Artem Poromov ORCID iD: 0000-0002-2004-3935

Umifenovir Susceptibility Monitoring and Characterization of Influenza Viruses Isolated during ARBITR Clinical Study

Running title: Umifenovir susceptibility of influenza viruses

Irina A. Leneva[1*], Irina N. Falynskova[1], Nailya R. Makhmudova[1], Artem A. Poromov[1,2], Svetlana B. Yatsyshina[3], Viktor V. Maleev[3]

[1] I. Mechnikov Research Institute for Vaccines and Sera, Moscow, Russia;

[2] Lomonosov Moscow State University, Moscow, Russia;

[3] Central Research Institute for Epidemiology, Moscow, Russia

* Corresponding author: Irina A. Leneva, Department of Experimental Virology, I. Mechnikov Research Institute for Vaccines and Sera, Moscow, Russia;

E-mail address: wnyfd385@yandex.ru

Antiviral drugs can play a significant role in the control of influenza. Umifenovir (Arbidol) is licensed and widely used in Russia for the prophylaxis and/or treatment of influenza. We evaluated the susceptibility to umifenovir of reference influenza A and B viruses and influenza A viruses isolated from patients in the ARBITR clinical trial in 2012-2014 seasons. Using an MDCK cell-based ELISA, we showed that the replication of antigenically dominant human influenza A and B viruses was efficiently inhibited by umifenovir. The wild-type A/Perth/265/2009 (H1N1)pdm09, A/Fukui/45/2004 (H3N2) and B/Perth/211/2001 viruses and their oseltamivir-resistant counterparts were susceptible to umifenovir among in vitro laboratory assays. All 18 clinical isolates of influenza A viruses

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obtained before and during therapy were susceptible to umifenovir with EC_{50} ranging from 8.4 ± 1.1 to 17.4 ± 5.4 μM. No molecular markers of umifenovir resistance were identified in influenza viruses isolate d from patients at 3, 5 and 7 days after initiation of therapy. None of the viruses isolated before and during umifenovir therapy displayed reduced susceptibility to neuraminidase (NA) inhibitors. Thus, umifenovir is effective against influenza viruses circulating in 2012-2014 seasons, and therapy did not lead to the emergence of drug-resistant variants.

Keywords: influenza virus, resistance, chemotherapy, drug specificity

1. INTRODUCTION

Influenza virus infections can lead to serious social and economic consequences around the world. Vaccination is the primary intervention against influenza, however, vaccines lose effectiveness in case of antigenic mismatch between the circulating influenza viruses and vaccine strain. Therefore, antiviral treatment and prophylaxis is an additional line of defense for the control of influenza. A limited number of antiviral drugs are approved for both treatment and prophylaxis of influenza. Although the membrane (M)2 ion channel inhibitors, amantadine and its derivative rimantadine, are licensed for influenza treatment, their clinical usefulness is limited because of the widespread emergence of transmissible drug-resistant seasonal influenza viruses.[1,2]

Knowledge of the crystallographic structure of the influenza virus neuraminidase (NA) glycoprotein complex with sialic acid, the natural substrate of the NA enzyme, permitted the design of NA inhibitor zanamivir, which was quickly followed by development of oseltamivir.[3,4] These two NA inhibitors were licensed for influenza in 1999 and have been shown their efficacy in the prophylaxis and treatment of influenza infections as well as their safety.[5,6] The prevalence of NA inhibitor resistant influenza A and B viruses is <1% in adults and 4 - 8% in children during antiviral treatment.[1,2] The increased number

of resistant influenza variants (18% and 27%) among children treated with NA inhibitors was reported in some clinical observations.[7,8]

Unexpectedly, during the 2007–2008 season, oseltamivir-resistant influenza A(H1N1) viruses with a common H275Y NA substitution (N1 numbering) were became widespread first the northern and then in the southern hemispheres.[9] During the 2009 influenza A(H1N1) pandemic, and to date, almost all tested viruses have remained susceptible to NA inhibitors.[10] Although, oseltamivir- and peramivir-resistant influenza A(H1N1)pdm09 variants have been isolated from a few community clusters in a period from November 2013 to February 2014 in Japan,[11,12] China,[13] and the United States.[14]

The RNA-dependent RNA polymerase inhibitor favipiravir (T-705) (Toyama Chemical Co. L. The New Drug Application Approval of AVIGAN®, Tablet 200 mg)[15] and acidic polymerase (PA) endonuclease inhibitor baloxavir marboxil (Shionogi & Co., Ltd. XOFLUZA, Tablets 10 mg/20 mg)[16] were approved in Japan in 2014 and 2018, respectively. It was shown that favipiravir therapy did not lead to emergence of seasonal influenza A and B viruses with reduced drug susceptibility.[17,18] Variants (I38T, I38F, E23K) with reduced susceptibility to baloxavir marboxil were isolated from treated patients during phase II clinical trials.[19] The same I38T PA substitution also caused resistance of influenza A(H1N1) viruses serially passaged in MDCK cells with RO-7, an investigational endonuclease inhibitor that is similar but not identical to baloxavir marboxil.[20]

