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EPIGENETIC CHANGES IN CELLS OF EXPERIMENTAL LEUKAEMIA UNDER THE BLOCKING OF POLYAMINE SYNTHESIS

Summary. ***Objective*****:** *the early changes of histondeacetylase and ribonuclease activity and the level of total DNA methylation in cells of experimental leukemia L1210 under the blocking of polyamine synthesis has been studied.* ***Objects and methods*****:** *experimental leukemia L1210 cells, methods of global DNA methylation, evaluation of histondeacetylase activity, enzymogram method of RNase activity.* ***Results*****:** *it was found that the blocking of polyamine synthesis at both the ornithine decarboxylation step (synthesis of putrescine) and the S-adenozyl methionine decarboxylation (step of spermidine and spermine synthesis) results in inhibition of histondeacetylaze and growth of RNase activity even during the first 2–4 hours of experiment. The level of total DNA methylation in leukemic cells varied mostly downward.* ***Conclusions*****:** *utilization of inhibitors of polyamine synthesis with different mechanisms of action is a promising direction in the development of targeting tumor growth.*

INTRODUCTION

In recent years, the attention of researchers of tumor growths has been attracted by the peculiarities of functioning of genome, which are connected with epigenetic mechanisms. The most recent data argue for the existence of special system of regulation of gene expression and preservation of genetic information, which is located outside the limits of structural rebuilding of DNA. Epigenetic modifications are convertible rebuilding of chromatin, which modulate gene expression without alteration of DNA sequences and provide variability of transcriptional activity of particular genes, course of cellular cycle, replication and reparation of DNA, as well as phenomena of imprinting. Two independent epigenetic mechanisms – methylation of DNA and acetylation of histones are often involved in the process of malignant transformation of cell [1, 2].

In addition to the data on genetic nature of cancer, it is known that cancer is epigenetic disease [2–5]. The main epigenetic modification at malignant neoplasms is DNA methylation. In particular, one of the most important modifications is inactivation of gene-suppressors of tumor growth through hypermethylation of CpG-islands in their promoter regions [6–8]. These areas (among others) are targets for the DNA-demethylating agents as possible antitumor agents [1, 6, 7].

Second group of epigenetic regulatory mechanisms (on the transcription level) realizes through modifications of structure of histone proteins. It is known that structure of nuclear chromatin, density of its packaging significantly depends on total charge of histones. Changes of processes of acetylation, phosphorylation, methylation, ubiquitination and other modifications essentially influence the level of this charge, and thus – ability of histones to bind with DNA, deactivate function of particular genes or their groups [9, 10].

Histone code regulating gene expression is formed from differently acetylated residues of lysine in histones. Significant increase of part of deacetylated histones in malignant cells is usually associated with hypermethylation of DNA and deactivation of suppressor genes of tumor growth. These mechanisms were named «Histone language» [11]. Series of compounds known as antitumor drugs are capable to inhibit histone deacetylases (HDAC) [1, 12, 13]. HDAC are group of enzymes regulating transcription and series of specific cellular functions, in particular, suppression of tumor growth with the assistance of protein p53. Inhibitors of HDAC (iHDAC) show antitumor activity, in most cases are well endured and were successfully introduced into the clinical experiments [14, 15, 19]. They are classified by chemical structure and ability to suppress HDAC of I, II and IV classes. The most powerful among iHDAC are derivatives of hydroxamic acid, in particular, SAHA that was recently used for the therapy of skin lymphomas. Other classes of iHDAC: short chains of fat acids (SCFA), benzamides, epoxy ketones, non-epoxy ketones that contain cyclic tetrapeptides, and hybrid molecules [8–10, 12, 13, 16–18], — were also low-toxic and showed antitumor activity against solid and hematological tumors [10, 13, 19]. Moreover, iHDAC are sensitizers for the radiation influence. At the same time, molecular basis of selective antitumor action of this group of substances remains unknown. The idea is based on mentioned data that deep study and application of iHDAC (as

independent agents or their synergetic influence with hypermethylating agents as well as agents of chemo- and radiotherapy) forms significant interest and may become one of the strategies for the treatment of malignant tumors [10, 14, 16, 17].

