Activity of ultra-low doses of antibodies to gamma-interferon against lethal influenza A(H1N1)2009 virus infection in mice

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ABSTRACT

Background: The influenza A virus is a highly infective agent that causes acute pulmonary diseases. In serious cases, it causes pneumonia which is particularly fatal in patients with cardiopulmonary diseases, obesity, young children and elderly people. The present study shows a protective effect of ultra-low doses of purified antibodies to gamma-interferon (Anaferon for children®, AC®) against lethal influenza virus infection caused by pandemic influenza virus A(H1N1) in mice.
Methods: Balb/c mice were infected with mouse-adapted pandemic influenza virus A/California/07/09 (H1N1)v. Mortality, weight loss, infectious titer of the virus in lungs and lung morphology were monitored in the groups of AC®-, oseltamivir- and placebo-treated animals.
Results: The protective action of AC® was demonstrated by prolongation of life of the infected animals, reduction of infectious titer of the virus in the lung tissue, normalization of weight dynamics in the course of disease, decrease in mortality of treated animals compared to a placebo control and normalization of lung tissue structure. The protective activity of AC® was similar to that of the reference compound oseltamivir. Combination of AC® with oseltamivir resulted in a higher protective effect comparing to oseltamivir alone.
Conclusion: Based on the results obtained, AC® should be considered as an important part of anti-influenza prophylaxis and therapy, in particular in severe cases of the disease.

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1. Introduction

The influenza A virus is a highly infective agent that causes acute pulmonary diseases. In serious cases, influenza A causes pneumonia which is particularly fatal in patients with cardiopulmonary diseases, obesity, young children and elderly people. Outbreaks of highly pathogenic influenza virus infections and appearance in 2009 of a new pandemic influenza A virus have triggered the renewal of interest in influenza infection. As of May 2010, more than 214 countries and overseas territories or communities worldwide have reported on laboratory confirmed cases of pandemic influenza H1N1 2009, including more than 18,097 deaths (WHO, 2010). Antiviral drugs occupy an important niche in the management of the disease (Moscona, 2005, 2008). They target virus-specific components and are effective for treatment when administered at the early stage of infection or soon after virus exposure (Moscona, 2008).

Two main classes of anti-influenza drugs are currently accepted for chemotherapy of influenza. Adamantane derivatives (amanta-dine and rimantadine) target M2 ion channel of influenza A virus and are not effective against influenza B virus (Hayden, 1996). Moreover, rapid emergence of drug-resistance among influenza viruses since mid-90s has greatly compromised the effectiveness of these compounds (CDC, 2008). All pandemic H1N1 viruses tested so far also showed to be drug-resistant (Dawood et al., 2009).

Inhibitors of neuraminidase (oseltamivir, zanamivir and peramivir) have a wider spectrum of activity which includes influenza A and B viruses (Hayden, 2009). Nevertheless, since 2007 rapid emergence and transmission of drug-resistant viruses have been observed (CDC, 2008; Hauge et al., 2009; Dharan et al., 2009). Several strains resistant to inhibitors of neuraminidase were also isolated from pandemic H1N1 virus (Chan et al., 2010). There is therefore a need for both searching for new effective antivirals and development of optimal regimens and combinations of antiviral compounds used in clinics.

Anaferon for children (AC®) is an antiviral drug with a wide range of activity (Kudin et al., 2009; Shishkina et al., 2008; Vasil'ev et al., 2008; Epstein, 2005; Sergeev et al., 2004; Martyushev-Poklad et al., 2004) containing ultra-low doses of antibodies to interferon-gamma, it has been successfully used in medical practice for

10 years and is permitted for use in children, including those of 1 year old. Research organizations in Russia, EU countries and USA, which studied AC® on various experimental models of viral infections, have come to the same conclusion concerning AC® antiviral activity: the drug reduces mortality of laboratory animals, increases average lifespan and decreases viral titers. In clinical trials, AC® reduced total morbidity and incidence of acute respiratory viral infections and shortened the duration of fever, intoxication, and catarrhal syndromes. These data proved that AC® is a potential agent for prophylaxis and treatment of influenza virus infection both in experiment and in clinics.

