ISSN 2347-6893
Volume 9 Number 3
Journal of Advances in Biology

# Antiviral and apoptosis modulating effect of methylenebisphosphonic acids

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## ABSTRACT

The research of the molecular and genetic characteristics of Epstein-Barr virus are going for years and its ability to develop lymphoproliferative diseases. However, the search for effective antiviral and anticancer drugs remains relevant today. The aim was to investigate the biological properties methylenebisphosphonic acids (10s20-22), including cytotoxicity, apoptosis stimulating and antiviral activity. For this we used MTT-metod, PCR and flow cytometry. Also bioinformatic analysis was conducted using compounds PASS. It was shown 10s-20 compound effectively inhibits the replication of EBV in low concentrations. Compound 10c-21 showed a significant effect on apoptosis stimulating B95-8 cells. Data analysis showed prediction that this class of compounds may be a substrate for cytochrome C (CYP2H), which in turn may indicate the involvement of mitochondrial ways of inducing apoptosis. The data indicate that the group methylenebisphosphonic acids are perspective for further research.

**Keywords**: Epstein-Barr virus; apoptosis; methylenebisphosphonic acids

**Academic Discipline And Sub-Disciplines:** Virology

**SUBJECT CLASSIFICATION:** Antiviral drugs

**TYPE (METHOD/APPROACH):** In vitro Experiment

## INTRODUCTION

Epstein-Barr virus belongs to the *Herpesviridae* family, members of which after initial infection can cause lifelong persistent infections. EBV is present in 90% of human population [1]. Lytic EBV infection leads to the formation of new virions and to the dissemination of the virus while latent infection provides lifelong persistence in human organism. The reservoirs for latent virus are memory B-cells, which express only some of the latent viral proteins [2, 3]. It is the latent form of EBV-infection that is associated with emergence of several lymphoproliferative diseases [4] and carcinomas such as Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma and post-transplant disorder [5]. EBV, as well as other herpesviruses, affects central and peripheral nervous system, causing severe meningoencephalitides [6]. Recently, Epstein-Barr virus has been associated with the development of autoimmune diseases such as multiple sclerosis and systemic lupus erythematosus [7].

To date, drugs are necessary to prevent and to treat diseases. However, the search and creation of a commercial drug is a very lengthy, expensive and complicated process, which is dependent on many factors (comparative evaluation of safety and clinical effectiveness) [8]. *In silico* methods based upon the use of computer programming are being increasingly used to solve these problems. This approach is very efficient, because it helps to conduct screening for biological activity of a large number of compounds in a short time [9]. However, these methods do not provide 100% result in further studies *in vitro.*

A combination of results from computer modelling and *in vitro* research is optimal for more effective screening of compounds, creation of new antiviral drugs and study of their action mechanisms.

Thereby, the objective of this work was to examine the biological and anti-EBV activity of bisphosphonic acid derivatives with further study of the apoptosis stimulation effect of the most promising compounds on the EBV infection model.

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## MATERIALS AND METHODS

### Cell culture

Raji is a human Burkitt's lymphoma-derived cell line, harboring the latent form of EBV cycle. B95-8 is a lymphoblastoid cell line, EBV-transformed and chronically producing viruses. CHO is a epithelial cell line established from chinese hamster ovary cell.

All cell lines were grown in 90 % of RPMI 1640 ("Sigma", USA) supplemented with 10% fetal bovine serum (FBS, "Sigma", USA), 2 mM L-glutamine and gentamicin (100 µg/ml) in tissue culture flasks. Cultivation was performed at 37 $^{O}$C in a 5 % $CO_2$ atmosphere. For the induction of lytic cycle, cells were stimulated with 3 mM n-byturate and 20 mg/ml TPA.


DOI : 10.24297/jab.v9i3.634

## Chemical substances

Derivates of methylenebisphosphonic scids: tert-butyl ether N- (2,2,3,3-tetraftortiopropionil) alanine (10S-20), mol. wt. 289,29; tert-butyl ether N-(2,2,3,3-tetraftortiopropionil) alanine, methyl ester N-(2,2,3,3 tetraftortiopropionil) phenylalanine methyl ester (10S-21), mol. wt. 323,31 and N- (2,2,3,3-tetraftortiopropionil) tryptophan (10S-22), mol. wt.: 362,34 were synthesized at the Institute of Organic Chemistry of the National Academy of Sciences of Ukraine [10]. The substances dissolved in DMSO and filtered through a filter with a pore diameter of 0.22 microns (Sarstedt, USA). Working solutions were prepared to culture medium.

