



# 1st International Electronic Conference on Medicinal Chemistry

2-27 November 2015

chaired by Dr. Jean Jacques Vanden Eynde

sponsored by



pharmaceuticals

## **Study of peculiarities of viruses' interactions and effectiveness of antiviral drugs in the model of mixed infection**

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**Abstract:** Human adenovirus and herpes simplex virus cause infectious diseases of eyes, respiratory, enteric and urogenital tracts, of central and peripheral nervous system and are capable to persistence in latent form and are activated under the influence of endogenous and exogenous factors.

A high spread of infections caused by herpes virus and adenovirus and their mixed infections is the problem of modern medicine. During mixed infections, an absence of viruses' interaction as well as a mutual influence of viruses may originate.

Search of drugs with a relatively high activity against the co-associated viruses by inhibiting their reproduction and transmission is an important task. Activity of antiviral drugs under condition of co-infection is not studied enough. The changes of the nature of the pathological processes were found, as a result of the interference of the viruses and the differences of the drugs activities against the co-associated viruses in conditions of the mono- and co-infections in MDBK cells. Both the increase and inhibition of the drugs activities were detected that may lead to the formation of resistant strains of viruses.

**Key words:** human adenovirus serotype 5, herpes simplex virus type 1, mixed infections, abnormal nucleosides, antivirus performance



# Introduction

Today there is a large number of viruses pathogens of humans, which affect various tissues of macroorganism and have the levels of manifestations from latent to lethal forms. Viruses use different ways to preserve their genetic information in the body, starting from the integration into the genome of host cells and ending by the formation of episomes. Activation of persistent form of viral infection takes place both under action of external environmental factors and under inhibitory action of other viruses on the immune system. Modern epidemiological studies show that, as a rule, the clinical picture of the infection involves multiple etiological agents. Typically, the range of viral and bacterial pathogens, which are in the same tissues and can interact, simultaneously coexists in macroorganism. Choice of the true etiotropic treatment for mixed infections requires a fundamental knowledge on the molecular biology of viruses, in particular, the features of their reproduction in permissive and non permissive conditions with regards to their influence on different components of the human immune system.



