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ANTIVIRAL ACTIVITY OF EXTRACTS FROM WILD GRASSES AGAINST EPSTEIN-BARR VIRUS AND INDUCTION OF APOPTOSIS IN EPSTEIN-BARR VIRUS-POSITIVE LYMPHOBLASTOID CELLS

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Epstein-Barr virus (EBV) belongs to the human herpesvirus family that infects more than 90 % of the population. EBV is associated with a number of lymphoproliferative and autoimmune diseases. Usage of the drugs, which would not only inhibit the reproduction of the virus, but also would stimulate the elimination of tumor cells, is important for the treatment of virus-associated tumors. ***The purpose****. In the current research the antiviral effects and apoptotic activity of the herbal drugs proteflazid and neoflazid were studied on the models of latent, acute and chronic EBV infection in Raji and B95-8 lymphoblastoid cells.* ***Methods****. The investigations were performed in Raji (virus non-productive) and B95-8 (virus-productive) lymphoblastoid cells. Proteflazid and neoflazid, plant drugs, were studied, acycloguanosine was used as a reference drug. Trypan blue staining (TBS) method and MTT-assay were used to study of cell viability. Antiviral activity was estimated by real time polymerase chain reaction (RT-PCR). Apoptotic cells were detected using flow cytometry method.* ***Results.*** *The investigations showed that neoflazid was more toxic towards Raji cells than proteflazid: the cytotoxic concentration (CC_{50}) indexes were 8 μg/ml and 36 μg/ml respectively. Toxicities of these compounds in B95-8 cells were almost the same and their CC_{50} indexes were close to 25 μg/ml. Both drugs showed high antiviral activity against EBV lytic infection in Raji cells and effective concentration (EC_{50}) was 0.02 and 0.083 μg/ml for proteflazid and neoflazid, respectively, and selectivity indexes (SI) were 1800 and 96. These compounds were less effective in B95-8 cells and even at the concentration of 10 μg/ml inhibited virus replication only by 10–19 %. We checked an ability of proteflazid to induce apoptosis and found that the drug at cytotoxic concentration (30 μg/ml) stimulated the apoptotic death of 70 % cells in latent and lytic EBV infection. The non-toxic concentration (5 μg/ml) induced apoptosis 30 % of cells.* ***Conclusions.*** *Thus, our research showed that both plant preparations proteflazid and neoflazid possessed high antiviral activity under acute EBV infection. Proteflazid, in addition, induced dose-depending apoptosis in EBV-positive lymphoblastoid cells.*

Keywords: Epstein-Barr virus, proteflazid, neoflazid, antiviral activity, apoptosis.

Epstein-Barr virus (EBV) is a human gamma-herpesvirus, which infected about 95% of people in the world, and is able to affect almost all organs and systems of the host, causing latent, acute and chronic forms of infection. EBV is associated with a number of cancers mainly lymphoproliferative (lymphoma, nasopharyngeal carcinoma, Burkitt's lymphoma) and autoimmune diseases (rheumatic diseases, vasculitis, ulcerative colitis, etc.) [1–4].

Drugs based on plant substances are widely used for treatment of herpes diseases. For example, flavonoids, which are the derivatives of phenolic compounds, are the yellow and brown plant pigments. They show wide therapeutic action. Flavonoids have a broad range of biological activity and involved in redox processes by performing an antioxidant function; some flavones possess a vitamin *P* activity, may reduce toxicity of some substances, and show antimicrobial, antiviral and antitumor effects [5–9].

Proteflazid and neoflazid drugs generally used to treat herpes infections caused by HSV type 1 and type 2, as well as hepatitis, papilloma virus diseases and influenza [10–11].

The advances in knowledge of fundamental ideas of molecular mechanisms of viral oncogenesis, of operation and interaction between viral and cellular oncogenes, resulted in the development of a new approach that helps to search for antiviral drugs that are able not only effectively inhibit the

replication of the virus, but also to stimulate cell renewal or cause the elimination of infected cells, in particular by initiation of the programmed cell death program (apoptosis). Currently, most drugs that influence the replication of EBV are able to inhibit the replication of other herpesvirus as well, but none of them are licensed for the treatment of EBV infection in hospital. Therefore, an important issue for the treatment of EBV-associated diseases is to search for the drugs, which will stimulate the process of apoptosis in the virus-transformed cells in addition to the inhibitory effect on virus replication.

The **aim** of the current study was to show the antiviral activity and the apoptosis inducing action of the plant preparations proteflazid, neoflazid and an oil form of proteflazid in models of lytic and latent EBV infection *in vitro.*

Materials and methods

Cell cultures

Limphoblastoid cell cultures from the European Collection of Authenticated Cell Cultures (ECACC) [12] were used in the studies. Cell line B95-8 is the lymphocytes of Tamarin monkey *Saguinus oedipus* that transformed with EBV and chronically produce complete infectious virus particles. Cell line Raji is the undifferentiated human lymphoblastoid cells of B-type from Burkitt's lymphoma, which contain 55–64 copies of EBV in episomal form and express only latent viral proteins. Cell cultures store in the Cell Culture Bank of the Ivanovsky Institute of Virology, RAMS. Cell line CHO is the epithelial cells of gray hamster (*Cricetulus griseus*) oocytes. Cell line MDBK is the bovine kidney epithelial cells.

