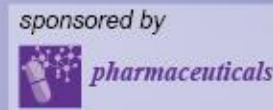




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Antiviral Activity of Extracts from Wild Grasses against Epstein-Barr Virus and Induction of Apoptosis in EBV-positive Lymphoblastoid Cells

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Abstract: Epstein-Barr virus (EBV) is associated with a number of lymphoproliferative and autoimmune diseases. Use 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. In the current research the antiviral effects of the herbal extracts Proteflazid and Neoflazid were studied on the models of latent, acute and chronic EBV infections in Raji and B95-8 lymphoblastoid cells. Neoflazid was more toxic towards Raji cells than Proteflazid: the CC_{50} indices were 8 $\mu\text{g/ml}$ and 36 $\mu\text{g/ml}$ respectively. Toxicities of these compounds in B95-8 cells were almost the same and their CC_{50} indices were close to 25 $\mu\text{g/ml}$. Both drugs showed high antiviral activity against EBV lytic infection in Raji cells and EC_{50} was 0.02 and 0.083 $\mu\text{g/ml}$ for Proteflazid and Neoflazid, respectively, and selectivity indices were 1800 and 96. They were less effective in B95-8 cells and even at a concentration of 10 $\mu\text{g/ml}$ these compounds inhibited virus replication by only 10-19%. We checked the ability of Proteflazid to induce apoptosis and found that the drug stimulated the apoptotic cell death in latent and lytic EBV infections at cytotoxic concentrations (30 $\mu\text{g/ml}$). The non-toxic concentration (5 $\mu\text{g/ml}$) induced apoptosis more actively (by 10%) during EBV lytic infection in cells B95-8 than in the case of latent infection in Raji cells.

Keywords: Epstein-Barr virus; Proteflazid; Neoflazid; antiviral activity; apoptosis.



Introduction

EBV is associated with a number of cancers mainly lymphoproliferative (lymphoma, nasopharyngeal carcinoma, Burkitt's lymphoma) and autoimmune diseases. Drugs based on plant preparations are widely used for treatment of herpes diseases. For example, flavonoids, which are the derivatives of phenolic compounds. They show wide phytotherapeutic 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 and antiviral effects. The advances in knowledge of fundamental ideas of molecular mechanisms of viral oncogenesis, of operation and interaction between viral and cellular oncogenes, resulted in development of a new approach that help to search antiviral drugs suitable not only effectively inhibit the replication of the virus, but also stimulate cell renewal, or cause the elimination of infected cells, in particular by initiation of the programmed cell death program (apoptosis). 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 extracts in models of lytic and latent EBV infections *in vitro*.



Results and discussion

Cytotoxicity and AntiEBVactivity of plant extracts to lymphoblastoid cells

Cytotoxicity tests: Trypan blue staining (TBS) method and MTT-assay for study of cell viability. Determination of antiviral activity was performed using RT-PCR tests were performed with "AmpliSens®EBV-screen-FL» (AmpliSens, Russia) according to the manufacturer's recommendations. The amplicator device was Thermocyclers qTOWER 2.2 (Analytic Jena, Germany).

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 (Table), that was much more than SI of Acyclovir (23). The SI of Neoflazid was higher than that of Acyclovir, but too low in comparison to Proteflazid. Both plant extracts and Acyclovir as well were ineffective to treat chronic EBV infection.

Samples	Raji			B95-8		
	CC ₅₀ , μg/ml	EC ₅₀ , μg/ml	SI,	CC ₅₀ , μg/ml	EC ₅₀ , μg/ml	SI,
Proteflazid	36	0.02	1800	25	> 10	<2.5
Neoflazid	8	0.083	96	24	> 10	<2.4
Acyclovir	5000	220	23	3337	> 500	<6.6