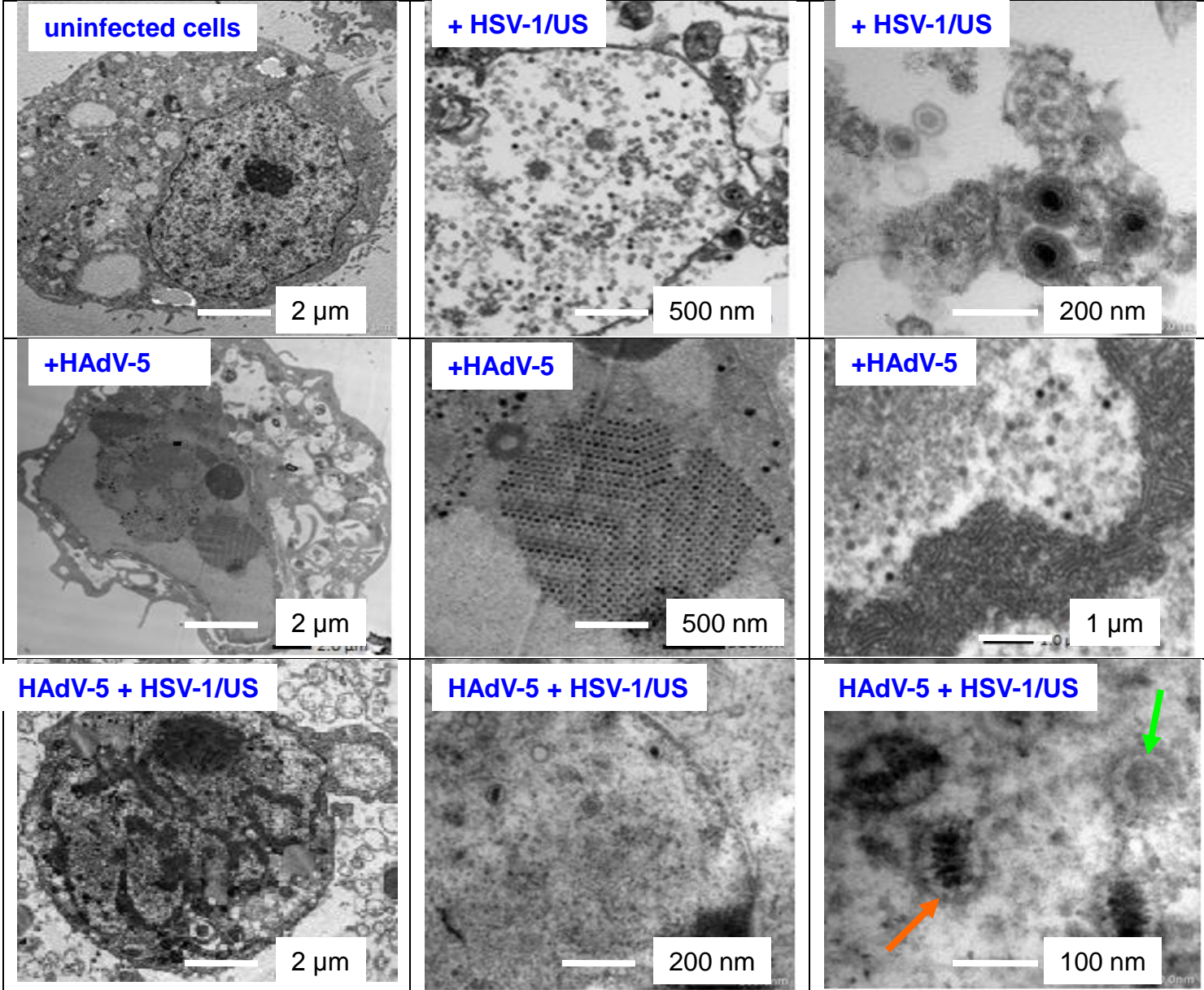
## Results and discussion



Human adenovirus serotype 5 (HAdV-5) and herpes simplex virus type 1 (HSV1/US) were used as the model viruses. MDBK cells were infected with both viruses simultaneously.

The created model of mixed infection in the populations of MDBK cells was confirmed by the electron microscopy.

Infection of MDBK cells with HAdV-5 or HSV-1/US caused formation of some subcellular structures, which were well seen in the electron microscope. These were: the nuclear enlargement and the chromatin aggregation along the nuclear; the formation of the virus-specific inclusions, of mostly the granular or fibrillar type periphery (margination), which were the crystal-like intranuclear complexes composed of complete and incomplete nucleocapsids; the presence of the paracrystals; the hypertrophy of the nuclear membrane, that resulted of an enlargement of the perinuclear space; the cytoplasm with the severe vacuolization in comparison with the uninfected cells; there were numerous rounded mitochondria and tubular-reticular formations, EPR structures were enlarged and had dense membranes; cell membranes were hypertrophied with an irregular form. These morphological changes resulted in complete destruction of cell after 72-96 h.





 HSV-1/US capsid  
 HAdV-5 capsid



By contrast a mixed infection of the cells with HSV-1/US and HAdV-5 characterized by a decreased level of the cytomorphological changes of the cells and only a small amount of viral capsids was detected within the cells.