Despite this activity, influenza infection in humans, especially in its severe cases, cannot be treated only with anti-interferon antibodies in ultra-low doses. For this reason, optimal schemes for combined application of preparations with different mechanisms of activity should be developed in order to reach maximum effective treatment of the disease. Here we present the study results on protective activity of combination of AC® with etiotropic antiviral oseltamivir on the model of lethal influenza pneumonia caused by pandemic influenza virus A(H1N1)2009.

2. Materials and methods

2.1. Compounds

AC® was supplied as a ready-to-use solution by OOO "NPF "MATERIA MEDICA HOLDING" (Russia, Moscow). Affinity purified rabbit polyclonal antibodies to recombinant human interferon gamma were manufactured in accordance with current European Union requirements for Good Manufacturing Practice for starting materials (EU Directive 2001/83/EC as amended by Directive 2004/27/EC) by Angel Biotechnology Holdings plc (UK, Edinburg) as a starting material for commercial production of AC® for therapeutic oral application. Ultra-low doses of antibodies to interferon gamma were obtained using routine methods described in the European Pharmacopoeia (6th Edition, 2007). All dilutions were prepared in glass vials. Antibodies to interferon gamma (2.5 mg/ml) were mixed with a solvent (ethanol–water solution) and shaken for 1 min to produce C1 dilution. All subsequent dilutions consisted of one part of the previous dilution to 99 parts of solvent (ethanol–water solution for intermediate dilutions and distilled water for preparation of the final dilution), with succession between each dilution. Solutions were prepared in sterile conditions, avoiding direct intense light, and were stored at room temperature. Distilled water was used as a control. Tamiflu (oseltamivir phosphate, F. Hoffmann–La Roche Ltd., Switzerland) was used in experiments as a reference drug.

2.2. Virus

Influenza virus A/California/07/09 (H1N1) was obtained from the collection of viruses of Influenza Research Institute. Prior to experiment, the virus was adapted to mice by three serial passages in mice lung tissue with a subsequent passage in the allantoic cavity of 10–12 days old chicken embryos and a final passage in mice again (Narasaraju et al., 2009). Lung homogenate in nine volumes of sterile phosphate-buffered saline was used as an infecting material in further experiments.

2.3. Mice

Inbred female BALB/c mice, 12–16 g were obtained from the animal breeding facility of Russian Academy of Medicine "Rappopolovo" (Rappopolovo, Russia). The mice were quarantined 48 h prior to the experimental manipulation and were fed standard rodent chow and had *ad libitum* access to water. Animal experiments were conducted in accordance with the principles of laboratory animals care (Guide for the Care and Use of Laboratory Animals, National Academy Press, Washington, DC, 1996) and were approved by the Institutional Ethical Committee.

2.4. Virus titration

Prior to studies of the protective activity of compounds in animals, mouse-adapted influenza virus was titrated for lethal effect. For this purpose mice (10 in each experimental group) were inoculated intranasally under anesthesia with 50 μL of serial decimal dilutions (10^{-1}–10^{-5}) of the lung homogenate from virus-infected mice. The dilution that caused death of 50% of the animals in 21 days post infection (LD_{50}) was calculated as described previously (Reed and Muench, 1938) and was used for subsequent experiments.

2.5. General procedures

In order to evaluate anti-influenza activity of AC® *in vivo*, mice were infected with ten LD_{50} (30 mice) or one LD_{50} (35 mice) of previously titrated virus (see "Virus titration" section). AC® was applied orally via gavage twice a day in a volume of 0.2 mL for five days prior to infection and 21 days post infection (p.i.). The reference drug oseltamivir (final dose 20 mg/kg body weight) was dissolved in saline and applied to mice orally in a volume of 0.2 mL. Separate group of animals received both oseltamivir and AC® (20 mg/kg/day of oseltamivir in 0.2 mL of AC®). Control animals were treated with distilled water. Ten uninfected untreated mice were used as intact control.

For each dose of the virus, 20 mice were used for weight and mortality control. Additionally, in 1 LD_{50} experiments five mice were used for virus titration and five animals from each group were used for morphology analysis of the lungs.

The mortality in each group of animals was calculated. Each group was monitored daily on lethal cases for 3 weeks post inoculation. Based on the data received, percent of mortality, index of protection (ratio of mortality in the control group over mortality in the experimental group) and mean day to death (MDD) were calculated.

On day three p.i. five mice from each group infected with 1 LD_{50} of the virus were sacrificed, their chest opened and lungs isolated. Five lungs were used for virus titration and five others – for histological examination (see below).