## Determination of cell cultures viability

The viability of cell cultures was determined by staining them with 0,4% solution of trypan blue («Sigma», USA), which was used to detect dead cells.

## MTT-assay

The MTT staining method as described by Mosmann was used [11]. The absorbance was determined spectrophotometrically at 538 nm on an Multiskan FC universal microplate reader (Thermo scientific, USA).

## Real time PCR detection of viral DNA

A real time PCR (polymerase chaine reaction), assay was performed to assess the antiviral activity of various drugs against EBV [12]. DNA isolation of virus from samples conducted using «innuPREP Virus DNA Kit» (Analityk Jena AC, Germany). DNA concentration was measured by Biophotometer (“Eppendorf”, Germany). To detect DNA of EBV using a set «Amplisens®EBV-FL» (FGYN CNIIE, Russian) ccording to manufacturer's recommendations with detection in real time (qTOWER 2.2., Germany).

## Flow cytometry

Flow cytometry was performed to quantitatively detect the apoptotic cells [13]. The sub-G1 peak was measured with a flow cytometer Beckman Coulter Epics XL (USA) and analyzed using Flowing Software, version 2,5 (USA).

## PASS prediction

PASS (Prediction of Activity Spectra for Substances) is a computer based program used for the prediction of different types of biological activity for different substances. Prediction of this spectrum by PASS based on structural activity relationship analysis of the training set containing more than 250000 compounds exhibiting more than 3750 kinds of biological activities [14, 15, 16].

## Statistical analysis

Statistical analysis was performed according to standard approaches of the calculation of the statistical error (standard deviation) using the computer program Microsoft Excel 2007 [17].

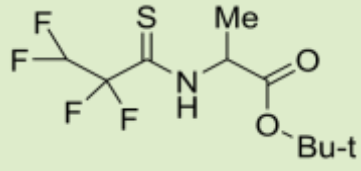
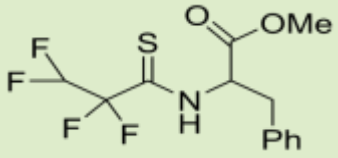
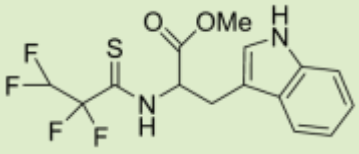
# Results and discussion

The activity analysis of the studied compounds that has been carried out *in silico* using PASS software helped to reveal similar characteristics, such as acrocylindropepsin inhibitor, chymosin inhibitor, saccharopepsin inhibitor, as well as different ones (Table 1). The potential activity of the studied compounds against bacterial, fungal and mammalian proteins shows that such direction of research exists now, but there is no research of antiviral activity.

Every derivative compound has an amino acid in its structure. Therefore, it can be assumed that it is the involvement of amino acids in these compounds that leads to appearance of the presented activities and their possible activity via proteins. The compound 10S-21 is also of interest since there is a high possibility that it is a substrate for the cytochrome c (CYP2H substrate).

ISSN 2347-6893
Volume 9 Number 3
Journal of Advances in Biology

**Table 1. Evaluation of cytotoxic concentration of the studied compounds**

| Substanses | Structure formula | Pa | Pi | Probable biological activity |
|---|---|---|---|---|
| 10S-20 |  | 0,763 | 0,029 | Acrocylindropepsin inhibitor |
| | | 0,763 | 0,029 | Chymosin inhibitor |
| | | 0,763 | 0,029 | Saccharopepsin inhibitor |
| | | 0,654 | 0,034 | Pro-opiomelanocortin converting enzyme inhibitor |
| 10S-21 |  | 0,810 | 0,018 | Polyporopepsin inhibitor |
| | | 0,729 | 0,037 | Acrocylindropepsin inhibitor |
| | | 0,729 | 0,037 | Chymosin inhibitor |
| | | 0,715 | 0,033 | CYP2H substrate |
| 10S-22 |  | 0,460 | 0,004 | ATP-binding cassette A1 stimulant |
| | | 0,454 | 0,004 | Anthranilate phosphoribosyltransferase inhibitor |
| | | 0,500 | 0,113 | Acrocylindropepsin inhibitor |
| | | 0,500 | 0,113 | Saccharopepsin inhibitor |

With a purpose of discovering new biological properties of methylenebisphosphonic acids we have studied the toxicity and antiviral activity of their modifications on different cell lines: CHO, Raji and B95-8. The results received show that the toxic effect on different cell lines differs significantly (Table 2).

