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Study of Antiviral Compounds in the Conditions of Mixed Infections

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Abstract:

Mixed viral infection is one of the current and unexplored issues of human infectious diseases. A special place in the development of these pathologies is occupied by adeno- and herpes viruses that are able to persist for a long time in a latent condition in the body. There is a huge lack of knowledge about antiviral activities of specific drugs during the mixed infections. 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.

Our work is related to the determination of the effectivity of the antiherpetic drug acyclovir (ACV) and the new fluorine-containing derivatives of L-phenylalanine on the model of simultaneous adeno-herpetic infection of MDBK cells.

Keywords: **mixed infection, antiviral activity, fluorine-containing compounds**

Introduction

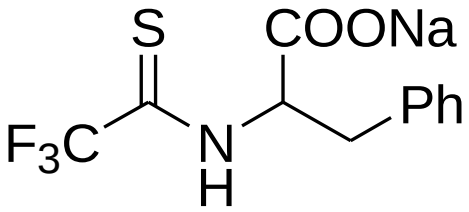
Adeno- and herpes viruses are among the most widespread human pathogens. They are agents of the high spectrum of diseases ranging from acute respiratory diseases to neoplastic symptoms. A considerable increase of the levels of herpetic and adenoviral infectious diseases among both adults and children causes a necessity of comprehensive studies of such infections and the development of effective methods for prevention and treatment of different forms of pathologies induced by these viruses.

The greatest success in the chemotherapy of the virus infections has been achieved with the using of abnormal nucleosides and non nucleosides that are inhibitors of viral polymerase and are included in the synthesis of a new chain of nucleic acids. Currently, *in vitro* and in *vivo* studies are ongoing to expand the range of existing effective antiviral drugs and the search for new antiviral compounds, but the activity of these drugs in the condition of mixed viral infection is almost not investigated. At the same time, clinical studies indicate that the use of a drug relative to a single virus can affect the reproduction of an associated virus.

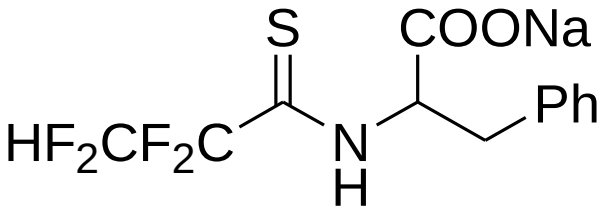
Results and discussion

Cells and viruses: Cell culture MDBK (bovine kidney), adenovirus serotype 5 (HAdV5), herpes simplex virus 1 (HSV-1/US) were used.

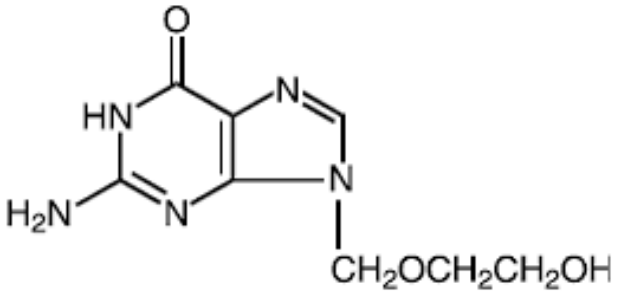
Tested substances: Antiherpetic drug acyclovir (ACV) and fluorinated derivatives - sodium (2,2,2- trifluoroethanethioyl)-L-phenylalaninate and sodium (2,2,3,3-tetrafluoropropanethioyl)-L-phenylalaninate (10S-23 and 10S-24) were synthesized in Institute of Organic Chemistry NAS of Ukraine by Nadiia V. Pikun.



