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ANALYSIS OF NEW POLYFLUOROTHIOACYLATED AMINO ACIDS DERIVATIVES BY *IN SILICO* AND *IN VITRO* METHODS

Aim. *The purpose of this work was to analyze the potential biological activity and the target of action of esters of tetrafluoropropanethioacylated amino acids derivatives and to investigate the obtained results in vitro.* ***Materials and methods.*** *PASS software, web-server PharmMapper, PCR, MTT assay, trypan blue and neutral red assay were used.* ***Results.*** *According to PASS prediction, two compounds (10S20 and 10S21) may possess antiviral activity, Pa/ Pi was 0,294/0,005 and 0,214/0,084, respectively. Also, all compounds may possess a cytochrome c as the substrates that might play an important role in the induction of apoptosis. Several targets were identified by using molecular docking (PharmMapper). It was shown that a lot of possible targets are proteins, such as Gag-Pol protein (viral protein) and different kinds of protein kinases. Study in vitro showed that all compounds inhibited the replication of the Epstein-Barr virus. On the other hand, esters of fluorinated aminoacids have high levels of cytotoxicity in various lymphoblastoid cell lines, which may be due to the mechanism of action of the compounds.* ***Conclusions.*** *Our results let to relate the compound tert-butyl (2,2,3,3-tetrafluor opropanethioyl)alaniate to a perspective anti-EBV agent. Analyzed data showed, that polyfluoroalkanethioacylated amino acids derivatives (10S21 and 10S22) may possess apoptosis modulating properties.*

Key words: Epstein-Barr virus, tetrafluoropropanethioacylated amino acid esters, in silico, in vitro.

Epstein-Barr virus (EBV) infection is the most common and persistent virus infection in humans, with approximately 95% of the world‘s population sustaining an asymptomatic life-long infection. EBV was the first detected human tumor virus. It is estimated that EBV causes more than 200,000 cases of cancer annually and 1.8% of all cancer deaths are due to EBV-induced malignancies [14, 6].

Computer-aided drug design approaches have emerged as attractive and

complementary approaches to traditional high throughput screening [5]. Virtual screening has been applied to the successful identifications of biologically active molecules.

Fluorine is often included to drug molecules as even a single atom can greatly change the chemical properties of the molecule in desirable ways. Of all commercialized pharmaceutical drugs, twenty percent contain fluorine, including important drugs from different pharmacological classes [11]. Adding of fluorine atom(s) into a compound result in increasing of hydrophilicity of the whole molecule and thus might help to penetrate through the cell membrane [8]. The use of fluorinated aminoacids to modulate peptide and protein structure has become more prevalent in recent years. The replacement of hydrophobic amino acid residues with polyfluorothioacylated amino acids has provided peptides with clear differences to the native peptide in terms of structure and activity.

The purpose of this work was to analyze the potential biological activity and the target of action of derivatives of esters of tetrafluoropropanethioyl amino acids by using in silico methods and examined received results by in vitro study. Therefore, in a first step, we used the PASS software and PharmMapper online tool by which we could identify potential biological activity and possible targets of polyfluoroalkanethioacylated amino acids esters. In a second step, we used in vitro methods for study cytotoxicity and antiviral potential study of the new compounds.

Materials and methods

Cell cultures. Raji cells are a human Burkitt's lymphoma-derived cell line, containing the latent form of EBV cycle. B95-8 cells are a lymphoblastic cell line, transformed by EBV and chronically producing virus. Raji and B95-8 cell lines were obtained from the Bank of Cell Cultures of the Institute of Virology of the RAMS (Russia). All cell lines were grown in the culture medium containing 90% of RPMI 1640 with L-glutamine ("Sigma", USA), 10% fetal bovine serum (FBS, "Sigma", USA) and gentamicin (100 μg/ml) ("Sigma", USA). Cultivation was performed at 37 ^{o}C in a 5% CO_2 atmosphere. For detection of antiviral activity of studied compounds, Raji cells were infected with EBV.

Chemical substances. Amino acids derivatives: tert-butyl (2,2,3,3-tetra fluoropropanethioyl)alaninate (10S-20), methyl (2,2,3,3-tetrafluoropropaneth ioyl)phenylalaninate (10S-21) and methyl (2,2,3,3-tetrafluoropropanethioyl) tryptophanate (10S-22) were synthesized at the Institute of Organic Chemistry of the National Academy of Sciences of Ukraine [8]. The substances were dissolved in DMSO (dimethyl sulfoxide) and filtered through a filter with a pore diameter of 0.22 microns (Sarstedt, USA). Working solutions were prepared to the culture medium.