One more important epigenetic mechanism of regulation of genome expression is activity of numerous intracellular ribonucleases (RNases). Last ones are large group of vitally important for the cells enzymes, which functioning provides the necessary balance between processes of synthesis, functioning and destruction of RNA; their role is known in mechanisms of processing and splicing of different classes of RNA [20–22]. Numerous RNases are important for the expression of products of different genes. In particular, they play important role in the processes of apoptosis [23], destruction of viral and extracellular RNA [24]. Today it is known that some types of RNases show significant antitumor activity and in the near future may be used as antitumor drugs [25-28]. The level of activity of RNases in cells of malignant neoplasms is low [28, 29, 30, 33, 34]. Their activity increases in biological fluids in organism with tumor. The cause of this could be the increased level of "flowing out" of the enzymes from the transformed cells that is caused, in particular, by weakening of protein-protein connections with the assistance of polyamines [32, 35].

At present time, the interrelation between RNases and polyamines has not been studied enough. It has been showed that spermine and spermidine in micromolar concentrations are able to increase activity of various RNases suppressed by inhibitors in several times (up to 7 times) [36]. Effect of such RNase inhibitors as poly-A, poly-G, double helix RNA or DNA, may be fully stopped if small concentrations of spermidine, spermine and, to a lesser degree, putrescine are present [36–38]. At the same time, large concentrations of polyamines are able to suppress the activity of RNases even to the full blocking. Data are worth of notice concerning the fact that under the effect of polyamines, the substrate specificity of these enzymes, which is connected with the sequence of one or another nucleotides in RNA molecule, can be changed [38]. All above-mentioned shows that RNases, along with polyamines, form the important mechanism of regulation of genetic activity and have to be the subject of active study.

Taking into account the above-mentioned, the aim of the work was to study series of the early epigenetic changes in tumor cells under the blocking of the synthesis of polyamines by specific inhibitors.

OBJECT AND METHODS

High-specific inhibitor of ornithine decarboxylase (ODC; EC4.1.1.17) difluoromethyl ornithine (DFMO) (R.M.I., France) and inhibitor of S-adenosyl methyl decarboxylase (SAMDC, EC4.1.1.50) methylglyoxal bis (guanylhydrazone) – dihydrochloride (MGBG) (Janssen, Belgium) have been used in study.

Adult, sexually mature male mice of line CDF_1 of breeding of experimental base of IEPOR of NAS of Ukraine have been used in study. The experiments with them have been carried out according to the International regulations concerning the working with experimental animals [39]. Cells of experimental leukemia L1210 have been transplanted via administration in abdominal cavity of $5•10^5$ cells in the volume of 0.25 ml of sterile isotonic solution of sodium chloride.

For the *in vitro* studies, samples from suspension of cells L1210 have been selected ($20•10^6$ cells), which were put in the nutrient medium 199, where the studied inhibitors of synthesis of polyamines have been added. Samples have been divided as follows: 1 – control; 2 – DFMO (360 μM); 3 – MGBG (34μM); 4 – DFMO + MGBG (360 and 34 μM correspondingly). Cell suspensions have been incubated at t $37\ ^0$C during 60 min or 4 hrs whereupon have been centrifuged on the low speed (1500 r/min, 5 min). From sediment of cells, the HDAC and RNase have been extracted by appropriate buffer solutions (for determination of activity of enzymes, according to the manufacturer's instructions) or DNA has been extracted for the evaluation of the level of its methylation.

In *in vivo* studies, on the 5^{th} day of leukaemia, animals have been administered DFMO (800 mg/kg), MGBG (75 mg/kg) or combination of these inhibitors of synthesis of polyamines in mentioned doses. The administration has been repeated in 24 hrs. Control animals have been administered by 0.25 ml of isotonic solution of sodium chloride. In 24 hrs after the last administration of inhibitors of synthesis of polyamines, all animals have been euthanized under the light ether narcosis, the ascitic fluid has been washed off the abdominal cavity, the number of leukaemia cells has been calculated. Out of the total volume of suspension, samples have been selected in such way that each contained $5•10^6$ cells. Cells have been sedimented by low-speed centrifugation, washed off one time by chilled 0.14 M solution of NaCl and the extraction of enzymes has been carried out or DNA has been extracted.

At quantitative determination of activity of HDAC, the fluorogenic substrate «HDAC Assay Kit Fluorimetric» (SIGMA-ALDRICH) has been used; the level of fluorescence has been determined at $\lambda = 450$ nm upon the conditions of excitation at $\lambda = 360$ nm in plates Nunclon TM.

