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INHIBITORS OF EPSTEIN-BARR VIRUS REPRODUCTION - RIBONUCLEOSIDES OF 3-SUBSTITUTED 1,2,4-TRIAZINO[5,6-b][1,4] BENZOTHIASINES

Studying of antiviral effect of condensed triazine derivatives - 3-oxo- triazinobenzothiazine (the preparation №1), its ribonucleoside (the preparation №3) and ribonucleoside of its 3-thio-analogue (the preparation №2) on reproduction of Epstein-Barr virus (EBV) in lymphoblastoid Raji cells is reported in present work. The cytotoxic action of these preparations on test cells was determined and CC_{50} index was 125 μg/ml for the preparation № 1, for preparation №2 - 625 μg/ml, and for preparation № 3 - 750 μg/ml. Antiviral activity of the preparations was tested by inhibition level of accumulation of viral DNA in the cell culture, and effective concentration (EC_{50} index) was determined for each preparation. EC_{50} constituted 1μg/ml for each triazinobenzothiazines. Selectivity index (SI) was 125 for preparation №1, for preparation №2 - 625, and for preparation №3 - 750. The obtained results of studying of triazinobenzothiazines as inhibitors of EBV-infection support their high antiEBV activity and can be interesting for further investigations.

Keywords: Epstein-Barr virus, antiviral effect, ribonucleosides.

High morbidity caused by herpesviruses is one of actual problems of modern medicine. Herpesviruses are capable to infect practically all organs and systems of a host organism causing latent, acute and chronic forms of infection [9]. Epstein-Barr virus (EBV) is related to *Gammaherpesvirinae* subfamily but differs by its lymphotropism. Primary infection of human by the virus occurs more often at childhood with following development of infectious mononucleosis [8]. The virus is able to persist in a host organism and then to cause lymphoproliferative diseases, stomach and parotis adenocarcinomas, lesions of central and peripheral nervous system of human [5,11,14,16].

Nowadays, the main group of antiherpetic drugs with direct antiviral activity is most widely represented by a set of acyclic nucleosides preparations. Acyclovir, Ganciclovir and their derivatives, as well as acyclic nucleotide analogues Cidofovir and Adefovir are used for inhibition of EBV-infection in medical practice worldwide [2,17]. Only Acyclovir (trade mark "Zovirax") and Ganciclovir (trade mark "Cymevene") are registered in Ukraine [3].

Formation of resistance to known drugs stimulates the search for new effective antiherpetic substances. The condensed triazine bases (triazinoindoles, pyridotriazines, triazinobenzimidazoles and their derivates) provoke significant interest in connection with their broad spectrum of antibacterial [12,13] and antiviral [1,6,8,10] activities. The new series of the condensed triazine - 1,2,4-triazinobenzothiazines were synthesized at the Institute of Molecular Biology and Genetics of NAS of Ukraine.

The purpose of present study is the comparative examination of the effect of a series of 1,2,4-triazinobenzothiazines using Acyclovir as reference preparation on Epstein–Barr virus reproduction in Raji cell culture.

Materials and methods

Cell cultures. Lines of lymphoblastoid B-phenotype Raji cells (human B-lymphocytes isolated from Burkitt's lymphoma) and B 95-8 cells (lymphocytes of peripheral blood of marmoset monkeys) were used as *in vitro* model of EBV-infection.

The cells have been received from bank of cell cultures of D.I. Ivanovsky Institute of virology of AMS of Russia (Moscow).

The cells were cultured in RPMI 1640 medium (Sigma, USA) containing 10% (v/v) fetal bovine serum (Sigma, USA) with antibiotics and incubated with 5 % CO_2 at 37^0C.

Epstein–Barr virus was isolated from EBV-producing lymphoblastoid cell line B95-8. The virus purification was carried out by Wolls, Kroford method [19].

