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Prophylactic and therapeutic combination effects of rimantadine and oseltamivir against influenza virus A (H3N2) infection in mice

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ABSTRACT

The combined effect of rimantadine and oseltamivir in a prophylactic context (therapy beginning 4 h previrus infection) and therapeutic context (therapy started at 24 h post-viral inoculation) course on influenza H3N2 virus infection in mice was studied. In the prophylactic course 5 and 10 mg/kg/day rimantadine with 0.2 and 0.4 mg/kg/day (25:1 dose ratio) oseltamivir showed a protection index (PI) of 79.6% and 75%, respectively and a mean survival time (MST) of 13.1 and 12.9 days. The individual effects of the same doses ranged from 0% to 33.3% PI and 8.2 to 10.3 days MST, respectively. Lung virus titers were decreased 630-fold in the combination-treated groups as compared to monotherapy and placebo groups. The reduction of surface lung pathology in combination-treated groups demonstrated a protective effect for the combination of both antivirals. In the therapeutic course 5 and 10 mg/kg rimantadine combined with 0.2 and 0.4 mg/kg oseltamivir showed no beneficial effect. At higher dosage (0.8, 1.6, 3.2 mg/kg oseltamivir and 20, 40, 80 mg/kg rimantadine) preserving the 25:1 ratio, the resultant PI ranged from 57.6% to 80.5% and the MST was 12.8–13.4 days. Used alone at the same doses the compounds' protection varied between 10.7% and 71.8% PI, MST 9.8–12.8 days (8.7 days in PBS control). Compared to vehicle and individual treatment, a decrease in infectious viral titers of up to 1000-fold and other viral pneumonia parameters were also recorded. The therapeutic effect of the drugs' optimal effective doses combinations was characterized as synergistic. Survival of animals was 81.2–100% and MST was extended by 5–7 days compared to placebos. Monotherapy protection was from 9.1% to a maximum of 56.5%, MST being prolonged only by 1.3–4.2 days compared to 7.5 days in the PBS control group. Lung viral titers were decreased 1445-fold for the most efficacious combination groups and a significant reduction in lung parameters was observed. These data emphasize that prophylactic and therapeutic courses using a combination of oseltamivir and rimantadine have a significant protective effect in mice experimentally infected with drug-sensitive influenza virus A (H3N2).

1. Introduction

Influenza viruses are major causative agents of severe respiratory diseases leading to huge medical and economical losses in annual epidemics and periodic pandemics. Currently two classes of antivirals are approved for prophylaxis and treatment of influenza: M2 ion channel blockers and neuraminidase inhibitors. However, the emergence of influenza virus strains resistant to these both classes of antivirals has become a global health concern in the last decade.

Adamantane derivatives (amantadine and its derivative rimantadine) bind M2 proton channel and blocks the influx of H+ ions into the virion, a process essential for the uncoating stage of the viral replication cycle (Horimoto and Kawaoka, 2005). Particular amino acid substitutions (mainly S31N) in the M2 protein significantly reduce susceptibility to this class of drugs (Hay et al., 1985, 1986; Belshe et al., 1988). Resistance acquired either in response to treatment or due to natural variation has increased dramatically over the last 15 years from 0.8% in 1995 to 100% S31N (H3N2) of substituted mutants being resistant to adamantanes in some Asian countries and the USA (Deyde et al., 2007; Nelson et al., 2009). In seasonal epidemics 2009–2011 the predominantly circulating H1N1v strains were also 100% resistant to amantadine and rimantadine (Dawood et al., 2009; Zaraket et al., 2011). However, the majority of H1N1 seasonal strains which emerged in 2008 and were naturally resistant to oseltamivir (a neuraminidase inhibitor) were susceptible to M2 blockers (Lackenby et al., 2008; Puzelli et al., 2011) but amantadine-resistant variants have also been detected (Cheng et al., 2009). Amantadine has considerable side effects on the CNS, including nausea, vomiting, and loss of concentration which are significantly reduced by the treatment with rimantadine

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due the fact that it does not pass through the blood–brain barrier. Although adamantanes are currently not widely recommended because of the resistance rates, they might still be considered as a possible treatment alternative for seasonal H1N1 viruses and are recommended to WHO for treatment of the highly pathogenic avian influenza H5N1 (Kable, 2009). Moreover, there is no summarized data till the moment regarding susceptibility of the highly- pathogenic avian type, isolated from patients. A study conducted with samples of H5N1 human infections from Turkey indicated that the Turkish viruses are sensitive to both classes of antiviral drugs, including oseltamivir and amantadine (WHO, 2006).

Neuraminidase inhibitors (NAIs) - oseltamivir and zanamivir are structural analogues of the *N*-acetylneuraminic acid of cellular glycoprotein receptors and bind the viral neuraminidase (N) blocking the cleavage and release of the influenza progeny from the surface of the infected cell (Moscona, 2005a, b).

Since the introduction of NAIs in 1999 and until 2007, less than 1% of viruses tested have demonstrated natural resistance to them (NISN) (McKimm-Breschkin et al., 2003; Monto et al. 2006; Sheu et al., 2008). Limited development of resistance to oseltamivir has been observed in persons treated, with little evidence of onward transmission of resistant viruses (Aoki et al., 2007). However, oseltamivir-resistant viruses emerged in 18% (9/50) of Japanese children treated for influenza virus A (H3N2) infection and 16% (7/43) of treated Japanese children with influenza virus A (H1N1) infection (Kiso et al., 2004). In late January 2008 an unexpectedly high level and spread of oseltamivir-resistant influenza viruses A (H1N1) occurred in Europe, caused by an H275Y amino acid substitution in the neuraminidase (NA) of these viruses (Lackenby et al., 2008). They remained susceptible to zanamivir because of the principle difference in the mechanisms of binding the active site of the viral neuraminidase. The frequency of H1N1v strains currently circulating has also been investigated. Oseltamivir-resistant H275Y variants emerged in 2009 (www.who.int; Chen et al., 2009; Harvala et al., 2010; Lackenby et al., 2011) showing a recent increase in resistant variants up to 30% resistant variants in some countries (Australia, Singapore). The substitution S247N in the neuraminidase has been detected conferring a reduced susceptibility to both oseltamivir and zanamivir. When combined with the H275Y mutation, as found in an oseltamivir-treated patient, the double S247N/H275Y mutants exhibited an extremely high oseltamivir resistance (Hurt et al., 2011). An additional obstacle to oseltamivir therapy is represented by the adverse drug reactions – occurring in over 1% of clinical trials' participants. The latter include nausea, vomiting, diarrhea, abdominal pain, and headache. Several cases of impaired consciousness, abnormal behavior and hallucinations particularly in teenagers, associated with oseltamivir have been reported in Japan. (http://www.timesonline.co.uk; http://www.medscape.com).