Lympoblastoid cells were grown in RPMI 1640 medium that contained glutamine ("Sigma", USA). Monolayer cells were grown in the medium that contained 50 % of RPMI 1640 with glutamine and 50 % of DMEM ("Sigma", USA) and were supplemented with 10 % of fetal bovine serum (FBS) ("Sigma", USA) and antibiotics (100 μg/ml of penicillin and 100 μg/ml of streptomycin). All cells were grown at 37° C in a 5 % CO_2 atmosphere. Passages were performed every three days by adding fresh cell culture medium.

Virus accumulation

For the accumulation of virus the cells-producers (B95-8) were grown for 10 days at a concentration of $1x10^6$ cells/ml without changing the medium. Induction of EBV synthesis was performed with TFA (12-O-tetradecanoylphorbol-13-acetate) ("Sigma", USA) [13, 14]. After incubation, the cell suspension was sedimented by centrifugation at 1500 rpm for 10 min and the supernatant that contained EBV was used to infect cells. Aliquots of the supernatant were stored at -70° C until demand.

Plant preparations

Plant preparation proteflazid was supplied by the manufacturer SPC "Ekopharm", Kyiv, Ukraine. The active drug compounds are flavonoid glycosides that were isolated from the wild grass *Deschampsia caespitosa* L. and from *Calamagrostis epigeios* L. It solved in ethanol 96 %. The solvent phase for neoflazid is propylene glycol.

Acyclovir (2-amino-9-[(2-hidroksyetoksy) methyl]-1,9-dihydro-6H-purine-6-one, Mr = 225.21) in a form of acycloguanosine provided by "Sigma", USA, was used as a reference drug.

Cytotoxicity tests

Trypan blue staining (TBS) method. Cytotoxicity of the plant preparations was determined in Raji and B95-8 cells. Cells in concentration of 5×10^5 cells/ml were plated in 96-well plates in medium that contained 10 % of FBS and the preparations under study in known concentrations. Each concentration of preparations was tested in 3–4 replicas. Cells that were not treated with plant preparations were used as a control. After 2 days of incubation at 37° C in the 5 % CO_2 atmosphere the equal volumes of 0.4 % trypan blue ("Sigma", USA) solution was added to each well, and after resuspension, the 25 μl of cell mixture was used to count the total number of cells and an amount of alive (unstained) cells. The portion of dead cells (in %) was estimated by the ratio dead cells to the total number of cells in the sample. A ratio of alive to dead cells was determined for each sample and was compared to the control samples.

MTT-assay for study of cell viability. This method is based mainly on the activity of dehydrogenases in mitochondria, which can convert 3,(4,5-dymetyltriazol-2-yl)-2,5-dyfeniltetrazolium bromide (MTT) to formazan. The quantity of the product of this reaction is determined by spectrophotometry. The conversion of MTT to formazan decreases with cell death and under an influence of toxic substances.

Cell suspensions with density of 5×10^5 cells/ml were cultured in 96-well plates in the corresponding medium that contained 10 % of FBS and the plant preparations in known concentrations. In case of the study of cytotoxicity of preparations on cell monolayers, the nutrient medium was removed from the wells of the 96-well plates and fresh

medium with known concentrations of the plant preparations was added.

An untreated with plant preparations cells were used as a control. Each concentration of the preparations was tested in 3–4 replicas. The plates with cells were kept at 37° C in 5 % CO_2 atmosphere, for 48 h. After that a filtered MTT solution was added to each well achieving a final concentration of MTT 0.5mg/ml, and plates additionally were incubated for 3 h at 37° C in 5 % CO_2 atmosphere [15]. An optical density of the solutions was determined with spectrophotometer Multiskan FC ("ThermoScientific", USA) at 538 nm. Percentage of inhibition of cell viability under the influence of drugs was determined according to quantity of formazan that was synthetized in the experimental samples and compared with the control ones.

Cytotoxicity of solvent. A 96 % ethanol at 1:80 dilution was used in proteflazid studies and therefore its toxicity was evaluated as well. Cytotoxicity of ethanol was estimated equal to 16.2±3.5% by TBS method and as a nontoxic by MTT-assay. Detected effect was taking into account in all experiments and data presented in Results section describe effects of plant preparations alone.

Determination of the antiviral activity of the plant preparations in vitro

Raji cells that infected with EBV were used as a model of acute EBV infection, while cell culture B95-8 without additional EBV infection was used as a model of chronic infection. The ability of compounds to inhibit the virus replication was determined as an effective concentration (EC_{50}) [16], which is the concentration of the drug that decrease by 50% the number of genome-equivalents of viral DNA per cell.

Infection of Raji cells

Infection of Raji cells was performed with the viral material in the supernatant of cells B95-8. The cells were pelleted by centrifugation at 1500 rpm for 10 min and washed twice with RPMI 1640 medium without serum to completely remove FBS, which can prevent adsorption of the virus on the cell surface. The precipitate was dissolved in minimal amount of medium without FBS and 0.2–0.4 ml of virus containing supernatant was added per 1×10^6 cells [13]. Cells were incubated with virus for 1 h at 37° C. After that cells were washed twice with RPMI 1640 medium without serum by centrifugation at 1000 rpm for 10 min and diluted in supportive nutrient medium that contained 5% FBS to achieve initial concentration of cells 5×10^5 per ml [13, 14].

