Progress in Chemical and Biochemical Research

Journal homepage: www.pcbiochemres.com

Influence of arabinofuranosylcytosine-5`-monophosphate and its emoxipin salt on viability and functional status of peripheral blood lymphocytes subpopulations

Aliaksei Sysa*, Maryna Labai, Eugeni Kvasyuk, Uliana Ivyts, Maksim Khanchevskii[1]

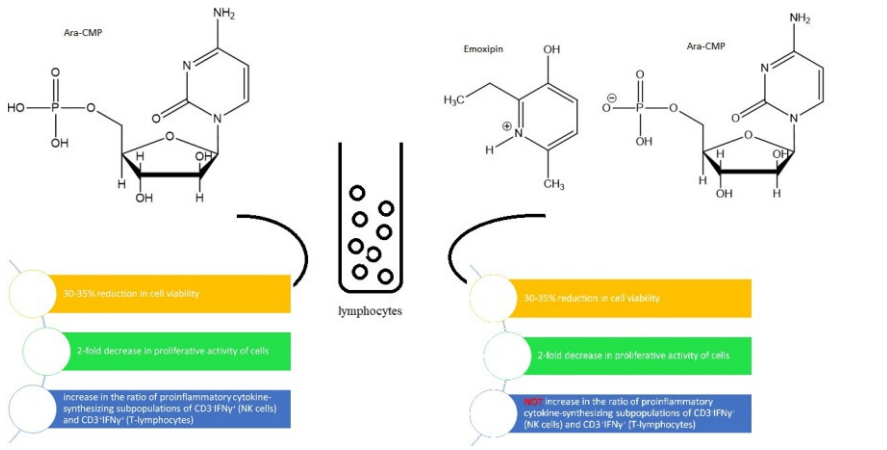
Belarusian State University, ISEI BSU, Minsk, Belarus

ARTICLE INFO

Article history:

Submitted: 2019-07-22
Revised: 2019-09-30
Accepted: 2019-10-19
Available online: 2019-10-31
Manuscript ID: PCBR-1907-1042

GRAPHICAL ABSTRACT



KEYWORDS

Antimetabolites
Ara-CMP
Antioxidants
Emoxipin
Proliferation

ABSTRACT

The influence of the modified nucleotide arabinofuranosylcytosine-5`-monophosphate in the form of the free acid (Ara-CMP) and its salts with emoxipin (Ara-CMP+Em) on the viability of peripheral blood mononuclear cells, the lymphocyte proliferation of mitogen-stimulated cells was studied. It was shown that emoxipin - the compound with strong antioxidant properties - does not lead to inhibition of the main function of the antimetabolite Ara-CMP, namely, cell growth arrest (p = 0.96) and lysis of target cells (p = 0.97). Under the conditions of nonspecific stimulation of lymphocytes, Ara-CMP at high concentrations (10^{-4} M) increased in both IFNγ-secreting T-lymphocytes (five times) and IFNγ-secreting other lymphocytes (three times). Emoxipin led to almost complete leveling of the detected effect.

* Corresponding author: Aliaksei Sysa
Tel: 00375-29-3034931; Fax: 00375-17-3989953, E-mail: aliaksei.sysa@iseu.by
DOI: 10.33945/SAMI/PCBR.2019.4.3

1. Introduction

Nucleobase/nucleoside analogues realize their cytotoxic effects by mimicking natural endogenous nucleosides (after phosphorylation - nucleotides) [1-5]. The mechanism of action can be associated either with inhibition of enzymes or with substitution of endogenous nucleosides as substrates in nucleic acid synthesis, which leads to damage to DNA and RNA and disruption of DNA methylation [6-8]. It should be emphasized that for the realization of its pharmacodynamic effect nucleobase/nucleoside analogues need to fulfill many conditions: to achieve tumour cells in sufficient concentration (delivery), not to be destroyed (stability), to penetrate the membrane inside the tumour cells (usually through protein-carriers), to turn into their active forms (bioactivation), to interact with the molecular target (pharmacodynamic activity) [9].

Often the process of triphosphorylation of nucleoside analogues is difficult or impossible because of the high specificity of cellular nucleoside- and nucleotidekinases. The nucleoside-5'-monophosphates can not be used directly due to the fact that their transport into cells is extremely limited; in addition, they are rapidly destroyed to the corresponding nucleosides at the cell membrane.