Zanamivir is an effective agent against all types and subtypes of influenza virus and zanamivir resistance levels are still quite low. Despite the drug's minimal side effects and its lack of teratogenicity, limitations are associated with its aerosol route of administration which makes it inapplicable in patients with chronic respiratory illnesses such as asthma, pneumonia and others because of the risk of bronchospasms.

An experimental alternative strategy has been developed in the last decades based on synergistic combination of anti-influenza inhibitors with different mode of action. Such an approach overcomes selection of drug resistant variants, increases efficacy and decreases drug adverse reactions by reduction of doses (Govorkova and Webster, 2010). It also has an advantage in cases of several co-circulating influenza viruses susceptible to different drugs. Depending on the virus strain, the combination treatment with oseltamivir and rimantadine is recommended as an acceptable alternative and might be necessary for patients who cannot receive zanamivir (CDC, 2008).

A number of studies have been conducted with different combinations of adamantanes and neuraminidase inhibitors. *In vitro* studies were performed by Govorkova et al. (2004) demonstrating additive to synergistic effect of combinations of neuraminidase inhibitors (zanamivir, oseltamivir carboxylate, peramivir) and rimantadine against influenza A (H1N1) and (H3N2) and B variants in MDCK cells. Amantadine combined with oseltamivir also demonstrated enhanced inhibitory effect against H3N2, H1N1 and H5N1 (Ilyushina et al., 2006) and synergy in another study against the amantadine-sensitive H5N1 virus (Smee et al., 2009). The first investigations of the *in vivo* effect of oseltamivir in combination with rimantadine were performed in mice experimentally infected with the avian flu virus A (H9N2), in which a marked beneficial effect of the combined drugs was observed (Leneva et al., 2000). The synergistic effect of rimantadine and oseltamivir against H3N2 subtype in mice was first established by Galabov et al., 2006. When combined with oseltamivir, amantadine in laboratory animals demonstrated an enhanced protection as compared to monotherapy against H1N1, H3N2, A/Duck/MN/1525/81 (H5N1) (Masihi et al., 2007; Ilyushina et al., 2007; Smee et al., 2009). Peramivir in combination with rimantadine synergistically protected mice infected with H3N2 virus (Bantia et al., 2010). Combination therapy with oseltamivir and amantadine also appears safe and without pharmacokinetic consequences in healthy subjects as established by Morrison et al. (2007).

Recently triple combinations with amantadine, ribavirin and oseltamivir have also been tested *in vitro* demonstrating highly synergistic effect against 13 influenza strains A (H1N1), A (H3N2), A(H5N1) viruses (Nguyen et al., 2009, 2010) and is currently under ongoing clinical testing as TCAD (triple combination of antiviral drugs).

Encouraged by the optimistic results so far, we performed a study with a combination of rimantadine and oseltamivir in two courses of application – prophylactic and therapeutic, to prove the potency of combination therapy with M2 blockers and neuraminidase inhibitors against H3N2 viruses.

2. Materials and methods

2.1. Compounds

Rimantadine hydrochloride was kindly provided by Olainfarm, SA, Riga, (Latvia) and oseltamivir phosphate (the ethyl ester prodrug of oseltamivir) was purchased from Hoffman-La Roche (Switzerland). Compounds were diluted *ex tempore* in sterile bidistilled water (rimantadine) and in phosphate-buffered saline (PBS) (oseltamivir) for *in vivo* experiments.

2.2. Virus

Influenza virus A/Aichi/2/68 (H3N2) was obtained from the collection of the D. I. Ivanovsky Institute of Virology, Moscow (Russia), adapted to mice and then propagated in 10-day-old chicken embryos through serial intraallantoic passages.

2.3. Cells

Madin-Darby canine kidney (MDCK) cells were obtained from the ATCC (Manassas, VA, USA) and were grown in DMEM (Gibco BRL, Paisley, Scotland, UK) supplemented with 10% fetal bovine serum (Gibco BRL, Paisley, Scotland, UK), 3.7 mg/ml sodium bicarbonates, 10 mM HEPES buffer (AppliChem GmbH, Darmstadt, Germany), 100 IU/ml of penicillin, 100 μg/ml of streptomycin and 50 μg/ml of gentamycin in 5% CO_2 incubator Thermo Scientific 311 (Thermo Fisher Scientific, USA).

2.4. Mice

Male white mice of the ICR line with body weight 16–18 g were delivered by Slivnitza Animal Pharm (BAS, Bulgaria). They were subjected to an acclimation period of 5 days (prior to inoculation) and were observed for any signs of disease and/or physical abnormalities.

2.5. General procedure for in vivo antiviral experiments

Mice were anesthetized by ether inhalation and were inoculated intranasally with 0.05 ml/mouse of diluted virus, containing 10 MLD_{50}. Treatment course was started 4 h before or 24 h after virus inoculation and lasted for 5 days with compounds being administered twice a day (individually or in combination). Experimental groups consisted of 9–12 or 14–15 mice per drug dosage (20–25 mice in the placebo group). Two or three repetitions were performed. Parameters of antiviral activity such as protection index (PI) based on cumulative mortality rate and the mean survival times (MST) were evaluated until 14 days after infection.

2.6. Determination of lung parameters: viral titer, lung pathology surface score and index

In another set of experiments, three infected mice were sacrificed at various days of infection, their body weight was measured and their lungs were removed and weighed. The lung index was calculated following the multiplication of lung weight/body weight ratio by 100 (%). Lung pathology surface score was estimated following a range from 0 (normal lungs) to 4 (maximal hepatization) based on the percentage of damaged lung area with characteristic plum-like coloration (Sidwell et al., 1968).

Three mice from each experimental group were sacrificed at various days after infection. Under sterile conditions, the lungs were removed, washed three times in phosphate-buffered saline (PBS), homogenized and suspended in a total volume of 1 ml of PBS (10% w/v suspension). After centrifugation at 2000 rpm for 10 min, the supernatants were diluted in 10-fold steps, and the virus titers were determined in MDCK cells in 96-well plastic microplates (Cellstar, Frickenhausen, Germany) following the end-point dilution design. Twenty-four-hour cell monolayers were inoculated with 100 μl of diluted virus per well followed by 60-min adsorption at room temperature (four wells per dilution). Maintenance medium (200 μl per well) consisted of DMEM containing 0.5% fetal bovine serum, 3 μg/ml trypsin (Gibco BRL, Paisley, Scotland, UK), 3.7 mg/ml sodium bicarbonate, 10 mM HEPES buffer (AppliChem GmbH, Darmstadt, Germany) and antibiotics. Titers of infectious virus were presented as $\log_{10}$ of 50% cell culture infectious doses ($CCID_{50}$)/0.1 ml by recording the virus cytopathic effect (CPE) following 48–72 h of incubation at 37 °C.