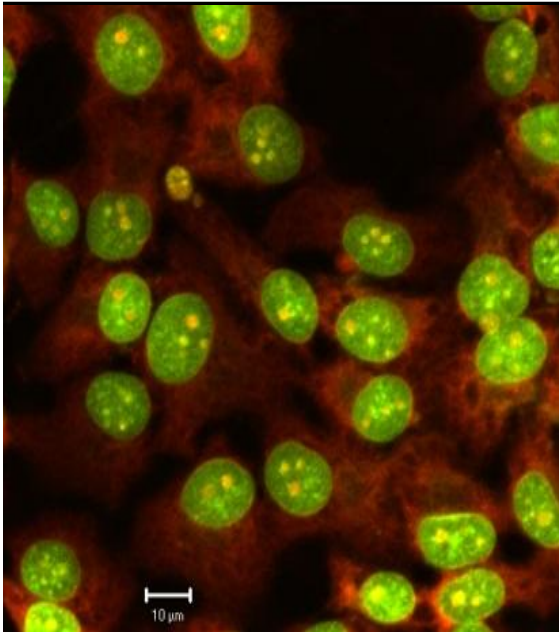
A cytomorphological method developed in our laboratory is based on the calculation of infected cells with DNA-containing intranuclear inclusion bodies induced by the viruses. A characteristic feature of reproduction of HAdV and HSV within the cell is the formation of specific intranuclear inclusions that can be detected with fluorescent microscopy after staining of fixed cells with acridine orange.

Adenoviral inclusions look grainy or granular, or like bright green glow in the center of nuclei surrounded with a dark homogeneous zone.

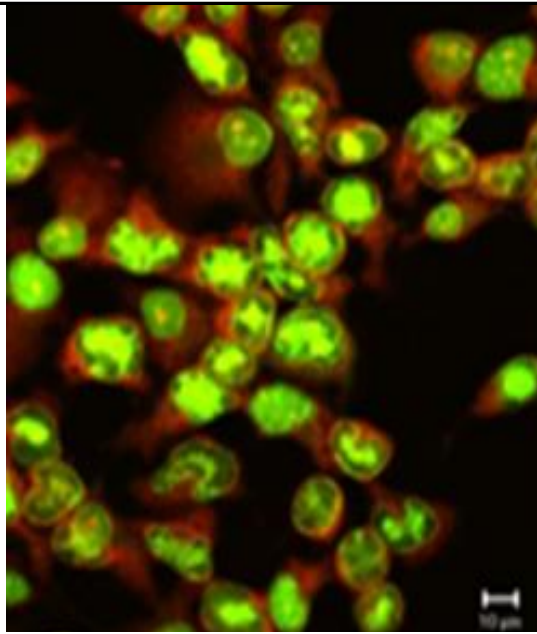
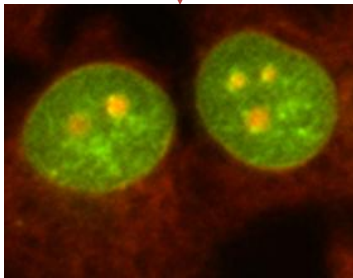
The herpes virus inclusions look like smoky uniform diffuse green glow of nucleus with a pronounced orange-green nuclear membrane. It is well known that the inclusions of both viruses composed of viral DNA, specific viral antigens, clusters of viral kapsomers (which form so-called “paracrystals”) and crystal-like structures composed of complete and incomplete nucleocapsids.



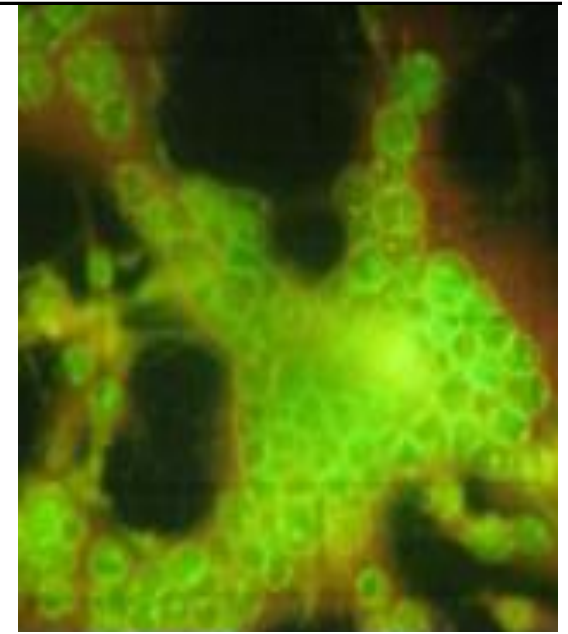
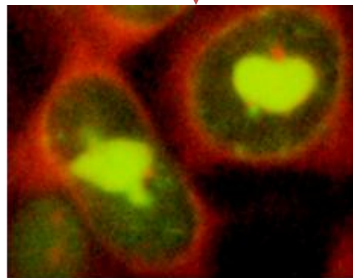
# Cytomorphological features of virus infection in MDBK cells (acridine orange staining)



