

UDC 578.825.:578.222

DOI: 10.15587/2519-8025.2018.124689

ANTIVIRAL AND APOPTOSIS MODULATING POTENTIAL OF FLUORINATED DERIVATIVES OF URACIL

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*Першим вірусом класифікованим, як онкологічний був вірус Епітейна-Барр. Метою роботи було дослідити біологічні властивості фторованих похідних на основі урацилу. Згідно прогнозування PASS, сполуки G26 та G27 можуть мати противірусну активність. Анти-ВЕБ активність *in vitro* була показана для обох досліджуваних сполук. Таким чином, похідні урацилу є перспективними для створення нових анти-вірусних агентів*

Ключові слова: вірус Епітейна-Барр, фторовані аналоги нуклеозидів, апоптоз, *in silico* та *in vitro*

1. Introduction

Epstein-Barr virus (EBV) is a member of the herpesvirus family. As with other herpesviruses, EBV is an enveloped virus that contains a DNA core surrounded by an icosahedral nucleocapsid and a tegument [1, 2]. Although herpesviruses are ubiquitous in nature, humans serve as the only natural host for EBV. It is now known that EBV infects >90 % of the adult human population [3]. Upon infection, the individual remains a lifelong carrier of the virus [4].

Approximately 2 million cases of cancer each year are caused by infectious agents [5]. EBV was the first tumor virus identified from cultured lymphoblasts of Burkitt's lymphomas and its potential role as a causative agent of EBV-associated tumors has been the important subject of such investigation approximately for the last 40 years. EBV is classically associated with infectious mononucleosis (IM), Burkitt's lymphoma [6, 7]. The role of EBV in the pathogenesis of epithelial tumors – namely, undifferentiated nasopharyngeal and gastric carcinoma – remains unclear but is thought to result from the aberrant establishment of latent viral infection [1]. The range of EBV-associated diseases has included oral hairy leukoplakia from AIDS patients, Hodgkin's disease, T-cell lymphoma, lymphoproliferative diseases (LPD) of primary and secondary immunodeficiency and lymphoepithelioma-like carcinoma of the stomach, thymus, lung and salivary gland [8].

In the past few decades, human malignancies associated with Epstein-Barr virus (EBV), including a variety of malignant lymphomas and carcinomas, have been well documented. According to the World Health Organization (WHO) classification of lymphoid tumors, disease entities, such as endemic Burkitt lymphoma, lymphomatoid granulomatosis, reveal a high prevalence of EBV positivity [9].

2. Literature review

In the past decades, computational techniques like virtual screening have proven to be of greater use to make the drug development process faster and less expensive. The most commonly targeted protein in herpesviruses is the thymidine kinase; other targets like viral helicases, proteases or the protein-protein interfaces of replication proteins are also being investigated [10].

The importance of nucleoside drugs for clinical use and the clear-cut molecular mechanisms underlying

their biological activities alongside the increasing concern about drug resistance encountered with antiviral and anticancer chemotherapy has led to an intensified search by bio-organic and medicinal chemists for novel nucleoside analogs as more efficacious drug candidates [11]. The use of unnatural heterocycles as nucleobases in the design of novel nucleoside analogs not only enhances *in vivo* stability but also confers novel biologically interesting activities [12]. Analogs of nucleoside have been playing a major role in treating tumor and virus either as selective inhibitors of certain obligatory enzymes for cancer or viral replication or as nucleic acid chain terminators which interrupt the replication of cancer cells or a virus. They are chemically modified analogs of natural nucleosides, nucleotides, and bases, which are endogenous metabolites involved in many essential cellular processes, such as DNA and RNA synthesis, and purinergic signaling [13]. The selective introduction of a fluoro group into biologically active molecules has received much attention by medicinal chemists. Fluorinated nucleosides exhibit a wide variety of biological activity and have been used extensively as anti-tumor and antiviral agents [14]. In many cases, the stability of the nucleoside analog, particularly the stability of the glycosyl bond, is an important factor determining the biological activity as well as the therapeutic usefulness of nucleosides as a drug candidate. Fluorine substitution also has a favorable effect of increasing the metabolic stability. These findings suggest the importance of a fluorine moiety in the nucleoside as therapeutic agents. Thymidylate synthase, ribonucleotide diphosphate reductase (RDPR) and viral polymerases are the major targets by fluoronucleosides and fluoroheterocyclic bases [15].