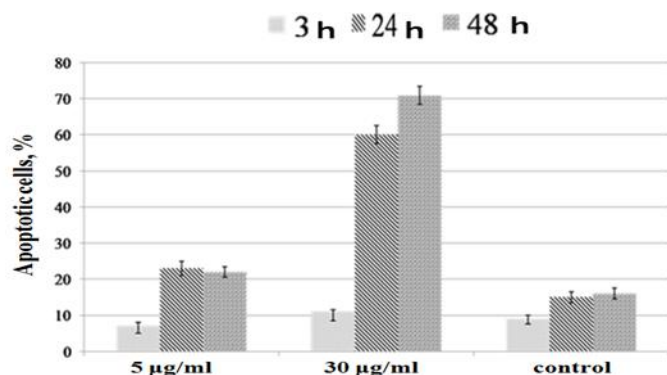


Induction of apoptosis in lymphoblastoid cell cultures

Detection of apoptotic cells analyzed with flow cytometer (50 $\mu\text{g/ml}$ PI, 0.1% (w/v) sodium citrate, 0.1% (w/v) Triton X-100, in sdH₂O) 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.

Raji cells, treated with Proteflazid at concentration 5 $\mu\text{g/ml}$, showed a bit higher than 20% level of apoptosis after 24 h and 48 h), at concentration 30 $\mu\text{g/ml}$ increased the percentage of apoptotic cells to 60% after 24 h and 71% after 48 h. B95-8 cells treated with Proteflazid at concentration 5 $\mu\text{g/ml}$ showed a 30% level of apoptosis after 48 h. Nevertheless, the higher concentration of Proteflazid 30 $\mu\text{g/ml}$ increased the level of apoptosis to 50% and 70% after 24 h and 48 h, correspondingly. This data almost coincide with the cytotoxicity studies. This indicates that apoptosis is the main way of cell death in cells treated with Proteflazid (Figure)

A



B

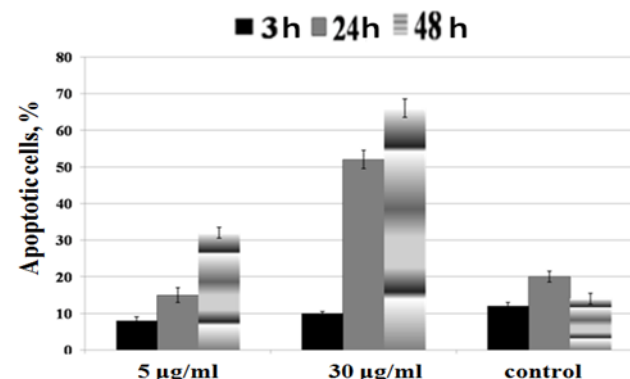


Figure Apoptosis-inducing action of Proteflazid of latent EBV-infected Raji (A) and B95-8 (B) cell.



Discussion

In the current work we showed that Proteflazid and Neoflazid showed high antiviral activity against EBV lytic infection in Raji cells. 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 occurs that requires 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 expression of viral DNA. This may be due to a low level of induction of synthesis of interferon or due to neutralization of interferon by viral mechanisms. It was shown that during lytic EBV infection a negligibly low level of synthesis of interferon took place. 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.



Discussion

Induction of cell death limits viral production and reduces or eliminates viral progeny, but many viruses, including Epstein-Barr virus, can blockade apoptosis in 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 compounds. Previously, it was studied an ability of Proteflazid to increase the cytotoxicity of anticancer drug Etoposide in cell culture MT-4 , but there were not registered any apoptotic effect. Ability of Proteflazid to induce apoptosis in Raji and B95-8 cell cultures may be connected to their EBV-positivity: it is likely that the mechanism of induction of apoptosis with Proteflazid is associated with it direct action on latent or lytic EBV-proteins and as a result the process of apoptosis is switched on.

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



Conclusions

- 1. Proteflazid and Neoflazyd showed high antiviral activity against EBV lytic infection in Raji cells and EC_{50} was 0.02 $\mu\text{g}/\text{ml}$ and 0.083 $\mu\text{g}/\text{ml}$, respectively. IS was 1800 and 96, correspondingly. In case of B95-8 cells, even at highest of the analyzed concentrations 10 $\mu\text{g}/\text{ml}$, these extracts inhibited virus replication by 10-19% only**
- 2. Thus, we can assume that Proteflazid induces apoptotic cell death of B95-8 and Raji cells.**



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