**Table 2. Evaluation of cytotoxic concentration of the studied compounds**

| Name | chemical substances | The $CC_{50}$ in cell cultures, µg/ml | | |
|---|---|---|---|---|
| | | CHO | Raji | B95-8 |
| 10S-20 | $C_{10}H_{15}F_4NO_2S$ | 648 | 114 | 100 |
| 10S-21 | $C_{13}H_{13}F_4NO_2S$ | 442 | 85 | 10 |
| 10S-22 | $C_{15}H_{14}F_4N_2O_2S$ | 275 | 85 | 50 |

The study was conducted on cell lines: CHO – a model cell line which does not contain viral transforming agent; Raji and B95-8 – B-cell lines transformed by EBV. The use of several cell lines allowed receiving results of toxicity of the studied compounds which was 275 – 648 µg/ml. At the same time in B95-8 cell line, which produces virions of EBV, the toxic concentration was 10-100 µg/ml. In Raji cell line, in which an example of other type of viral latency can be observed and by which only some of viral proteins are expressed, the toxic concentration was 85 – 114 µg/ml. Since growth and proliferation of the lymphoblastic cells used in the study depends only upon the presence of virus, it is the influence of compounds that leads to elimination of virus and to cell death.

Due to such impact of the compounds on B95-8 cell line, which shows an example of lytic infection, this cell line was taken for further research of antiviral activity of bisphosphonic derivatives.

The antiviral activity of compounds was determined by qPCR using appropriate commercial kits. When comparing the data on antiviral activity of compounds in B95-8 cells it should be noticed that a more intense inhibition is observed when adding 10S-20 (Fig. 1).

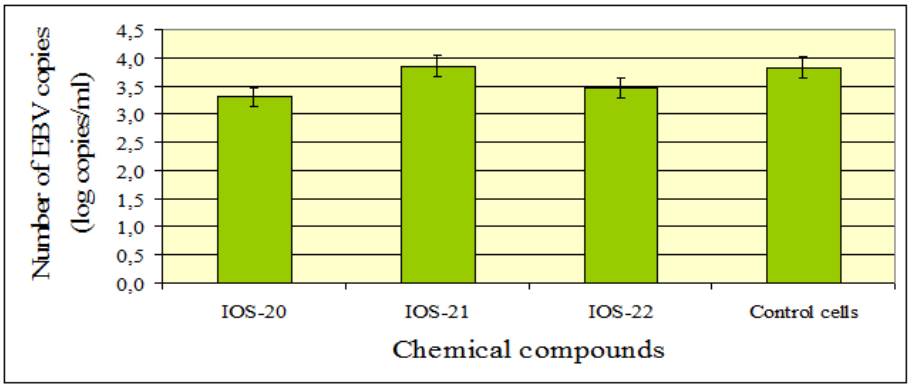



**Fig. 1. Inhibition of EBV replication after 48 hours of incubation (with compounds in concentration of 1 µg/ml).**

Fig. 1. Inhibition of EBV replication after 48 hours of incubation (with compounds in concentration of 1 µg/ml).

The concentrations that inhibited virus replication were within 1 – 10 µg/ml. The selectivity index of 10S-20 is 100, which shows the perspectives of its use against EBV.

One of the aspects of modern anticancer therapy is the search for apoptosis-stimulating compound and one of the ways to stimulate apoptosis is mitochondrial path (internal), through the release of cytochrome. The analyzed results provide reasons for further research of the compound 10S-21. Basing on data received from PASS prediction and high

level of cytotoxicity we used MTT assay to study the mitochondrial activity of B95-8 cell line under the influence of 10S-21 (Fig. 2).

