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Effectiveness of Antiviral Compounds at The Adeno-Herpetic Co-Infection

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Abstract: Viral co-infection is one of the current and unexplored issues of human infectious diseases. A special place in the progression of these pathologies is occupied by adeno- and herpes viruses being able to long persist in the body. There is a huge lack of knowledge about antiviral activities of specific drugs during the mixed infections. For the testing of the antiviral medication acyclovir (ACV) and for research of new fluorine-containing analog of L-phenylalanine (10S-24), the model of simultaneous adeno-herpetic infection of MDBK cells was used. Determination of the antiviral effect using cytomorphology method, infectious virus yield reduction assay and cell cycle analysis demonstrated the inhibitory effect of the 10S24 on the late phase of the HSV-1 and HAdV5 reproduction. Decreased activity of the substance was resulted by 16-57% compared to HSV-1 through the application of 10S-24 at mixed infection. The use of the ACV at mixed infection, reduces its activity against HSV-1 by 16 - 46%. It was shown that compounds were not effective against HAdV5 at the mixed infection of cells. Further investigation is needed to explore the mechanisms of the loss of antiviral activity of compounds.

Keywords: mixed infection, antiviral activity, fluorine-containing compound, inclusion bodies, cell cycle

INTRODUCTION

The widespread of viral infections with the development of mixed infection of patients is an actual and important problem of medicine (Da Palma,et al.2010). Adeno- and herpes viruses are among the most prevalent human pathogens. They are agents of the high spectrum of diseases ranging from acute respiratory diseases to neoplastic symptoms (Arvin, A,et al.2007, Ghebremedhin, B.2014). The viruses' tropism to the identical tissues creates the possibility of simultaneous infections of the host organism with the forming of a mixed viral infection (Tucker,et al.1975, Spector,et al.1978, Waner, J.1994). A significant increase of the levels of herpetic and adenoviral infectious diseases among both adults and children cause a necessity of comprehensive studies of such infections and the elaboration of effective methods for prevention and treatment of various forms of pathologies induced by these viruses.

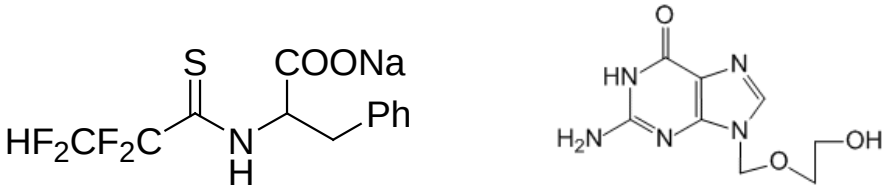
Synthetic nucleoside and non-nucleoside analogs have a broad of biological activities; the effective anticancer and antiviral agents were found among them (De Clercq,et al.2016, Strand, M.2014). Further progress in the creation of efficient antiviral drugs is linked to the synthesis of the fluorinated analogs and the studying of the mechanism of their actions are the pressing issue of our time, because as we know the introduction of fluorine atoms in the molecule significantly affects the physical, and biochemical properties of the molecule (Qiu,et al.2004, Ojima, I.2009). Currently, in vitro and in vivo studies are ongoing to expand the spectrum of action of existing effective antiviral drugs and the search for new antiviral compounds, but the effectiveness of these

drugs at the mixed viral infection is almost not investigated. Whereas, clinical studies indicate that the use of a drug relative to a single virus can affect the associated-viruses reproduction (Audsley,et al.2009, McMahon,et al.2008). The study of known drugs and new compounds using not only standard mono-infections but also created mixed infections is a topical and a new direction in antivirus screening (Biliavska,et al.2017). If the models of mixed infection are used for researching of the modern of antiviral medicaments and for the preclinical research of new compounds with potential antiviral activity; a new data on the role of virus-virus interactions in the development of virus's resistance to the known antiviral compounds will be obtained.

Materials and Methods

Virus and cells: The Madin-Darby Bovine Kidney (MDBK), the type 5 of human adenovirus (HAdV5) and the strain US of herpes simplex virus type 1 (HSV-1/US) were used in this study. The cells and viruses were cultured according to standard methods.

