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# Virus susceptibility and clinical effectiveness of anti-influenza drugs during the 2010–2011 influenza season in Russia

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SUMMARY

*Background:* Antiviral drugs are critical adjuncts to influenza vaccination. This study determined the in vitro susceptibilities of influenza A and B viruses isolated in the 2010–2011 season in Russia to the neuraminidase inhibitor oseltamivir and the hemagglutinin fusion inhibitor umifenovir and clinical efficacy of this antiviral drugs in this season.
*Methods:* The antiviral potency of these drugs against A(H1N1)pdm09 virus in mice was assessed. Importantly, the clinical effectiveness of oseltamivir and umifenovir was evaluated in a retrospective study conducted in 26 regions of Russia.
*Results:* All tested viruses ($n = 36$) were susceptible to oseltamivir and umifenovir in vitro. Oseltamivir (10 mg/kg/day) and umifenovir (60 mg/kg/day) significantly increased the survival of mice challenged with A/California/04/2009 (H1N1)pdm09 virus ($p < 0.05$). Influenza infection was laboratory-confirmed in 442 patients among 1462 patients hospitalized with acute respiratory infections. The treatment of influenza-infected patients within 48 h of symptom onset with oseltamivir and umifenovir was associated with a significant decrease in the duration of illness (2–3 days) and symptoms ($p < 0.001$). Pneumonia was observed in none of the patients treated with oseltamivir and in 0.3% of the patients treated with umifenovir, compared to 23.7% of patients who did not receive antiviral therapy ($p < 0.001$).
*Conclusions:* This study provided experimental and clinical evidence of the efficacy of oseltamivir and umifenovir against influenza viruses, representatives of which have continued to circulate in post-pandemic seasons.


## 1. Introduction

Influenza A and B viruses are the most common human respiratory pathogens that cause annual epidemics with high morbidity and significant mortality. Occasionally influenza A viruses have caused pandemic outbreaks affecting millions of people worldwide. On June 11, 2009, the World Health Organization (WHO) raised the global pandemic alert level to phase 6, the pandemic phase, in response to the emergence and global spread of a novel influenza A(H1N1)pdm09 virus that contained a previously unseen combination of genes of swine origin.[1] In Russia, from 2009 through 2011, it is estimated that the influenza virus caused approximately 30.8 million cases of influenza annually.[2,3]

The first pandemic of the 21st century emphasized the limited available strategies for the control of influenza infections. The prevention and treatment of influenza currently relies on vaccines and antiviral agents. Although vaccines are the better option for influenza control, their composition must be updated regularly to reflect changes in the circulating viruses. The lesson learned from the 2009 A(H1N1) pandemic is that, despite improvements in the preparation of influenza vaccines, the current strategies for the preparation of either inactivated or live-attenuated influenza vaccines require more than 6 months. Consequently, vaccines were not available to control the first wave of the pandemic.[3,4] In Russia, a vaccine to the new A(H1N1)pdm09 virus was available for use only at the beginning of 2010. Additionally, some people are not adequately protected by vaccination.[5,6] Consequently, effective

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anti-influenza drugs, of which several are available, comprise an important adjunct to vaccination.

There are some anti-influenza drug classes that are licensed in Russia. The membrane (M)2 ion channel inhibitors, including rimantadine, are active against the influenza A virus, but their clinical use is limited because they are not effective against the influenza B virus and lead to the emergence of transmissible drug-resistant strains.[6,7] At the beginning of the pandemic, it was shown that the A(H1N1)pdm09 viruses were resistant to amantadine and rimantadine. The neuraminidase inhibitor oseltamivir (oral drug, 75 mg/dose; Tamiflu) is active against influenza A and B viruses and binds to the neuraminidase (NA) surface glycoprotein of newly formed virus particles, preventing their efficient release from the host cell.[6,7]

Umifenovir (arbidol; ethyl 6-bromo-4-[(dimethylamino)-methyl]-5-hydroxy-1-methyl- 2-[(phenylsulfanyl)methyl]-1*H*-indole-3-carboxylate hydrochloride monohydrate) was developed by a joint consortium of Russian scientists from the Centre for Drug Chemistry, Moscow, the Medical Radiology Scientific Research Institute, Obninsk, and the Pasteur Scientific Research Institute for Epidemiology and Microbiology, St. Petersburg.[8,9] Umifenovir is a broad-spectrum antiviral compound that blocks viral fusion.[10–13] It is manufactured in both the Russian Federation and China, where it is licensed for use for the prophylaxis and treatment of influenza A and B infections. Studies on its mechanism of action against the influenza virus have shown that umifenovir falls within a class of inhibitors that interact with hemagglutinin (HA) to stabilize it against the low pH transition to its fusogenic state; consequently, it inhibits HA-mediated membrane fusion during influenza virus infection.[11,12] Umifenovir has strong antiviral activity against both influenza A and B viruses in cell culture and in virus-infected mice.[14–16] Clinical trials conducted on more than 30 000 patients have shown that umifenovir is well tolerated, and no side effects have been revealed.[8,9] Arbidol-resistant mutants have been generated in cell culture,[13] but to date, arbidol-resistant viruses have not been isolated from humans. There is also no evidence of naturally occurring resistance to umifenovir in any influenza virus isolates.[17,18]

On August 10, 2010, the WHO declared a worldwide post-pandemic period. Similar to most other countries, the peak influenza season during 2010–2011 in Russia was associated with the co-circulation of three influenza viruses: A(H1N1)pdm09, A(H3N2), and B. These three viruses were different in prevalence and geographic location. A(H3N2) dominated in the Far East, A(H1N1)pdm09 and B were observed in Siberia, and A(H1N1)pdm09 was observed in the European region. On average, the influenza virus activities during the 2010–2011 epidemic period in Russia were as follows: A(H1N1)pdm09, 53.0%; A(H3N2), 7.0%; B, 40.0%. A detailed study of the epidemic antigenic properties of the strains showed that most of the strains were similar to the reference strains that were recommended for vaccine composition for the 2010–2011 epidemic season, which included A/California/07/2009 (H1N1)pdm09, A/Perth/16/2009 A(H3N2), and B/Brisbane/60/2008 (8.0% of strains were similar to another evolutionary line, B/Yamagata/16/88).[17,18]

The purpose of this study was to provide detailed information regarding virus susceptibility and clinical effectiveness in a retrospective pharmacoepidemiological study of oseltamivir and umifenovir, which were licensed and used widely during the first post-pandemic 2010–2011 influenza season in Russia. The experimental part of this study aimed to make recommendations for the treatment and prophylaxis of influenza viruses that circulated during this period, including the swine influenza A(H1N1) virus. These data will also contribute to the monitoring of antiviral resistance. The aim of the clinical observational individual patient data study was to analyze the effectiveness of antiviral use on influenza severity and outcomes.