The neutral red uptake assay (NRU) method was used as previously described [3]. 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. The trypan blue (0,4 %) exclusion test was used as previously described [10].

MTT assay. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide) staining method as described by Mosmann [7] was used with minor modifications. The absorbance was determined at 538 nm on a Multiskan FC universal microplate reader (Thermo Scientific, USA) [12].

A real-time PCR (polymerase chain reaction). The assay was performed to assess the antiviral activity of various drugs against EBV [9]. DNA of virus from the samples was isolated using «innuPREP Virus DNA Kit» (Analityk Jena AC, Germany). DNA concentration was measured by Biophotometer ("Eppendorf", Germany). To detect DNA EBV in real time was using a set «Amplisens®EBV-FL» (FGYN CNIIE, Russia) according to manufacturer's recommendations (qTOWER 2.2., Germany).

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. The proposed web server is freely available at http:// www.pharmaexpert.ru/passonline/. PASS works on the basis of structural activity relationship (SAR) analysis [2, 4]. 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 proposed web server is freely available at http://lilab.ecust.edu.cn/pharmmapper/ [13]. Statistical analysis was performed according to standard approaches of the calculation of the statistical error (standard deviation) using the computer program Microsoft Excel 2010 [1].

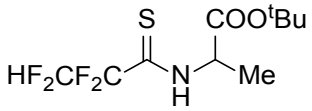
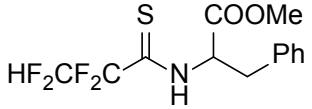
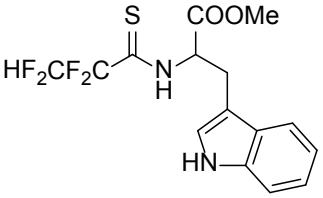
Result and discussion

With the view of finding the specific activity of these compounds, they were exploited for prediction of activity, using PASS (Prediction of Activity Spectra for Substances). The predicted activity spectrum of a compound is estimated as Pa (probably activity) and Pi (probable inactivity).

In the present study, PASS predicted that the antiviral activity was inherent in the compound 10s20 and 10s21, Pa/ Pi was 0,294/0,005 and 0,214/0,084, respectively (table 1).

Table 1

Predicted biological activity for compounds using PASS

	Substance	Pa	Pi	Biological activity
10S20		0,485	0,134	CYP2H substrate
		0,308	0,005	Histone deacetylase SIRT1 inhibitor
		0,294	0,005	Antiviral *(Picornaviruses)*
10S21		0,715	0,033	CYP2H substrate
		0,341	0,057	Atherosclerosis treatment
		0,214	0,084	Antiviral *(Hepadnaviruses)*
10S22		0,397	0,219	CYP2H substrate
		0,234	0,012	Histone deacetylase SIRT1 inhibitor

List of activity included antiviral activity against Picornaviruses and Hepadnaviruses (compound 10S20 and 10S21). The obtained results suggests, that studied compounds might inhibit RNA- or DNA-containing viruses, such as Epstein-Barr virus. According to PASS, all studied compounds may be a substrate for cytochrome c, which might play an important role in the induction of apoptosis. Also, all studied compounds may be a histone deacetylase inhibitors, that might effect on cell cycle.

Identification of biomolecular targets of molecules is essential for unraveling their underlying causes of effects at the molecular level. PharmMapper is a web server for identification of potential drug target based on the use of a pharmacophore mapping approach. It functions on the ligand-protein reverse docking strategy and reports potential target on the basis of normalized fit score. It was established, that the majority of the targets are enzymes, such as protein kinases and apoptotic proteins (table 2). It was shown, that compound 10S20 could interact with heat shock protein and other proteins. Both compounds, 10S21 and 10S22, might play an important role in the induction of apoptosis by interacting with a mitogen-activated protein kinase.

