list of known genes undergoing epigenetic regulation in MS will make a significant contribution to our understanding of the disease's pathogenesis. In addition, we may expect the identification of the genes whose methylation levels either differ in different MS courses or change upon exposure to immunomodulatory drugs, which may facilitate the development of effective prognostic tests and the identification of new therapeutic targets. ●

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Poly(ADP-Ribosyl) Code Functions

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ABSTRACT **Poly(ADP-ribosyl)ation plays a key role in cellular metabolism. Covalent poly(ADP-ribosyl)ation affects the activity of the proteins engaged in DNA repair, chromatin structure regulation, gene expression, RNA processing, ribosome biogenesis, and protein translation. Non-covalent PAR-dependent interactions are involved in the various types of cellular response to stress and viral infection, such as inflammation, hormonal signaling, and the immune response. The review discusses how structurally different poly(ADP-ribose) (PAR) molecules composed of identical monomers can differentially participate in various cellular processes acting as the so-called "PAR code." The article describes the ability of PAR polymers to form functional biomolecular clusters through a phase-separation in response to various signals. This phase-separation contributes to rapid spatial segregation of biochemical processes and effective recruitment of the necessary components. The cellular PAR level is tightly controlled by a network of regulatory proteins: PAR code writers, readers, and erasers. Impaired PAR metabolism is associated with the development of pathological processes causing oncological, cardiovascular, and neurodegenerative diseases. Pharmacological correction of the PAR level may represent a new approach to the treatment of various diseases.**
KEYWORDS **poly-ADP-ribose, PARP, PARG, PAR code, NAD+, phase separation.**
ABBREVIATIONS **ADPR – ADP-ribose; ADPRr – ADP-ribose residue; PAR – poly(ADP-ribose); PARG – poly(ADP-ribose) glycohydrolase; PARP – poly(ADP-ribose) polymerase.**

INTRODUCTION

The existence of a third nucleic acid, poly(ADP-ribose) (PAR), has been known for more than half a century. Unlike DNA and RNA, PAR has a rather simple structure composed of repeating ADP-ribose (ADPR) units, but it encodes neither proteins nor RNA (*Fig. 1*) [1]. However, involvement of PAR in cell death and metabolism, as well as highly regulated synthesis, metabolism, and degradation of PAR, indicates the crucial role it plays in the cell [2–4]. Usually, PAR covalently binds to proteins and changes their activity; for this reason, poly(ADP-ribosyl)ation is often considered as a post-translational protein modification [3, 4]. This covalent modification is known to regulate the functions of the proteins involved in a number of key nuclear and cytoplasmic events, such as DNA damage repair, chromatin structure regulation, gene expression, RNA processing, ribosome biogenesis, and protein translation [4–7]. In addition, there are non-covalent PAR-mediated interactions due to the presence of PAR-recognition domains in a number of proteins. Non-covalent interactions with PAR play an important role in the events determining the types of cellular response to a viral infection and stress: e.g., inflammation, hormonal signaling, and immune response [2, 8–11]. A lot of evidence of PAR involvement in diseases has been accumulated. For example, β-amyloid-mediated oxidative stress in Alzheimer's is accompanied by an increase in the PAR level; PAR also interacts with the α-synuclein that accelerates toxic fibril formation in Parkinson's disease [12]. Numerous studies have demonstrated that there is a relationship between PAR and the processes involved in tumorigenesis [13–17]. As early as in 1979, poly(ADP-ribosyl)ation inhibition by nicotinamide analogs was shown to increase the sensitivity of cancer cells to cytotoxic damage [18]. To date, more than 200 similar compounds are undergoing preclinical and clinical studies as antitumor agents and four poly(ADP-ribose) polymerase (PARP) inhibitors have already been used in practice [15, 19–22]. PAR is involved in cell reprogramming: intense poly(ADP-ribosyl)ation is observed in induced pluripotent stem cells, while inhibition of PAR synthesis reduces the ability of somatic cells transfected with Yamanaka factors (c-Myc, Sox2, and Oct4) to dedifferentiate [23–25]. These observations, as well as the fact

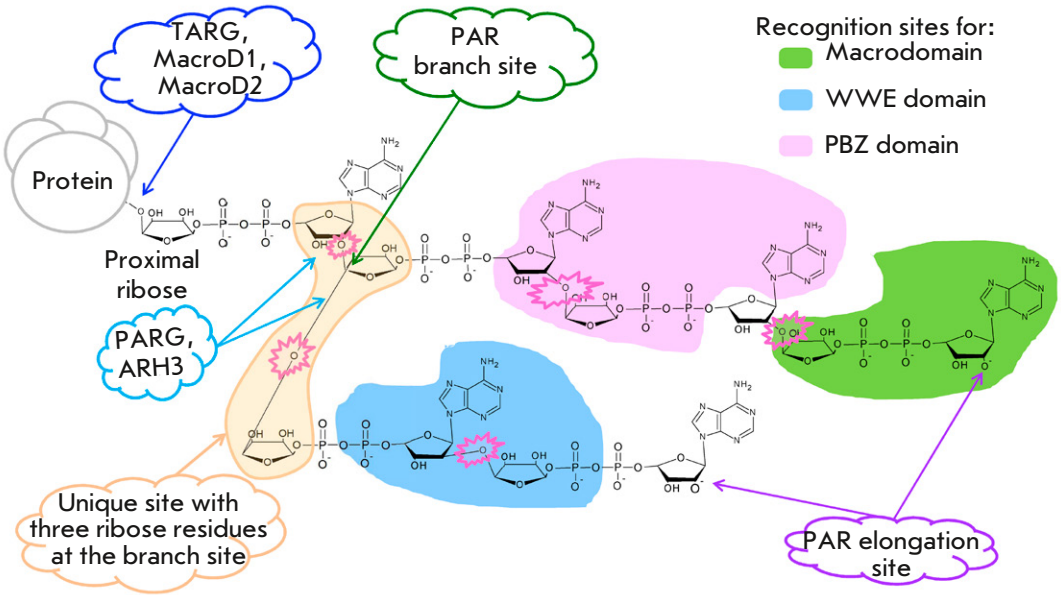

Fig. 1. Structure of PAR on the protein globule surface and recognition sites for PAR readers and erasers. The sites for ADP-ribose elongation and branching are shown. The O-glycosidic bonds of adjacent ADP-ribose residues are encircled by a pink sawtooth line. The blue and dark blue arrows denote the sites for PAR hydrolysis by various PAR erasers. The yellow contour shows a unique site at the branch point containing three ribose residues. The areas of interaction with PAR readers containing different PAR-binding domains are denoted by green, pink, and blue contours. The green contour shows the region of the binding macrodomains that preferably interact with terminal ADP-ribose. The pink contour denotes the interaction area with PBZ domains capable of simultaneous binding to adenines in two adjacent PAR ADP-ribose units. The blue contour encircles the area of interaction with the WWE domain that recognizes iso-ADP-ribose containing a specific 2', 1"-O-glycosidic bond

that the PAR-synthesizing enzyme PARP-1 recruits the KLF4 protein to activate telomerase expression and induce stem cell pluripotency, indicate that disruptions in the PAR regulation system may lead to a more aggressive tumor stem cell phenotype. Studies on the effect of poly(ADP-ribosyl)ation on life expectancy [26–28] and progeria (Werner [29] and Cockayne [30] syndromes of premature aging) deserve special consideration. Interestingly, oxidative damage to the cell causes PARP-1 activation, which promotes cardiac and vascular dysfunction under various pathophysiological conditions [31, 32]. Pharmacological inhibition of PAR is considered a promising approach to the treatment of non-oncological diseases, such as ischemic stroke, acute pancreatitis, septic shock, asthma, and acute lung injury [19, 31–34].

In general, the cellular PAR level is tightly controlled by enzymes and maintained at a low level through a finely tuned balance between the activities of poly(ADP-ribose) polymerases (PARPs) and poly(ADP-ribose) glycohydrolases (PARGs). Certain stress stimuli can rapidly increase PAR levels and trigger PAR-dependent pathways.

How can PAR molecules consisting of identical ADP-ribose monomers perform such diverse functions? How does the so-called PAR code work? This review considers the mechanisms of PAR code action, which depend on the polymer length and branching pattern, and discusses the proteins involved in code establishment, editing, and functioning.

PROTEINS INVOLVED IN PAR SYNTHESIS

Poly(ADP-ribosyl)ation begins with PARP-mediated attachment of the first ADP-ribose moiety to an acceptor protein (usually at glutamate, aspartate, lysine, asparagine, serine, and cysteine residues). PARPs are unique glycosyltransferases that catalyze the transfer of ADP-ribosyl residues (ADPRrs) from NAD+ to available protein groups and subsequent chain elongation through the formation of glycosidic bonds (1''-2', rarely 1'''-2'') between the ribosyl moieties of ADP-ribose monomers. Thus, a polymer composed of two to several hundred monomers and attached covalently to the protein forms (*Fig. 1*) [35–37]. A number of chromatin-associated proteins, including core and linker histones, topoisomerases, DNA ligases, DNA polymerases, and PARPs, can act as PAR chain acceptors [5].

PAR-synthesizing proteins are often referred to as PAR writers. PARPs are the main enzymes providing the PAR structural diversity that is the basis of

Table 1. PARP subfamilies

PARP subfamilies	Subfamily members and their features
DNA-dependent PARPs	DNA-dependent PARPs are activated upon DNA damage due to the presence of DNA-binding domains. The main representative, PARP-1 (ARTD1), has three DNA-binding domains (so-called zinc fingers) for damage recognition. Other subfamily members are PARP-2 (ARTD2) and PARP-3 (ARTD3).
Tankyrases	Tankyrases contain ankyrin repeats and highly specific sterile alpha motifs (SAMs) responsible for protein-protein interactions. Representatives include tankyrase-1 (PARP-5a, ARTD5) and tankyrase-2 (PARP-5b, ARTD6).
CCCH PARPs	CCCH PARPs contain a zinc finger domain with a $CX_{7-11}CX_{3-9}CX_3H$ CCCH motif interacting with RNA. These PARPs share a common WWE domain. Representatives include TIPARP (PARP-7, ARTD7), PARP-12 (ARTD12), and PARP-13 (ARTD13).
Macro PARPs	Macro PARPs contain macrodomains and mediate the association of poly- (and, possibly, mono-) ADP-ribosylated proteins. Representatives are BAL1 (PARP-9, ARTD9), BAL2 (PARP-14, ARTD8), and BAL3 (PARP-15, ARTD7).
Other PARPs	PARP proteins not included in the above subfamilies. Representatives are PARP-4 (ARTD4), PARP-6 (ARTD17), PARP-8 (ARTD16), PARP-10 (ARTD10), PARP-11 (ARTD11), and PARP-16 (ARTD15).

the PAR code. Bacterial ADP-ribosyl transferases (ADPRTs) (e.g., cholera and diphtheria toxins) and members of different yeast and animal protein families, such as arginine-specific ectoenzymes (ARTCs) and sirtuins (SIRTs), can also catalyze ADP-ribosylation.

The human PARP family includes 17 known proteins that differ in their polypeptide chain length, non-catalytic domain structure, ability to modify acceptor proteins, expression level, and intracellular distribution [2, 4, 5, 13, 38, 39]. A feature of all members of the family is a rather conserved C-terminal amino acid sequence containing a catalytic center that is a PARP signature. Most PARPs (PARP-3, 4, 6–8, 10–12, and 14–16) mono-ADP-ribosylate proteins, and only four PARPs (PARP-1, 2, 5a, and -5b), are capable of poly(ADP-ribosyl)ation. High evolutionary conservation of the primary structure of the PARP catalytic site shows that the functions of these enzymes are extremely important for the cell and the whole body. A unique feature of the PARP catalytic pocket is the ART domain, whose key motif is either the histidine-tyrosine-glutamate (HYE) triad in PARP-1–4, 5a, and 5b or the histidine-tyrosine-hydrophobic (HYφ) amino acid triad in PARP 6–8, 10–12, and 14–16 [36]. In both triads (HYE and HYφ), the conserved histidine forms a hydrogen bond with a 2-OH-ribose of the NAD+ adenosine, while conserved tyrosine residues form π–π stacking interactions with the NAD+ nicotinamide moiety. Probably, variation in the last amino acid residue in the triads controls the ability for either poly(ADP-ribosyl)ation [40] or mono-ADP-ribosylation [41]. The PARP family is currently divided into five subfamilies, based on their structural and functional features (*Table 1*).

PAR synthesis is mainly performed by PARP-1 and PARP-2 (75%–95% and 5%–15%, respectively) in response to DNA damage [42–44]. Studies *in vivo* and in cell cultures have shown that a decrease in the level of PARP-1 or PARP-2 increases cell sensitivity to ionizing radiation, oxidative stress, and alkylating agents [45].

PROTEINS HYDROLYSING PAR POLYMERS

PAR polymers are actively synthesized and hydrolyzed in the cell [6, 46]. ADP-ribosyl hydrolase 3 (ARH3), PAR glycohydrolases (PARGs), TARG/C6orf130, MacroD1, MacroD2, and NUDIX family hydrolases [2, 3, 6, 41] remove ADPR covalently bound to proteins and modulate the PAR code. All these proteins are termed PAR erasers. Many of these enzymes contain a macrodomain fold motif that allows for interaction with ADP-ribosylated substrates. PAR degradation occurs in two steps: the polymer chain is first cleaved to single ADPRrs, and the protein-bound proximal residue is then hydrolyzed (*Fig. 1*). The hydrolases PARG and ARH3 effectively cleave unique 2'–1"-glycosidic ribose–ribose bonds and release free ADPR fragments, with the proximal ADPR remaining attached to the acceptor protein [47]. Some enzymes, namely TARG, MacroD1, and MacroD2, hydrolyze an ester bond between the remaining ribose and protein acceptor amino acids, finally removing the ADPRr. The complex system of hydrolase functioning that changes the local concentration and length of PAR (i.e. modulates the PAR code) is complemented by fine regulation of specific recognition of ADPR complexed with various amino acid residues: in particular, ARH1 with Arg, ARH3 with Ser, and MacroD1, MacroD2, and TARG1 with Glu and Asp [3].

Table 2. PAR-recognizing modules

Module	Description	Recognition mechanism	Representatives	Functions	References
PBM	~20 a.a. [HKR] xx[AIQVY[KR]$_2$[AILV] [FILPV] (where x stand for any amino acid)	Binding is mediated by electrostatic interactions between negatively charged PAR residues and a positively charged PBM consensus sequence; it can achieve high affinity with the complex dissociation constant (K_d) values in the submicromolar and nanomolar ranges. Interactions are enhanced by tandem arrangement of PBM modules within a protein	H1, H2A, H2B, H3, H4, p21, p53, XRCC1, XPA, MSH6, ERCC6, ATM, MRE11, DNA-PKcs, KU70, DNA ligase 3, NF-kB, TERT, DEK, CAD, CENP-A, CENP-B, lamin A/C, BUB3, hCAP-D2, HK1, HKDC1, G3BP1, hnRNPA1, hnRNPK, hnRNPH, hnRNPG, hnRNPM, iNOS hnRNPA2B1, hnRNPC1C2, AURKAIP1, RECQL5, WRN, and TOP1	PBMs are found in many proteins participating in the cellular response to DNA damage, as well as in replication, transcription, and chromatin rearrangements	[54, 55, 57–59]
Macrodomains	Evolutionarily conserved structural modules composed of ~130–190 a.a. packed into a characteristic core sandwich fold consisting of a six-stranded β-sheet surrounded by five α-helices. It is found in proteins with various cellular functions. MacroD motif: Nx(6)GG[V/L/I]D and G[V/I/A][Y/F]G	Recognition of terminal ADP-ribose residues. K_d values are in the micromolar range. ADPR-binding sites are located in the macrodomain internal cavity	Macrodomains are widespread among all kingdoms, including eukaryotes, prokaryotes, and archaea. The families are MacroH2A, MacroD, Macro2, ALC1, PARG, and SUD-M. Protein members are GDAP2, TARG1 (c6orf130), PARP-9, PARP-14, and PARP-15	Macrodomains have a regulatory effect on inter- and intracellular signaling, transcription, DNA repair, genomic stability maintenance, telomere dynamics, differentiation, proliferation, and cell death. The macrodomains of a number of proteins have catalytic activity. PARG uses a macrodomain for PAR binding and hydrolysis. MacroD and C6orf130 are involved in deacetylation of O-acetyl-ADP-ribose (a metabolite of sirtuin-mediated deacetylation of Lys). Catalytically active macrodomains in Coronaviridae, Togaviridae, and Hepeviridae viruses counteract the innate immune response, interfering with PARP-mediated antiviral protection	[60–64]
PBZ	~30 a.a. C2H2 type: [K/R] xxCx[F/Y] GxxCxbbxxxxHxxx[F/Y] xH	PBZ lacks secondary structure; substrate recognition is achieved through hydrogen bonds. One PBZ module is supposed to contain two binding sites that simultaneously recognize adenines in two adjacent ADPRrs in PAR, which is a distinctive feature of interaction with PBZ.	APLF, CHFR, and SNM1A	DNA damage signaling. APLF promotes retention of specific NHEJ subunits in repair of double-stranded DNA breaks and stimulates the rate of NHEJ repair. CHFR is involved in regulation of the onset of mitosis	[55, 65, 66]
WWE	~80–100 a.a. Six antiparallel β-strands of the WWE domain form a half barrel structure with an α-helix in its center	Interaction occurs through phosphate groups on each iso-ADP-ribose side, which binds to a positively charged edge of the WWE domain. The interaction is accompanied by penetration of the adenine aromatic ring into the binding pocket. Binding is characterized by high affinity (K_d ~370 nM) and specificity	RNF146/Iduna	RNF146 is an E3 ubiquitin ligase that specifically recognizes PAR-conjugated protein substrates and targets them for proteasomal degradation	[67, 68]
FHA/BRCT	~80–100 a.a.	Phosphate-binding pockets interact with ADP- and iso-ADP-ribose residues	APTX, PNKP, XRCC1, NBS1, BARD1, and DNA ligase 4	DNA damage signaling and repair	[69]

Module	Description	Recognition mechanism	Representatives	Functions	References
RRM	~60–80 a.a.	The canonical RRM structure consists of four antiparallel β-strands and two α-helices located on one side of the β-sheet. The RRM domain is characterized by the presence of either 6 a.a. or 8 a.a. consensus with an exposed aromatic residue forming π–π stacking with RNA bases	Families: BRUNO, CPEB, DAZ, EIF, ELAVL, ENOX, G3BP, HNRP, IGF2BP, MSI, PABPC, PPARGC, PTBP, RALY, RAVER, RBM, RBMS, RBMY1, SAF, SF3B, SFRS, SNRP, and U2AF. Proteins: ASF/SF2, NONO, SPEN, SR140, SRRP35, SSB, SYNCRIP, TARDBP, THOC4, RBMX, TAF15, PARP-10, and PARP-14	RNA metabolism, DNA damage signaling and repair. Targets include heterogeneous nuclear ribonucleoproteins, the proteins involved in the regulation of alternative splicing of proteins comprising small nuclear ribonucleoproteins and proteins regulating RNA stability and translation	[70–72]
SR- and KR-rich motifs	Variable	Presumably electrostatic interactions	ASF/SF2 and dMi-2	Gene expression and RNA metabolism	[54]
OB fold	~70–150 a.a. Oligonucleotide/oligosaccharide binding	Interactions with iso-ADP-ribose residues	SSB1 and BRCA2	DNA damage signaling and repair	[73]
PIN domains	~130–150 a.a.	Presumably electrostatic interactions	EXO1	DNA damage signaling and repair	[74]
RG/RGG repeats	Tri-RGG: $RGG(X_{0-4})RGG(X_{0-4})RGG$ Di-RGG $RGG(X_{0-4})RGG$ Tri-RG $RG(X_{0-4})RG(X_{0-4})RG$ Di-RG $RG(X_{0-4})RG$	Presumably electrostatic interactions. In addition, aromatic residues are often found between RGG repeats; they enable hydrophobic interactions with nitrogenous bases	Tri-RGG: FUS/TLS, EWS/EWSR1, TAF15, nucleolin, fibrillarin, SERBP1, hnRNP U, hnRNP A1, LSM14/Scd6, CHTOP, GAR1, MLL4. Di-RGG: Sam68, RPS2, hnRNP K, SYNCRIP, BRWD3, PSF, FMRP, SPRN, RasiP1 NSD1, Aven, hnRNPUL1. Tri-RG: MRE11/A, Sm-D1/D3, KDM4E, PABP1, CIRBP, ING5, SHANK1, BAZ1A, MBD2, DDX5, DDX5, TDRD3, ILF3, 53BP1, Coilin, DHX9. Di-RG: ADAM20, E2F-1, E2F-1, Gemin 5, HMGA1, DGCR14, PDGFRB, FXR2; SRSF1, ABL2, SETD5, CPSF, BRD4, MBP, MBNL1, TGFbR, NFKBIL1, and RBBP6	Binding of various secondary RNA structures (G-quadruplexes and guanine tetrads), snRNA biogenesis, alternative splicing, translation repression (LSM14A/Scd6), DNA damage signaling, apoptosis, G-quadruplex folding, stress granule assembly, and formation of protein condensates	[75–79]

Proteins regulating PAR degradation are considered attractive therapeutic targets [6]. The first group of compounds modulating PARG activity consisted of DNA intercalators capable of association with PARs, protecting them from hydrolysis by PARGs [48]. Intercalators affect PARG activity not through direct interaction with the enzyme but by hindering its access to the substrate. Later, natural polyphenolic compounds, such as tannins directly inhibiting PARG activity, were discovered [49]. In particular, gallotannin was shown to inhibit PARG and trigger synthetic lethality in BRCA2-deficient tumors [50]. Several classes of PARG inhibitors have been studied and described so far: ADP–HPD, rhodamine inhibitors, and PDD00017273. Approaches aimed at stabilizing PARG mRNA through interaction with RNA-binding proteins (HuR) are also being developed [6, 51–53].

PROTEINS RECOGNIZING PAR STRUCTURAL FEATURES

Proteins containing modules capable of recognizing ("reading") the PAR structures by binding different ADPR polymer forms and acting as the so-called PAR readers have been identified over the past decade [3, 39, 54–56]. Hundreds of proteins interact with PAR directly or indirectly, thus causing subcellular redistribution of proteins and affecting many cellular processes. The structures of PAR-binding protein modules vary from highly structured domains to disordered structures (*Table 2*).

PRINCIPLES OF PAR CODE FUNCTIONING

Thus, a complex system of PAR synthesis, functioning, and degradation exists in the cell. This system regulates protein functions using the code determined by the PAR structure. The PAR code is controlled by both the

PAR polymer length and the branching pattern. How does the PAR code work?

PAR length

PAR can be cytotoxic to cells under certain conditions [9]. A decrease in PARG expression, leading to PAR accumulation in the cell, enhances cell death in the presence of damaging agents both *in vitro* and *in vivo*; PARG knockout mice die on day 3.5 of embryonic development [80]. PAR-mediated cytotoxicity was previously explained by a suicide hypothesis based on cellular energy collapse caused by PARP-dependent depletion of NAD+ stores [81, 82]. Since the synthesis of a NAD+ molecule requires four ATP molecules, robust PARP activity can deplete reserves of high-energy molecules, suppress cellular energy-dependent processes such as glycolysis and mitochondrial respiration, and ultimately cause cell death [83]. However, PAR polymers themselves can be cytotoxic to cells, with the cytotoxicity level, as shown in cortical neurons, climbing with an increase in the polymer chain length and being dose-dependent (*Fig. 2*) [81]. At the same time, intracellular administration of anti-PAR antibodies significantly reduces cytotoxicity. The mechanisms of high-molecular-weight PAR cytotoxicity are being studied. The apoptosis-inducing factor (AIF) was found to be released from mitochondrial membranes in response to the treatment of isolated mitochondria with purified PARs [84]. This process also occurs in the cell's cytoplasm, causing AIF translocation to the nucleus and cell death initiation through the mechanism of caspase-independent apoptosis. This type of programmed cell death, caused by hyperactivation of PAR synthesis, is called parthanatos. Parthanatos can be activated by severe DNA damage due to the action of alkylating agents, as well as by oxidative stress, hypoxia, hypoglycemia, and inflammation.

Depending on its length, PAR can interact with different regulatory proteins (*Fig. 2*). The human tumor suppressor protein p53 non-covalently binds to PAR and has three potential binding sites [56]. PARs longer than 50 ADPRrs are capable of high-affinity interaction with p53, while 38- to 50-mer and 5- to 38-mer PARs display moderate and weak affinity for p53, respectively [85]. Furthermore, 16- and 55-mer PARs form one and three types of complexes with the p53 protein, with dissociation constants of 250 and 130 nM, respectively [85].

Another protein interacting with PAR is the nucleotide excision repair factor XPA. XPA contains a zinc finger domain; the protein recognizes a damaged DNA region and interacts with other components of the DNA repair system. XPA does not bind to short (16-mer) PARs but forms a 1 : 1 complex with 55-mer

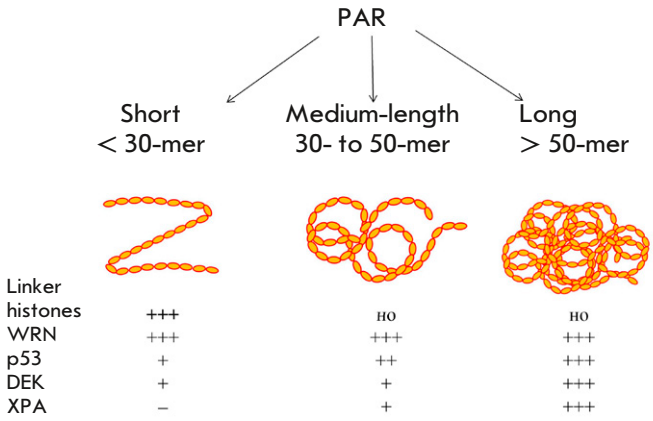

Fig. 2. PAR length determines its association with PAR-binding proteins. The relative strength of interaction between a particular protein and PAR of a specific length is indicated by a series of crosses: "+++" – high, "++" – medium, "+" – low interaction strength, "–" – no interaction

PAR molecules (K_d ~370 nM) [85]. A PAR-binding site overlapping with the TFIIH-recognizing region was identified in the C-terminus of the XPA protein; the TFIIH factor is involved in the initiation of transcription [86] and, together with DNA repair proteins, nucleotide excision repair [56]. It is possible that interaction with PAR may, thus, regulate XPA activity during nucleotide excision repair.

The interaction of the DEK oncoprotein with PAR also turns out to be dependent on the polymer length. DEK is involved in various intracellular processes: replication [87, 88], DNA repair [89], RNA processing [90], and transcription regulation [91–93]. High DEK levels were shown to contribute to cell immortalization, as well as suppress aging and apoptosis [94, 95]. DEK is also associated with several autoimmune disorders [96]. A number of DEK functions are regulated by either direct poly(ADP-ribosyl)ation or non-covalent interaction with PAR. Mapping of PAR-binding sites in DEK showed that the DEK region of a.a. 195–222 efficiently binds PAR, while the other two DEK regions exhibit a weaker affinity for PAR [97]. PAR chains longer than 57 ADPRrs form complexes with DEK, with a K_d ~60 nM. PAR chains containing 34–54 ADPRrs exhibit moderate affinity for DEK; the interaction is weaker in the case of shorter polymers. Poly(ADP-ribosyl)ation disrupts the ability of DEK to bind DNA through the SAP domain, while non-covalent interactions with PAR polymers very weakly inhibit the DEK–DNA interaction [89].

Some proteins, on the contrary, efficiently interact with short PAR polymers (*Fig. 2*). For instance, histone H1 actively binds to 15- to 19-mer polymers [97].

PAR non-covalently interacts with histone H1 through the protein's C-terminal domain, which is enriched in lysine residues [98]. Furthermore, PAR and DNA compete for binding to histone H1. PAR is suggested to be able to displace histone H1 from chromatin, preserving it in the immediate vicinity of the chain break site and, thus, implementing the "histone shuttle" mechanism [99].

We should note that the linker and core histones not only can interact non-covalently with PAR, but can also undergo covalent poly(ADP-ribosyl)ation upon PARP activation. PARP-1 and PARP-2 were shown to modify the C- and N-termini of histones H1 and H2B, respectively, causing chromatin relaxation and facilitating the recruitment of repair proteins to the damage site [100–103].

The WRN factor binds equally effectively both short (10–50-mer) and long (> 50-mer) PAR polymers [104]. Interaction with PAR directly affects the WRN functions [104] that are associated with such aspects of DNA metabolism as replication, repair, and telomere length maintenance [105, 106]. A mutation in the *WRN* gene causes the hereditary Werner syndrome that is characterized by premature aging and a high risk of tumors [106], which may be explained by a high susceptibility to genotoxic stress at the cellular level. PAR can also compete with DNA for binding to the WRN N-terminal region comprising both the DNA-binding domain and the PBM domain [104]. PAR at a concentration of 10 μM inhibits WRN helicase activity, while > 50 μM PAR inhibits WRN exonuclease activity. These effects can be caused by conformational changes in WRN upon PAR binding, which lead to allosteric inhibition of the enzyme.

Why do different proteins prefer PARs of different lengths? The molecular basis for PAR recognition has not been established yet. It is possible that PAR polymers form different secondary structures, depending on their length and branching pattern (*Fig. 2*). Molecular modeling shows that five-mer PARs have a compact disordered structure, and ≥ 25-mer PARs can form several globular subdomains linked by unfolded regions [107]. As shown by circular dichroism experiments, PAR polymers (~32 units) can adopt helical conformations either in the presence of 0.1 mM spermine, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, > 3 M NaCl, or at pH > 5 [38].