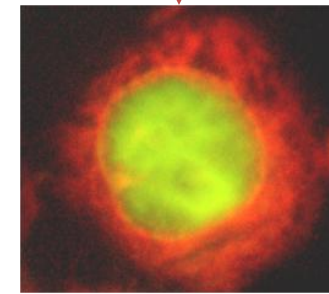
uninfected cells



+ HAdV-5



+ HSV-1/US



Simultaneous infection of cells caused inhibition of reproduction of both viruses. The level of inhibition depended on the multiplicity of infection of cells by the adenovirus. The virus reproduction activity in the cells was studied by determining the percentage of infected cells in monolayer. Infected cells were detected by the presence of virus-induced DNA-containing inclusions in their nuclei.

### Analysis of the formation of specific virus-induced DNA-containing inclusions in the nuclei infected MDBK cells

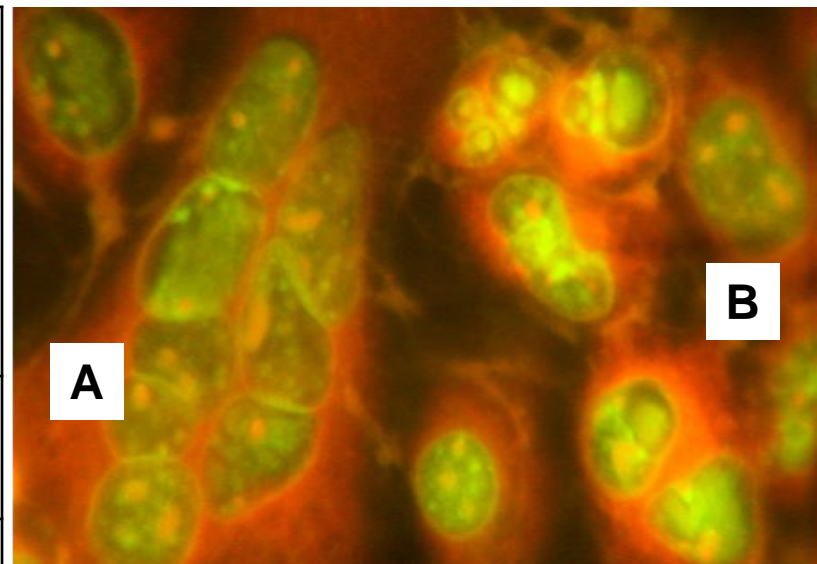
Viruses/dose		Infected cells, %	
		HAdV-5	HSV-1/US
<b>HAdV5 (12 IFU/cell)</b>	-	<b>47</b>	-
<b>HAdV5 (6 IFU/cell)</b>	-	<b>26</b>	-
-	<b>HSV1/US (0,02 IFU/cell)</b>	-	<b>100</b>
-	<b>HSV1/US (0,01 IFU/cell)</b>	-	<b>82</b>
<b>HAdV5 (12 IFU/cell)</b>	<b>HSV1/US (0,02 IFU/cell)</b>	<b>6</b>	<b>67</b>
<b>HAdV5 (6 IFU/cell)</b>	<b>HSV1/US (0,02 IFU/cell)</b>	<b>12</b>	<b>67</b>
<b>HAdV5 (12 IFU/cell)</b>	<b>HSV1/US (0,01 IFU/cell)</b>	<b>5</b>	<b>25</b>
<b>HAdV5 (6 IFU/cell)</b>	<b>HSV1/US (0,01 IFU/cell)</b>	<b>17</b>	<b>50</b>





The increase of portion of cells with inclusions was proportional to the values of the infectious virus titers. The 3 and 2 orders reduction of virus infectious titers was marked in the mixed infections compared with mono infections for adenovirus and herpes virus, correspondingly.