## 2. Materials and methods

### 2.1. Compounds

Oseltamivir carboxylate ((3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid) and the pro-drug oseltamivir phosphate (oseltamivir) (ethyl(3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate) were generous gifts from Chemical Diversity Inc. (San Diego, USA). The compounds were dissolved in sterile distilled water for the in vitro and in vivo experiments.

Umifenovir (arbidol; ethyl 6-bromo-4-[(dimethylamino)methyl]-5-hydroxy-1-methyl-2-[(phenylsulfanyl)methyl]-1*H*-indole-3-carboxylate hydrochloride monohydrate) was a generous gift from Prof. Glushkov. Umifenovir was dissolved to completion in 0.5 ml of 96-proof ethanol at 37 °C for 10 min, followed by dilution in 4.5 ml of sterile distilled water. For each experiment, a freshly made stock was used. This stock (1800 μM) was used for the preparation of the required umifenovir concentrations for the cell culture experiments. For oral delivery to mice, umifenovir was suspended in 1% starch.

### 2.2. Cells and viruses

Madin–Darby canine kidney (MDCK) cells were grown in minimal essential medium (MEM) supplemented with 10% foetal bovine serum (FBS), 5 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin sulphate, and 100 μg/ml kanamycin sulphate in a humidified atmosphere of 5% $CO_2$.

Influenza A/Aichi/2/68 (H3N2) (mouse-adapted), as a reference prototype strain, was obtained from the D.I. Ivanovsky Research Institute of Virology, Moscow, Russian Federation. The A/California/04/2009 (A/CA/04/09) (H1N1)pdm09, A/Victoria/361/2011 (H3N2), and B/Wisconsin/1/2010 influenza viruses were provided by the WHO Collaborating Centre for Reference and Research on Influenza (St. Petersburg, Russia). Oseltamivir-resistant A/Perth/265/2009 (H1N1pdm09) with the H275Y NA substitution (N1 numbering) and B/Perth/211/2010 with the D197E NA substitution (B numbering) were obtained from the NIS Panel of Influenza A and B Viruses for Assessment. Laboratory and vaccine strains of influenza A and B viruses were grown in 9-day-old embryonated chicken eggs. Clinical isolates were isolated and grown in MDCK cells.

### 2.3. Virus susceptibility to an NA inhibitor, in vitro

A fluorometric assay using the fluorogenic substrate 20-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA) (Sigma-Aldrich) was used to determine viral NA activity.[19] The fluorescence of the released 4-methylumbelliferone was measured in a Varioskan multi-mode microplate reader (BioTek) using excitation and emission wavelengths of 360 and 460 nm, respectively. Serial 10-fold concentrations of oseltamivir carboxylate (0.01 nM to 10 000 nM) were studied. The drug concentration that inhibited 50% of the NA enzymatic activity ($IC_{50}$) was determined from the dose–response curve using GraphPad Prism 5.0 software. The results were reported as the average of three experiments for each virus.

### 2.4. Umifenovir antiviral activity by ELISA assay

A modified ELISA was used to measure the inhibition of influenza A and B virus replication in MDCK cells with umifenovir.[20] This assay detected the expression of viral proteins (M+NP) on infected cells. Briefly, MDCK cells were seeded in 96-well plates at 3000 cells per well in MEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin sulphate, and

100 μg/ml kanamycin sulphate. The cells were incubated at 37 °C with 5% $CO_2$ until 90% cell confluency was reached. Then, the cells were washed twice with serum-free MEM before infection. Each microtitre plate included uninfected control wells, virus-infected control wells, and virus-infected wells to which umifenovir was added. Cells were overlaid with MEM (100 μl) containing 2.5 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin and serial 2-fold umifenovir dilutions (final concentration range, 1 μM to 30 μM). After incubation for 1 h at 37 °C, 100 μl of virus-containing allantoic fluid (approximately 0.1 PFU/cell) was added to all wells, except the uninfected control wells. After incubation for 18 h at 37 °C in a humidified atmosphere of 5% $CO_2$, the cells were washed and fixed by adding 50 μl of cold 0.05% glutaraldehyde in phosphate-buffered saline (PBS). Viral protein expression was measured by ELISA, as described previously.[20,21] The percentage inhibition of virus replication by umifenovir was calculated after correcting for background (cell control) values as follows: percentage inhibition = $100 \times [1 - (OD_{450})$ treated sample/$(OD_{450})$ virus control sample]. The $IC_{50}$ value (i.e., the concentration of compound required to inhibit virus replication by 50%) was determined by plotting the percentage inhibition of virus replication as a function of the compound concentration. The results were reported as the average of three experiments for each virus.

### 2.5. Genetic analysis of influenza A and B viruses

Identification of molecular markers of drug resistance was carried out by direct sequencing of the NA (neuraminidase), HA (hemagglutinin), and M2 (membrane ion channel protein) gene segments of the influenza viruses from the biological influenza-infected patient samples. The species were collected from the Central, North-Western, Southern, and Volga federal districts of Russia during the 2010–2011 influenza season. Autopsy material from influenza patients and randomized nasopharyngeal swabs were taken for surveillance and sent to the Central Research Institute for Epidemiology (CRIE) reference centre for confirmation and additional investigation, from the laboratories of 23 Russian Federal Centres of Hygiene and Epidemiology of Rospotrebnadzor. Nasopharyngeal swabs and autopsy material (e.g., trachea, bronchus, lung, and spleen fragments) were stored frozen (−70 °C) until they were studied.