In order to determine infectious titer of the virus in lung tissue, lungs were homogenized in ten volumes of sterile phosphate-buffered saline. Serial dilutions (10^{-1}–10^{-7}) were prepared from each homogenate. MDCK cells grown in 96-well plates were inoculated with 0.2 mL of each dilution and incubated at 36 °C for 48 h in 5% CO_2. After incubation, supernatant was harvested and tested for presence of influenza virus by mixing the fluid in round-bottom wells with equal volume of 1% suspension of chicken erythrocytes in saline. Virus titer in the lungs was considered as the final dilution causing positive hemagglutination reaction in the well and expressed in decimal logarithm of 50% infection dose ($\log_{10}ID_{50}$) per 20 mg tissue. The activity of the compounds was evaluated by their ability to decrease the infectious titer of the virus in lung tissue.

2.6. Histological examination

Lungs of mice were placed into 4% PBS-buffered formaldehyde, dehydrated in graded ethanol and embedded in paraffin. Four-micrometer pieces were cut off and stained with haemotoxylin-eosin. The severity of the pathological process was determined by evaluation of the relative size of virus-induced foci of inflamma-

tion (per cent of total lung surface). The morphometric values were evaluated by two independent observers.

2.7. Statistical analysis

The statistical analysis included the comparison of AC® versus control, AC® versus oseltamivir, and AC® plus oseltamivir versus oseltamivir alone. The log-rank test was used to compare survival times, which were expressed in number of days since inoculation. Survival times beyond the end of the follow-up period at 21 days were treated as censored observations. Secondary outcomes for virus titer in lungs and foci of pneumonia were analyzed by one-way ANOVA. Weight was analyzed by two-way ANOVA, with treatment condition and time (before versus after inoculation) as independent variables. Assessment of whether changes in weight were statistically significant was based on the P-value for the interaction between time and treatment condition. All data were analyzed using the SAS statistical package. Each pair of treatment conditions was compared separately. P-values <0.05 were regarded as statistically significant.

3. Results

Inoculation of animals with an adapted virus led to development of influenza pneumonia. The clinical signs of the disease were typical for severe influenza infection and included ataxia, tremor, short breath, as well as decrease of water and food consumption leading to weight loss. On day 21 p.i. death of 48–88% of infected animals was observed, depending on infecting dose of the virus.

No non-specific mortality was observed in control groups of intact animals and non-infected mice treated with AC®. Application of AC®, as well as of oseltamivir, was shown to result in decrease of specific mortality (0–90%) and in increase of mean day of death (up to 4.3 days) comparing to control values depending on the dose of virus and compound(s) used. Mice treated with a reference compound oseltamivir in combination with AC® demonstrated the lowest mortality (index of protection 90%) and prolongation of mean day of death (up to 5.4 days) comparing to control values (Table 1).

In the 1 LD_{50} experiment, survival was significantly greater for the AC® group relative to the control group ($\chi^2(1) = 9.44$, $P = 0.0021$). In the 1 LD_{50} experiment differences in survival were not significant for AC® versus oseltamivir ($\chi^2(1) = 0.32$, $P = 0.57$), or for AC® plus oseltamivir versus oseltamivir alone ($\chi^2(1) = 0.39$, $P = 0.53$). In the 10LD_{50} experiment, survival was significantly greater for the AC® plus oseltamivir condition relative to the oseltamivir alone condition ($\chi^2(1) = 3.96$, $P = 0.047$). In the 10LD_{50} experiment differences in survival were not significant for AC® versus control ($\chi^2(1) = 2.51$, $P = 0.11$), or for AC® versus oseltamivir alone ($\chi^2(1) = 1.51$, $P = 0.21$).

These data corresponded to weight dynamics in the course of disease. Treatment of animals with AC® as well as with oseltamivir (alone or in combination with AC®) resulted in normalization of weight dynamics in animals (Fig. 1). Changes in weight were statistically significant for the AC® group relative to the control group ($F(1,389) = 26.56$, $P < 0.0001$) and for AC® versus oseltamivir ($F(1,272) = 7.75$, $P = 0.0058$). Changes in weight for AC® plus oseltamivir versus oseltamivir alone approached statistical significance ($F(1,281) = 2.68$, $P = 0.10$).