Umifenovir ($C_{22}H_{25}BrN_2O_3S$,ethyl 6-bromo-4-[(dimethylamino)methyl]-5-hydroxy-1-methyl-2-[(phenylsulfanyl) methyl]-1H-indole-3-carboxylate) is an oral antiviral drug licensed for the treatment and prophylaxis of influenza A and B virus infections in Russia (Arbidol®, OJSC "Pharmstandard-Leksredstva") and in China in 1993 and 2006, respectively. Umifenovir interacts with the hemagglutinin (HA) glycoprotein of influenza viruses, stabilizes it against the low pH transition to its fusogenic state, and prevents HA-

mediated membrane fusion during influenza virus infection.[21] Co-crystal structure of umifenovir with the influenza virus HA glycoprotein indicates that umifenovir binds in a hydrophobic cavity at the interface of the HA protomers in the upper region of the stem.[22] Umifenovir inhibits the replication of a wide range of influenza A, B and C viruses, including highly pathogenic avian A(H5N1) viruses with EC_{50} ranging from 7.2 to 23.0 μM.[23–26] It also maintains antiviral activity against oseltamivir- and rimantadine-resistant influenza viruses.

Influenza viruses with reduced susceptibility to umifenovir (EC_{50} > 36 μM) were generated through several passages of influenza A(H7N7) viruses under drug exposure in MDCK cells. These variants possessed a single amino acid substitution in HA2 subunit (K51N, Q42H, Q27N, or K117R)[21] which lead to reduced ability of umifenovir to stabilize the acid-induced HA conformational changes. The sequence analysis of 108 clinical isolates obtained during 2010-2011 influenza season in Russia did not identify substitutions in the HA2 subunit, that can cause umifenovir resistance among influenza A viruses.[23] This finding supports an earlier report[24] on the absence of naturally occurring variants with low susceptibility to umifenovir among human influenza A and B viruses isolated during 2002–2005.

In Russia, umifenovir has been studied for over 30 years. The clinical trials of umifenovir performed in the former USSR during 1980-1995 influenza seasons were based on more than 14,000 patients. In a randomized, double-blind, placebo-controlled study among 232 adults carried out in China, umifenovir (200 mg three times daily for 5 days) significantly reduced the duration of fever (72.0 hours, ranged from 66.0 to 78.0 hours) in umifenovir-treated group compared to control untreated patients (96.0 hours, ranged from 87.5 to 104.5 hours), and reduced the risk of complications.[27] An observational study conducted among hospitalized laboratory-confirmed influenza patients (n = 5287) during the 2010-2014 influenza seasons in Russia demonstrated the effectiveness of umifenovir

therapy on reducing the length of fever and risk of complications, especially in patients from high-risk groups. In a clinical study when umifenovir was administered within 48 hours after the onset of symptoms, the duration of fever and frequency of complications in umifenovir-treated patients was lower than those in patients who were not treated by antiviral therapy.[28] Despite wide-spread clinical usage, there are currently no studies addressing the occurrence of umifenovir-resistant variants during treatment of influenza-infected patients.

A double-blind, randomized, placebo-controlled Phase IV ARBITR clinical trial was conducted during 2011-2016 influenza seasons (ClinicalTrials ID: NCT01651663) to assess the safety of umifenovir, Arbidol®), its effectiveness in the clinical management of influenza and other acute respiratory viral infections. The preliminary results of the ARBITR clinical trial were described by Kiselev et al.[29] Here, we examined the antiviral susceptibility of seasonal influenza A viruses isolated from patients before and during administration of umifenovir in ARBITR clinical trial.

2. MATERIALS AND METHODS

2.1. Cells and influenza viruses. Madin Darby canine kidney (MDCK) cells (American Type Culture Collection, Manassas, VA) were grown in minimal essential medium (MEM, source) supplemented with 10% fetal bovine serum (FBS, HyClone, Thermo Scientific), 5 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 100 μg/ml kanamycin sulfate. The cells were incubated at 37°C with 5% CO_2 until 90% cell confluency. A/California/04/2009 (H1N1)pdm09, A/Victoria/361/2011 (H3N2), B/Brisbane/60/2008, B/Massachusetts/2/2012 and B/Wisconsin/1/2010 influenza viruses were provided by the WHO National Influenza Centre of Russia (St. Petersburg, Russia). A panel of NA inhibitor-resistant viruses was

kindly provided by the Antiviral Group, International Society for Influenza and Other Respiratory Virus Diseases.

2.2. Compounds. Umifenovir (Arbidol®, Pharmstandart, Russia) was dissolved as a 10 mM stock in 96% ethanol at 37°C for 10 min followed by dilution in sterile distilled water. For each experiment a freshly made stock was used. Oseltamivir carboxylate and zanamivir (Sequoia Research Products, United Kingdom) were dissolved in distilled water as 5 mM stocks and stored in aliquots at -20 C.