Confirmation of influenza infection and the identification of influenza virus A(H1N1)pdm09 was done by real-time RT-PCR with the AmpliSens Influenza Virus A/B-FRT PCR Kit and the AmpliSens Influenza Virus A/H1-swine-FRT PCR Kit (CRIE, Russia),[22] in accordance with the manufacturer's instructions, on a Rotor-Gene 6000 instrument (Corbett Research, Sydney, Australia). Total RNA from the influenza-infected patient samples (nasopharyngeal swabs or autopsy samples) was extracted using reagents from the RIBO-prep Nucleic Acid Extraction Kit (AmpliSens, CRIE, Russia). The REVERTA-L RT Reagents Kit (AmpliSens, CRIE, Russia) was used for reverse-transcription of RNA. Amplification of viral cDNA from 108 influenza patient biological samples was conducted using the primers listed in Table 1 on a Tercyc thermocycler (DNA-Technology, Russia). PCR product sequencing reactions were done with the same primers as used for amplification (Table 1) using the ABI PRISM Big Dye v.3.1 Cycle Sequencing Reaction Kit, in accordance with the manufacturer's instructions, and were analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA). All sequences were assembled using Lasergene version 10.1 (DNASTAR Inc., USA).

### 2.6. Assessment of drug efficacy, in vivo

Female BALB/c mice weighing approximately 12–14 g were quarantined and acclimated for 3 days prior to use. Mice were group-housed in cages and used at a quantity of 10 mice per treatment group. Mice were lightly anesthetized and inoculated intranasally with 10 $MLD_{50}$ (mouse lethal dose) of mouse-adapted A/California/04/2009 (H1N1)pdm09 virus in PBS ($10^4$ $EID_{50}$ (50% Egg Infective Dose)). Compounds were prepared in a 0.2-ml volume. The treatments were administered to the mice for five consecutive days, beginning 6 h before viral inoculation, by oral gavage, using doses of 20, 30, and 60 mg/kg of body weight/day of umifenovir, which was administered once daily (in the morning), or doses of 2.5 and 10 mg/kg of body weight/day of oseltamivir, which was given twice daily. The placebo was administered in parallel with the antiviral treatments. Survival and weight changes were observed for 21 days after virus inoculation. Animals that showed signs of severe disease and weight loss of 25% were humanely euthanized. The mice were weighed on days 0, 1, 2, 3, 4, 5, 7, 9, 11, 13, and 15 after infection, and the weight loss or gain was calculated for each mouse as a percentage of its weight on day 0 before virus inoculation. The reported values are average percentage changes in weight ± standard errors (SEs). All studies were approved by the Mechnikov Research Institute of Vaccines and Sera Committee on the Ethics of Animal Experiments and were conducted in strict accordance with the applicable laws and guidelines.

**Table 1**
List of primers for PCR/sequencing of the influenza virus target genes

| Target gene | Coverage of amino acid region with potential mutations | Primer direction | Primer sequence |
|---|---|---|---|
| NA | Ile 223 Arg<br>His 274 Tyr<br>Arg 292 Lys<br>Asn 294 Ser | F<br>R | ttgcttggtcggcaagtgc<br>ttttttgaacaaactacttgtcaa |
| NA | Glu 119 Val | F<br>R | atgaatccaaatcaiaaiataaya<br>caattcigactctigigtyct |
| HA | Gln 27 Asn<br>Gln 42 His<br>Lys 51 Asn<br>Asn 117 Arg | F<br>R | attgccggtttcattgaag<br>ctgcactgcaaagacccattggagcaca |
| M (M2) | Leu 26 Phe<br>Val 27 Ala<br>Ala 30 Thr<br>Ser 31 Asn<br>Gly 34 Glu | F<br>R | agcaggtagatattgaaaaatga<br>gtagaaacaaggtagttttttac |

NA, neuraminidase; HA, hemagglutinin; F, forward; R, reverse.

### 2.7. Clinical effectiveness of antiviral use on influenza outcomes

#### 2.7.1. Study design

The observational case–control clinical study was set up in the 2010–2011 season. The aim of the study was to analyze individual patient data for the effectiveness of antiviral use on influenza disease severity and outcomes.

#### 2.7.2. Participants

The study population included adults (older than 18 years of age) admitted to hospitals with acute respiratory viral infections (ARVI) between July 2010 and May 2011 from 26 regions of the Russian Federation. A total of 1462 patients were included in the study. Data were collected from the routine medical records of ARVI patients. The collected data included demographic information, clinical observations (such as symptoms, chronic medical conditions, treatment, and temperature), and laboratory test results.

#### 2.7.3. Case definition

A human ARVI case was clinically defined as a combination of fever (>37 °C) and one or more of the following symptoms: cough,

sore throat, rhinorrhoea, and/or nasal congestion. The ARVI cases were defined as FLU-positive when the PCR (AmpliSens® Influenza virus A/B, Russia) or ELISA (NovaLisa®, NovaTec, Germany) results from the throat swab samples were positive for influenza A or B virus.

#### 2.7.4. Statistical analyses

The data were analyzed using Microsoft Excel 2007; simple proportions, means and standard deviations (SD) were calculated. Unpaired *t*-tests were applied. In other cases, the Mann–Whitney *U*-test and Chi-square test were used. A *p*-value below 0.05 was considered to indicate significance in all tests.

## 3. Results

### 3.1. In vitro drug susceptibility of human influenza viruses circulating during the 2010–2011 season

Based on the results received from two Russian national influenza centres, 969 influenza strains were isolated during the 2010–2011 season in different regions of Russia, of which 586 (60.4%) were typed as influenza A viruses and 383 (39.6%) as influenza B viruses. Of the sub-typed influenza A viruses, 521 (88.9%) were influenza A(H1N1)pdm09 and 65 (11.1%) were influenza A(H3N2). Among these, 36 strains were studied with respect to their susceptibility to umifenovir and 30 strains were studied for their susceptibility to oseltamivir carboxylate.

