Original article

Activity of ergoferon against lethal influenza A (H3N2) virus infection in mice

Maria A Skarnovich[1], Alexandra G Emelyanova[2], Nataliia V Petrova[2], Alena A Borshcheva[3], Evgeniy A Gorbunov[3], Oleg Yu Mazurkov[1], Maksim O Skarnovich[1], Sergey A Tarasov[3], Larisa N Shishkina[1], Oleg I Epstein[2]*

[1]Federal Budgetary Research Institution 'State Research Center of Virology and Biotechnology VECTOR', Federal Service for Surveillance on Consumer Rights Protection and Human Well-being, Koltsovo, Russian Federation
[2]The Institute of General Pathology and Pathophysiology, Moscow, Russian Federation
[3]Research and Analytical Department, OOO "NPF "MATERIA MEDICA HOLDING", Moscow, Russian Federation

*Corresponding author e-mail: agemelianova@gmail.com

Background: The influenza A virus accounts for serious annual viral upper respiratory tract infections. It is constantly able to modify its antigenic structure, thus evading host defence mechanisms. Moreover, currently available anti-influenza agents have a rather limited application, emphasizing the further need for new effective treatments. One of them is ergoferon, a drug containing combined polyclonal antibodies – anti-interferon gamma, anti-CD4 receptor and anti-histamine – in released-active form. The purpose of the study was to assess ergoferon antiviral efficacy in mice challenged with the A/Aichi/2/68 (H3N2) influenza virus.

Methods: The virus was inoculated intranasally at a 90% lethal dose. Ergoferon was administered at 0.4 ml/day per os in a preventive and therapeutic regimen – daily for 5 days prior to and for 16 days after the challenge. The reference product, Tamiflu (oseltamivir), was used as a positive control treatment – at 20 mg/kg/day for 5 days after the challenge. Mice in the negative control group received distilled water which had been utilized for test sample preparation; untreated control animals received no treatment. Antiviral efficacy was assessed by an increase in survival rate, average life expectancy and virus titre reduction in the challenged mouse lungs.

Results: Survival rate and average life expectancy values were increased significantly in groups treated with ergoferon and Tamiflu, as compared with controls. Lung virus titres were reduced in these groups as observed on days 2 and 4 post-inoculation.

Conclusions: Ergoferon demonstrated antiviral activity by reducing the severity and duration of the major signs of induced influenza infection.

Introduction

Between 3 and 5 million cases of influenza infection are reported every year worldwide, 250–500 thousand of which are fatal. The infection may be associated with severe progression and serious complications that may lead to hospitalization or death, especially in patients at high risk (for example, children, the elderly and those with chronic diseases) [1].

Annual epidemics occur as a result of changes in the virus's two surface antigens – haemagglutinin (H) and neuraminidase (N). High genetic variability contributes to antigenic drifts that can be observed every 2–5 years, which eventually allow the virus to evade human immune defence mechanisms and cause new outbreaks of the disease including pandemics when there is no immunity in humans to the influenza virus H and N antigens.

Thus, pandemic influenza viruses are able to create global rises in disease incidence worldwide and result in high mortality rates among those infected [2].

Amongst the many influenza virus types and subtypes that circulate globally each year, influenza A (H3N2) causes the largest number of infection outbreaks [3]. The first pandemic of modest severity occurred in 1889–1891. This virus then emerged in the human population in 1968, causing a pandemic known as 'Hong Kong fever' which led to 1 to 4 million deaths [4]. It has been shown to be associated with the most severe illness and the highest mortality rate, causing even more serious diseases in children and great socio-economic consequences [3].

Today, there are two classes of influenza antiviral agents recommended by the WHO: adamantanes, or

M2 ion channel blockers (amantadine and rimantadine), and neuraminidase inhibitors (oseltamivir and zanamivir as well as peramivir and laninamivir, which are approved in a limited number of countries). However, the effective use of these agents is limited: to achieve therapeutic effect, they should be administered within 48 h of the onset of infection symptoms [1], while a laboratory confirmation of influenza disease aetiology cannot be performed during such a short period of time [5]. Moreover, the majority of the influenza virus rapid diagnostic tests available have low sensitivity and lack the capacity to determine the pathogen subtype which may cause inefficiency in antiviral therapy [5]. For example, the widespread use of amantadine in some countries has led to an increase in the resistance to this agent among A(H3N2) viruses (from 12.3% in 2003 to 90.6% in 2005–2006) [5]. At the same time, although most influenza A (H3N2) viruses are sensitive to oseltamivir, oseltamivir-resistant variants can nevertheless be identified, especially in immunocompromised patients undergoing antiviral treatment [3]. Furthermore, during influenza seasons with several pathogens circulating simultaneously, cases of coinfections may occur, which are commonly accompanied by a recombination of several viral genotypes, contributing to the diversity of influenza genotypes. This variety of influenza viruses increases their potential to evade selective pressures or adapt to new host environments which finally may lead to influenza resistance to antiviral measures [6]. Therefore, there is a need for further effort to develop new, effective anti-influenza compounds.