These causes make the wide interest to the synthesis of pronucleotides, i.e. chemically modified nucleoside monophosphates and their analogues, which would have the ability to penetrate into the cell and turn into appropriate antimetabolites after chemical or enzymatic transformations.

Another aspect that limits the use of cytostatic drugs is that these drugs have undesirable side effects due to possible impact on the genetic machinery of the host cell. In this regard, it is attractive to search for substances or their combinations, the use of which will lead to decrease the intoxication rates in organismes of tumor carriers (with antioxidants, in particular).

Thus, most authors point to the ability of antioxidants to improve the tolerability of chemotherapy and long-term results of treatment [10, 11], supporters of a different point of view believe that antioxidants inhibit the antitumor effect of chemotherapy[12]. However, all these studies relate to the use of nutraceuticals with mild antioxidant action (β-carotene; vitamins A, C, E; selenium; melatonin, cysteine; b vitamins; vitamin D3; vitamin K3; glutathione, coenzyme Q10).

The aim of this work was to evaluate the influence of the modified nucleotide arabinofuranosylcytosine-5`-monophosphate in the form of the free acid (Ara-CMP **5**) and its salts (Ara-CMP+Em **7**) with 6-methyl-2-ethylpyridinium-3 (emoxipin **6**) (a synthetic derivative of 3-hydroxypyridine with strong antioxidant action) on the viability of peripheral blood mononuclear cells and the mitogen-induced lymphocytes proliferation.

Experimental

Reagents and Solutions.

Phosphorous oxychloride ($POCl_3$), trimethyl phosphate ($(CH_3)_3PO_4$), acyl chloride of acetylsalicylic acid ($C_9H_7O_7Cl$), acetonitrile (C_2H_3N), sodium hydroxide (NaOH), hydrochloric acid (HCl) and all other chemicals and solutions for cells preparapion and treatment were of analytical grades.

Synthesis of Ara-CMP 5 and Ara-CMP+Em 7.

Synthesis of 5`-monophosphate of arabinofuranosylcytosine 5 (Ara-CMP) was carried out according to the scheme (**Fig. 1.**)

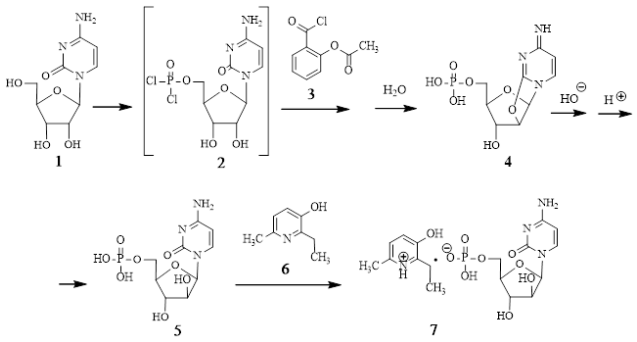


Figure 1. Scheme of synthesis of Ara-CMP **5** and Ara-CMP+Em **7**

The reaction between cytidine **1** with phosphorus oxychloride in trimethyl phosphate resulted in formation of cytidine-5'-phosphodichloridate **2**, which without isolation was treated with acyl chloride of acetylsalicylic acid **3** in acetonitrile and then with water. The resulting mixture was boiled until the intermediate products were completely transformed into cyclo-CMP **4**, which was isolated in crystalline form by ion exchange chromatography on a column with anion exchange resin Dowex 1x8 in acetate form.

The isolated cyclo-CMP **4** was purified by recrystallization from a mixture of water/alcohol. The interaction of cyclo-CMP **4** with a sodium hydroxide solution and subsequent treatment with a hydrochloric acid solution to pH = 2-4 led to Ara-CMP **5**, whose purification was carried out by its recrystallization from water.

Synthesis of emoxipin **6** was carried out according to the method presented in[13]. Ara-CMP+Em **7** was obtained by evaporation of a solution of equimolar amounts of Ara-CMP **5** and emoxipin **6** into water. The resulting powder Ara-CMP+Em **7** was dried in a vacuum at 60°C to a constant weight.

Cultivation of PBMC

PBMCs (peripheral blood mononuclear cells) were isolated from the peripheral blood of healthy volunteers using the Ficoll-Verografin density gradient (Sigma, Germany) method and seeded in 10% FBS (HyClone, UK) RPMI-1640 media (Lonza, Belgium) in concentration 10^6 cells/ml. Percentage of lymphocytes and monocytes of total events was analysed by flow cytometry (CytoFLEX; Beckman Coulter, USA) after 72 hours.