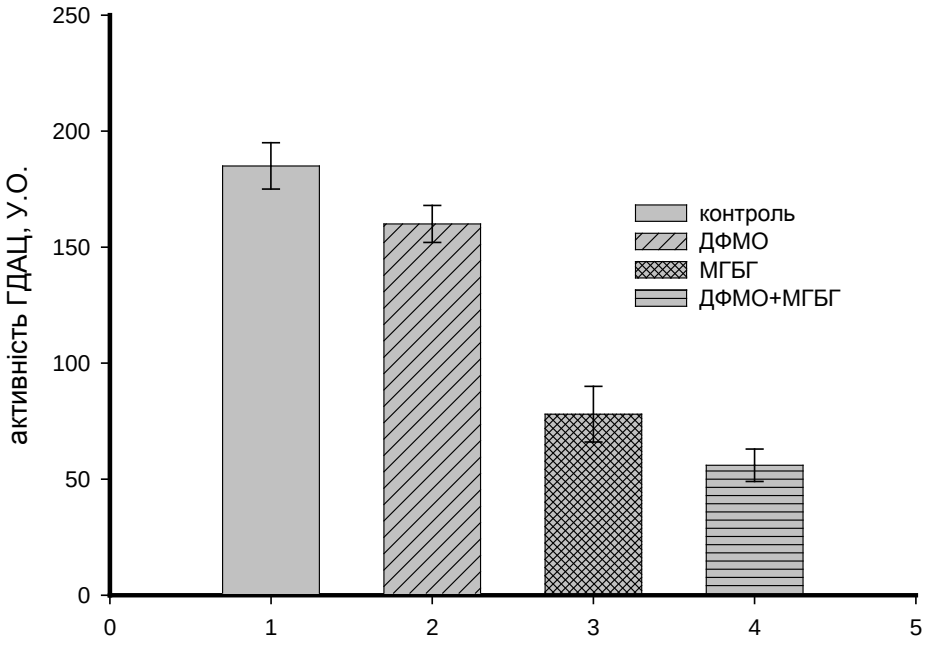
Studying the activity of RNases, their extraction has been carried out by 8 M buffered urea solution. The determination of activity has been carried out using electrophoresis in blocks of polyacrylamide gel. Cell extract has been enhanced to the 1% by sodium dodecyl sulfate, separated in 12% polyacrylamide gel block by Laemmli [40]. To the content of dividing gel, polyuridylic acid as substrate (400 mkg/ml) has been added. After carrying out of the electrophoresis during 4 hrs at voltage gradient 20 V/cm, gel was taken out of apparatus; after the extraction of sodium dodecyl sulfate, it has been incubated in 0.05 M acetate buffer (pH 5.5) during 60 min and stained by 0.1% solution of toluidine blue during 30 min. Gels have been photographed on digital camera and processed on the computer using programs Totallab. The results are represented in the form of histograms.

For determination of the level of total DNA methylation, cellular sediment has been twice washed off by cold 0.14 M solution of NaCl, whereupon from each sample ($5 \cdot 10^6$ cells) the separation of DNA by standard method of chloroform deproteinization or by Miniprep procedure has been conducted. After determination of DNA concentration, the level of total methylation has been studied by immunoenzyme method ELISA (DNA solution in concentration of 100 ng/ml was added in each well of plate by 50 μL) using standard kit Epigentek (USA) according to the procedure recommended by manufacturer, by measurement of inhibition at $\lambda = 450$ nm on the reader Synergy.

Statistical processing of the results has been carried out using Students t-criterion.

RESULTS AND DISCUSSION

The task of the first series of studies was to study the HDAC activity in cells of L1210 in the early stages of blocking *in vitro* of synthesis of polyamines (Fig. 1). Already at short-term (60 min) effect on L1210 cells of ODC inhibitor – DFMO, activity of HDAC in them decreases ($p = 0.05$); greater decrease ($p < 0.05$) has been marked under the effect of SAMDC inhibitor – MGBG. Most apparent decrease ($p < 0.01$) of HDAC activity has been observed at combined action of both inhibitors (see Fig. 1 *a*). Thus, changes of HDAC activity may be referred to the earliest manifestation connected with inhibiting of metabolism of polyamines. At longer effect of inhibitors (4 hrs), the HDAC activity in L1210 cells continues to decrease and stays on the low level (compared with control $p < 0.05$ in all variants of experiment) (see Fig. 1 *b*). The obtained data coincide with the results of many researchers [9, 10], who have showed the direct correlation between the level of suppression of activity of HDAC and antitumor action of inhibitors of synthesis of polyamines.