Determination of antiviral activity

Plant preparations were added at various concentrations to the cell culture infected with virus. Samples were incubated for 48 h in an incubator at 37° C in 5 % CO_2 atmosphere. Material for analysis was collected after 48 h since the moment of cells infection. Cells in concentration of 5×10^5 cells/ml were washed with fresh RPMI 1640 medium and were resuspended in 100 µl of this medium. The level of suppression of EBV reproduction was determined by polymerase chain reaction (PCR). Cells that were infected with virus and not treated with the preparations were used as a control. The percentage of inhibition of viral DNA accumulation in the samples treated with the preparations was determined in relation to the control, in which the expression was considered as 100 %.

Polymerase chain reaction (PCR)

DNA was isolated from cells with "DNA-sorb-B DNA kit" ("AmpliSens", Russia) and "innuPREP Virus DNA Kit" ("Analityk Jena AC", Germany) according to the instructions of the manufacturer. DNA concentration was determined spectrophotometrically with Biophotometer ("Eppendorf", Germany). Real time polymerase chain reaction (RT-PCR) tests were performed with "AmpliSens®EBV-screen-FL" ("AmpliSens", Russia) and "EBARPOL" ("Litech", Russia) according to the manufacturer's recommendations. The amplificator device was Thermocyclers qTOWER 2.2 ("Analytic Jena", Germany).

Detection of apoptotic cells

Cells treated and untreated (control) with the plant preparations of known concentrations were collected at various time points, centrifuged at 1500 rpm for 5 min and were resuspended in 70 % ethanol. Samples were stored at 4° C. Before analysis the stored samples were washed with PBS by centrifugation and the pellet was resuspended in 0.5 ml of hypotonic fluorochrome solution of propidium iodide (PI) (PI – 50 µg/ml, sodium citrate – 0.1% (w/v), Triton X-100 – 0.1% (w/v), in sdH_2O). Samples were incubated overnight at 4°C in the dark and analyzed with flow cytometer Beckman Coulter Epics XL ("Beckman", USA). Log-FL2, SS and FS values were recorded and analyzed with Flowing Software version 2.5.1 (Turku Centre for Biotechnology, University of Turku, Finland). The cell fraction with low values of DNA fluorescence and positioned on the left of cell cycle peaks was considered as apoptotic [17–18].

Statistical analysis

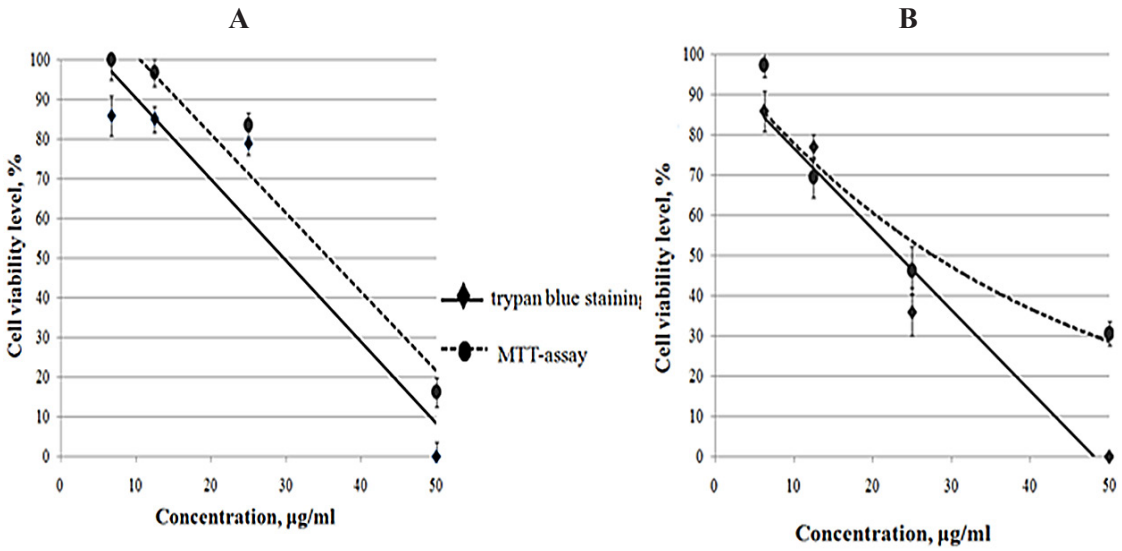
All tests were performed at least in three replicas. Statistical data processing was carried out with the appropriate statistical methods available in the software Microsoft Excel 2010. The differences between mean values were evaluated according to *t*-test at $p \leq 0.05$ [19,20]. Means, medians and coefficient of variation values for statistical analysis of the results obtained with flow cytometry was got with Flowing Software version 2.5.1 (Turku Centre for Biotechnology, University of Turku, Finland).