10S-23



10S-24



Acyclovir

Structure of compounds

Cellular toxicity

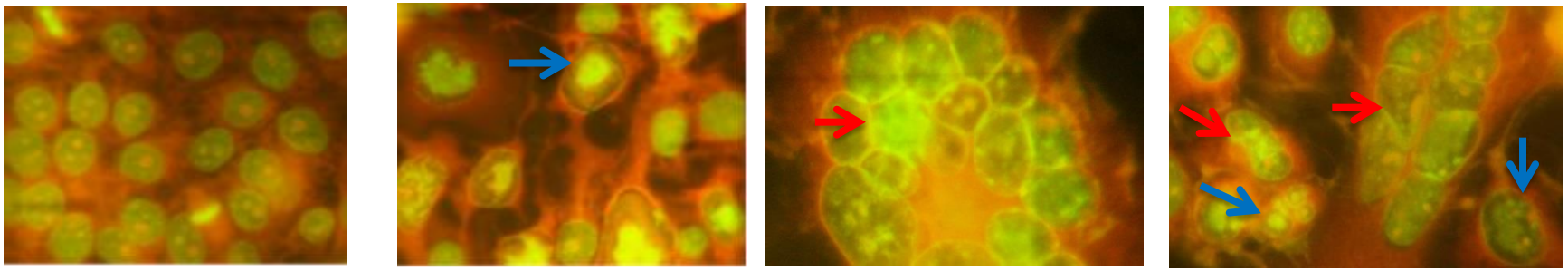
At the first stage of the antiviral assay it is necessary to determine the concentration of the compounds that is not toxic to the cells.

Cellular toxicity of compounds was tested *in vitro* according to a cell viability MTT-assay. Monolayers of MDBK cells in 96-multiwell plates were incubated with the compounds at concentration of 1000 – 15.6 µg/ml for 48 h. Next, 20 µl of a 5 mg/ml MTT solution (Sigma) was added into the medium. The plates were analyzed using an automatic plate reader Multiskan FC (Thermo Scientific, USA) with a 538 nm test wavelength. The concentrations of substances that inhibit 50% of cell viability compared to control cells (CC_{50}) were measured using a linear regression method in Microsoft Excel 10.

The compounds 10S-23 and 10S-24 at a concentration of 500 µg/ml exhibited little cytotoxic effect, and ~96% of cells survived. The CC_{50} values of the compounds were 1000.3±11.8 and 1004.2±9.7 µg/ml, respectively. The CC_{50} value of drug acyclovir (ACV) was more than 1000 µg/ml

Antiviral Assay

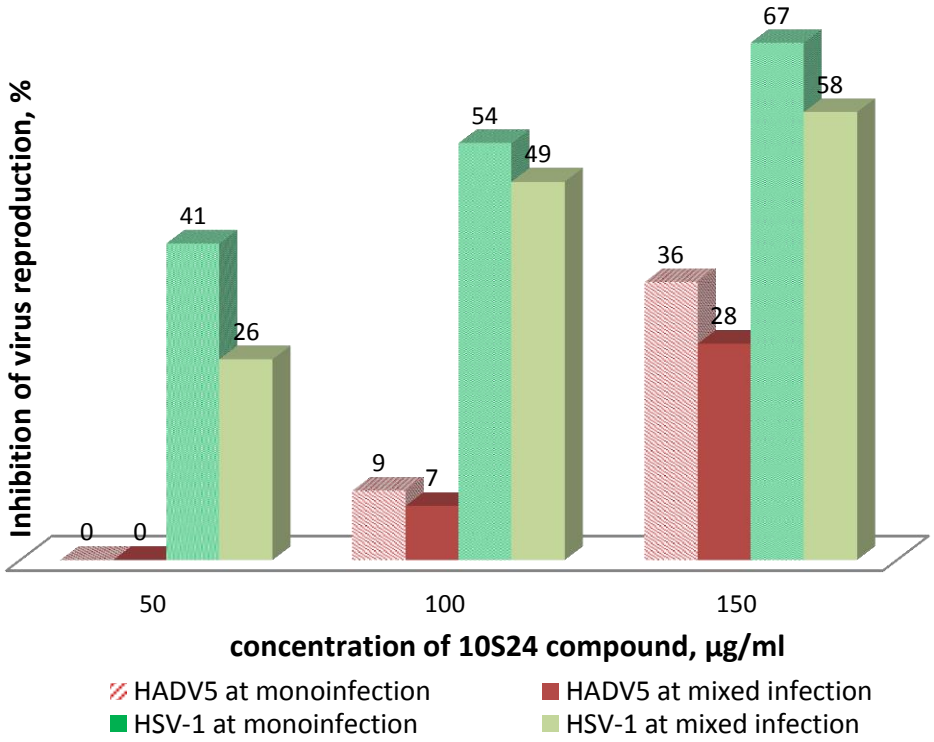
The antiviral effects of compounds against viruses under the conditions of mono- and mixed infections were evaluated by a cytomorphological method. It was used to identify infected cells containing specific intranuclear inclusion bodies induced by the viruses, that can be detected with fluorescent microscopy after staining of fixed cells with acridine orange (according to standard methodic previously reported). Cells were treated with the compounds in the growth medium at non-toxic concentrations after virus adsorption.