Table 2

Potential targets of esters of polyfluoroalkanethioacylated amino acids

Substance 10S20		**Substance 10S21**		**Substance 10S22**	
Target name	Fit score	Target name	Fit score	Target name	Fit score
Acetylinesterase	4.771	Gag-Pol polyprotein	3.963	Leukotriene hydrolase	4.533
Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	4.320	Cell division protein kinase 2	3.639	Gag-Pol polyprotein	4.468
Heat shock protein Hsp90-α	4.184	Serine/threonine-protein kinase	3.607	Purine nucleoside phosphorylase	4.288
Tyrosine-protein phosphatase	4.182	Mitogen-activating protein kinase 10	3.479	Protein-glutamine glutamyltransferase	4.078
Aspartate aminotransferase	3.904	Mitogen-activating protein kinase 14	3.291	Mitogen-activating protein kinase 1	3.910

The resulting list of prospective targets includes enzymes involved in the replication of viruses and cells. One of the features of the compounds studied is the amino acid in the structure. Thus, according to the pharmacophore mapping and the presence of amino acids, it can be assumed that these compounds can block the replication of viruses and affect various enzymes such as phosphatase, hydrolases, leading to a disturbance of their normal functioning.

Also, PharmMapper show pharmacophore model for each target from a list and helps to understand the interaction between the target and studied compound (fig. 1). Thus, hydrophobic (blue) interaction is essential for complex target and studied compounds. Also, there is less presented acceptor binding site (pink). This knowledge may help to understand the way of forming of the complex.

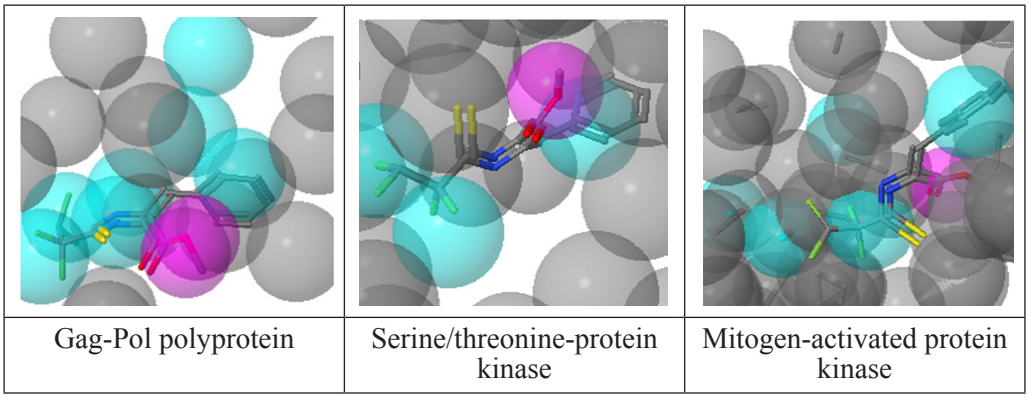


Fig. 1. The pharmacophore models of three targets for studied compounds

Identification of targets and prediction of possible biological activity allows to screen a large number of compounds and decided which methods to use. Any predicted property must be confirmed or disproved in the biological model. Accordingly, *in vitro* analysis of these compounds was carried out.

Determination of cytotoxicity of molecules is an integral component of any drug development process. The research was carried out by using MTT-assay, neutral red uptake assay (NRU) and trypan blue exclusion test of cell viability. Our results on Raji cell line model clearly show, that all polyfluoroalkanethioacylated amino acid esters are quite toxic (fig. 2) ($p<0.05$).