Virus isolate susceptibility to anti-influenza drugs (oseltamivir carboxylate and umifenovir) was studied and compared to laboratory and vaccine reference strains from the 2010–2011 season. A/Puerto-Rico/8/1934(H1N1), A/Solomon Islands/03/2006(H1N1), A/California/04/2009(H1N1)pdm09, and A/California/07/2009(H1N1)pdm09 were used as controls for the H1N1 influenza A virus isolates; A/Aichi/2/1969(H3N2) and A/Victoria/361/2011(H3N2) were used as controls for the H3N2 influenza A virus isolates; B/Brisbane/60/2008, B/Wisconsin/2010, and B/Perth/211/2001 were used as controls for the influenza B virus isolates. In addition, the oseltamivir-resistant viruses A/Perth/265/2009 (H1N1)pdm09, which possesses an H275Y substitution, and B/Perth/211/2001, which possesses a D197E substitution, were used as controls. The $IC_{50}$ values for oseltamivir carboxylate and umifenovir of the tested viruses are listed in Table 2.

Susceptibility of the virus isolates to oseltamivir carboxylate was assessed with a fluorescent NA inhibition assay. All tested virus isolates exhibited $IC_{50}$ values that were characteristic of oseltamivir carboxylate-susceptible influenza viruses (Table 2). The $IC_{50}$ for oseltamivir carboxylate ranged from $0.94 \pm 0.13$ nM to $10.12 \pm 0.22$ nM for the influenza A strains and from $20.44 \pm 2.44$ to $25.61 \pm 0.22$ nM for the influenza B strains. Nevertheless, the $IC_{50}$ values for the influenza B strains were 2.5- to 20-fold higher than those obtained for the influenza A strains, and they fell within the range that was previously observed for susceptible laboratory and clinical isolates. In contrast, the A/Perth/265/2009(H1N1)pdm09 (H275Y) and B/Perth/211/2001 (D197) viruses had a significantly reduced susceptibility to oseltamivir carboxylate (359- and 12-fold increases, respectively) compared with the susceptibility of the A/Perth/265/2009 (H1N1)pdm09 and B/Perth/211/2001 wild-type strains and all of the studied clinical isolates.

Susceptibility of the isolated viruses to umifenovir was evaluated using an ELISA-based cell assay, which is considered a standard protocol for testing antiviral activity of umifenovir in vitro (Table 2). All isolates were equally susceptible to umifenovir, with $IC_{50}$ values ranging from $7.2 \pm 0.75$ to $23.0 \pm 2.9$ μM, and similar $IC_{50}$ values were observed for the susceptible laboratory and vaccine reference strains of the influenza A and B viruses. Importantly, in these experiments, the wild-type A/Perth/265/2009 (H1N1pdm09) and B/Perth/211/2001 influenza viruses and their oseltamivir-resistant mutants were susceptible to umifenovir at similar levels.