Results

Cytotoxicity of plant preparations to lympho-blastoid cells

Evaluation of plant preparations by their toxicity to suspensions of Raji and B95-8 lymphoblastoid cells showed that compounds decreased cell viability and thus were toxic. Considering that cell viability is the most important indicator of an effectiveness of any drug we used two different viability tests in the current study. CC_{50} values, which is the concentration of drug at which a 50% inhibition of cell viability in the population is achieved [16], of proteflazid were 29 μg/ml and 36 μg/ml, determined respectively by TBS-method and MTT-assay in Raji cells (Fig. 1A). However, there was no difference between CC_{50} values of proteflazid determined by both these methods in B95-8 cells, and the common value was 25 μg/ml (Fig. 1B).

Neoflazid was found to be more toxic for cells in comparison to proteflazid. Analysis of cell viability by TBS and MTT-assay showed that the lowest studied concentration of neoflazid (6.75 μg/ml) caused death of more than half of cells in the population (71 % and 50 %, respectively), while a 100% cell death was observed at concentration of 100 μg/ml of the same drug, determined by TBS method.



F i g. 1. Cytotoxic effect of the plant preparation proteflazid on the cultures of Raji (A) and B95-8 (B) cells

The CC_{50} of neoflazid was equal to 8 μg/ml in Raji cells (Fig. 2A). However neoflazid was less toxic in B95-8 cells and corresponding CC_{50} values were 28 μg/ml and 24 μg/ml, determined by TBS and MTT-assay, respectively (Fig. 2B). Thus, neoflazid and proteflazid possess almost the same toxicity against B95-8 cells.

Cytotoxicity of plant preparations to epithelial cells

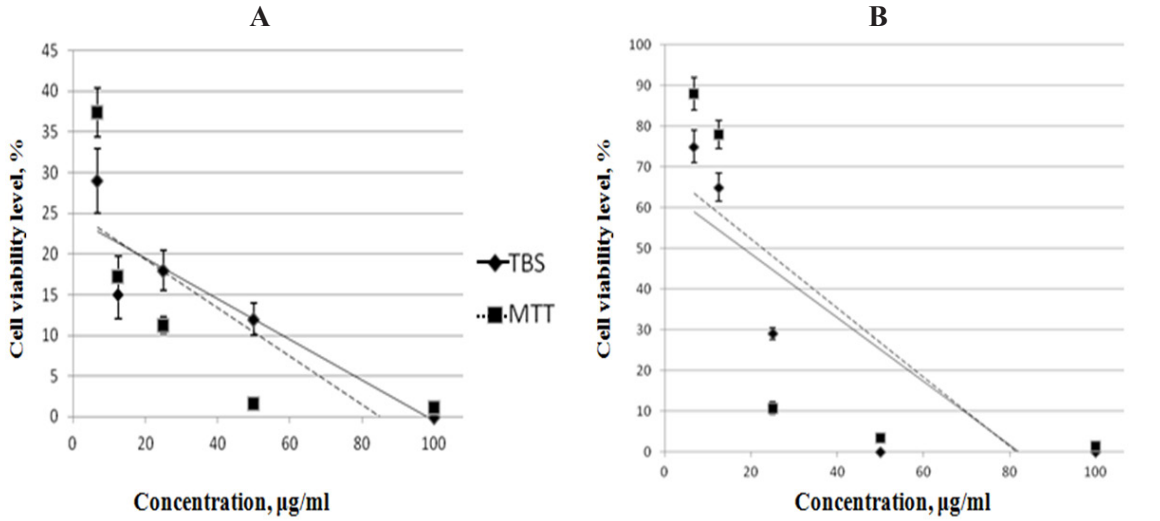
Both compounds (proteflazid and neoflazid) showed less toxicity to monolayers of CHO and MDBK epithelial cells. However, proteflazid was least toxic than neoflazid (Table 1).

Thus, proteflazid was less toxic against lymphoblastoid and epithelial cells in comparison to neoflazid. However, both compounds showed low toxicity to epithelial cells.

Antiviral activity of plant preparations in vitro

Both plant preparations proteflazid and neoflazyd showed high antiviral activity during acute EBV infection in Raji cells (Fig. 3).

Even at lowest concentration proteflazid and neoflazid (0.01 μg/ml), the portion of viral DNA decreased by 40 % and 30 % under the action of proteflazid and neoflazid, respectively. The EC_{50} value for proteflazid was 0.02 μg/ml and for neoflazid it was 0.083 μg/ml.