PAR branching

Although PAR branching chains were identified about 40 years ago [108], their biological functions and interactions with other cell nucleus components are still the subject of discussion. Branching PAR chains are formed with involvement of PARP-1 and PARP-2 [40, 109–111]. The unique branching pattern is achieved due to the fact that three ADP-ribose residues become linked to each other (*Fig. 1*), while known PAR-binding protein modules can recognize either one or two residues [3]. Thus, several PAR-binding domains must be coordinated to interact with the branched PAR site. Indeed, the APLF protein, which possesses two tandem PBZ domains, is capable of such binding, while the loss of the second PBZ domain switches APLF recognition from branched to linear PARs. APLF functions as a histone chaperone that preferentially binds to an H3/H4 tetramer and promotes histone release for chromatin relaxation [66, 112]. PAR chain branching provides APLF recruitment for DNA damage repair; PARP-2-deficient cells exhibit impaired kinetics of APLF recruitment to DNA damage sites. Other candidates for interaction with branched PAR sites are PARP family proteins, many of which contain tandems of PAR recognition domains [4, 38]. PARP-2 was found to interact with PAR via its N-terminal region, the so-called NTR, which lacks any specific structure [43, 113]. The PARP-2 NTR shares homology with the SAP domains of other proteins involved in chromatin organization and DNA repair, such as Ku70 and APE1 [44, 113, 114]. NTR deletion disrupted the PARP-2 ability to interact with PAR and suppressed its enzymatic activity in [109]. Since PARP-2 binds to PAR, the question arises as to whether this binding plays a significant role in the recruitment of PARP-2 to a damage site in the cell. Summarizing the data from various laboratories, we may suggest the following mechanism: PARP-1 is the first ($T_{1/2}$ ~1.6 s) to occur at the damage site [7, 110, 111, 115–118] and to synthesize the first PAR chains (*Fig. 3*). PARP-2 binds later (after ~30 s), accumulates at the DNA damage site (~2 min), and synthesizes secondary, predominantly branched PARs [109]. Treatment of cells with olaparib (PARP inhibitor) inhibits PARP-2 recruitment, while PARP-2 recruitment to the damage site in PARP-1-deficient cells occurs with a low efficiency [42]. These results suggest that PARP-2 recognizes PAR synthesized by PARP-1; PAR, in turn, mediates PARP-2 recruitment to the DNA damage site. In addition, PARP-1 and PARP-2 are characterized by short-term and long-term accumulation at the damage site, respectively [118].

It is also possible that branched PAR functions include recruitment of unique proteins and creation of the high-molecular-weight condensates involved in certain intracellular processes.

PAR participation in the formation of subcellular liquid-phase structures

Many subcellular compartments lack membranes. They form by separation of liquid phases and enable the cell to spatially separate different biochemical processes

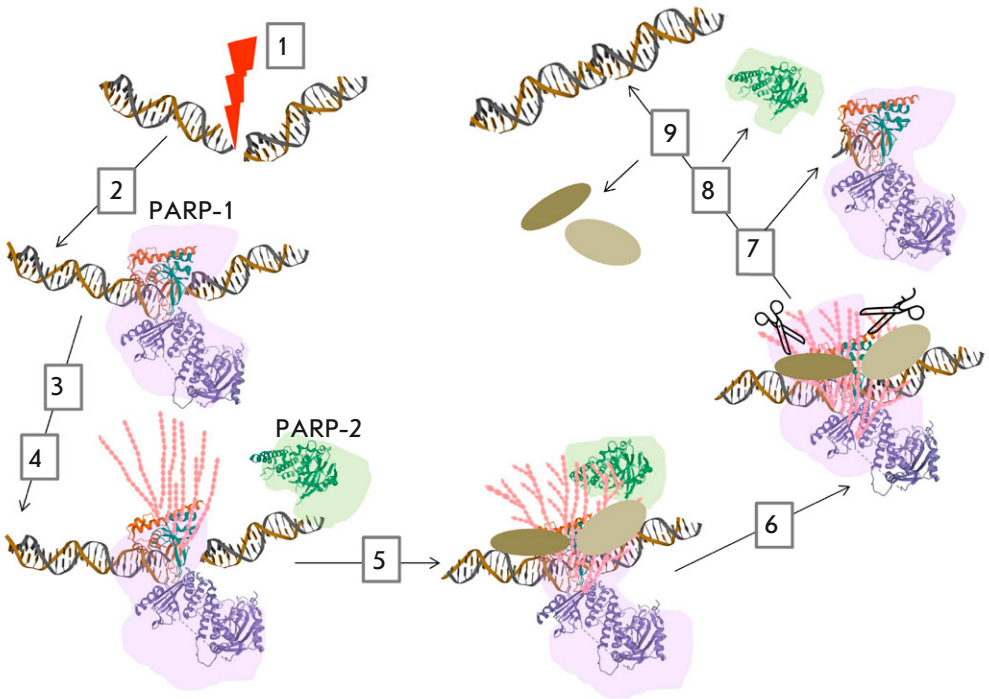

Fig. 3. Schematic representation of the combined action of PARP-1 and PARP-2 (PAR writers) during DNA damage repair: 1) DNA damage; 2) PARP-1 is the first protein to be bound at the damage site ($T_{1/2}$ ~1.6 s); 3) synthesis of primary PAR chains by PARP-1; 4) PARP-2 recruitment (after ~30 s) and accumulation (within ~2 min) at a DNA damage site; 5) synthesis of secondary PAR chains by PARP-2 and recruitment of repair factors (PAR readers); 6) degradation of PAR polymers by hydrolases (PAR erasers); 7) dissociation of PARP-1 and 8) PARP-2; 9) DNA repair and dissociation of repair factors

[119, 120]. Membraneless organelles (biomolecular condensates) resulting from phase transitions of macromolecular complexes include the nucleolus, nuclear bodies, Cajal bodies, DNA foci, PML bodies, and stress granules. Polymers composed of nucleic acids and proteins and containing disordered domains or, as they are usually called, low-complexity domains, play the most important role in the formation of these condensates. These domains are characterized by a tendency towards energetically favorable condensation due to weak but multivalent interactions between polymers [110, 121–123]. Single-stranded nucleic acids represent an ideal multivalent scaffold for the formation of numerous bonds with disordered protein domains and the production of biomolecular condensates [124, 125]. Currently, there is growing evidence of the important role of PAR in the initiation of the formation of these condensates (*Fig. 4*) [3]. PAR has a rather simple structure composed of repeating monomers, with a large binding surface area recognized by various proteins. PAR adenine bases occur in the anti-conformation, which exposes them to potential interaction with other molecules [126]. Furthermore, PAR is characterized by active synthesis and degradation kinetics, which allows PAR to serve as a temporary scaffold for both initiation of molecular condensates and destruction of these structures, which provides fast phase transitions "on demand," i.e. in response to changes in the microenvironment. A number of researchers have shown that PAR induces regulated formation of molecular condensates by recruiting proteins containing disordered domains [38, 59, 127, 128]. It is possible that the PAR length, branching pattern, and concentration affect the formation of these molecular condensates through a change in the scaffold area accessible to protein binding. The electrostatic interaction between PAR and proteins, which is crucial for phase separation, can be disturbed by introducing a negative charge into the proteins (e.g., through their regulatory phosphorylation) [75].

PAR is involved in the organization of liquid-phase membraneless organelles, such as the nucleolus, stress granules, and DNA foci (DNA damage sites) [3, 38, 129]. A mechanism for the formation of membraneless repair compartments, which is mediated by interaction of disordered FUS domains with PAR, has been proposed [127]. These compartments provide highly effective repair thanks to local accumulation of repair proteins and separation of damaged DNA from intact DNA [75, 127, 130].

Other liquid-phase membraneless compartments associated with PAR are ribonucleoprotein structures: stress granules and P-bodies (*Fig. 4*). These structures are involved in RNA metabolism, including control of mRNA stability and translation [131]. Poly(ADP-ribosyl)ation serves as an important regulator of the dynamics of ribonucleoprotein complexes. Formation of ribonucleoprotein complexes during prolonged stress

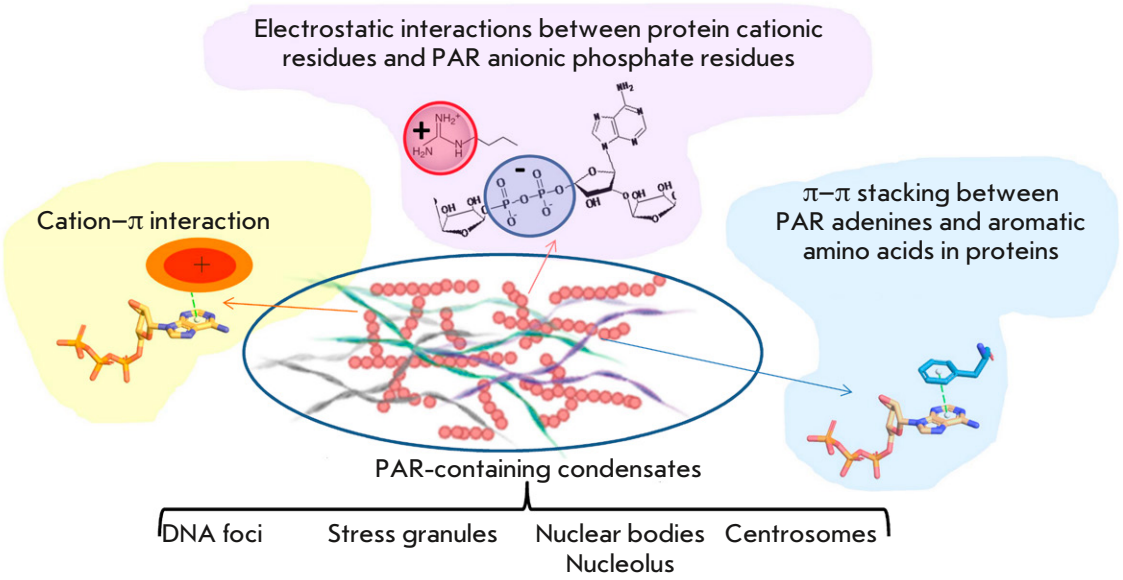

Fig. 4. PAR-dependent biomolecular condensates formed by phase separation; the PAR–protein interactions stabilizing these condensates

and excessive activation of PAR synthesis becomes pathological and leads to the formation of insoluble aggregates.

The PAR-mediated mechanism of phase transition provides for the formation of transient transcriptional complexes at expressed genes through the C-terminal domain (CTD) of RNA polymerase II, which contains a disordered domain capable of multivalent interaction [132–134]. CTD phosphorylation releases RNA polymerase II from these transcriptional complexes. PARP-1 is found at the promoters of actively transcribed genes, its activity stimulating post-translational modifications, promoting transcription; PARP-1 also displaces histone H1, thereby increasing the accessibility of DNA promoters [135, 136]. Thus, formation of transient condensates of transcriptional complexes promotes local formation of an active transcriptional environment.

CONCLUSION

Synthesis of PARs, namely nucleic acid-like polymeric structures of varying lengths, is one of the mechanisms of adaptation and initiation of the necessary cellular processes in response to various stress stimuli. Despite the fact that, unlike DNA and RNA, the PAR sequence does not encode any information, the length and structure of PAR polymers determine the PAR code. This code is recognized by a variety of the proteins involved in repair, transcription, and organization of the chromatin structure. The cellular PAR level is inconstant; it is strictly controlled by enzymes that synthesize, recognize, and hydrolyze PARs. Liquid-phase biomolecular compartments, in which PAR acts as a scaffold for the condensation of proteins containing disordered domains, and their partners, are assembled to increase the effectiveness of certain biochemical processes: e.g., transcription, repair, and RNA biogenesis. These complexes are quickly disassembled after PAR hydrolysis. Impaired PAR metabolism is associated with the development of pathological processes, leading to oncological, cardiovascular, and neurodegenerative diseases, as well as premature aging. Therefore, PAR code-modulating proteins are considered important therapeutic targets. Indeed, several PARP inhibitors are already successfully used as anticancer agents, while others are being developed and tested. PAR-hydrolyzing enzymes are another promising target. What is more, compounds capable of controlling the PAR level may be used in the therapy of non-oncological diseases. ●

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The authors declare that they have no conflict of interests.

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Parkinson's Disease Associated with *GBA* Gene Mutations: Molecular Aspects and Potential Treatment Approaches

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ABSTRACT **Parkinson's disease (PD) is a multifactorial neurodegenerative disease. To date, genome-wide association studies have identified more than 70 loci associated with the risk of PD. Variants in the *GBA* gene encoding glucocerebrosidase are quite often found in PD patients in all populations across the world, which justifies intensive investigation of this gene. A number of biochemical features have been identified in patients with *GBA*-associated Parkinson's disease (GBA-PD). In particular, these include decreased activity of glucocerebrosidase and accumulation of the glucosylceramide substrate. These features were the basis for putting forward a hypothesis about treatment of GBA-PD using new strategies aimed at restoring glucocerebrosidase activity and reducing the substrate concentration. This paper discusses the molecular and genetic mechanisms of GBA-PD pathogenesis and potential approaches to the treatment of this form of the disease.**
KEYWORDS **Parkinson's disease, *GBA*, glucocerebrosidase, treatment.**

INTRODUCTION

Parkinson's disease (PD) is a polyetiological neurodegenerative disease belonging to the class of synucleinopathies that also includes dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) [1]. In synucleinopathies, neurodegeneration is caused by the accumulation and aggregation of the alpha-synuclein protein in the neuronal (PD, DLB) and glial (MSA) cells of the brain [1].

Pathomorphologically, PD is a neurodegenerative disease predominantly affecting the dopaminergic neurons of the substantia nigra and leading to the formation of protein aggregates in the cytoplasm of survived neurons; the so-called Lewy bodies, the main component of which is the alpha-synuclein protein [3–5].

PD is the most common synucleinopathy, with its incidence rate 1–3% in adults over 60 years of age [2]. Motor symptoms manifest after a loss of about 50–60% of the dopaminergic neurons of the substantia nigra [3–5]. However, the neurodegeneration process begins many years before the development of motor symptoms and can be characterized by a wide range of non-motor symptoms, such as constipation, olfactory disorders, depression, various sleep disorders (including rapid eye movement sleep behavior disorder (RBD)), etc. [6].

Despite the accepted term synucleinopathy, a number of genetically determined forms of PD have been recently found not to be associated with Lewy body formation. During autopsy, Lewy bodies were not found in more than 50% of patients with PD associated with *LRRK2* gene mutations [7]. Aggregated alpha-synuclein forms were also not found in the brain cells of patients with *PRKN* gene mutations [8]. Furthermore, Lewy bodies are absent in 8% of patients with sporadic PD (sPD) [9].

PD is known to be multifactorial in nature, and both genetic and environmental factors promote the development of the disease. To date, a number of genes associated with the development of PD have been identified [10]. The risk of PD is primarily associated with variants of the glucocerebrosidase (*GBA*) gene [11–13]. Mutations in the *GBA* gene are found in 5–20% of PD patients (depending on the population), with the highest rate being observed in Ashkenazi Jews [11]. Importantly, *GBA* gene mutations, despite their rather

high rate in PD, have low penetrance. For example, 9–30% of carriers of *GBA* gene mutations at the age of 80 years and older develop clinical signs of the disease [14–16]. Of particular importance is the fact that *GBA* gene mutations are also associated with the development of other synucleinopathies, in particular DLB [17]. The data on the association of variants in the *GBA* gene with MSA remain controversial [18–20]. Recently, an association of *GBA* gene mutations with the development of RBD was established [21, 22]. More than 80% of patients with this disease develop PD or other synucleinopathies (DLB, MSA) [23].

This review discusses the molecular basis of GBA-PD pathogenesis and therapeutic approaches to the treatment of this form of the disease.

GENETIC RELATIONSHIP BETWEEN PARKINSON'S DISEASE AND GAUCHER DISEASE

Gaucher disease (GD) is the most common lysosomal storage disease [24]. The development of this disease is associated with homozygous point mutations or heterozygous compound mutations in the *GBA* gene, which reduce the activity of glucocerebrosidase (GCase) [25, 26]. To date, more than 400 *GBA* gene mutations are known [27]. It should be noted that homozygous variants leading to a complete loss of GCase activity are lethal [28, 29]. Residual activity of the enzyme is required for the development of the body. Depending on the extent of a GCase activity decrease, both "favorable" and "unfavorable" variants of the gene are distinguished. The residual activity of GCase with "favorable" homozygous mutations (p.N370S, p.V394L, and p.R463C) accounts for 20–35% of the wild-type enzyme activity, while the residual activity of "unfavorable" variants is 5–10% (p.L444P) or absent (c.84dupG) [30, 31]. There are also polymorphic variants of the gene (p.E326K, p.T369M) associated with a decrease in GCase activity by up to 50% [30, 32], which do not lead to the development of GD in a homozygous state [33, 34].

There are three types of GD [35]; of these, type I with a favorable prognosis is the most common. At the end of the 20th century, there appeared a number of clinical case reports of patients with parkinsonism symptoms who were relatives of GD patients [36–39].

In 2004, an association between *GBA* gene mutations and PD was first identified [40]. Later, this association was confirmed in a large-scale multicenter study [13]. The rate of *GBA* gene mutations in PD patients was found to vary in different populations [12, 41–43], prevailing among Ashkenazi Jews (up to 20%) [44]. Later, a 6- to 10-fold increase in the risk of PD in heterozygous carriers of *GBA* gene mutations was shown in many populations [12, 13, 43]. The carriage of p.E326K and p.T369M variants was found to increase the risk of PD 1.5- to 2-fold [12, 45, 46]. In this case, the risk of PD does not depend on the homozygous/heterozygous carrier status of *GBA* gene mutations [16]. However, the PD phenotype and the age of disease onset were shown to be associated with the type of mutation [11, 47, 48].

PHENOTYPIC FEATURES OF GBA-PD PATIENTS

GBA-PD patients are characterized by a special phenotype: the disease begins earlier than in sporadic PD (sPD) [48]; non-motor symptoms, including cognitive deficit, are more pronounced, and the rate of disease progression is higher than in sPD [49–54]. Also, GBA-PD patients are characterized by more frequent hallucinations and a higher risk of depression and anxiety [47, 53, 55–57]. In this case, cognitive impairments and mental symptoms are more typical of carriers of "unfavorable" mutations (p.L444P, c.84dupG, 370Rec) than carriers of more "favorable" alleles (p.N370S) [47]. Interestingly, cognitive impairments also prevail in carriers of gene variants associated with a slight increase in the risk of PD (p.E326K, p.T369M) in comparison with sPD patients [58].

FUNCTION OF GCase IN HEALTH AND DISEASE

The *GBA* gene encodes the lysosomal enzyme GCase that cleaves glucosylceramide (GlcCer) into glucose and ceramide. GCase is a membrane-bound protein with five glycosylation sites [27, 59]. A decrease in the enzyme activity is accompanied by lysosomal accumulation of GlcCer and the lysosphingolipid glucosylsphingosine (GlcSph) formed during deacetylation of GlcCer. Accumulation of these substances in lysosomes of GD patients leads to the formation of phenotypically altered macrophages, the so-called Gaucher cells. Accumulation of Gaucher cells in various organs and tissues leads to the development of GD symptoms (changes in bones, hepatosplenomegaly, anemia) [60]. Synthesis of the protein encoded by a mutant *GBA* gene in the endoplasmic reticulum (ER) is accompanied by misfolding as well as changes in the native conformation of the enzyme and its transport into lysosomes (*Fig. 1*). After maturation in the ER, the protein binds to the lysosomal integral membrane protein 2 (LIMP-2). The LIMP-2 protein encoded by the *SCARB2* gene provides GCase transport from the ER to lysosomes, where the proteins dissociate under acidic conditions [61]. Altered *LIMP-2* expression in PD model mice was shown to lead to a decrease in GCase activity and damage to dopaminergic neurons, mediated by the accumulation of alpha-synuclein [62].

Transport of the GCase–LIMP-2 complex into the lysosome is facilitated by various proteins. In particular, these include the heat shock protein HSP70 with

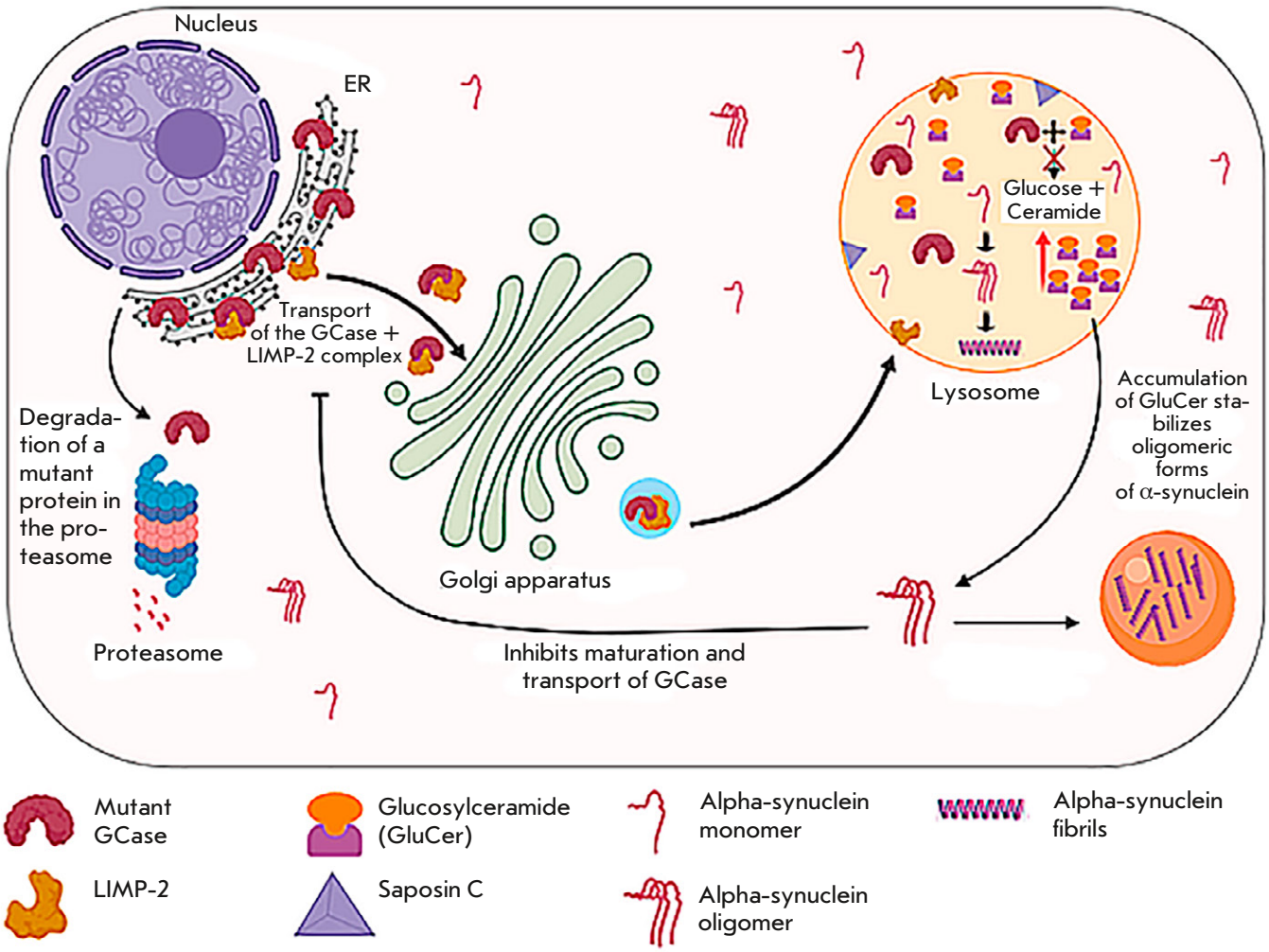

Fig. 1. Metabolism of GCase and possible interaction with alpha-synuclein

progranulin, as a cochaperone [63]. Furthermore, progranulin was shown to modulate GCase activity [64, 65]. Interestingly, the locus of the *GRN* gene, which encodes progranulin, and variants in the *SCARB2* gene are associated with the development of PD [66–68].

Co-factor proteins are required for functional activity of GCase. An acidic environment in lysosomes is favorable for the functioning of GCase; however, the saposin C protein is required to increase the catalytic activity of the enzyme [69]. The lysosomal protein saposin C provides maximum GCase activity and prevents proteolysis of the enzyme [70]. Saposin C is supposed to bind the protein with GlcCer and directs the substrate to the enzyme active center [69]. Saposin C is one of three proteins encoded by the *PSAP* gene. Rare mutations in this gene lead to the development of GD [71]. However, no association between variants in the *PSAP* gene and PD has been found [72].

The pathogenesis of GBA-PD is unclear. A decrease in GCase activity could cause lysosomal dysfunction and, subsequently, a reduction in alpha-synuclein degradation. Studies, including *in vitro*, in animal models and *post mortem* have revealed a number of features of the interaction between GCase and alpha-synuclein, which suggest a molecular basis of GBA-PD pathogenesis. A physical interaction between GCase and alpha-synuclein was found in an acidic environment *in vitro* [73, 74]. As mentioned, GCase is a membrane-bound protein. The interaction between GCase and alpha-synuclein can lead to the formation of a membrane GCase–alpha-synuclein complex. This structure is supposed to increase the efficiency of alpha-synuclein cleavage by proteases [59]. Also, impaired degradation of alpha-synuclein in lysosomes can lead to a decrease in GCase activity [75, 76] and an increase in alpha-synuclein aggregation [75, 76]. In this case,

lipids of the lysosomal membrane and sphingolipids, in particular, can affect alpha-synuclein aggregation [77, 78]. Furthermore, *in vitro* and *in vivo* studies have shown an interaction between GlcCer and GlcSph sphingolipids and alpha-synuclein, which can lead to the accumulation of neurotoxic forms of the protein, due to its oligomerization [75, 79, 80]. Experiments on a neuronal cell culture have also demonstrated that sphingolipids promote alpha-synuclein aggregation [81]. Accordingly, a decrease in the synthesis of glucosylceramide leads to a reduction in the alpha-synuclein concentration [82]. Recently, an inverse correlation was uncovered between the GCase protein level and the ratio of alpha-synuclein phosphorylated at Ser129 to total alpha-synuclein [83]. Modeling of potential pathogenic pathways suggested that the effect of GCase dysfunction on an increase in the phosphorylated alpha-synuclein level is partly due to an increase in the glucosylsphingosine level in the substantia nigra [83].

While a decrease in blood GCase activity and accumulation of lysosphinglipids are considered GD biomarkers [35], no changes in these parameters in heterozygous carriers of *GBA* gene mutations could be detected for a long time. By using modern methods for determining GCase activity and metabolite concentrations (liquid chromatography with tandem mass spectrometry), we and other authors have uncovered a decrease in blood GCase activity in GBA-PD patients [32, 84]. An increase in the blood lysosphingolipid concentration was shown in GBA-PD [85, 86]. A decrease in GCase activity was also established in blood cells of sPD patients [32]; however, these data could not be confirmed in a number of studies [84, 87, 88]. A decrease in GCase activity in the cerebrospinal fluid and substantia nigra of sPD patients was also shown [89–91]. But it should be noted that GCase activity decreases with age [92].

Therefore, according to the most circulated hypothesis of the PD developmen mechanism in carriers of *GBA* gene mutations, accumulation of GlcCer and GlcSph is related to a decrease in the enzymatic activity of GCase (loss of function), which leads to impaired autophagy and oligomerization of alpha-synuclein [75].

Earlier, we identified an increase in the concentration of oligomeric forms of alpha-synuclein in the blood plasma of patients with both GD and GBA-PD [84, 93, 94]. Also, accumulation of alpha-synuclein and a decrease in GCase activity were found in various parts of the brain in sPD [90]. Accumulation of sphingolipids and alpha-synuclein aggregates in the brain and their co-localization were demonstrated in animal models of parkinsonism [79]. An inverse correlation among GCase activity, cognitive dysfunction, and motor deficits was found in model animals [82]. Therefore, a slight, but long-term decrease in the enzymatic activity of GCase may be a trigger for the accumulation of alpha-synuclein. As already mentioned, GBA-PD patients have a special clinical phenotype [49–51, 53, 56, 57] with a predominance of cognitive impairment, anxiety, and depression [53, 56, 95]. A similar phenotype is characteristic of patients with mutations and multiplications of the *SNCA* gene encoding alpha-synuclein [96, 97]. Probably, GBA-PD and *SNCA*-associated PD develop in a similar pathogenic pathway and have a similar phenotypic picture.

However, there exist data inconsistent with the hypothesis discussed above. For example, autopsy material of the substantia nigra from GBA-PD patients was characterized by a decrease in GCase activity [89, 98, 99] and no increase in the concentration of sphingolipids [100]. According to an alternative hypothesis (gain of function), due to mutations, GCase acquires a toxic function and disrupts the ER and protein transport in the cell [101].

There exist also data on the impact of inflammation on alpha-synuclein aggregation and PD development [102]. Alpha-synuclein was shown to be capable of directly provoking an inflammatory response [103, 104]. We and other authors have found that the blood concentration of cytokines in GBA-PD patients is increased compared to that in sPD [105, 106].

POTENTIAL THERAPEUTIC APPROACHES FOR GBA-PD

To date, PD therapy remains completely symptomatic and fails to slow down the rate of neuron loss in the brain. Today, there are no drugs capable of preventing or slowing down the development of the disease. Levodopa, proposed in 1961, remains the gold standard of treatment [107]. The search for drugs or compounds that have a therapeutic or neuroprotective effect is considered a priority in PD research.

The known molecular features of GBA-PD were used to hypothesize a possible preventive and therapeutic effect of drugs aimed at increasing GCase activity and reducing the concentration of sphingolipids. Clinical trials of several drugs are currently under way (*Table 1*). It should be noted that a prerequisite for the use of these drugs in the treatment of PD is their ability to pass through the blood-brain barrier (BBB).

Currently, treatment of GD involves enzyme replacement therapy (ERT) and substrate reduction therapy [108, 109]. In the former case, intravenous administration of a recombinant GCase enzyme is employed [109]. ERT drugs are successfully used in type I GD. However, these drugs do not pass through the BBB; so, they do not exhibit a therapeutic effect on neurological symptoms in patients with type II and type III GD and cannot be effective in PD.