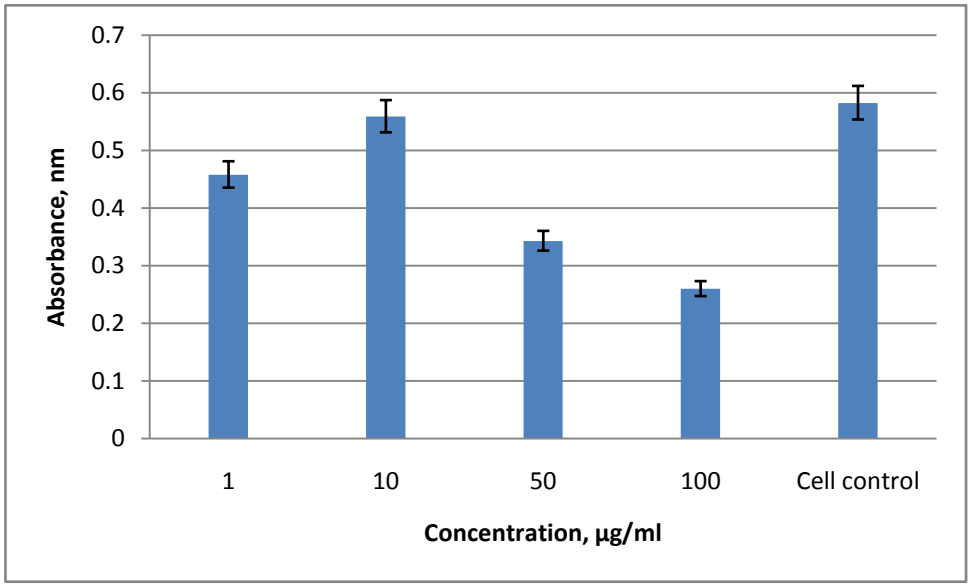



**Fig. 2.Absorbance of cell suspension under the influence of 10S-21 determined by MTT assay.**

High optical density indicates high mitochondrial activity of the cells in culture. An abrupt increase in absorbance after the addition of the compound 10S-21 in concentration 10 µg/ml may be caused by a considerable increase of mitochondrial activity. Since the 10 µg/ml concentration is near to $CC_{50}$ the activation of cell defense mechanisms may be possible as a result of the cytotoxicity increasing. After increasing the concentration we observed a dose-dependent decrease of mitochondrial activity which may be caused by the cytotoxic effect of the compound. Mitochondria provide the cells with energy and they are also responsible for one of the paths of apoptosis activation. This is why the results that show decrease of mitochondrial activity after the addition of 10S-21, the high level of the compound's cytotoxicity and the prediction from PASS help to study the apoptosis-stimulating activity of this compound.

One of the special characteristics of herpesviruses, including Epstein-Barr virus, is their lifelong persistence in host's organism. To date there are no drugs that would affect the latent virus [18]. The search for new effective drugs is topical

ISSN 2347-6893
Volume 9 Number 3
Journal of Advances in Biology

since EBV is the cause of some lymphoproliferative diseases [19]. The research of induction of lytic infection by drugs that have apoptosis-stimulating effect is one of the possible ways to treat diseases caused by EBV [20].

The analysis of the compound 10S-21 for the apoptosis-stimulating effect was conducted since it has an antiviral effect and a significant influence on the growth of transformed cells.

Using flow cytometry the condition of the B95-8 cells was evaluated. The cells were stained with fluorophore propidium iodide and analyzed by flow cytometry. This method allows to study the condition of cell population (apoptotic, necrotic and dividing cells). The study was conducted in dynamics on 48$^{th}$ and 72$^{nd}$ hour and the compound was added in two concentrations (10 and 100 µg/ml).

We have established the percentage of apoptotic cells (Table 3). The results received show that the compound 10S-2 has an apoptosis-stimulating effect. It was also shown that on 48$^{th}$ hour after the addition of the compound 10S-21 in concentration 10 µg/ml the percentage of apoptotic cells has exceeded the control and was 35,7%, while on 72$^{nd}$ hour it was 40%.

After the addition of common inducers of viral replication (TPA and Na-butyrate) [21] the percentage of apoptotic cells increased in two times, up to 79.24% (Table 4).