<b>Viruses</b>	<b>Infectious titer of HAdV-5 <i>IFU/ml</i></b>	<b>Infectious titer of HSV-1/US <i>IFU/ml</i></b>
<b>HAdV-5</b>	<b><math>3.5 \times 10^7</math></b>	<b>-</b>
<b>HSV-1/US</b>	<b>-</b>	<b><math>3 \times 10^5</math></b>
<b>HAdV-5+ HSV-1/US</b>	<b><math>8.5 \times 10^4</math></b>	<b><math>6.8 \times 10^3</math></b>



**Co-infection MDBK cells with (A) herpesvirus and adenovirus (B) inclusions**



The created model of mixed infection in the populations of MDBK cells was confirmed by immunofluorescence analysis. The levels of synthesis of the major proteins of adenoviruses and herpes viruses during the mixed infection were studied using flow cytometry. Virus proteins were studied using mono-specific antibodies to adenovirus hexon obtained by the standard Keller method and rabbit monospecific antibodies to main capsid protein of herpes simplex virus type 1 (VP5) ("Abcam", Great Britain). The anti rabbit immunoglobulins labeled with FITC (Sigma, USA) were used as the secondary antibodies.

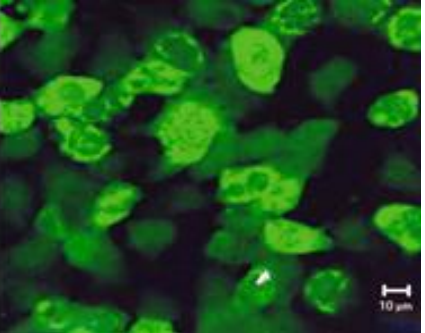
Flow cytometer (Beckman Coulter Epics LX, USA) with laser wavelength 530 nm and the corresponding filter/detector was used for the quantitative analysis of the capsid proteins of viruses. The processing of results was carried out in the program Flowing Software, version 2.5



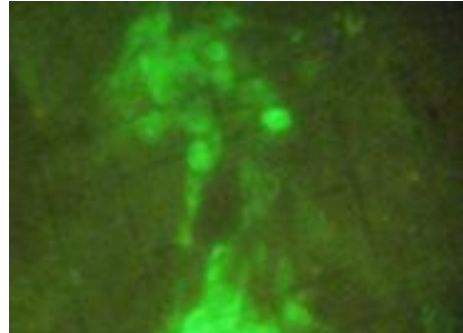
# Blocking of the synthesis of major structural protein HAdV-5 (hexon) and late proteins of HSV-1/US under co-infection ( the fluorescent antibodies technique).