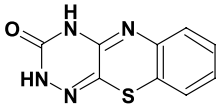
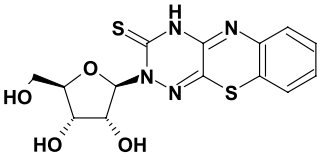
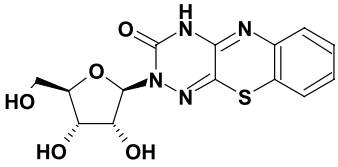
Preparations. 1,2,4-triazinobenzothiazines were dissolved in minimal volume of dimethylsulfoxide (Sigma, USA) with following dilution in whole RPMI 1640 medium to required concentrations. Range of 1000 – 0,1 μg/ml concentrations of these preparations was used in experiments for definition of cytotoxic effect and antiviral activity. Acyclovir – 2-amino-9-[(2-hydroxyetoxy)-methyl]-1,9-dihydro-6H-purine-6-on («Acycloguanosine», the substance, Sigma, USA) at the concentration range of 250 – 0,5 μg/ml was used as the reference preparation in antiviral activity studies.

Chemistry. 3-oxo- and 3-thio-1,2,4-triazinobenzothiazines were synthesized as described by Alexeeva et al. [1]. The synthesis of 3-oxo- and 3-thio-triazinobenzothiazines ribonucleosides proposed for study was carried out by two alternative manners. Namely: by direct glycosilation of corresponding tricyclic aglycon by tetraacetylribose under condition of "silyl condensation" with following deblocking of intermediate acylnucleoside, or annelation of triazine cycle of N2-azanucleoside (6-azauridine) by 2-aminothiophenol. The first mode was more effective for obtaining of desired compounds (**2** and **3**) and was suggested for their preparative development. Individuality and structure of the given substances were confirmed by chromatography, UV-, ^{1}H-NMR- spectroscopy and mass–spectral methods [1].

Some structural characteristics of synthesized substances are demonstrated below (Table 1).

Table 1

Structural formulae of tested preparations

Preparation	Structural formulae
3-oxo-base (preparation №1)	
N_2-glycoside of 3-thio-base (preparation №2)	
N_2-glycoside of 3-oxo-base (preparation №3)	

Preparations	Chemical name	Formula	MW
1	2,4-dihydro-3-oxo-1,2,4-triazino[5,6-b] [1,4]benzothiazine	$C_8H_6N_4OS$	218,24
2	2-β-D-ribofuranosyl-3(4H)-thio-1,2,4-triazino[5,6-b][1,4] benzothiazine	$C_{14}H_{14}N_4O_4S_2$	366,4
3	2-β-D-ribofuranosyl-3(4H)-oxo-1,2,4-triazino[5,6-b][1,4] benzothiazine	$C_{14}H_{14}N_4O_5S$	350,35

Cytotoxicity testing. Cytotoxic analysis of investigated substances at which viability of cellular population of Raji line reduced by 50 % (CC_{50} index) was carried out by MTT-method. The method is based on measuring of activity of living cells via mitochondrial dehydrogenase activity as processing of the MTT artificial substrate (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) ("Sigma", USA) in formazan. 25 µl of solution, containing 5 mg of MTT/ml, was added to each well maintained cells and corresponding preparations in respective concentrations of 100µl total volume on the 96-well microtitration plate with following incubation for 3h at 37° C at 5% CO_2. After incubation period experimental materials were centrifuged at 1000g for 10 min and then the contents of each well were removed and 200 µl of 96% ethanol was added to dissolve the dye. The measurement of optical density (OD) at 540 nm wavelength using multifunctional reader Dynatech (Sweden) was preceded by 10 min of gentle shaking at 37^0C. The percentage of MTT conversion in formazan was accounted for each well comparing the optical density (OD) of experiment with control. Each concentration was investigated in three repetitions with calculation of average value.

Antiviral assays. A level of inhibition of Epstein–Barr virus reproduction in Raji cell culture was evaluated by quantitative PCR method using "AMPLY-Sens-100R" primers and reagents (Russia) with following evaluation using "Biotest A" software. The analyses were carried out according to the instruction provided with the test-system. The level of EBV reproduction was determined as genome equivalents of EBV DNA per cell. Tests were carried out in three repetitions.

Statistical data processing was carried out according to standard approaches with calculation of the statistical error and analysis of correlation dependence using Origin 6.0 software [15,18].