2.7. Statistical analysis

The survival time included the period from the day of virus inoculation until the day before the animal's death. Mortality was recorded until day 14. The protection index (PI) was calculated using the equation PI = [(PC–1)/PC] ×100, where PC is the coefficient index =% mortality in placebo group /% mortality in the drug-treated group. The number of survivors was compared by Fisher's exact test. For the comparisons of MST, body and lung weights, one-way ANOVA test with Bonferroni's post-test was used. Viral titers were compared by two-way ANOVA with Bonferroni's post-test. Kruskal–Wallis test with Dunn's multiple comparisons post-test was performed for estimation of lung pathology surface scores. *P*-values of less than 0.05 were considered as statistically significant. Standard errors (SE) were calculated for the parameters where applicable. Data were analyzed by Graph Pad Prism® (Version 5.03 for Windows, Graph Pad Software Inc., USA). The character of the combined antiviral effect of optimal doses for *in vivo* studies of rimantadine hydrochloride and oseltamivir in influenza virus A (H3N2) infection in mice was determined according to the three-dimensional model (Prichard and Shipman, 1990) for analysis of drug-drug interactions, modified for *in vivo* trials (Nikolaeva and Galabov, 2000). The experimental data were evaluated by the MacSynergy™ II program (Prichard et al., 1992) which calculates the theoretical additive interactions of the drugs based on the Bliss independence mathematical definition of expected effects for drug-drug interactions. The Bliss independence model is based on statistical probability and assumes that the drugs act independently to affect virus replication. The calculated theoretical additive interactions were determined from the dose–response curves of the individual drugs. The calculated additive surface, which represents the predicted additive interaction, was then subtracted from the observed surface to reveal regions of statistically significant greater-than-expected (synergy) or less-than-expected (antagonism) interactions. The theoretical additive surface obtained was subtracted from the experimentally determined dose–response surface. The 95% confidence interval around the experimental dose–response surface was used to evaluate data statistically. Positive values, i.e. peaks above the horizontal plane at 0% inhibition, indicated synergy while negative ones, i.e. depression in the plane – antagonism.

3. Results

3.1. Combined effect of prophylactic course with rimantadine and oseltamivir in a 25:1 dose ratio on animals' survival

In our previous study we demonstrated additive to synergistic combination effect of rimantadine and oseltamivir with the highest protection value of doses at a ratio of 100:1 (Galabov et al., 2006). Here we examined the protective effect of the combination of oseltamivir and rimantadine at a dose ratio of 1:25 on the survival of mice, infected with 10 MLD_{50} influenza A (H3N2) virus. Selected doses were again lower or close to minimal and effective according to published data, i.e. 10 mg/kg/day rimantadine (Galabov et al., 1991) and 1 mg/kg/day oseltamivir (Sidwell et al., 1998). As seen in Table 1, individual treatment with each of the compounds did not significantly affect the survival rate of animals nor was their life lengthened. Combinations showed a PI of 54.5%, 75% and 79.6%, MST being prolonged by 4.9 days for the combination of 0.2 mg/kg oseltamivir + 5 mg/kg rimantadine, by 4.7 days for 0.4 mg/kg oseltamivir + 10 mg/kg rimantadine and 3.3 days for 0.8 mg/kg oseltamivir + 20 mg/kg rimantadine. At the same time, groups subjected to monotherapy had 0 to maximum 2.2 days of MST difference.

Dynamics of mortality also demonstrated a beneficial effect of the combined application of rimantadine and oseltamivir but individual treatment followed the dynamics of the PBS control group (Fig. S1A and B).

3.2. Combined effect of the prophylactic course with rimantadine and oseltamivir on animals' lung viral titers and lung pathology surface score

In another set of experiments the titers of influenza virus A (H3N2) in the lungs of infected mice treated with combinations of oseltamivir (0.2 and 0.4 mg/kg) and rimantadine (5 and 10 mg/kg) were determined on the first and fourth days post virus inoculation (Fig. 1A and B). Animals treated with a combination of 0.2 mg/kg oseltamivir and 5 mg/kg rimantadine showed 46-fold reduction on the first day and almost 158-fold decrease on day 4

Table 1
Efficacy of prophylactic course with rimantadine and oseltamivir combinations (25:1 dose ratio) on influenza A/Aichi/2/68 (H3N2) virus infections in mice at 10 MLD_{50}[a].

Oseltamivir	Rimantadine (mg/kg/day)	Survivors/Total[c]	Mortality (%)	PI (%)	MST(Days) ± SE[b,d]
0.4	10	24/30[***,□□,μμμ]	20	75	12.9 ± 0.7[**,μ]
0.4	–	11/30	63.3	23.1	9.8 ± 0.05
–	10	2/29	93	–	8.2 ± 0.15
0.2	5	25/30[***,λλλ,χχ]	16.7	79.6	13.1 ± 0.15[**,λλ,χ]
0.2	–	13/29	55.2	33.3	10.3 ± 0.05[*]
–	5	6/30	80	0	8.2 ± 0.1
PBS control		9/45	80.5		7.5 ± 0.7

[a] There were 14–15 animals in each treatment group, 20 and 25 mice in the PBS placebo group. Experiments were done in duplicate: data presented are from two experiments as follows: number of animals is cumulated, mortality percentages and protection index (PI) being evaluated, means are presented for mean survival time (MST).
[b] Statistical analysis: SE – standard error.
[c] Fisher's exact test.
[d] One-way ANOVA (Bonferroni's multiple comparison post-test.
□□ $P < 0.01$ vs 0.4 mg/kg oseltamivir alone.
μ $P < 0.05001$ vs 10 mg/kg rimantadine alone.
μμμ $P < 0.001$ vs 10 mg/kg rimantadine alone.
χ $P < 0.05$ vs 0.2 mg/kg oseltamivir alone.
χχ $P < 0.01$ vs 0.2 mg/kg oseltamivir alone.
λλ $P < 0.01$ vs 5 mg/kg rimantadine alone.
λλλ $P < 0.001$ vs 5 mg/kg rimantadine alone.
* $P < 0.05$ vs PBS control.
** $P < 0.01$ vs PBS control.
*** $P < 0.001$ vs PBS control.