uninfected cells

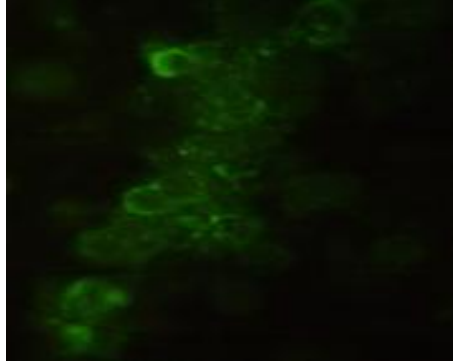


HAdV-5

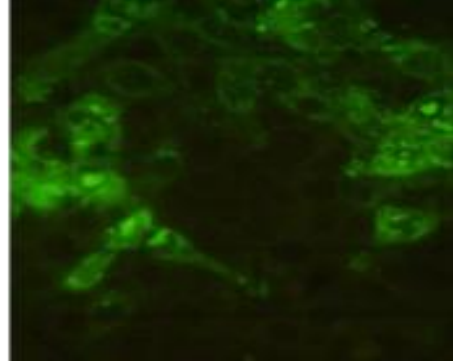


HSV-1/US

late proteins  
(hexon) of HAdV-5



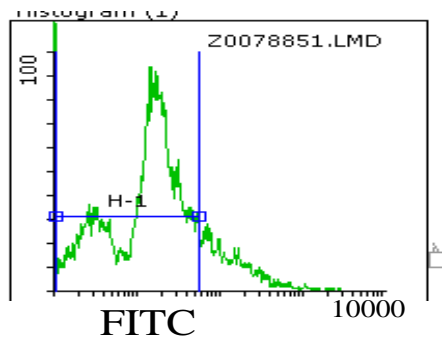
HAdV-5 + HSV-1/US



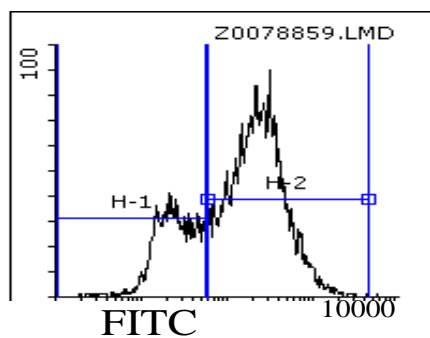
late proteins  
of HSV-1/US



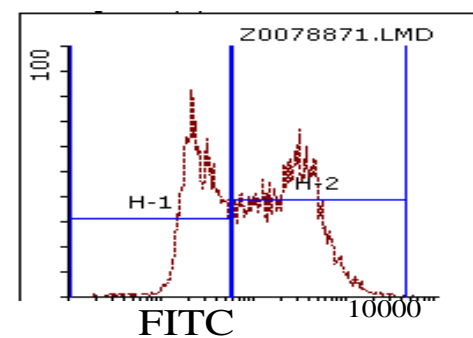
# Comparative analysis of hexon synthesis of the HAdV-5 (a) and the main capsid protein of the HSV-1 /US (b) in MDBK cells during mono- and co-infection (flow cytometry)



**Uninfected cells**

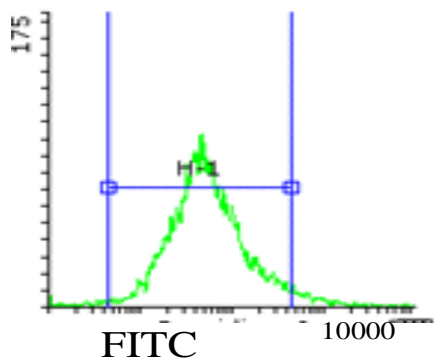


**HAdV-5  
(72,3 %)**

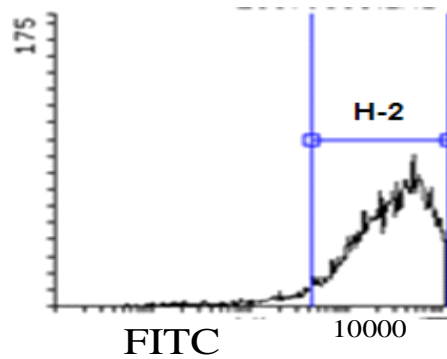


**Co-infection  
(59,9 %)**

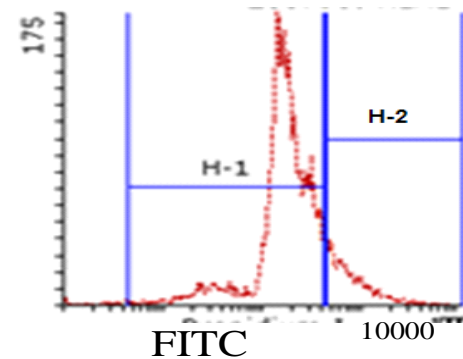
**a**



**Uninfected cells**



**HSV-1/US  
(68,9 %)**



**Co-infection  
(16 %)**

**b**



In case of the mixed infection of the cells, a decrease in the histogram peak that corresponds to cells infected by adenovirus (zone H-2) and an increase of the peak in uninfected cells (zone H-1) were observed, indicating the inhibition of the synthesis of hexon by 17%. Analysis of the structural proteins of herpes virus under mixed infection showed a significant decrease of the level of the main capsid protein (83%).