Drugs containing antibodies in released-active form (RA Ab) represent a new class of drugs with a high safety [7] and efficacy profile. An RA Ab is a biotechnological product containing antibody-related supramolecular structures which emerge after a technological treatment of the initial antibodies' substance and acquire the ability to modify conformational properties of both their target (such as various endogenous bioregulators including enzymes, receptors, cytokines, etc.) and associated molecules [8]. The RA Ab efficacy has been demonstrated in both non-clinical and clinical studies in the treatment of various diseases including severe ones such as diabetes mellitus, rheumatoid arthritis, bronchial asthma and viral infections [9–19]. Anaferon is one of several innovative Russian drugs with antiviral activity created on the basis of released-active antibodies to interferon-gamma. This medicine has been successfully used for the treatment of many infectious diseases for more than 15 years and is approved for use in children and in adults [20–22]. Among the experimental infectious models which were used previously to show high antiviral efficacy of anaferon was the model of influenza infection in mice inoculated with influenza virus strain A/Aichi/2/68 (H3N2) [23]. The high efficacy shown for anaferon in the treatment of viral infections provided grounds for the development of a second generation drug, ergoferon, consisting not only of released-active antibodies to interferon-gamma (IFN-γ) but also to CD4 and histamine, which were demonstrated to have the ability to enhance T-cell immune response and to provide anti-inflammatory [24] and anti-allergic effects, respectively [25]. Thus, the aim of this study was to investigate the antiviral efficacy of ergoferon in the same model that was used to assess anaferon: a mouse experimental model of influenza infection with A/Aichi/2/68 (H3N2) virus [23].

Methods

Compounds

Ergoferon was supplied as a ready-to-use water solution by OOO "NPF "MATERIA MEDICA HOLDING" (Moscow, Russian Federation).

Affinity purified rabbit polyclonal antibodies to recombinant human IFN-γ, affinity purified rabbit polyclonal antibodies to recombinant human histamine and affinity purified rabbit polyclonal antibodies to the human CD4 receptor, were manufactured in accordance with current European Union requirements for Good Manufacturing Practice (GMP) for drug substances (EU Directive 2001/83/EC as amended by Directive 2004/27/EC) by AB Biotechnology (Edinburgh, UK), an MHRA licensed GMP manufacturing facility. Antibodies have been certified by MHRA as antibodies which can be used as a substance for the production of medicinal products for therapeutic application per os. Affinity purified polyclonal antibodies were produced by affinity purification of serum from specific pathogen free rabbits immunized with the respective antigen in accordance with Note for Guidance on Production and Quality Control of Animal Immunoglobulins and Immunosera for Human Use (CPMP/BWP/3354/99), current GMP regulations and part II of Volume 4 of the Rules Governing Medical Products in the EU titled 'Basic Requirements for Active Substances used as Starting Materials' issued 3 October 2005.

Antibodies have been characterized based on the requirements of the general European Pharmacopoeia Monograph on Immunosera for Human Use, Animal and as indicated in Note for Guidance on Production and Quality Control of Animal Immunoglobulins and Immunosera for Human Use (CPMP/BWP/3354/99) in accordance with the status approved by MHRA. All testing methods (for example, Competitive ELISA, Reducing and Non reducing SDS-PAGE, SEC-HPLC) have been developed in compliance with European Pharmacopoeia procedures and have been validated.

Released-active forms of rabbit polyclonal antibodies have been manufactured based on a novel patented biotechnological platform (US Patent 7,572,441 B2, 2009) using routine procedures described in the European Pharmacopoeia (6th Edition, 2007). Briefly, RA Ab have been prepared by consecutive reduction of antibodies to IFN-γ (2.5 mg/ml), to histamine (2.5 mg/ml) and to CD4 (2.5 mg/ml) concentration via their multiple dilutions under specific conditions in water–ethanol solutions as described previously [26]. Solutions were prepared avoiding intense direct light in sterile conditions and were stored at room temperature. Vehicle (distilled water) was used as a control. Tamiflu (oseltamivir phosphate, F Hoffmann–La Roche Ltd, Basel, Switzerland) was used as reference drug. All samples (except Tamiflu) were coded by the manufacturer and used blinded in the studies.