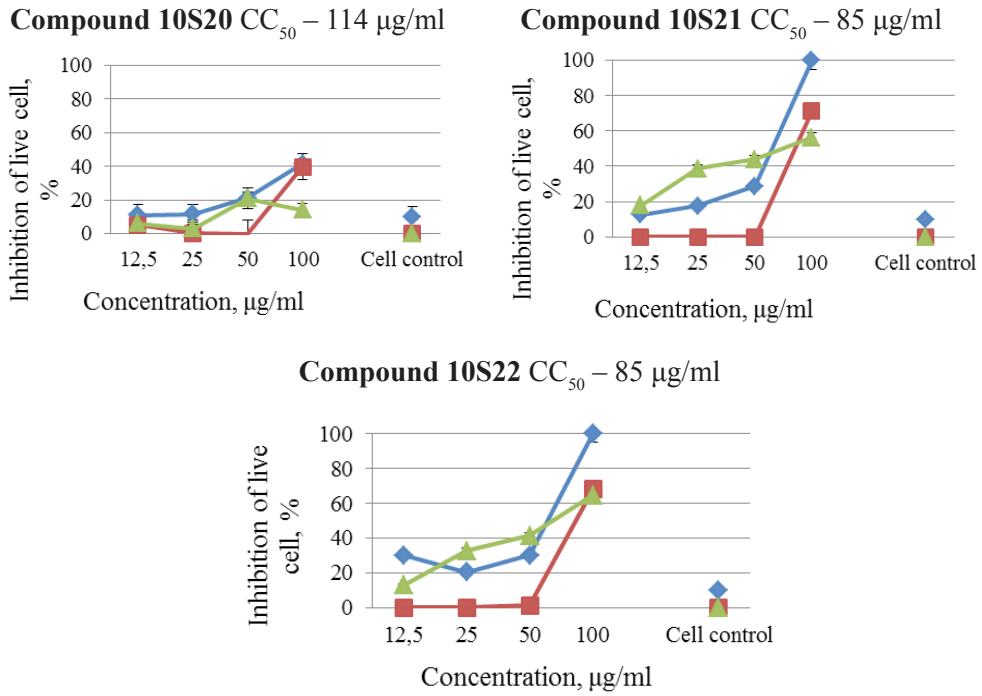


Fig. 2. The cytotoxicity of studied compounds on the model of Raji cells line (trypan blue (blue), MTT (red) and NRU (green) assay were used; $p<0.05$)

A less toxic compound 10S20 at a high concentration of 100 µg / ml showed a 40% percent inhibition of cells. Compounds 10S21 and 10S22 had shown a high level of cytotoxicity. The 50% cytotoxic concentration of studied compounds for Raji cell line was 114, 85 and 85 µg/ml, respectively. The different assays showed activity on different compartments of the cell. Thus, the present work reports, that studied compounds inhibit all essential processes in Raji cells.

The cytotoxicity evaluation of studied compounds revealed a variety of cytotoxic level on the model of B95-8 cell line (fig. 3). The present study showed a high level of cytotoxicity for esters of tetrafluoropropanethioyl amino acids on model of B95 cell line. All studied compounds inhibited living cells. The study of cytotoxicity showed inhibition of all essential processes of the cell. Thus, all compounds inhibit mitochondrial compartment of cell MTT assay.

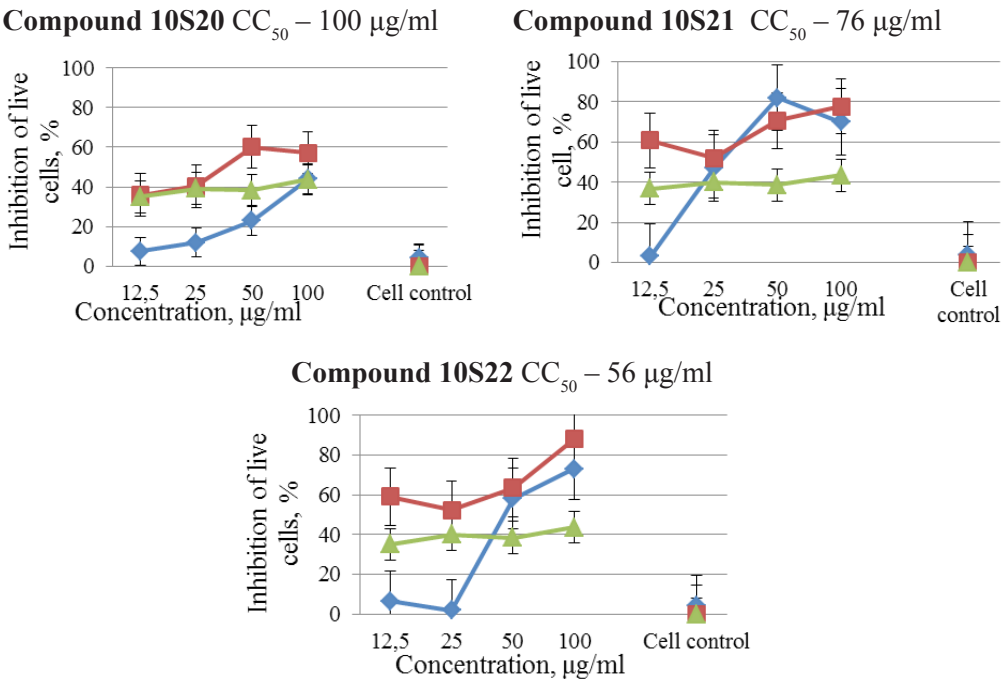


Fig. 3. The cytotoxicity of studied compounds on the model of B95 cell line (trypan blue (blue), MTT (red) and NRU (green) assay were used; $p<0.05$)

Also, trypan blue exclusion test showed a significant alteration of the integrity of the cell membrane. The neutral red uptake assay detected increasing of activity of lysosomes. Thus, high level of cytotoxicity might affect on antiviral activity, by killing transformed cells.