Proliferation assay

PBMCs were diluted in complete RPMI 1640 medium to a final concentration of 10^6 cells/ml. The PBMC suspension was then transferred to 96-well flat-bottom culture plates (Costar), and 100 µl of RPMI 1640 medium containing 10^4 to 10^6 M of Ara-CMP/Ara-CMP+Em was added. Cells were stimulated in the presence of PHA (2,5 µg/ml) for 6 days at 5% CO_2 and 37°C. Analysis of prolifetation was performed by flow cytometry. Proliferation and viability of PBMCs or monocyte subsets were controlled by flow cytometry (CytoFLEX; Beckman Coulter, USA) analysis using 7 µM carboxyfluorescein succinimidyl ester (CFSE; Sigma, Germany), FITC-labeled anti-IFNγ monoclonal antibody (MAb) (Becton Dickinson), and PE-labeled anti-CD3 MAb (Becton Dickinson).

Statistical analysis

Statistical (i.e., based on the average trend) dependence of the effect value φ on the level of influence x is described by models, which in general can be represented as $\varphi(x; b, c, d, e,...) = C + (d - c)\ \psi\ (x; b, e,...)$, where the parameters c and d are the lower and upper limits of the response, and ψ is some given nonlinear function with parameters b and e. The list and description of widely used models is given in [14]. In this paper we used a log-logistic model with four parameters (b, c, d, e) LL.4, which has the form:

$$\varphi(x) = c + \frac{d - c}{1 + e^{b\ (\log x - \log e)}}$$

The estimated parameters of the models have a definite physical meaning. In particular, for the log-logistic model, the parameters c and d determine the lower and upper horizontal asymptotes of the sigmoid curve, e corresponds

to the position of the inflection point, and *d* – to the angle of inclination in the transition region. Fitting of model parameters to the analyzed empirical data was carried out using the generalized method of minimizing the sum of squares of deviations of model forecasts from the observed values, taking into account specially selected weight coefficients.

Statistical analysis of the estimated parameters was carried out using Student's t-test, which tested the hypothesis of the equality of each coefficient to zero and calculated *p*-values that determine the achieved level of significance. The statistical significance of the model as a whole was verified by comparing it with a simple regression with a zero slope coefficient (the horizontal regression line corresponds to the absence of dose-effect dependence) by ANOVA.

Results and Discussion

As can be seen from the data presented at Fig. 1, Ara-CMP **5** is a synthetic analogue of cytidine. The chemical structure of Ara-CMP differs from the known nucleotides cytidine and deoxycytidine by the carbohydrate fragment of its molecule. It is represented in Ara-CMP not by ribose or deoxyribose, but arabinose, i.e., there are differences in the configuration of hydroxyl group at the C2-atom in sugar part of the molecule. The existence of C2-hydroxyl in the molecule of arabinozylcytosine in the *cis*-position relative to cytosine causes resistance of arabinosylcytosine to phosphorylases. *In vivo* under the action of deoxycytidinekinases the transformation into arabinosylcytosine triphosphate (Ara-CTP) is occured.

Emoxipin **6** is an analogue of vitamin B_6 – a derivative of 3-hydroxypyridine – with strong antioxidant properties. In medicine, emoxipin is used as a drug from the group of antiplatelets and antioxidants and is a corrector of microcirculation. The active fragment 2-methyl-6-ethylpyridine-3 after penetration into the blood flow strengthens blood vessels, prevents their fragility, thins the blood, thus preventing the development of destructive processes in the lumen of the vessels. The antioxidant effect emoxipin allows to stimulate natural processes, provides neutralization of free radicals, and thus prevents damage to vital biological molecules.

The addition of Ara-CMP **5** and its emoxipin salt Ara-CMP+Em **7** at concentrations of 10^{-6} to10^{-4} M affected the viability and proliferative activity of PBMCs (**Fig. 2 and 3**).

As can be seen from the data presented at Fig. 2, both Ara-CMP **5** and Ara-CMP+Em **7** lead to 30-35% reduction in cell viability compared to control in the studied concentration range. Analysis of variances (ANOVA) showed no statistically significant differences between the effects of Ara-CMP **5** and Ara-CMP+Em **7** (p = 0.97). The ratio of calculated values of ED_{50} for Ara-CMP and Ara-CMP+Em (see equation) was 1.97, which may indicate the potentiation of the cytostatic action of the antimetabolite Ara-CMP **5** by emoxipin.