**Table 2**
Drug susceptibility of human influenza viruses that circulated during the 2010–2011 season in Russia

| Influenza virus | Susceptibility to antiviral drugs | |
|---|---|---|
| | Oseltamivir carboxylate (mean $IC_{50} \pm SD$, nM)[a] | Umifenovir (mean $IC_{50} \pm SD$, μM)[a] |
| *Influenza A(H1N1) subtype* | | |
| A/Puerto-Rico/8/1934[b] | $2.02 \pm 0.75$ | $10.8 \pm 0.55$ |
| A/Solomon Islands/03/2006[b] | $1.23 \pm 0.56$ | $10.8 \pm 0.75$ |
| A/California/04/2009[b] | $0.94 \pm 0.13$ | $7.2 \pm 0.75$ |
| A/California/07/2009[b] | $0.99 \pm 0.43$ | $7.5 \pm 0.68$ |
| A/Perth/265/2009 (H1N1)pdm09 | $1.00 \pm 0.068$ | $9.1 \pm 0.75$ |
| A/Perth/265/2009 (H1N1)pdm09 H275Y oseltamivir-resistant | $359.9 \pm 87.2$ | $13.5 \pm 0.75$ |
| A/IIV-Vladimir/68/2011 | $8.02 \pm 0.5$ | $12.9 \pm 0.7$ |
| A/IIV-Vladimir/69/2011 | ND | $16.5 \pm 0.7$ |
| A/IIV-Vladimir/35/2011 | $5.02 \pm 0.43$ | $9.0 \pm 0.9$ |
| A/IIV-Vladimir/67/2011 | ND | $11.3 \pm 0.5$ |
| A/IIV-Moscow/27/2011 | $1.02 \pm 0.25$ | $16.7 \pm 1.1$ |
| A/IIV-Moscow/40/2010 | $2.22 \pm 0.55$ | $9.7 \pm 0.9$ |
| A/IIV-Moscow/88/2011 | $4.16 \pm 0.12$ | $10.8 \pm 1.0$ |
| A/IIV-Moscow/70/2011 | $5.34 \pm 0.67$ | $12.0 \pm 0.8$ |
| A/IIV-Moscow/38/2010 | $3.42 \pm 0.15$ | $13.3 \pm 2.2$ |
| A/IIV-Moscow/13/2011 | ND | $16.2 \pm 2.1$ |
| A/IIV-Moscow/30/2011 | $4.56 \pm 0.45$ | $14.2 \pm 1.5$ |
| A/IIV-Moscow/75/2011 | $2.15 \pm 0.71$ | $9.9 \pm 0.8$ |
| A/IIV-Moscow/74/2011 | $2.86 \pm 0.325$ | $9.5 \pm 0.1$ |
| A/IIV-Moscow/39/2010 | $7.34 \pm 0.33$ | $9.9 \pm 0.3$ |
| A/IIV-Moscow/1/2011 | $8.32 \pm 0.75$ | $11.3 \pm 1.6$ |
| A/IIV-Moscow/3/2011 | $7.51 \pm 0.77$ | $13.6 \pm 2.4$ |
| A/IIV-Moscow/26/2011 | $2.99 \pm 0.23$ | $9.0 \pm 0.7$ |
| A/IIV-Moscow/38/2011 | $10.12 \pm 0.22$ | $12.8 \pm 2.1$ |
| A/IIV-Moscow/37/2011 | $9.56 \pm 0.73$ | $9.0 \pm 0.5$ |
| A/IIV-Moscow/28/2011 | ND | $9.0 \pm 0.2$ |
| A/IIV-N. Novgorod/66/2011 | $8.15 \pm 0.22$ | $13.5 \pm 1.7$ |
| A/IIV-N. Novgorod/60/2011 | $7.08 \pm 0.87$ | $13.55 \pm 0.4$ |
| A/IIV-Moscow/45/2011 | $6.09 \pm 0.35$ | $13.6 \pm 1.2$ |
| A/IIV-Moscow/43/2011 | $6.99 \pm 0.77$ | $19.8 \pm 2.0$ |
| A/IIV-Moscow/4/2011 | ND | $10.6 \pm 1.0$ |
| A/IIV-Cheboxary/71/2011 | $5.12 \pm 0.34$ | $13.5 \pm 1.8$ |
| *Influenza A(H3N2) subtype* | | |
| A/Victoria/361/2011[b] | $1.89 \pm 0.19$ | $8.1 \pm 0.4$ |
| A/Aichi/2/1968[b] | $0.43 \pm 0.24$ | $13.5 \pm 0.3$ |
| A/Perth/16/2009[b] | $0.92 \pm 0.01$ | $9.5 \pm 2.1$ |
| A/Vladivostok/28/2011 | $0.98 \pm 0.05$ | $13.5 \pm 1.4$ |
| A/Vladivostok/22/2011 | $2.31 \pm 0.15$ | $11.3 \pm 1.1$ |
| A/Vladivostok/21/2011 | $2.56 \pm 0.23$ | $16.5 \pm 2.3$ |
| A/Vladivostok/27/2011 | ND | $23.0 \pm 2.9$ |
| A/Vladivostok/4/2010 | $0.99 \pm 0.07$ | $21.8 \pm 2.7$ |
| A/Vladivostok/1/2010 | $1.91 \pm 0.11$ | $13.5 \pm 0.9$ |
| *Influenza B viruses* | | |
| B/Brisbane/60/2008[b] | $25.42 \pm 0.51$ | $17.1 \pm 0.4$ |
| B/Wisconsin/2010[b] | $21.29 \pm 0.48$ | $14.4 \pm 1.2$ |
| B/Perth/211/2001 | $19.24 \pm 2.43$ | $9.0 \pm 0.7$ |
| B/Perth/211/2001 (D197E) oseltamivir-resistant | $230.3 \pm 62.6$ (4) | $10.8 \pm 0.5$ |
| B/Vladivostok/21/2011 | $25.61 \pm 0.22$ | $18.1 \pm 1.3$ |
| B/Vladivostok/27/2011 | $20.44 \pm 2.44$ | $19.8 \pm 2.4$ |
| B/Moscow/70/2011 | $25.31 \pm 0.68$ | $17.4 \pm 0.8$ |
| B/Moscow/38/2011 | $23.46 \pm 0.55$ | $18.7 \pm 2.3$ |

$IC_{50}$, the drug concentration that inhibits 50% of the NA enzymatic activity; SD, standard deviation; ND, not done.
[a] The results are reported as the average of three experiments for each virus.
[b] Reference laboratory strain or reference vaccine strain.

### 3.2. Efficacy of oseltamivir and umifenovir in mice inoculated with the A/California/07/2009 (H1N1)pdm09 virus

At the beginning of the pandemic, there were no data regarding the efficacy of antivirals against the new pandemic

A(H1N1)pdm09 influenza virus in laboratory animal models. During the 2010–2011 influenza season, influenza A(H1N1)pdm09, A(H3N2), and B viruses co-circulated, and A(H1N1)pdm09 was absolutely prevalent. It was shown that without prior host adaptation, the A/California/07/2009 (H1N1)pdm09 and A/California/04/2009 (H1N1)pdm09 influenza viruses did not kill mice,[23,24] which displayed only a transient weight reduction (up to 11%). To estimate the efficacy of the antivirals, the A/California/07/2009 (H1N1)pdm09 virus was adapted to mice through three passages in the mouse lungs, and this virus was then used in the experiments. The efficacy of oseltamivir and umifenovir was studied in mice infected with the 10 $MLD_{50}$ mouse-adapted A/California/07/2009 (H1N1)pdm09 virus. A high mortality rate (100%) and loss of body weight (21%) were observed after viral infection.

Of the three umifenovir regimens (20, 30, and 60 mg/kg/day), significantly enhanced survival ($p < 0.05$) was observed only in mice that received 60 mg/kg/day (Figure 1A). The survival rate rose constantly when the dose was gradually increased. Umifenovir administration in 60 mg/kg/day doses significantly increased the survival rate (50%) and time to death compared with untreated control mice. Additionally, umifenovir treatment at all doses studied prevented the weight loss (Figure 1B).

Oseltamivir was effective against A/California/07/2009 (H1N1)pdm09 virus infections at all dosages investigated. Oseltamivir treatments at 2.5 mg/kg/day protected 40% of the infected animals, prevented weight loss (Figure 1), and resulted in a mean survival time of 13.9 days compared with 7.5 days in the untreated control mice. The most profound therapeutic effect was observed for oseltamivir at 10 mg/kg/day. Oseltamivir administration (10 mg/kg/day) completely protected the infected mice from death and prevented weight loss (Figure 1).

