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Analysis of Antiviral Properties of Hexoral *In Vitro* against Some Viruses that Cause Acute Respiratory Infections and Herpes

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Antiviral properties of Hexoral (0.1% solution and 0.2% aerosol for local application) and its constituent hexetidine against viruses causing human respiratory tract infections and herpes virus were studied *in vitro*. It was found that non-cytotoxic concentrations of hexetidine (alone and as a component of Hexoral) attenuated infectious properties of highly virulent influenza virus A/H5N1, pandemic influenza virus A/H1N1pdm, respiratory syncytial virus, and herpes simplex virus type 1 after a short-term exposure (30 sec) by 100 or more times. It was found that hexidine mostly contributes to the virucidal effect of Hexoral.

Key Words: *influenza virus; Hexoral; cell cultures; antiviral properties*

The search for new drugs actively suppressing replication of viral pathogens that cause acute respiratory diseases [1,5,7-9], including pandemic influenza A/H1N1pdm [5], highly virulent influenza virus A/H5N1 [1,4] that causes death of 60% infected individuals, respiratory syncytial virus (RSV), and herpes simplex virus type 1 (Herpesviridae family) [6,10], including Epstein–Barr virus causing infectious mononucleosis in children remains a pressing problem of modern virology. The State Collection of Viruses organized on the basis of D. I. Ivanovsky Research Institute of Virology includes collection of all these viral strains suitable for antiviral drug screening.

Here we studied the ability of the two forms of Hexoral (0.1% solution and 0.2% spray for local use) and hexetidine (a component of this drug) to suppress *in vitro* infectious activity of viruses that infect human respiratory tract, and the herpes virus.

MATERIALS AND METHODS

Hexetidine substance (1,3-bis (2-ethylhexyl)hexahydro-5-methyl-5-pyrimidinamine, certified by Famar Orleans) was provided by Johnson & Johnson, as well as Hexoral (McNeil Manufacturing). Acyclovir (acycloguanosine; Zovirax; GlaxoSmithKline) was used as the reference drug.

Cell cultures. Cytotoxic properties and antiviral activity of hexetidine and Hexoral were analyzed by using confluent Vero-E6 cell cultures in 96-well plastic plates with minimum Eagle medium (PanEco) supplemented with 7% fetal calf serum (Sigma) pre-heated on a water bath (20 min at 56°C), glutamine (150 μg/ml) and antibiotics (100 U/ml penicillin and streptomycin) at 37°C and 4% CO_2. Dulbecco's modified minimal Eagle medium containing glutamine and antibiotics in the same concentration and 1% fetal calf serum (Sigma) served for maintenance medium after virus adsorption.

Canine kidney cell cultures (MDCK) were grown in 96-well plastic plates using growth medium and support medium of the same composition with the addition of 2 μg/ml TPCK-trypsin (Sigma). Porcine embryo kidney cells (SPEV) were cultured in 96-well plastic plates in medium 199 (PanEco) supplemented with 100 U/ml penicillin and streptomycin and 10% inactivated cattle serum. Cell lines were obtained from the Cell Culture Collection of D. I. Ivanovsky Research Institute of Virology.

Viruses. The following viruses were used in the study: influenza A/duck/Novosibirsk/56/05 vi-

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rus (H5N1) isolated from poultry in the Novosibirsk region in 2005 [7]; pandemic influenza A/Moscow/01/2009 (H1N1)pdm strain isolated in 2009 in Moscow; RSV strain Long; herpes simplex virus type 1 strain L2 (HSV-1/L2) were used. All strains were obtained from the State Collection of Viruses. The medium collected from cultures infected with these viruses at the peak of infectious process served as virus-containing material.

To study cytotoxic activity of the test drugs, we used Vero-E6, SPEV, and MDCK cultures. The cell suspension in growth medium (4×10^5 cells/ml) was incubated in 96-well plastic plates for 24 h at 37°C and the cell monolayer was washed twice with Hanks saline; the test drugs in various concentrations in maintenance medium were added (the total volume of medium per well was 200 µl). Treated and untreated cell cultures were incubated for 72 h and then, the cells were stained with methylene blue and counted using Cauntess slide cytometer (Invitrogen). Then the cytotoxic dose (CD) was calculated as the minimum drug concentration inducing death of 50% monolayer cells within 72 h after drug exposure (CD_{50}).