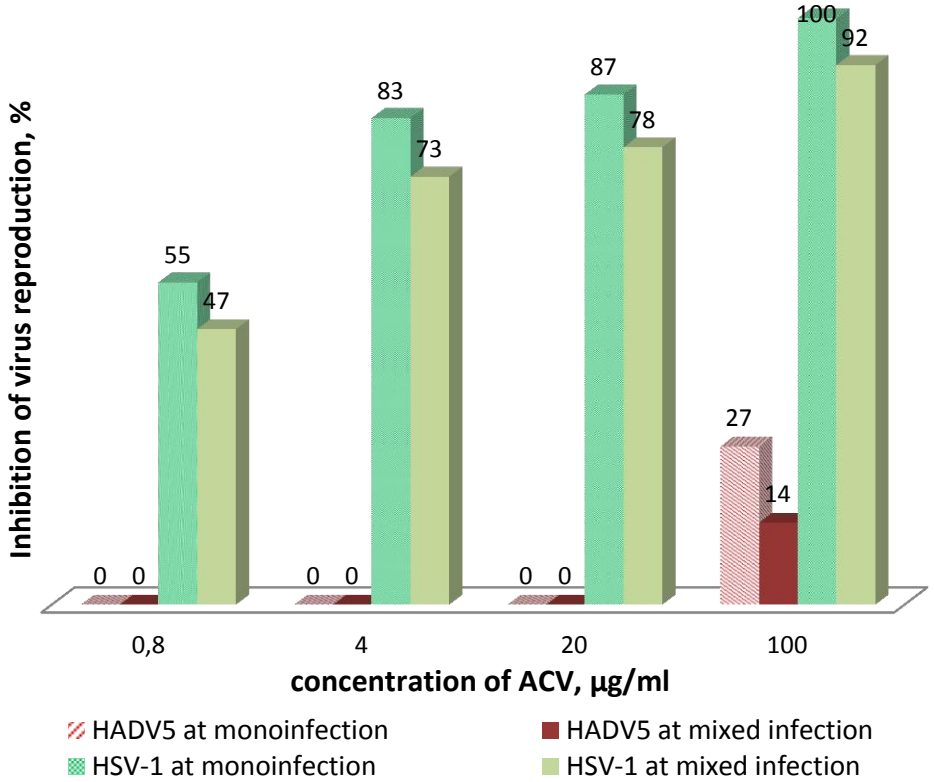


Cytomorphological features of virus infection in MDBK cells

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

Effects of compounds on viruses reproduction at the condition of mono- and mixed infections





The results revealed that 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 mixed infection led to the inhibition of reproduction of adenovirus up to 28% and of herpes virus up to 58%.

The application of 10S-23 at mixed infection induced a decrease of effectiveness of the compound by 58-73% and 100% against HSV-1 and HADV 5, respectively.