### 3.3. Genetic analyses of influenza viruses

Data from 108 patients with influenza A(H1N1)pdm09 virus infection during the 2010–2011 influenza season were analyzed. Sequences are available from the GenBank database (**JN185093.1**–**JN185139.1**, **JN714484.1**–**JN714487.1**, **JN714492.1**–**JN714550.1**). All specimens tested contained the S31N mutation in the M2 protein, which confers cross-resistance to the adamantane class of anti-influenza drugs.[25] Mutations that led to an amino acid substitution at H275Y in the NA protein, which cause resistance of the A(H1N1)pdm09 influenza virus to oseltamivir, were found in three of the 108 samples (2.8% of patients). No mutations that led to amino acid substitutions in the HA2 protein, which cause resistance of the influenza A virus to umifenovir,[11] were found in the 108 samples tested.

### 3.4. The clinical effectiveness of antiviral medications

#### 3.4.1. Clinical characteristics of acute respiratory tract infection (ARTI) patients who tested positive for influenza virus

Of the 1462 hospitalized patients who met the ARTI case definition criteria, 442 (30%) were positive for influenza viruses (FLU-positive); influenza viruses were not detected in 1020 cases (70%) (FLU-negative). The presence of influenza A viruses was confirmed in 339 cases, which included 177 with the A(H1N1)pdm09 virus and 162 with the H3N2 virus. One hundred and three patients were positive for the influenza B virus. The other cases ($n = 1020$) included patients with ARTIs of an aetiology other than viral (38/1020, 3.7%), cases in which a viral aetiology was not detected (282/1020, 27.7%), and those for whom laboratory confirmation was not performed (699/1020, 68.5%). All variables and analyzed data of the FLU-positive patients are shown in Table 3.

#### 3.4.2. Clinical effectiveness of antiviral medications on symptom duration and influenza outcome

Only 24.8% of patients (110/442) received antivirals (umifenovir 200 mg four times a day for 5 days, or oseltamivir 75 mg twice daily for 5 days) within 48 h of symptom onset. Fifty-seven percent of patients (252/442) received antiviral drugs at more than 48 h after symptom onset. A comparative analysis of the effectiveness of early and late antiviral treatment revealed that patients with non-complicated influenza who received antiviral drugs within 48 h of symptom onset had a shorter overall illness duration by a mean of 2.8 days (8.9 days vs. 11.7 days, $p < 0.001$), a shorter duration of fever by a mean of 2.2 days (3.5 vs. 5.7 days, $p < 0.001$), and a shorter duration of common symptoms by a mean of 2.6 days (4.6 vs. 7.2 days, $p < 0.001$) and catarrhal symptoms by a mean of 3.3 days (5.5 vs. 8.8 days, $p < 0.001$), compared with patients who received antiviral drugs at later than 48 h ($n = 252$) (Table 4 ).

The effectiveness of therapy in groups of patients who received early treatment with umifenovir (55 of 110 patients) or early treatment with oseltamivir (55 of 110 patients) were compared

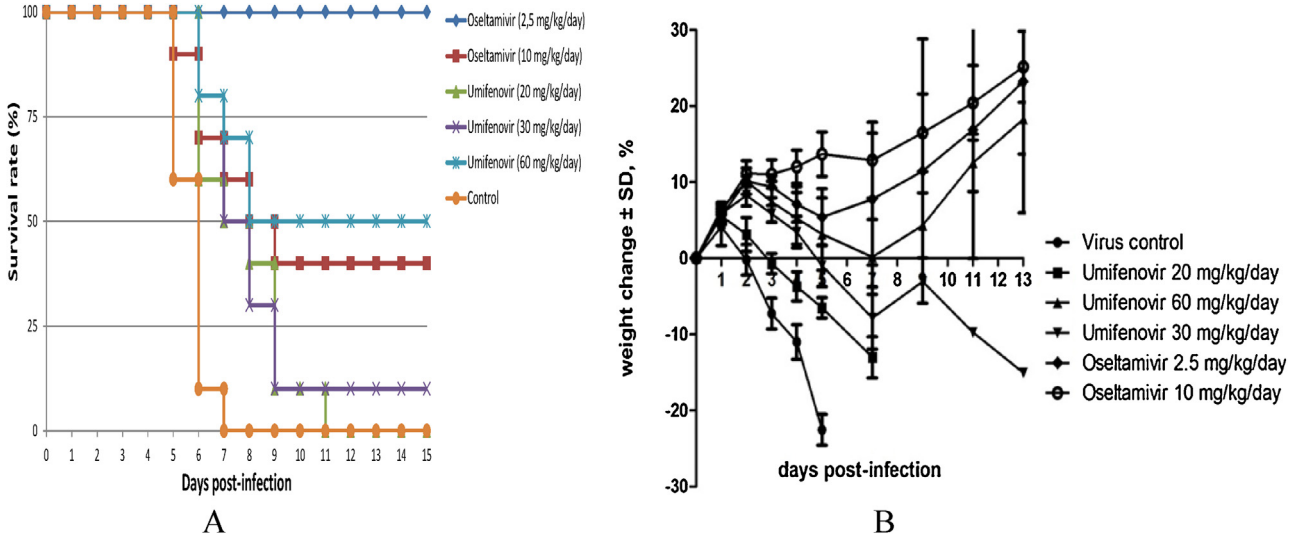


**Figure 1.** The effect of oseltamivir and umifenovir treatments on survival rates (A) and weight loss (B) in mice infected with the mouse-adapted A/California/07/2009 (H1N1) virus.