3. Aim and objectives of the study

Aim – Analyze the potential antiviral and apoptosis modulating activity of fluorinated derivatives of uracil by using *in silico* and *in vitro* methods.

To achieve this aim, it was necessary to solve the following problems:

1. Identify potential biological activity and possible targets of fluorinated derivatives of uracil by using the PASS software and PharmMapper online tools.

2. Study potential antiviral and apoptosis modulating the activity of the new compounds using *in vitro* methods.

4. Material 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. Raji, B95-8 cell lines obtained from the Bank of Cell Cultures of the Institute of Virology of the Russian Academy of Medical Sciences. 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) ("Sigma", USA) in tissue culture flasks. Cultivation was performed at 37 °C in a 5 % CO₂ atmosphere.

Chemical substances. Derivates of fluorinated analogs of 5-(p-tolilsulfonil)-6(polyfluoroalkyl)uracil (compounds G26 and G27) were synthesized at the Institute of Organic Chemistry of the National Academy of Sciences of Ukraine. As reference drug was used ganciclovir (Cymeven, Roche, Switzerland). 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 the culture medium.

Neutral Red Uptake assay (NRU). The NRU method was used as previously described [16]. After the neutral red had dissolved, the absorbance of the plate was determined spectrophotometrically at 538 nm on a Multiskan FC universal microplate reader (Thermo Scientific, USA).

Trypan Blue Exclusion Test of Cell Viability. The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude trypan blue, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. The trypan blue exclusion test was used as previously described [17].

MTT assay. The MTT staining method as described by Mosmann was used with minor modifications [16]. The absorbance was determined at 538 nm on a Multiskan FC universal microplate reader (Thermo Scientific, USA).

Real-time PCR detection of viral DNA. A real-time PCR, the assay was performed to assess the antiviral activity of various drugs against EBV [18]. For detection of antiviral activity of studied compounds, Raji cells were infected with EBV 0,3 ml/cell. DNA isolation of virus from samples conducted using «innuPREP Virus DNA Kit» («Analytik Jena AC», Germany). DNA concentration was measured by Biophotometer («Eppendorf», Germany). To detect DNA EBV was using a set «Amplisens®EBV-FL» («Amplisens», Russian) according to manufacturer's recommendations for detection in real time (qTOWER 2.2., Germany).

Analysis apoptosis by flow cytometry. Flow cytometry was performed to quantitatively detect the apoptotic cells processed by a hypotonic solution of propidium iodide (50 µg/ml, Sigma C113A) [19]. The sub-G1 peak was measured with a flow cytometer Beckman Coulter Epics XL (USA) and analyzed using Flowing Software, version 2,5 (USA).

Evaluation of nuclear condensation by Hoechst 33342 staining. Cells were seeded at a concentration of 5×10^5 cells/ml in a 24-well plate and treated with different concentrations of compounds. After incubation, the cells were collected by centrifugation and suspended in 1 ml of phosphate buffer solution (PBS, pH-7.4). PBS was then removed by centrifugation, and the cells were incubated for 30 min at 37°C with 100µl of the DNA-specific dye Hoechst 33342 (0.05mg/ml). After incubation, the cells were suspended in 1 ml of PBS. PBS was then removed by centrifugation. Cells were suspended in 50 % glycerin ("Sigma", USA) with 4 % paraformaldehyde ("Sigma", USA). Morphological changes were observed under Ulab LW300TF fluorescent microscope x100 (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. PASS works on the basis of structural activity relationship (SAR) analysis. The proposed web server is freely available at <http://www.pharmaexpert.ru/pas-online/> [20].

PharmMapper. The PharmMapper online tool is a web server for potential drug target identification by reversed pharmacophore matching the query compound against an in-house pharmacophore model database. The PharmMapper web server is freely available at <http://lilab.ecust.edu.cn/pharmmapper/> [21].

Statistical analysis. Statistical analysis was performed according to standard approaches (parametric criteria) using the computer program Microsoft Excel 2010 [22]. Data are presented as means ± standard deviation (SD). All studies were conducted in three replies. The difference between the mean values was estimated according to the t-criterion and was considered to be valid at $p < 0.05$. The selective index (SI), a marker of antiviral activity, was determined as the ratio of CC₅₀ to EC₅₀.