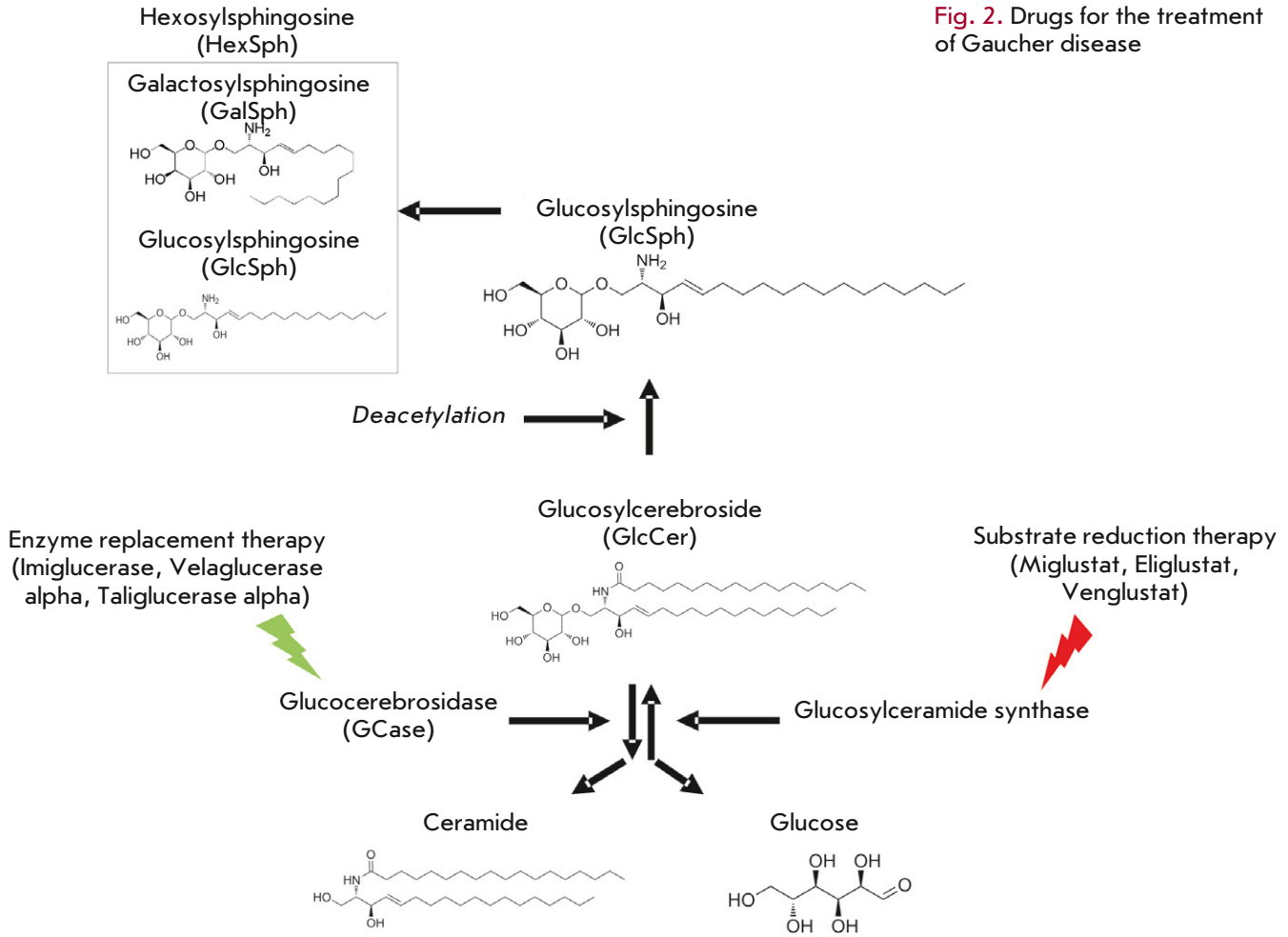

Fig. 2. Drugs for the treatment of Gaucher disease

Substrate reduction therapy could potentially relieve the symptoms of PD. Currently, miglustat and eliglustat are used for the treatment of GD [110, 111] (*Fig. 2*). The action of these drugs is based on a selective inhibition of GlcCer biosynthesis through the inhibition of glucosylceramide synthase, which decreases the GCase substrate level [108, 109]. It should be noted that miglustat, despite its ability to penetrate the BBB, was ineffective in neuropathic forms of GD [112]. In this case, the development of therapeutic agents of this class passing more efficiently through the BBB should modify the clinical course of neuropathic forms of GD and GBA-PD [82, 113]. The first clinical trial of a drug in this group is currently underway in GBA-PD patients. Phase I clinical trials have shown that venglustat can penetrate into the central nervous system; phase II trials are underway (https://www.clinicaltrials.gov/ct2/show/study/NCT02906020).

In the case of GBA-PD, the most promising area is the search for small chemical compounds, pharmacological chaperones, which bind to enzymes, facilitating their folding and transport to organelles. This strategy is considered as a potential approach to increasing the enzymatic activity of GCase, because most *GBA* gene mutations result in amino acid substitutions outside the enzyme active site, which disrupt GCase activity, affecting the maturation of this protein. The action mechanism of pharmacological chaperones involves their binding to GCase, which promotes the correct assembly of the enzyme in the ER and its transport to lysosomes, where dissociation of a substance and the GCase enzyme occurs under low pH conditions [114].

One of these substances is ambroxol hydrochloride (ambroxol), which is registered as a drug that reduces mucus hypersecretion in the respiratory tract and is used in the treatment of the hyaline membrane disease in newborns. The modulating effect of ambroxol on GCase was reported in 2009 [115]. The effectiveness of ambroxol in restoring the enzymatic activity of GCase has been demonstrated both in cell lines and in animal models of parkinsonism. Ambroxol has been repeatedly tested *in vitro* [115–119] and *in vivo* [120–123].

Our team and other authors have shown that a primary culture of macrophages derived from the pe-

Clinical trials of drugs targeting GBA-PD

Drug	Pharmacological group	Mechanism	Phase
Ambroxol	Pharmacological chaperone	Activation of GCase	II
Venglustat (GZ/SAR402671)	Substrate reduction therapy	A decrease in the substrate concentration (inhibition of glucosylceramide synthase)	II
LTI-291	Pharmacological chaperone	Allosteric activator of GCase	Ib

ripheral blood monocytes of GBA-PD and GD patients can be used for personalized screening and assessment of the effectiveness of pharmacological chaperones [124, 125]. Peripheral blood macrophages from GD and GBA-PD patients, which were cultured in the presence of ambroxol, demonstrated an increase in GCase activity and a decrease in the concentration of lysosphingolipids [124–126]. Recent data have demonstrated that the effects of ambroxol can depend on the type of *GBA* gene mutations. Ambroxol was less effective in a line of fibroblasts from GD patients with "unfavorable" *GBA* gene mutations (e.g., L444P/L444P or D409H/L444P) than in GD patients with the N370S/N370S mutation [124]. The ability of ambroxol to pass through the BBB and increase GCase activity, and reduce alpha-synuclein aggregation, was shown in PD animal models [127].

The first clinical trial of ambroxol for the treatment of GBA-PD was recently completed. This open-label, non-randomized, non-controlled study included 18 PD patients (8 GBA-PD, 10 PD) who received oral ambroxol [119]. The drug proved safe and had the ability to pass through the BBB. The patients had improved clinical symptoms; however, it should be noted that a small sample of patients and the absence of a placebo control group complicate any interpretation of the results [119]. Currently, the effectiveness of ambroxol in the treatment of PD with dementia is under study [128].

Another pharmacological chaperone of GCase is the iminosugar isophagomine [129]. *In vitro* and *in vivo* studies have shown the effectiveness of isophagomine in restoring mutant GCase activity, reducing the level of substrates, and decreasing the rate of neurodegeneration [114, 130, 131].

Clinical studies of isophagomine for the treatment of GD have revealed the safety and satisfactory tolerability of the drug. However, the clinical effect was minimal, and the third phase of the studies was not performed (https://ir.amicusrx.com/news-releases/news-release-details/amicustherapeutics-announces-preliminary-results-phase2-study).

Also, a clinical study of another GCase molecular chaperone (LTI-291 (LTI/Allegran)) has been registered. This study, assessing the effectiveness of the drug in the treatment of GBA-PD, is undergoing phase 1b testing (https://www.trialregister.nl/trial/7061) (*Table*).

We have constructed an *in silico* model of mutant GCase with allowance for the enzyme glycosylation sites [132]. Using molecular docking methods, we have searched for possible modifications of allosteric pharmacological chaperones of GCase which increase their binding to the enzyme and, as a consequence, their effectiveness in restoring the enzymatic activity of GCase (unpublished data).

CONCLUSION

An investigation of the pathogenic basis of GBA-PD has identified new therapeutic targets in a short time. The challenge is the expansion of a GBA-PD patient cohort for clinical trials. Of great importance is the screening of *GBA* gene mutations in PD patients for their potential enrollment in clinical trials. The scale of research to identify new GCase activators and the increasing number of compounds approved for clinical trials suggest that GBA-PD may become the first form of parkinsonism for which new therapeutic approaches are developed. ●

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Figure 1 was created with BioRender.com

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Pathogenesis, Diagnosis, and Treatment of Hemostatic Disorders in COVID-19 Patients

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ABSTRACT **The novel coronavirus infection named COVID-19 was first detected in Wuhan, China, in December 2019, and it has been responsible for significant morbidity and mortality in scores of countries. At the time this article was being written, the number of infected and deceased patients continued to grow worldwide. Most patients with severe forms of the disease suffer from pneumonia and pulmonary insufficiency; in many cases, the disease is generalized and causes multiple organ failures and a dysfunction of physiological systems. One of the most serious and prognostically ominous complications from COVID-19 is coagulopathy, in particular, decompensated hypercoagulability with the risk of developing disseminated intravascular coagulation. In most cases, local and diffuse macro- and microthromboses are present, a condition which causes multiple-organ failure and thromboembolic complications. The causes and pathogenic mechanisms of coagulopathy in COVID-19 remain largely unclear, but they are associated with systemic inflammation, including the so-called cytokine storm. Despite the relatively short period of the ongoing pandemic, laboratory signs of serious hemostatic disorders have been identified and measures for specific prevention and correction of thrombosis have been developed. This review discusses the causes of COVID-19 coagulopathies and the associated complications, as well as possible approaches to their early diagnosis, prevention, and treatment.**

KEYWORDS **coronavirus, hemostatic disorders, thrombosis, anticoagulants, cytokine storm, COVID-19.**

ABBREVIATIONS **COVID-19 – coronavirus disease 2019; SARS-CoV-2 – severe acute respiratory syndrome coronavirus 2; DIC – disseminated intravascular coagulation; IL – interleukin; G-CSF – granulocyte colony-stimulating factor; MCP-1 – monocyte chemotactic factor-1; TNF-α – tumor necrosis factor-α; aPTT – activated partial thromboplastin time; AT – antithrombin; FDP – fibrinogen/fibrin degradation product; PT – prothrombin time; INR – international normalized ratio; TT – thrombin time; LMWH – low molecular weight heparin; NOAC – novel oral anticoagulant; PE – pulmonary embolism.**

INTRODUCTION

Coronaviruses (CoVs) are large, pleomorphic, and unsegmented RNA viruses that are abundant in mammals, especially in humans [1–3]. To date, six types of human coronavirus have been identified (HCoV-229E, -OC43, -NL63, -HKU1, MERS-CoV, SARS-CoV). They can cause upper respiratory-tract infection of varying severity, including the severe acute respiratory syndrome (SARS) [3]. At the end of 2019, a novel coronavirus was isolated from the epithelial cells of the human respiratory tract, which was named severe acute respiratory syndrome-associated coronavirus 2 (SARS-CoV-2) [4].

From the moment the novel pneumonia, defined as coronavirus disease 2019 (COVID-19), started spreading in China and other countries, the number of patients worldwide has steadily increased, including patients with severe pneumonia [2]. COVID-19 can lead to critical condition, with an acute respiratory distress syndrome and multiple-organ failure, which are in many cases caused by systemic coagulopathy [5]. Patients with the viral infection can develop sepsis

that causes disseminated intravascular coagulation (DIC) in 30–50% of cases [6]. The DIC syndrome is an acquired clinical-biological syndrome characterized by a systemic intravascular activation of coagulation, which is induced by various causes, and thrombosis in the microvasculature, leading to organ dysfunction [7]. Clinical variants of the DIC syndrome are diverse, and its pathogenesis is very complex and not yet fully understood. In particular, in the sepsis-associated DIC syndrome, monocytes and endothelial cells are activated, which is accompanied by the release of cytokines, expression of the tissue factor, and secretion of the von Willebrand factor. Massive thrombi formation leads to the consumption of fibrinogen, antithrombin III, and other blood coagulation factors, as well as to thrombocytopenia, which are collectively referred to as "consumption coagulopathy" and can manifest itself in the form of hemorrhagic diathesis. The later stages of the DIC syndrome are associated with fibrinolysis activation aimed at recanalization of blood vessels, which can aggravate bleeding. Typical laboratory signs of the DIC syndrome include hypofibrinogenemia, thrombocytopenia, antithrombin III deficiency, and prolonged clotting tests, in combination with the clinical picture of blood circulatory disorders. The typical features are increased levels of the D-dimer and fibrin degradation products (FDPs), which are markers of fibrin deposition and secondary fibrinolysis [8]. A number of studies have indicated that the DIC syndrome is characteristic of COVID-19 and is, especially, often associated with mortality; however, the bleeding component, unlike in septic DIC, is absent in COVID-19 [8].

There is a close relationship between hemostatic disorders and the systemic inflammatory response to viral infection [9]. Clinical and laboratory signs of thrombotic conditions and their severity correlate directly with the production of inflammatory cytokines such as IL-2, IL-6, IL-7, IL-10, G-CSF, IP10, MCP-1, MIP-1A, and TNF-α, although the causes and mechanisms of "cytokine storm" development in either COVID-19 or other viral infections are not yet fully understood [10]. The relationship between inflammation and thrombosis and the ability of these two processes to exacerbate each other have been described in many pathological conditions [11, 12]. Physiological pro- and anticoagulants, as well as platelets, have pro-inflammatory properties independent of their hemostatic functions [13–17]. The interdependence of thrombotic complications and the systemic inflammatory response is one of the main links in COVID-19 pathogenesis [18–20].

This review provides data on the changes in the laboratory parameters of hemostasis in COVID-19 patients. According to the published data, routine laboratory tests enable the identification of threatening and existing hemostatic disorders and the development of adequate and relevant approaches to the prevention and treatment of hemostatic disorders in COVID-19 patients. All the data on coagulopathies in COVID-19 reported to date have been obtained in relatively small patient cohorts. The findings obtained at the peak of the pandemic are preliminary and require a careful retrospective analysis.

COVID-19 AND BLOOD COAGULATION DISORDERS

A study by Guan *et al.*, who reported data on 1,099 patients with a laboratory-confirmed COVID-19 infection, showed that blood D-dimer levels in COVID-19 patients were significantly higher than the normal values and were consistent with high levels of the C-reactive protein. In severe cases, deviations of laboratory parameters (leukopenia, lymphopenia, thrombocytopenia) were more pronounced than those in mild symptoms of the disease [20].

Researchers from a Chinese clinical hospital examined 94 patients diagnosed with COVID-19 and 40 individuals in the control group, in accordance with the "pneumonia diagnosis protocol for novel coronavirus infection" that included coagulation tests [21]. The coagulation tests included the following laboratory parameters: activated partial thromboplastin time (aPTT), antithrombin (AT), fibrinogen/fibrin degradation products (FDP), fibrinogen, prothrombin time (PT), international normalized ratio (INR), thrombin time (TT), and D-dimer. Then, the COVID-19 patients were divided into three subgroups with mild, severe, and critical clinical symptoms of the disease, respectively. No significant differences in aPTT, PT, and INR were found between the three subgroups and the control group. The antithrombin value in all three subgroups was lower than that in the control group, but there was no difference among the COVID-19 subgroups. The blood D-dimer level in the patients with severe symptoms was significantly higher than that in the control group [21]. Tang *et al.* conducted an analysis of coagulation tests in 183 COVID-19 patients. It revealed that the D-dimer value in patients with severe symptoms who died was almost 3.5-fold higher, on average, than the normal values. The FDP, PT, and aPTT values were also higher than those in the survived patients. These results showed that the coagulation parameters in the deceased patients were similar to those in the DIC syndrome [8]. Thus, excessive activation of blood coagulation leads to the development of the DIC syndrome, which is an unfavorable prognostic factor in COVID-19 [22].

The D-dimer is a product of fibrinolytic degradation of fibrin cross-linked by factor XIIIa; therefore, an increase in the blood D-dimer concentration is used

in clinical laboratory diagnostics of micro- and macrothrombosis [23]. Examination of 191 COVID-19 patients showed that D-dimer values in non-survived patients were almost 9-fold higher [24]. Clinical data, laboratory parameters, and results of chest-computed tomography of 248 COVID-19 patients were retrospectively analyzed. The D-dimer level was high (≥ 0.5 mg/L) in 75% of the patients. In hospitalized patients, the D-dimer level climbed significantly as the severity of COVID-19 increased. In moderately severe patients, the median level of D-dimer was approximately 7-fold higher than the normal values and increased to critical values in severe patients. Other researchers have also identified changes in hemostasis; in particular, an increase in the blood D-dimer level in COVID-19 patients [25, 26]. Higher D-dimer levels are found in patients with concomitant critical diseases (chronic heart failure, respiratory diseases, malignant neoplasms, etc.); therefore, the D-dimer level may be used as a prognostic marker of mortality in COVID-19 [27].

The clinical and laboratory data of 41 patients hospitalized with a confirmed diagnosis of COVID-19 were reported. Higher PT values and D-dimer levels were noted in patients requiring transfer to an intensive care unit [28].

Zhang *et al.* reported three COVID-19 cases with severe pneumonia and coagulopathy. All the patients had a hypertension history; two patients had a coronary heart disease; one patient had a stroke. On examination, there were signs of ischemia in the lower extremities on both sides. Laboratory tests showed increased PT, aPTT, fibrinogen, and D-dimer levels, leukocytosis, and thrombocytopenia [29]. The presence of antiphospholipid antibodies in the blood indicates development of the antiphospholipid syndrome; however, these antibodies can be temporarily produced in patients with various infections [30]. The presence of these antibodies can lead to thrombotic complications that, in critical patients, are difficult to differentiate from other types of diffuse microthrombosis, such as DIC, heparin-induced thrombocytopenia, and thrombotic microangiopathy.

Therefore, COVID-19 is associated with pronounced changes in the laboratory parameters of hemostasis; an elevated D-dimer level (≥ 1 μg/mL) is considered an unfavorable prognostic factor [24, 31–33].

COVID-19 AND THROMBOCYTOPENIA

A meta-analysis by Lippi *et al.* revealed a decrease in the platelet count in patients with severe COVID-19 (mean 31×10^9/L, 95% CI: 29×10^9 to 35×10^9/L), with thrombocytopenia being associated with a five-fold increase in the risk of a severe form of the disease [34]. Thrombocytopenia often occurs in patients with a critical course of the disease and is usually combined with multiple-organ pathology and coagulopathy in the form of the DIC syndrome [35]. Thrombocytopenia, which is considered a mortality risk factor, was found in 55% of the patients with the severe acute respiratory syndrome [36].

Along with consumption of platelets for the formation of thrombi, thrombocytopenia in COVID-19 is associated with the ability of the coronavirus to directly affect the bone marrow, which leads to abnormal hematopoiesis or triggers an autoimmune response to hematopoietic and stromal bone marrow cells [36, 37]. The platelet count in COVID-19 is a simple and readily available biomarker associated with the clinical picture and mortality risk [38, 39]. It should be noted that a low blood platelet count correlates with elevated indicators of disease severity and multiple organ dysfunction, such as the New Simplified Acute Physiology Score II (SAPS II) and Acute Physiology and Chronic Health Evaluation II (APACHE II) [39].

"CYTOKINE STORM" IN COVID-19

There is growing evidence of "cytokine storm" development in severe COVID-19 [40], as a response to systemic inflammation [9]. Inflammation is an integral part of an effective immune response, which enables the neutralization and elimination of the infectious agent. Massive formation of inflammatory cytokines accompanies a pronounced inflammation and leads to a high permeability of blood vessels, multiple-organ failure, and, probably, death at very high blood cytokine concentrations [41]. The term "cytokine storm" in relation to infectious diseases was introduced for the first time in the early 2000s during a study of the cytomegalovirus infection [42], Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis [43], group A streptococcus [44], influenza virus [45], hantavirus [46], variola virus [47], and the severe acute respiratory syndrome coronavirus (SARS-CoV) [48].

Cytokines are a diverse group of small proteins that are secreted by cells for intercellular communication [49]. A complex cytokine response is considered as a series of overlapping reactions, each with its own degree of redundancy and alternative pathway. This combination of overlap and redundancy is important in identifying key steps in the cytokine response to the infection and in identifying specific cytokines for therapeutic intervention.

There have been many studies in humans and experimental models that have convincingly proven the pathogenic role of inflammatory cytokines/chemokines derived from inflammatory monocyte-macrophages and neutrophils. The effect of coronavirus on cytokine production in the acute phase of the disease

was characterized by measuring the levels of the plasma cytokines IL-1B, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (known as CXCL8), IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, eotaxin (known as CCL11), basic FGF2, G-CSF (CSF3), GM-CSF (CSF2), IFN-γ, IP10 (CXCL10), MCP-1 (CCL2), MIP-1A (CCL3), MIP-1B (CCL4), PDGFB, RANTES (CCL5), TNF-α, and VEGFA [28]. Critical care patients were found to have higher plasma levels of IL-2, IL-7, IL-10, G-SCF, IP10, MCP-1, MIP1-A, and TNF-α. These findings suggest that the "cytokine storm" is associated with a severity of the disease [28]. Therefore, therapeutic interventions targeting pro-inflammatory cytokines can attenuate excessive inflammatory responses. It is also important to note that high viral titers at the early and later stages of the infection are strongly correlated with the severity of the disease. Therefore, strategies aimed at controlling the viral load and attenuating the inflammatory responses are very important in the treatment and management of patients. This approach requires more research to identify the specific signaling pathways that mediate inflammatory responses in coronavirus patients [50].

OTHER HEMATOLOGICAL CHANGES IN COVID-19

The most common hematologic findings include lymphocytopenia [51–53], neutrophilia [54], eosinopenia [55], mild thrombocytopenia [53], and, less commonly, thrombocytosis [34]. The leukocyte counts can be normal, decreased [28], or increased [24]. According to a meta-analysis [56], leukocytosis, lymphopenia, and thrombocytopenia in a COVID-19 infection are associated with a more severe course of the disease and even death. According to Terpos *et al.*, during the first days of the disease, when non-specific symptoms are present, the leukocyte and lymphocyte counts are normal or slightly reduced [57]. Later, on days 7–14 of the infection, the disease affects organs with a higher expression of the angiotensin-converting enzyme 2 (ACE2) [58], a SARS-CoV-2 receptor, such as the lungs, heart, and gastrointestinal tract. At this stage, more pronounced hematological changes, in particular a significant decrease in the lymphocyte count, are present. This is more typical of non-survived patients. In survived patients, the lowest lymphocyte count was encountered around day 7 of symptoms onset, followed by recovery [24]. Thus, the dynamics of the lymphocyte count, i.e. their serial count over time, may be a predictor of the disease's clinical outcome. An analysis of the published data showed that, among all hematological changes, lymphopenia is one of the most frequent indicators of a lethal outcome. Ratios of blood cell counts are of great clinical importance: e.g., a reduced lymphocyte/leukocyte ratio indicates severe symptoms and/or a lethal outcome [59]. Similarly, increased neutrophil/lymphocyte and neutrophil/platelet ratios may indicate myocardial damage and increased mortality [60]. Therefore, it is important to monitor hematological parameters to assess COVID-19 progression and prognosis.

PROPHYLAXIS AND TREATMENT OF COAGULOPATHY IN COVID-19

A high rate of thrombotic complications has spurred interest in thromboprophylaxis and anticoagulant therapy in COVID-19. Data on systemic hypercoagulability, in particular massive thrombinemia and diffuse microthrombosis accompanied by multiple organ failures, are used as a pathogenic rationale for treatment. Therefore, inhibition of thrombin formation and/or activity in the blood may potentially decrease the risk and prevalence of thrombosis and reduce mortality in COVID-19 [23, 37].

The most common method for the prophylaxis and treatment of thrombosis in COVID-19 patients is the use of low-molecular-weight heparin (LMWH) [61]. LMWH should be administered to all patients (including non-critical ones) who require hospitalization for COVID-19 in the absence of contraindications (active bleeding and a platelet count of less than 25×10^9/L). The efficacy of prophylactic heparin therapy was shown in a study of 449 patients with severe COVID-19; of those, 99 patients received heparin (mainly LMWH) at prophylactic doses [62]. Although there were no differences in the 28-day mortality in patients untreated and treated with heparin, LMWH in patients with more pronounced hemostatic disorders (sepsis-induced coagulopathy score ≥ 4) reduced significantly the mortality rate (40% versus 64%, $p = 0.029$). Heparin therapy reduced mortality in patients with a 6-fold or more elevated level of D-dimer (33% versus 52%, $p = 0.017$) [62]. In addition, LMWH administration reduced the risk of pulmonary embolism in critical patients.

The possible effect of other drugs received by patients should be considered when evaluating the dose of LMWH. Approximately 50% of the patients who died from COVID-19 in Italy had multiple comorbidities, such as atrial fibrillation or coronary heart disease, which required anticoagulant or antiplatelet treatment. The treatment of these patients is particularly challenging due to potential interactions between heparin and other drugs, such as new oral anticoagulants [63] that have proven themselves well in the prophylaxis and treatment of venous thromboembolism; these drugs may also be promising for reducing the risk of thrombosisin in COVID-19 patients [41].

CONCLUSIONS

COVID-19 patients often develop hemostatic disorders: in particular, hypercoagulability of varying severity. Typical laboratory signs of these disorders are thrombocytopenia, increased D-dimer and fibrinogen concentrations in the blood, and prolonged PT and aPTT, especially in patients with severe COVID-19. Dynamic monitoring of these hemostatic parameters may reflect a transformation of the clinical course of the disease into a more severe case. The most pronounced changes in hemostasis in COVID-19 have an unfavorable prognostic value. Given the increased risk of thromboembolic complications in COVID-19 patients, prophylactic and therapeutic use of anticoagulants, primarily low-molecular-weight heparins, is justified. ●

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Molecular Mechanisms of Muscle Tone Impairment under Conditions of Real and Simulated Space Flight

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ABSTRACT **Kozlovskaya *et al.* [1] and Grigoriev *et al.* [2] showed that enormous loss of muscle stiffness (atonia) develops in humans under true (space flight) and simulated microgravity conditions as early as after the first days of exposure. This phenomenon is attributed to the inactivation of slow motor units and called reflectory atonia. However, a lot of evidence indicating that even isolated muscle or a single fiber possesses substantial stiffness was published at the end of the 20th century. This intrinsic stiffness is determined by the active component, i.e. the ability to form actin-myosin cross-bridges during muscle stretch and contraction, as well as by cytoskeletal and extracellular matrix proteins, capable of resisting muscle stretch. The main facts on intrinsic muscle stiffness under conditions of gravitational unloading are considered in this review. The data obtained in studies of humans under dry immersion and rodent hindlimb suspension is analyzed. The results and hypotheses regarding reduced probability of cross-bridge formation in an atrophying muscle due to increased interfilament spacing are described. The evidence of cytoskeletal protein (titin, nebulin, etc.) degradation during gravitational unloading is also discussed. The possible mechanisms underlying structural changes in skeletal muscle collagen and its role in reducing intrinsic muscle stiffness are presented. The molecular mechanisms of changes in intrinsic stiffness during space flight and simulated microgravity are reviewed.**
KEYWORDS **skeletal muscle, gravitational unloading, atonia, hindlimb suspension, dry immersion, muscle stiffness, intrinsic stiffness, passive stiffness, cytoskeleton, sarcomeric cytoskeletal proteins, titin, collagen, signaling.**
ABBREVIATIONS **CSA – cross-sectional area; Akt – protein kinase B; GSK3β – glycogen synthase kinase 3β; HSP – heat shock protein; 17-AAG – 17-(allylamino)-17-demethoxygeldanamycin.**

INTRODUCTION

The mysterious mechanisms of maintaining and decreasing muscle tonus have always attracted the attention of physiologists. The tone is usually referred to as mechanical tension in the relaxed muscle, which provides a biomechanical basis for performing directed movements. A change in the tone can be assessed by changes in muscle stiffness. Reflex control of muscle tone has been known for a long time. Whether the muscle possesses molecular and cellular mechanisms to maintain its tone still remains a controversial issue.

Kozlovskaya *et al.* [1] and Grigoriev *et al.* [2] used tensometric and vibrometric methods to assess transverse stiffness in human muscles *in vivo* and observed a significant loss of muscle stiffness as early as in the first days of exposure under both true (space flight) and simulated microgravity conditions. This phenomenon is called hypogravity-induced atonia. The loss of stiffness is associated mainly with changes in the performance of extensor motor units: i.e., inactivation of a pool of slow-twitch motor units during gravitational unloading [3, 4] (*Fig. 1*). These concepts are supported by several observations indicating a significant decrease or complete cessation of electrical activity in the rat postural soleus muscle under support withdrawal during both ground-based experiments with hindlimb suspension and real microgravity created by Kepler orbit flight [5–8]. Therefore, we suggest that stiffness is lost largely due to the inactivation of the slow muscle fibers that maintain baseline mechanical

activity in the muscle even at rest on Earth, which, in turn, influences muscle stiffness parameters *in vivo*. This stiffness component may be called reflectory stiffness (*Fig. 2*).

Are there any intrinsic peripheral mechanisms for reducing muscle stiffness during its inactivation?

By the end of the 20th century, there was a lot of evidence indicating that even an isolated muscle or an isolated (and permeabilized) fiber has functionally significant stiffness that is gradually lost after cessation of contractile activity. This intrinsic muscle stiffness (*Fig. 2*) is controlled by both the active component, i.e. the ability to form some of the actin-myosin bonds (cross-bridges) during stretching and contraction, and the parallel elastic component, i.e. structural proteins of the cytoskeleton and extracellular matrix, which are capable of exerting mechanical resistance during muscle/fiber stretch and contraction (*Fig. 3*).

Stiffness is an increase in the mechanical tension, i.e. the tensile force per cross-sectional area (CSA), in response to deformation (relative elongation) of muscle fibers. Since a muscle cell, especially one that is activated, exhibits not only elastic, but also viscoelastic properties, the result of determining the stiffness depends on the method of measurement used. There is dynamic or instantaneous stiffness, which can be measured by applying a very rapid deformation, and static stiffness, which is characterized by the level of tension established long after the end of length change. There are stepwise (rectangular), sawtooth or sinusoidal patterns of muscle length changes used for stiffness measurements. In the first case, the muscle is subjected to step length changes lasting about 0.1 ms in the best experimental conditions, which enables measuring of instantaneous stiffness. In the second case, the muscle length is changed linearly, which enables direct measurement of the length-tension curve during loading or unloading. Sinusoidal or harmonic stretching allows for the best use of available equipment in order to achieve maximum time resolution. Due to the nonlinearity of the muscle stress-strain diagram in response to as small as a few percents stretching, the tangent and secant or chordal stiffness types are different. Active stiffness of an intact muscle can be caused by background electrical potential, and that of an isolated muscle is associated either with the presence of a suprathreshold concentration of calcium ions causing partial activation of the troponin–tropomyosin regulatory system or with defects in this system: e.g., partial loss of troponin complexes resulting in activation of some regulatory units even in the absence of calcium ions. The active stiffness component can be eliminated by adding blebbistatin, a specific myosin II inhibitor that penetrates the cell through the sarcolemma [9], binds myosin, and inhibits its transition to the strong actin-myosin complex [10]. The active stiffness component can be precisely measured by applying sufficiently rapid stretching, with deformation rates of at least several muscle lengths per second. Otherwise, the stiffness value is underestimated due to stress relaxation. Since passive stiffness is nonlinear, the entire length-tension curve (tensile force per CSA) should be recorded.