Antiviral activity of the drugs was studied by the method of routinely used in pre-clinical trials [4]. For evaluation of the capacity of the test drugs to attenuate infectious properties of the viruses, the virus-containing medium with initial titer was mixed 1:1 with the studied drugs (experimental samples) or with maintenance medium (control) and shaken vigorously for 30 or 60 sec at room temperature. Then, serial 10-fold dilutions of these mixtures were immediately prepared and 100 µl of each dilution was added to washed monolayer of cells susceptible to each virus. After 72-96-h incubation, when the monolayer in control (untreated) wells was complete destructed, the infectious virus titer was determined by the method of Reed and Muench. The antiviral effect of hexetidine (sole and in Hexoral) was assessed by the difference in infectious virus titers between the control and experimental wells.

Statistical analysis was performed by conventional biological methods, and also using Spearman–Kerber method with assessment of significance of differences (p=0.05) for 95% confidence level.

RESULTS

Hexetidine, the active component of Hexoral, is characterized by broad spectrum of antibacterial and antifungal activity, particularly against gram-positive bacteria and fungi as Candida, Pseudomonas aeruginosa, and Proteus. Hexetidine destructs bacterial cell walls and inhibit oxidative metabolic reactions (thiamine antagonist). In a concentration of 100 µg/ml hexetidine actively inhibits the growth of the majority of bacterial strains; no resistance was observed. At the same time, the antiviral effect of hexetidine on viral agents that affect the human respiratory tract is poorly studied.

Analysis of *in vitro* antiviral (virucidal) activity of hexetidine alone and as a component of commercial topical formulation showed its cytotoxic properties in different cell cultures (PK cells, Vero-E6, MDCK). Thus, CD_{50} of hexetidine alone and in 0.1% Hexoral solution was the same for Vero-E6, MDCK, and PK cell cultures (5.0 µg/ml). CD_{50} of aerosol for these types of cell lines was 10 µg/ml.

Noncytotoxic concentrations of hexetidine attenuated the infectious properties of viruses infecting human respiratory tract: highly pathogenic influenza A/H5N1, pandemic influenza A/H1N1pdm, RSV and HSV-1/L2 virus (Tables 1-3).

Virucidal properties of hexetidine substance and aerosol, as well as Hexoral solution manifested within

TABLE 1. Inhibition of Infectious Activity of A/H5N1 and A/H1N1pdm Influenza Viruses in Vero-E6 and MDCK Cell Cultures, Respectively, by Hexetidine Alone and as a Component of Hexoral

Drug		Virus titers (lg CD_{50}) after drug application in concentration of					
		5.0 µg/ml		2.5 µg/ml		control (without drug)	
		A/H5N1	A/H1N1pdm	A/H5N1	A/H1N1pdm	A/H5N1	A/H1N1pdm
Hexoral, 0.1% solution	30 sec of exposition	2.5±0.3	1.3±0.2	2.3±0.1	1.5±0.2	4.2±0.1	3.5±0.2
	60 sec of exposition	2.4±0.2	1.2±0.3	2.1±0.4	1.4±0.3	4.2±0.1	3.6±0.2
Hexoral, 0.2% aerosol	30 sec of exposition	2.0±0.2	1.1±0.3	2.2±0.4	1.3±0.2	4.2±0.1	3.5±0.2
	60 sec of exposition	2.0±0.1	1.0±0.1	2.3±0.1	1.2±0.4	4.2±0.1	3.6±0.2
Hexetidine	30 sec of exposition	2.1±0.3	1.0±0.2	2.7±0.2	1.2±0.2	4.2±0.1	3.5±0.2
	60 sec of exposition	2.2±0.1	1.0±0.1	2.3±0.2	1.3±0.1	4.2±0.1	3.4±0.2

TABLE 2. Inhibition of Infectious Activity of RSV for Vero-E6 cells by Hexetidine Alone and as a Component of Hexoral