Results

The cytotoxicity of preparations *in vitro* was studied in lymphoblastoid Raji cell culture. Investigations were carried out in 96-well culture plates using three parallel wells for each concentration. Acyclovir was used as reference preparation. All substances were tested at the range of concentrations from 1000 to 10 µg / ml. The level of the viability of Raji cells was counted relative to the control of untreated cells which was accepted as 100%. The values of optical density (OD) obtained as a result of the MTT-test were statistically processed and the standard error of three repetitions was calculated. Its value did not exceed 0,05 confirming the reliability of the obtained results. The data of cytotoxic activity for investigated samples are shown in Table 2.

Table 2

Cytotoxic activity of triazinobenzothiazines in Raji cell culture

Preparation	**3-oxo-base (the preparation №1) ***	**N_2-glycoside of 3-thio-base (the preparation №2) ***	**N_2-glycoside of 3-oxo-base(the preparation №3) ***
	Level of cell culture viability, %		
Concentration, µg/ml			
1000	0	31	47
500	5	53	54
100	56	83	78
50	71	87	80
10	85	94	82

The note: * a standard error ±0,05

Table data shows that the preparation №1 led to almost 100 % of cell death at range of concentrations 1000 - 500 µg/ml, and at the same when testing of 3-thio- and 3-oxo-nucleosides (№2 and №3) 1/3 of cells remained alive at maximum concentration of 1000 µg/ml stand. Further decrease of concentration led to such level of viable cells that confirmed about dose-dependent effect of the studied substances on the viability of Raji cell culture.

Origin 6.0 software was used for processing obtained data and construction of linear regression graphs that allowed characterizing exact reverse dependence numerically between the concentration of substance and corresponding optical density. The correlation coefficients (r) were as followed: -0.92 for №1, -0.96 for №2, and -0.93 for №3, demonstrating considerable degree of reliability and the reverse character of this dependence (Fig.1). Obtained mathematic characteristics allowed calculating the CC_{50} - i.e. the concentrations of preparations that reduced proliferating activity of tested cell population by 50%. An embedded statistic function based on the straight line equation was used for calculation of the indexes.

Thus, studying the level of proliferating activity of Raji cells under the effect of different concentrations of tested triazinobenzothiazine derivatives established that 50% death of the cellular population (CC_{50} index) was achieved at concentration of the preparation № 1 – 125 μg/ml, №2 – 625 μg/ml, № 3 – 750 μg/ml.

Acyclovir was low-toxicity preparation; its CC_{50} was 5000 μg/ml.

When analyzing the obtained data, it should be noted that comparing to Acyclovir 3-thio- and 3-oxo-nucleosides have 10 times higher toxicity (CC_{50}), when the toxicity of the 3-oxo-base №1 was almost 40 times higher.

Studying of anti-EBV activity

Antiviral activity of test agents was estimated by level of inhibition of Epstein-Barr reproduction in Raji cell culture by quantitative PCR method at a concentration range from 10 to 0,1 μg/ml at three repetitions. Reference preparation Acyclovir was tested at similar mode in concentrations from 250 to 0,5 μg/ml.

The investigated samples were taken in 48 hours since this period was optimal considering Raji cell line growth dynamics and EBV reproductive cycle. The levels of inhibition of viral DNA accumulation in infected cells treated with various concentrations of substances were determined in relation to control infected but untreated cells in which viral DNA accumulation was accepted for 100% (Fig.2).

Fig.2 shows, that investigated preparations had pronounced antiviral effect in *in vitro* conditions, their effective concentrations (EC_{50}), i.e. the concentration reducing the level of EBV reproduction by 50 % for all preparations was 1 μg/ml. Taking into consideration the molecular weight, it corresponds to 4.6 μM for 3-oxo-base (№ 1); 2.8 μM for 3-thio-nucleoside (№ 2); 2.7 μM for 3-oxo-nucleoside (№ 3).

It should be noted that Acyclovir was not effective against EBV infection – its EC_{50} concentration was 220 μg/ml that corresponded to 0.98 M and was almost 1000 times higher than that for triazinobenzothiazines.