Antiviral activity of the tested agents was assessed by the degree of inhibition of EBV reproduction by quantitative PCR (polymerase chain reaction) method at concentrations 1–100 µg/ml for Raji cell culture (fig. 4). The samples for the analysis were taken after 48 h since this time interval was an optimum both for the growth dynamics of cells lines and for the EBV reproductive cycle. Study of an antiviral

action of 10S22 against EBV in infected Raji cells showed that this compound at a maximum concentration of 100 μg/ml could effectively inhibit the viral DNA accumulation by 50%. A slightly different response to increasing concentrations of the compound 10S20 was observed in the model Raji cell line. 50% inhibition of EBV replication was detected at a minimum concentration of 1 μg/ml.

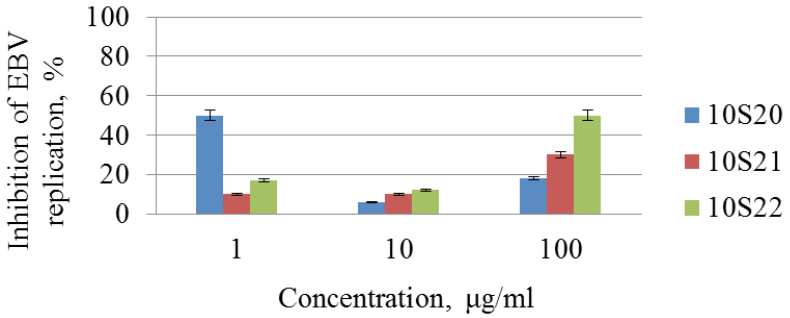


Fig. 4. Antiviral activity of esters of tetrafluoropropanethioyl amino acids derivatives on Raji cell line

It was shown, that all esters of polyfluoroalkanethioacylated amino acids derivatives have anti-EBV activity. The most perspective is the compound 10S20. Levels of antiviral activity of the compounds 10S21 and 10S22 were lower, EC50 were 20 μg/ml and 100 μg/ml, respectively. Increased concentrations of the compounds may lead to an activation of protection systems of the cell, such as DNA repair, for example.

Selectivity index (SI) is used to estimate the therapeutic effect of a drug and to identify drug candidates for further studies. In terms of SI values, all investigated compounds can be sorted into two groups: inactive (SI<4) and active (SI≥4) (table 3).

Table 3

The effectiveness of the compounds relative to Epstein-Barr virus

Studied compounds	CC_{50} [a]	EC_{50} [b]	SI[c]
10S20	114	1	114
10S21	85	20	4
10S22	85	100	1

a) The 50% cytotoxic concentration of studied compound for Raji and B95-8 cells in μg/ml
b) Concentration of compounds (μg/ml) producing 50% inhibition of EBV reproduction
c) SI = CC_{50}/ EC_{50}

Consequently, the compounds 10S20 and 10S21 can be considered as the active agents. The present study makes it possible to assign the compound 10S20 to a group of promising antiviral compounds, according to the high value of the selective index of 114. The compounds 10S21 and 10S22 showed anti-EBV activity and high level of cytotoxicity. EBV is associated with lymphoproliferative disorders

and one of the ways of medical treatment is the elimination of transformed cells. Thus, the compounds 10S21 and 10S22 may play an important role in treatment of lymphoproliferative disorders. Studied compounds may be substrates for cytochrome c and histone deacetylase inhibitor which might be potential inducers of apoptosis in transformed cells, too.

In the present study, the antiviral activity of esters of polyfluoroalkanethioacylated amino acids derivatives was evaluated *in silico* and *in vitro* to understand their poten-tial spectrum as anti-EBV agents. According to PASS prediction and this study, it was shown, that derivative of esters of tetrafluoropropanethioyl amino acids 10S20 has a good selective index and may be a potential antiviral drug. The study demonstrates the ability of investigated compounds to inhibit the mechanism of virus replication. All compounds contain amino acids in their composition, in particular essential. Thus, the effect of these compounds, can be attributed to the change in the activity of the target of viral protein.

Obtained and analyzed data let to relate the compound 10S20 to a perspective anti-EBV agent, and the 10S21 and 10S22 derivatives to apoptosis-inducing compounds that can be used in further research on the antitumor action.