5. Result and discussion

With the view of finding the specific activity of these compounds, they were exploited for prediction of activity, using PASS. The predicted activity spectrum of a compound is estimated as Pa (probably activity) and Pi (probable inactivity). Accordingly, the activities of compounds taken into consideration for the possibility of particular pharmacological activity are the ones showing more Pa value than Pi. Two compounds exhibited a number of biological activities as in Table 1.

In the present study, PASS predicted that the antiviral (poxvirus, picornavirus, herpesvirus), nucleotide metabolite regulator, DNA polymerase I inhibitor, antineoplastic (non-Hodgkin's lymphoma), CDK9/cyclin T1 inhibitor activity were expressed by compound G26. The TP53 expression enhancer, DNA polymerase I inhibitor, thymidylate 5'-phosphatase inhibitor, antiviral (poxvirus, herpesvirus), antineoplastic (non-Hodgkin's lymphoma, carcinoma), RNA directed DNA polymerase inhibitor was expressed by compound G27.

Table 1

Predicted biological activity for compounds using PASS			
Compound	Pa	Pi	Possible activity
	0,649	0,012	Antiviral (Poxvirus)
	0,599	0,019	Nucleotide metabolism regulator
	0,550	0,032	Antiviral (Picornavirus)
	0,495	0,011	Antineoplastic antimetabolite
	0,478	0,010	DNA polymerase I inhibitor
	0,464	0,055	Antineoplastic (non-Hodgkin's lymphoma)
	0,445	0,019	Antiviral (Herpes)
	0,394	0,107	Antineoplastic
	0,758	0,016	TP53 expression enhancer
	0,458	0,013	Antineoplastic antimetabolite
	0,449	0,013	DNA polymerase I inhibitor
	0,448	0,036	Thymidylate 5'-phosphatase inhibitor
	0,425	0,026	Antiviral (Poxvirus)
	0,412	0,097	Antineoplastic (non-Hodgkin's lymphoma)
	0,400	0,018	RNA directed DNA polymerase inhibitor
	0,397	0,047	CDK9/cyclin T1 inhibitor
	0,372	0,049	Antiviral (Herpes)

In silico methods allows for screening of a large number of compounds. Any predicted property must be confirmed or disproved in the biological model. Accordingly, *in vitro* analysis of these compounds was carried out. The cytotoxic effect of compound G26 and G27 were analyzed by trypan blue; MTT and NRU assay (Fig. 1). Samples for analysis were taken after 48h incubation with a treatment of studied compounds.

The cytotoxicity test of two compounds showed CC_{50} value ranged from 206 $\mu\text{g/ml}$ to 1316 $\mu\text{g/ml}$ (Table 2). These compounds G26 and G27 are considered to be non-cytotoxic at Raji cell line, as the value ranged from 502 $\mu\text{g/ml}$ to 1316 $\mu\text{g/ml}$. Compound G26 is considered to be more cytotoxic at B95-8 cell line, as the value ranged from 206 $\mu\text{g/ml}$. B95-8 is B-lymphocyte cell culture, which transformed by EBV and chronically producing viruses. Thus, a higher level of cytotoxic of studied compounds may be explained by producing viruses and the effect of the compounds on the reproduction of viruses in cells.

Thus, compounds are an important value for use in the antiviral study due to their low toxic effect. Thus, mechanism of action may be considered at blocking replication of EBV. Results from antiviral screening showed that both fluorinated analogs of the uracil exert good activity, as shown in Table 3. In particular, compound G26 and G27 showed a high level of antiviral activity for Raji and B95-8 cell line.

According to PASS prediction, it may block the synthesis of DNA as an inhibitor of the polymerase. Also, this compounds may play an important role in blocking enzymes necessary for transcription and replication of virus DNA, such as CDK9/cyclin T1 and

thymidylate 5'-phosphatase. Moreover, SI is used to estimate the therapeutic effect of a drug and to identify drug candidates for further studies. In this study, both compounds could be considered as promising new anti-EBV drug candidates for lytic infection of EBV.

To further understand the mechanism of action of the synthesized compounds, molecular modeling and docking studies of G26 and G27 were performed using web-server PhrmMapper (Table 4).