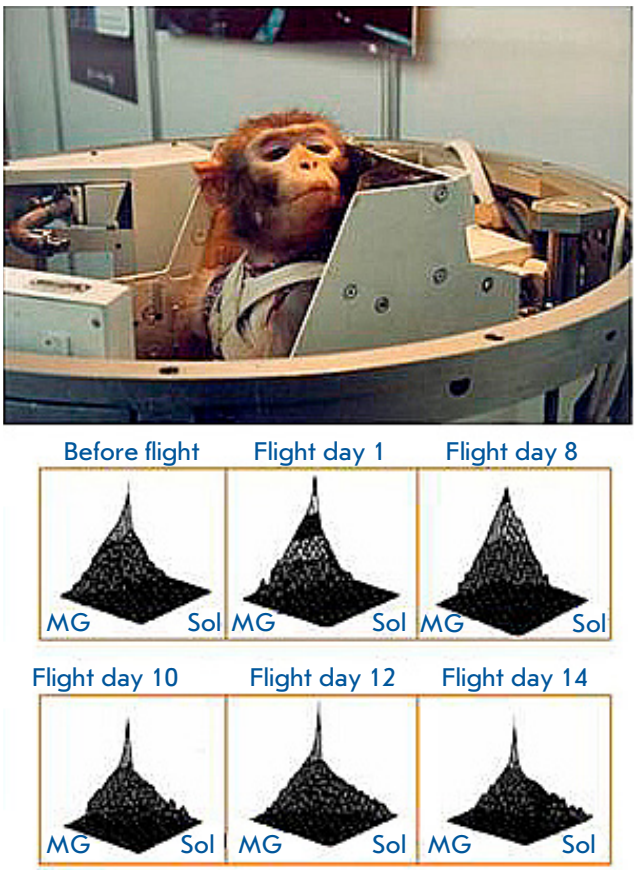

Fig. 1. Changes in the recruitment order of the rhesus monkey gastrocnemius and soleus muscles in a foot lever pressing task with sustained load during space flight aboard a biosatellite. Slow-twitch fiber comprise up to 95% of the soleus muscle. The percentage of slow-twitch fiber in the gastrocnemius muscle does not exceed 40–50%, the rest of the fibers are fast-twitch ones. Monitoring of the EMG activity of these two muscles during a lever-pressing task in the biosatellite capsule showed that the movement was performed mainly by the soleus muscle before flight. The pattern changed from day to day during the space flight: soleus activity decreased, while gastrocnemius activity increased. Thus, the task was performed almost completely by the gastrocnemius muscle by the end of the 2-week flight

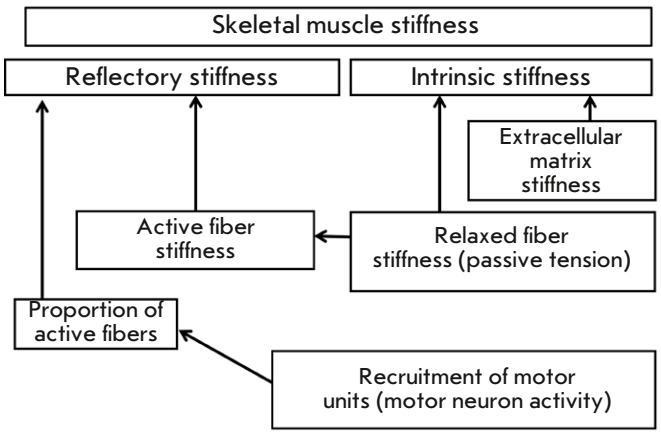

Fig. 2. Physiological classification of skeletal muscle stiffness characteristics

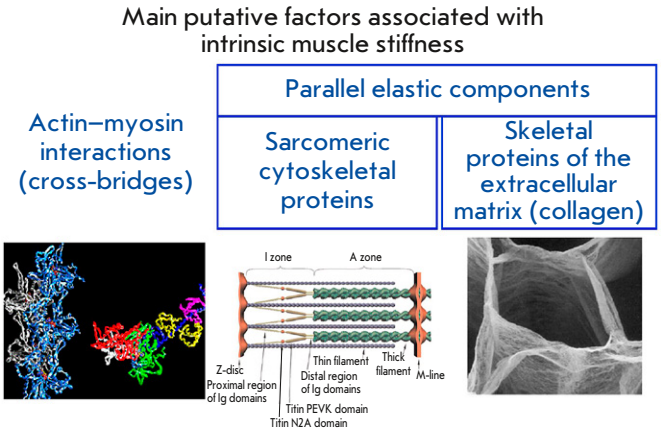

Fig. 3. Main putative factors associated with intrinsic muscle stiffness

This review discusses the central data on the changes in intrinsic muscle stiffness under conditions of gravitational unloading that mainly result in deep inactivation of many muscles. We will primarily analyze the data obtained under support withdrawal conditions, i.e. in experiments using a dry immersion model (with the participation of volunteers, *Fig. 4*) and, then, hindlimb suspension (using laboratory rodents, *Fig. 5*). We will also discuss the putative mechanisms of a decline in intrinsic muscle stiffness and the role of this decline in muscle atrophy.

Prior to discussing the issue at hand, we would like to briefly describe the experimental approaches mentioned above.

Dry immersion is a model developed in Russia in the 1970s [11]. It involves complete water immersion of the subject in an open bath. The subject's body surface is separated from the water by a waterproof piece of fabric covering the water surface and bath edges, with the subject head only exposed to air (*Fig. 4*).

Hindlimb suspension [12, 13] remains one of the most commonly used microgravity models in laboratory rodents. The animal is suspended below the cage ceiling either by the tail, back skin, or a cloth vest so that the forelimbs rest on the ground, while the hindlimbs hang at an angle of 30–40 degrees to the floor (*Fig. 5*). If the model is used correctly, the animal can move freely inside the cage. The level of corticosterone indicating the degree of animal stress rarely exceeds that of an intact control rodent [14].

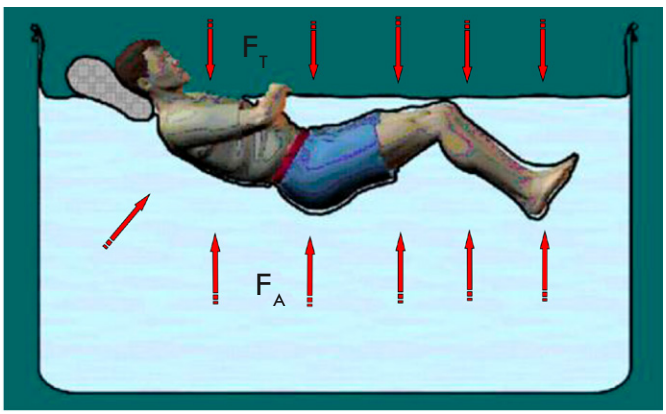

Fig. 4. An experimental model of dry immersion. When the body is immersed in water, the resulting force of hydrostatic pressure (Archimedean force, F_A) balances the force of gravity (F_T). However, the Archimedean force is distributed over the entire body surface. Because of that, the pressure on each point per unit body surface area is much lower than the support reaction force in the contact area in an upright, sitting, or prone position

PASSIVE AND ACTIVE STIFFNESS OF ISOLATED MUSCLE AND FIBER DURING GRAVITATIONAL UNLOADING

Gravitational unloading is known to decrease significantly both the passive and active stiffness of muscle and muscle fiber. Goubel *et al.* demonstrated that passive tension of the rat postural soleus muscle significantly reduces after 3–4 weeks of suspension [15]. As early as in their first work, the authors attributed a decline in the series elastic component to both the active mechanisms (cross-bridges) and the passive (in the authors' opinion, mainly tendon) elements. However, a decline in the passive tension was also established in single permeabilized soleus muscle fiber after 14-day suspension [16]. Furthermore, as shown

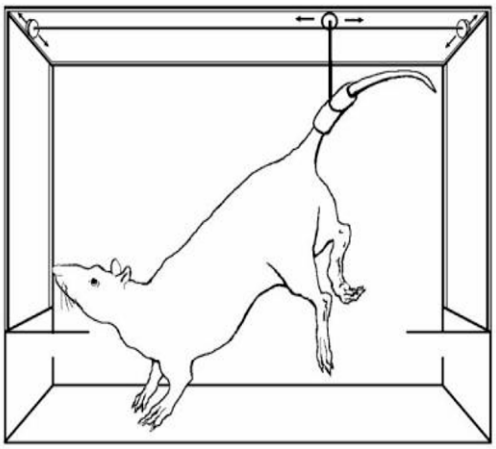

Fig. 5. An experimental simulation model of rodent hindlimb suspension. After detachment of the animal's foot from the ground support, afferents are activated and the animal turns out to be under unloading conditions

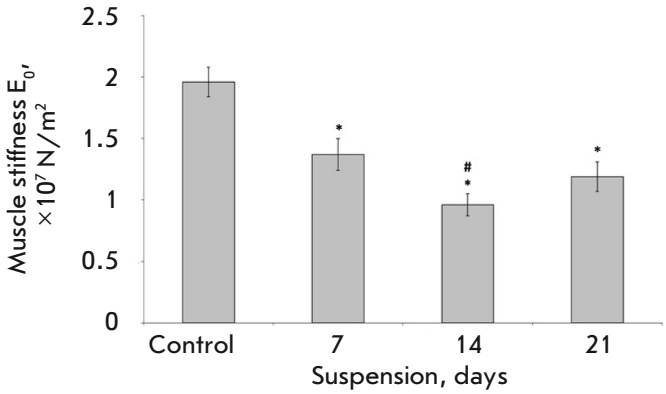

Fig. 6. Changes in the dynamic elastic Young's modulus (stiffness index) of fully activated permeabilized fiber of the rat soleus muscle during hindlimb suspension [17] after 7, 14, and 21 days. * – significant difference from the control group ($p < 0.05$), # – significant difference from the 7-day unloading group ($p < 0.05$)

in an experiment with elimination of the effect of actin-myosin bonds, this decline may be, for the most part, associated with a decrease in the relative content of titin, an elastic cytoskeletal protein. The time course of the changes in the dynamic stiffness of fully activated muscle fibers under simulated gravitational unloading (suspension) was investigated by McDonald and Fitts [17]. The Young's modulus decreased by 30% after seven days of unloading and by 50% after two weeks of suspension compared to that in the control animals (*Fig. 6*). Interestingly, the modulus value after three-week suspension remained the same as after two weeks of unloading. Transverse stiffness of permeabilized soleus muscle fiber in suspended rats was evaluated by atomic force microscopy in the laboratory of one of the authors of the current review. An analysis of the contractile apparatus with this method, following detergent-based removal of membrane structures, revealed that transverse stiffness of the myofibrillar apparatus in the area from the M-line to the Z-disc was statistically significantly reduced by 35% only on the third (but not on the first) day of suspension. The stiffness then decreased slower, but transverse stiffness was 68% lower than in the controls by day 12 of suspension [18]. Transverse stiffness in the Z-disc region dropped more than two-fold by day three of suspension and further continued to decrease. Interestingly, measuring the transverse stiffness of the contractile structures of a muscle fiber activated by a high concentration of Ca^{2+} ions (pCa 4.2) revealed a much more pronounced decline in the stiffness after suspension: an almost two-fold reduction in the region between the Z-disc and the M-line after three days and a more than 63% decrease after 12 days. It should be noted that, since activated fiber stiffness was almost two-fold higher than that of relaxed fiber in an intact animal, the absolute value of a decline in activated fiber stiffness was significantly higher. Similar data were obtained for the human soleus muscle in an experiment with volunteers after seven days of dry immersion [19]. When considering these data, one has to take into account the limited capabilities of atomic force microscopy: the inability to capture the longitudinal resistance of a sample, as well the stiffness of the whole fiber/muscle due to the limited depth of cantilever penetration.

Thus, the data available to date do not question the decline in intrinsic longitudinal and transverse, dynamic and static, as well as passive and active, stiffness of the muscle, its fibers, and their components upon simulated gravitational unloading of mammals. However, the molecular mechanisms underlying this decline in stiffness remain unclear.

MOLECULAR FACTORS AFFECTING MUSCLE STIFFNESS: CROSS-BRIDGES

Cross-bridges [20–22], as well as cytoskeletal (titin, nebulin, obscurin, and myosin-binding protein C) and regulatory proteins, determine passive muscle stiffness during stretching. These proteins constitute the passive parallel elastic component of the muscle [23, 24] and affect the probability of cross-bridge formation [25–28].

Interfibrillar matrix components, in particular collagen fibrils, also determine the stiffness of the entire muscle or its fiber bundles [29]. Extracellular matrix stiffness was recently shown to be significantly higher than that of isolated fiber [30]. Studying the effect of gravitational unloading on these proteins is of great interest. Passive stiffness is higher in muscle predominantly composed of fiber expressing slow myosin heavy chains [15]. Therefore, one would expect that stiffness should decrease under gravitational unloading due to a change in the expression pattern of myosin heavy chain isoforms in favor of fast-twitch isoforms, provided that all the other parameters are equal [31, 32].

The probability of cross-bridge formation is higher if the interfilament spacing in the myofibrillar apparatus is optimal. A decrease in the relative number of normally arranged actin filaments (in the absence of structural disturbances) should increase the interfilament spacing and should reduce the probability of cross-bridge formation. Fitts and Riley noted a reduced amount of actin filaments and shortening of some of them in the soleus muscle after 14 days of suspension in rats [33], 17 days of bed rest, and 17 days of space flight [34–36]. These changes are accompanied by a decrease in the maximum force and power of contraction of single permeabilized fibers, as well as in their calcium sensitivity. The discovered phenomenon may be directly associated with reduced active muscle stiffness. The cause of these changes has not yet been established. Previously, we noted a decrease in the content of nebulin, a thin filament protein, in the rat soleus muscle after 7–14 days of suspension [37, 38]. A possible cause of the "loss" of actin filaments may be a decrease in the relative nebulin content. Meanwhile, it has recently been established that the number of strong actin–myosin bonds in a genetically atrophied muscle decreases, while the number of weak actin–myosin bonds in the muscle increases during isometric contraction (based on EPR data) [39]. In an experiment with hindlimb suspension in rats, we have recently shown that the specific and effective inhibitor of myosin II blebbistatin has the same effect on passive stiffness of the soleus muscle in both an intact animal and an animal with reduced passive stiffness, after three days of gravitational unloading. These results suggest that a possible change in the parameters of a small number of the cross-bridges formed in a resting muscle after gravitational unloading does not affect its passive stiffness [40]. However, one cannot exclude the possibility that increasing interfilament spacing, decreasing the number of thin filaments, and changing the parameters of cross-bridges in unloading and hypogravity-induced atrophy may significantly affect active dynamic stiffness. This issue is a challenge for future research.

SARCOMERIC PROTEINS AND MUSCLE STIFFNESS

Among sarcomeric cytoskeletal proteins, titin attracts the most attention; its contribution to passive muscle stiffness is considered to be very significant [23, 41]. Several domains of a giant titin molecule have, to greater or lesser extent, spring-like properties and can compress and stretch (*Fig.* 7). A decrease in the relative content of titin during hindlimb unloading was first discovered by Christine Kasper in 2000 [42]. Similar data were obtained in a laboratory of the University of Lille in 2002 [16]. In the same year, we found a decrease in the level of titin-1 (T1) and an increase in the level of its proteolytic fragment T2 in the rat soleus muscle after 14-day hindlimb unloading [43]. Given that titin is one of the constituents of the parallel elastic component determining the value of fiber passive stiffness that reduces during unloading, one might expect either a decrease in the content of this protein or an increase in its compliance as early as 2–3 days after hindlimb unloading (when passive muscle stiffness is already decreased). However, this turned out to be not entirely true. Goto *et al.* found no changes in the connectin (titin-1) content after three days of hindlimb unloading [44]. In this case, an elastic region of the titin molecule that is located between the Z-disk and the N2A-domain (including PEVK spring region) was found to lose its elasticity instead of increasing it, thus showing less elastic properties after hindlimb unloading [44]. These data have recently been explained in a study by Nishikawa *et al.* [45], who demonstrated that an increase in the calcium ion level in a fiber (which takes place during gravitational unloading [46–48]) results in rigid binding of a titin molecule to thin filaments in the N2A domain. In 2008, we also found no decrease in the content of a N2A titin-1 isoform, typical of skeletal muscles, in the rat soleus muscle after three days of hindlimb unloading [49]. A significant decrease in the titin-1 content was noted after seven days of hindlimb unloading [50]. A statistically significant increase in titin expression in the rat soleus muscle during three days of unloading (hindlimb suspension) was recently revealed in the laboratory of one of the authors of this review [51]. It is possible that this increased expression compensates for the breakdown of some titin molecules, which leads to the lack of visible changes in its content. Interestingly, the titin expression level did not exceed the control after seven days of hindlimb unloading [50], which made it possible to register a decrease in the titin content at this time interval, which is probably due to its enhanced calcium-dependent proteolysis.

Thus, there is good evidence to suggest that the destruction of titin and nebulin during exposure of an animal to simulated gravitational unloading for more

than three days can contribute significantly to a decline in passive muscle stiffness. However, the question of whether alterations in this protein can be associated with changes in the stiffness properties of an unloaded muscle in the early period of unloading (up to three days) remains open. Likely, a change in the degree of protein phosphorylation may contribute to a change in the stiffness of the titin molecule and, respectively, the entire muscle in the early period of unloading. There are grounds for this suggestion. Phosphorylation/dephosphorylation of PEVK and N2B domains in cardiac muscle titin is known to alter the stiffness properties of the molecules, leading to a change in the titin-based passive stiffness of cardiomyocytes and the entire muscle [52]. These changes, in turn, play an important role in the regulation of myocardium contractile activity. There is evidence of phosphorylation of skeletal muscle titin [53, 54]. The role of this post-translational modification in changing the stiffness properties of the titin molecule is unclear. However, these changes have been suggested to play a role in reducing titin-based passive stiffness, as based on data demonstrating a decreased level of PEVK region phosphorylation in titin in the rat vastus lateralis muscle after 15-minute physical activity (treadmill running) [54]. The role of titin hypophosphorylation in the decrease in the stiffness of its molecules and the compromising of the contractile ability of the rat diaphragm after 18-hour mechanical unloading (mechanical lung ventilation), leading to muscle atrophy, is also discussed [55–57]. We found an increase in the total T1 and T2 phophosphorylation level resulting in a decreased T1 content in the mouse gastrocnemius muscle after a 30-day space flight [58]. Reduced titin and nebulin contents under gravitational unloading would undoubtedly decrease the passive stiffness developed by titin molecules upon stretching, as well as general muscle stiffness. However, titin stiffness can both decrease and increase. depending on which molecule regions are phosphorylated.

A hypothesis linking the breakdown in some cytoskeletal proteins (presumably affecting muscle stiffness) to phosphorylation of specific sites in their molecules cannot be excluded. This hypothesis has recently been confirmed in studies on the mechanisms of desmin (intermyofibrillar and intermyofilament cytoskeletal protein) breakdown. Cohen *et al.* showed that phosphorylation of desmin by the well-known kinase GSK3β triggers ubiquitination and calpain-mediated depolymerization of desmin [59]. The kinase can be inhibited via negative phosphorylation by kinase Akt1 [60] and NO-dependent kinase of the guanylate cyclase cascade [61]. Thus, phosphorylation/dephosphorylation of desmin can affect both the protein content and the degree of intrinsic muscle stiffness.

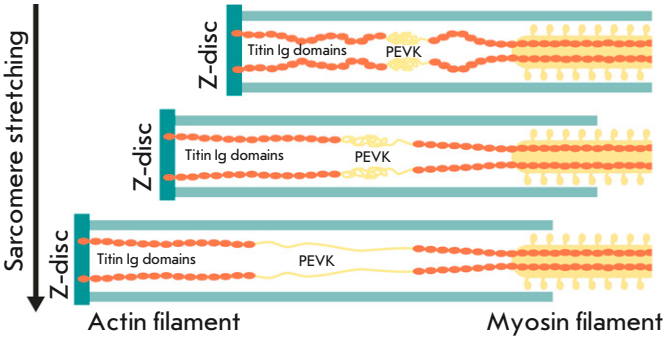

Fig. 7. Spring-like properties (compliance) of the titin PEVK domain

The phosphorylation level of myosin light chains, primarily in fast-twitch fiber, is of great importance for cross-bridge formation. Phosphorylation of myosin light chains by light chain kinase promotes cross-bridge formation and enhances the calcium sensitivity of permeabilized fiber [62, 63]. However paradoxical it may sound, the phosphorylation level of myosin light chains in the rat soleus muscle increases, and does not decrease, under simulated gravitational unloading (hindlimb suspension model), as it was shown at the beginning of this century [64]. Thus, an elevated phosphorylation level of myosin light chains under gravitational unloading can, to some extent, compensate for a decline in muscle stiffness caused by an increase in intermyofilament spacing, a decrease in the number of thin filaments, and a decrease in the content of the sarcomeric cytoskeleton protein titin.

The myosin-binding protein C plays the most important role in cross-bridge formation. A phosphorylated (at three sites) protein acts as a scaffold in the actin–myosin cross-bridge assembly [65]. However, we failed to find any data describing this protein's state during unloading. The same can be said for another important sarcomeric protein, obscurin.

Another protein, telethonin, anchors adjacent titin filaments in the Z-disc and, therefore, plays an important role in maintaining the Z-disc structure and integrity, as well as titin cytoskeleton integrity. Taillandier *et al.* showed that hindlimb suspension causes telethonin ubiquitination and breakdown in the rat soleus muscle [66]. Interestingly, the telethonin content decreases significantly after three days of hindlimb unloading [40].

One of the authors of this review found that gravitational unloading leads to a degradation of alpha-actinin-2, a characteristic Z-disc protein [67]. This degra-

dation becomes statistically significant only after seven days of hindlimb suspension. Interestingly, the content of alpha-actinin-3 in the rat soleus muscle decreases by 20% already after three days of hindlimb unloading [40]. Probably, a decreased content of alpha-actinins-2 and -3 may, to some extent, lead to Z-disc disintegration. This, in turn, may compromise interfilament spacing stability and reduce the chance of cross-bridge formation, which contributes to a decreased active muscle stiffness. It should also be noted that, like telethonin, alpha-actinins anchor titin in the Z-disc [68]. Their destruction can result in disintegration of the entire sarcomeric cytoskeleton and reduced muscle stiffness.

Collagen

Passive stiffness of the extracellular matrix and connective tissue of the skeletal muscle is an important component of the whole muscle stiffness. This stiffness significantly exceeds passive stiffness of muscle fiber and exhibits a pronounced nonlinear dependence [30, 69, 70]. The main factor determining the mechanical properties of the extracellular matrix and muscle connective tissue is the number and properties (such as the number of hydroxyproline cross-links) of collagen fibrils. Several different collagen isoforms are present in skeletal muscles. Collagens I and III make the greatest contribution to the muscle's mechanical properties [71].

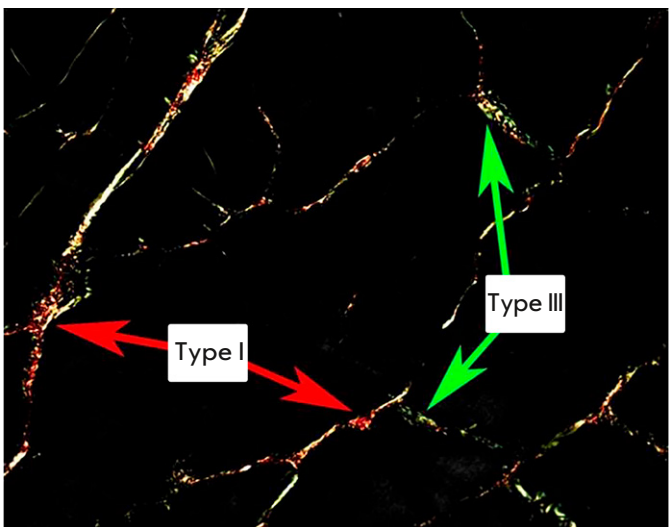

Fig. 8. Collagen isoforms: collagen I (red) and collagen III (green). A transverse section of the human soleus muscle is shown. The sample was stained in picrosirius red and examined by polarization microscopy

Of these, collagen III has lower stiffness and greater elasticity (*Fig. 8*).

The contribution of collagen to passive stiffness of the whole muscle is undeniable. However, it is currently unclear to which extent breakdown and reduced synthesis of collagen during unloading affect a decline in stiffness. Despite a progressive increase in the connective tissue volume under conditions of gravitational unloading [72, 73], no increase in the collagen content was recorded in muscle during these experiments [74]. On the contrary, a significant decrease in the collagen content was observed in the soleus, plantar, and some other hindlimb muscles in rats after a 7-day space flight [75]. Similar data were obtained during immobilization of the soleus muscle in a shortened position [76]. A pronounced decrease in the level of type I and III collagen mRNAs was observed on day three of an experiment simulating gravitational unloading by hindlimb suspension in rats [77]. The collagen mRNA level reached its control level by day seven of the experiment [77]. The expression of collagen III mRNA in the soleus muscle decreases after seven days of hindlimb suspension [78]. At the same time, a significant drop in the expression of all muscle collagen isoforms was revealed mainly in the fast-twitch gastrocnemius muscle after 3-week hindlimb suspension [79]. Analysis of collagen expression in the human vastus lateralis and soleus muscles after 90-day bed rest showed no significant changes [80]. An interesting phenomenon was observed after 14 days of hindlimb unloading: a shift in the expression ratio of type I collagen (a stiffer isoform) and type III collagen (a more elastic isoform) in favor of type III collagen [81]. It is unknown how this phenomenon can affect muscle stiffness. Considering the above, it is clear that the collagen state in a postural muscle under gravitational unloading has not been studied enough yet. Therefore, it is difficult to evaluate the role of collagen types in the decrease in passive muscle stiffness during unloading.

Molecular mechanisms of reducing intrinsic muscle stiffness

The available data indicate that intrinsic muscle stiffness is mainly associated with the state of sarcomeric cytoskeletal proteins. In this regard, we are considering here the concepts on the mechanisms of a decrease in inactivated muscle stiffness, based on knowledge on the breakdown of these proteins.

Degradation of a number of cytoskeletal proteins, in particular titin, is known to involve calcium-dependent cysteine proteases: calpains [82]. Murphy *et al.* demonstrated that treatment of a permeabilized fiber specimen with a μ-calpain solution results in a rapid decline in passive force: i.e., stiffness. In addition, rapid prote-

olysis of titin was observed. The role of calpains during gravitational unloading has been intensively studied in recent years. For instance, calpain activity was shown to significantly increase in the first days of suspension (albeit measured in a lysate in the presence of calcium ions at a supraphysiological concentration), while desmin underwent rapid decomposition [18, 83–85]. Interestingly, calpain activation is associated with structural abnormalities in the Z-disc in muscle fiber [86]. We found that prevention of excessive accumulation of calcium ions in muscle fiber using a calcium-binding agent or an inhibitor of dihydropyridine calcium channels (nifedipine) reduces μ-calpain activity [85]. Another interesting finding is that inhibition of calcium channels decreases the level of μ-calpain mRNA, which is elevated under unloading conditions [87].

All these data indicate the high activity of calpain during unloading, which should contribute to rapid breakdown of cytoskeletal and regulatory sarcomeric proteins and decreased muscle stiffness. Indeed, the use of the specific calpain inhibitor PD150606 not only prevented degradation of cytoskeletal proteins that stabilize titin (α-actinin-2 and telethonin), but also reduced passive stiffness of the soleus muscle [40].

Endogenous calpain inhibitors include calpastatin and nitric oxide. Mice overexpressing the calpastatin gene showed no atrophic changes during hindlimb unloading [88]. Calpastatin expression in healthy animals, on the contrary, decreases during hindlimb unloading [84]. Unfortunately, no physiological mechanisms depending on the level of muscle activity and regulating calpastatin expression are known to date. Another endogenous calpain inhibitor is nitric oxide [89]. Its production depends on the muscle contractile activity [90]. The production of nitric oxide decreases during muscle unloading [91]. At the same time, administration of *L*-arginine to increase the level of nitric oxide in an atrophied muscle prevents breakdown of a number of cytoskeletal proteins and, to some extent, reduces the severity of muscle atrophy [91]. We have recently obtained data indicating prevention of titin breakdown during gravitational unloading upon *L*-arginine administration [50]. Thus, we may suggest that a reduced level of nitric oxide during gravitational unloading contributes to decreased muscle stiffness thanks to calpain-mediated breakdown of cytoskeletal proteins.

Another group of factors preventing proteolysis of cytoskeletal proteins is the heat shock proteins (HSPs) that activate neuronal NO synthase and ensure titin integrity [92, 93]. The degradation of contractile proteins can be enhanced by breakdown of Hsp90 and 70 heat shock proteins, which are usually present at very high concentrations in a muscle. However, their level drops by 50–70% during gravitational unloading due to muscle atrophy [94, 95]. Some authors believe that decreased Hsp expression in muscles during unloading may be of significant importance in muscle atrophy. A sharp rise in the level of Hsp90 and Hsp70 proteins was obtained using the 17-AAG inhibitor during gravitational unloading [96]. The Hsp90 inhibitor 17-AAG prevented an increase in the calpain level and intensification of protein ubiquitination. The active Hsp90–neuronal NO synthase interaction and its protective effect on titin suggest that decreased HSP90 expression during gravitational unloading may be associated with reduced muscle stiffness.