Selectivity index (SI) for researched preparations was determined for the purpose of estimating their potential anti-EBV properties. According to existing normative documents, the substance is considered perspective for creation of drugs if its SI is over 16 [17]. The SI of the studied preparations was calculated by the ratio of cytotoxic and effective concentrations: selectivity index of base was 125, N_2-glycosides (preparations №2 and №3) – 625 and 750, accordingly. At the same time SI of Acyclovir was 23 in this model system.

The received data demonstrate clearly the sufficient essential influence of 2-β-D-ribofuranosyl fragment. Its presence in the molecule of nucleoside increases the level of antiviral action twice and decreases its toxicity toward Raji cells in 6 times comparison with the base.

Based on the toxicity indexes (CC_{50}), the effective concentrations (EC_{50}), selectivity indexes (SI) of triazinobenzothiazines and Acyclovir (Table 3) it is possible to conclude that triazinobenzothiazines have high antiviral potential toward EBV and are interesting for further investigations.

Table 3

Indices of anti-EBV activity of triazinobenzothiazines obtained from *in vitro* experiments in lymphoblastoid Raji cell line

Index	3-oxo-base	N_2-glycoside 3-thio-base	N_2-glycoside 3-oxo-base	Acyclovir
CC_{50}	125 μg/ml	625 μg/ml	750 μg/ml	5000 μg/ml
EC_{50}	1 μg/ml	1 μg/ml	1 μg/ml	220 μg/ml
SI	125	625	750	23

The data demonstrated in Table 3 allow considering investigated triazinobenzothiazines as perspective antiviral substances. Taking into account the results obtained by another research group about the inhibition of reproduction of Herpes simplex virus type 2 by these nucleosides [1], it is possible to assume the wide spectrum of their applications. This characteristic is important since herpetic infections usually reactivate in mix-form in clinic practice.

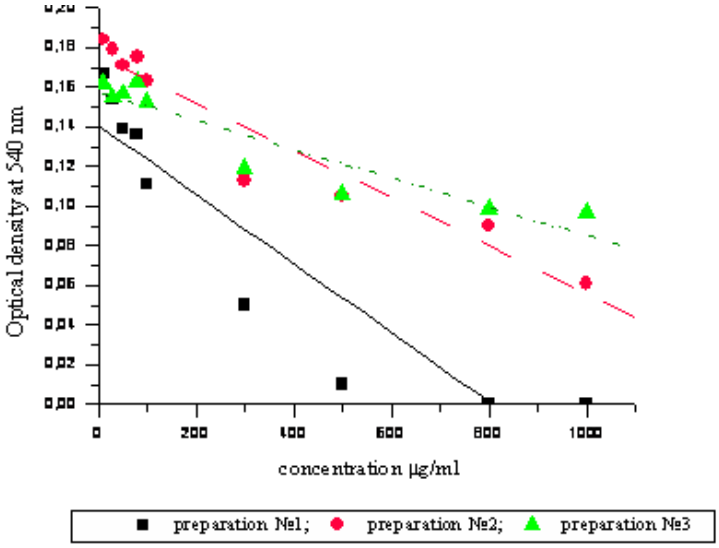


Figure 1. Alterations in the level of proliferating activity of Raji cells under effect of tested triazinobenzothiazines. A linear regress method.