Mice

196 female Balb/c mice weighing 16–18 g at the age of 6–8 weeks were obtained from the animal facility at the State Research Center of Virology and Biotechnology 'Vector' (SRC VB 'Vector', Novosibirsk, Russian Federation). In the animal facility, the animals were kept in ventilated UNI-PROTECT cabinets (EHRET, Germany) with an air flow of 10 changes per h and temperature and relative humidity of 23–25°C and 40–60%, respectively. The cabinets were located in a specially equipped room licensed for experiments with influenza virus. The mice were housed in type II polysulfone cages (20 in each) containing fine wood shavings as a bedding material and equipped with a G4 filter, with a natural light cycle and 60–70 dB sound level, standard maintenance diet with the use of boiled and granulated feed and unlimited access to tap water in the drinking bowls. The experimental procedures and animal housing conditions were in accordance with the principles of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes [27], and the Guide for the Care and Use of Laboratory Animals [28]. All procedures performed on the animals in the course of the study were reviewed and approved by Institutional Animal Care and Use Committee (IACUC) under bioethical protocol #1-01.2014 dated 28 January 2014.

Virus

A mouse-adapted influenza strain A/Aichi/2/68 (H3N2) employed for the experiments was originally obtained from the State Collection of Viral Infections and Rickettsioses Agents of SRC VB 'Vector' and had been passed 12 times in mice and twice in chicken embryos. Virus titre in allantoic fluid (AF) was determined by titration in MDCK culture (Madin–Darby Canine Kidney) [29] and then calculated and expressed as $\log_{10}$ 50% tissue culture infectious dose ($TCID_{50}$)/ml using the Spearman–Kärber method [30]. The calculated AF virus titre was 8.2 ±0.2 $\log_{10}$ $TCID_{50}$/ml. The AF with virus concentrations (titre designated) to be used in the study were stored at -70°C.

Virus titration

Prior to the studies of the compounds' protective activity in animals, an influenza virus was titrated for lethal effect. For this purpose lightly ether-anaesthetized mice (6 animals in each experimental group) were intranasally inoculated with 0.04 ml of the appropriate AF virus dilution ($10^{0.8}$, $10^{1.8}$, $10^{2.8}$, $10^{3.8}$, $10^{4.8}$ and $10^{5.8}$ $TCID_{50}$/mouse) using a single-channel automatic pipette. The dilution causing death of 50% of the animals within 16 days post infection (LD_{50}) was calculated by the Spearman-Kärber method using the levels of virus utilized and corresponding mortality rates [30]. Calculated LD_{50} corresponded to the dose of 3.0 lg $TCID_{50}$/mouse. Based on LD_{50} data and a dose-response regression generated with the use of probit method as well as experimental verification, a 90% lethal dose (LD_{90}) of influenza virus was determined, which was 20 LD_{50} (4.3 lg $TCID_{50}$/mouse) for control animals.

General procedures

In order to evaluate the test samples' antiviral activity *in vivo*, mice were intranasally infected with 20 LD_{50} of the previously titrated virus (see *Virus titration* section). Groups of animals (n=40) were generated randomly by weight prior to the initiation of treatment. There were four treatment groups within the study. Mice in group 1 received ergoferon ("NPF "MATERIA MEDICA HOLDING", Russia). In order to boost host immune defence to the viral impact, ergoferon was given as a solution per os using the therapeutic and preventive regimen: 0.2 ml/animal twice daily, starting 5 days prior to infection with the virus and for 16 days post infection starting at 1 h after inoculation (total amount 0.4 ml per animal a day). Previous studies with anaferon (which is one component of ergoferon) demonstrated that a dose of 0.4ml/mouse/day was effective and well tolerated and was therefore used in this study [23,26]. Animals in the second group were dosed with the reference compound oseltamivir (Tamiflu) which is the drug of choice for the treatment of human influenza infections [31]. For this, a suspension was prepared: 1 Tamiflu capsule containing 75 mg of oseltamivir was dissolved in 80 ml distilled water. Oseltamivir was given using the therapeutic regimen: 0.2 ml/per animal twice daily (total amount 0.4 ml per animal a day), equivalent to a daily dose of 20 mg/kg per animal, for 5 days post infection starting at 1 h after inoculation. Additionally, to ensure the same treatment regimen as group 1,

mice in group 2 were dosed with 0.2 ml/animal of distilled water (0.4 ml per animal a day) starting 5 days prior to inoculation and for 11 days after oseltamivir treatment. Animals in group 3 received distilled water according to ergoferon's regimen, in a total amount of 0.4 ml per animal a day. Mice in group 4 (untreated) did not receive any treatment after virus inoculation.