Table 1
Protective activity of AC® against influenza A(H1N1)2009-caused lethal pneumonia in BALB/c mice. When P < 0.05 values are indicated in bold.

| Treatment	Virus dose	Survival/total(% survival)	Mean day to death ± SEM	Index of protection (%)	Lung data	
					Virus titer ($\log_{10}EID_{50}$/20 mg tissue ± SEM)	Medium size of foci of pneumonia (%)
AC®	1 LD_{50}	19/20 (95%)[*]	20.1 ± 0.9[*]	89.5	5.1 ± 0.9[*]	17.2 ± 4.7[*]
	10 LD_{50}	7/20 (35%)[*]	11.3 ± 1.7	25.7	nd[a]	nd[a]
Oseltamivir(20 mg/kg/day)	1 LD_{50}	18/20 (90%)[*]	19.7 ± 0.9[*]	78.9	3.4 ± 0.6[*]	9.2 ± 3.0[*]
	10 LD_{50}	2/20 (10%)	7.9 ± 1.0	-2.9	nd[a]	nd[a]
AC® + Oseltamivir(20 mg/kg/day)	1 LD_{50}	19/20 (95%)[*]	20.9 ± 0.1[*]	89.5	3.1 ± 1.2[*]	16.5 ± 4.5[*]
	10 LD_{50}	10/20 (50%)[*]	13.3 ± 1.8[*]	42.9	nd[a]	nd[a]
Control(no treatment)	1 LD_{50}	21/40 (52.5%)	15.8 ± 0.9	0	6.3 ± 0.4	34.5 ± 4.6
	10 LD_{50}	5/40 (12.5%)	7.9 ± 0.9	0	nd[a]	nd[a]
Uninfected(no treatment)	0	10/10 (100%)	–	–	–	–

[a] Not done.
[*] P < 0.05 vs. control.
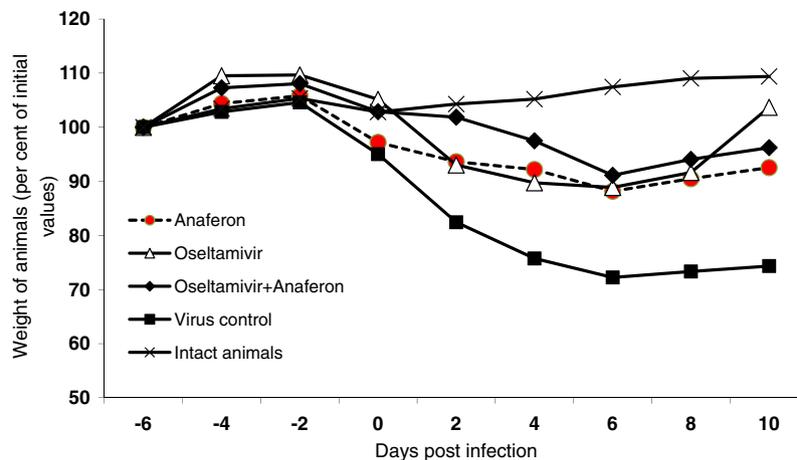
Fig. 1. Dynamics of body weight of mice in the course of pneumonia caused by influenza virus A/California/7/09 (H1N1)v.
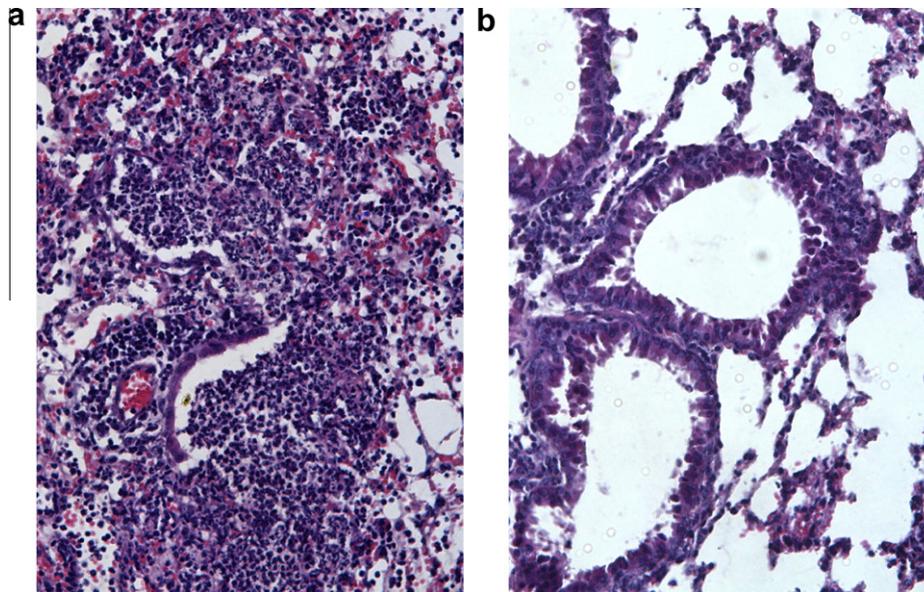
Fig. 2. Pathologies of mice intranasally inoculated with mouse-adapted influenza virus A/California/7/09 (H1N1)v. (a) Lungs of a placebo-treated mouse. Large area of pneumonia with intense exudates in bronchial lumen, severe neutrophilic-to-histiocytic alveolitis. (b) Mild lymphocytic peribronchitis with mild alveolitis in a mouse treated with ultralow doses of antibodies to interferon-gamma. Cells of bronchial epithelium are intact, mild exudates in bronchial lumen. Hematoxylin-eosin, ×200.