Although most authors agree that extracellular matrix proteins, in particular collagen isoforms, significantly contribute to the control of intrinsic passive muscle stiffness, changes in these proteins during unloading have been studied much less than changes in sarcomeric cytoskeletal proteins. Thus, investigation of the mechanisms regulating collagen expression depending on muscle contractile activity is at its very beginning. Elucidating the mechanism of function-dependent inhibition of collagen expression in interstitial fibrogenic cells is of prime importance. Regarding this issue, miR-206 function is of great interest. Increased expression and secretion of miR-206 (in the form of exovesicles) was recently shown to inhibit collagen expression in muscle fibroblasts present in the interstitial space between fibers [97]. Interestingly, a serum miR-206 level increases upon hindlimb suspension in mice [98]. Decreased collagen content during unloading can be possibly due to changes in this microRNA expression and transport. There is little information on miR-206 expression and vesicular secretion during gravitational unloading so far. Further research will elucidate the mechanisms regulating the collagen content in a muscle and its stiffness during unloading.

THE ROLE OF SUPPORT AFFERENTATION IN MAINTAINING THE STIFFNESS PROPERTIES OF A POSTURAL MUSCLE

The direct effect of support afferentation on human motor functions was first shown in a joint Soviet-Cuban experiment aboard a Soviet spacecraft. Plantar mechanical stimulation was used in the experiment [99]. Modified devices were further used in dry immersion experiments, which enabled prolonged sessions of plantar stimulation. These studies revealed that support stimulation during immersion maintains a normal level of electrical activity and reflectory transverse stiffness in the soleus muscle [100].

The following protocol for plantar stimulation was used in our experiments: daily plantar pressure of 40 kPa. Stimulation was carried out for 6 h in total, with 20-min exposure sessions at the beginning of each

hour using natural modes of locomotion: slow walking (75 steps/min) for 10 min and fast walking (120 steps/min) for 10 min. No significant decrease in the CSA of slow-twitch muscle fiber and no noticeable change in the percentage ratio of fiber expressing slow- and fast-twitch isoforms of myosin heavy chains were noted in the soleus muscle after 7-day immersion using plantar stimulation [101]. Thus, atrophy was prevented without the use of intense running or resistive loads. The use of plantar stimulation prevented a decrease in the maximum isometric tension and the calcium sensitivity of permeabilized fiber [19, 101, 102]. The obtained results indicate that muscle activity induced by stimulation of support afferents makes it possible to avoid disruptions in cross-bridge formation.

The studies on the transverse stiffness of the myofibrillar apparatus (atomic force microscopy following pretreatment of permeabilized fiber with Triton X-100) using application of plantar stimulation during 7-day immersion demonstrated a significant decrease (by 30%) in stiffness only in the Z-disc plane in relaxed fiber. Transverse stiffness in all other sarcomere regions did not differ statistically significantly from the pre-immersion values [19]. The use of plantar stimulation did not completely prevent stiffness reduction in activated fibers (pCa, 4.2). However, the resulting stiffness drop varied within a range of 15%–25% in different sarcomere regions. Thus, the decrease in the activated fiber stiffness was significantly less pronounced after plantar stimulation compared to that after immersion alone [19]. Apparently, muscle activity enabled preservation of the stiffness of the myofibrillar apparatus by preventing both disruption in cross-bridge formation and breakdown of sarcomeric cytoskeletal proteins. The latter suggestion is supported by the data on the titin and nebulin contents in the human soleus muscle, which were obtained using plantar stimulation during dry immersion. The titin and nebulin contents in individuals in the group of plantar stimulation during dry immersion showed only a slight tendency to decrease, while the same parameters in the group with dry immersion only decreased by something like 40% [101, 102]. A reduced desmin content was not observed during plantar stimulation, either. Since a breakdown of the above cytoskeletal proteins is usually ascribed to the activity of μ-calpain, we may suggest that muscle activity induced by afferent stimulation initiates an endogenous mechanism of calpain inhibition. This mechanism may be associated with maintenance of a high activity of nitric oxide synthase, which is known as an endogenous inhibitor of calpain activity (see above). In our study, plantar mechanical stimulation not only prevented a decrease in the content of neuronal nitric oxide synthase, but also slightly increased its content compared to the pre-immersion level [103]. Further studies will show whether our suggestions about the mechanism underlying support afferentation are valid. These are the mechanisms by which support afferentation, providing a constant (albeit low) activity level in a postural soleus muscle, maintains the normal state of the cytoskeleton and actin–myosin motor mobilization system.

STIFFNESS AND ATROPHY

Skeletal muscle stiffness is not only the mechanical basis for antigravitational stability in mammals, but also an integral component of the mechanotransduction system: i.e., the transformation of mechanical alteration of muscle fiber structures into a metabolic signal regulating gene expression, protein synthesis, and protein degradation. Over the years, numerous publications (e.g., [104]) have discussed a potential signaling role for titin. However, for a long time, there have been almost no experimental data to substantiate these suggestions. The only evidence of a signaling role for titin was translocation of E3 ubiquitin ligase MuRF2 bound to the kinase domain of the titin M-line region to the muscle nucleus during gastrocnemius muscle denervation [105]. In addition, the same research group reported increased ATPase activity and phosphorylation of the titin kinase domain upon titin stretching *in vitro* [106].

The following questions remain open. The first relates to how the titin kinase domain localized in the sarcomere M-line region and involved in dimerization of titin molecules bound to two adjacent myosin filaments can serve as a mechanosensor. The second question is about exactly what mechanical signal it perceives. It was hypothesized that this domain may serve as a sensor for disordering myosin filaments and that it is the sarcomere structure disruption that triggers sarcomeric protein synthesis [107]. This hypothesis is based on a mathematical model of sarcomere mechanics, which also takes into account the contribution of some extra-sarcomeric cytoskeletal proteins of the M-line (mainly obscurin). The suggestion on the involvement of obscurin in the stabilization of thick filaments in sarcomeres was further confirmed in experiments with the flight muscle of obscurin-knockdown Drosophila [108].

Recent experiments on hemidiaphragm denervation compared the signaling properties of muscles in two mutant mouse lines with either increased or decreased titin stiffness. Denervation atrophy was prevented by muscle mechanical stretching stimulating anabolic processes. The anabolic effect of stretching was found to be more pronounced in mice with increased titin stiffness [109]. According to this report, the anabolic signal was transmitted using a specific ankyrin repeat

protein bound to titin. This protein was released from the complex with titin and entered muscle nuclei upon stretching. It is believed to stimulate the expression of the genes regulating anabolic processes in fiber. Thus, the mechanical signal of muscle stretching could transform into a chemical signal that further stimulated protein synthesis.

Based on numerous reports on the anabolic effect of stretching, as well as eccentric and resistive loading in general, a number of authors believe that the source of muscle atrophy during gravitational unloading is not the cessation of fiber contractile activity but rather a decreased tension, i.e. load capacity, resistance of muscle contraction [11, 110]. This conclusion is mainly supported by experiments with chronic low-frequency electrical stimulation combined with suspension [110–112]. Even partial prevention of atrophy in the soleus muscle was not achieved in these experiments. Interestingly, the use of repeated electrical stimulation instead of continuous stimulation prevents not only muscle weight loss, but also a decrease in passive muscle stiffness [113–115]. We used 7-day immersion, combined with mechanostimulation of support afferents, and obtained a significant decrease in the muscle atrophy degree without creating additional tension in the soleus muscle [6, 101]. The use of plantar mechanostimulation in experiments with short-term (1–3-day) hindlimb suspension in rats fully prevents an elevation in proteolytic enzyme expression and partially prevents a decrease in the protein synthesis rate [116]. We may suggest that, at least at the initial stage of unloading, the contractile activity caused by activation of support afferents counteracts the breakdown of the rigid cytoskeletal network and overcomes its intrinsic resistance, thus allowing partial or complete prevention of muscle atrophy.

CONCLUSION

Thus, the facts known to date indicate the following:

– Intrinsic muscle stiffness in human and rodent limbs, both transverse and longitudinal, as well as dynamic and static, naturally decreases as early as during the first week under support withdrawal; the most pronounced stiffness decrease is observed in the Z-disc zone;

– The decrease is accompanied by a reduction in the content of sarcomeric cytoskeletal proteins, both giant ones (titin and nebulin) and the Z-disc proteins stabilizing titin filaments; the contribution of changes in the nature of actin–myosin interactions to a stiffness decrease during gravitational unloading seems insignificant;

– Cytoskeletal proteins are degraded by calpains, members of the family of calcium-dependent cysteine proteases, which are regulated by nitric oxide synthase and some heat shock proteins;

– Activation of muscle contractions under support afferentation reduces the cytoskeletal protein breakdown rate and maintains the level of muscle stiffness close to its native level; and

– Intrinsic muscle stiffness and activity of cytoskeletal proteins are a prerequisite for preventing the atrophy of inactive muscles.

The current state of the issue of the molecular mechanisms reducing the passive stiffness of a postural muscle in simulated gravitational unloading leaves a number of important problems unresolved, which include:

– What sarcomeric component (cross-bridges, giant cytoskeletal proteins, as well as minor and regulatory proteins) changes are responsible for decreased stiffness in an isolated muscle at different time intervals of animal exposure to gravitational unloading?

– What processes lead to breakdown/inactivation of sarcomeric proteins during unloading?

– What role does a decrease in the intensity of defense mechanisms play in these processes?

– Does extracellular matrix proteins (mainly collagens) affect the processes of reducing isolated muscle stiffness?

– What are the mechanisms of cytoskeletal protein influence on the signaling pathways regulating anabolic processes in fiber, and does a decrease in muscle stiffness affect these mechanisms?

The search for answers to these questions could prove exhilarating for future research. ●

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The Role of the MCTS1 and DENR Proteins in Regulating the Mechanisms Associated with Malignant Cell Transformation

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ABSTRACT **The mutations associated with malignant cell transformation are believed to disrupt the expression of a significant number of normal, non-mutant genes. The proteins encoded by these genes are involved in the regulation of many signaling pathways that are responsible for differentiation and proliferation, as well as sensitivity to apoptotic signals, growth factors, and cytokines. Abnormalities in the balance of signaling pathways can lead to the transformation of a normal cell, which results in tumor formation. Detection of the target genes and the proteins they encode and that are involved in the malignant transformation is one of the major evolutions in anti-cancer biomedicine. Currently, there is an accumulation of data that shed light on the role of the MCTS1 and DENR proteins in oncogenesis.**
KEYWORDS **MCTS1 and DENR proteins, malignant cell transformation, translation initiation factors, signaling pathways, apoptosis, cell cycle.**

INTRODUCTION

It is a generally accepted fact that mutations associated with malignant cell transformation disrupt the expression of a significant number of genes whose protein products are involved in the regulation of the activity of many signaling cascades. These cascades are associated with the mechanisms responsible for differentiation, proliferation, as well as sensitivity to apoptotic signals, growth factors, and cytokines.

Abnormalities in the balance of signaling cascades can lead to cell transformation, and subsequent tumor formation. The search for the target genes – and their encoded proteins – which are involved in malignant cell transformation is one of the main challenges of modern cancer biomedicine. Currently, a growing body of data indicates that these genes include *MCTS1* and *DENR*.

MCTS1 AND DENR EXPRESSION

The *MCTS1* (Malignant T-cell-amplified sequence 1) gene, located on the long arm of the X chromosome (Xq22-24), was first described in 1998, at the same time the hypothesis about its involvement in the development of malignant diseases, in particular, the malignant transformation of T-cells, was proposed [1]. Later, the MCTS1 protein was shown to possess the RNA-binding domain PUA, which is characteristic of some tRNA- and rRNA-binding proteins [2]. Next, the PUA domain of MCTS1 was found to be involved in the interaction with the cap-binding complex, one of the components of which, namely the DENR protein, contains the SUI1 domain, which is responsible for translation initiation [3–5].

It is now known that both proteins are normally expressed in almost all tissues; however, the mechanisms they regulate have not been established yet. MCTS1 is assumed to be involved in the regulation of various processes, including cell cycle modulation and apoptosis induction. The gene coding for the DENR (Density-regulated re-initiation and release factor) protein is located on the long arm of chromosome 12 (12q24.31). DENR got its name after a close correlation was uncovered between its level and cell density in culture [6].

The 3'-untranslated region (3'-UTR) of DENR mRNA contains adenine- and uracil-rich sequences. These sequences often serve as binding regions for some of the proteins involved in mRNA turnover. In particular, the AUF1 ribonucleoprotein can bind adenine/uracil-rich regions of the DENR mRNA 3'-UTR, and inhibition of AUF1 expression by RNA interference increases the DENR protein level in cells [7–9].

It has been established relatively recently during ribosomal profiling of NIH3T3 cells with *DENR* knockdown that this protein can bind to the upstream open reading frame (uORF) of *CLOCK* mRNA, one of the key regulators of circadian rhythms [10, 11]. This led to the conclusion that DENR may also be one of the proteins potentially involved in regulating cyclic fluctuations in the biological processes associated with alteration of day and night. Laboratory mice studies showed that the DENR and MCTS1 proteins are involved in neuronal migration during brain development. Furthermore, the *DENR* mutations p.C37Y and p.P121L, resulting in abnormal protein forms, are found in the neuronal cells of patients with autism and Asperger's syndrome, respectively [12].

MCTS1 AND DENR IN TRANSLATION REGULATION

As mentioned above, the involvement of the MCTS1 and DENR proteins in translation regulation has been studied the most. Recently, it has been shown that the MCTS1–DENR complex is highly homologous to the translation initiation factor eIF2D [13]. The MCTS1–DENR complex plays an important role in translation re-initiation [14–18]. Eukaryotic translation re-initiation can occur when the ribosome initiates translation at the uORF. This results in translation termination, with its subsequent re-initiation at the main ORF [19]. However, the molecular mechanisms regulating translation re-initiation are still poorly understood. There are several factors known to be involved in re-initiation; they include the canonical translation initiation factors eIF1, eIF2, and eIF3, which remain associated with ribosomes after termination at uORFs [20]. Later, it was found that eIF2D, a larger protein with a MCTS1 and a DENR homology domains in the N-terminal and C-terminal regions, respectively, is involved in translation re-initiation [14, 15].

Figure 1 shows a schematic representation of the homologous domains between the proteins.

The involvement of DENR and MCTS1 in translation re-initiation was demonstrated in various models, including human cells [18, 21]. Translation re-initiation is known to be accompanied by the formation of a heterodimeric MCTS1–DENR complex and its binding to tRNA [22]. During translation re-initiation, the MCTS1–DENR heterodimer binds to the small (40S) ribosomal subunit, with direct interaction between MCTS1 and the h24 helix of 18S rRNA and between the DENR C-terminal region and the h44 helix of 18S rRNA. This interaction is believed to result in tRNA recruitment to the P-site of the 40S ribosomal subunit. X-ray crystallography studies of the C-terminal region in DENR revealed a high degree of homology between this protein and initiation factor eIF1 [23], which also indicates the involvement of DENR in translation regulation.

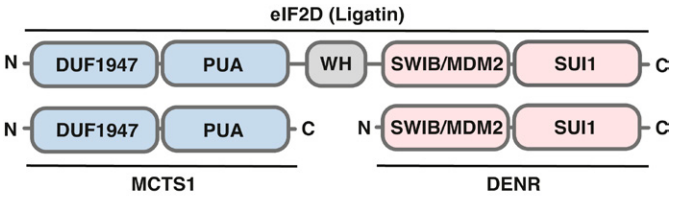

Fig. 1. Domain structure of DENR, MCTS1, and eIF2D. DUF1947 – domain with unknown function; PUA – RNA-binding domain; SWIB/MDM2 – regions homologous to the SWIB protein involved in chromatin remodeling and the p53 inhibitor MDM2; SUI1 – protein region functionally similar to the initiation factor eIF1; WH (winged helix) – DNA-binding domain. MCTS1-homologous regions are highlighted in blue. DENR-homologous regions are highlighted in pink

MCTS1 IN THE REGULATION OF CELL CYCLE AND CDK4/6 ACTIVITY

The MCTS1 protein is involved in cell cycle regulation. *MCTS1* overexpression was shown to increase the proliferation rate of NIH3T3 cells; in particular, by accelerating the progression of the G1 phase of the cell cycle. Meanwhile, this stimulates cell growth [1]. Analysis of cell growth in a semi-liquid medium showed that only cells overexpressing *MCTS1* can form viable colonies [1, 24, 25]. Ectopic expression of *MCTS1* in interleukin-2-(IL-2-)-dependent human EC155 T-cells sensitize them to apoptotic signals [24]. G1 phase progression involves type D- and E-type cyclins, as well as cyclin-dependent kinases (CDKs). Cyclins-D forms a complex with either CDK4 or CDK6 (*Fig. 2*) [26–30]. Ectopic expression of *MCTS1* in NIH3T3 cells increases the level of cyclin D and the efficiency of cyclin D/CDK4 and cyclin D/CDK6 complex formation [24].

A region with a degree of homology to the sequence encoding for cyclin H, namely, a domain responsible for protein-protein interactions, was found in the *MCTS1* nucleotide sequence [31]. This homology between MCTS1 and cyclin H may indirectly indicate the in-

volvement of the MCTS1 protein in cell cycle regulation, particularly the mitotic phase.

MCTS1 AND REGULATION OF APOPTOSIS

MCTS1 is known to reduce the intracellular level of the p53 and p21 proteins, which can also contribute to malignant cell transformation and promote tumorigenesis [31]. Treatment of human MCF-7 cells with bleomycin, which induces double-strand breaks in the DNA of rapidly dividing cells, increases the expression of *TP53* encoding the p53 protein. Ectopic *MCTS1* expression decreases the level of p53 activation in cells treated with bleomycin and, hence, the efficiency of apoptosis of damaged cells [31].

Cells with ectopic expression of *MCTS1* contain higher levels of ubiquitinated p53 (Ub–p53) and phosphorylated MDM2. This suggests that a decrease in the p53 level due to high *MCTS1* expression may be associated with MDM2-dependent degradation of p53 in proteasomes [32]. Treatment of these cells with the proteasome inhibitor MG132 increased the p53 level, which indicates the involvement of MCTS1 in the regulation of its stability [31].

Treatment of cells with ectopic expression of *MCTS1* with bleomycin resulted in a less efficient synthesis of the p21 protein, one of the major targets of p53, compared to control cells. Small interfering RNA-mediated suppression of *MCTS1* increased the expression levels of not only p53, but also p21 (*Fig. 3*) [31]. The MEK/ERK signaling cascade is known to be involved in the regulation of p53 activity and p21 expression [33, 34]. MCTS1 enhances phosphorylation of the ERK1/2 protein kinase (pMAPK) [35], which is part of one of the main signaling pathways involved in malignant cell transformation and associated with sensitivity to chemotherapeutic drugs [36–39]. Inhibition of *MCTS1* expression by RNA interference in MCF-10A breast cancer cells, and A549 lung cancer cells, results in caspase-3 activation and cell death. Suppression of *MCTS1* expression in lung and breast tumors xenografts significantly suppresses tumor development [35, 40].

ASSOCIATION OF MCTS1 WITH CHROMOSOME INSTABILITY

Cytogenetic analysis demonstrated that MCTS1 affects genome integrity. In particular, irradiated MCF-7 cells overexpressing *MCTS1* were found to increase the number of chromosomal breaks by 20%, formation of larger derivative chromosomes by 28%, and reduction in chromatid gaps b by 62% compared to control samples [31]. Thus, chromosomal aberrations are more likely to occur in *MCTS1*-overexpressing cells.

MCTS1 is known to reduce cell sensitivity to etoposide, an inhibitor of topoisomerase II. In order to

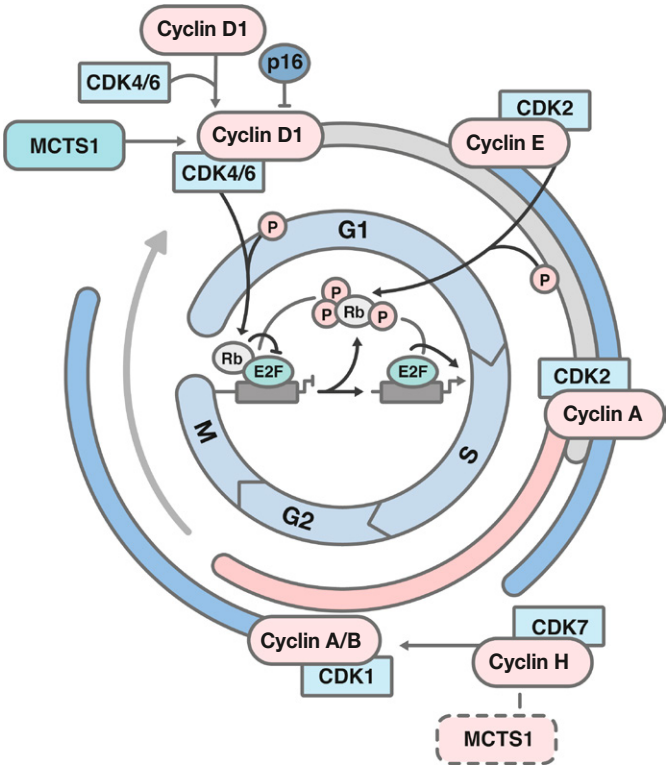

Fig. 2. Schematic representation of the cell cycle. CDK – cyclin-dependent kinase; it is involved in cell cycle progression. Phosphorylation of the Rb (retinoblastoma protein) protein leads to transition through the G1/S stages. E2F – transcription factor; p16 (CDKN2A) – a CDK inhibitor; it impedes cell division while inhibiting G1/S transition; G1/S/G2 – interphase, M – mitosis

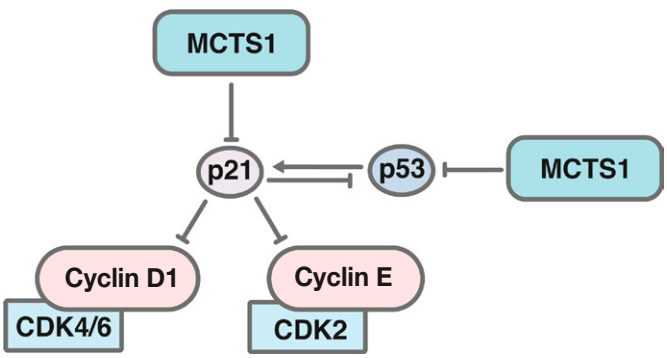

Fig. 3. Effect of MCTS1 on the pro-apoptotic protein p53 and its inhibitor p21. Formation of a complex between cyclin D1 and CDK4/6 and a complex between cyclin E and CDK2 regulates the transition through the G1 stage of the cell cycle

compare the sensitivity of *MCTS1*-overexpressing cells to the genotoxic effect of etoposide, the DNA comet assay, which allows one to determine the frequencies of DNA double-strand breaks and its repair, was used. Etoposide-treated cells overexpressing *MCTS1* turned out to have a shorter DNA comet tail, which indicates a more efficient path of repair processes compared to control cells expressing low levels of *MCTS1* [31]. A decreased *MCTS1* expression was also shown to activate proteolytic cleavage of poly (ADP-ribose) polymerase (PARP) and reduce its activity. PARP is one of the main proteins responsible for DNA repair, including those associated with the effect of chemotherapeutic drugs [41]. It should be noted that PARP inhibitors are considered promising agents against a number of malignancies [42–44].

EFFECT OF MCTS1 ON AKT AND SRC SIGNALING

Protein phosphatase PTEN is one of the main elements in the negative regulation of the AKT signaling pathway (protein kinase B). PTEN damage resulting from mutations or a significant decrease in protein expression can cause malignant cell transformation [45–49]. Ectopic expression of *MCTS1* in the human breast cancer cells MCF-10A decreases the levels of PTEN mRNA and protein [40]. An increase in *MCTS1* expression is accompanied by PTEN degradation. MCTS1 also stimulates the interaction between the Src and p190B proteins, resulting in the formation of a complex inhibiting RhoA, one of the main factors regulating cytokinesis (*Fig. 4*) [50].

MCTS1 is known to regulate not only Src, but the Shc–Ras–ERK signaling pathway as well. Shc (transforming protein 1 with an Src homology domain) is an adaptor protein involved in signal transduction upon activation of certain receptors [51]; in particular, the epidermal growth factor receptor (EGFR) [52], erbB-2 receptor [53], and insulin receptor [54]. Several isoforms of the Shc protein are usually present in cells. An excessive Shc level is associated with abnormal activation of the ERK signaling pathway [55], which, in turn, significantly affects the development and progression of malignancies, including the sensitivity of malignant cells to chemotherapeutic drugs. Suppression of *MCTS1* expression in immortalized cell lines of breast and lung cancer by RNA interference decreases the levels of p66, p52, and p46 isoforms of the Shc protein [35]. The direct effect of MCTS1 on the signaling pathway involving Shc may partially explain how the increase in *MCTS1* expression associated with the induction of cyclin D1 accumulation and activation of the Rb protein phosphorylation impact on the acceleration of the G1 phase progression (*Fig. 2*).

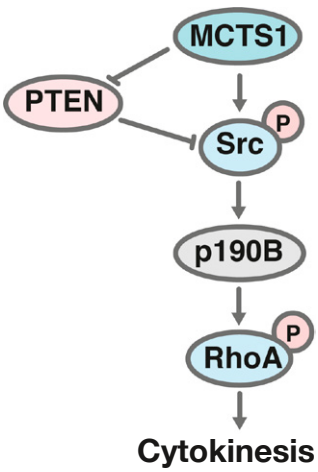

Fig. 4. Effect of MCTS1 on the PTEN/Src signaling. PTEN – an inhibitor of the PI3K/AKT/mTOR signaling pathway; Src – a protein kinase of the Src kinase family; RhoA – a transforming protein of the Ras family of GTPases

MCTS1 ROLE IN THE IL-6/IL-6R SIGNALING PATHWAY

The IL-6/STAT3 signaling pathway is known to be involved in the regulation of breast cancer cell stemness [56]. Ectopic expression of *MCTS1* in the human breast cancer cells MDA-MB-231 stimulates the formation of malignant, discrete clusters of cells, namely mammospheres, upon cell growth under certain conditions. It is important that elevation of *MCTS1* expression increases the level of CD44, a tumor stem cell marker [57]. Treatment of the cells ectopically expressing *MCTS1* with the IL-6 cytokine leads to an even more rapid formation of mammospheres; therefore, MCTS1 may be involved in the regulation of IL-6 signaling (*Fig. 5*). Treatment of cells with tocilizumab, a monoclonal antibody that inhibits the IL-6 receptor, reduces the intensity of mammosphere formation under *MCTS1* induction and also significantly decreases the number of cells CD44+/CD24- subpopulation to a control level [57]

A study of the relationship of MCTS1 and IL-6 with the clinical path of the disease revealed a positive correlation between the proteins levels in all patients with triple-negative breast cancer with a deficient expression of the epidermal growth factor receptor (HER2), estrogen receptor (ER), and progesterone receptor (PR). Moreover, high *MCTS1* and *IL-6* levels were found to correlate with the risk of metastases [57].

Cytokines and growth factors produced by cells of the tumor microenvironment play an important role in tumor progression [58–60]. Triple-negative breast cancer cells with enhanced *MCTS1* expression secrete significantly more of the pro-inflammatory cytokines IL-6, MCP-1, and GM-CSF than cells with a relatively lower *MCTS1* expression level [57].

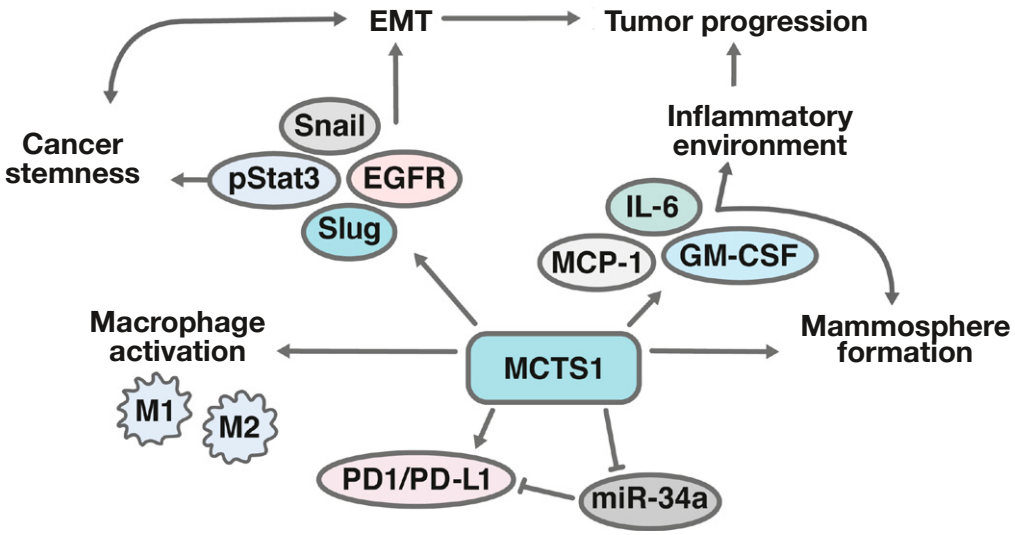

Fig. 5. Schematic illustration of the contribution of MCTS1 to EMT, tumor escape from immune surveillance, and activation of pro-inflammatory factors by tumor cells. EMT – epithelial-mesenchymal transition; Snail and Slug – transcription factors involved in EMT; EGFR – epidermal growth factor receptor; pro-inflammatory cytokines IL-6 – interleukin-6, MCP-1 – monocyte chemoattractant protein, and GM–CSF – granulocyte-macrophage colony-stimulating factor; M1 – classically activated macrophages providing the production of pro-inflammatory cytokines; M2 – macrophages responsible for anti-inflammatory response

MCTS1 AND IMMUNE SURVEILLANCE OF A TUMOR

When developing approaches to the immunotherapy of malignancies, various methods, such as the receptors and ligands regulating immune surveillance, are used to inhibit immune checkpoints [61]. Currently, one of the most studied mechanisms is based on inhibiting the PD1 receptor and its ligand, PD-L1. An increased PD-L1 level is observed in many oncological diseases. An abnormally high expression of this ligand on the surface of malignant cells is considered to be associated with their evasion of immune surveillance [62, 63]. Anti-PD1/PD-L1 antibodies have been approved for the treatment of certain cancers (melanoma, non-small cell lung cancer, Hodgkin lymphoma, bladder cancer, renal cell carcinoma, squamous cell carcinoma of the head and neck, breast cancer, Merkel cell carcinoma, hepatocellular carcinoma, and stomach cancer) [62]. However, the use of anti-PD1/PD-L1 antibodies turned out to be effective only in some patients and does not always lead to the desired result. MicroRNA miR-34a is involved in the regulation of the PD-L1 signaling pathway [64, 65]. An increase in miR-34a expression in cancer cells causes a pronounced antitumor effect [65].