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АНАЛІЗ НОВИХ ПОЛІФТОРТІОАЦИЛЬОВАНИХ ПОХІДНИХ АМІНОКИСЛОТ МЕТОДАМИ *IN SILICO* ТА *IN VITRO*

Реферат

Мета роботи *– проаналізувати потенційну біологічну активність та мішень дії похідних ефірів поліфтороалкантіоацильованих амінокислот за допомогою методів in silico, а також вивчити прогнозовані властивості in vitro.* ***Матеріали та методи.*** *Були використані: програмне забезпечення PASS, веб-сервер PharmMapper, ПЛР, МТТ-аналіз, забарвлення трипановим синім та метод нейтрального червоного.* ***Результати.*** *Згідно з прогнозом PASS, дві сполуки (10S20 та 10S21) можуть мати противірусну активність, Pa/Pi становить 0,294/0,005 та 0,214/0,084, відповідно. Крім того, всі сполуки можуть бути субстратами цитохрому с, який відіграє важливу роль в індукції апоптозу. Кілька мішеней були визначені за допомогою молекулярного стикування (PharmMapper). Було показано, що більшість прогнозованих мішеней є білками, зокрема, вірусний білок Gag-Pol та різні види протеїнкіназ. Дослідження in vitro показало, що всі сполуки інгібують ре-*

плікацію вірусу Епштейна-Барр. З іншого боку, похідні ефірів поліфтороалкантіоацильованих амінокислот мали високий рівень цитотоксичності на різних лініях лимфобластоїдних клітин, що може бути зумовлено механізмом дії сполук. ***Висновки.*** *Дослідження in silico є перспективним підходом до розробки нових антивірусних препаратів. Наші результати дозволяють віднести сполуку трет-бутил (2,2,3,3-тетрафторопропантіоїл)аланінат до перспективних анти-ВЕБ агентів. Отримані дані показали, що похідні ефірів поліфтороалкантіоацильованих амінокислот (10S21 та 10S22) можуть мати апоптоз-модулюючий вплив, що є ключовим аспектом при розробці препаратів для онкологіних захворювань, індукованих вірусними інфекціями.*

Ключові слова: вірус Епштейна-Барра, ефіри тетрафторопропантіоацильованих амінокислот, in silico, in vitro.

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АНАЛИЗ НОВЫХ ПОЛИФТОРТИОАЦИЛИРОВАННЫХ ПРОИЗВОДНЫХ АМИНОКИСЛОТ МЕТОДАМИ *IN SILICO* И *IN VITRO*

Реферат

Цель работы *– проанализировать потенциальную биологическую активность и мишень действия производных эфиров полифторалкантиоацилированных аминокислот с помощью методов in silico, а также изучить прогнозируемые свойства исследуемых соединений in vitro.* ***Материалы и методы.*** *Были использованы: программное обеспечение PASS, веб-сервер PharmMapper, ПЦР, МТТ-анализ, окраска трипановым синим и метод нейтрального красного. Результаты. Согласно прогнозу PASS, два соединения (10S20 и 10S21) могут иметь антивирусную активность, Pa/Pi составляет 0,294/0,005 и 0,214/0,084, соответственно. Кроме того, эти соединения могут быть субстратами цитохрома с, который играет важную роль в индукции апоптоза. С помощью молекулярного докинга (PharmMapper) были определены потенциальные мишени. Было показано, что большинство из возможных мишеней являются белками, такими как белок Gag-Pol (вирусный белок) и различные виды протеинкиназ. Исследования in vitro показали, что все соединения ингибируют репликацию вируса Эпштейна-Барр. С другой стороны, производные эфиров полифторалкан-тиоацилированных аминокислот имели высокий уровень цитотоксичности на разных линиях лимфобластоиднх клеток, что может быть обусловлено механизмом действия этих соединений.* ***Выводы.*** *Исследования in silico являются хорошим подходом для разработки новых антивирусных препаратов. Наши результаты позволяют отнести соединение трет-бутил (2,2,3,3-тетрафторпропантиоил)аланинат к перспективным анти-ВЭБ агентам. Полученные данные показали, что производные эфиров полифторалкантиоацилированных ами-*

нокислот (10S21 и 10S22) могут иметь апоптоз-модулирующие свойства, что является ключевым аспектом при разработке препаратов для онкологических заболеваний, индуцированных вирусными инфекциями.
Ключевые слова: вирус Эпштейна-Барр, эфиры полифторалкантиоацилированных аминокислот, in silico, in vitro.

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