As shown by virus titration from lungs of mice, on day 3 p.i. the virus replicated in the lung tissue up to $10^{5,1}$ EID_{50}/20 mg tissue. Application of the reference compound oseltamivir decreased the viral titer approximately 320-fold ($10^{2.6}$ EID_{50}/20 mg tissue). Treatment of animals with AC® also resulted in decrease of virus titers (Table 1). These decreases were statistically significant for the AC® group relative to the control group ($F(1,13) = 10.76$, $P = 0.006$) and for AC® versus oseltamivir ($F(1,8) = 10.70$, $P = 0.011$). Effects for AC® plus oseltamivir versus oseltamivir alone were not statistically significant ($F(1,8) = 0.20$, $P = 0.66$).

In order to evaluate the effect of AC® on structure of the lung tissue, morphology analysis was performed on day 3 p.i. As revealed by visual examination, lungs of virus-infected mice were consolidated and edematous. All infected mice had exudative diffuse alveolar damage with interstitial edema, fibrinous exudates in alveoli, inflammatory infiltration, bronchiolar epithelial necrosis and desquamation. Cells of bronchial and bronchiolar epithelium contained viral inclusions or were absent denuding basal membrane (Fig. 2a).

Application of AC®, similar to the reference compound oseltamivir, resulted in normalization of lung tissue structure, particularly in restriction of edema and alveolar damage, decrease of amount of debris in bronchial lumen and protection of bronchial epithelium from death (Fig. 2b, Table 1). The medium size of foci of pneumonia were significantly lower in the AC® group relative to the control group ($F(1,27) = 5.35$, $P = 0.029$). Differences in sizes of pneumonia foci were not statistically significant for AC® versus oseltamivir ($F(1,35) = 1.89$, $P = 0.18$) or for AC® plus oseltamivir versus oseltamivir alone ($F(1,35) = 1.67$, $P = 0.20$).

4. Discussion

In the present study, we showed a protective effect of orally applied ultra-low doses of anti-interferon gamma antibodies against lethal influenza virus infection caused by pandemic influenza virus A(H1N1) in mice. Results of the present study confirm the conclusions of previously conducted studies Apcis S.A. (France, Maisons-Alfort), State Research Center of Virology and Biotechnology "Vector" (Russia, Novosibirsk), Utah State University (USA,

Logan) with a model of influenza A/H1N1 (A/California/07/09 and A/California/04/09 strains) in mice (Shishkina et al., 2008; Tarasov et al., 2007, 2010); as well as the conclusions of clinical trials and of a long experience of AC® usage in medical practice.

The protective action of AC® was demonstrated by reduction of infectious titer of the virus in the lung tissue, prolongation of life of infected animals, normalization of weight dynamics in the course of disease, decrease of mortality among treated animals compared to a placebo control and normalization of lung tissue structure. These effects were most expressed at low inoculating dose (1 LD_{50}). The antiviral activity of AC® was in general statistically comparable to the reference compound oseltamivir (20 mg/kg/day).