Tested substances: Antiherpetic medicament acyclovir (ACV) and fluorinated derivative - sodium (2,2,3,3-tetrafluoropropanethioyl)-L-phenylalaninate (10S-24) were synthesized in Institute of Organic Chemistry NAS of Ukraine (fig.1). It was purified by chromatography on silica gel, eluting with a mixture (9:1) of hexane and ethyl acetate (Yield: 84%). The general characteristic of compound was shown by Pikun et al (2016). The compounds were dissolved in RPMI medium and sterilized by filtration through 0.2 μm Millipore filters. Only fresh solutions were used.



10S-24 *Acyclovir*

Fig.1. Structure of compounds

Cellular toxicity: The cellular toxicity of compounds was tested *in vitro* according to a MTT assay previously reported (Bag, P,et al.2012). Monolayers of MDBK cells were incubated with the compounds at concentration of 1000 – 15.6 μg/ml for 48 h. Next, 20 μl of MTT solution (Sigma) was added into the medium. The plates were detected using an automatic plate reader Multiskan FC (Thermo Scientific, USA) with a 538 nm test wavelength. Value of CC_{50} was detected.

Antiviral Assay: Cells were grown in tubes with strips of cover glasses for 24 h. Subsequently, the cells were infected with HSV-1 (at a MOI of 3.2), HAdV-5 (at a MOI of 7), and a suspension of both viruses and incubated for 2 h at room temperature (Biliavska,et al.2017). The unabsorbed virus was removed and the substances under study were added at the appropriate concentrations (150 – 4 μg/ml). After 48 h, the infected cells were fixed with 96% ethanol, washed with Henks Solution, and stained with 0.01% acridine orange solution. The number of infected cells with virus inclusion bodies was counted using a fluorescence microscope (Alexeeva,et al.2000).

Infectious virus yield reduction assay: MDBK cells were infected with HSV-1 (at a MOI of 3.2), HAdV-5 (at a MOI of 7), and a suspension of viruses. After adsorption, the compounds at concentrations of 150 - 4 μg/ml were added. After 72 h, viruses' titers were studied by a cytomorphological method in MDBK cells. Confluent MDBK cell monolayers were then infected with 200 μl of ten-fold serial dilutions of the virus-containing suspension, and allowed to adsorb for 2 hours. Then 800 μl of medium was added, and incubated for 48 hours. The quantity of inclusion-forming cells was explored and the decrease of virus titer was calculated:

$$\% \text{ Percentage inhibition of virus reproduction} = (1 - T/C)*100\%$$

where T is the compounds-treated viral titers and C is the control (viral titers without compounds).

Cell Cycle Analysis: Mono- and co-infected cells treated and not treated of the substances (1×10^6) were harvested by centrifugation at 300 g (2000 rpm) for 7 min, resuspended in 96% ice-cold ethanol, resuspended in 300 µl solution of PBS that contained RNAse (100 µg/ml) and PI (50 µg/ml), and incubated at 20°C for 1 h (Kim K.H,et al.2015). The cell fluorescence intensity was measured by a flow cytometer (Beckman Coulter Epics LX, USA) with laser wavelength 488 nm. Cell cycle profiles were analyzed with the program Flowing Software, version 2.5

Results and discussion

The concentrations of substances used for the antiviral activity of the compounds did not induce any morphological changes in MDBK cells. The ACV and 10S-24 at a concentration of the 500 µg/ml have shown a little cytotoxic effect, and ~96% of cells survived. The CC_{50} value of the compound10S-24 for cells was 1004.2±10.8 µg/ml and for acyclovir, it was more than 1000 µg/ml.

Previously in our department, the models of adeno-herpetic infections in cells of different origins were created and the features of the viruses' reproduction in these systems were studied (Biliavska,et al.2014, Zagorodnya,et al.2016). The antiviral effects of compounds against viruses under the conditions of mono- and mixed infections were evaluated by a cytomorphological method, used for identify infected cells containing virus-specific inclusion bodies that can be found with fluorescent microscopy after staining cells with acridine orange (fig.2).