It is well known that analog nucleosides exert its antiviral activity by binding to DNA and blocking their synthesis. But it's important to find out what target use this compounds. Because for more and more viruses become resistant to analog nucleosides. PhrmMapper is a web server for potential drug target identification based on the use of a pharmacophore mapping approach. PhrmMapper server works by 'probing' the ligand into a database of pharmacophore models of binding sites. It functions on the ligand-protein reverse docking strategy and reports a potential target on the basis of the normalized fit score. It was established, that majority of the targets are enzymes that participate in the synthesis of nucleic acids and apoptotic proteins. It was shown that compound G27 could be an inhibitor of the synthesis of nucleic acids by interacting with DNA topoisomerase 2, DNA primase/helicase and other. Compound G26 could inhibit the synthesis of DNA or RNA by interacting with thymidylate synthase and DNA mismatch repair protein mutL.

Also, the list of targets presents proteins, included at apoptotic cascade (Table 4, Fig. 2). It is important, because EBV is associated with cancer, so induction of apoptosis might be one of the ways of treatment of disorders.

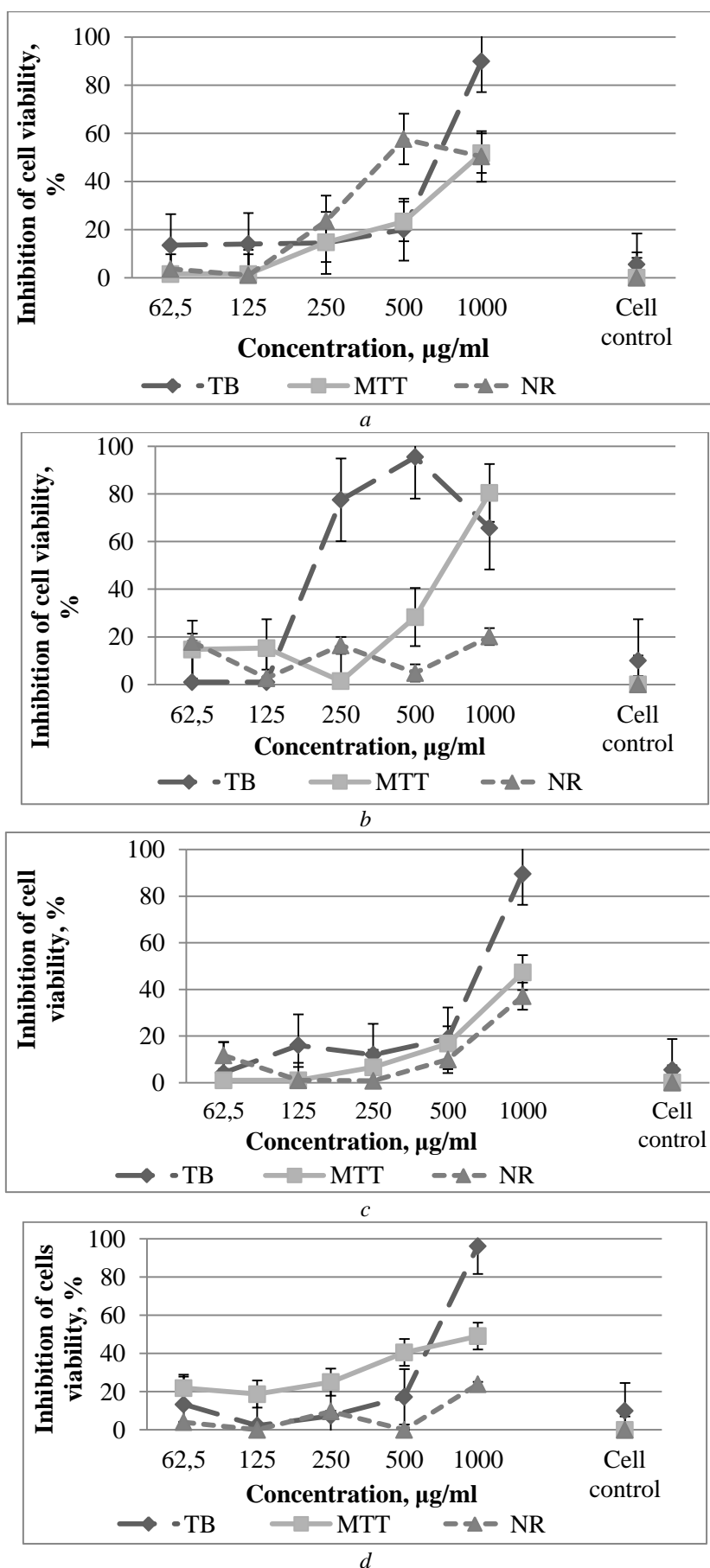


Fig. 1. The cytotoxicity of fluorinated analogs of uracil on model lymphoblastoid cell line: a – the cytotoxic effect of compound G26 on Raji cells; b – the level of cytotoxicity of compound G26 on B95-8 cell line; c – the cytotoxic effect of compound G27 on Raji cells; d – the level of cytotoxicity of compound G27 on B95-8 cell line, $p < 0.05$ in comparison with untreated cells in all cases