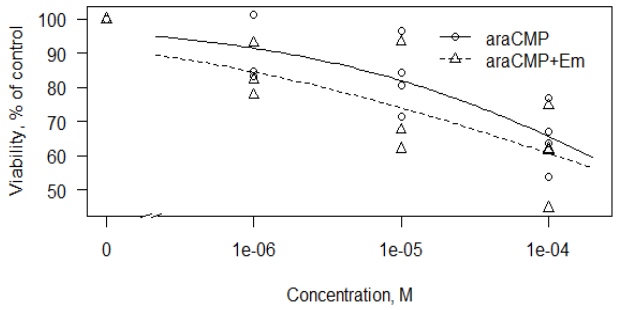


Figure 2. Impact of Ara-CMP **5** and Ara-CMP+Em **7** on viability of PBMCs

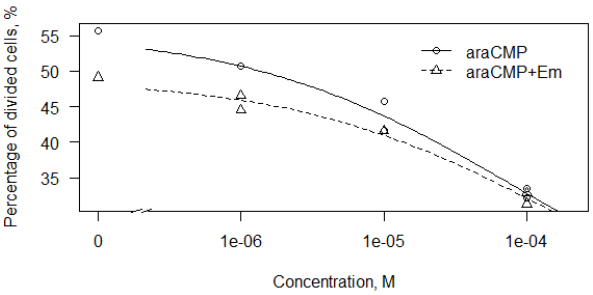


Figure 3. Impact of Ara-CMP **5** and Ara-CMP+Em **7** on proliferation of PBMCs

The results of estimation of nucleotide influence on lymphocyte proliferation under mitogen-specific stimulation showed that both Ara-CMP **5** and its emoxipin salt Ara-CMP+Em **7** lead to a twofold decrease in proliferative activity of cells compared to the control in the studied range of concentrations (10^{-6} – 10^{-4} M) (Fig. 3). ANOVA analysis of the data showed no statistically significant differences between the effects Ara-CMP **5** and Ara-CMP+Em **7** ($p = 0.96$). The ratio of calculated values of ED_{50} for Ara-CMP and Ara-CMP+Em (see equation) was 1.74, which may also indicate the potentiation of antiproliferative action of Ara-CMP **5** by emoxipin.

Our results shows that antioxidant emoxipin not only does not affect the main function of antimetabolite Ara-CMP, namely: to interrupt the cell division and growth, but also modulates its action.

Next the functional state of peripheral blood lymphocyte subpopulations under the influence of the studied compounds was analyzed. The dynamics of those cell fractions that are associated with a response to oxidative stress, which inevitably occurs under the action of cytostatic drugs and is accompanied by an increased accumulation of toxic products of lipid peroxidation and imbalance of the links of antioxidant protection is of particular interest [15].

Normally, all mechanisms of regulation are set up in a such way that an increase in the content of reactive oxygen species leads to an increase in the activity of antioxidant systems, which returns the level of free radicals to normal. With the development of pathological condition, these mechanisms of regulation are violated. There is a growing number of publications in the literature on the relationship between oxidative stress and immune protection in various pathologies. Oxidative stress products alter immune responses, causing an increase in the content of proinflammatory cytokines, including IFNγ [16].

We assumed that with the increase in the content of proinflammatory cytokines, the proliferation of the corresponding cytokine-synthesizing cells increases, as well as evidenced by the change in the weight of $IFN\gamma^+$-lymphocyte fractions. Indeed, cultivating of mononuclear cells with Ara-CMP changed the ratio of subpopulations of $CD3^-IFN\gamma^+$ (NK cells) and $CD3^+IFN\gamma^+$ (T-lymphocytes) in dose-dependent manner (**Fig. 4 and 5**).

As can be seen from the data presented at Fig. 4, Ara-CMP **5** led to 2-4-fold increase in the content of $CD3^-IFN\gamma^+$-cell subpopulation compared to the control. While cultivation in a medium containing Ara-CMP+Em **7**, no such effect was observed.