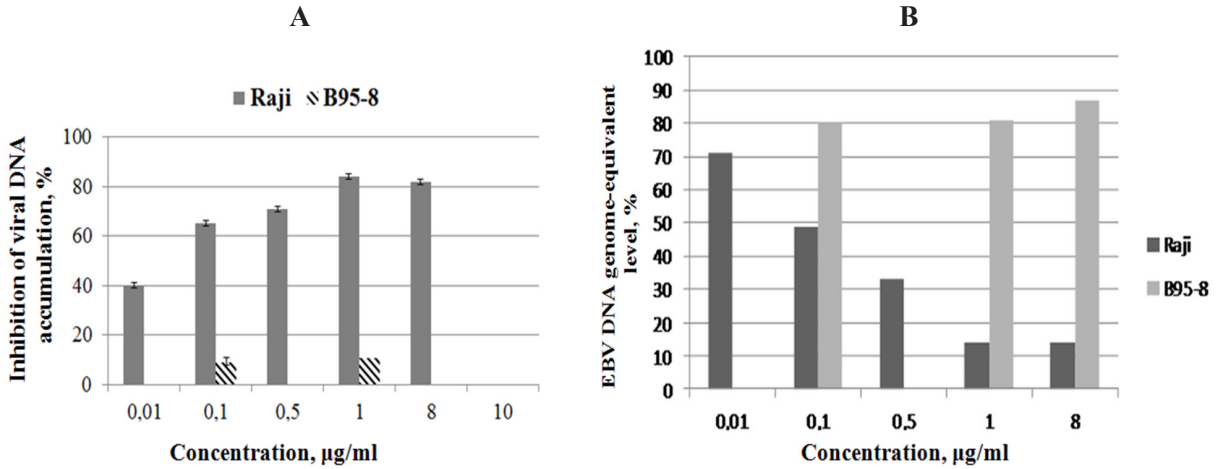


F i g. 2. Cytotoxic effect of the plant extracts neoflazid on Raji (A) and B95-8 cells (B)

Table 1

Cytotoxicity of proteflazid and neoflazid in CC_{50} (in µg/ml) to monolayers of CHO and MDB epithelial cell cultures K

Plant preparation	Cell culture	
	CHO	MDBK
proteflazid	96±2	131±4
neoflazid	74±3	102±2



F i g. 3. AntiEBV action of proteflazid (A) and neoflazid (B): inhibition of viral DNA accumulation in EBV-infected Raji and B95-8 cells

Same time, both preparations were ineffective and could not decrease the replication of viral DNA during chronic EBV infection, at which there was not marked any significant influence of the preparations.

Selectivity indexes

The potential of any compound to be used in medicine is assessed according to their selectivity indexes (SI), which is the ratio of CC_{50} to EC_{50} and, thus, is a "distance" between the concentration, at which the drug is affective as an antiviral, and the concentration, at which the drug is ineffective because of its high cytotoxicity [16]. More prospective compounds should have longer distances (higher SI). All new compounds should be compared with a reference drug like acyclovir.

In our study, proteflazid showed the highest antiviral activity against EBV acute infection and although it was much more cytotoxic in comparison to acyclovir, nevertheless, it SI was 1800 that is higher than SI of acyclovir – 23 (Table 2).

Table 2

Antiviral activity of the compounds obtained *in vitro* in Raji and B95-8 cell cultures

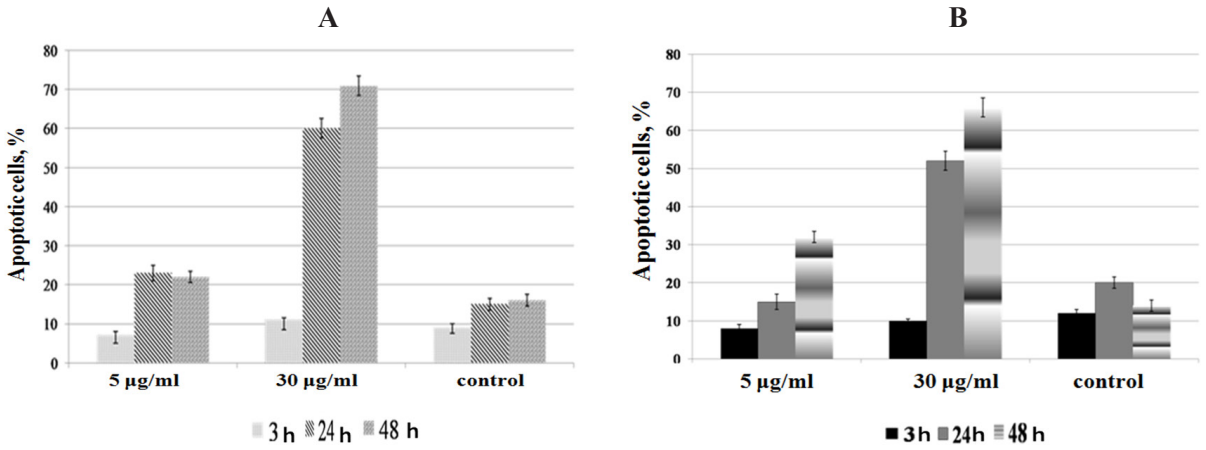
Compound	Raji cells			B95-8 cells		
	CC_{50}, μg/ml	EC_{50}, μg/ml	SI,	CC_{50}, μg/ml	EC_{50}, μg/ml	SI
proteflazid	36±2	0.02±0.001	1800±2	25±3	> 10	<2.5
neoflazid	8±1	0.083±0.001	96±2	24±2	> 10	<2.4
acyclovir	5000±54	220±15	23±2	3337±13	> 500	<6.6

The SI of neoflazid was higher than that of acyclovir, but too low in comparison to proteflazid. Both plant preparations and acyclovir as well were ineffective to treat chronic EBV infection.

Induction of apoptosis in lymphoblastoid cell cultures

Plant preparation proteflazid showed the highest potency to be used in medicine. But it demonstrated the relatively high toxicity for lymphoblastoid Raji and B95-8 cells. The corresponding CC_{50} values were 36 μg/ml and 25 μg/ml. Its higher toxicity for B95-8 cells, in which a lytic EBV reproduction occurs, may indicate that proteflazid is able to induce apoptosis, which, as known, is connected to lytic EBV proteins production [3–4].