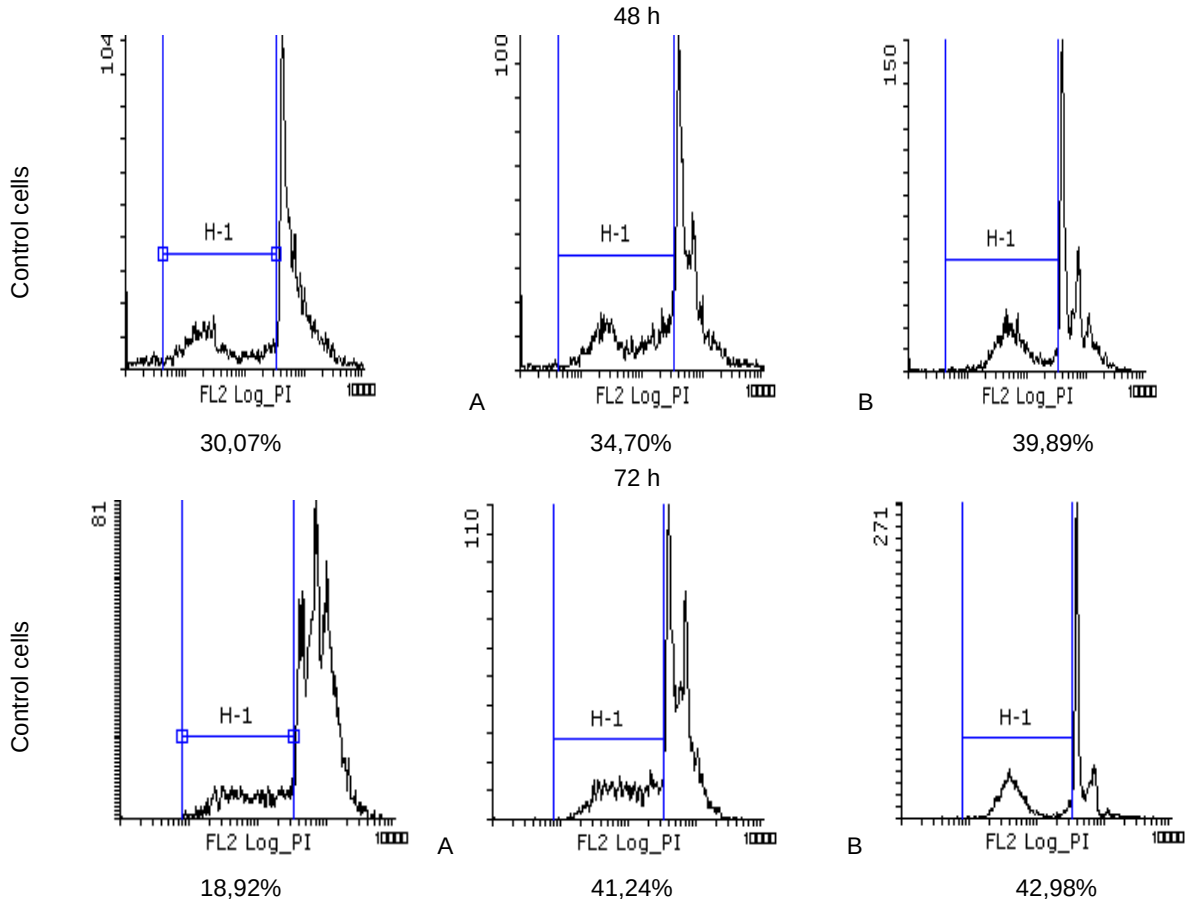



**Fig. 3: Analysis of apoptosis by hypotonic PI staining method. In this experiment B95-8 cells were cultured in the presence of chemical compound 10S-21 in concentration 10 (A) and 100 µg/ml (B) for 48 h and 72 h to induce apoptosis. The H-1 region encompasses the apoptosis cells.**

The histograms of induced cells and non-induced ones differ significantly. For the non-induced cell culture the peak of apoptotic cells are much lower that for the induced ones. In the cell control we observed a considerable increase in the number of apoptotic cells on 48 hour and decrease on 72 hour.