Table 2

Substances	The cytotoxicity of the studied compounds on the CC ₅₀ index (µg/ml)					
	Raji			B95-8		
	Methods					
	Trypan blue	MTT assay	Neutral red assay	Trypan blue	MTT assay	Neutral red assay
G26	1250	952	544	262	317	1081
G27	1316	1038	952	520	206	1192
GCV	962	560	502	833	1020	1042

Table 3

Compounds	CC ₅₀ ^a	Antiviral activity fluorinated derivatives of uracil on EBV and EC ₅₀				
		Raji		CC ₅₀	B95-8	
		Antiviral activity (EC ₅₀ ^b)	Selectivity index (SI ^c)		Antiviral activity (EC ₅₀)	Selectivity index (SI)
G26	952	50	19	317	75	4
G27	1038	100	10	206	65	3
GCV	560	5	112	1020	10	102

Note: ^a – The 50 % cytotoxic concentration for Raji and B95-8 cell in µg/ml; ^b – Concentration of compound (µg/ml) producing 50 % inhibition of EBV reproduction; ^c – Selectivity index (SI) = CC₅₀/ EC

Table 4

Compound G26		Compound G27	
Target name	Fit score	Target name	Fit score
Thymidylate synthase	5.351	DNA topoisomerase 2	3.837
DNA mismatch repair protein mutL	4.372	Mitogen-activated protein kinase 14	3.827
Gag-Pol protein	4.304	DNA primase/helicase	3.789
Serine/threonineprotein kinase	4.2	Tyrosine-protein kinase	3.722
Neuraminidase	4.2	Gag-Pol polyprotein	3.711
DNA topoisomerase 6 sub. B	4.086	Deoxyribonucleotidase, mitochondrial	3.696
Death-associated protein kinase	4.084	Thymidine kinase	3.662
Nucleoside diphosphate kinase, cytosolic	4.078	Apoptotic protease-activating factor	3.656

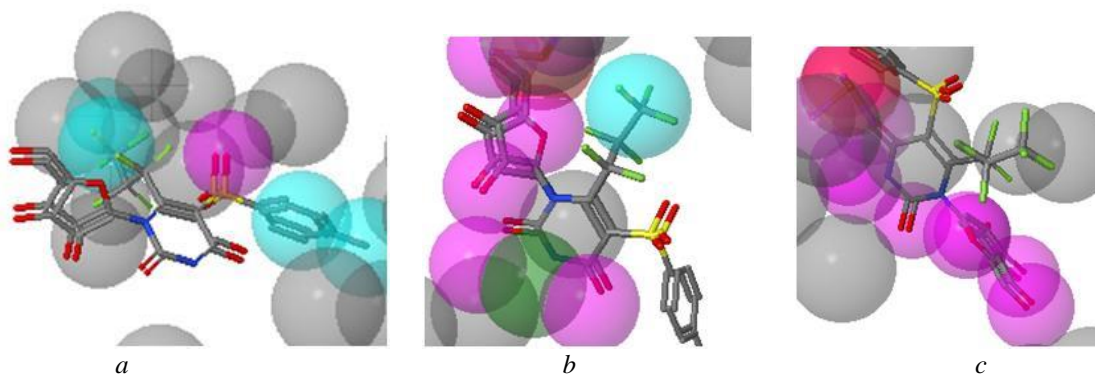
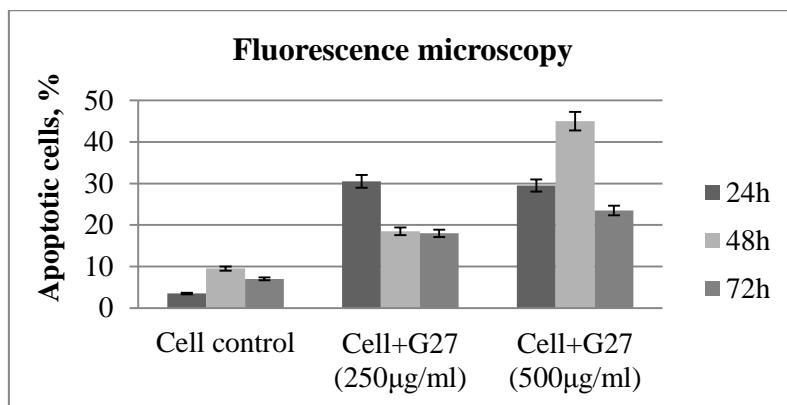