Raji cells, treated with proteflazid at concentration of 5 μg/ml, showed a bit higher than 20 % level of apoptosis after 24 h and 48 h that was significantly by 5–7 % higher than in control cells where the average apoptosis level was 15 % (Fig. 4 A).



F i g. 4. Apoptosis-inducing action of proteflazid in latent EBV-infected Raji cells (A) and B95-8 cells (B)

Proteflazid at concentration of 30 μg/ml increased the percentage of apoptotic cells to 60 % after 24 h and 71 % after 48 h. This data almost coincides with the cytotoxicity studies, in which 18 % of dead cells in the population was detected at low concentration and over 50 % of dead cells were observed at higher concentrations of proteflazid. This indicates that apoptosis is the main way of cell death in Raji cells treated with proteflazid.

B95-8 cells treated with proteflazid at concentration of 5 μg/ml showed a 30 % level of apoptosis after 48 h (Fig. 4B). This result was in agreement with the MTT-assay values, by which 44 % decrease of population viability was detected. Nevertheless, the higher concentration of proteflazid (30 μg/ml) increased the level of apoptosis to 50 % and 70 % after 24 h and 48 h, respectively. Thus, we can assume that proteflazid induces apoptotic cell death of B95-8 and Raji cells.

Discussion

Plant preparations proteflazid and neoflazid both are recommended for use in medical practice as a comprehensive preparations with direct antiviral action (they can inhibit activity of virus specific enzymes – thymidine kinase and DNA polymerase) and immunocorrective action (they can induce interferon synthesis in cells modified with virus) [10–11, 21, 22].

In the current work we showed that proteflazid and neoflazid are more toxic towards lymphoblastoid cells (Raji and B95-8) rather than epithelial cells (MDBK and CHO), towards

which they showed almost 3 times lower toxicity. This toxicity may originate of the nature of these preparations: they are the plant extracts and thus contain a mixture of biologically active components, which may exhibit cytotoxic effects.

Proteflazid and neoflazyd showed high antiviral activity against EBV lytic infection in Raji cells (SI were 1800 and 96, respectively). In case of B95-8 cells, antiviral effect was minimal. The different sensitivity of Raji cells in comparison to B95-8 cells may be due to the mechanisms of homeostasis, repair and detoxification, which are more active just in virus-producing B95-8 cells, in which a chronic production of virus occur that is require permanent work of mechanisms involved in leveling of cytolytic action of virus. One of the mechanisms of antiviral action of these compounds is the induction of endogenous interferon, however, this feature was not realized in B95-8 cells considering that there were not observed an inhibition of viral DNA expression. This may be due to a low level of induction of interferon synthesis or due to neutralization of interferon by viral mechanisms [23–25]. It was shown that during lytic EBV infection a negligibly low level of interferon synthesis took place [26, 27]. However, the ability of these compounds to inhibit the replication of EBV in Raji cells may indicate the presence of other mechanisms of antiviral action that does not relate to the induction of interferon.

Induction of cell death limits viral production and reduces or eliminates viral progeny, but many viruses, including EBV, can blockade apoptosis of infected cells. For this purpose, the viral genome encodes specific proteins, most of which focused on one of the key regulatory elements that lead to apoptosis. Proteflazid was found be able to induce apoptosis during the lytic and latent EBV infections at cytotoxic concentrations of the compounds. Previously, it was studied an ability of proteflazid to increase the cytotoxicity of anticancer drug "Etoposide" in cell culture MT-4 [10], but there were not registered any apoptotic effect. Ability of proteflazid to induce apoptosis in cell cultures Raji and B95-8 may be connected to their EBV-positivity: it is likely that the mechanism of apoptosis induction by proteflazid is associated with its direct action on latent or lytic EBV-proteins and as a result the process of apoptosis is switched on.

Thus, both plant preparations proteflazid and neoflazid possess high antiviral activity under acute EBV infection. Proteflazid, in addition, may induce apoptosis in EBV-positive lymphoblastoid cells.

АНТИВІРУСНА АКТИВНІСТЬ ЕКСТРАКТІВ З ДИКИХ ТРАВ ПРОТИ ВІРУСУ ЕПШТЕЙНА-БАРР ТА ІНДУКЦІЯ АПОПТОЗУ У ВІРУС ЕПШТЕЙНА-БАРР-ПОЗИТИВНИХ ЛІМФОБЛАСТОЇДНИХ КЛІТИНАХ