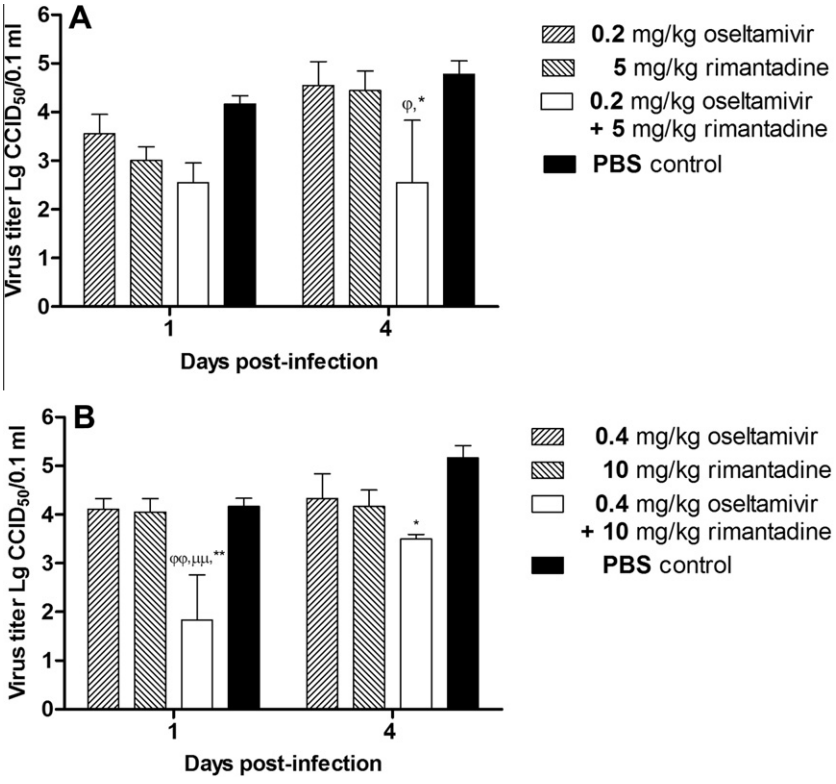

Fig. 1. (A and B) Effect of prophylactic course with combination of 5 mg/kg rimantadine with 0.2 mg/kg/day oseltamivir (A) and 10 mg/kg rimantadine with 0.4 mg/kg/day oseltamivir (B) on infectious virus titer in lungs of mice inoculated with influenza virus (H3N2) on 1st and 4th day after infection. Statistical analysis: Two-way ANOVA analysis of variance with Bonferonni's multiple comparisons post-test. Error bars – SE. $^{*}P < 0.05$, $^{**}P < 0.01$ vs PBS control, $^{\varphi}P < 0.05$, $^{\varphi\varphi}P < 0.01$ vs oseltamivir alone, $^{\mu\mu}P < 0.01$ vs rimantadine alone.

compared to the placebo group but these were without significance. Statistical comparisons of the 0.2 mg/kg oseltamivir + 5 mg/kg rimantadine combination group to individually- and the placebo-treated ones showed significance only versus PBS control on the 4th day (Fig. 1A). For the combination 0.4 mg oseltamivir with 10 mg rimantadine the viral load in the lungs was significantly reduced, especially on the first day, when compared to the monotherapy and placebo control (Fig. 1B).

On day 6 after viral inoculation lungs of mice from each treated group were removed and photographed for estimation of the lung pathology as seen from the Fig. S2. Additionally, an intact mouse was sacrificed as a healthy lung tissue control.

3.3. Combined effect of the therapeutic course with rimantadine and oseltamivir (25:1 dose ratio) on animals' survival

To study the efficacy of the combination therapy as an ongoing infection the protective effect of the combination of oseltamivir and rimantadine at a dose ratio of 1:25 was examined in a series of experiments. The application of a 5-day treatment course started 24 h after an infection with influenza A (H3N2) virus and the survival of mice was followed (Table 2). Lower doses applied individually and in combinations (0.2 and 0.4 mg/kg oseltamivir with 5 and 10 mg/kg rimantadine) did not demonstrate a significant protection of animals compared to the vehicle-treated group. Higher dosages (0.8, 1.6 and 3.2 mg/kg oseltamivir with 20, 40 80 mg/kg rimantadine) protected mice more effectively with up to 80.5% PI and MST of up to 13.4 days (3.2 mg/kg oseltamivir with 80 mg/kg rimantadine). The individual efficacy of compounds ranged from no protection and 7.7 days MST (8.7 days MST in the PBS control) in the 0.5 mg/kg oseltamivir treated group to 71.8% PI and 12.8 days MST in the 3.2 mg/kg oseltamivir-treated group.

Survival comparison demonstrated dynamics differences in mortality between individually and combination-treated groups but also a dose-dependent increase in the rate of protection (Fig. S3A–E).

3.4. Combined effect of the therapeutic course with rimantadine and oseltamivir (25:1 dose ratio) on lung parameters: viral titers, lung index and lung pathology surface score

For combinations which showed beneficial effect on the survival of treated animals, infectious virus titers in the lungs of three mice from each group on day 2, 4 and 6 post infection were determined in MDCK cell cultures. On day 2 a peak of viral titer of 4.66 $CCID_{50}$ lg was recorded in the PBS control group. When compared with the data for combinations of 0.8 mg/kg oseltamivir + 20 mg/kg rimantadine showed a 2-fold decrease, 1.6 mg/kg oseltamivir + 40 mg/kg rimantadine – a 21-fold reduction and 3.2 mg/kg oseltamivir + 80 mg/kg rimantadine demonstrated a 1000-fold decrease. Statistical significance, however, showed only the group treated at the highest doses of the compounds (3.2 mg oseltamivir with 80 mg rimantadine) compared to individually- and PBS-treated groups (Fig. 2A–C).

On day 6 after the influenza A (H3N2) infection animals' weight, lung weight and the lung pathology surface scores of 2–4 mice in each group were determined and the lung index was evaluated. Data are represented in Table 3. Combination treatment influenced parameters of viral pneumonia by preserving mice body weight (no statistically significant difference was evaluated), preventing infiltration in the lungs, decreasing lung index and lung pathology score compared to monotherapy and placebo-treated mice. Highest protection rates were again observed in the group treated with 3.2 mg/kg oseltamivir and 80 mg/kg/rimantadine, where body weight was 6.1 g higher than in the PBS control and 2.2 to 3.5 g higher than in individually treated animals. The mice weight of 23.5 g was close to that of the intact healthy control. Lung indices of 0.9% and 0.4% were recorded and the lung pathology score demonstrated differences of 2 and 3.3, respectively compared to placebos.