MCTS1 can induce PD-L1 expression while decreasing miR-34a levels. miR-34a can inhibit the epithelial-mesenchymal transition (EMT) induced upon activation of the TGF-β (transforming growth factor β) signaling pathway [66]. In addition, miR-34a negatively affects the expression of the genes coding for the proteins involved in EMT (Snail, Slug, and ZEB1), as well as the proteins associated with the maintenance of tumor stem cells (BMI1, CD44, CD133, OLFM4, and c-MYC) [67]. In addition, miR-34a is directly involved in the regulation of macrophage activation in the tumor microenvironment and closely related to the immune response to tumor cells. All of this suggests that *MCTS1* suppression, combined with *miR-34a* gene activation, can be considered as a promising strategy in breast cancer therapy.

ROLE OF MCTS1 AND DENR IN MALIGNANCIES

The hypothesis on the involvement of *MCTS1* in the malignant transformation of lymphoid cells was suggested almost immediately after the discovery of this gene. Abnormal *MCTS1* amplification was noted in various malignant lymphoid cell lines. In normal lymphoid tissues, the *MCTS1* gene is expressed at a low level [67].

An increase in *MCTS1* expression was found in IL-2-independent, but not in IL-2-dependent, T-cell lines, including IL-2-stimulated peripheral blood lymphocytes (PBLs) [67]. A high level of *MCTS1* expression

was also observed in a number of transformed B-cell lines derived from patients with non-Hodgkin lymphoma [67].

Thus, an increased level of *MCTS1* was found in 41% of diffuse large B-cell lymphoma patient samples. However, expression of *MCTS1* was not observed in chronic lymphocytic leukemia cells [67].

Increased *MCTS1* expression was later shown to be typical not only of malignant lymphoid diseases.

The study using the Kaplan–Meier method (kmplot.com) demonstrated that high *MCTS1* expression in breast cancer samples is associated with a lower overall survival rate in patients compared to relatively lower *MCTS1* levels. This is typical of *TP53*-positive breast cancers, lymph node metastases-free breast cancers, *HER2*-negative breast cancers, luminal-A, and luminal-B breast cancers. Patients with relatively high *MCTS1* levels in biopsies have lower recurrence-free survival rates compared to patients with a low *MCTS1* expression.

Elevated *MCTS1* levels were also detected in lung cancer samples. Moreover, high expression levels were noted for all four stages of the disease [57].

Bioinformatic analysis of the transcriptome of tumor cells derived from patients with lung cancer, stomach cancer, hepatocellular carcinoma, and kidney cancer showed that low *DENR* levels correlate with a more favorable disease path and better prognosis [68]. Gene Set Enrichment Analysis (GSEA) showed that *DENR* can be associated with the regulation of the signaling cascades responsible for cell cycle progression, DNA repair, and splicing [68]. Analysis of *DENR* expression in lung cancer metastases showed that a higher gene expression level is characteristic of lymph node metastases.

Detection of the tumor marker alpha-fetoprotein in the blood serum is widely used in the diagnosis of malignancies. An increased level of alpha-fetoprotein is found in blood serum for liver, breast, stomach, and sometimes lung cancer [69, 70]. High serum alpha-fetoprotein levels are associated with a poor prognosis in patients with hepatocellular carcinoma [71]. A bioinformatic analysis of the transcriptome databases of patients with various oncological diseases revealed that a high level of *DENR* expression in tumor cells correlates with a high serum level of alpha-fetoprotein [68].

Higher *DENR* levels are characteristic of later stages of various tumors, including hepatocellular carcinoma, lung, breast, kidney, and rectal cancer. One study noted that a relatively higher *DENR* expression might indicate an increased risk of glioma in dogs [72]. This was established by a comparative analysis of the transcriptomes of brain samples derived from dog breeds with an elevated risk of developing glioma and breeds less prone to this disease.

The data above indirectly suggest that *DENR* may be associated with the onset and development of oncological diseases and can be directly involved in tumor development. However, it should be noted that most of the data supporting this assumption are obtained using bioinformatics analyses. At the same time, there is nary data to indicate the functional effect of this protein on cellular growth and their sensitivity to chemotherapeutic drugs. It should be also noted that, currently, there is a relatively scarce amount of data describing the involvement of *DENR* in the regulation of the expression of the genes involved in the development of malignant diseases.

These data indicate that the DENR and MCTS1 proteins can be considered promising diagnostic and therapeutic targets. ●

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At the Crossroads: Mechanisms of Apoptosis and Autophagy in Cell Life and Death

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ABSTRACT **Apoptosis and autophagy are conserved processes that regulate cell survival and death under stress conditions. Apoptosis aims to remove cells from the body with minimal damage to surrounding tissues. Autophagy promotes removal of damaged organelles, protein aggregates, and cellular pathogens, stimulating cell survival. The signaling pathways involved in the regulation of apoptosis and autophagy largely overlap, leading to both competition and unidirectional interaction, which is of particular interest in investigating them as potential targets for cancer, autoimmune, and neurodegenerative disease therapies. This review analyzes the main pathways of molecular interactions between autophagy and apoptosis, which is necessary for understanding the mechanism maintaining the balance between cell death and survival under unfavorable conditions.**
KEYWORDS **apoptosis, autophagy, telomerase, signaling pathways, regulation.**

INTRODUCTION

Autophagy is a process that is stimulated by intracellular or environmental stress. The formation of autophagosomes and their fusion with lysosomes result in targeted degradation of damaged organelles, protein aggregates, and intracellular pathogens [1]. Investigating autophagy has become of great importance in the last decade, because the process is involved in the regulation of the metabolism of both the cell and the body. Dysregulation of autophagy affects the basic metabolic functions of cells, which can lead to the development of various diseases [2]. Now, there is reliable evidence that activation of autophagy by anticancer drugs can protect cancer cells from death, and that a decrease in the autophagy level is associated with the development of neurodegenerative and autoimmune diseases and general aging of the body [3].

Apoptosis is an evolutionarily conserved programmed mechanism of cell death, which selects cells during the normal development of eukaryotes and the maintenance of body homeostasis. Apoptosis is accompanied by morphological changes in the cell structure, which are associated with enzyme-dependent biochemical processes, as well as by the removal of cells from the body with minimal damage to the surrounding tissues [4].

Decreased cell apoptosis, coupled with a high proliferation level, can provoke the development of diseases such as cancer, while an accelerated rate of cell death promotes pathologies such as Alzheimer's disease, Parkinson's disease, and rheumatoid arthritis [4]. As we age, the efficiency of autophagy decreases [5, 6] while apoptosis increases in intensity [7]. Therefore, this review explores the molecular mechanisms regulating the cross-talk between apoptosis and autophagy.

APOPTOSIS

Apoptosis is a process of controlled death of the cell without spillage of its contents into the surrounding environment, which is called programmed cell death [4]. This process is regulated by proteins of the Bcl-2 family, which include both pro-apoptotic and anti-ap-

optotic components. The balance of these components determines cell life or death [8]. Stimulation of apoptosis leads to the activation of the pro-caspases that are the precursors of the cysteine-aspartic proteases known as caspases. There are two categories of caspases: initiator caspases and executioner caspases [9]. Specific signals indicative of cell damage stimulate the initiator caspases (caspases 8 and 9) that are activated by autoproteolysis and hydrolyze precursors of the executioner caspases (caspases 3, 6, and 7), ensuring that they remain active. Activation of the executioner caspases initiates a cascade of events that lead to the destruction of nuclear and cytoskeletal proteins, to protein crosslinking, the expression of ligands recognized by phagocytic cells, the formation of apoptotic bodies, and cell death [10, 11]. Apoptosis is accompanied by DNA fragmentation by endonucleases. The apoptosis process is highly conserved in multicellular organisms and is genetically controlled [12]. There are two pathways of apoptosis initiation: intrinsic and extrinsic. *Figure 1* shows the pathways of apoptosis.

The intrinsic pathway depends on factors released from mitochondria [13]; it includes various stimuli that act on several targets in the cell. The lack of cytokines, hormones, and growth factors leads to the activation of intracellular apoptosis activators from the Bcl-2 family, such as the p53 upregulated modulator of apoptosis (PUMA), Noxa, and BAX [4]. Under normal conditions, these proteins usually interact with anti-apoptotic proteins of the Bcl-2 family. In the absence of signals for survival and proliferation and upon exposure to hypoxia, toxins, radiation, reactive oxygen species, and viruses [14], the PUMA protein usually accumulates and its excess interacts with pro-apoptotic proteins of the Bcl-2 family, such as BAK and BAX. Their translocation into the mitochondrial membrane causes opening of the mitochondrial pore and relocation of pro-apoptotic proteins, such as cytochrome *c*, Smac/Diablo, and HtrA2/Omi, into the cytoplasm. Cytochrome *c*, a component of the mitochondrial respiratory chain, enters the cytoplasm, interacts with the apoptotic protease activating factor 1 (Apaf-1), and forms an apoptosome [15] that promotes the activation of initiator caspase 9, which triggers a cascade of apoptotic reactions. The mitochondrial proteins Smac/Diablo and HtrA2/Omi enter the cytoplasm and interact with inhibitors of apoptosis (IAP proteins), which promotes the release of caspases and their activation [4].

The extrinsic pathway of apoptosis activation is regulated by signaling cascades triggered by the death receptor (DR) [13]. Binding of death ligands, which are secreted by patrolling natural killer cells (NK cells) and macrophages or anchored on the surface of lymphocytes, to the DRs promotes interaction between the DR

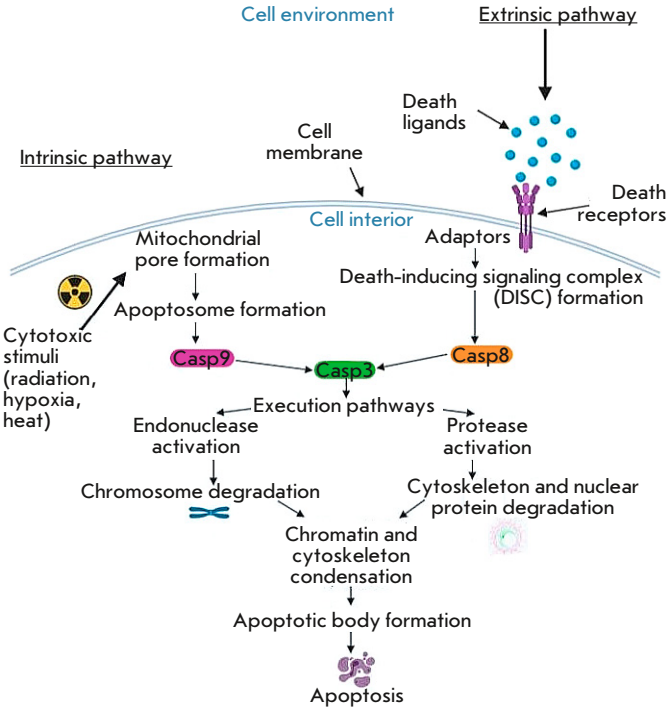

Fig. 1. Scheme illustrating the intrinsic and extrinsic pathways of apoptosis activation (according to D'Arcy [4])

cytoplasmic death effector domain (DED) and monomeric procaspase 8 [16]. The resulting death-inducing signaling complex (DISC) provides proteolytic activation of caspase 8. The processed caspase induces the apoptosis-stimulating activity of endonucleases and proteases [4, 16].

The p53 transcription factor plays a key role in the regulation of apoptosis. p53 has a short lifetime, and its concentration in mammalian cells remains low through constant ubiquitination and subsequent degradation. But under stress conditions (DNA damage, hypoxia, cytokines, etc.), ubiquitination of p53 is inhibited and p53 is stabilized and accumulates in the nucleus. Various kinases provide for activated phosphorylation of p53. Depending on the conditions, these can be the kinases involved in cell cycle control (checkpoint kinases (Chk)) and cAMP-dependent protein kinase A (PKA), a regulator of lipid metabolism and glucose and glycogen levels; cyclin-dependent kinase 7 (CDK7) involved in cell cycle control and regulation of the transcriptional activity of RNA polymerase II; DNA-dependent protein kinase (DNA-PK), a mediator of the cellular response to DNA damage; and mitogen-activated protein kinases (MAPKs) such as Jun N-terminal kinase (JNK). Phosphorylation stimulates the oligomerization

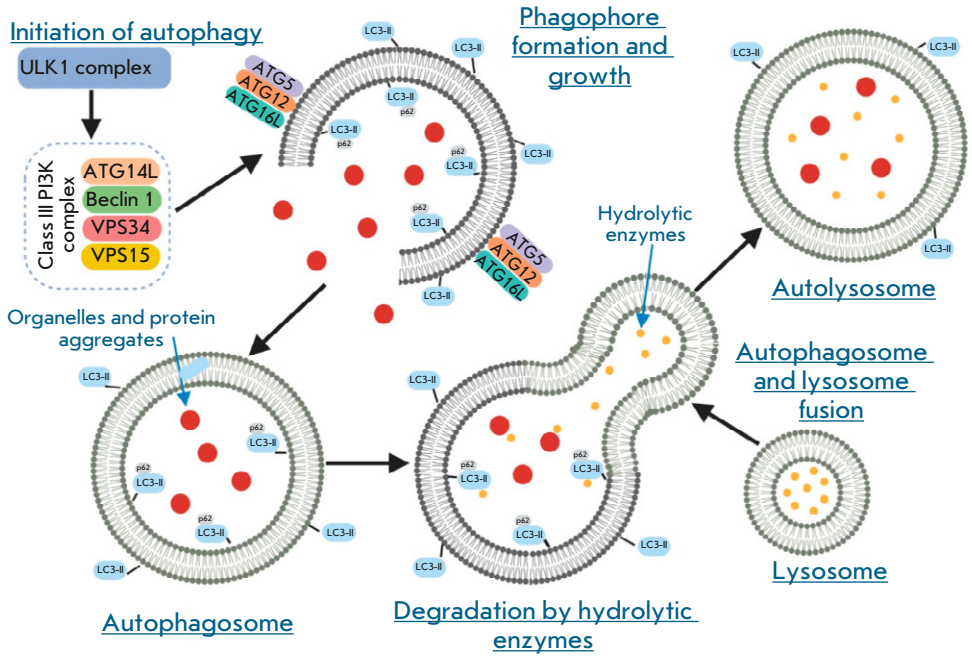

Fig. 2. General scheme of autophagolysosome formation (according to D'Arcy [4]). Activation of ULK1 and class III PI3K complexes stimulates autophagophore formation. A complex consisting of ATG5, ATG12, and ATG16L stimulates, together with LC3II, phagophore lengthening and is necessary for autophagosome formation. The p62 protein associates with LC3II and ubiquitinated degraded proteins and is engulfed by the autophagosome. Lysosomal enzymes hydrolyze the autophagosome contents after fusion

of p53, resulting in the formation of a tetramer. Tetrameric p53 activates the expression of genes whose promoter regions contain sites for interaction with p53 [17, 18]; e.g., Fas ligand genes [19, 20] and the *DR5* gene encoding a death receptor interacting with tumor necrosis factor family cytokines TRAIL (TNF-associated apoptosis-inducing ligand). Involvement of p53 in the intrinsic pathway of apoptosis is associated with the Bcl-2 family proteins that regulate the release of cytochrome *c* from mitochondria. The key pro-apoptotic Bcl-2 genes include *BAX*, *Noxa*, *PUMA*, and *BID*, which are targets for p53 [21].

AUTOPHAGY

During autophagy, various cellular components or even entire organelles enter lysosomes that contain enzymes that hydrolyze engulfed components [4]. Autophagy is stimulated in response to various factors such as ATP and nutrient deficiency or signals originating on the surface of damaged organelles or regulating cell differentiation during embryogenesis [22]. The autophagy process underlies adaptive and innate immunity. For example, destruction of intracellular pathogens, delivery of antigens to MHC class II holding compartments, and transport of viral nucleic acids to Toll-like receptors involve autophagosomes [23]. Although autophagy is often used to recycle cellular components, it can also lead to cell destruction. Therefore, autophagy is associated with the removal of senescent cells from tissues and the destruction of tumor lesions [22]. A low efficiency of autophagy is associated with the development of cancers and, especially in old age, the accumulation of protein aggregates in neurons and the development of neurodegenerative conditions, including Alzheimer's [24]. Autophagy activation in rapidly proliferating cells facilitates the overcoming of deficiency in the intracellular components necessary for biosynthesis [25]. An increased autophagy level, often present in cancer cells, enables the cells to function more efficiently under nutrient deficiency and also reduces their sensitivity to cytotoxic substances [26].

There are three different forms of autophagy: macroautophagy, microautophagy, and selective autophagy. In macroautophagy, whole regions of the cell are enclosed in double-membrane vesicles called autophagosomes. Autophagosomes fuse with lysosomes to form autophagolysosomes, the contents of which are degraded by hydrolytic enzymes [27]. *Figure 2* presents a general schematic of autolysosome formation [4].

Pathways of autophagy regulation

At the first stages of autophagy, the ULK1 complex consisting of Unc-51-like autophagy-activating kinase 1 (ULK1), autophagy-related protein 13 (ATG13), focal adhesion kinase family interacting protein of 200 kDa (FIP200), and ATG101 is translocated to the autophagy initiation sites and regulates the recruitment of the VPS34 (vacuolar protein sorting 34) complex. VPS34,

composed of class III phosphoinositide 3-kinase (PI3K), VPS34, ATG14L, VPS15, and Beclin 1 (*Fig. 2*), provides for the formation of phosphatidylinositol-3-phosphate (PI3P) at phagophore formation sites. PI3P initiates the binding of a number of proteins that form the autophagosome. The formed phagophores gradually increase through two ubiquitin-like conjugation cascades: ATG5-ATG12 and MAP-LC3/ATG8/LC3. The phagophore, as it elongates, engulfs part of the cytoplasm, forming a double-membrane autophagosome by self-closure. Finally, fusion of the autophagosome with the lysosome leads to the formation of an autolysosome and degradation of the contents, and the produced macromolecular blocks are released into the cytosol and can be re-used by the cell as building blocks [28]. The central regulator of autophagy is the mammalian target of rapamycin (mTOR) kinase. Suppression of mTOR activity stimulates ULK1 complex formation and activates autophagy.

Activation of autophagy by internal or external stimuli is subject to multistep regulation that involves the main cellular signaling cascades. The most studied modulators of autophagy are the PI3K, AKT, and AMPK kinases that regulate cell proliferation, metabolism, and survival. Activation of the PI3K/AKT/mTOR-mediated signaling pathway usually inhibits autophagy [29]. This signaling cascade is modulated by the phosphatase and tensin homolog (PTEN), insulin, Sirt1, 5'-AMP-activated protein kinase (AMPK), p38 mitogen-activated protein kinase (p38 MAPK), and p53. Stimulation of autophagy is facilitated by the activation of the MAPK signaling pathway Ras/Raf/ERK that regulates the activity of the JNK kinases involved in the modulation of proliferation, differentiation, inflammation, and apoptosis. Activating mutations in the *Ras* or *B-Raf* oncogenes are often associated with a malignant transformation of cells, and JNKs regulate apoptosis through post-translational phosphorylation of Bcl-2 [30, 31].

Figure 3 displays the mechanisms regulating autophagy.

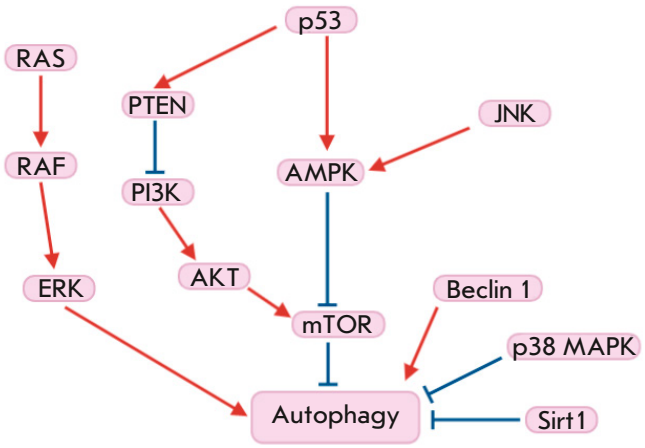

Fig. 3. Scheme illustrating the regulatory mechanisms of autophagy (according to Jiang [1])

AUTOPHAGY AND APOPTOSIS: MOLECULAR INTERACTIONS

Both autophagy and apoptosis play an important role in the development processes, maintenance of tissue homeostasis, and the pathogenesis of many diseases. To date, there is growing evidence that the main molecular components of the signaling pathways of autophagy and apoptosis are in complex cross-talk and are often induced by similar stimuli. For example, experiments have demonstrated that both apoptosis and autophagy are activated in response to metabolic stress [32] or exposure to reactive oxygen species [33]. Interesting data on the cross-talk between autophagy and apoptosis were obtained by the analysis of the molecular mechanisms of endoplasmic reticulum stress. The adaptive response of cells to the disruption of calcium homeostasis or endoplasmic reticulum dysfunctions is an enhancement of autophagy and apoptotic cell death [34].

There are several key variants of functional interactions between apoptosis and autophagy. In the case of "partnership relationships," apoptosis and autophagy act in the same way, leading to cell death. In the case of an "antagonistic relationship," apoptosis and autophagy are processes with different goals. In this case, autophagy does not lead to cell death and, moreover, reduces the efficiency of apoptosis, providing conditions favorable to cell survival. In the case of "activating Relationships", autophagy promotes the apoptotic program, ensuring certain stages, without leading to cell death in itself [35].

Therefore, autophagy and apoptosis can interact, counteract, or facilitate each other, affecting the cell's fate in different ways. In this case, there are several main molecular pathways that provide for complex functional interactions between autophagy and apoptosis.

Beclin 1 regulates the choice between autophagy and apoptosis

An important component of the pre-autophagosome is the Beclin 1 protein that plays a regulatory role in choosing the stress response mechanism. The Bcl-2 protein family (Bcl-2, Bcl-xL, and Mcl-1) includes

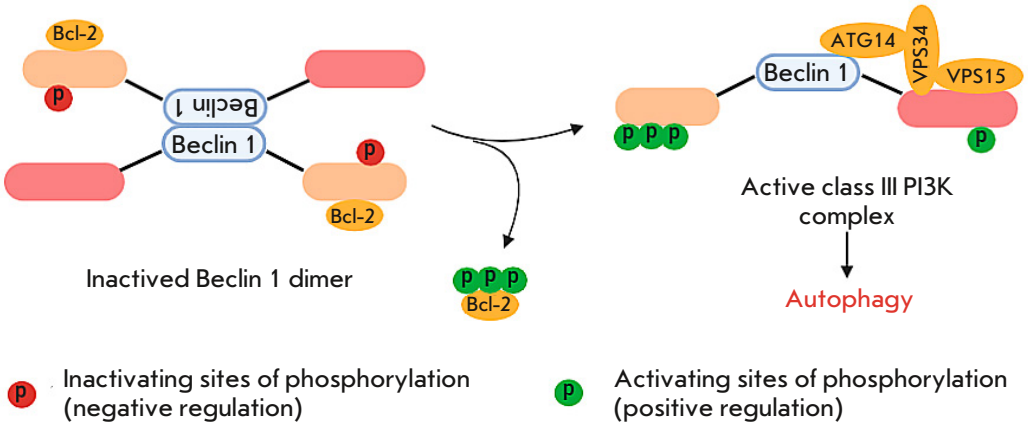

Fig. 4. Scheme illustrating the conversion of an inactive Bcl-2–Beclin1 complex into the active PI3K complex (according to Menon [41])

well-known anti-apoptotic mediators whose role in suppressing autophagy is under study. The cytoprotective function of Bcl-2 proteins is related to their ability to interact with BAX and BAK and, thus, prevent apoptosis [36]. The Beclin 1 protein contains a BH3 domain homologous to the Bcl-2 domains. This protein determines the fate of cells under stress by modulating the interaction of autophagy and apoptosis. Beclin 1 recruits key autophagic proteins into the pre-autophagosomal structure [4]. The Beclin 1 BH3 domain is responsible for interaction with anti-apoptotic members of the Bcl-2 family (*Fig. 4*), which interferes with the assembly of the pre-autophagosomal structure and leads to the inhibition of autophagy [36]. Under starvation stress, JNK kinase phosphorylates Bcl-2, which promotes dissociation of the Bcl-2–Beclin 1 complex, followed by pre-autophagosomal structure assembly and autophagy [37]. Prolonged activation of the JNK cascade and Bcl-2 phosphorylation lead to apoptosis, due to caspase 3 activation [36]. Kinases, such as the cell-death-associated protein kinase (DAPK), Rho-associated kinase 1 (ROCK1) involved in the regulation of cell proliferation, inflammation, and adhesion [38, 39], as well as MK2 and MK3, which serve as substrates for p38 MAPK [40], were shown to perform inhibitory phosphorylation of the Beclin 1 BH3 domain and block the assembly of the pre-autophagosome. The stimulating effect is exerted by kinase Mst1, a regulator of effector T cell activity and regulatory T cell differentiation. Phosphorylation of the Beclin 1 BH3 domain by Mst1 kinase promotes the Bcl-2–Beclin 1 interaction [38], thereby preventing the assembly of the class III PI3K complex, which leads to the inhibition of autophagy. *Figure 4* presents a schematic of activated class III PI3K complex formation.

mTOR kinase signaling pathways

One of the points of molecular interaction between the autophagy and apoptosis pathways is mTOR kinase, a serine/threonine kinase from the phosphatidylinositol kinase family, which plays an important role in the regulation of growth and aging processes. The mTOR kinase activity changes depending on external and internal factors: presence/absence of nutrients, ATP, growth factors, and stress factors [1].

mTOR is known to be involved in two complexes: mTORC1 consisting of mTOR, mLST8, DEPTOR, RAPTOR, and PRAS40; mTORC2 consisting of mTOR, mLST8, DEPTOR, RICTOR, mSIN1, and PROTOR. mTORC1 phosphorylates ribosomal protein S6 kinase (p70 S6K1) and translation initiation factor 4E-binding protein 1 (4EBP1) and stimulates protein biosynthesis. mTORC1 performs regulatory phosphorylation of ULK1, which inhibits autophagy, and is involved in lipid metabolism via a modification of Lipin 1 phosphatidate phosphatase. The mTORC2 complex was discovered relatively recently. mTORC2 is activated in response to growth factors, and its substrates are AKT kinase, serum and glucocorticoid-inducible kinases (SGKs), and protein kinase C (PKC), a component of the regulatory cascade activated by G-protein-coupled growth factor receptors [42].

mTOR activity is regulated by the small GTP-binding protein Rheb. After binding to GTP, Rheb activates mTOR. GTP hydrolysis stimulated by the TSC1/TSC2 (tuberous sclerosis) complex leads to the inactivation of Rheb and mTOR, respectively. Inhibition of autophagy by the regulatory phosphorylation of TSC1/TSC2 is mediated by various factors. For example, AKT and MAP kinases, extracellular signal-regulated kinases (ERK), and p90 ribosomal S6 kinase (RSK), which

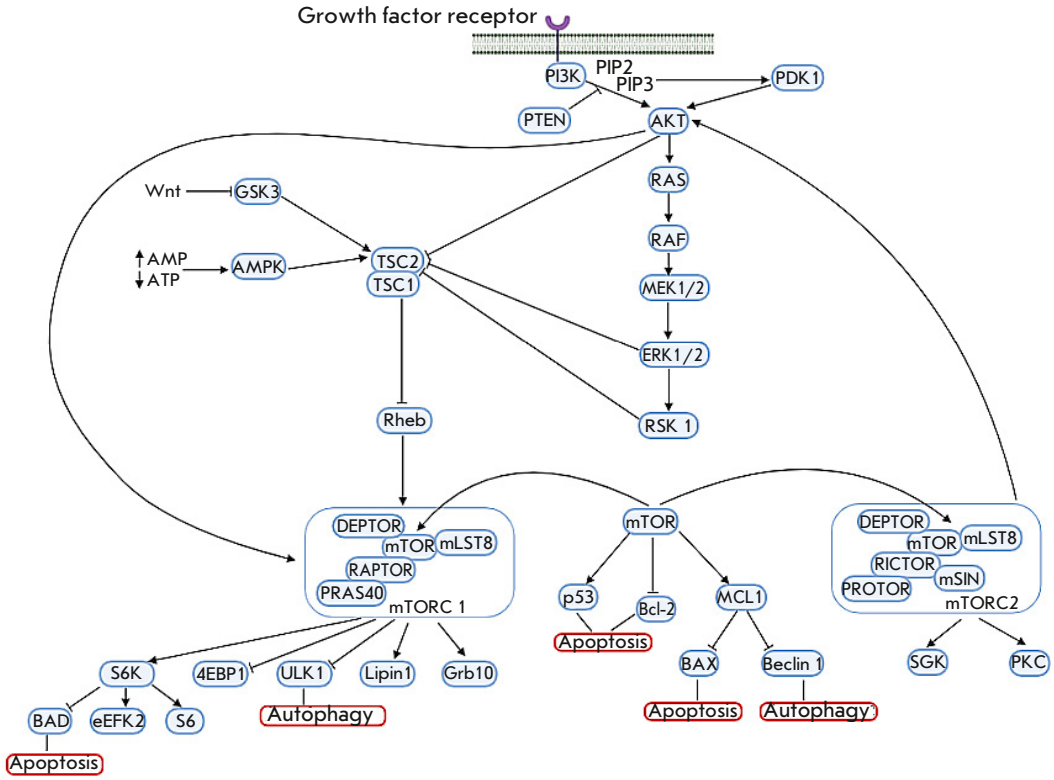

Fig. 5. Scheme illustrating the mTOR-regulated pathways of autophagy and apoptosis (according to [35, 42, 48, 49])

perform inactivating phosphorylation of Ser939 TSC2, inhibit autophagy; AMPK, which phosphorylates Ser1387 TSC2, stimulates autophagy [35].

Stress or nutrient and energy deficiency in the cell leads to the inhibition of mTOR activity and, therefore, to the induction of autophagy [1]. However, prolonged starvation leads to mTOR reactivation and, consequently, to the inhibition of autophagy [43].

In addition, mTOR has a pleiotropic effect on apoptosis, in particular through the p53, BAD, and Bcl-2 proteins [44]. The interaction between Bcl-2 and Beclin 1 inhibits autophagy and prevents the regulation of the expression of pro-apoptotic protein genes by the p53 protein [45]. MCL1, one of the Bcl-2 family proteins, has been shown to act as a stress sensor that simultaneously controls both autophagy and apoptosis in neurons [46, 47].