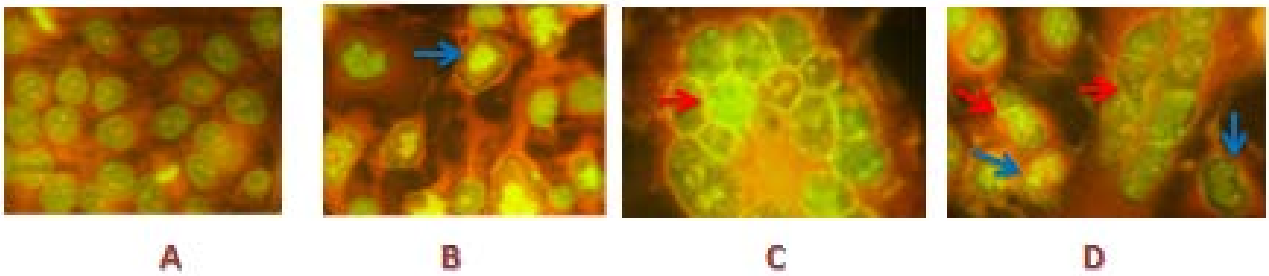


Fig.2. Cytomorphological features of virus infection in MDBK cells

Luminescent microscopy of cells stained with 0.01% acridine orange solution. A – uninfected cells, B – HADV5 infected cells, C – HSV-1 infected cells, D – co-infected cells with herpesvirus (red arrow) and adenovirus (blue arrow) inclusions (48 p.i.)Cells were processed with the compounds in the growth medium at non-toxic concentrations after virus adsorption. The data on inhibition of virus reproduction by the substances under study in mono- and co-infected cells are given in figure 3.

Both 10S-24 and ACV inhibited viruses' reproduction in a dose-dependent manner. 10S-24 at concentrations of 50 – 150 µg/ml inhibited HSV-1 and HAdV-5 inclusions formation by 41 – 67 % and 36%, respectively.

The use of 10S-24 in conditions of co-infection has caused inhibition of the adenovirus reproduction up to 28% and of herpes virus up to 58%. The analysis of antiviral activity of Acyclovir in the model of monoinfections showed the reduction of reproduction of HSV-1 /US by 55 – 100% and HAdV-5 by 27%. The efficacy of the compound against adenovirus during the co-infection increased by 48%.

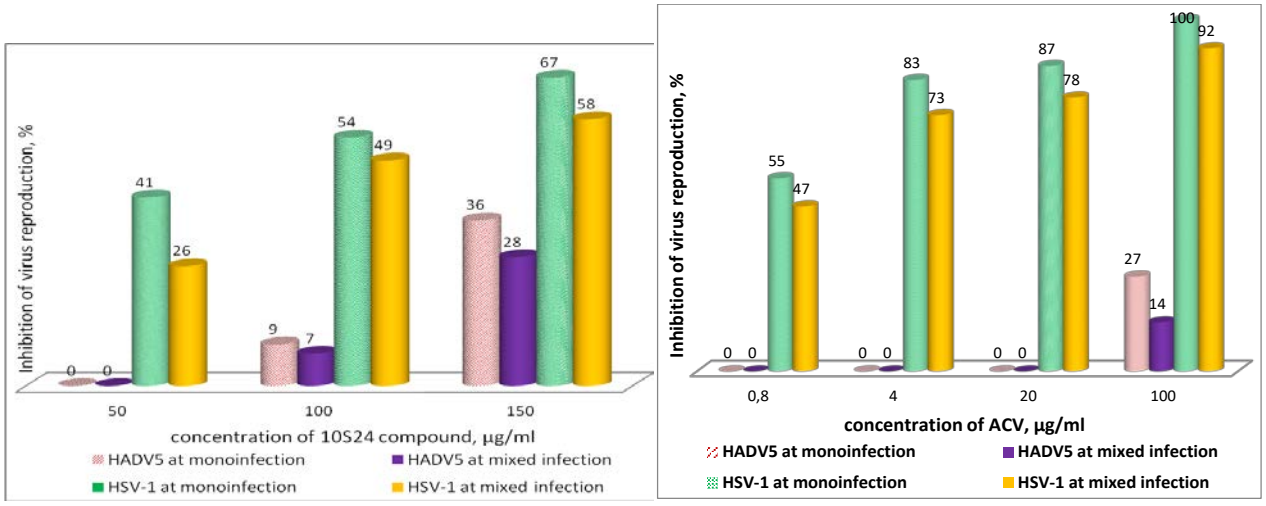


Fig.3. The influence of compounds on the infected cells under the condition of mono- and mixed infections