3.5. Combined effects of the therapeutic course with rimantadine and oseltamivir at optimal dosages on survival of mice

In the next series of experiments we studied the combination effect of 5 and 10 mg/kg/day oseltamivir with 40 and 80 mg/kg/day rimantadine – doses considered in the literature as half the optimal and optimal, respectively (Galabov et al., 1991; Sidwell et al., 1998). Doses were applied individually or in combination in a chess-board order according to the design for evaluating the character of the interaction between compounds. Efficacy results of the efficacy of combinations on survival are presented in Table 4. All four combinations demonstrated significant protective effects reaching values of PI from 76.2% to 100% and MST from 12.9 to more than 14 days.

Table 2
Combined effects of therapeutic course with oseltamivir and rimantadine in 1:25 dose ratio on survival of albino mice experimentally infected with influenza virus A (H3N2)[a].

Oseltamivir Rimantadine (mg/kg/day)		Survivors/Total[c]	Mortality (%)	PI (%)	MST(Days) ± SE[b,d]
0.2	–	3/29	89.7	–	7.7 ± 0.5
0.4	–	12/30	60.0	15.3	9.7 ± 0.8
0.8	–	13/30	56.7	20.0	10.1 ± 0.5
1.6	–	14/30	53.3	24.8	11.2 ± 0.7
3.2	–	24/30***	20.0	71.8	12.8 ± 0.3*
–	5	9/30	70.0	1.0	8.9 ± 1.0
–	10	9/30	70.0	1.0	9.8 ± 0.2
–	20	11/30	63.3	10.7	9.8 ± 0.8
–	40	11/30	63.3	10.7	10.0 ± 0.5
–	80	21/30***	30.0	57.6	12.5 ± 0.3
0.2	5	11/30φ	63.3	10.7	9.8 ± 0.3
0.4	10	15/30	50.0	29.1	10.7 ± 1.4
0.8	20	21/30***,ψ	30.0	57.6	12.8 ± 0.9*
1.6	40	25/30***,μμ,λλλ	16.7	76.4	12.9 ± 0.6*
3.2	80	25/29***	13.8	80.5	13.4 ± 0.4**
PBS control		17/58	70.7		8.7 ± 0.9

[a] There were 9–11 animals in each treatment group and 15–20 mice in the PBS placebo group. Data are from three separate experiments: number of animals is cumulated, percentages mortality and protection index (PI) being evaluated on the base of cumulated numbers; means are presented for mean survival time (MST).
[b] Statistical analyis: SE – standard error
[c] Fisher's exact test
[d] One-way ANOVA (Bonferroni's multiple comparison post-test).
* $P < 0.05$ vs PBS control.
** $P < 0.01$ vs PBS control.
*** $P < 0.001$ vs PBS control.
φ $P < 0.05$ vs 0.2 mg/kg oseltamivir alone.
ψ $P < 0.01$ vs 20 mg/kg rimantadine alone.
μμ $P < 0.01$ vs 1.6 oseltamivir alone.
λλλ $P < 0.001$ vs 40 mg/kg rimantadine alone.

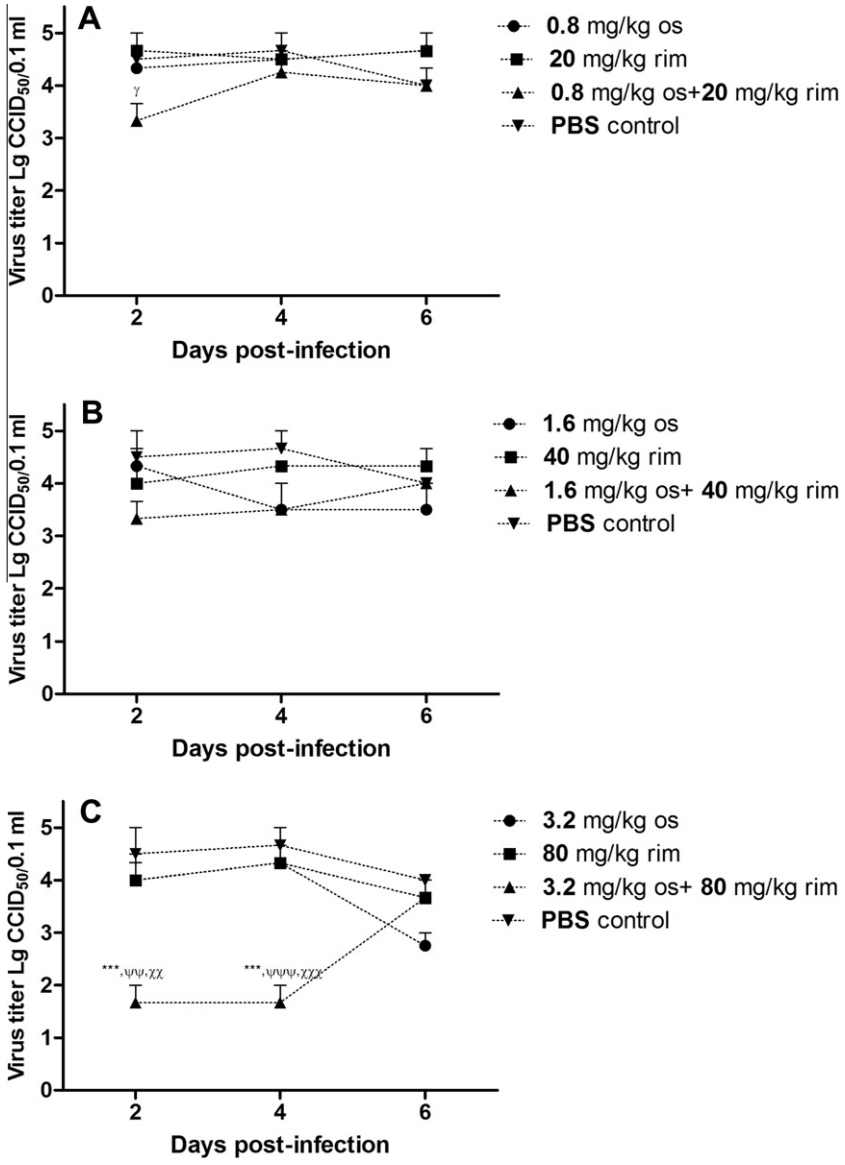

Fig. 2. (A–C) Effect of therapeutic course with oseltamivir and rimantadine combination in 1:25 dose ratio on infectious virus titers in lungs of mice inoculated with influenza virus A/Aichi/2/68 (H3N2). Statistical analysis: Two-way ANOVA with Bonferonni's multiple comparisons post-test. Error bars – SE. $^{***}P < 0.001$ vs PBS control, $^{\chi\chi}P < 0.01$, $^{\chi\chi\chi}P < 0.001$ vs oseltamivir alone, $^{\Psi}P<0.05$, $^{\Psi\Psi}P < 0.01$, $^{\Psi\Psi\Psi}P < 0.001$ vs rimantadine alone.