2.3. Enrollment and design. The study protocol was approved by The Ministry of Health, Moscow, Russia. The design of ARBITR study was described previously.[29] Briefly, adults aged 18-65 years were eligible for inclusion if they presented to the unit within 36 hours after onset of symptoms suggestive of influenza-like illness. Patients were treated with umifenovir (Arbidol®) (2 x 100 mg capsules 4 times daily for 5 days). Nasal swabs were stored in 2 mL of virus transportation medium and frozen at -80°C until use. Rapid diagnostic test was used to identify influenza virus in the nasal swabs. Semi-quantitative real-time PCR was used to identify virus type and, subtype for influenza A viruses.

2.4. Patients and virus isolation. The schematic representation of study design is shown in the Figure 1. Nasal swabs (n = 32) were obtained from 16 adult patients with laboratory-confirmed influenza during 2012-2014 influenza seasons. The specimens were collected before (day 1, screening and treatment start) and during administration of umifenovir (days 3, 5, 7 after umifenovir therapy started). Viruses were isolated from the clinical respiratory samples by passaging 3 times in MDCK cells as recommended by the World Health Organization Manual on Animal Influenza Diagnosis and Surveillance.[30]

2.5. Umifenovir antiviral activity by cell-based ELISA assay. Susceptibility of influenza A and B viruses to umifenovir was assessed by a modified enzyme-linked immunoassay (cell ELISA)[31]Briefly, MDCK cells were seeded in 96-well plates (3,000

cells/well), washed twice with serum-free MEM, and overlaid with MEM (100 µl) containing 2.5 µg/ml N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich) and umifenovir (final concentration range, 1 - 30 µM). After incubation for 1 hour at 37°C, 100 µl of virus inoculum containing approximately 0.1 PFU/cell was added to all wells, except the uninfected control cells. After incubation for 18 h, the cells were washed and fixed by adding 50 µl of cold 80% acetone in PBS. Viral nucleoprotein (NP) expression was measured by ELISA and the 50% effective concentration (EC_{50}) was then calculated, as previously described.[21,31]

2.6. Virus susceptibility to NA inhibitors *in vitro*. Viral NA activity of influenza viruses was determined in a fluorescence-based assay using the fluorogenic substrate 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA) (Sigma-Aldrich)[32]. 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. The concentrations of oseltamivir carboxylate and zanamivir ranged from 0.01 to 1000 nM. The drug concentration that inhibited 50% of the NA enzymatic activity (IC_{50}) was determined from the dose-response curve using R-Studio software (version 1.0.143).[23,33]

2.7. Sequence analysis. Identification of molecular markers of drug resistance was carried out by sequencing of the NA, HA and M2 gene segments of the influenza viruses isolated from clinical samples. Total RNA was extracted using RIBO-prep nucleic acid extraction kit (AmpliSens, CRIE, Russia). The REVERTA-L reagents kit (AmpliSens, CRIE, Russia) was used for reverse-transcription of RNA. Amplification of viral cDNA was conducted using primers listed in Table 1 on a Tercyc thermocycler (DNA-Technology, Russia). Sequencing reactions of overlapping PCR products were made with the same primers used for amplification (Table 1) with the ABI PRISM Big Dye™ v.3.1 Cycle Sequencing Reaction Kit according to the manufacturer's instructions on an ABI-3100

PRIZM™ Genetic Analyzer (Applied Biosystems, USA). All sequences were assembled with the Lasergene version 10.1 package (DNASTAR Inc, USA).

2.8. Statistical analysis. Statistical analyses were performed using R-Studio software (version 1.0.143). Grubb's test was used to detect significant outliers. Statistically significant differences between groups were determined by using Mann-Whitney *U* test. A *P* value of 0.05 was prospectively chosen to indicate that the findings of these analyses were not the result of chance alone.

3. RESULTS

3.1. Umifenovir susceptibility of reference vaccine strains and NA inhibitor-resistant influenza viruses. The WHO Global Influenza Surveillance and Response System (GISRS) and the Russian Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing reported that influenza A/California/07/2009 (H1N1)pdm09-like and A/Victoria/361/2011 (H3N2)-like viruses and influenza B/Massachusetts /2/2012-like, B/Wisconsin/1/2010-like and B/Brisbane/60/2008-like viruses were circulating in different regions of Russia during the 2012-2014 seasons.[34,35] Our results demonstrated that umifenovir inhibited replication of antigenically dominant influenza A and B viruses (reference viruses) that circulated in 2012-2014 seasons (Table 2). The influenza B viruses were less susceptible to umifenovir, although the EC_{50} values were in a range reported previously for drug susceptible laboratory and clinical isolates.[23,36] We confirmed that influenza A and B viruses from an antiviral panel [A/Perth/265/2009 (H1N1)pdm09 with H275Y NA change, A/Fukui/45/2004(H3N2) with E119V NA change, and influenza B/Perth/211/2001 virus with D197E NA change] exhibited reduced inhibition by oseltamivir carboxylate, but all were susceptible to zanamivir (Table 2). These NA inhibitor-resistant and matched wild-type viruses were also equally susceptible to umifenovir, with EC_{50} values ranging from 12.9 ± 1.4 to 24.3 ± 1.3 μM. Similar EC_{50} values