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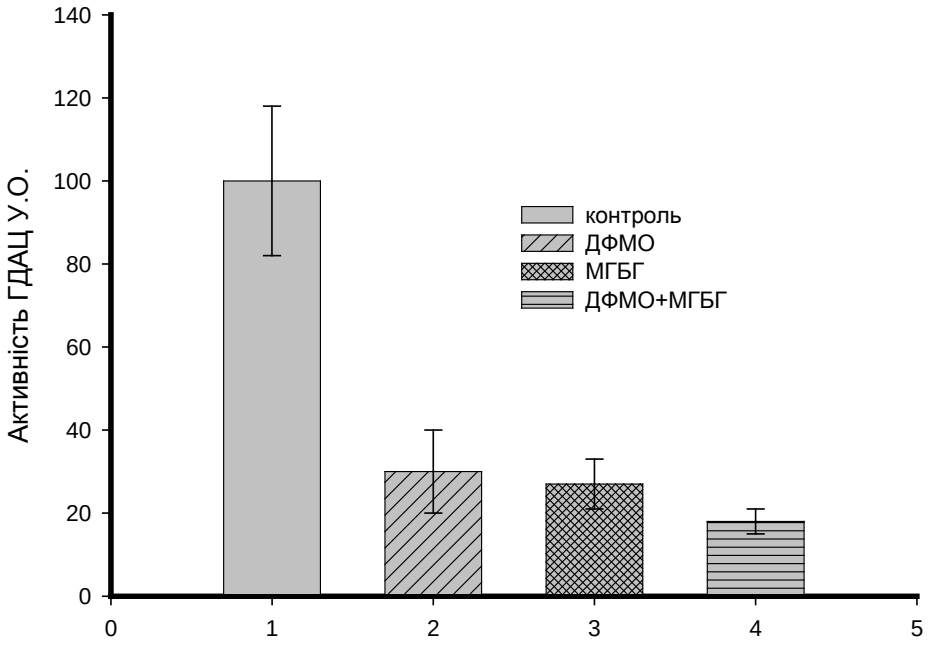

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Fig. 1. Effect of DFMO, MGBG and their combination on the activity of HDAC in cells of experimental leukaemia L1210 at influence *in vitro* during 60 min (a) and 4 hrs (b)

In the next series of studies, the changes of HDAC activity upon the conditions *in vivo* have been studied, when inhibitors have been administered to the animals with experimental leukaemia L1210 (Fig. 2). The obtained results have confirmed results of studies *in vitro*, according to which, the blocking of synthesis of polyamines both on the level of decarboxylation of ornithine and on the level of suppression of activity of S-adenosyl methionine decarboxylase, causes the prolong decrease of HDAC activity.