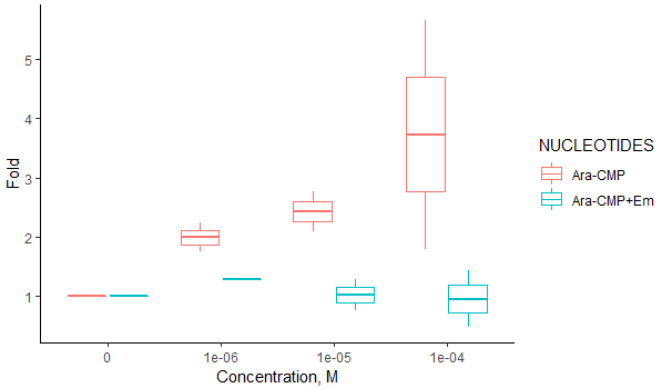


Figure 4. Impact of Ara-CMP **5** and Ara-CMP+Em **7** on the proportion of $CD3^-IFN\gamma^+$ subpopulation (the fold of increase of specific weight compared to the control)

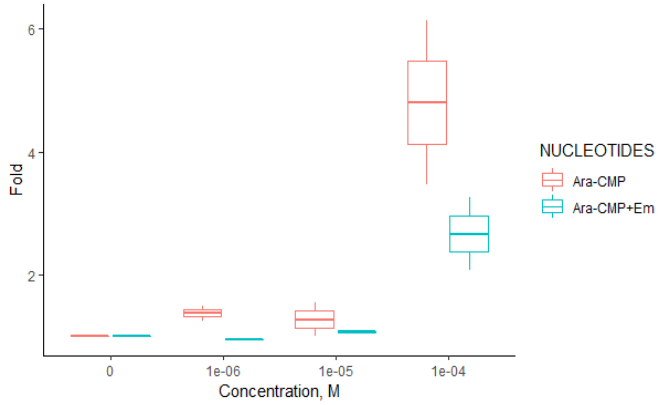


Figure 5. Impact of Ara-CMP **5** and Ara-CMP+Em **7** on the proportion of $CD3^+IFN\gamma^+$ subpopulation (the fold of increase of specific weight compared to the control)

As can be seen from the data presented at Fig. 5, in the studied range of concentrations Ara-CMP **5** did not lead to a change in the specific weight of $CD3^+IFN\gamma^+$ T-lymphocyte subpopulation compared to control. Only in the maximum concentration (100 mM) there was a fivefold increase in the case of Ara-CMP **5** and a threefold increase in the case of Ara-CMP+Em **7**.

Thus, under conditions of the nonspecific stimulation lymphocytes responsed to the introduction into the cultural media of antimetabolite Ara-CMP **5** not only with stopping cell growth and proliferation, but with increasing in fractions of secreting pro-inflammatory cytokines cells, as a systemic reaction to the increased ROS level resulted from the destruction of cells. Note that the presence of moxipin (a compound with antioxidant properties) in the media almost completely leveled the detected effect.

Conclusion

Antimetabolites, in particular nucleobase/nucleoside analogues, are cytotoxic drugs that in combination with other chemotherapeutic agents have significantly transformed clinical oncology and turned cancer into a curable disease. However, even taking into account the fact that in the case of combining the use of chemotherapy with radiotherapy, surgery or immunotherapy can now cure almost all types of cancer, for a significant proportion of patients, cancer is still incurable. Understanding the differences in metabolism, pharmacokinetics, pharmacodynamics, and tumor biology of patients who can be cured and patients who cannot creates a scientific basis for improving rational therapy.

The emoxipin – compound with strong antioxidant properties – does not lead to inhibition of the main functions of the antimetabolite arabinofuranosylcytosine-5`-monophosphate: growth supression and lysis of target cells. Moreover, the obtained results indicate the possible increase with emoxipin the cytotoxic and antiproliferative action of antimetabolite.

The obtained results not only strengthen the position of supporters of the combined use of antimetabolites with antioxidants in chemotherapy of tumor diseases, but also contribute to a better understanding of the molecular mechanisms of the effect of nucleic antimetabolites on biochemical processes, which can serve as the basis for the targeted search and creation of new anticancer drugs.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The work was supported by the Belarusian Republican Foundation for Fundamental research (grant X18MB-019).

References:

[1] F. Cividini, R. Pesi, L. Chaloin, S. Allegrini, M. Camici, E. Cros-Perrial, C. Dumontet, L.P. Jordheim and M.G. Tozzi, The purine analog fludarabine acts as a cytosolic 5′-nucleotidase II inhibitor. *Biochemical pharmacology*, 94 (2015) 63-68.