were observed for the candidate vaccine strains previously[23], indicating that they are susceptible to umifenovir. Thus, we showed that the wild-type influenza A(H1N1)pdm09, A(H3N2) and B viruses and their oseltamivir-resistant counterparts were susceptible to umifenovir, indicating its potential for controlling NA inhibitor-resistant viruses.

3.2. Isolation of clinical viruses. To examine whether umifenovir-resistant variants emerged in influenza virus-infected patients treated with umifenovir, we evaluated level of susceptibility of viruses isolated from patients before and during umifenovir therapy. Overall, influenza A viruses were isolated from 18 nasal swabs from 14 patients with laboratory-confirmed influenza (Table 3). Viruses were typed as influenza A/Victoria/361/2011 (H3N2)-like (7 patients) and A/California/7/2009 (H1N1)pdm09-like (7 patients). Influenza B viruses were not isolated in this study. From patient #290, viruses were isolated at 1 and 3 days after initiation of therapy. From patients #89, 616, 654 and 715, viruses were isolated at 3 day after initiation of therapy. In this group of patients, fever (>37^{o}C) lasted 4 - 5 days longer than average for all patients of ARBITR clinical trial.[29] Complete recovery of all patients was at 9 - 10 days after treatment had started. For patient #718 (female, 36 years old), influenza A viruses were isolated from clinical samples obtained at 1, 3, 5, 7 days after initiation of umifenovir therapy. Patient #718 had prolonged fever (>37^{o}C) that lasted until 8 day, and the patient recovered by 9 day. Chronic diseases or influenza complications were not recorded for this group of patients. In 8 patients (# 93, 274, 671, 683, 226, 235, 239 and 277), viruses were isolated only before initiation of antiviral therapy (day 1 sample).

3.3. Umifenovir susceptibility of influenza viruses isolated from patients before and during therapy. The pattern and frequency at which umifenovir-resistant influenza viruses may arise during therapy is unknown. To explore the activity of umifenovir against influenza viruses isolated in a course of antiviral therapy, we determined and compared the EC_{50} values of the viruses isolated before or during drug administration. All clinical isolates

studied were susceptible to umifenovir, with EC_{50} ranging from 8.4 ± 1.1 to 17.4 ± 5.4 μM. The means of the EC_{50} values for the viruses isolated before or during umifenovir therapy ranged from 8.5 ± 1.1 to 11.8 ± 3.6 μM and from 8.4 ± 1.1 to 17.4 ± 5.4 μM for A(H1N1)pdm09 and A(H3N2) viruses, respectively (Table 4). We could not analyze susceptibility of the influenza viruses from swabs of 8 patients. The virus titers were below level of detection (0.01 $\log_{10}TCID_{50}$/ml) in these clinical samples. In 4 patients, we isolated viruses from clinical samples collected only at a single time point (3 days after initiation of umifenovir therapy). The EC_{50} of these viruses were not differ from previously detected susceptible values for clinical and laboratory isolates of influenza A viruses.[23] One matched pair of isolates (patient #290) showed no significant difference between samples taken before or after 3 days of treatment. Analysis of the samples obtained at 1, 3, 5 and 7 days after initiation of therapy (patient #718) showed similar umifenovir susceptibility pattern, and EC_{50} were similar to previous values which estimated for influenza A viruses and umifenovir.[23,26] The EC_{50} of viruses obtained from clinical samples collected at 5 and 7 days were comparable to those before initiation of treatment and to those collected at day 3 ($P >$ 0.05). This data showed no susceptibility decreasing to umifenovir during antiviral therapy for all analyzed isolates (Table 4).

3.4. NA inhibitor susceptibility of influenza viruses isolated from patients before and during umifenovir therapy. To determine whether umifenovir therapy has a potential to affect virus susceptibility to another class of anti-influenza drugs (i.e. NA inhibitors), we determined NA inhibitor IC_{50} values for influenza viruses isolated during umifenovir therapy. The IC_{50} values of 10 A(H1N1)pdm09 and 8 A(H3N2) influenza viruses are shown in Table 4. The IC_{50} values for oseltamivir carboxylate ranged from 0.9 ± 0.4 to 1.8 ± 0.2 nM and from 1.4 ± 0.2 to 2.7 ± 0.3 nM for A(H1N1)pdm09 and A(H3N2) viruses isolated before or during umifenovir therapy, respectively (Table 4). The IC_{50} values for zanamivir ranged from 0.3 ± 0.2 to 0.6 ± 0.2 nM and from 0.4 ± 0.2 to 1.3 ± 0.3 nM for