Figure 5 shows the pathways of interaction between autophagy and apoptosis with the involvement of mTOR.

p38 MAPK signaling pathway

p38 MAPK plays an important role in the regulation of apoptosis, cell cycle, and growth and differentiation processes and serves as a target for a number of drugs (e.g., cyclophosphamide, oxaliplatin). But under certain conditions, p38 MAPK can also mediate resistance to apoptosis (through activation of COX-2, etc.) [50]. p38 MAPK-regulated signaling pathways are activated in response to a wide range of stimuli, such as mitogenic factors (e.g., growth factors or cytokines), environmental signals, and genotoxic stress. After exposure to these stimuli, p38 MAPK is activated by the upstream kinases MKK3 and MKK6. Sometimes, p38 can also be phosphorylated by MKK4 kinase, which is well known as a JNK activator [51].

In addition to apoptosis, p38 MAPK is involved in the regulation of autophagy in response to chemotherapeutic agents [52]. The molecular mechanisms of the interaction between p38 and autophagy remain largely unknown. By phosphorylating Atg5, p38 MAPK is known to be able to inhibit the autophagy caused by a lack of nutrients [53]. Also, p38 MAPK can negatively regulate macroautophagy during cell growth in a normal medium containing amino acids and serum (basal autophagy) [54], and autophagy caused by nutritional deficiencies [55, 56]. Activation of p38 MAPK signaling induces autophagy to maintain cell survival through phosphorylation of GSK3β kinase from the serine/threonine kinase family,

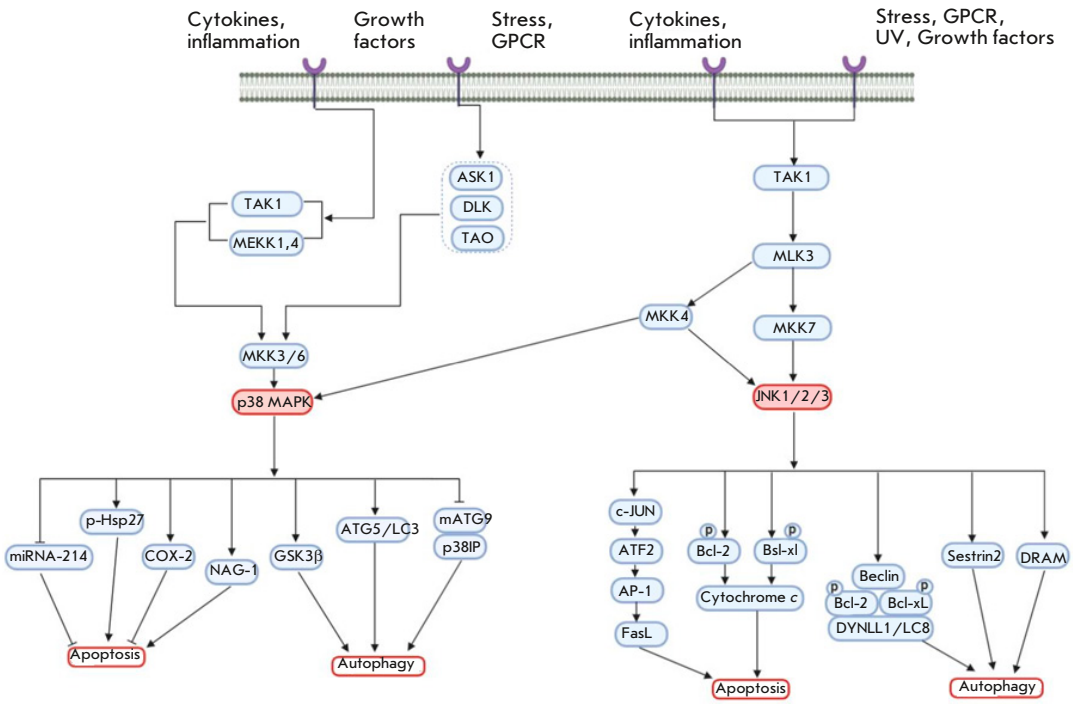

Fig. 6. Scheme of p38 MAPK and JNK signaling pathways, which illustrates their role in the regulation of autophagy and apoptosis (according to [50])

which is involved in the regulation of energy metabolism [57].

p38 MAPK is believed to be the main factor involved in maintaining a balance between p53-dependent apoptosis and autophagy under genotoxic stress induced by 5-fluorouracil [58].

However, there are contradictory data on the potential role of p38 MAPK in autophagy and apoptosis processes. Reactive oxygen species can induce oxidative stress that enhances autophagy and decreases apoptosis [59]. MAPK was found to play a vital role in the transition from autophagy to apoptosis in human colon cancer cells treated with MS-275, a histone deacetylase inhibitor. A high level of *p38* expression is associated with the activation of autophagy, while low expression of this gene induces apoptosis. Therefore, the p38 MAPK signaling pathway can play a critical role in choosing one of the two cellular processes triggered by chemotherapy-induced genotoxic stress [50].

JNK signaling pathway

JNK kinase, also known as a stress-activated protein kinase (SAPK) of the MAPK family, is initially activated in response to various stress signals and is involved in many cellular processes, including apoptosis and autophagy. Under genotoxic stress conditions, JNK is a positive regulator of both apoptosis and autophagy [50].

JNK regulates apoptosis through two different mechanisms. On the one hand, it promotes phosphorylation of c-Jun and the transcription factor ATF2, which activates the transcription factor AP-1 (activator protein 1) and expression of the genes associated with the signaling pathway regulated by Fas death receptors. Binding of the FasL ligand to the Fas receptor can mediate the activation of caspase 8 that processes effector caspase 3, initiating apoptosis. On the other hand, JNK provides phosphorylation of Bcl-2/Bcl-xL anti-apoptotic proteins, which changes the mitochondrial membrane potential and promotes the release of cytochrome *c*, activation of caspases 9 and 3, and induction of apoptosis [60].

Bcl-2/Bcl-xL phosphorylation stimulates autophagy through the dissociation of the Beclin 1–Bcl-2/Bcl-xL complex [61]. On the other hand, JNK activates the damage-regulated autophagy modulator (DRAM). DRAM is a target of p53, and DRAM induction under genotoxic stress conditions [62] stimulates autophagy by blocking the fusion of autophagosomes with DRAM-containing lysosomes [63, 64].

In general, the results of the studies carried out to date indicate a significant overlap or mutual dependence of the intracellular signaling mechanisms involved in the regulation of JNK-mediated apoptosis and autophagy. However, the question of how JNK

controls the balance between apoptosis and autophagy in response to genotoxic and oxidative stress remains open [50].

Figure 6 illustrates the role of JNK and p38 MAPK signaling pathways in the regulation of autophagy and apoptosis.

AUTOPHAGY AND APOPTOSIS IN AGING

Aging of an organism is a complex process involving a disruption of and decrease in the functions of many systems both at the whole organism level and at the cellular level [65]. All these processes ultimately lead to the death of the body and the development of many diseases, including the metabolic syndrome, neurodegenerative diseases, and cancer [6]. Aging of cells is accompanied by shortening of telomeres [66, 67], a decrease in the efficiency of autophagy [5, 6], and excessive activation of apoptosis [7]; however, the mechanisms of these processes remain not fully understood.

Telomeres can be lengthened by a specialized complex, telomerase. The complex includes reverse transcriptase (TERT), RNA telomerase (TERC), and additional proteins involved in the assembly of the enzyme and regulating its activity. The complex is active in cells characterized by a high proliferation rate, such as bone marrow cells, activated lymphocytes, gametes, and cancer cells, while telomerase is inactive in most somatic cells [66]. Expression of the *TERC* gene in cells lacking telomerase activity suggests that RNA telomerase performs some additional functions unrelated to telomerase activity and telomere elongation. Under stress conditions, TERT shuttles from the nucleus into mitochondria and promotes the protection of cells [68]. Increased expression of the telomerase component genes stimulates expression of the hexokinase 2 gene and activates autophagy through mTOR inhibition [69, 70]. Deletion of the *mTERC* gene in mice leads to mTOR activation and a constantly increased level of S6K1 phosphorylation. Inhibition of mTORC1 by rapamycin reduces the lifespan of these mice, but not that of wild-type mice [71].

Transcription of the human telomerase RNA gene produces an elongated precursor [72] that contains an open reading frame encoding the hTERP protein [73]. An increased level of the hTERP protein protects cells under conditions of apoptosis induction, while hTERP mutations affect processing of the LC3 protein, one of the main participants in autophagosome formation [72–74]. hTERP is involved in the regulation of molecular interactions between autophagy and apoptosis, as well as in the adaptation of cells to stress conditions [73]. The molecular mechanisms of the influence of telomerase components on autophagy are under active study.

CONCLUSION

To conclude, it is clear that autophagy and apoptosis are in a complex functional relationship that varies from cooperation to antagonism in different tissues and under different conditions. The balance between autophagy and apoptosis is maintained by a complex system of interactions between many signaling pathways, which involve both key proteins of autophagy and apoptosis (Beclin 1, caspase, p53, etc.) and polyfunctional regulatory molecules (e.g., mTOR, p38 MARK, or JNK). But it should be noted that clinical and experimental data on the autophagy and apoptosis ratio in normal tissues and various pathological conditions, including malignant tumors, are for the most part contradictory, and that the topic of balance between apoptosis and autophagy needs further investigation. ●

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Efficacy of (*R*)-6-Adamantane-Derivatives of 1,3-Oxazinan-2-One and Piperidine-2,4-Dione in The Treatment of Mice Infected by the A/California/04/2009 influenza Virus

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ABSTRACT **The World Health Organization (WHO) recommends antivirals as an additional line of defense against influenza. One of such drugs is rimantadine. However, most of the circulating strains of influenza A viruses are resistant to this drug. Thus, a search for analogs effective against rimantadine-resistant viruses is of the utmost importance. Here, we examined the efficiency of two adamantane azaheterocyclic rimantadine derivatives on a mouse model of pneumonia caused by the rimantadine-resistant influenza A virus /California/04/2009 (H1N1). BALB/c mice inoculated with the virus were treated with two doses (15 mg and 20 mg/kg a day) of tested analogs via oral administration for 5 days starting 4 hours before the infection. The efficacy was assessed by survival rate, mean day to death, weight loss, and viral titer in the lungs. Oral treatment with both compounds in both doses protected 60–100% of the animals, significantly increased the survival rate, and abolished weight loss. The treatments also inhibited virus titer in the lungs in comparison to the control group. This treatment was more effective compared to rimantadine at the same scheme and dosage. Moreover, the study of the sensitivity of the virus isolated from the lungs of the treated mice and grown in MDCK cells showed that no resistance had emerged during the 5 days of treatment with both compounds.**

KEYWORDS **influenza virus, antiviral drugs, rimantadine, mouse model of influenza viral pneumonia.**

ABBREVIATIONS **MDD – mean day to death; IC_{50} – 50% inhibitory concentration; $TCID_{50}$ – 50% tissue cytopathic infective dose; MLD – mouse lethal dose; PSB – Phosphate buffered saline; MEM – Minimum Essential Medium; MDCK – Madin-Darby canine kidney; WHO – World Health Organisation; ELISA – enzyme-linked immunosorbent assay; DMSO – Dimethyl sulfoxide; RMT – rimantadine; pdm – pandemic.**

INTRODUCTION

Influenza A viruses are a diverse group of respiratory pathogens that cause acute infections in humans, mammals, and birds [1]. Despite the availability of vaccines and antiviral drugs, influenza viruses cause annual epidemics and pandemics accounting for up to 650,000 deaths each year over the world, with up to 40,000 deaths in the United States alone [2]. In the past 10–15 years, from 27.3 to 47.2 million cases of acute respiratory viral infections have been registered annually, with the influenza infection responsible for 25–60% of all cases, depending on the intensity of the epidemics. The emergence of influenza pandemics, usually occurring every 20-30 years, is of particular concern. Along with the direct impact on public health, especially on populations from high-risk groups [2], infections lead to a huge, hard-to-measure, negative economic effect, as follows from the current COVID-19 pandemic. Vaccination is considered by the WHO as the mainstay in the prophylaxis of an influenza virus infection. However, due to the high and unpredictable variability of the influenza virus surface proteins, the composition of the vaccine is constantly changing depending on the antigenic structure of the circulating strains of influenza

viruses. Therefore, the WHO, in addition to vaccination, recommends the use of small molecule antivirals that are especially important in a pandemic caused by new strains of the influenza A virus.

Currently, there are two classes of anti-influenza drugs that have been approved worldwide [1, 3, 4]: **M_2 channel blockers** – aminoadamantanes – amantadine and rimantadine (**RMT**) (*Fig. 1*) and **neuraminidase inhibitors** – oseltamivir, zanamivir, peramivir and lanamivir (only in Japan) (*Fig. 1*). M_2 channel blockers belong to the first generation of antivirals effective against the influenza A virus. Although they have been successfully used for the treatment of influenza for more than 30 years [3, 4], their use has not been recommended since 2006 due to the widespread drug resistance of circulating strains [5]. The drug resistance has formed as a result of both evolutionary changes in the influenza virus and direct mutations during patient treatment with rimantadine and amantadine. Amantadine and **RMT** have a lower genetic barrier to drug resistance (1–2 passages) that has been shown in numerous experiments on animals or in cell cultures, and the drug resistance in humans can develop within 2–4 days after the start of treatment with these drugs [6]. The genetic basis of the resistance is mutations in gene 7 in the spliced second reading frame encoding the M_2-protein and is associated with the replacement of amino acids at positions L26, V27, A30, S31 and G34 [7]. Mutation S31N (serine-arginine) is the most common case of resistance to aminoadamantanes in humans, avians and pigs [8]. Nevertheless, the unique and extensive experience in the successful clinical use of adamantane-type drugs worldwide leaves them in the arsenal of antiviral therapy as reserve drugs used to treat the appearing sensitive influenza strains that can be resistant to other influenza drugs: in particular, neuraminidase inhibitors. It should be noted that the emergence of oseltamivir-resistant strains has been continuously reported and was prevalent in the 2008–2009 seasonal influenza, when almost all circulating H1N1 strains had the H275Y mutation in the neuraminidase gene [9] while maintaining sensitivity to adamantanes.

As a result of efforts to overcome the existing resistance of influenza viruses to the first two classes of drugs, baloxavir marboxil, an endonuclease inhibitor has been elaborated, which is highly effective against various strains of influenza A and B viruses (approved in Japan, undergoing the last stage of trials in the USA) [10–12]. In addition, there are two drugs approved in Russia and China: umifenovir ("Arbidol"), which is an inhibitor of the fusion induced by hemagglutinin [13,14], as well as riamilovir ("Triazavirin", Russia), an RNA-replicase inhibitor (*Fig. 1*).

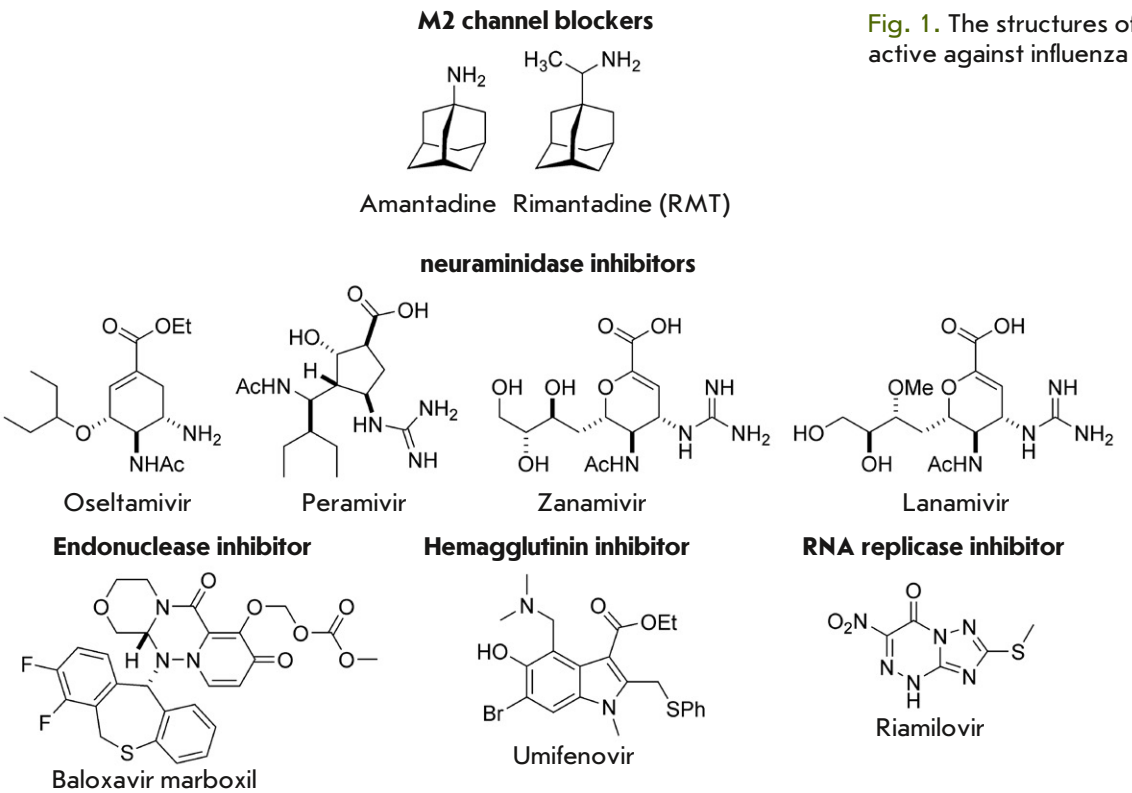
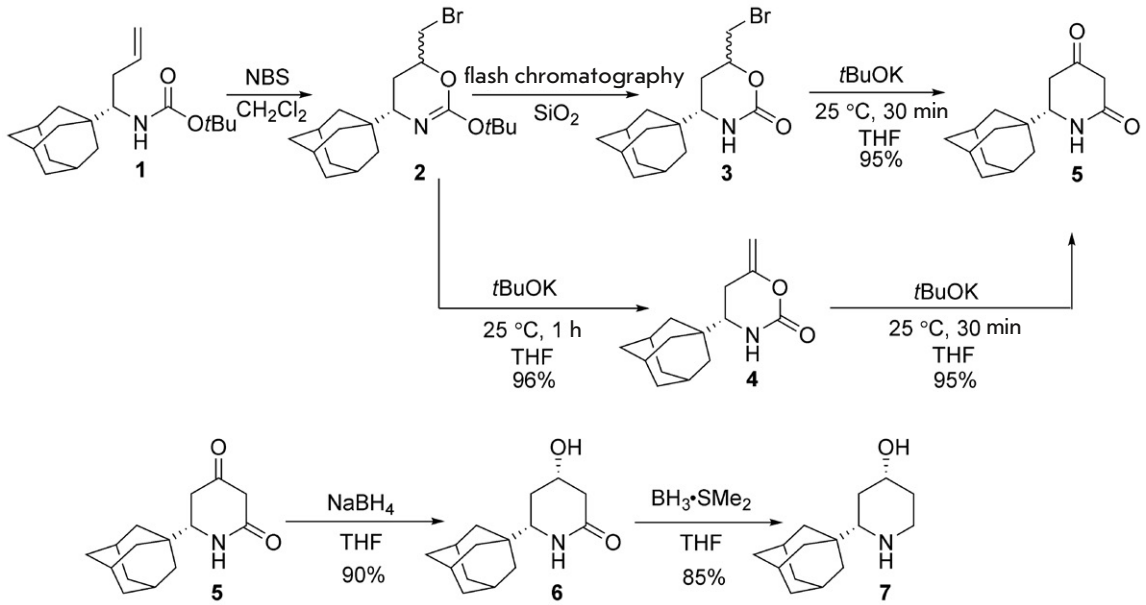


Fig. 1. The structures of the drugs active against influenza viruses



Scheme. The structures of new adamantane derivatives active against rimantadine-resistant strains of the H1N1 influenza virus

To ensure reliable protection of the population in the face of an influenza epidemic, it is essential to have a set of antivirals acting through different mechanisms [15]. Unfortunately, there are currently no approved effective M_2-blockers for the S31N virus. The influenza M_2 channel is a highly conserved virus region, and, according to recent studies, experimental M_2-blockers are quite sustained for resistance development [16]. Moreover, in the case of occurrence of such mutated strains, most of them [16, 17] do not remain in the viral population, suggesting that the elaboration of M_2-blockers is a promising avenue.

Previously, we developed a convenient method for the synthesis of new enantiomerically pure 6-adamantyl derivatives of 1,3-oxazinan-2-ones and piperidines **3**–**7** from corresponding enantiomeric homoallylamines **1** (*Scheme*). The key steps in the process were bromocyclocarbamation (**1** into **2** and **3**), dehydrobromination by *t*BuOK (**2** into **4**), and enolate-isocyanate rearrangement (**4** into **5**). The last two reactions are "one pot" in the case of bromide **3**. Diketone **5** was then reduced stepwise to 4-hydroxylactam **6** and to 4-hydroxypiperidine **7**. The obtained compounds **3**–**7** were found to inhibit *in vitro* replication of the pandemic strains A/California/7/2009 and A/IIV-Orenburg/29-L/2016 bearing the S31N mutation [18]. In each pair of enantiomers, (*R*)-isomers (asymmetric center at the adamantyl group) of **3**–**5** and **7** inhibited *in vitro* replication of the influenza viruses most effectively (*Scheme*, *Table 1*).

Table 1. Inhibition of influenza A H1N1 virus replication by inhibitors **3**–**7** *in vitro*

Virus strain	IC_{50}, μM			
	3	4	5	7
A/California/7/2009 (H1N1)	11.3	8.1	20.6	18.4
A/IIV-Orenburg/29-L/2016 (H1N1)	20.1	7.7	27.1	17.7

Since the *in vitro* inhibitory activity of the compounds was quite promising, their effectiveness *in vivo* had to be tested. However, compound **3** was excluded from the study due to its low solubility in aqueous solutions, as well as compound **7**, which was rather difficult to synthesize in diastereomerically pure form. Thus, (*R*)-6-adamantyl derivatives of 1,3-oxazinan-2-one **4** and piperidin-2,4-dione **5** were selected, due to their simplicity of synthesis and acceptable solubility in aqueous solutions. Evaluation of the activity of the compounds **4**, **5** was carried out on a mouse model of pneumonia induced by the rimantadine-resistant influenza virus A/California/04/09 (H1N1).

EXPERIMENTAL PROCEDURES

Compounds and their preparation

(*R*)-isomers of compounds **4** and **5** were synthesized from the corresponding (*R*)-*N*-Boc-derivative of ada-

mantyl homoallylamine **1**, according to the procedures described in [18]. For each experiment, freshly made solutions of compounds **4**, **5** and **RMT** in 50% DMSO were used. The studied solutions were administered orally to mice in a volume of 200 μl, and the animals were treated by compounds **4**, **5**, and **RMT** in doses of 15 and 20 mg/kg/day.

Cells and viruses

MDCK cells were grown in a modified Eagle's medium (MEM; CellGro, Manassas, VA) supplemented with 10% FBS and 5 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 100 μg/ml kanamycin sulfate in a humidified atmosphere of 5% CO_2. The influenza A/California/04/2009 (H1N1) virus was provided by the WHO National Influenza Centre of Russia (St. Petersburg, Russia) and mouse-adapted by three lung-to-lung passages. The virus stock grown in the allantoic cavity of 9-day-old embryonated chicken eggs for 48 h at 37°C was used to modulate the influenza infection in the animals according to the conventional technique [19].

Animals

Inbred female mice (12–14 g) were obtained from the Andreevka Research Centre for Biomedical Technology (Moscow Region). Animal maintenance and care were performed in accordance with the Guide for the Care and Use of Laboratory Animals. The mice were fed with briquetted feed following the approved standards. All studies were approved by the I.I. Mechnikov Research Institute of Vaccines and Sera Committee on the Ethics of Animal Experiments.

Assessment of drug efficacy in a mouse model

The mice were group-housed in cages and used at a quantity of 8–13 mice per treatment group. On the day of experiment, the mice were weighed and then infected intranasally under light anesthesia. In the first series of experiments, a high infection dose of 10 MLD_{50} (mouse lethal dose of 50) was used, corresponding to 4.5 $lgTCID_{50}$ (tissue cytopathic infectious dose of 50); in the second series of experiments – a low dose of MLD_{90}, corresponding to 4.0 $lgTCID_{50}$. Compounds **4**, **5** and **RMT** (control drug) were administered by oral gavage in a 0.2 ml volume to every animal 4 h before and after infection, and the treatment continued for 5 days twice daily. The placebo was administered in parallel with the antiviral treatments (PBS in experiment 1 or 50% DMSO in experiment 2). The survival rate and weight change were observed for 16 days after virus inoculation. The animals that showed signs of severe disease and weight loss of 30% were humanely euthanized. The efficacy of the compounds in the mouse model of influenza pneumonia was estimated by the following criteria: survival rate; mean day to death (MDD); weight loss and viral titer reduction in the lungs in the treated animal groups compared to the control. MDD was calculated by the following formula

$$MDD = \sum f(d-1)/n,$$

where f is the number of dead mice on day d (survivors on day 16 were included in f for that day) and n is the number of mice in the group. The weight loss or gain was calculated for each mouse as a percentage of its weight on day 0 before virus inoculation. The weight of an animal before inoculation was considered to be 100%. For all the mice in one group, an average value of their weight loss and gain was calculated. Four days after inoculation, three mice from each group were sacrificed: their lungs were removed under sterile conditions to be thoroughly rinsed with 0.01 M sterile PBS, homogenized, and suspended in 1 mL of cold PBS. After separation of the cell debris by centrifugation at 2000 *g* for 10 min, the supernatant was used to determine the viral titer in the MDCK cell culture by the generally accepted method. Virus titers in mouse lungs were calculated as the mean $lgTCID_{50}/mL \pm SD$.

Statistical processing of the data was carried out using the log-rank Mantel-Cox test in the Statistica 8.0 program with the $p < 0.05$ value considered a statistically significant difference from the control.

Antiviral activity by cell-based ELISA assay

Stock-solutions (1 mg/ml) of samples and **RMT** prepared in DMCO were used to prepare final concentrations. MDCK cells were seeded in 96-well plates (3,000 cells/well, "Costar") and grown as a confluent monolayer, washed twice with serum-free MEM, and overlaid with MEM (100 μl) containing 2.5 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich) with a final concentration range of 1–10 μg/mL. After incubation for 2 h at 37°C, 100 μl of the virus isolated from the lungs of the treated animals containing approximately 0.1 PFU/cell was added to all wells, except the uninfected control cells. After a 24-hour incubation period, the cells were washed and fixed by adding 50 μl of cold 80% acetone in PBS. Viral expression was measured by ELISA, as previously described. For a point in the experiment, four wells of a plate were used and each value represented a mean calculated from three independent experiments.

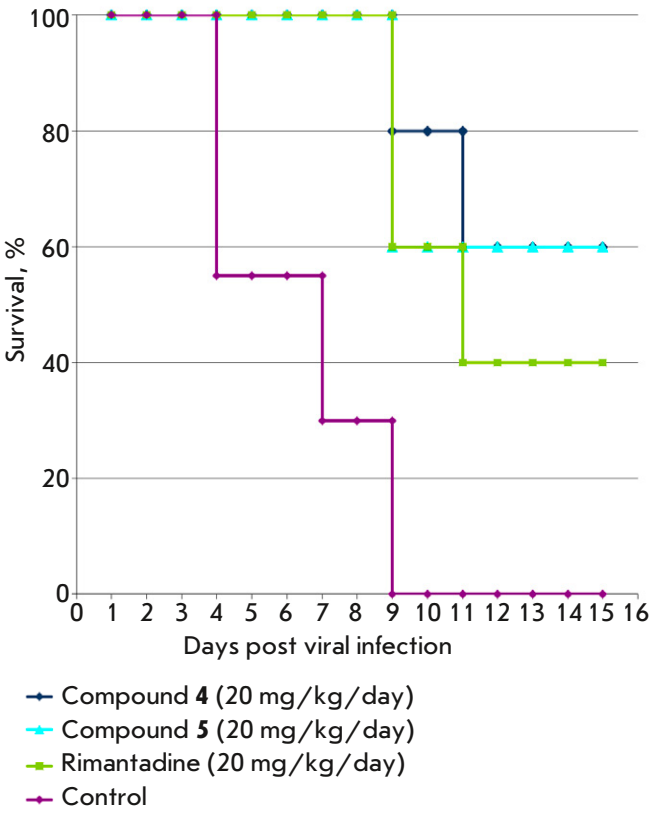


Fig. 2. Survival rates of mice treated with compounds **4**, ***5*** in a murine model of influenza pneumonia induced by a high dose of the virus

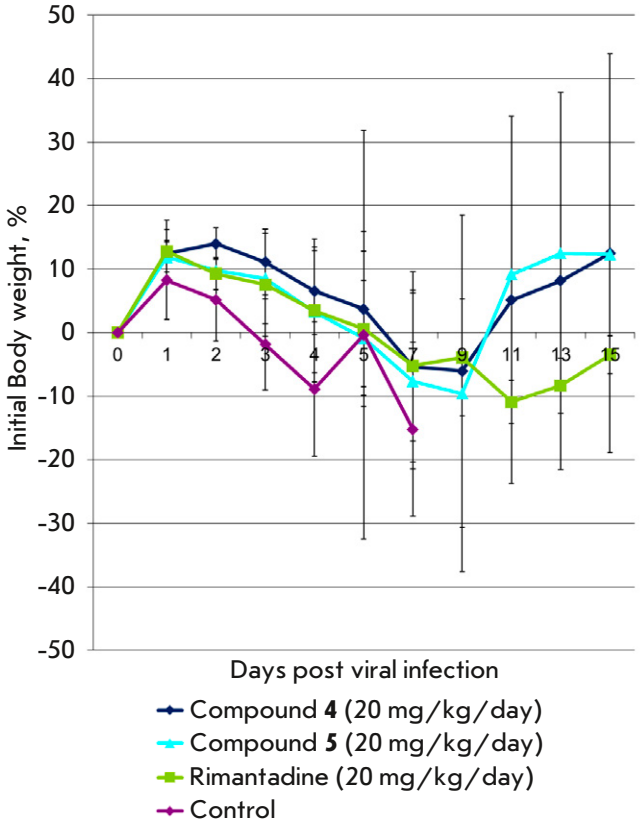


Fig. 3. Change in the body weight of mice during treatment with compounds **4**, ***5*** in a murine model of influenza pneumonia induced by a high dose of the virus

Sequence analysis of the M gene

Identification of the molecular marker of drug resistance was carried out by sequencing of the *M2* gene of the influenza A/California/04/2009 (H1N1) pdm09 virus that was used to infect the animals. Total RNA was extracted using a RIBO-prep nucleic acid extraction kit (AmpliSens, CRIE, Russia). A REVERTA-L reagents kit (AmpliSens, CRIE, Russia) and 5′agcaaaagcagg primer were used for reverse-RNA transcription. Amplification of viral cDNA was conducted using such primers as M 1F agcaaaagcaggtagatgtt; M 1027R agtagaaacaaggtagttt on a Tercyc thermocycler (DNA-Technology, Russia). Sequencing reactions of overlapping PCR products were conducted with the same primers used for amplification with an ABI PRISM Big Dye™ v.3.1 Cycle Sequencing Reaction Kit according to the manufacturer's instructions on an ABI-3100 PRIZM™ Genetic Analyzer (Applied Biosystems, USA). All sequences were assembled with the Lasergene version 10.1 package (DNASTAR Inc, USA).