The note: a standard error ±0,05

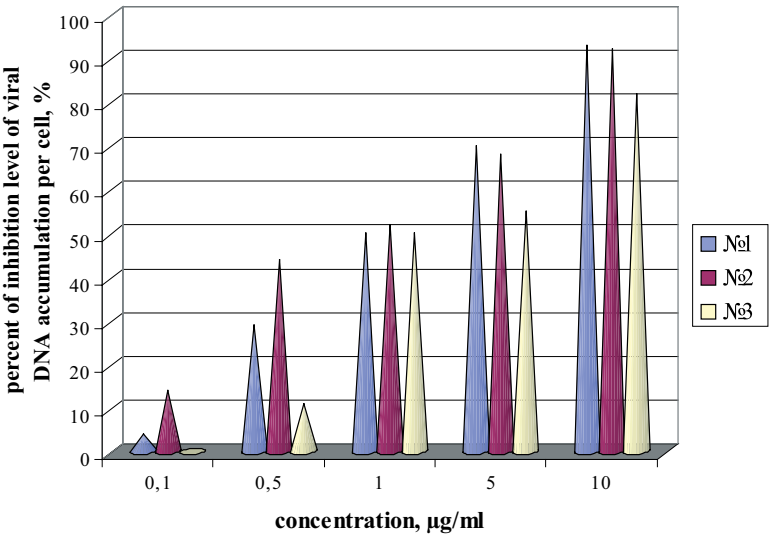


Figure 2. The levels of inhibition of EBV DNA accumulation as effected by triazinobenzothiazines.

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ІНГІБІТОРИ РЕПРОДУКЦІЇ ВІРУСУ ЕПШТЕЙНА-БАРР – РИБОНУКЛЕОЗИДИ 3-ЗАМІЩЕНИХ 1,2,4-ТРИАЗИНО[5,6-B][1,4] БЕНЗОТІАЗИНІВ

Р е з ю м е

В роботі представлені дані по антивірусній дії похідних конденсованого триазину – 3-оксо-триазинобензотіазину (препарат №1), його рибонуклеозиду (препарат №3) та рибонуклеозиду 3-тіо-аналогу (препарат №2) на репродукцію вірусу Епштейна-Барр (ВЕБ) в лімфобластоїдних клітинах Raji. Визначена цитотоксична дія препаратів на досліджувані клітини – показник CC_{50}, який був для препарата №1 – 125 мкг/мл, №2 – 625 мкг/мл, №3 – 750мкг/мл. Досліджена антивірусна дія препаратів по рівню пригнічення накопичення вірусної ДНК в культурі клітин та визначена ефективна концентрація (показник EC_{50}) для кожної речовини. EC_{50} для 3-оксо триазинобензотіазину та обох рибонуклеозидів був 1 мкг/мл. Індекс селективності (SI) для препарата №1 сстановив 125, для препарата №2 – 625, препарата №3 – 750. Отримані результати дослідження триазинобензотіазинів як інгібіторів ВЕБ-інфекції свідчать про їх високу антиВЕБ активність та можуть представляти інтерес для подальших досліджень.

Ключові слова: вірус *Епштейна-Барр*, антивірусна дія, рибонуклеозиди.

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ИГНИБИТОРЫ РЕПРОДУКЦИИ ВИРУСА ЭПШТЕЙНА-БАРР РИБОНУКЛЕОЗИДЫ 3-ЗАМЕЩЕННЫХ 1,2,4-ТРИАЗИНО[5,6-B][1,4] БЕНЗОТИАЗИНОВ

Р е з ю м е

В работе представлены данные по антивирусному действию производных конденсированного триазина – 3-оксо-триазинобензотиазина (препарат №1), его рибонуклеозида (препарат №3) и рибонуклеозида 3-тио-аналога (препарат №2) на репродукцию вируса Эпштейна-Барр (ВЭБ) в лимфобластоидных клетках Raji. Определено цитотоксическое действие препаратов на исследуемые клетки – показатель CC_{50} для препарата № 1 составил 125 мкг/мл, для препарата №2 - 625 мкг/мл, для препарата № 3 - 750 мкг/мл. Исследовано антивирусное действие препаратов по уровню угнетения накопления вирусной ДНК в культуре клеток и определена эффективная концентрация (EC_{50}) для каждого вещества. Показатель EC_{50} для 3-оксо-триазинобензотиазина и обоих рибонуклеозидов был 1 мкг/мл. Индекс селективности (SI) для препарата №1 составил 125, для препарата №2 – 625, препарата №3 – 750. Полученные результаты исследования триазинобензотиазинов как ингибиторов ВЭБ-инфекции свидетельствуют об их высокой антиВЭБ активности и могут представлять интерес для дальнейших исследований.

Ключевые слова: вирус *Эпштейна-Барр*, антивирусное действие, рибонуклеозиды.

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