A(H1N1)pdm09 and A(H3N2) viruses, respectively (Table 4). The mean IC_{50} values for oseltamivir carboxylate of viruses isolated before and during administration of umifenovir ranged from 1.4 to 1.5 nM and from 2.3 to 1.8 nM for A(H1N1)pdm09 and A(H3N2) viruses, respectively. The mean IC_{50}s for zanamivir ranged from 0.5 to 1 nM and from 0.3 to 1.7 nM for A(H1N1)pdm09 and A(H3N2) viruses, respectively. The IC_{50} values for oseltamivir carboxylate and zanamivir of viruses obtained before and during umifenovir treatment from patients #290 and #718 were equally similar. There were no differences ($P >$ 0.05) in the susceptibility to oseltamivir carboxylate and zanamivir of viruses isolated from patient #290 before and at day 3 of therapy, as well as of virus isolated from patient #718 at 1, 3, 5 and 7 days. These results show that therapy with umifenovir does not lead to decrease of influenza viruses isolates susceptibility to NA inhibitors oseltamivir carboxylate and zanamivir.

3.5. Sequence analyses of clinical virus isolates. To obtain genotypic data in relatedness to phenotypic data, we sequenced HA genes from viruses isolated before and after completion of umifenovir therapy. A total 10 pairs of closely matched viruses were studied, including 4 isolates from patient #718 (samples before and at 3 or 5 and 7 days of treatment) and 2 isolates from patient #290 (samples before and at 3 day of treatment). It was reported previously that K51N, Q42H, Q27N, or K117R amino acid changes in HA were associated with reduced susceptibility to umifenovir.[21] We did not detect any of these HA changes in the tested samples (Table 5).

Additionally, sequences of NA genes from matched pairs of A(H1N1)pdm09 viruses obtained before and during umifenovir treatment from patients #718 and #715 (at 3 day of initiation of therapy) were compared to identify possible treatment-induced changes. H275Y amino acid substitutions, which caused resistance of A(H1N1)pdm09 influenza virus to oseltamivir were not found in the tested samples (Table 5). The sequences of M genes

showed that all influenza A viruses isolated from patients in this study contained the S31N change in the M2 protein, which confers cross-resistance to amantadine and rimantadine.

4. DISCUSSION

The double-blind, randomized, placebo-controlled ARBITR clinical trial was aimed to obtain novel data about the safety and efficacy of umifenovir (Arbidol®) for the control of influenza and other acute respiratory viral infections.[29] The aim of our study (which was conducted as a part of ARBITR) was to determine whether umifenovir therapy could lead to development of resistance in influenza-infected patients. For umifenovir resistance testing, we used phenotypic and genotypic assays, which are complementary. First, we conducted a MDCK cell-based ELISA assay to evaluate the umifenovir potency against influenza viruses. As reported previously,[37] this assay was found to be objective, reliable and rapid to study the effect of umifenovir on virus yield in cell culture. The data showed that antigenically dominant influenza A and B viruses (reference viruses) circulated in 2012-2014 seasons were highly susceptible to umifenovir. Our results demonstrated also that oseltamivir-resistant viruses are susceptible to umifenovir, and thus suggests that umifenovir may be a suitable alternative for the clinical management of infection caused by these viruses.

Susceptibility of 18 influenza A(H1N1)pdm09 and A(H3N2) viruses isolated from patients before and during therapy with umifenovir were examined. Importantly, all viruses retained susceptibility to umifenovir with EC_{50} values ranging from 8.4 ± 1.1 to 17.4 ± 5.4 μM. This data correlated with those previously reported for susceptible laboratory and clinical isolates.[23] While studying 10 isolates of influenza viruses that were obtained before umifenovir therapy, we did not detect resistant variants. This is consistent with the available publications, where naturally occurring resistance to umifenovir was not reported.[23,24]

Analysis of viruses isolated from samples collected at 3, 5 and 7 days after initiation of therapy revealed that all viruses were susceptible to umifenovir, and known HA molecular markers associated with resistance phenotypes were not identified. Thus, this study provides evidence of the lack of emergence of umifenovir resistance during 5 days of therapy of acute influenza infection. According to analysis of the sequential samples collected in oseltamivir-treated patients, resistant viruses were detected by 2 - 4 days after initiation of oseltamivir therapy.[38] Actually, we did not find viruses resistant to umifenovir at least by 3 days after initiation of umifenovir therapy. It is possible to suggest that the frequency of emergence of antiviral-resistant viruses in clinical settings is most likely lower for umifenovir compared with that for oseltamivir. However, the low number of influenza viruses evaluated in the current study calls for additional experiments.