**Table 3**
Clinical characteristics of patients admitted to hospitals with influenza between July 2010 and May 2011

| Clinical characteristics | Influenza-positive patients ($n = 442$) |
|---|---|
| Demographics | |
| Age, years, mean ± SD | 34.2 ± 14.6 |
| Sex, male | 37.6% |
| Comorbidity | |
| Patients with BMI >30 kg/m$^2$ | 10.2% |
| Patients with chronic diseases | 43.7% |
| Pregnant women | 19.6% |
| Clinical presentation | |
| Common symptoms | |
| Temperature, °C, mean ± SD | 38.4 ± 0.8 |
| Temperature >39 °C | 16.2% |
| Headache | 63.1% |
| Myalgia | 46.6% |
| Ocular injection | 33.0% |
| Respiratory symptoms (catarrhal) | |
| Cough | 89.1% |
| Rhinorrhoea | 33.9% |
| Sore throat | 45.0% |
| Laboured breathing | 12.0% |

ARTI, acute respiratory tract infection; SD, standard deviation; BMI, body mass index.

with a propensity-matched control group that received no antiviral treatment (Table 5). The overall duration of illness (8.47 vs. 11.31 days) and duration of main symptoms, including fever (3.67 vs. 4.97 days) and catarrhal symptoms (5.25 vs. 7.75 days), was lower in the umifenovir treatment group than in the group of patients who received no antiviral therapy ($p < 0.001$). Similar data were observed for patients treated with oseltamivir: oseltamivir therapy within 48 h of symptom onset significantly reduced the total disease duration (8.35 vs. 11.31 days) and the main symptoms, fever (3.05 vs. 4.97 days) and catarrhal phenomena (4.4 vs. 7.75 days), compared with the patients did not receive any antiviral therapy ($p < 0.001$). No significant differences were found in the duration of illness and main symptoms of influenza between the umifenovir and oseltamivir treatment groups.

Pneumonia as a complication of influenza was observed in 0.3% of the patients treated with umifenovir, in 23.7% of the patients who did not receive antiviral therapy ($p < 0.001$), and in none of the patients treated with oseltamivir.

The results showed that antiviral treatment with umifenovir and oseltamivir within 48 h of symptom onset significantly decreased the durations of illness and symptoms by 2–3 days.

## 4. Discussion

In Russia, antiviral drugs are approved by the Ministry of Health to treat or prevent influenza virus infections. The most widely used drugs are the NA inhibitor oseltamivir and the fusion inhibitor umifenovir. In this study, the in vitro susceptibility of influenza A and B viruses that circulated during the 2010–2011 season to oseltamivir carboxylate and umifenovir was evaluated. Similar to most other countries, the peak influenza season from 2010–2011 in Russia was associated with the co-circulation of A(H1N1)pdm09, A(H3N2), and B viruses. The data from the present study showed that the $IC_{50}$ values of oseltamivir carboxylate and umifenovir for all tested A(H1N1)pdm09, A(H3N2), and B virus isolates from the 2010–2011 influenza season were comparable to those for the human susceptible viruses. Notably, umifenovir inhibited the replication of both oseltamivir-susceptible and oseltamivir-resistant viruses. Therapy with oseltamivir resulted in a lower frequency of emergence of drug-resistant variants.[26] However, during the 2008–2009 influenza season, oseltamivir-resistant A(H1N1) viruses with an H275Y NA substitution spread globally.[27] Since the first wave of the 2009 pandemic, the overall level of oseltamivir resistance among A(H1N1)pdm09 variants has remained relatively low (1%).[28] However, as drug-resistant variants continue to emerge naturally and through the selective pressure applied by antiviral drug use,

**Table 4**
Clinical effectiveness of antiviral therapy on overall symptom duration

| Clinical presentation (mean ± SD) days | Early antiviral therapy (umifenovir or oseltamivir therapy started at ≤48 h from disease onset) ($n = 110$) | Late antiviral therapy (umifenovir or oseltamivir therapy started at >48 h from disease onset) ($n = 252$) | $p$-Value[a] |
|---|---|---|---|
| Time from symptom onset to antiviral treatment order | 0.79 ± 0.41[b] | 3.37 ± 2.06 | <0.001 |
| Length of hospital stay | 8.09 ± 2.79 | 8.63 ± 3.84 ($n = 249$) | 0.694 |
| Overall duration of illness | 8.88 ± 2.82[b] | 11.72 ± 4.51 ($n = 251$) | <0.001 |
| Duration of fever | 3.49 ± 1.47[b] | 5.67 ± 3.13 | <0.001 |
| Duration of catarrhal symptoms | 5.51 ± 3.18[b] | 8.77 ± 5.08 | <0.001 |

SD, standard deviation.
[a] Level of significant differences between groups.
[b] Statistically significant difference between early and late antiviral therapy groups.

**Table 5**
Influence of antiviral drugs on the duration of influenza symptoms[a]

| Clinical presentations | Umifenovir ($n = 55$) | Oseltamivir ($n = 55$) | No antiviral treatment ($n = 48$) | $p$-Value 1[b] | $p$-Value 2[b] | $p$-Value 3[b] |
|---|---|---|---|---|---|---|
| Time from symptom onset to antiviral treatment order, days | 0.82 ± 0.39 | 0.75 ± 0.44 | - | - | - | 0.358 |
| Length of hospital stay, days | 8.47 ± 1.83[c] | 8.35 ± 2.47[c] | 11.31 ± 4.36 | 0.003 | <0.001 | 0.125 |
| Overall illness duration, days | 3.67 ± 1.59[c] | 3.05 ± 1.22[c] | 4.96 ± 2.31 | 0.019 | <0.001 | 0.023 |
| Fever duration, days | 4.51 ± 1.46[c] | 4.27 ± 1.72[c] | 6.73 ± 3.79 ($n = 41$) | 0.001 | <0.001 | 0.5 |

[a] Data are presented as the mean ± standard deviation.
[b] $p$-Value 1: umifenovir-treated group compared to placebo-treated control group; $p$-Value 2: oseltamivir-treated group compared to placebo-treated control group; $p$-Value 3: oseltamivir-treated group compared to umifenovir-treated group.
[c] Statistically significant difference.

the efficacy of oseltamivir may wane over time. The present data demonstrated that oseltamivir-resistant viruses are susceptible to umifenovir, and this suggests that umifenovir may be a good alternative for the clinical treatment of infection caused by oseltamivir-resistant viruses. These findings are in correlation with reports that have shown umifenovir to effectively inhibit the replication of A/California/4/2009, A/California/7/2009 A(H1N1), and other A(H1N1)pdm09 viruses that were isolated from patients in 2009.[15,29,30]