The analysis of antiviral activity of Acyclovir in the model of mono infections showed the reduction of reproduction of HSV-1 /US by 55-100% and HAdV-5 by 27%. The effectiveness of the drug against adenovirus during the co-infection increased by 46%.

Infectious virus yield reduction assay

MDBK cells were cultured in 24 well plates for 24 hours. The cell monolayer was then infected with HSV-1 (at a MOI of 3.2), HAdV-5 (at a MOI of 7), and a suspension of both viruses and was incubated for a further 2 h at 37°C and 5% CO_2. Cells were washed with medium and the compounds at concentrations ranging between 150 and 4 µg/ml in a supportive medium were added immediately after adsorption. At 72h after virus inoculation, supernatant consisting of culture medium and cell lysate was obtained by centrifugation at 400 × g for 10 min at 4°C. Viruses titers were determined by a cytomorphological method in MDBK cells. Ten-fold serial dilutions of cell lysates were prepared prior to infection. Confluent MDBK cell monolayers were then infected with 200 µL of viral dilutions ranging from 10^{-1} - 10^{-4} in full log increments, and allowed to adsorb for 2 hours at 37˚C and 5% CO_2. After 2 hours, unabsorbed virus was aspirated and 800 µl of medium was added to each test tube, and incubated for 48 hours. The number of inclusion-forming cells was determined and decrease of virus titer was calculated:

% Percentage inhibition of virus reproduction = (1 – T/C) *100%

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

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	$7,3x10^5$	62,00	$2,8 x10^3$	97,86	$3,5 x10^4$	13,78
	20	$6,5 x10^3$	99,97	$1,2 x10^6$	36,16	$2,1 x10^4$	83,60	$3,6 x10^4$	12,22
	4	$2,0 x10^4$	99,91	$1,3 x10^6$	34,95	$5,2 x10^4$	60,02	$3,6 x10^4$	11,71
	0,8	$5,3 x10^4$	99,78	-	-	$6,1 x10^4$	53,82	-	-
10S-24	150	$1,4 x10^4$	99,94	$8,4 x10^4$	95,67	$3,6 x10^4$	72,25	$3,9 x10^4$	5,21
	100	$1,8 x10^4$	99,93	$3,1 x10^5$	83,91	$6,2 x10^4$	52,97	$4,5 x10^4$	0
	50	$1,9 x10^4$	99,92	$7,2 x10^5$	62,91	$7,5 x10^4$	43,10	$5,4 x10^4$	0
Control of virus		$2,4x10^7$	-	$1,9x10^6$	-	$1,3x10^5$	-	$4,0x10^4$	-

An activity of 10S-24 and ACV, determined by yield reduction assay, was observed against HSV-1. The significant delays of HSV-1 reproductions were observed, 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%.

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 conditions of co-infection of cells.

Cell Cycle Analysis

Cells (1×10^6) were harvested by centrifugation at 300 g (2000rpm) for 7 min, resuspended in 96% ice-cold ethanol, washed with PBS, resuspended in 500 μl solution of PBS (Sigma) that contained RNAse (100 μg/ml) and PI (50 μg/ml), and incubated at room temperature for 1 h.

The cell fluorescence intensity was measured by an 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

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. 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 G1 to S phase, in which productivity is greatest. HADV infection of a range of epithelial cell lines including a primary cell line causes G2 phase synchronization and arrest. This synchronization in the G2 phase may well be a significant factor contributing to the cell-size increase.

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.