Reduced susceptibility of influenza viruses to umifenovir refers to a substitution in HA2 subunit of HA glycoprotein.[21] It was shown that some oseltamivir-resistant variants generated in cell culture and in animals possess substitutions not only in HA but also in NA glycoproteins.[39] To address whether influenza A viruses resistant to oseltamivir carboxylate and zanamivir emerged after umifenovir therapy, IC_{50} values for viruses isolated from patients before and during therapy were compared. It has been shown that sensitivity to NA inhibitors was not reduced, thus suggesting that umifenovir administration does not result in the emergence of NA inhibitor-resistant variants. Additionally, sequence analysis of isolates from patient #718 did not identify NA inhibitor resistance-associated substitutions, including H275Y change.

This study has some limitations. Due to the acute nature of influenza infection and the efficacy umifenovir on reduction of virus yield in the nasal cavities of patients, the number of virus pairs was low. It was shown in the clinical section of ARBITR that the number of patients shedding influenza virus 4 days after initiation of therapy was reduced in the umifenovir-treated group compared to placebo ($P < 0.5$).[29]

The development of resistance has been documented at a higher frequency in immunocompromised patients treated with NA inhibitors.[1] The design of the ARBITR clinical study, that was a placebo-controlled trial, did not allow to enrollment of such patients. Only healthy adults aged 18 - 65 years were included into ARBITR clinical trial, and it could contribute to a lack of detection of resistant variants in these patients. Further, large scale clinical research trials, including those that target high-risk patients, is needed to estimate the occurrence of umifenovir-resistant variants during therapy. Additionally, conducting antiviral surveillance studies with the aim to establish the patterns of naturally occurring resistance to umifenovir is also of importance.

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CONFLICT OF INTERESTS

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Figure 1. Schematic representation of antiviral susceptibility assays of virus isolates obtained from patients of experimental group (treated by umifenovir, 800 mg/day) during the ARBITR clinical trial (2012 – 2014). *Nasal swabs from patients with laboratory-confirmed influenza. ** Sequence analysis of viruses (n=10) isolated from nasal swabs collected from patients at day 3 or later after umifenovir therapy started.

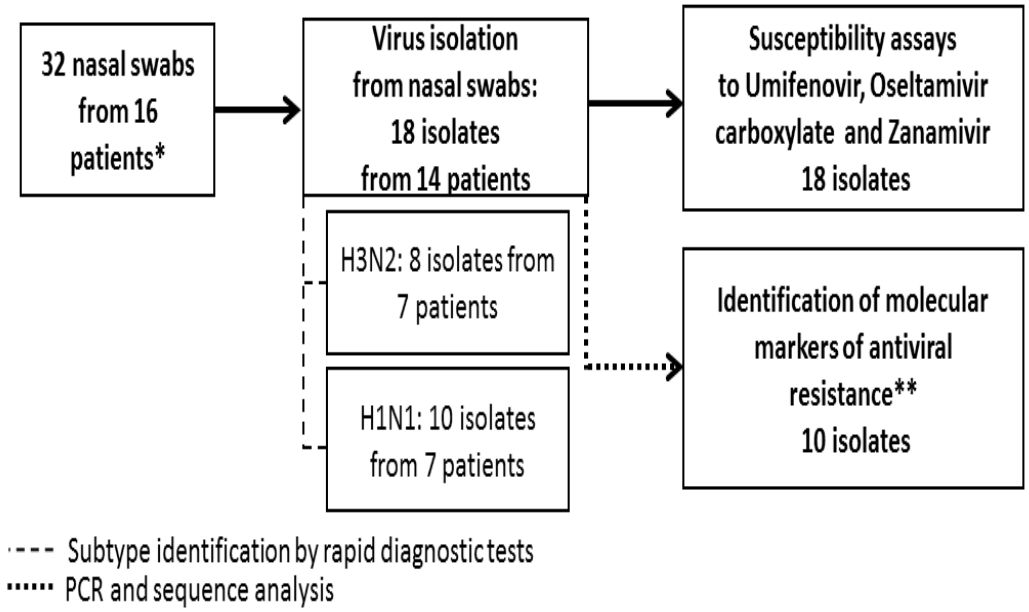

Table 1. List of primers for RT-PCR and sequencing of the HA, NA and M2 gene segments of influenza viruses.

Influenza virus	Gene segment	Primer designation	Primer sequence
A(H1N1)pdm09	HA	HA1-1F	5′**ATGAAGGCAATACTAGTAGT** -3′
		HA1-1R	5′**CATAGCACGAGGACTTCT** -3′
		HA1-2F	5′**GGAAATTCATACCCAAAGCT** -3′
		HA1-2R	5′**GATGGTGATAACCGTACCA** -3′
		HA1-3F	5′**ATTGCCGGTTTCATTGAAG** -3′
		HA1-3R	5′**CTGCACTGCAAAGACCCATTGGAGCAC** A-3′
	NA	NA1/2-1F	5′ ATGAATCCAAATCAIAAIATAAYA-3′
		NA1/2-1R	5′ CAATTCIGACTCTIGIGTYCT-3′
		NA1-2F	5′ TTGCTTGGTCGGCAAGTGC-3′
		NA1-2R	5′ TTTTTTGAACAAACTACTTGTCAA-3′
	M2	MF	5′ AGCAGGTAGATATTGAAAAATGA-3′