[2] C. Harrington and F.W. Perrino, The effects of cytosine arabinoside on RNA-primed DNA synthesis by DNA polymerase α-primase. *Journal of Biological Chemistry*, 270 (1995) 26664-26669.

[3] T. Mikita and G.P. Beardsley, Functional consequences of the arabinosylcytosine structural lesion in DNA. *Biochemistry*, 27 (1988) 4698-4705.

[4] F. Perrino and H. Mekosh, Incorporation of cytosine arabinoside monophosphate into DNA at internucleotide linkages by human DNA polymerase

alpha. *Journal of Biological Chemistry*, 267 (1992) 23043-23051.
[5] M. Tsuda, K. Terada, M. Ooka, K. Kobayashi, H. Sasanuma, R. Fujisawa, T. Tsurimoto, J. Yamamoto, S. Iwai and K. Kadoda, The dominant role of proofreading exonuclease activity of replicative polymerase ε in cellular tolerance to cytarabine (Ara-C). *Oncotarget*, 8 (2017) 33457.
[6] P.A. Dijkwel and F. Wanka, Enhanced release of nascent single strands from DNA synthesized in the presence of arabinosylcytosine. *Biochimica et Biophysica Acta (BBA)-Nucleic Acids and Protein Synthesis*, 520 (1978) 461-471.
[7] D.D. Ross, S.-R.S. Chen and D.P. Cuddy, Effects of 1-β-d-Arabinofuranosylcytosine in DNA Replication Intermediates Monitored by pH-Step Alkaline Elution. *Cancer research*, 50 (1990) 2658-2666.
[8] P. Huang, S. Chubb and W. Plunkett, Termination of DNA synthesis by 9-beta-D-arabinofuranosyl-2-fluoroadenine. A mechanism for cytotoxicity. *Journal of Biological Chemistry*, 265 (1990) 16617-16625.
[9] N. Tsesmetzis, C.B. Paulin, S.G. Rudd and N. Herold, Nucleobase and nucleoside analogues: resistance and re-sensitisation at the level of pharmacokinetics, pharmacodynamics and metabolism. *Cancers*, 10 (2018) 240.
[10] K.I. Block, A.C. Koch, M.N. Mead, P.K. Tothy, R.A. Newman and C. Gyllenhaal, Impact of antioxidant supplementation on chemotherapeutic toxicity: a systematic review of the evidence from randomized controlled trials. *International journal of cancer*, 123 (2008) 1227-1239.
[11] H. Pan, P. Mukhopadhyay, M. Rajesh, V. Patel, B. Mukhopadhyay, B. Gao, G. Haskó and P. Pacher, Cannabidiol attenuates cisplatin-induced nephrotoxicity by decreasing oxidative/nitrosative stress, inflammation, and cell death. *Journal of Pharmacology and Experimental Therapeutics*, 328 (2009) 708-714.
[12] A. babaei and A. Taheri, Direct Electrochemistry and Electrocatalysis of Myoglobin Immobilized on a Novel Chitosan-Nickel Hydroxide Nanoparticles-Carbon Nanotubes Biocomposite Modified Glassy Carbon Electrode. *Anal. Bioanal. Electrochem.*, 4 (2012) 342 - 356.
[13] R. Jalilian and A. Taheri, Synthesis and application of a novel core-shell-shell magnetic ion imprinted polymer as a selective adsorbent of trace amounts of silver ions. *e-Polymers*, 18 (2018) 123-134.
[14] V.K. Shitikov, V.A. Terekhova, B. A. Uzbekov, K.A. Kydralieva and B.M. Khudaybergenov, *Principles of ecology*, 4(3) (2015) 73 (in Russian).
[15] V.S. Yu, *Journal of Siberian Medical Sciences*, 2 (2013) 1 (in Russian).
[16] A. Agita and M.T. Alsagaff, Inflammation, immunity, and hypertension. *Acta Med Indones*, 49 (2017) 158-165.

How to cite this manuscript:

A. Sysa, M. Labai, E. Kvasyuk, U. Ivuts and M. Khanchevskii, Influence of arabinofuranosylcytosine-5`-monophosphate and its emoxipin salt on viability and functional status of peripheral blood lymphocytes subpopulations. *Progress in Chemical and Biochemical Research*, (2019) 178-184.

DOI: 10.33945/SAMI/PCBR.2019.4.3