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IN VITRO STUDY OF ANTI-INFLUENZA ACTIVITY OF PARA-AMINOBENZOIC ACID AND PROSPECTS OF NASAL DRUG DEVELOPMENT ON ITS BASE

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Introduction

Viral diseases are widely spread in the living world, e.g. more than two hundred pathogenic types of viruses for human are currently described [1]. Among different types of human viral diseases, a special role belongs to acute respiratory viral infections (ARVI) due to their easy spreading among people, strong transmissibility, and frequent recurrences, especially among children. Thus, according to the literature data, an adult on the average has up to three ARVI recurrences per year, and children have up to eight ARVI recurrences per year. Moreover, ARVI may initiate different complications both, in upper and lower respiratory tract, and in other organs (ears, heart, brain, kidneys, joints etc.) [2, 3].

Currently, medical practice has a very limited number of drugs with antiviral activity for treatment of upper respiratory tract infections. Each of these drugs influences on different stages of virus life cycle (in particular, influenza virus) in human cells.

Drugs of the first group of inactivate virus particles outside the cell, such as cation-active antiseptics.

Drugs of the second group influence the processes of adsorption and penetration of the virus into cell, as well as its removal from the cell, such as membrane protein M2 inhibitors and neuraminidase inhibitors.

Drugs of the third group influence replication of genetic material, assembling of viruses, such as interferons, interferonogens, anomalous nucleotides, protease inhibitors, reverse transcriptase inhibitors, etc. [4].

In addition, it should be noted that drugs for etiotropic and symptomatic treatment of influenza / ARVI are in general represented by products for systemic administration (tablets, capsules, suppositories, injections) and there are very few drugs for local administration (drops, sprays).

One work (5) presents data about drugs for nasal administration in Ukrainian pharmaceutical market: about 55 % of them are vasoconstrictors, 14 % are interferons, 8% are antiseptics, and 1 % is a fibrinolysis inhibitor. All these drugs have their own advantages and disadvantages, but none of them has universal antiviral properties and, furthermore, there are no complex drugs with concomitant antipathogenic and symptom relief effects.

One of the promising substances that exhibit antiviral activity is a vitamin-like substance, para-aminobenzoic acid (PABA). From literature data, we know about its activity against herpes simplex virus and adenovirus, as well as about interferonogenic activity of this substance [6]. Drug “Actipol” for local treatment of viral eye conjunctivitis and keratitis based on this substance has been developed in the Russian Federation [7].

Another promising substance with antiviral activity is ε-aminocaproic acid (ACA). From the literature sources, we know about antiviral activity of ACA and, in particular, against influenza virus [8]. This substance has an effect on some life cycle processes of influenza virus in the cell: penetration of virus into the cell, assembling of virus proteins and some other. Drug “ACA” for local and systemic treatment of influenza based on this substance has been developed and introduced into practice in Ukraine [9].

These substances have good physical-chemical indexes of stability, low price, and they are practically nontoxic. All these facts encouraged of authors to carry out a more detailed study of antiviral activity of these substances in order to ground their inclusion into formulation of a complex nasal drug for local treatment and prevention of influenza / ARVI.

The **aim** of this work is study antiviral activity of para-aminobenzoic acid, ε-aminocaproic acid, and their mixture against influenza virus by *in vitro* methods.

Materials and Methods

PABA test solution is prepared by dilution of accurately weighed portion of the substance in physiological solution with concentration of 5,000 ug/ml, 500 ug/ml, and 50 ug/ml.

As a reference drug, we have chosen “ACA”: nasal solution of ε-aminocaproic acid 50 mg/ml in 5-ml container, No. 10, “Yuria-Pharm” LLC, CP206/1, expiration date 10/2019, Kiev, Ukraine.

Solution of PABA and ε-ACA mixture (1:100) is prepared by dilution of PABA (50 mg) in 100 ml solution of ε-ACA for infusion (50 mg/ml).

For evaluation of cytotoxic concentration (CC_{50}) of the test substance, we used MDCK culture cells. In the study, we used at least ten rows of wells in plates with culture cells for every dilution of the substance in the nutrient medium. We incubated plates with culture cells at 37 ^{0}C with 5.0 % CO_2 supply for 5 days. Every day, we carried out monitoring of culture cells inoculated with test and reference drugs for evaluation of presence or absence cytopathic effects (CPE).

We evaluated the degree of CPE by cell morphology changes (sphericity, cell shrinkage, rejection from surface wells of degenerate cells) with 4-plus system from 0 to ++++. Cytotoxic concentration of the test substance was the one that caused cell degeneration.

For determination of anti-influenza activity of PABA, ACA, and their mixture *in vitro*, we used 24-hour

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inoculated passaged monolayer MDCK culture cells of dog's kidney, influenza virus strain A/FM/1/47 (H_1N_1) with infective titer in MDCK culture cells from 4.0 to 9.0 $lgID_{50}$. The cells were grown in plates on nutrient medium RPMI-1640 with 10% fetal serum at 37 ^{0}C in the thermostat with 5.0 % CO_2 supply. For increasing of cell sensitivity to influenza virus, we treated them with trypsin enzyme. We prepared stock trypsin solution by adding 3 g of enzyme to 3 ml of DMEM nutrient medium. We treated the cells with this solution three times, 50 uL per well. The nutrient medium was decanted; influenza virus at a dose of 100 of 50% tissue cytopatic dose (TCD_{50}) was added to the cells, than test drugs at different concentrations were added. The culture cells were incubated in the thermostat at 37 ^{0}C with 5.0 % CO_2 supply for three days; their state was daily monitored with a microscope. After 48-72-hour incubation of cells, we collected culture liquid and determined its infection titer of influenza virus by titration in culture cells.