The antiviral efficacy of the tested compounds was assessed according to the survival rate (SR), the average life expectancy (ALE) of infected mice and the viral load in the lungs at 2 and 4 days post infection. The ALE was calculated as described previously [32], briefly: the post-infection life expectancy of each animal was calculated, the life expectancy of those still alive until the end of the observation period was assumed to be 16 days, and then all the data were averaged. Lung viral load was determined in five mice from each group (at 2 and 4 days post infection) by titration of lung homogenates in MDCK cells. Calculated (Spearman-Kärber procedure) virus titres were expressed as $\log_{10}$ $TCID_{50}$/ml [30].

Statistical analysis

The results were statistically analysed by conventional methods accepted in biological research, using the R language and software packages stats, survival and Posthoc Comparison of Mean Rank (PMCR) [33].

Survival in animal groups was estimated using the Kaplan–Meier procedure followed by the pairwise log rank test with Benjamini–Yekutieli correction for multiple comparisons [34]. The probability of error (p) and significance of difference (P) were estimated for the comparisons. Differences were statistically significant at $p<0.05$ ($P\geq95\%$).

The comparison of lung viral loads and ALE data between treatment groups was performed with the Kruskal–Wallis test in combination with post-hoc Dunn's test and Benjamini–Yekutieli correction [35].

Results

For the study, influenza virus A/Aichi/2/68 (H3N2) was prepared in chicken embryos and its LD_{50} was determined for intranasal inoculation of mice with virus dilutions. The obtained LD_{50} was 3.0 $\log_{10}$ $TCID_{50}$ per mouse.

According to our experimental and literature data, a 90% mortality in mice challenged intranasally with influenza virus can be observed for a very large range of doses – from 5 LD_{50} to 100 LD_{50} [18,19]. The LD_{90} used in these experiments to induce influenza infection in mice was 20 LD_{50} (hereinafter LD_{90}), that is, 4.3 $\log_{10}$ $TCID_{50}$/mouse.

Mortality rates in the control group treated with distilled water and the group of untreated control after the challenge with LD_{90} of virus were 80% and 90%, respectively, which is within the probable range defined for 20 LD_{50} induced lethal infection in mice (Figure 1 and Table 1). Kaplan–Meier survival curves (Figure 1) showed a significant difference between the control groups (including distilled water treated group) and groups that received medications ($p=0.001$). The survival rate in the Tamiflu-treated group was 96.7% ($p<0.0001$ versus either of the control groups), even though some of the animals demonstrated apparent signs of illness. The survival of animals in the ergoferon group was significantly increased (60%, $p=0.0057$, $p<0.0001$) as compared to the group of distilled water and untreated control, respectively (Figure 1 and Table 1).

At the same time, the ALE values of mice infected with LD_{90} were 8.9 days in the distilled water group and 7.5 days in the group of untreated control, respectively. The greatest ALE (15.73 days) was obtained in the Tamiflu treated group ($p<0.0001$ versus either of the control groups). The ALE of ergoferon-treated mice was 12.3 days ($p=0.0334$ versus distilled water control; $p=0.0001$ versus untreated control; Table 1).