Experimental data were analyzed by three-dimensional method of Prichard and Shipman, and the effect was determined as synergistic for all studied combinations. The total volume of synergy was 40.13 μM^2 unit% (95% confidence interval, Bonferroni adjustment) which is considered as minor but significant synergy. The peak on the graph in Fig. 3 represents the combination of optimal doses (10 mg/kg/day oseltamivir with 80 mg/kg/day rimantadine) which fully protected infected animals. The synergy was 21.53 μM^2 unit% (95% confidence interval, Bonferroni adjustment). The second smaller peak on the graph corresponds to the combination of 10 mg/kg/day oseltamivir with 40 mg/kg/day rimantadine (synergy volume was 9.81 μM^2 unit%). Kaplan–Meier curves' analysis also clearly differentiated the survival of treated groups and demonstrated again the advantage of combined application of the tested compounds (Fig. S4A and B).

3.6. Effect of the therapeutic course with rimantadine and oseltamivir combinations at optimal dosages on lung parameters: viral titers, lung index and lung pathology score

The titers of influenza virus A/Aichi/2/68 (H3N2) in the lungs of infected mice (10 MLD_{50}) subjected to a 5-day treatment course with the combination of 5 and 10 mg/kg oseltamivir with 40 and 80 mg/kg rimantadine daily, and with the two compounds administered individually, were recorded on the 2nd, 4th and 6th day post virus inoculation. Fig. 4A and B illustrate the summarized data. A sharp 14-fold decrease in the infectious virus content was found in the group of mice treated with the synergistic combinations (5 mg/kg oseltamivir + 40 mg/kg rimantadine) and a 144-fold (5 mg/kg oseltamivir + 80 mg/kg rimantadine) on day 4, compared to the PBS treated control group. The combination of 10 mg/kg

Table 3
Effect of the therapeutically applied combinations of oseltamivir and rimantadine in 1:25 ratio on influenza A/Aichi/2/68 (H3N2) virus infected mice (6th day post-infection): lung parameters.

Oseltamivir	Rimantadine (mg/kg/day)	Mean mouse weight ± SE[a,b] (g)	Mean lungweight ± SE[a,b] (g)	Lung index (%)	Lung score ± SE[c]
0.8	–	18.9 ± 0.3	0.38 ± 0.02	2.0	3.5 ± 0.2
1.6	–	16.7 ± 0.1	0.42 ± 0.01	2.5	3.1 ± 0.4
3.2	–	21.4 ± 1.2	0.44 ± 0.03	2.1	2.3 ± 0.8
–	20	18 ± 0.4	0.39 ± 0.02	2.2	3.1 ± 0.2
–	40	18.7 ± 1.6	0.48 ± 0.04	2.6	3.3 ± 0.3
–	80	20 ± 0.5	0.39 ± 0.01	1.9	2.4 ± 0.1
0.8	20	22 ± 1.5	0.3 ± 0.05*	1.4	1.6 ± 0.8
1.6	40	20.2 ± 3.8	0.28 ± 0.02*	1.4	1.7 ± 0.8
3.2	80	23.5 ± 0.6	0.21 ± 0.04***,χχ	0.9	0.4 ± 0.2**
PBS control		17.4 ± 0.7	0.5 ± 0.01	2.9	3.7 ± 0.1
Uninfected and untreated		24.1 ± 0.1	0.2 ± 0.03	0.8	0

There were 12 animals in each treatment group. On day 2, 4 and 6 three mice from each group were sacrificed, their lungs assayed for viral titers and lung parameters were evaluated.
Statistical analysis:
[a] SE – standard error.
[b] One-way ANOVA (Bonferroni's multiple comparison post-test).
[c] Kruskal–Wallis test with Dunn's multiple comparison post-test.
* $P < 0.05$ vs PBS control.
** $P < 0.01$ vs PBS control.
*** $P < 0.001$ vs PBS control.
χχ $P < 0.01$ vs 3.2 mg oseltamivir alone.

Table 4
Combined effect of optimal doses rimantadine and oseltamivir orally administered 24-h post viral inoculation with 10 MLD_{50} influenza virus A/Aichi/2/68 (H3N2) on survival of albino mice[a].

Compound Dosemg/kg/day Oseltamivir	Rimantadine	Survivors/ Total[c]	Mortality (%)	PI (%)	MST Days ± SE[b,d]
5	–	20/32***	37.5	52.4	11.0 ± 0.2**
10	–	21/32***	34.4	56.5	11.8 ± 0.2***
–	40	10/32	68.8	9.1	8.8 ± 1.2
–	80	20/32***	37.5	52.4	11.7 ± 0.8**
5	40	29/32***,φφφ,μ	9.4	88	13.6 ± 0.4***,φφφ
5	80	30/32***,ψψ,μμ	6.3	91.9	13.7 ± 0.2***
10	40	26/32***. φφφ	18.8	76.2	12.9 ± 0.2***,φφ
10	80	32/32***,ψψψ,λλλ	0	100	⩾14.0 ± 0.0***
PBS control		13/60	78.3		7.5 ± 0.6

[a] There were 9–12 animals in each treatment group and 20 mice in the PBS placebo groups. Data are from three separate experiments: number of animals is cumulated, percentages mortality and protection index (PI) being evaluated on the base of cumulated numbers; means are presented for mean survival time (MST). Statistical analysis.
[b] SE – standard error.
[c] Fisher's exact test.
[d] One-way ANOVA with Bonferroni's multiple comparison post-test).
** $P < 0.01$ vs PBS control.
*** $P < 0.001$ vs PBS control.
φφ $P < 0.01$ vs 40 mg/kg rimantadine alone.
φφφ $P < 0.001$ vs 40 mg/kg rimantadine alone.
ψψ $P < 0.01$ vs 80 mg/kg rimantadine alone.
ψψψ $P < 0.001$ vs 80 mg/kg rimantadine alone.
μ $P < 0.05$ vs 5 mg oseltamivir alone.
μμ $P < 0.01$ vs 5 mg oseltamivir alone.
λλλ $P < 0.001$ vs 10 mg oseltamivir alone.

oseltamivir with 40 mg/kg rimantadine showed statistically significant difference of a 457-fold decrease as compared the placebo on the 4th day. In the 10 mg/kg oseltamivir + 80 mg/kg rimantadine group the reduction was a 45710-fold on day 2 and a 1445-fold on day 4 (viral peak in the placebo control). It is well seen in Fig. 4 that the courses with oseltamivir and rimantadine used alone were without an effect on the lung virus titer and the titer dynamics was similar to that in the placebo control group.