Drug		RSV titers after drug application in concentration of		
		5.0 μg/ml	2.5 μg/ml	control (without drug)
Hexoral, 0.1% solution	30 sec of exposition	4.00±0.05	4.2±0.1	6.3±0.1
	60 sec of exposition	3.9±0.1	4.0±0.2	6.3±0.1
Hexoral, 0.2% aerosol	30 sec of exposition	3.2±0.2	4.0±0.1	6.2±0.1
	60 sec of exposition	3.1±0.1	3.9±0.3	6.1±0.1
Hexetidine	30 sec of exposition	4.1±0.3	3.80±0.05	6.3±0.1
	60 sec of exposition	4.2±0.1	4.1±0.2	6.3±0.1

TABLE 3. Virucidal effect of Hexoral (Topical Formulation) and Hexetidine on HSV-1/L_2 Model

Experimental conditions	Virus titer*		Mean virus titer after two trials		Decrease in virus titer in comparison to control			
	lg PFU/ml		lg PFU/ml		abs.		%	
Control	6.70		6.63±0.10		–		–	
	6.51							
	6.44							
	6.88							
Exposure, 30 sec	F	S	F	S	F	S	F	S
	4.87	6.44	4.64±0.09	6.49±0.07	1.99	–	98.99	–
	4.51	6.70						
	4.48	6.40						
	4.70	6.40						
Exposure, 60 sec	4.40	4.65	4.19±0.09	4.73±0.05	2.44	1.76	99.65	98.29
	4.00	4.70						
	4.24	4.68						
	4.10	4.88						

Note. F: Hexoral formulation; S: hexetidine substance; *results of two independent experiments.

a short time after exposure of a material containing herpes and influenza viruses with the drug (~30 sec). Comparative studies on the antiviral activity of the hexetidine substance and Hexoral preparation against influenza viruses, RSV, and HSV-1/L2 showed that antiviral properties of hexetidine in Hexoral solution and spray did not differ from the properties of the substance alone. Their antiviral activity was stable and high: in non-toxic concentrations they suppressed virus activity by 100 or more times (Tables 1-3).

It was shown that hexetidine substance and Hexoral demonstrated similar inactivation of the studied viruses. The preparations did not affect virus replication in cells.

These findings suggest that the virucidal effect of Hexoral is determined by hexitidin. The decrease in viruses concentration from the initial level (titer) was ≥99% after exposure to Hexoral in a concentration of 2.5 μg/ml and >90% after exposure to 1.25 μg/ml.

REFERENCES

1. P. G. Deryabin, G. A. Galegov, A. G. Botikov, *et al*., *Vopr. Virusol.*, **56**, No. 1, 21-24 (2011).
2. D. K. L'vov, I. T. Fedyakina, M. Yu. Shchelkanov, *et al*., *Vopr. Virusol.*, **51**, No. 2, 20-22 (2006).
3. D. K. L'vov, M. Yu. Shchelkanov, P. G. Deryabin, *et al., Vopr. Virusol.*, **51**, No. 1, 11-14 (2006).
4. A. N. Mironov and N. D. Bunatyan, *Manual for Preclinical Testing of Drugs* [in Russian], Moscow (2012).

5. I. T. Fedyakina, M. Yu. Shchelkanov, P. G. Deryabin, *et al*., *Antibiot. Khimioter.*, **56**, No. 3-4, 3-9 (2011).
6. E. De Clercq, J. Descamps, G. Verheist, *et al*., *J. Commun. Dis*., **141**, No. 5, 563-573 (1980).
7. R. Dolin, R. C. Reichman, H. P. Madore, *et al*., *N. Engl. J. Med.* **307**, No. 10, 580-584 (1982).
8. I. I. Fedorov, E. M. Kazmina, G. V. Gurskaya, *et al*., *J. Med. Chem*, **40**, No. 4, 486-494 (1997).
9. F. G. Hayden, R. L. Atmar, M. Schilling, *et al*., *N. Engl. J. Med.* **341**, No. 18, 1336-1343 (1999).
10. R. T. Sarisky, T. T. Nguyen, K. E. Duffy, *et al*., *Antimicrob. Agents Chemother.*, **44**, No. 6 1524-1529 (2000).