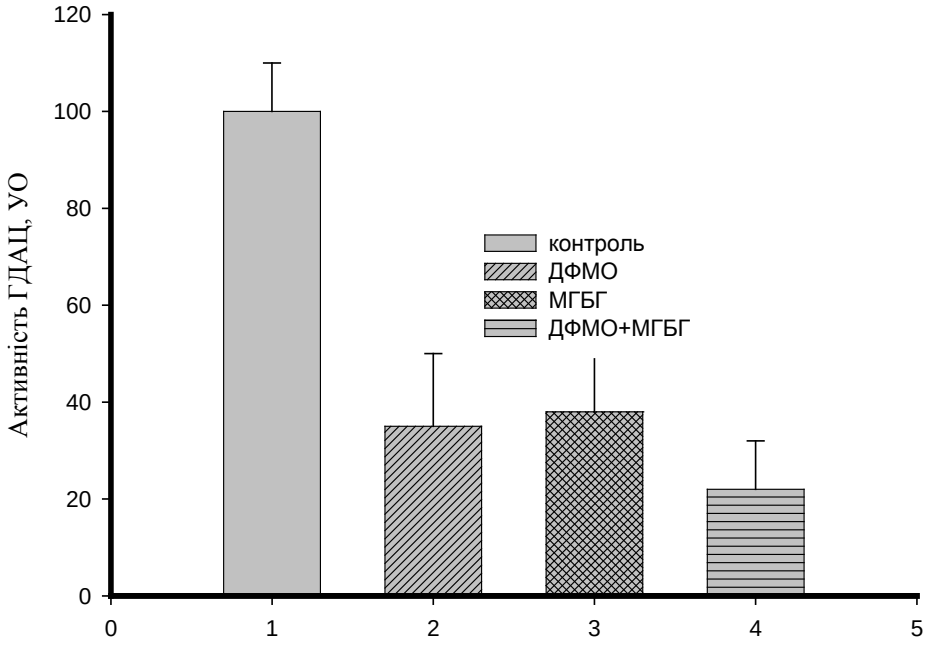
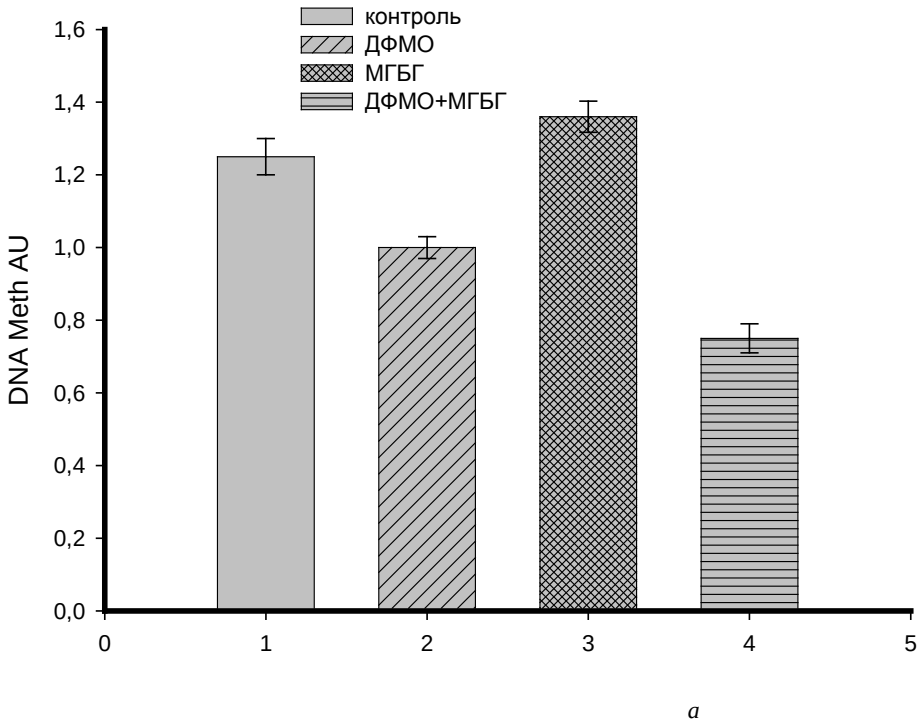


Fig. 2. Effect of DFMO, MGBG and their combination on the activity of HDAC in cells of experimental leukaemia L1210 at influence *in vivo* during 28 hrs

Study of methylation of DNA in the early stages of blocking of the synthesis of polyamines *in vitro* has showed that at short-term (60min) action on L1210 cells of DFMO and DFMO combined with MGBG, the level of methylation of DNA significantly decreases; most apparent decrease has been observed at combination of both inhibitors. At the same time, effect of MGBG does not change the level of methylation (which even becomes significantly higher than in control) (Fig.3

a). At longer (4 hrs) action of DFMO combined with MGBG, the level of DNA methylation in L1210 cells continues to decrease and stay stable on the low level. At the same time, changes of DNA methylation under isolated effect of each inhibitor have opposite (compared with described above) direction: at long action of DFMO, the level of methylation increased to the same in control; at action of MGBG – significantly ($p < 0.05$) decreased at comparison both with control and data of short-term (60 min) incubation (Fig. 3 *b*). In study *in vivo*, when the inhibitors of synthesis of polyamines have been administered to animals with transplanted leukaemia, the level of total methylation of DNA in leukaemia cells was lower than in control both at action of each inhibitor and of their combination (Fig. 4).