The influence of the treatment courses on lung pathology surface scores and the lung indices is presented in Table 5. The treatment course with the combinations significantly diminished the surface hemorrhaging rates and the lung index values as compared to the placebo control, whereas courses with the compounds applied individually showed markedly lower protection at selected dosages.

4. Discussion

In the present study we demonstrate that when applied as both prophylactic and therapeutic courses, the combination of rimantadine hydrochloride and oseltamivir manifests a higher protective effect on experimental infection with influenza virus A/Aichi/2/68 (H3N2) in albino mice as compared to monotherapy. Several studies have been conducted with M2 blockers and oseltamivir combinations as prophylactic courses. Our previous investigations on this combination have demonstrated that when therapy begins 4 h pre-viral exposure a marked synergistic effect is exerted with the strongest inhibition of the 1:100 oseltamivir/rimantadine dose ratio (Galabov et al., 2006). Here low doses of 0.2 and 0.4 mg/kg oseltamivir combined with 5 and 10 mg/kg rimantadine in different ratio, 1:25, are also observed to increase survival and decreased

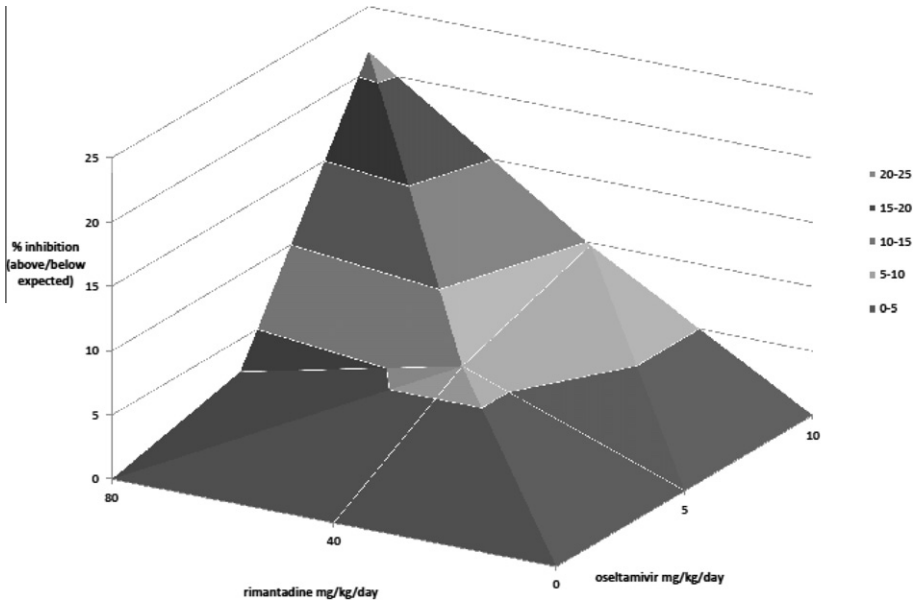

Fig. 3. Combination effect of therapeutic course with 5 and 10 mg/kg/day oseltamivir with 40 and 80 mg/kg/day rimantadine on experimental infection in albino mice with 10 MLD_{50} influenza virus A/Aichi/2/68 (H3N2) demonstrated by the three-dimensional model of Prichard and Shipman. The total volume of synergy was found to be 40.13 μM^2 unit% (Synergy plot 95% confidence interval, Bonferroni adjustment). The guidelines for the volumes of synergy/antagonism determinations are as follows: 0–25 μM^2 unit% insignificant synergy or antagonism; 25–50 μM^2 unit% minor but significant synergy or antagonism; 50–100 μM^2 unit%, moderate synergy or antagonism;>100 μM^2 unit% strong synergy or antagonism. Synergy plot was made at the 95% confidence limit (Ilyushina et al., 2008).

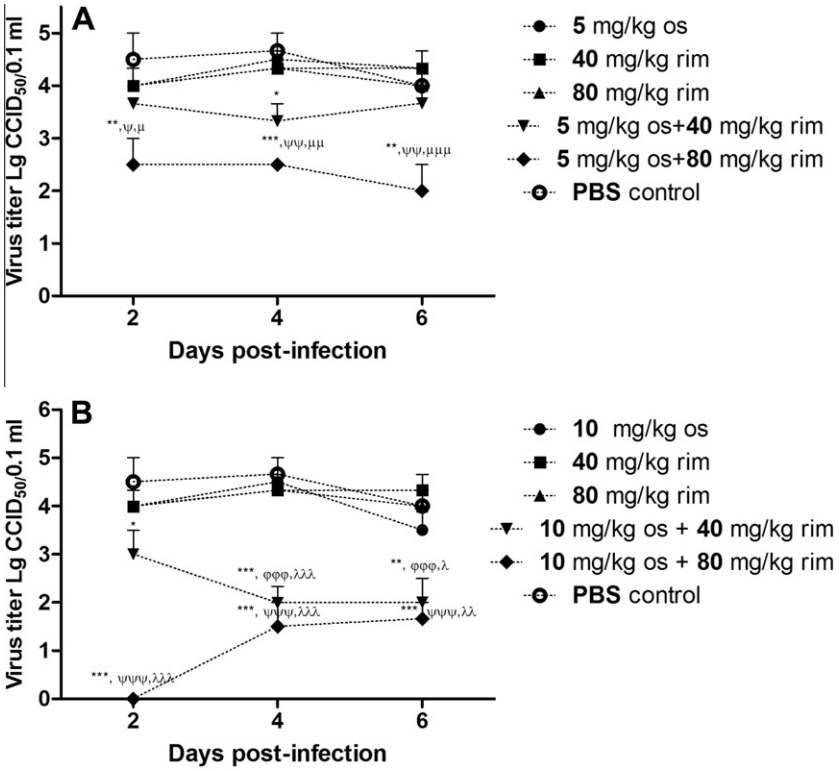

Fig. 4. (A and B) Effect of orally administered optimal doses oseltamivir and rimantadine individually and in combination on infectious virus titer in lungs of mice inoculated with influenza virus A/Aichi/2/68 (H3N2) Statistical analysis: Two-way ANOVA with Bonferonni's multiple comparisons post-test. Error bars – SE.$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs PBS control, $^{\varphi\varphi\varphi}P < 0.001$ vs 40 mg rimantadine alone, $^{\psi}P < 0.05$, $^{\psi\psi}P < 0.01$, $^{\psi\psi\psi}P < 0.001$ vs 80 mg rimantadine alone, $^{\mu}P < 0.05$, $^{\mu\mu}P < 0.01$, $^{\mu\mu\mu}P < 0.001$ vs 5 mg oseltamivir alone, $^{\lambda}P < 0.05$, $^{\lambda\lambda}P < 0.01$, $^{\lambda\lambda\lambda}P < 0.001$ vs 10 mg oseltamivir alone.