In clinics, one of the main reasons for severe and complicated influenza pneumonia, including fatal cases, is late and/or inadequate treatment (Lapinsky, 2010; Rello et al., 2009). In such cases, the course of the disease is driven by mechanisms that are initially induced by the virus but ultimately realized by the host, including, in particular, severe inflammation ("cytokine storm") (Woo et al., 2010; Garigliany et al., 2010; To et al., 2010). Experiments using influenza virus-infected knockout mice with inactivated genes in the inflammatory pathways, such as interleukin 1α/β, macrophage chemokine receptors CCR5 and CCR2, cyclooxygenase 1 and 2 (Carey et al., 2005; Schmitz et al., 2005; Dawson et al., 2000) have clearly demonstrated that in addition to level of virus replication in the lungs, the intensity of the host reactions contributes significantly to the course and the outcome of the disease. Therefore in severe cases of influenza pathogenetic approaches should be applied in addition to direct antiviral treatment (Alleva et al., 2010). For example, recently a high protective activity of 7-hydroxycoumarin (7-HC) has been demonstrated (Kurokawa et al., 2010). 7-HC was proved to possess antiviral properties owing to its ability to decrease the level of proinflammatory cytokines in infected animals, thus alleviating severe influenza infection.

One of AC® mechanisms of action is induction of endogenous interferons α/β or γ, proved by both experimental (Sherstoboev et al., 2003) and clinical studies (Epstein, 2008). AC® administration is followed by significant increase of lymphocytes ability to produce interferon α/β and interferon γ on 1–2 days of the disease onset (Obraztsova et al., 2009; Tarasov et al., 2008), normalization of subpopulational content of immunocompetent cells (CD3, CD4,

CD8, CD16, CD20) in peripheral blood, decrease of total sensibilisation level (IgE) and enhancement of generation of the main resistance factor – secretory IgA (Epstein et al., 2010).

Previously, Euroscreen S.A. (Belgium, Gosselies) and Cerep S.A. (France, Paris) revealed a range of receptors, which are involved in realization of AC® pharmacological activity (sigma-1 receptor and interferon gamma receptor). As a positive allosteric modulator of interferon gamma receptor AC® increases by more than 50% the amount of endogenous interferon gamma, bound with receptor (data not published). AC® enhances subpopulation of lymphocytes, in cell membrane of which interferon gamma receptors are expressed (CD119+ lymphocytes) (Tarasov et al., 2008a).

AC® effects the system of natural autoantibodies to interferon gamma, which are the main bioregulatory antibodies involved in formation of normal immune response, homeostatic regulation, in establishment of idiotypic repertoire and in regulation of cytokines production. AC® increases concentration of natural autoantibodies to interferon gamma and their functional activity (U-CyTech bioscience B.V. (the Netherlands, Utrecht) (Tarasov et al., 2008b).

Combination of AC® with oseltamivir, the drug of direct antiviral mode of action, gives the highest values of protection affecting both virus replication and host reactions.

Thus the conducted study confirmed the antiviral activity of AC® shown in previous experimental and clinical studies (Shishkina et al., 2010), which can be effectively used in treatment of influenza infection caused by pandemic strain of the virus A/California/07/09 (H1N1). Interestingly, despite lack of the direct antiviral activity in anti-IFNγ antibodies, application of AC® led to suppression of virus' replication in lung tissue. One of the possible explanation might be the following. Recently, the host inflammatory pathway triggered by nuclear factor κB (NF-kB) was shown to be proviral, i.e. playing virus-enhancing role during influenza infection. Despite being a key factor in triggering influenza A virus-induced inflammatory cytokine production (Bernasconi et al., 2005) it is used by the virus to enhance its production (Ludwig, 2009; Wei et al., 2006; Nimmerjahn et al., 2004). Interfering with inflammatory pathways in vivo, AC® might suppress the level of NF-κB thus restricting indirectly the virus' replication. The exact mechanism of regulation of influenza virus infection by anti-IFNγ antibodies remained to be further elucidated.

In addition to previously obtained results, AC was shown to dramatically decrease the level of lung infiltration with inflammatory cells, the property making it prospective in terms of restriction of reactive damage of the tissue. Furthermore, its application led to protection of the layer of bronchial epithelium thus inhibiting one of the main reasons of severe influenza and its complications (Sanders et al., 2011). It has been proved that the antiviral efficacy of oseltamivir can be increased by its combination with AC®.

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