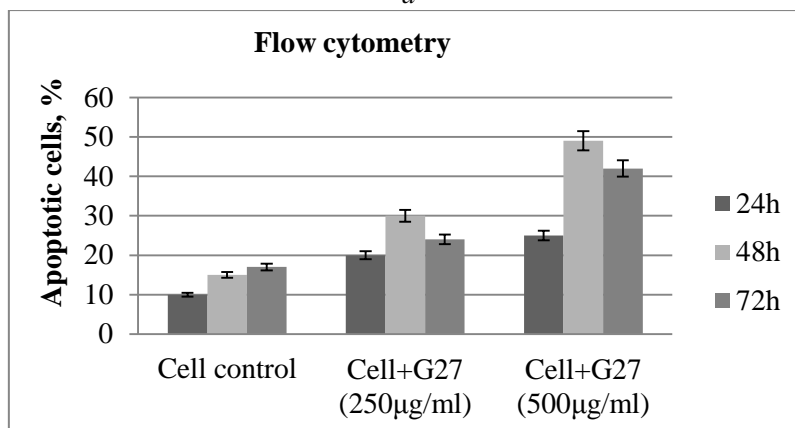
Fig. 2. The pharmacophore models of three targets for compound G27: *a* – Mitogen-activated protein kinase 14; *b* – 5(3)-deoxyribose nucleotidase, mitochondrial; *c* – Apoptotic protease-activating factor 1

EBV persists for life in low number metabolically quiescent memory-B-cells as a methylated and transcriptionally silenced nuclear dsDNA episome, expressing only non-coding small RNAs and several latent proteins and being invisible to immune responses. Also, EBV has been thought to counter apoptosis during latency indirectly through LMP1-mediated activation of NF- κ B-regulated genes. The Bcl-2 homologs encoded by the EBV BHRF1 and BALF1 ORFs are expressed only during the viral lytic cycle, presumably to prevent premature cell death during active EBV replication [23]. New strategies are emerging for the virus-targeted therapy to

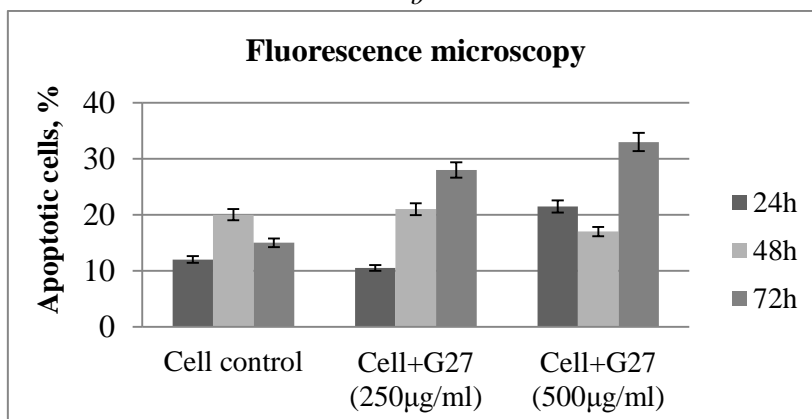
treat EBV-associated malignancies, generally consisting of two components. The first step is aimed at initiating virus reactivation and triggering recognition of the tumor cells by the immune system. The second step is the administration of an antiviral drug which can be metabolized exclusively in cells expressing viral lytic cycle proteins [24]. Another way it is the elimination of tumor cells by apoptosis-inducing by different chemical or nature compounds. For studies of induction of apoptosis by derivatives of uracil, flow cytometry and fluorescence microscopy were used (staining by Hoechst 33342) (Fig. 3).