		MR	5′ GTAGAAACAAGGTAGTTTTTTAC-3′
A(H3N2)	HA	HA3-1F	5′ GCAGGGGATAATTCTATTAACCATG-3′
		HA3-1R	5′ GITTGTTGGCTTCTTTTGGTAG-3′
		HA3-2F	5′ ACTGGAGTTTAICIATGAAAGCTTC-3′
		HA3-2R	5′ CTCCCAACCATTTTCTATGAAACC-3′
		HA3-3F	5′ ATCACTCCAAATGGAAGCATTCC-3′
		HA3-3R	5′ CAAGGGTGTTTTTAATTAATGCACTC-3′
	M2	MF	5′ AGCAGGTAGATATTGAAAAATGA-3′
		MR	5′ GTAGAAACAAGGTAGTTTTTTAC-3′

Note. HA, hemagglutinin; NA, neuraminidase; M2, matrix protein 2; F, forward primer; R, reverse primer.

Table 2. Antiviral susceptibility of human influenza viruses antigenically similar to the viruses circulating in Russia during the 2012-2014 seasons.

Influenza virus[a]	Susceptibility to oseltamivir carboxylate (NA substitution[b])	Antiviral drugs susceptibility:		
		Umifenovir (EC_{50} ± SD, μM)[c]	Oseltamivir carboxylate (IC_{50} ± SD, nM)[d]	Zanamivir (IC_{50} ± SD, nM)[d]
Influenza A (H1N1)pdm09 viruses				
A/California/04/2009	S[e]	12.9 ± 1.4	0.9 ± 0.1	1.0 ± 0.1
A/California/07/2009	S	13.5 ± 1.3	1.0 ± 0.4	0.7 ± 0.0
A/Perth/265/2009	S	16.3 ± 1.4	1.0 ± 0.1	0.3 ± 0.0
A/Perth/265/2009	R[f] (H275Y)	24.3 ± 1.3	359.9 ± 87.2	0.5 ± 0.0
Influenza A (H3N2) viruses				
A/Victoria/361/2011	S	14.5 ± 0.7	1.9 ± 0.2	0.3 ± 0.0
A/Fukui/45/2004	S	19.2 ± 0.5	3.0 ± 0.0	1.4 ± 0.1
A/Fukui/45/2004	R (E119V)	16.3 ± 1.6	220.4 ± 23.1	1.7 ± 0.2

Influenza B viruses				
B/Brisbane/60/2008	S	30.9 ± 0.7	25.4 ± 0.51	0.9 ± 0.1
B/Wisconsin/1/2010	S	25.9 ± 2.2	21.3 ± 0.5	1.1 ± 0.1
B/Massachusetts /2/2012	S	18.9 ± 4.1	20.0 ± 0.8	2.4 ± 0.11
B/Perth/211/2001	S	16.2 ± 1.3	19.2 ± 2.4	1.9 ± 0.1
B/Perth/211/2001	R (D197E)	19.4 ± 0.9	230.3 ± 62.6	6.0 ± 0.1

[a]A panel of NA inhibitor-resistant viruses was kindly provided by the Antiviral Group, International Society for Influenza and Other Respiratory Virus Diseases. The panel included oseltamivir-susceptible A/Perth/265/2009 (H1N1)pdm09, A/Fukui/20/2004 (H3N2), B/Perth/211/2001 viruses, and oseltamivir-resistant A/Perth/265/2009 (H1N1)pdm09 virus with H275Y NA substitution, A/Fukui/45/2004 (H3N2) virus with an E119V NA substitution, B/Perth/211/2001 with E197D NA substitution.

[b]Amino acid position numbering are A subtypes and B type specific.

[c]Concentration of umifenovir that reduced viral replication by 50% relative to that without inhibitor. Values represent the mean ± SD from 3 independent experiments performed in triplicate using MDCK cell-based ELISA.

[d]Concentration of NA inhibitor that reduced viral NA activity by 50% relative to NA activity without inhibitor. Values represent the mean ± SD from 3 independent experiments performed in triplicate using phenotypic fluorescence-based assay.

[e]S – oseltamivir-susceptible virus.

[f]R– oseltamivir-resistant virus.

Table 3. Clinical features and demographic characteristics of influenza A virus infection in patients.