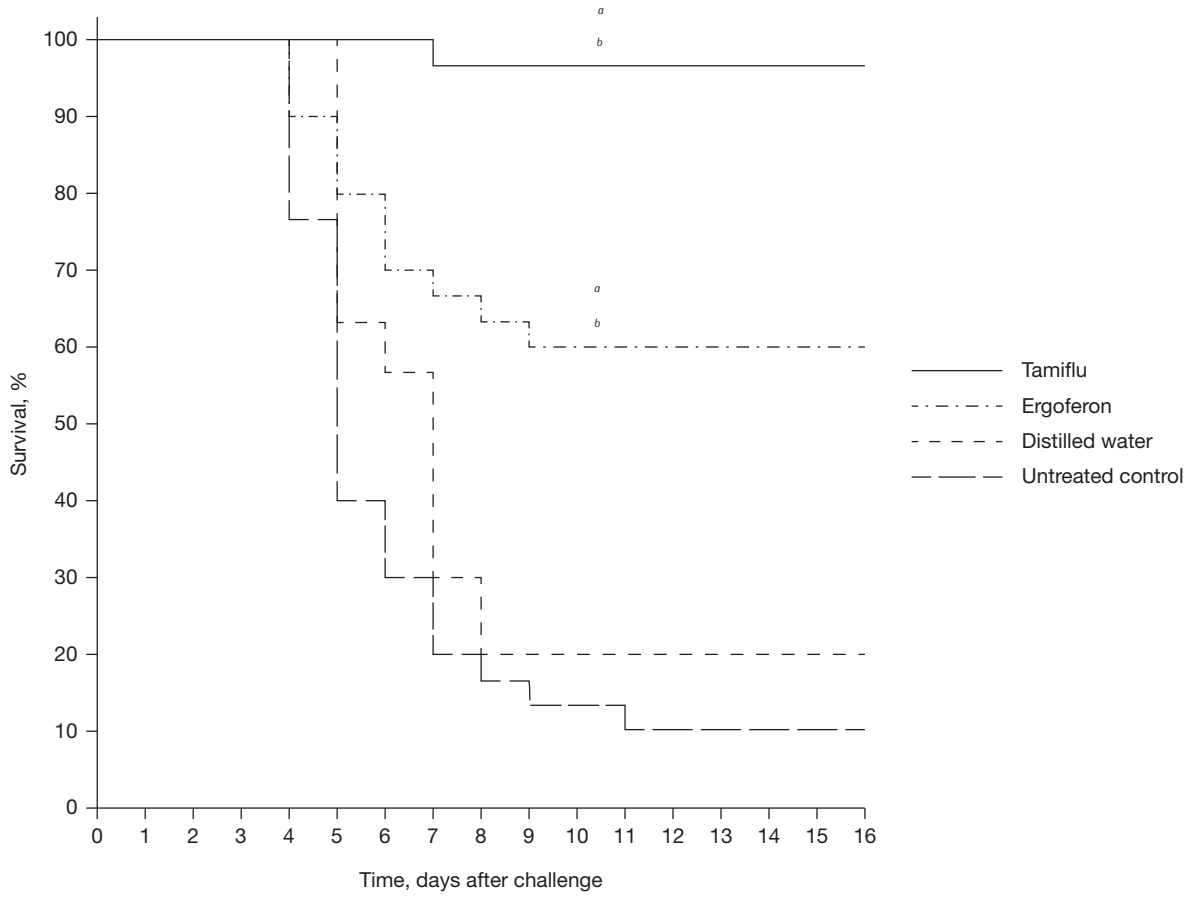
Antiviral effects of tested compounds were also assessed by changes in viral load in the lungs of infected mice at 2 and 4 days after inoculation with virus (Table 2). Significant differences in respiratory viral load between the ergoferon group and either of the control groups (distilled water control and untreated control) were observed as soon as day 2 after infection ($p=0.0349$; $p=0.0170$, respectively). In 4 days, a significant difference in lung virus titre of mice treated with ergoferon was only observed in comparison with untreated control ($p=0.0322$). In the group of reference compound oseltamivir, significant differences in virus titre were identified both at 2 and 4 days post infection as compared to the group of distilled water ($p=0.0255$; $p=0.0157$, respectively) and untreated control ($p=0.0170$; $p=0.0013$, respectively).

Discussion

Analysis of survival, average life expectancy data and viral load in the lungs of mice receiving study treatments suggests a beneficial effect of ergoferon. Furthermore, in these experiments the antiviral efficacy of ergoferon was higher than that of its single-component predecessor anaferon, as demonstrated by an increase from 42.5% to 60% in the number of surviving animals, with similar average life expectancy values – approximately 12 days [23].

The main factors that ensure defence against viral airway infections, including those caused by different influenza virus strains, are the neutralizing activity of upper respiratory tract secretory factors and pulmonary surfactant, interferon induction, pro-inflammatory

Figure 1. Survival of mice challenged with 20×LD_{50} of influenza virus A/Aichi/2/68 (H3N2) and receiving different treatment



[a]Difference versus distilled water control, at $p<0.05$. [b]Difference versus untreated control, at $p<0.05$.