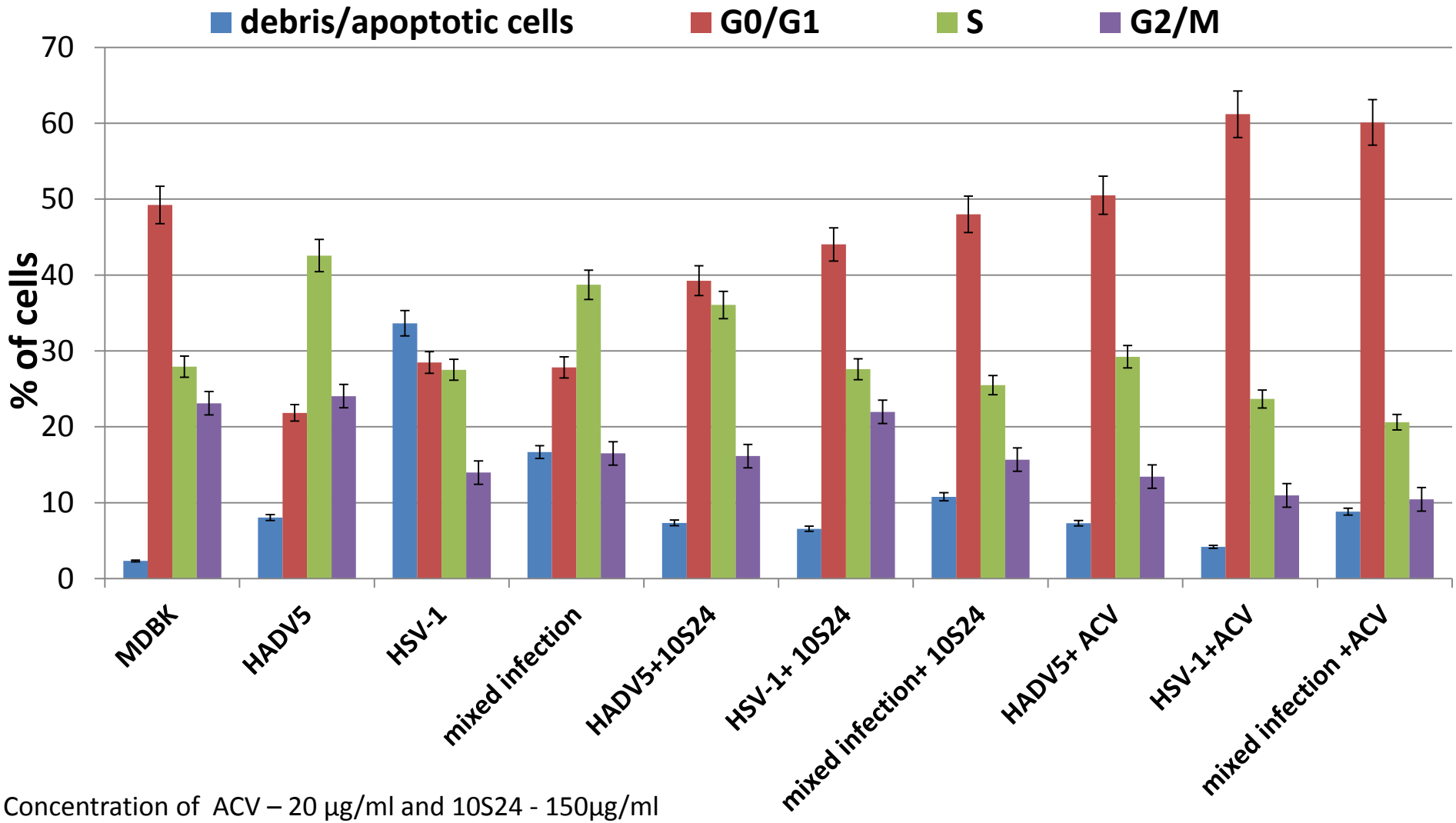
To determine the influence of growth state following viral infection, growing uninfected culture of cells were examined by FCM.

There was a significant number of cells in the G0/G1 (49%) and S (28%) phases of the cell cycle (24 h). 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,/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.

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 G0/G1 cells increases to 38 - 61% and amount of the apoptotic cells decreased to 4 – 11%.

Influent of compounds on the cell cycle under condition of mono and mixed infections



Concentration of ACV – 20 µg/ml and 10S24 - 150µg/ml

Conclusions

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 relevant. 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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