severity of infection in murine lungs. Leneva et al. (2000) have demonstrated that the combination of 1 mg/kg per day rimandatine with 0.1 mg/kg per day oseltamivir significantly increases the number of survivors and the survival time in mice infected with influenza virus A (H9N2). Later, Masihi et al. (2007) studied the effect of the combination of low doses oseltamivir with amantadine in mice infected with H3N2 (A/Hongkong/1/68) or H1N1 (A/PR/8/34). In that study the combination of amantadine with osel-

Table 5
Effect of the orally administered 5 and 10 mg/kg/day oseltamivir and 40 and 80 mg/kg/day rimantadine individually and in combination on influenza A/Aichi/2/68 (H3N2) virus infected mice (6th day post-infection): lung parameters.

Compound dose (mg/kg/day) Oseltamivir	Rimantadine	Mean mouse weight ± SE[a,b] (g)	Mean lung weight ± SE[a,b] (g)	Lung index (%)	Lung score ± SE[a,c]
5	–	21 ± 1.2	0.39 ± 0.02	1.9	2.6 ± 0.1
10	–	23.8 ± 0.4	0.38 ± 0.05	1.6	1.5 ± 0.4
–	40	18.7 ± 1.6	0.48 ± 0.04	2.6	3.3 ± 0.3
–	80	20 ± 0.5	0.39 ± 0.01	1.9	2.4 ± 0.1
5	40	23.6 ± 0.2	0.38 ± 0.03*	1.6	1.1 ± 0.2
5	80	20.1 ± 0.0	0.27 ± 0.07*	1.5	0.8 ± 0.6*
10	40	23.4 ± 7.0	0.29 ± 0.05*	1.2	0.4 ± 0.2**
10	80	23.1 ± 1.9	0.28 ± 0.02*	1.2	0.8 ± 0.1*
PBS control		17.4 ± 0.7	0.5 ± 0.01	2.9	3.7 ± 0.1
Uninfected and untreated		24.1 ± 0.1	0.2 ± 0.03	0.8	0

There were 12 animals in each treatment group. On day 2, 4 and 6 three mice from each group were sacrificed and their lungs assayed for viral titers and lung parameters were evaluated.
[a] Statistical analyis: SE – standard error.
[b] One-way ANOVA (Bonferroni's multiple comparison post-test).
[c] Kruskal–Wallis test with Dunn's multiple comparison post-test.
* $P < 0.05$ vs PBS control.
** $P < 0.01$ vs PBS control.

tamivir required 15-fold less oseltamivir than monotherapy to confer complete protection and was also effective against the amantadine-resistant H1N1 (A/PR/8/34) virus. In contrast, in another study, the efficacy of amantadine-oseltamivir combination against amantadine-resistant H5N1 virus was comparable to that of oseltamivir alone. In the same study, amantadine and oseltamivir in prophylactic course (treatment started 24 h before viral inoculation) combination treatment provided greater protection than monotherapy against the amantadine-sensitive recombinant H5N1 virus, with up to 90% survival. Spread of the virus to the brain was prevented by both combinations (Ilyushina et al., 2007). An enhanced protective effect was reported also when amantadine-oseltamivir combinations were tested against amantadine-sensitive A/Duck/MN/1525/81 (H5N1) virus in mice when the treatment course started 4-h prior to infection (Smee et al., 2009).

Fewer investigations have been conducted on combination effects with onset of treatment after the viral challenge. It was demonstrated that a 48 h delay in monotherapy with 1 mg/kg/day oseltamivir significantly decreases the protective effect against 10 MLD_{50} of A/HK/156/97 (H5N1) virus (Leneva et al., 2000). Smee et al. (2010) showed that favipiravir (T-705) and oseltamivir individual and combination effects on survival of mice infected with H1N1, H3N2 and H5N1 virus were higher when treatment started at −2 h than at +24 h. Recently, a study with triple combination with amantadine, oseltamivir and ribavirin showed high protection (over 90% survival and reduced weight loss) of mice, infected with amantadine-resistant H1N1 or low-pathogenic H5N1 strain. The efficacy was observed in both – prophylactic and delayed to +72 h course of application (Nguyen et al., 2012).

To our knowledge the present data are the first reported on the effects of the double combination therapy with oseltamivir and rimantadine against H3N2 virus type when treatment started with a 24-h delay after infection. These effects are demonstrated at a lower dosage 1:25 and optimal effective doses combination. It is clearly seen that in lower dosage both antivirals either used alone or in combination did not protect animals. However, doses of 0.8, 1.6, 3.2 mg/kg oseltamivir combined with 20, 40, 80 mg/kg rimantadine, respectively, applied therapeutically (24 h after the onset of infection) increased the survival. In addition, lung viral titers were decreased to 1000-fold. The highest protective and synergistic effects were observed when optimal doses were combined, both on mortality reduction (100% survival) and on parameters of viral pneumonia (1445-fold – titers difference, 3.3 lung pathology rates decrease, 1.7% reduction in the lung index, and preservation of body weight as compared to the PBS placebo group). Interestingly, monotherapy with 1.6, 3.2, 5 and 10 mg/kg oseltamivir and 80 mg/kg rimantadine had some beneficial effect on survival rates, lung pathology score and indices reduction but did not affect viral content in murine lungs and similar titers were recorded, compared to the PBS control. It is important to study the efficacy of the delay in any treatment due to the fact that in clinical practice chemotherapy is usually applied after the manifestation of the first symptoms of the disease, i.e. within the first 48 h after infection.

Extensive research and optimistic data from pre-clinical and clinical trials with novel compounds strongly support the idea that influenza infection will be prevented and treated much more successfully in the next years with a significant reduction in disease severity and mortality rates. Nevertheless, whatever efficacious a certain monotherapy is, the risk of drug resistance development still exists. Monitoring of viral drug-resistance rates and alternative approaches for counteracting the resistance should be applied globally. In fact, the neuraminidase inhibitors are the recommended antiviral drugs for treatment currently because the circulating viruses type H3N2 are adamantane-resistant. However, how would the situation change with this type in the future remains unclear. Last but not least, susceptibility of H5N1 clinical isolates is not summarized globally and in cases of infection with the avian strain in humans the combination therapy with a M2 blocker and neuraminidase inhibitor could be a possible effective strategy.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2012.05.004.

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