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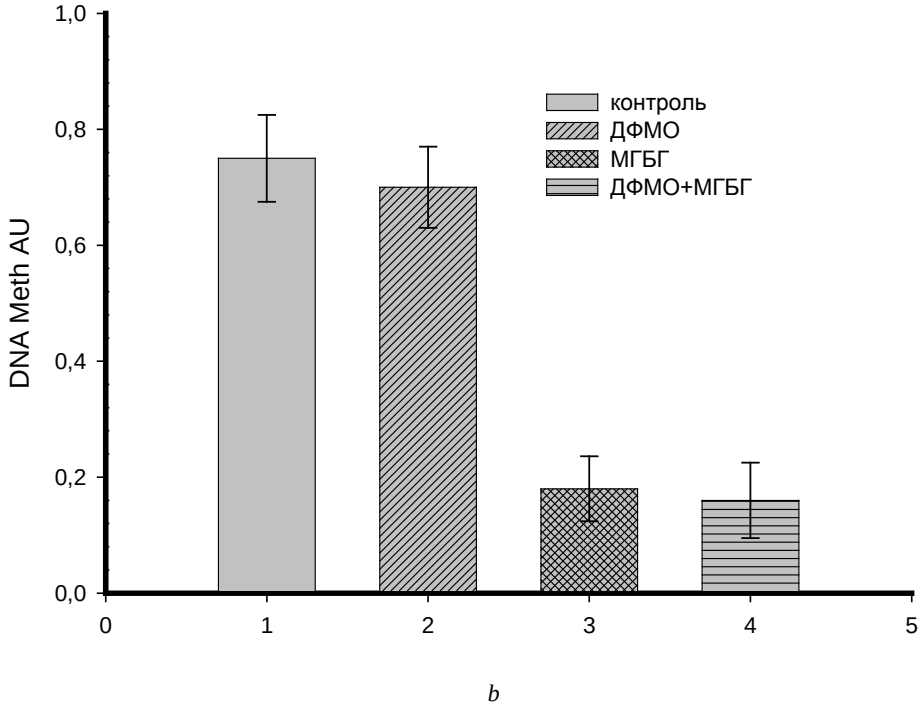

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Fig. 3. Effect of DFMO, MGBG and their combination on the level of DNA methylation extracted from cells of experimental leucosis L1210, at action *in vitro* during 60 min (*a*) and 4 hrs (*b*)