Results

Table 1 presents the results on antiviral activity in the form of dependency of influenza virus infective titer ($lgID_{50}$) on concentration of test substances.

Table 1. Determination of anti-influenza activity of test substances

PABA		**PABA+ ε-ACA (1:100)**		**"ACA"**	
Concentration, ug/ml[1]	**$lgID_{50}$[2]**	**Concentration, ug/ml**	**$lgID_{50}$**	**Concentration, ug/ml**	**$lgID_{50}$**
0.0625	2.0	1.25	5.0	25.0	<1.0
0.0313	2.0	**0.62**	**5.0**	6.25	<1.0
0.0156	3.0	0.31	6.0	1.56	<1.0
0.00781	**4.0**	0.15	6.0	**0.78**	**3.0**
Control of virus	9.0	Control of virus	9.0	Control of virus	6.0

Note. 1. Relative error of concentration for substances in the solution was not more than 5 %.
2. Absolute error of infective titer $lgID_{50}$ was 0.5 units with repeat count n=3 and confidence interval P=95 %.

Discussion

As it can be seem from data of Table 1, anti-influenza activity of PABA solution is almost one hundred times higher than those of the reference drug "ACA", and its mixture with ε-aminocaproic acid (in ratio 1:100) demonstrates synergetic effect *in vitro*, which can be seen when comparing component concentration that decreases infective titer of virus ($lgID_{50}$) by 50 ± 7% from the initial value.

For evaluation of synergetic effect degree of antiviral activity of PABA and ACA mixture in the solution, we calculated fractional inhibitory coefficient (FIC) by the following formula (1) [10]:

$$FIC = \frac{IC_{50}(PABA + \varepsilon ACA)}{IC_{50}(PABA) + ID_{50}(\varepsilon ACA)} = \frac{0.62}{0.0078 + 0.78} = 0.79 \quad (1)$$

where *FIC* is a fractional inhibitory coefficient, *FIC* 0.5-1.0 indicates to moderate synergism for the mixture of substances, *FIC* 1.0-2.0 indicates to additive effect for the mixture of substances, and *FIC* more than 2.0 indicates to antagonism for the mixture of substances.

IC_{50} is inhibitory concentration for PABA, εACA, and their mixture, which is associated with decreasing virus infective titer ($lgID_{50}$) by 50 % from the initial value.

As it can be also seen, for combination of PABA and εACA (1:100), fractional inhibitory coefficient equals *FIC*=0.79, which indicates to moderate level of the synergetic effect of their mixture.

In the result of another set of our studies, it has been found out that PABA in the concentration range tested from 10 and up to 1,000 ug/ml, causes no cytopatic changes in MDCK culture cells.

It indicates to very low toxic properties of this substance. If we accept PABA maximum tolerance concentration as CC_{50}=1,000 ug/ml and effective concentration IC_{50}=0.0078 ug/ml, the selectivity index (*SI*) will be calculated by the following formula (2):

$$SI = \frac{CC_{50}}{IC_{50}} = \frac{1{,}000}{0.0078} = 128{,}205 \quad (2)$$

These facts proves usefulness of the combination of PABA and ACA, and demonstrates significant potential for development of a complex nasal drug for etiotropic and symptomatic treatment or prevention of influenza / ARVI.

Conclusions

1. In the result of our *in vitro* studies, we have determined high-level antiviral activity of para-aminobenzoic acid against influenza virus, and its combination with ε-aminocaproic acid demonstrates a synergetic effect.
2. We have shown that PABA does not exhibit any cytotoxic effects on MDCK culture cells in the concentration range studied and demonstrates high level of therapeutic safety.
3. In consequence of these studies, we have shown good prospects for development of a formulation and technology of a complex nasal drug for local treatment

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and prevention of influenza based on para-aminobenzoic acid and ε-aminocaproic acid mixture.

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One of the promising molecules having antiviral activity is a vitamin-like substance, para-aminobenzoic acid (PABA). The **aim** of this work is to study antiviral activity of para-aminobenzoic acid, ε-aminocaproic acid, and their mixture against influenza virus, to evaluate their cytotoxic effect and to calculate their selectivity index by *in vitro* methods. **Materials and Methods.** For evaluation of cytotoxic concentration (CC_{50}) of the substance tested, we used MDCK cell culture. For determination of anti-influenza activity of PABA, ε-ACA, and their mixture under conditions of *in vitro* experiment, we used 24-hour passaged MDCK culture cells of dog's kidney, influenza virus strain A/FM/1/47 (H_1N_1), infected titer of which in MDCK culture cells of was between 3.0 and 9.0 $lgID_{50}$. **Results**. As a result of our studies, we have determined that PABA in the concentration range studied from 10 to 1,000 ug/ml did not have any cytopahtic effect in MDCK culture cells. We calculated selectivity index (SI) for PABA, that was equal SI=128205, this fact confirm high level of therapeutic safety of this substance. The result of our experiments demonstrate that anti-influenza activity of PABA solution is almost 100 times higher than that of "ACA", furthermore in our *in vitro* experiment the mixture of PABA and ε-aminocaproic acid (in ratio 1:100) demonstrates a synergetic effect. In addition, for combination of PABA and εAKK (1:100) we calculated fractional inhibitory coefficient FIC=0.79, that indicates to moderate synergetic effect of these substances. **Conclusions**. As a result of our *in vitro* studies, we have determined a high-level antiviral activity of para-aminobenzoic acid against influenza virus, and in combination with ε-aminocaproic acid it demonstrated a synergetic effect. We have determined that PABA does not exhibit any cytotoxic effect on MDCK culture cells in the concentration range studied and demonstrates high level of therapeutic safety.

Keywords*: para-aminobenzoic acid, antiviral activity, influenza virus*.

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