Based on previously reported findings and taking into account the chemical nature of 10S-24, we supposed that compound might block the shaping of a full and infectivity virus progeny. Phenylalanine residues are aromatic and hydrophobic amino acids that are known to be involved in protein-protein interactions and have been shown to play an important role in virus assembly and a critical regulatory function in infectious virus production (Charles,et al.2014).

An activity of 10S-24 and ACV, determined by yield reduction assay, was found against both viruses (table.1). The significant delays of HSV-1 reproductions were observed in all used concentrations of compounds; the titer of virus obtained *de novo* reduces by >99%. It was shown that for adenovirus infection of the cells, all compounds reduced the titer of the virus by 34 – 96%.

Table 1. Effect of compounds on infectivity of viral offspring

Compound (µg/ml)		Monoinfection				Mixed infection			
		HSV-1		HADV5		HSV-1		HADV5	
		Virus titer IFU/ml	% of inhibition	Virus titer IFU/ml	% of inhibition	Virus titer IFU/ml	% of inhibition	Virus titer IFU/ml	% of inhibition
ACV	100	-	100.00	7.3×10^5	62.00	2.8×10^3	97.86	3.5×10^4	13.78
	20	6.5×10^3	99.97	1.2×10^6	36.16	2.1×10^4	83.60	3.6×10^4	12.22
	4	2.0×10^4	99.91	1.3×10^6	34.95	5.2×10^4	60.02	3.6×10^4	11.71
	0,8	5.3×10^4	99.78	-	-	6.1×10^4	53.82	-	-
10S-24	150	1.4×10^4	99.94	8.4×10^4	95.67	3.6×10^4	72.25	3.9×10^4	5.21
	100	1.8×10^4	99.93	3.1×10^5	83.91	6.2×10^4	52.97	4.5×10^4	0
	50	1.9×10^4	99.92	7.2×10^5	62.91	7.5×10^4	43.10	5.4×10^4	0

Control of virus	$2.4x10^7$	-	$1.9x10^6$	-	$1.3x10^5$	-	$4.0x10^4$	-

The use of the ACV at mixed infection led to the 16 – 46% loss of the drug activity against HSV-1. The application of 10S-24 at mixed infection induced a decrease of effectiveness of the compound by 16-57% compared to HSV-1. It was shown that compounds were not effective against HAdV5 under mixed infection of cells.

"Virus infection frequently results in the disturbance of key cellular processes within the host cell. The subversion of cell cycle pathways is a well-established mechanism by which viruses create the most suitable environment for their replication. Notably, the induction of S-phase is either mandatory or at least advantageous for lytic replication of a number of viruses" [21(e100004)]. "The prominent role of cellular factors from the DNA synthesis machinery in viral replication was demonstrated for adenoviruses. Adenoviral infection has been reported to have effects on the cell cycle. It is well-known that adenoviral E1 gene products interact with pRb (retinoblastoma protein), causing the release of E2F transcription factor, which potentiates transition from G_1 to S phase, in which productivity is the greatest. HADV infection of a range of epithelial cell lines including a primary cell line causes G_2 phase synchronization and arrest. This synchronization in the G_2 phase may well be a significant factor contributing to the cell-size increase" (Sandhu, K,et al.2008). "In contrast, herpesviruses encode their own DNA polymerase and accessory proteins, and thus theoretically do not require an S-phase environment to support their replication" [21(e100004)].

To study the influence of growth state following viral infection, growing uninfected culture of cells were examined by FCM. There was a significant number of cells in the G_0/G_1 (49%) and S (28%) phases of the cell cycle (24 h p.i.) (fig.4). The adenovirus-infected cells underwent DNA synthesis with the accumulation of 39% cells in the S phase of the cycle, while herpetic infection leads to increase of the number of apoptotic cells to 33%. At a point when infected cells move into the S and G_2/M phase of the cell cycle, the cells are making viral DNA, late protein, and virions. These cells are dying, detaching from the monolayer, and floating in the supernatant.