Thus, the reproduction of adenovirus and herpes virus under conditions of the mixed infection was studied. The simultaneous infection of cells with two viruses resulted in mutual inhibition of both viruses reproduction.



The model of co-infection of MDBK cells was used to evaluate the efficiency of the antiviral agents of the broad action spectrum:

- ganciclovir (Hoffman-LaRoche, Switzerland),
- cidofovir (Gilead Sciences Inc., USA)
- ribavirin (Institute Organic Synthesis of the Latvian Academy of Sciences).

The cytotoxic activity of the drugs was studied by the MTT-assay.

The antiviral effects of the drugs were studied using the medical scheme of introduction of preparation (as a component of supporting medium).

The analysis of the virus DNA synthesis in infected cells after treatment with the drugs was carried out by the RT-qPCR and the effectiveness of the drugs under mono- and mixed infections was studied by the cytomorphological method with acridine orange dye. Antiviral activity was determined by a method developed in our laboratory, and is based on the calculation of infected cells with DNA-containing intranuclear inclusion bodies induced by the viruses. Cells were grown in tubes with strips of cover glasses. In 48 hours, cells were infected with HAdV-5 and HSV-1/US and after 1 hour of the adsorption of viruses at room temperature, cell were washed with Hank's solution and pre-incubated in maintenance medium containing drugs. The infected cells, which were not treated with the assayed compounds under study, were used as a positive control of virus infection.



After 48 hours of incubation at 37°C, the cells were fixed with 96% ethanol, washed with Hank's solution, stained with 0,01% acridine orange solution, and the number of infected cells with DNA-containing inclusion bodies was counted using a luminescent microscope. On each of the three strips, 500 cells were counted and the percentage of infected cells was determined. The 50% effective concentration (EC<sub>50</sub>) was determined as the compound concentration decreasing the percentage of infected cells with intranuclear inclusion bodies by 50% in comparison to the virus control.

Ribavirin at a concentration 32 µg/ml was added into the medium after infection of the cells. Use of ribavirin in conditions of mono infection led to the inhibition of DNA replication of adenovirus by 100% and of herpes virus by 93%. Ribavirin was ineffective against HAdV-5 and its activity against herpes virus remained almost unchanged under conditions of co-infection.

The analysis of antiviral activity of ganciclovir (32 µg/ml) in the models of mono infections showed the reduction of reproduction of HSV-1/US by 60% and HAdV-5 by 4%. Ganciclovir inhibited the reproduction of herpes virus by 61% during the co-infection. The effectiveness of the drug against adenovirus increased 20%.



## Antiviral activity of Ribavirin and Ganciclovir during mono- and co-infections of MDBK cells

Variant of infection	Inhibition of virus reproduction by drugs , %			
	Ribavirin		Ganciclovir	
	HSV-1	HAdV-5	HSV-1	HAdV-5
Mono-infection + drug	93±3,2	100	60±3,0	4±0,6
Co-infection + drug	82±3,1	0*	61±2,9	25±1,9

\* – *no inhibition*





## Antiviral activity of Cidofovir during mono- and co-infections of MDBK cells

Variant of infection	Infective titer of viruses, IFU/ml	
	HAdV-5	HSV-1/US
Mono-infection	$3,5 \times 10^7$	$5,8 \times 10^3$
Co-infection	$8,5 \times 10^4$	$6,8 \times 10^3$
Mono-infection + drug	$1 \times 10^5$	$1,9 \times 10^3$
Co-infection + drug	$3,4 \times 10^4$	$1,5 \times 10^5$

Under conditions of the mixed Infection the cidofovir (32  $\mu\text{g/ml}$ ) activity against HAdV-5 and HSV-1/US decreased 3-fold and 79-fold, respectively, compared to the mono- infections.



## Conclusion

The changes of the nature of the pathological processes were found, as a result of the interference of the viruses and the differences of the drugs activities against the co-associated viruses in conditions of the mono- and co-infections of MDBK cells. Both the increase and inhibition of the drugs activities were detected that may lead to the formation of resistant strains of viruses.