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Резюме

Вірус Епштейна-Барр (ВЕБ) відноситься до родини герпесвірусів людини, що інфікує понад 90 % населення. Вірус викликає гостру інфекцію (інфекційний мононуклеоз) і асоційований з великою кількістю лімфопроліферативних та аутоімунних захворювань. Пошук лікарських засобів, які б інгібували репродукцію вірусу, а також могли б стимулювати елімінацію пухлинних клітин є важливим питанням для лікування вірус-асоційованих пухлин. **Мета.** Дослідити антивірусну дію та апоптотичну активність рослинних препаратів протефлазіду та неофлазіду на моделі латентної, гострої та літичної ВЕБ-інфекції в лімфобластоїдних клітинах Raji та B95-8. **Методи**. Дослідження були проведені в лімфобластоїдних лініях клітин Raji та B95-8, які продукують ВЕБ (B95-8) або не продукують його (клітини Raji). Досліджувані речовини: рослинні препарати протефлазід та неофлазід. Як референс препарат використовували ациклогуанозин. Цитотоксичну дію виявляли методом фарбування клітин барвником трипановим синім та МТТ-аналізом. Антивірусну активність визначали полімеразною ланцюговою реакцією в реальному часі. Детекцію апоптотичних клітин проводили методом проточної цитофлюориметрії. **Результати.** В результаті проведених досліджень виявили, що неофлазід був токсичнішим за протефлазід в клітинах Raji: показники цитотоксичної концентрації (CC_{50}) становили 8 мкг/мл та 36 мкг/мл відповідно. В клітинах B95-8 цитотоксичність цих препаратів була майже однаковою – їх показники CC_{50} становили 25 мкг/мл. Обидва препарати проявили високу антивірусну активність проти літичної ВЕБ-інфекції в клітинах Raji. Ефективна концентрація (EC_{50}) становила 0,02 і 0,083 мкг/мл для протефлазіда та неофлазіда відповідно, їхні індекси селективності (SI) склали 1800 і 96. Однак препарати не були ефективними

в клітинах B95-8: навіть за концентрації 10 мкг/мл інгібування вірусної реплікації становило тільки 10–19 %. На моделі латентної та літичної ВЕБ-інфекції через 48 год інкубації з протефлазідом в цитотоксичній концентрації (30 мкг/мл) нами було виявлена здатність препарату індукувати апоптотичну загибель клітинної популяції до 70 %. При використанні препарату в нетоксичній концентрації (5 мкг/мл) відсоток апоптотичних клітин не перевищував 30 %. **Обговорення.** Рослинні препарати протефлазід та неофлазід рекомендується використовувати в медичній практиці як комплексні препарати з прямою противірусною та імунокорекційною дією. У своїй роботі ми показали, що протефлазід та неофлазід є більш токсичними щодо вірус-асоційованих лімфобластоїдних клітин (Raji та B95-8), але не для епітеліальних клітин. Протефлазід і неофлазід виявляли високу противірусну активність в умовах літичної ВЕБ-інфекції в клітинах Raji (SI були 1800 і 96 відповідно). У випадку клітин B95-8 антивірусний ефект був мінімальним. Різниця у чутливості між клітинами Raji та B95-8 може бути обумовлена механізмами гомеостазу, відновлення та детоксикації, які активніші саме в клітинах B95-8, в яких відбувається хронічна продукція вірусу, тобто відбувається постійна робота механізмів, що беруть участь у нівелюванні цитолітичної дії вірусу. Одним з механізмів противірусної дії досліджених сполук є індукція ендогенного інтерферону, проте ця дія не була реалізована в клітинах B95-8, враховуючи, що не спостерігалося пригнічення експресії вірусної ДНК. Це може бути пов'язано з низьким рівнем індукції синтезу інтерферону або через нейтралізацію інтерферону вірусними механізмами. Було показано, що під час літичної ВЕБ-інфекції відбувався незначно низький рівень синтезу інтерферону. Однак здатність досліджених препаратів інгібувати реплікацію ВЕБ в клітинах Raji може свідчити про наявність інших механізмів противірусної дії, що не стосується індукції інтерферону. Індукція загибелі клітин обмежує вироблення вірусу і зменшує або усуває вірусне потомство, проте багато вірусів, включаючи вірус Епштейна-Барр, можуть блокувати апоптоз у заражених клітинах. З цією метою вірусний геном кодує специфічні білки, більшість з яких зосереджена на одному з ключових регуляторних елементів, що призводить до апоптозу. Було виявлено, що протефлазід у цитотоксичних концентраціях може викликати апоптоз під час літичної та прихованої ВЕБ-інфекції. Раніше була виявлена здатність протефлазіду підвищувати цитотоксичність протипухлинного препарату «Етопозид» у клітинній культурі MT-4 [10], але не було зафіксовано жодного апоптотичного ефекту. Здатність протефлазіду індукувати апоптоз в культурах клітин Raji та B95-8 може бути пов'язана з їхньою ВЕБ-позитивністю. Цілком ймовірно, що механізм індукції апоптозу протефлазідом пов'язаний з його прямою дією на приховані або літичні ВЕБ-білки і, як результат, вмикається процес апоптозу. Таким чином, рослинні препарати, як протефлазід, так і неофлазід, мають високу противірусну активність при гострій інфекції, що передається ВЕБ. Крім того, протефлазід може викликати апоптоз у ВЕБ-позитивних лімфобластоїдних клітинах. **Висновки.** В результаті проведених досліджень було показано, що протефлазід та неофлазід мають високу антивірусну активність проти гострої ВЕБ-інфекції. Протефлазід також індукує дозозалежну апоптотичну загибель ВЕБ-позитивних лімфобластоїдних клітин.

Ключові слова: вірус Епштейна-Барр, протефлазід, неофлазід, противірусна активність, апоптоз.