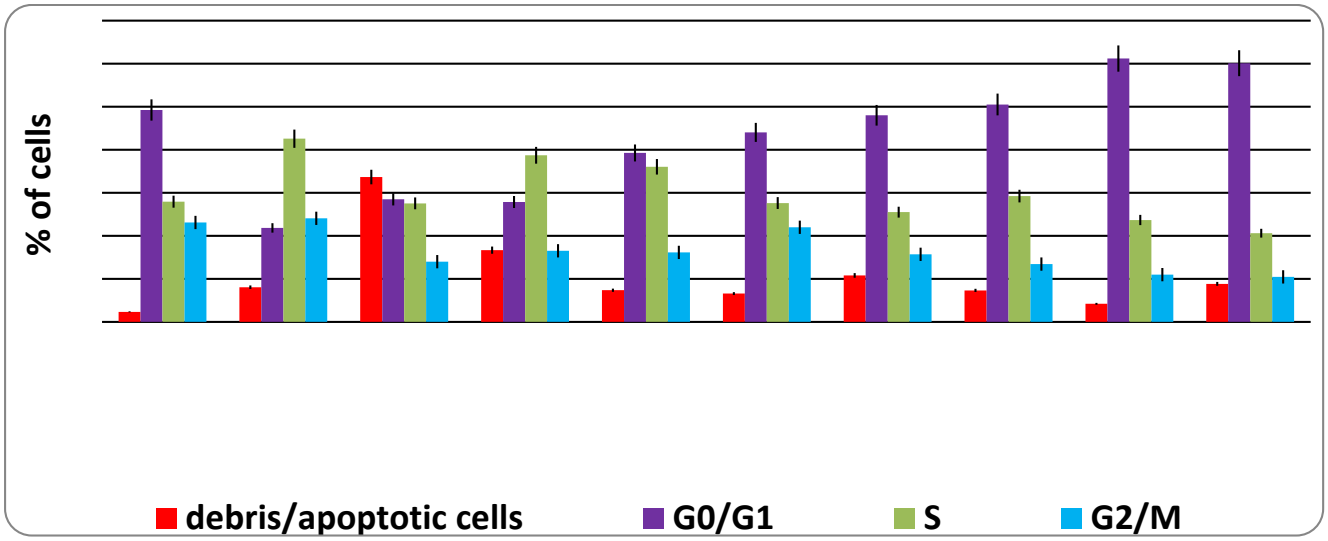


Fig.4. Influent of compounds on the cell cycle under condition of mono- and mixed infections

Cell cycle analysis of MDBK mono- and co-infected with HSV-1 and HAdV5 with the compounds. Cells were harvested 24 h pi and the cellular DNA content was analyzed by flow cytometry after staining with propidium iodide. The percentage of cells in the G_0/G_1, S, and G_2/M phases of the cell cycle is represented as bars.

The characteristic changes in DNA synthesis and content induced by HSV1 and HADV5 infection allow the use of flow cytometry to detect not only an infection but also the potential antiviral activities. It was found that under condition of drugs treated mono- and mixed infections, the number of G_0/G_1 cells increases to 38 – 61% and amount of the apoptotic cells decreases to 4 – 11%.

Conclusions

Our work is related to the investigation of the effectivity of the Acyclovir and the new fluorine-containing derivative of L-phenylalanine on the model of simultaneous adeno-herpetic infection of MDBK cells.

An abnormal activity of compounds in the case of virus co-infection of cells has been revealed, indicating that screening of drugs should take place not only on experimental mono infection but also on mixed infections. Because the formation of resistant strains of viruses in the process of their reproduction as a results of the formation of recombinant viruses, pseudotype viruses, mutants, etc., as well as inhibition of the functional activity of specific targets in the reproduction of co-infected viruses, occurs. Therefore, the further molecular-biology study of the regulatory elements of the viruses and cells continues to be important. It will enable the establishment of mechanisms for viruses' interaction and the development of viral infections in these conditions and identification of the causes and mechanisms of the ineffectiveness of drugs in conditions of mixed infection.

Acknowledgments

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