Table 1. Survival rate and average life expectancy of mice in experimental groups and the group of untreated controls after infection with influenza virus A/Aichi/2/68 (H3N2)

Mice treatment groups (are the same as in Figure 1)	Number of dead mice	Survival rate of infected mice, %	Mean ALE, days ±SD
Ergoferon (*n*=30)	12[a,b]	60[a,b]	12.30 ±4.71[a,b]
Tamiflu (*n*=30)	1[a,b]	96.7[a,b]	15.73 ±1.46[a,b]
Distilled water (*n*=30)	24	20	8.90 ±3.75
Untreated control (*n*=30)	27	10	7.50 ±3.30

n is the number of animals per group. [a]Difference versus distilled water control, at $p<0.05$. [b]Difference versus untreated control, at $p<0.05$. ALE, average life expectancy of mice calculated taking into account the maximum animal observation period – 16 days post infection.

Table 2. Accumulation of influenza virus A/Aichi/2/68 (H3N2) in lungs of mice in experimental groups and the group of untreated infected control

Mice treatment groups (are the same as in Figure 1)	Mean viral load in lungs of mice after 2 and 4 days post influenza virus infection, log_{10} $TCID_{50}$/ml ±SD	
	Day 2 (*n*=5)	Day 4 (*n*=5)
Ergoferon	6.57 ±0.28[a,b]	6.67 ±0.20[a,b]
Tamiflu	6.33 ±0.48[a,b]	6.07 ±0.52[a,b]
Distilled water	7.37 ±0.14	7.23 ±0.19
Untreated control	7.47 ±0.14	7.43 ±0.09

n is number of animals per group. [a]Difference versus distilled water control, at $p<0.05$ at respective time points post infection. [b]Difference versus untreated control, at $p<0.05$ at respective time points post infection. $TCID_{50}$, 50% tissue culture infectious dose.

cytokine activation and other factors of specific and innate immunity [36–39].

The composition of ergoferon, which comprises released-active antibodies raised against interferon-gamma, CD4 receptor and histamine, suggests that it has a combined antiviral, anti-inflammatory, and anti-histamine effect. The effects of ergoferon components have been investigated well: for RA Ab to IFN-γ it was demonstrated that their effect is capable of modifying the constant of IFN-γ interaction with its receptor. In particular, RA Ab to IFN-γ are able to increase efficacy of such interaction thus ensuring increased antiviral activity implemented via IFN-γ [22,26]. At the same time, for released-active form of antibodies to histamine, its capability to modify histamine-dependent activation of H1 receptors was demonstrated [8,25]. Moreover, *in vitro* studies have shown the antagonistic effect of RA Ab to histamine on H1 and H4 histamine receptors (data not shown). Considering H1 and H4 receptors' involvement in histamine-induced inflammatory reactions, it can be implied that RA Abs to histamine are able to mediate the anti-inflammatory effect of the drug. RA Ab to the human CD4 receptor, have shown a capacity to activate $CD4^+$ T-lymphocytes and to reinforce the adapter function of the CD4 receptor in terms of signal transduction from the T-cell receptor [24]. The drug's essentially triple formulation is intended to implement its pharmacological activity via stimulation of different immune system components such as the interferon-gamma system, cellular and humoral immunities and the anti-inflammatory mediator network [22,25,26]. Such stimulation reduces inflammation and lessens inflammation-mediated damage to vital organs which is observed with influenza infection.

The experiments performed to investigate the novel product ergoferon demonstrated its antiviral efficacy following therapeutic and preventive dosing in mice intranasally challenged with a lethal dose of influenza virus. Although ergoferon treatment effect is less than that of Tamiflu, ergoferon possesses several advantages over the latter. Such features of the drug as high safety profile, absence of side effects, reinforcement of host antiviral response, no necessity to identify the pathogen before its use as well as no risk of virus resistance development (since the target of the drug is the host organism and not the virus) [40–43] – allow its usage in cases when Tamiflu administration is limited or restricted. The high safety of the drug and its mechanism of action targeted boosting of the host's immunity, making its usage clinically expedient for an extended period of time. This includes drug administration for prophylactic purposes during the annual influenza seasons which is replaced with a therapeutic regimen in case of an illness. The efficacy of the prophylactic and treatment regimen of ergoferon administration used in the present study has been confirmed in clinical trials [40–43]. Furthermore, ergoferon's three-component formula ensures its complex antiviral, anti-inflammatory and anti-allergic effect which is essential for effective treatment of this infectious disease.

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