1. King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ. Virus Taxonomy. Classification and nomenclature of viruses. IXth Report of the International Committee on Taxonomy of Viruses. Elsevier Acad. Press; 2012. p. 111–123.
2. Lu Ch-Ch, Chen M-R. Lytic replication of Epstein-Barr virus. Future Virol. 2006; 1(4):435–46.
3. Kutok JL, Wang F. Spectrum of Epstein-Barr virus-Associated Diseases. Annu Rev Mech Dis. 2006; 4(1):375–404.
4. Ayee R, Ofori MEO, Wright E, Quaye O. Epstein Barr virus associated lymphomas and epithelia cancers in humans. J Cancer. 2020; 11(7):1737–50.
5. Mateeva N, Eyunni SVK, Redda KK, Ononuju U, Hansberry TD, Aikens C, et al. Functional Evaluation of Synthetic Flavonoids and Chalcones for Potential Antiviral and Anticancer Properties. Bioorg Med Chem Lett. 2017; 27(11):2350–6.
6. Zakaryan, H, Arabyan E, Oo A, Zandi Kl. Flavonoids: promising natural compounds against viral

infections. Arch Virol. 2017; 162:2539–51.
7. Chahar MK, Sharma N, Dophal MP, Joshi YC. Flavonoid: a versatile source of anticancer drugs. Pharmacogn Rev. 2011; 5(9):1–12.
8. Ben-Shabat S, Yarmolinsky L, Porat D, Dahan A. Antiviral effect of phytochemicals from medical plants: applicatiobs and drug delivery strategieg. Drug Deliv Transl Res. 2020; 10(2):354–67.
9. Lee M, Son M, Ryu E, Shin YS, Kim JG, Kang BW, et al. Quercetin-induced apoptosis prevents EBV infection. Oncotarget. 2015; 6(14):12603–24.
10. Rybalko SL, Dyadyun ST, Rudenko AV, et al. Proteflazid. Clearing materials on the properties and methods. Kiev: SPC "Ekofarm"; 2002.
11. Matias VI, Bobrovaya IA, Shevchuk VB. Pharmacotherapeutic mechanisms of Proteflazid with viral. Problems of epidemiology, diagnostic, clinical, treatment and prevention of infective disease. Kiev; 2002. p. 381–4.
12. European Collection of Animall Cell Cultures Cataloge https://www.phe-culturecollections.org.uk/collections/ecacc.aspx.
13. Lan K, Verma SC, Murakami M, Bajaj B, Robertson ES. Epstein-Barr Virus (EBV): Infection, Propagation, Quantitation, and Storage. Current Protocols in Microbiology. 2007; 14(14). doi: 10.1002/9780471729259.mc14e02s6.
14. Wolls E, Kroford D. Kultivirovanie kletok B95-8. Limfocyty. Metody. Moscow: Mir, 1990. p 230–49.
15. Sylvester PW. Optimization of the tetrazolium dye (MTT) colorimetric assay for cellular growth and viability. Methods Mol Biol. 2011; 716:157–68.
16. Guidance for Inductry antiviral product development – conducting and submitting virology studies to the Agency. U.S. Department of Health and Human Services Food and Drug Administration. 2006. Rockville, p. 17.
17. Ulukaya E, Acilan C, Ari F, İkitimur E, Yilmaz Y.A glance at the methods for detection of apoptosis qualitatively and quantitatively. Turk J Biochem. 2011; 36(3):261–9.
18. Nicoletti I, Migliorati G, Migliorati MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Meth. 1991; 139:271–9.
19. McDonald JH. Handbook in biological statistics. 3d ed. Baltimore: Sparky House Publishing; 2014.
20. Krzywinski M, Altman N. Error bars. Nature Meth. 2013; 10:921–2.
21. Rybalko CK, Dyadyun ST, Atamanyuk VP, et al. Vyvchennya antyherpetychnoyi aktyvnosti proteflazydu. Visnyk Vinnytskogo derzh univer. 2002; 2:350–3.
22. Usachova OV. Efektyvnist' preparatu "Proteflazyd" pry likuvanni ditey pershogo roku zhyttya z vnutrishn'outrobnoyu tsytomegalovirusnoyu infektsiyeyu. Reproduktivnoe zdorov'e zhenshtciny. 2005; 22(2):165–6.
23. Fuji N, Oguma A. Induction of Oligo-2',5'-adenylate Synthetase in Human Lymphoid Cells Treated with 5-Azacytosine and 5-Iododeoxyuridine . J gen Virol. 1986; 67:2521–6.
24. Bentz GL, Liu R, Hahn AM, Shackelford J, Pagano JS. Epstein-Barr virus BRLF1 inhibits transcription of IRF3 and IRF7 and suppresses induction of interferon-β. Virol. 2010; 402(1):121–8.
25. Wang JT, Doong SL, Teng SC, et al. Epstein-Barr virus BGLF4 kinase suppresses the interferon regulatory factor 3 signaling pathway. J Virol. 2009; 83(4):1856–69.
26. Kanda K, Kempkes B, Bornkamm GW, von Gabain A, T. Decker T. The Epstein-Barr virus nuclear antigen 2 (EBNA2), a protein required for B lymphocyte immortalization, induces the synthesis of type I interferon in Burkitt's lymphoma cell lines. Biol Chem. 1999; 380(2):213–21.
27. Malmgaard L. Induction and regulation of IFNs during viral. J INTERF CYTOK RES. 2004; 24: 439–54.

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