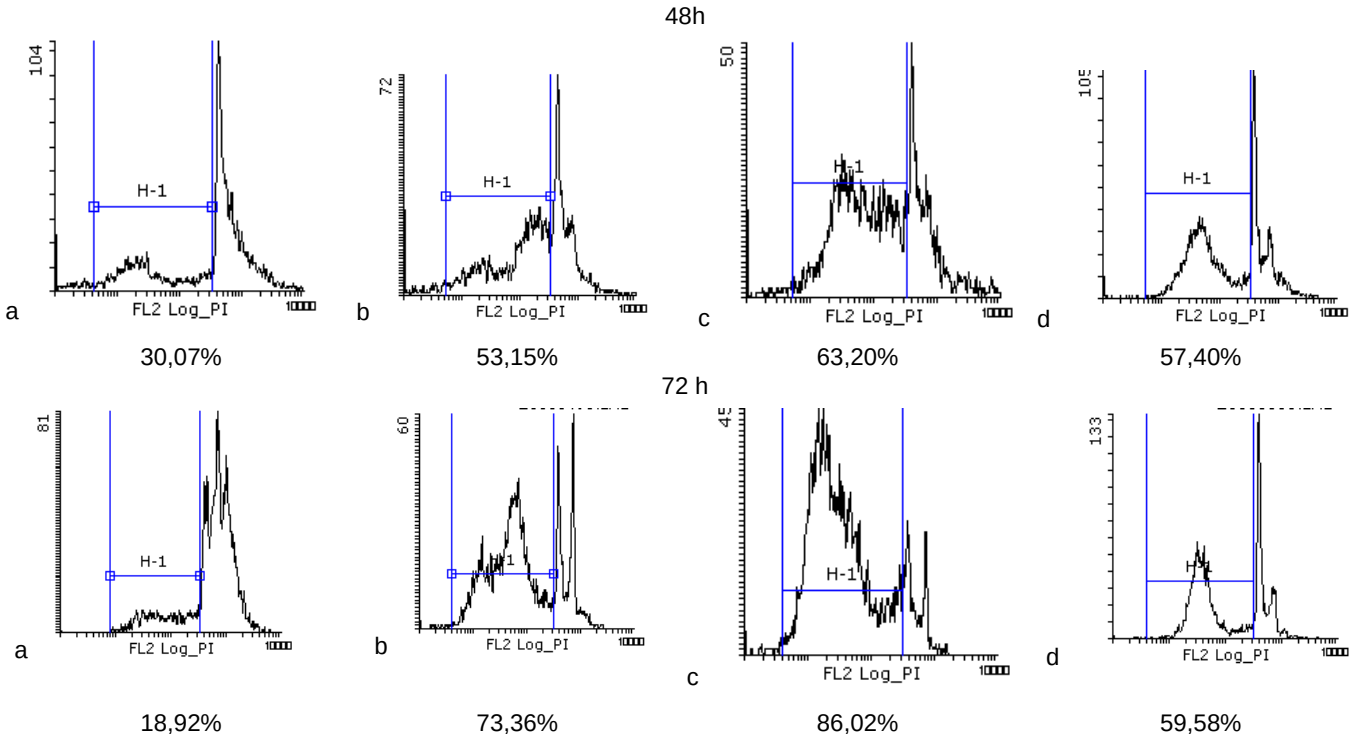



**Fig. 4: In this experiment B95-8 cells were induced apoptosis by treatment with inducers of viral replication (TPA and n-butyrate) and chemical compound 10S-21 to induce apoptosis. Untreated control B95-8 cells (a), B95-8 cells treated with inducers (b), B95-8 cells treated with 10S-21 10 µg/ml (c) and 100 µg/ml (d) for 48 h and 72 h. The H-1 region encompasses the apoptosis cells.**

The obtained and analyzed data allow to classify the compound 10S-21 as a perspective apoptosis-stimulating compound. Although the phenomenon of induction of the lytic infection by addition of apoptosis-stimulating compounds is not well-studied yet, it is of interest for further research.

The given ethers contain amino acids in their structure that make up the proteins which are necessary for every creature on our planet. It is the chemical structure and the presence of amino acid in the molecule that may cause such biological properties. The presence of fluorine atoms allows the compounds to effectively penetrate the cell membrane [22, 23] and due to amino acids to integrate into proteins. The substitution of the amino acid in the active site may cause changes in the structure and in the protein functions respectively.

Modern antiherpetic drugs are aimed at blocking/inhibiting the replication of viral DNA. Since these drugs are nucleoside analogues they integrate into the DNA and block further synthesis, whereas ether derivatives, in particular the compound 10S-20, due to Ala in their structure possibly integrate into proteins and disrupt their structure and functions. In this way the effect of the compound 10S-20 may be based exactly on interaction between viral proteins and the compound and on blockage of certain stages of EBV reproduction.

The compound 10S-21 (phenylalanine methyl ester) displayed a significant apoptosis-stimulating effect as well. Since, by the prediction of PASS, the compound may be a substrate for cytochrome, and the *in vitro* studies have shown considerable inhibition of mitochondrial activity (MTT assay) as well as increase in the percentage of the apoptotic cells (flow cytometry). It must also be mentioned that phenylalanine is an essential amino acid which is not synthesized in human body and is often included in active site of proteins. The analyzed data suggest that it is phenylalanine that, by integrating into the protein, causes the protein to lose its functions and leads to the launch of apoptosis in the cell.

This class of ester derivatives of amino acids is promising for further research and study of antiviral mechanisms and apoptosis-stimulating activity.

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DOI : 10.24297/jab.v9i3.634