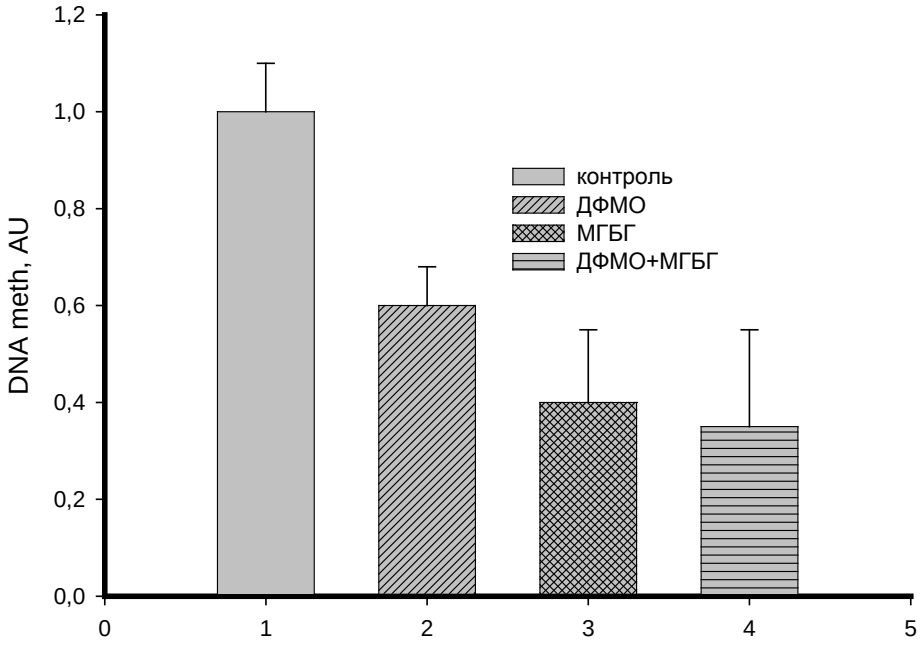
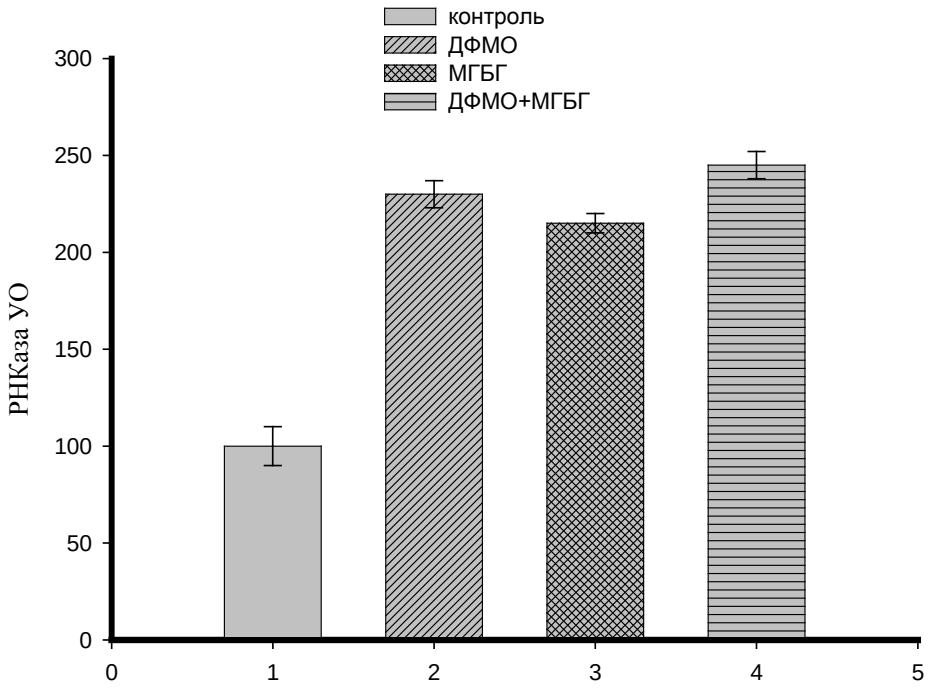


Fig. 4. Effect of inhibitors of synthesis of polyamines in *in vivo* study during 28 hrs on the level of total DNA methylation in cells of leukaemia L1210

Next stage of study was investigation of activity of intracellular RNases under the effect of inhibitors of synthesis of polyamines. At short (60 min) blocking of synthesis of polyamines in L1210 cells *in vitro*, the apparent (almost 2 times) increase of activity of RNases has been marked, especially at combination of two inhibitors – DFMO and MGBG (Fig.5 *a*). Increase of duration of effect of inhibitors up to the 4 hrs has been accompanied with more intensive increase of activity of intracellular RNases (Fig. 5 *b*). Similar results have been obtained *in vivo* (Fig. 6): apparent increase of activity of RNases in cells of L1210 leukemia under the effect of DFMO+MGBG, DFMO and to significantly lesser degree MGBG.



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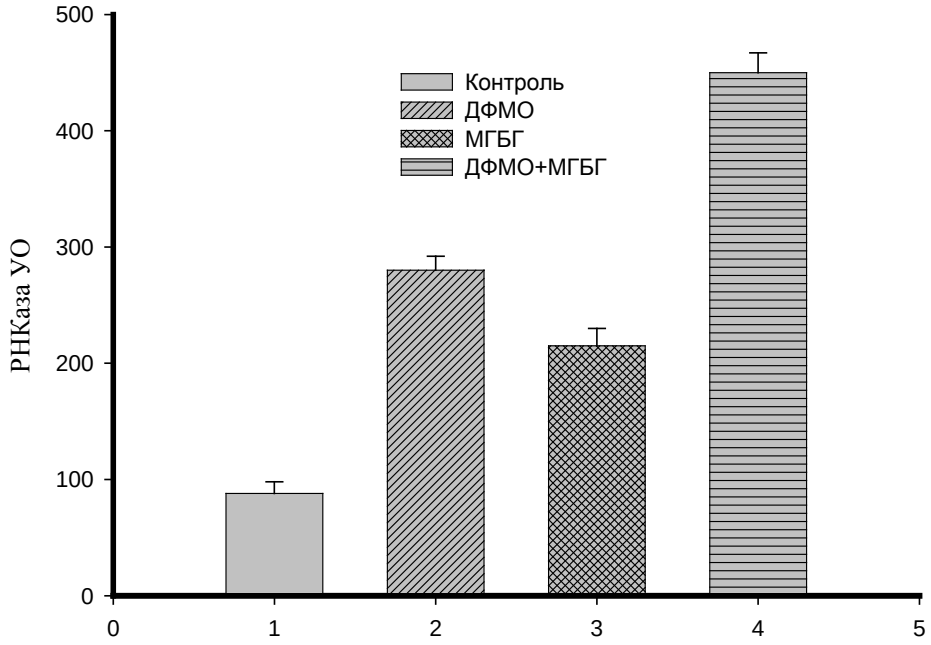