RESULTS AND DISCUSSION

The efficacy of compounds 4 and 5 at a dose of 20 mg/kg/day on a murine model of viral pneumonia induced by a high dose of the rimantadine-resistant influenza A/California/04/2009 virus

Preliminary experiments showed that the administration of the compounds under study in doses of up to 60 mg/kg/day according to the schemes used in the subsequent treatment of intact mice did not cause weight loss and mortality in any of the animals. To further study the effectiveness of compounds **4** and **5** in comparison with RMT, a dose of 20 mg/kg was chosen as an optimal dose for studying the effectiveness of RMT in mice [3].

In the control group of infected animals not receiving any treatment, cases of death were observed starting from day 7 and mortality reached 100% by day 9: the mean day to death (MDD) in this group was 5.1 days. The loss of body weight in the control began from the second day after infection and reached its maximum

value (18%) by day 5. Compounds **4** and **5** were equipotent, protecting 60% of the animals on the 15th day of observation. Treatment of the mice with **4** and **5** was more effective than with **RMT** at the same dose, which provided protection to 40% of the animals. The MDD was 10.1 days for **RMT**, while for **4** and **5** it was more than 12 days. In addition, in the groups treated with all tested adamantanes (**4**, **5**, **RMT**), the weight loss was less significant than in the control group (*Fig. 2–3*, *Table 2*).

Determination of the efficacy of compounds 4 and 5 at doses of 15 and 20 mg/kg/day on a mouse model of pneumonia induced by a low dose of the rimantadine-resistant influenza virus A/California/04/2009

To identify the differences in the actions of compounds **4** and **5**, in subsequent experiments the viral inoculation dose was reduced and two doses of 20 and 15 mg/kg/day of the compounds were selected.

In the control group of non-treated infected mice, death of animals by the 16th day of observation reached 90% and MDD in this group was 10 days (*Fig. 4*, *Table 3*). The oral administration of compound **4** in a dose of 15 mg/kg/day did not have a statistically significant effect on the survival rate;mortality in these groups was 50% (*Fig. 4*, *Table 2*). An increase of the dose to 20 mg/kg/day led to a significant decrease in mortality, to 20%. Compound **5** was more effective – with treatment at a dose of 15 mg/kg/day the mortality rate was 30%, and a dose of 20 mg/kg/day fully protected the animals from death.

Table 2. Efficacy of oral treatment with compounds 4 and 5 in a murine model of influenza pneumonia induced by a high dose of the influenza A/California/04/2009 (H1N1) pdm09 virus

Dose, mg/kg/day	Survival		Protection from mortality, %	MDD, days
	Alive/Total	Mortality, %		
Compound **4**				
20	3/5[a]	40	60	12.6
Compound **5**				
20	3/5[b]	40	60	12.2
RMT				
20	2/5[c]	60	40	10.1
Virus control				
	0/10	100		5.1

[a] – ($p = 0.003198$); [b] – ($p = 0.003198$); [c] – ($p = 0.031863$).

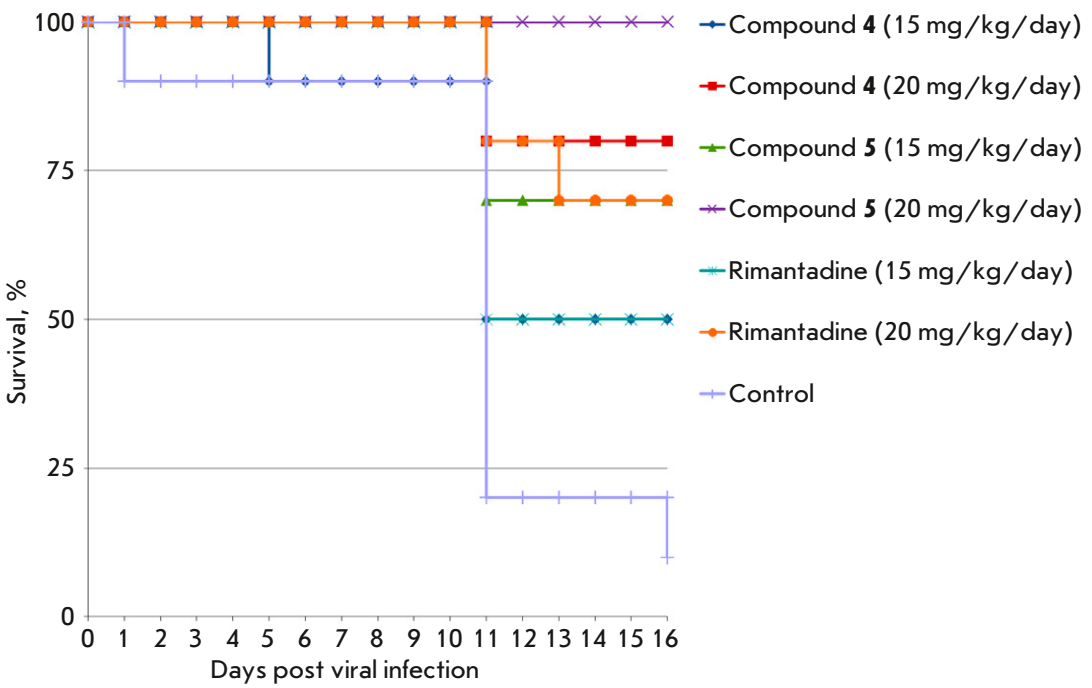


Fig. 4. Survival of mice in a model of influenza pneumonia induced by a low dose of the virus

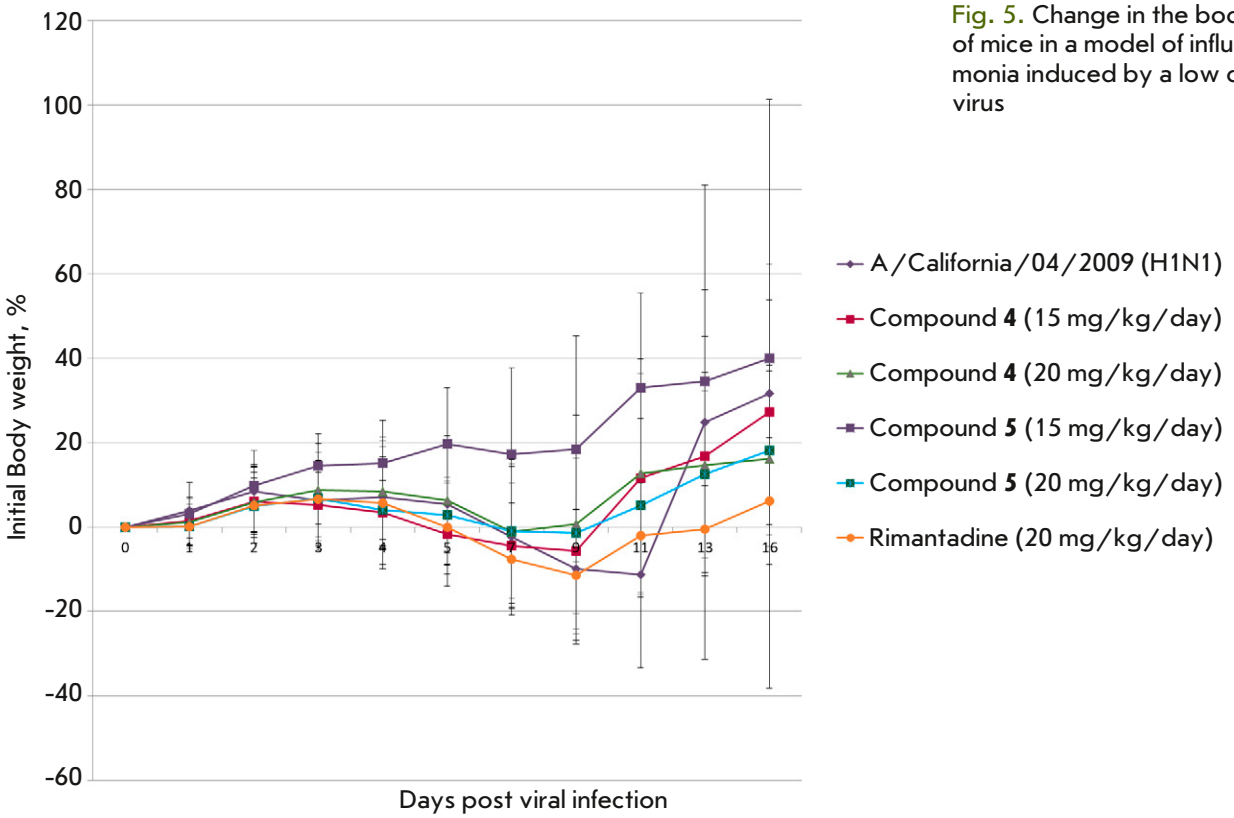


Fig. 5. Change in the body weight of mice in a model of influenza pneumonia induced by a low dose of the virus

Table 3. Efficacy of oral treatment with compounds **4** and **5** in a murine model of influenza pneumonia induced by a low dose of the influenza A/California/04/2009 (H1N1)pdm09 virus

Dose, mg/kg/day	Survival		Protection from mortality, %	MDD, days	Viral titre, lg $TCID_{50}$
	Alive/Total	Mortality, %			
	Compound **4**				
15	5/10[a]	50	40	12.4	4.5 ± 0.5
20	8/10[b]	20	70	14	1.16 ± 1.6
	Compound **5**				
15	7/10[c]	30	60	13.5	2.5 ± 2.3
20	10/10[d]	0	100	> 16	2.6 ± 2.3
	RMT				
20	7/10[e]	30	60	13.7	1.3 ± 0.3
	Virus control				
-	1/9	90	-	10	6.1 ± 0.3

[a] – ($p = 0.075134$); [b] – ($p = 0.001106$); [c] – ($p = 0.007137$); [d] – ($p = 0.000000168$); [e] – ($p = 0.007137$).

In the control group, body weight loss was observed starting from the 3rd day after the viral infection, reaching 11% on average by the 11th day. Survival data was confirmed by the most important criterion for the severity of the disease – weight loss. In the groups treated with compound **5** in both studied doses and with compound **4** at a dose of 20 mg/kg/day, a decrease in body weight was not observed *(Fig. 5)*. Treatment with **RMT** at a dose of 20 mg/kg/day led to a higher level of mortality (30%) and weight loss compared to the mice treated with the same dose of compounds **4** and **5** that correlated with the survival data.

The observed greater animal survival rate in the second series of experiments evidently was due to the reduced virus dose, since the effectiveness of the antiviral drug was inversely proportional to the dose of infection, as well as to the fact that for the initial screening of the compounds in the first experiment, the groups including a smaller number of animals were the ones studied. A dose-dependent increase in the effectiveness of the tested compounds was also observed. On the whole, the obtained data indicate a virus-specific effect of the studied compounds.

The effect of treatment with RMT and compounds 4, 5 in various doses on the viral titer in the lungs of a mouse model of pneumonia induced by the rimantadine-resistant influenza virus A/California/04/2009

The data on the increased survival rate were confirmed by a virological method. The viral titer reflects the replication of the virus in the lungs, its higher value corresponding to more severe pathological changes in the lungs. The highest viral titer (6.1 ± 0.3 lg $TCID_{50}$) measured was in the control group. The smallest suppression of the viral titer was observed during treatment with compound **4** at a dose of 15 mg/kg/day (4.5 ± 0.5 lg $TCID_{50}$). An increase in the dose of compound **4** to 20 mg/kg/day, as well as treatment with compound **5** at both doses, significantly inhibited the replication of the virus, reducing the titer by 2.4–4.9 lg $TCID_{50,}$ which corresponded to the clinical parameters of treatment efficiency obtained for these compounds. It is also important to note a significant suppression of virus replication in the lungs when treated with **RMT**. Although the mortality rate for **RMT** applied at a dose of 20 mg/kg/day in both series of experiments was higher than that with compounds **4** and **5** at the same dose, it was statistically significantly lower compared to the group of infected untreated animals. Since previously no **RMT** activity had been observed in the cell culture with the rimantadine-resistant influenza virus A/California/04/2009(H1N1), data demonstrating such activity in experiments with mice was somewhat unexpected. However, it must be stressed that the data obtained *in vivo* more adequately characterize antiviral activity, since they account for such features as compound bioavailability, toxicity, and pharmacokinetics directly in the body. Often, the drug concentrations reached in blood plasma can significantly exceed the necessary concentrations to suppress antiviral activity in *in vitro* experiments. This may explain the efficacy of the drugs in respect to viruses resistant to them. A similar effect was noted in the study of the efficacy of oseltamivir in ferrets [20], where oseltamivir was effective not only against oseltamivir-sensitive, but also against oseltamivir-resistant H1N1 influenza viruses with the H274Y mutation, though to a lesser degree. These data are also in agreement with the clinical studies that showed the efficacy of oseltamivir during the 2008–2009 epidemic season, when the oseltamivir-resistant strain H1N1 (H274Y) was in circulation: however, this efficacy was lower than that of another neuraminidase inhibitor, zanamivir, to which the virus strain was also sensitive [21]. Very similar results were

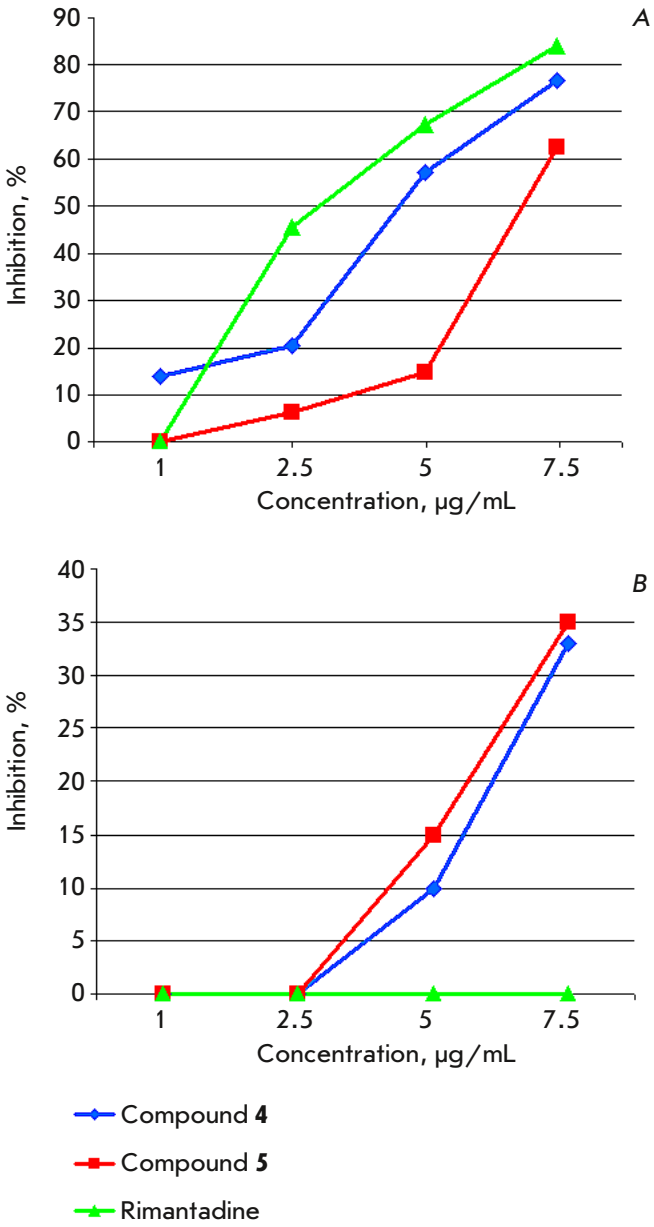


Fig. 6. Antiviral activity of compounds **4**, **5**, and **RMT** in a MDCK cell culture against influenza A / Aichi / 2/68 (H3N2) (*A*) and A / California / 04/09 (H1N1) viruses isolated from the lungs of treated animals (*B*)

reported by the authors of [22], where **RMT** efficacy in the treatment of influenza during seasons with the circulation of the rimantadine-resistant strain A/California/04/2009 (H1N1) was observed. However, the efficacy of such treatment was lower compared to that of oseltamivir, which was used in the same studies.

Sequence analysis of the mouse-adapted rimantadine-resistant influenza A/California/04/2009 virus

The influenza virus A/California/04/2009pdm (H1N1) has a mutation, S31N, in the M_2 protein, which is a molecular genetic marker of resistance to adamantanes. Although in our experiments we showed that treatment with novel adamantanes was more effective than treatment with **RMT**, the fact that **RMT** itself reduced animal mortality, weight loss, and virus replication in the lungs of mice infected by the rimantadine-resistant influenza A/California/04/2009pdm (H1N1) virus was notable. In actuality, the origin strain of A/California/04/2009pdm (H1N1) is not lethal for mice; therefore, in our experiments, we used a virus adapted to mice by passaging it into the lungs of the animals. We assumed that the mutation responsible for resistance to **RMT** could be lost in the process of adaptation. To verify this assumption, sequencing of gene 7 encoding the M_2 protein of the mouse-adapted virus was performed. The nucleotide sequence of the 7th gene found showed that, in our mouse-adapted strain, the S31N mutation responsible for virus resistance to rimantadine was, indeed, preserved.

The possibility of occurrence of resistance to compounds 4 and 5 in the course of their intake

Another important aspect in the development of antiviral drugs is that drug resistance occurs during infection treatment. As was mentioned before influenza A viruses develop resistance to adamantanes in a cell culture and in animals just after 2–3 passages; in a human population, such strains can appear within 2–4 days after the start of treatment [4–6]. To study the possible emergence of resistane to compounds **4** and **5**, the viruses were isolated from the lungs of treated (with both compounds or **RMT)** mice on the 4th day post-infection and their sensitivity studied in MDCK cells. For comparison, influenza A/Aichi/2/68(H3N2) virus sensitive to **RMT** was used (*Fig. 6*). It can be seen that both compounds **4**, **5,** and **RMT** were active against this virus. At the same time, the viruses isolated from the lungs of the mice infected with the rimantadine-resistant influenza A/California/04/2009pdm (H1N1) virus and treated with compounds **4** and **5** remained sensitive to them, which is an indication that no resistance to these compounds had developed during their repeated application. The results are in accordance with literature data demonstrating that, unlike **RMT**, no resistance to S31N-M_2-blockers occurs in the course of treatment [16].

Conclusions

According to the previously developed convenient and efficient method, the (*R*)-isomers of 6-(1-adamantyl)-1,3-oxazinan-2-one **4** and 6-(1-adamantyl) piperidin-2,4-dione **5** were synthesized in preparative gram-scale quantities to study the antiviral activity of a murine model of viral pneumonia induced by the influenza A virus. Both compounds, administered orally in doses of 15 and 20 mg/kg/day, protected mice, significantly reducing animal mortality, weight loss, virus replication in the lungs of the animals, and they increased survival of the animals (mean day to death). The treatment of mice with compounds **4** and **5** was more effective than treatment with the comparative drug rimantadine at the same doses and scheme. It is noteworthy that application of these novel adamantanes for 5 days did not lead to the development of resistance to them. The compounds effectively inhibit the replication of influenza A viruses, including rimantadin-resistant strains. The synthetic scheme of these adamantane derivatives is simple and contains easily available compounds. It is our hope that directed modification of the structures of adamantyl (hydroxylation) and heterocyclic (substitution in the 4th position of compounds **4** and **5**) fragments of these compounds would further enhance their antiviral activity and shed light on how they block the M_2 channel. Given the abovesaid, the studied heterocyclic adamantanes are promising for the development of new therapeutic agents for the treatment of the influenza A infection. ●

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Forces for Folding

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ABSTRACT Understanding the nature of the forces driving the folding of proteins, nucleic acids and the formation of their complexes absolutely requires thermodynamic data, in addition to structural information. In practical terms, this means the use of super-sensitive scanning and titration calorimeters for experimental determination of the heats (enthalpies) characterising these processes. Peter Privalov was both an experimental thermodynamicist and a calorimeter designer/manufacturer who followed and propagated this credo. The sum total of his many publications, every one of which addresses a fundamental question, is his lasting epitaph.
KEYWORDS thermodynamics, calorimeters, enthalpy, protein folding, hydration.

INTRODUCTION

The first views of the folded globin chains, with a heme group nestling among the many helices, as shown by Perutz and Kendrew in the late '50s, was a wonder to behold. Pauling's α-helix was celebrated beyond expectations. The question as to how a tetramer of such globules fulfilled its functions remained to be elucidated – and Perutz spent the rest of his life doing just that – but the structural underpinnings were very apparent. But not quite to everyone's full satisfaction, however. Peter Privalov immediately asked himself: "what are the forces that stabilise these helices and drive their folding to generate this complex but exquisite bundle"? Another question was posed by Oleg Ptitsyn: "by what route, i.e. pathway, does the disordered chain convert to the folded globule"? So, new issues were raised, moreover from new directions.

EXPOSITION

Peter Privalov was the quintessential experimental, scientist who realised that the only way to define the forces responsible was to make thermodynamic measurements, which meant determining the heats of protein folding. At the dilute protein concentrations for which intermolecular effects can be neglected, such heats are extremely small, best arrived at by measuring the heat capacity changes of the solution as the protein undergoes thermal denaturation. So he devised (one could say 'invented') a calorimeter of two identical compartments that measured the heat capacity of the protein solution in one, relative to plain buffer in the other as their temperature was raised. The differential scanning calorimeter was thus born [1, 2].

The indicative protein used was lysozyme, and its calorimetric heat of unfolding was measured [3]. The heat input required (by convention a *positive* enthalpy) could be explained by the breaking of H-bonds and van der Waals close contacts, and the rise in the concomitant entropy, a result of the increased conformational freedom of the polypeptide chain. That much seemed clear, but a simpler approach to a determination of the thermodynamic parameters of melting is possible; it is called the van't Hoff (vH) plot. The sigmoidal melting transition of proteins suggests some degree of cooperativity, and if passage through the transition is plotted against the inverse temperature, this yields the apparent enthalpy and entropy involved: so, why the necessity for a sensitive calorimeter? Unfortunately, the thermodynamic parameters derived from a vH plot indicate nothing about the degree of cooperativity and can be positively misleading. But when the calorimetric enthalpy of lysozyme denaturation was compared with the vH value, they turned out to be actually the same! The correct value from the vH plot meant that the denaturation transition involves *only* two states, which must be the fully folded form and the fully denatured form of the chain. So, lysozyme denaturation is a fully cooperative process in which no other thermodynamically relevant states participate, proving its unfolding to be a genuine "all or nothing" process. The physical description of such a two-state transition is illustrated by the state of the protein in the middle of the unfolding transition: half the sample is still fully folded, and the other half completely unfolded; i.e., it is *not* a homogeneous sample of half-folded macromolecules. Demonstration

of protein folding as such a highly cooperative process was a revelatory discovery at that time.

Privalov then turned to larger proteins, such as pepsinogen, and found that the calorimetrically measured melting enthalpy was about twice that obtained from the vH plot [4]. It followed that native pepsinogen, although apparently a unified single fold, in fact consists of two quasi-independent sub-domains, each with its hydrophobic core. Differential scanning calorimetry might have seemed a somewhat obscure methodology in the 1970s and not of prime importance, but Privalov's observations were direct support for his conviction that solving the 3D structures of proteins was not sufficient for understanding the nature of the forces driving their formation and providing the stability of their native folds: only thermodynamic studies could do that.

Peter Privalov was the quintessential experimental scientist, a thermo-dynamicist extraordinary, who sadly died in Baltimore on December 20th 2020.

His scientific career started in his native Georgia at the Institute of Physics in Tibilisi, doing his Ph.D. under the supervision of its director, the low-temperature physicist Elveter Andronikashvili. His first super-sensitive calorimeter for measuring temperature-induced heat capacity changes in dilute solutions appeared in 1964 [1]. Even at that time, however, he was not quite a lone wolf: Julian Sturtevant was already making calorimetric measurements [5] and was much admired by Privalov, who frequently spoke and wrote very warmly of his influence [6]. In 1967, Privalov was recruited as one of 6 team leaders at the founding of the Institute of Protein Research in Poushchino (a research town 60 miles south of Moscow) – to which Ptitsyn was also recruited. He finally assembled a team to develop a 'school of bio-thermodynamics', an essential prerequisite, as he saw it, for taking the subject forward. It also provided the opportunity for instrument development and, before long, the commercial manufacture of a scanning calorimeter (DASM-1). This instrument was unique and consequently sold widely even in Western countries. Export of sophisticated scientific equipment from the USSR at that time was very rare and in stark contrast to the high level of imports such as NMR spectrometers, ultracentrifuges and the like. For this achievement, Privalov won a State Prize in 1978.

The single defining principle of his 'School' was to understand the forces controlling the formation, stability, and interactions of biological macromolecules: take a pure sample of the object in question and subject it to a thorough experimental calorimetric analysis. Then, do your best to interpret the results obtained: don't start with a theoretical analysis, as this might lead to strong convictions as to the expected result and be very prejudicial to performing appropriate experiments. Meaning: theory should follow an experiment, not lead it!

One cause of Privalov's wariness regarding theoretical descriptions of the driving forces was uncertainty as to how the role of the solvating water was modeled. The significance of hydration was exemplified by noting the large increase in heat capacity (ΔCp) of proteins when they denature and expose the hydrophobic residues of the core to water – the explanation for which was the changed state of the hydrating water molecules. Privalov was always at pains to point out the importance of knowing ΔCp, a quantity that determines the temperature dependence of all the thermodynamic parameters. The role of water very often drove his thinking: in 1979, he published a seminal paper [7] explaining how hydration of the collagen triple helix leads to the temperature dependence of its stability and flexibility.

The dissolution of the Soviet Union as a political entity in 1991 soon led to the collapse of its scientific enterprise and a large-scale exodus of researchers. Privalov was no exception, but unlike the contract posts given to the majority, he was offered a tenured professorship at a top U.S. research university (Johns Hopkins) that he took up in 1992. Despite such a dramatic change of circumstances, he never wavered in his commitment to understanding the basic principles of macromolecular stability: he did not resort to 'opportunistic science' and go with the prevailing winds so as to attract funding. Only fundamental questions were asked, and the experiments designed to answer them were then conducted. Nor did he stop developing DSC instrumentation: calorimeters with capillary cells made from gold were constructed. The idea for this metal came to Privalov (he reported) as he sat in the dentist's chair having a gold crown fitted: what an ideal material, very high thermal conductivity, chemically inert and very malleable. Such instruments were then commercialized in the U.S. Several spectacular scientific achievements from the Hopkins Lab come to mind, such as the 'cold denaturation' of proteins [8], accompanied by heat release, and the energetics of folding of an individual α-helix [9]. To these must be added his experimental determination of the entropy associated with forming a dimeric protein from two separate monomers – the so-called translational entropy – that he determined to be much less than predicted; in fact, about one order of magnitude lower than proposed by theoreticians [10, 11].

Privalov's realization that precision is essential in the measurement of thermodynamic quantities meant that he was not always an easy-going task-master: he expected results of maximum precision and very small tolerance for errors – a challenging requirement. But

those results were trustworthy, and the data could be relied upon. Although demanding, Privalov most certainly did not lack a sense of humour. On a visit to a thermodynamics conference organised by the UK Institute of Physics in Salford, the birthplace of James Joule: we bemoaned the fact that whilst our data were always expressed in joules, many participants were still using calories. "Ah yes", Privalov remarked, "but remember that Joule himself used calories"!

When the 21st century dawned, Privalov was very successfully installed at Hopkins and had switched from individual proteins to DNA and its interactions with the binding domains (DBDs) of transcription factors. Measurements of binding enthalpies were confounded by concomitant refolding of DBDs: Privalov's important contribution was to demonstrate how a combination of DSC with titration calorimetry can overcome this problem and thereby define the forces giving rise to the fully folded complexes revealed by crystallography [12]. In recent years, the DNA duplex itself became the object of study, joining many other researchers in a popular topic – but a field in the firm grip of the conviction that duplex melting is not accompanied by any change in heat capacity: so, DNA energetics are temperature independent. However, using careful measurements with several short duplexes, Privalov showed this not to be the case, once again the hydrating water playing a critical role [13].

Privalov never let up on his determination to understand the basis of all the forces involved in macromolecular folding and stability, most recently the contribution of hydrogen bonding. Experiments to determine the energetics of formation of the DNA duplex led to the conclusion that the contribution of H-bonds is not enthalpic but comes from the entropy increase resulting from the release of the water molecules bound to the bases in the disordered state of the oligonucleotides [14]. If that is the case, then what can be said about the formation of the H-bonds in α-helices? Is that also an essentially entropic process? In fact, Privalov's article discussing this last, very fundamental point is currently in press [15].

CONCLUSIONS

Science is a conversation: its participants tell each other about their results, and the building rises from their combined efforts. Privalov was both diligent and masterful in presenting his results: firstly, in writing many individual articles, followed by several, very extensive reviews of his work on proteins [16, 17], then on protein/DNA interactions [18], and finally in 2012 publishing a whole book "Microcalorimetry of Macromolecules" that splendidly sums up his complete oeuvres [19]. There is so much to be admired in all that Privalov achieved: the sum total of his publications is his epitaph. We have lost a great experimentalist of the highest stature, devoted to basic science, and we are all much the poorer for it.

ACKNOWLEDGEMENT

An abbreviated obituary of Peter Privalov (having some overlap with the present article) has appeared in the Newsletter of the U.S. Biophysical Society. ●

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