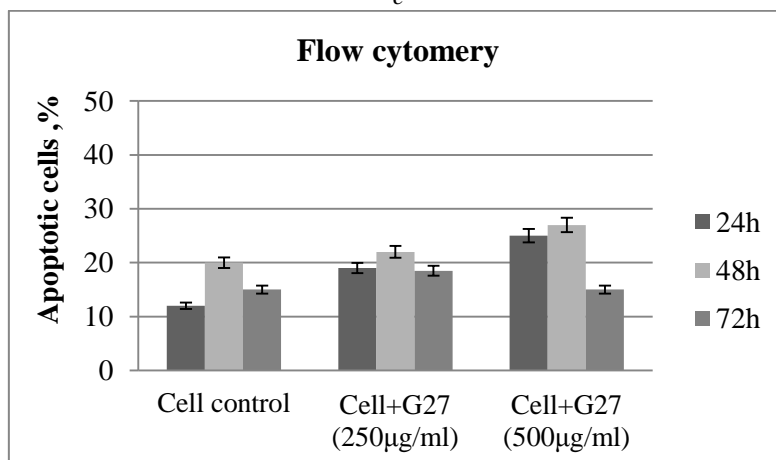
a



b



c



d

Fig. 3. Detection of apoptotic cell in lymphoblastoid cell line treated with compound G27: a – the apoptosis modulation effect on Raji cells; b – the percentage of apoptosis cells on B95-8 cell line; c – the apoptosis modulation effect on Raji cells; d – the percentage of apoptosis cells on B95-8 cell line, p<0.05 in comparison with untreated cells in all cases

The action of compound G27 has been studied at 500 µg/ml and 250 µg/ml concentrations for 24, 48 and 72 h. In both concentrations of 500 and 250 µg/ml the compound induced apoptosis of the non-infected transformed Raji and B95-8 cells. It was a significant increase in the percentage of apoptotic cells by 48 hours for Raji at 500 µg/ml concentration. It was established that the percentage of apoptotic cells exceeds control values and was 49 %, and it was 40 % after 72 h. It was showed less percentage of the apoptotic cell on B95-8 cell line. This effect may be due to the ability of the virus to block the various stages of the apoptotic cascade.

Induction of apoptosis might be caused by different targets, included in apoptosis cascades, such as apoptotic protease-activating factor or MAP kinase. So it can be assumed that the test compound possesses the antiviral activity against both forms of EBV infection and the destruction of cells infected with virus occurs through apoptosis.

EBV is gammaherpesvirus that is ubiquitous in humans, infecting more than 90 % of the world population [4, 8]. To date, there are no available drugs to target latent EBV, and the existing broad-spectrum antiviral drugs are mainly active against lytic viral infection. Thus, using computational molecular docking and *in vitro* methods, a new class of fluorinated nucleoside analogs of uracil was studied. Fluorinated nucleosides exhibit a wide variety of biological activity and have been used extensively as anti-tumor and antiviral agents. Viral polymerase is one of the most common and established targets of nucleoside and the presence of fluoro group makes it unique in terms of their chemical, biological and structural properties [15]. Our research showed that new derivatives of uracil with fluoro group have antiviral activity and might be affected at other enzymes of

replication, such as DNA topoisomerase 2, DNA primase/helicase. Also, it was established that fluorinated derivatives of uracil (compound G26 and G27) could induce apoptosis in EBV-transformed cell line Raji and B95-8. According to the literary data, nucleoside analogs mimic natural nucleosides in term of uptake and metabolism [15, 24]. Further, they can incorporate into newly synthesized DNA resulting in chain termination. Some of these drugs also inhibit key enzymes involved in the biosynthesis of purine and pyrimidine nucleotides and RNA synthesis as well as directly activate the apoptosis as an anti-cancer agent [15]. Also according to the prediction of targets these compounds may interact with different apoptotic proteins such as apoptotic protease-activating factor, MAP kinase, deoxyribonucleotidase (mitochondrial) and some classes of protein kinase.

6. Conclusion

1. This study indicates that the *in silico* virtual screening of different compounds may be of added value in the identification of new antiviral molecules. According to PASS prediction, all studied compounds might have antiviral and antineoplastic activity. There were also identified several classes of different proteins, which play an important role in the cell cycle and might be a potential target for the action of studied derivatives.

2. In vitro studies of fluorinated uracil derivatives showed their high antiviral activity against EBV in Raji cell culture (an acute form of infection). Selectivity indices were 19 (compound G26) and 10 (compound G27). It was established that only compound G27 had induced apoptosis at cell line Raji and B95-8 (B-lymphomas). Thus, the results of this study open new directions in the study of this class compounds.

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Дата надходження рукопису 16.01.2018

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