Clinical features and demographic characteristics	Influenza A virus	Influenza A virus subtype		
		H1N1	H3N2	P^a
No. of patients shedding virus	14	7	7	NA
No. of isolates	18	10	8	NA
Age (mean ± SD, years)	40.3 ± 13.3	35.9 ± 12.8	44.6 ± 13.7	>0.05
No. male/No. female	4/14	0/7	4/7	NA
Peak body temperature (mean ± SD, ^oC)	38.3 ± 0.29	38.2 ± 0.2	38.4 ± 0.3	>0.05
Time to the first administration of drug after the onset (mean ± SD, h)	13.7 ± 11.3	12.0 ± 13.9	15.4 ± 9.0	>0.05
Duration of fever (mean ± SD, h)	6.8 ± 2.86	7.6 ± 3.4	6.0 ± 2.2	>0.05
Duration of illness (mean ± SD, day)	11.2 ± 4.5	13.3 ± 4.8	9.1 ± 3.1	>0.05

[a] Statistical significance between patients age and influenza outcomes for H1N1 and H3N2 virus subtype was determined by Mann-Whitney U test.

NA - not applicable.

Table 4. Umifenovir and neuraminidase inhibitor susceptibility of viruses isolated from patients before and during umifenovir therapy.

Patient no.	Day of sample collection[a]	Virus titres ($log_{10}TCID_{50}/mL$)[b]	Subtype	Antiviral drugs susceptibility:		
				Umifenovir (EC_{50} ± SD, μM)[c]	Oseltamivir carboxylate (IC_{50} ± SD, nM)[d]	Zanamivir (IC_{50} ± SD, nM)[d]
89	3	0.4	H3N2	11.9 ± 3.1	2.3 ± 0.6	0.8 ± 0.2
93	1	1.4	H3N2	17.4 ± 5.4	2.5 ± 0.5	0.7 ± 0.1
226	1	1.4	H1N1	9.5 ± 1.6	1.5 ± 0.3	0.6 ± 0.2
235	1	0.5	H3N2	8.4 ± 1.1	2.5 ± 0.6	1.3 ± 0.3
239	1	1.1	H1N1	9.1 ± 1.4	1.3 ± 0.3	0.5 ± 0.1
274	1	1.6	H1N1	11.7 ± 2.5	1.5 ± 0.5	0.3 ± 0.2
277	1	0.9	H1N1	10.8 ± 3.2	1.4 ± 0.2	0.4 ± 0.1
290	1	1.3	H3N2	14.0 ± 0.0	1.6 ± 0.3	1.3 ± 0.1
	3	0.4		10.9 ± 1.6	1.4 ± 0.2	0.4 ± 0.2
616	3	1.3	H3N2	13.5 ± 0	1.5 ± 0.3	0.4 ± 0.2
654	3	0.5	H3N2	8.6 ± 0.5	2.3 ± 0.3	1.0 ± 0.3

671	1	1.7	H1N1	10.6 ± 3.6	0.9 ± 0.4	0.4 ± 0.1
683	1	1.4	H3N2	11.5 ± 4.3	2.7 ± 0.3	1.2 ± 0.3
715	3	0.4	H1N1	8.5 ± 1.1	1.3 ± 0.3	0.6 ± 0.2
718	1	2.0	H1N1	9.7 ± 1.4	1.8 ± 0.2	0.5 ± 0.1
	3	1.7		11.2 ± 1.8	1.7 ± 0.1	0.4 ± 0.1
	5	1.9		9.4 ± 0.7	1.5 ± 0.5	0.5 ± 0.1
	7	2.0		11.8 ± 3.6	1.5 ± 0.3	0.4 ± 0.1

[a] Day after initiation of antiviral therapy. Day 1 is indicated sample collected before initiation of therapy.

[b]Virus titers in nasal swabs from virus-infected and treated patients were determined at the indicated time points after the 1st passage in MDCK cells. The limit of detection for virus titer was 0.25 log10$TCID_{50/ml}$.

[c]Concentration of umifenovir that reduced viral replication by 50% relative to that without inhibitor. Values represent the mean ± SD from 3 independent experiments performed in triplicate using MDCK cell-based ELISA.

[d]Concentration of NA inhibitor that reduced viral NA activity by 50% relative to NA activity without inhibitor. Values represent the mean ± SD from 3 independent experiments performed in triplicate using phenotypic fluorescence-based assay.

Table 5. Identification of molecular markers of antiviral resistance in NA, HA and M2 gene segments.

Patient no.	Day of sample collection[a]	Subtype	Sequence analysis of gene segment:		
			HA	NA	M2
89	3	H3N2	None[b]	ND[c]	S31N
290	1	H3N2	None	ND	S31N
	3		None	ND	S31N
616	3	H3N2	None	ND	S31N
654	3	H3N2	None	ND	S31N
715	3	H1N1	None	None[d]	S31N
718	1	H1N1	None	None	S31N
	2		None	None	S31N
	5		None	None	S31N
	7		None	None	S31N

[a] Day after initiation of antiviral therapy. Day 1 is indicated sample collected before initiation of therapy. [b]None - no mutations responsible for resistance to umifenovir were determined. [c]ND - not done. [d]None- no mutations responsible for resistance to oseltamivir were determined.