The data obtained in vitro were confirmed in animal studies. It has been shown previously that oseltamivir is effective in mice and ferrets infected with the A/California/04/2009 (H1N1)pdm09 virus.[23,31] Prior to the current study, there were no data regarding the efficacy of umifenovir against the A(H1N1)pdm09 virus in animal models. The present study confirmed the findings regarding the high efficacy of oseltamivir at a dose 10 mg/kg/day in mice against the A(H1N1)pdm09 influenza virus.[31] It was also found that umifenovir at a dose 60 mg/kg/day was effective in mice infected with the A/California/04/2009 (H1N1)pdm09 virus, increased the survival rate (60%), and completely prevented weight loss as compared with untreated virus-inoculated control mice. In contrast, low doses of umifenovir only prevented weight loss and did not sufficiently affect the survival rate or mean days to death. This study is the first, to the authors' knowledge, to assess the effectiveness of umifenovir against pandemic A(H1N1) influenza virus infection in vivo.

Sequence analysis of the clinical samples identified the presence of the H275Y NA mutation, which is known to confer resistance to oseltamivir, in three specimens among the 108 specimens tested. The frequency of oseltamivir resistance in patients with the influenza A(H1N1) virus in the present study was consistent with that in a WHO report published during this period.[28] Among the 108 specimens from the 2010–2011 season, no HA amino acid changes that have previously been identified in vitro as being associated with reduced susceptibility to umifenovir were found. These data correlate with those of previous studies.[17,18] To date, umifenovir-resistant mutants have only been generated through serial passages in cell culture under drug pressure. All mutants had different amino acid substitutions in the HA2 subunit of the HA glycoprotein. Although umifenovir has been used clinically for 30 years in Russia, drug-resistant viruses have not yet been isolated in the clinic. Burtseva et al. tested over 700 clinical influenza A and B viruses that were isolated during 2002–2014 for their susceptibility to umifenovir in cell culture. There was no evidence of naturally occurring resistance to umifenovir in any of the tested isolates.[17,18]

The clinical retrospective observational study demonstrated that the timing of initiation of the antiviral treatment relative to the onset of illness is an important consideration in observational studies that assess antiviral efficacy. First, the treatment effectiveness was compared between patients who received the antiviral agents within 48 h of symptom onset and those who received the antiviral drugs later than 48 h after symptom onset. The priority of early treatment was demonstrated, and these data are in agreement with those reported from other experimental and clinical studies and the widely held concept that early initiation of antiviral therapy is important to obtain effective control of viral replication and, in turn, to shorten the duration of symptoms.[32]

Second, the effectiveness of oseltamivir and umifenovir was compared. For the primary endpoints, the time to symptom alleviation was not significantly different between the oseltamivir and umifenovir treatments. At present, oseltamivir is the gold standard and the most widely used anti-influenza drug, with proven efficacy against influenza A and B infections. Clinical trials with umifenovir in more than 30 000 patients were conducted in the former Soviet Union during 1980–1990.[8,9] The present study is the first to compare the clinical effectiveness of oseltamivir and umifenovir. It was demonstrated that the effectiveness of umifenovir treatment is comparable to that of the standard anti-influenza oseltamivir treatment and this is a welcome addition to the previous clinical trial data regarding umifenovir effectiveness for the treatment of influenza A and B infections. These data are encouraging for the current ongoing double-blind, randomized, placebo-controlled clinical trial for umifenovir that was initiated in 2012. The aim of that trial is to evaluate the efficacy and safety of umifenovir for the treatment and prophylaxis of influenza and other ARVIs.[33]

Recent meta-analysis studies have shown that there is no evidence that oseltamivir reduces the likelihood of complications, particularly hospitalization, pneumonia, or the combined outcome of pneumonia and mortality, in an intention-to-treat (ITT) population.[34–36] However, these studies have some limitations. These works excluded many observational studies that found oseltamivir to be helpful in normal clinical settings.[37] The evidence from randomized clinical trials (RCTs) was further limited by the lack of high-quality evidence for patient-important outcomes under the treatment of specific subgroups, including hospitalized and immune-compromised patients. Data obtained from RCTs among high-risk patients were limited by the actual small number of these patients.[36] Observational studies may provide more information in addition to data currently available from RCTs for certain elements of antiviral treatment. This benefit was reported in detail in a recent review.[37] To supplement the information from RCTs, the authors conducted a systematic review of observational studies of antiviral treatment for influenza. These observational studies suggested that oseltamivir may reduce mortality, hospitalization, and symptom duration. Evidence from some observational studies has also suggested that oral oseltamivir treatment results in fewer complications, such as pneumonia, otitis media, and any recurrent cardiovascular outcome.[38]

Similar data were obtained in the present observational study, which estimated the clinical effectiveness of oseltamivir and umifenovir during the 2010–2011 influenza season in Russia. There are some mismatches between the results of the meta-analysis studies with the highest level of evidence and the results of observational studies, such as the level of evidence, which is not notably high, but the conditions are close to those of real clinical situations. This condition does not allow definitive conclusions to be drawn about the benefit of antiviral treatments in individuals and on influenza outcomes. Further high-quality RCT evidence is needed to address important patient outcomes (e.g., mortality and complications) and include hospitalized influenza patients. The extrapolation of the results of such studies to the real clinical situation is limited. Thus, observational studies are important and additional tools that should be taken into consideration when estimating the effectiveness of antiviral therapy.

Overall, the observational study results regarding the effectiveness of oseltamivir and umifenovir during the 2010–2011 influenza season are concordant with the experimental data regarding the high susceptibility of influenza viruses that circulated during this period to these antivirals. The sequence analysis did not reveal mutations that are associated with resistance to umifenovir, but the H275Y NA mutation responsible for resistance to oseltamivir was found (3/108, 2%). As it is not possible to conduct placebo-controlled clinical trials for antiviral effectiveness in influenza-infected patients every season, such studies may be used to inform clinical and public health practices.

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