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Fig. 5. Effect of DFMO, MGBG and their combination on the level of activity of RNase in L1210 cells under the *in vitro* action during 60 min (a) and 4 hrs (b)

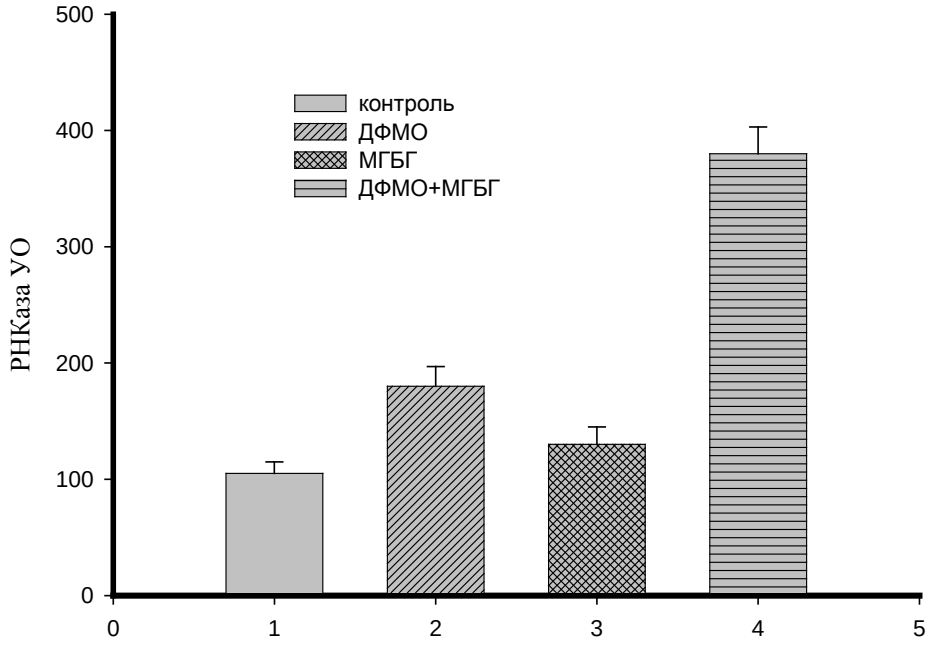

Fig. 6. Effect of inhibitors of synthesis of polyamines *in vivo* during 28 hrs on the activity of RNases in L1210 leukaemia cells.

Mechanism of increasing of activity of RNases under blocking of synthesis of polyamines is unknown. Increase of enzyme activity cannot be explained by destruction or blocking of action of natural inhibitor of enzyme, since the last one has significantly higher molecular mass and moves at electrophoresis in polyacrylamide gel much slower than enzyme. Thus, increase of activity of RNases may be connected with synthesis of enzyme *de novo* or with transition of enzyme from inactive (latent) form into the physiologically active form. Anyway, increase of activity of enzymes, which destroy different kinds of intracellular RNAs, may be the evidence of significant disorders of intracellular metabolism of leukaemia cells. It is known that in many cases, the action of cytostatics is accompanied with increase of activity of hydrolytic enzymes in tumor cells [31, 32].

CONCLUSIONS

Results of studies show that blocking of synthesis of polyamines activates the action of intracellular factors, which sharply change the processes of transcription, translation and structures of chromatin in tumor cells. Such changes in the most cases cause the apoptosis, autophagia or other forms of cellular death [23].

Application of inhibitors of synthesis of polyamines with different mechanism of action and their rational combination is important direction in the development of purposive influence on the tumor growth.

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