Rimantadine and oseltamivir demonstrate synergistic combination effect in an experimental infection with type A (H3N2) influenza virus in mice

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We studied the combination effect of rimantadine hydrochloride and oseltamivir phosphate on mice infected with influenza A/Aichi/2/68 (H3N2) virus. Compounds were simultaneously administered in a 5-day-treatment course, starting 4 h before intranasal infection with 10 or 20 viral 50% mouse lethal doses. Initially, we tested combinations of oseltamivir (0.05, 0.1 and 0.2 mg/kg/day) and rimantadine (2.5, 5.0 and 7.5 mg/kg/day). Significant differences were recorded between combination-treated groups, and groups with separately applied compounds and the placebo group, such as: protection index of oseltamivir with 5.0 or 7.5 mg/kg rimantadine varied between 34–41% and 43–87%, respectively, whereas the individual effects of oseltamivir, 5 mg/kg of rimantadine and 7.5 mg/kg of rimantadine were 0–10%, 0% and 18.7–29.6%, respectively; mean survival time in combination-treated groups was lengthened by 3.1–6.9 days, in oseltamivir groups by 0–1.9 days, and in rimantadine groups by 0.8–1.3 days at 5 mg/kg and 2.6–3.2 days at 7.5 mg/kg. The three-dimensional method of Prichard and Shipman characterized the combination effect as synergistic. Further, we studied the activity of 0.05 mg/kg/day of oseltamivir combined with 5 mg/kg of rimantadine. Lung virus titre in Madin Darby canine kidney cells, lung index and consolidation score proved the high effectiveness of the combination. When compared with the placebo group, a 2.8 log10 lower titre of 50% cell culture infectious dose (CCID50) was recorded in the combination-treated group at 48–60 h post-infection (the peak of lung virus growth). This is in contrast to the 0.1–1.0 log10 and 1.1–1.4 log10 reduction in CCID50 titre observed in the oseltamivir and rimantadine groups, respectively. These data emphasize the high anti-influenza A potential of the combination.

Keywords: combination effect, influenza virus A (H3N2), mice, oseltamivir phosphate, rimantadine hydrochloride

Introduction

The development of effective chemotherapy against flu is of paramount significance in the struggle against this disease, which frequently manifests a severe course with complications, and attacks huge contingents of the global population in periodic epidemics and pandemics. Currently, the emergence of drug resistance is a basic obstacle against the effectiveness of anti-flu chemotherapeutics. Besides the loss of the compound’s antiviral activity as a result of the spread of drug-resistant mutants, this phenomenon could possibly induce some other disadvantageous phenotypic manifestations. Recently, a study on more than 7,000 samples of influenza A virus-infected patients collected worldwide between 1994 and 2004 showed an increase of resistance to M2 blockers amantadine and rimantadine from 0.4 % in 1994–1995 to 12.3 % in 2003–2004 (Bright et al., 2005). Influenza virus A isolates resistant to oseltamivir were registered as well (McKimm-Breschkin, 2000), H5N1 variant included (de Jong et al., 2005). Some authors noticed a more rapid development of resistance to M2 blockers in comparison with neuraminidase inhibitors, that is, resistant mutants appearance necessitates a markedly lower number of passages of influenza A virus variants in Madin Darby canine kidney (MDCK) cells, grown in the presence of adamantanes (Hayden, 1996; Zambon and Hayden, 2001). Administration of anti-flu chemotherapeutic agents in children, immunocompromized patients and persons with chronic respiratory illnesses was found to facilitate the development of drug resistance (Zambon and Hayden, 2001).

The mechanisms of resistance to the two types of influenza virus inhibitors have been clarified (Hayden and Hay, 1992; Hayden, 1996; Zambon and Hayden, 2001). The theoretical base of resistance to antivirals was
primarily formulated by André Lwoff (1965) and later, resistance development was defined as a basic characteristic of the specific inhibitors of virus replication (Herrmann and Herrmann, 1977). Having this in mind, the development of resistance should not be expected in cases of antiviral substances acting via host cell mechanisms. Ribavirin, a broad-spectrum antiviral compound, which displays an anti-flu effect, is an example in this respect (Sidwell et al., 1985). The process of mutation rise is strongly dependent on the mutation rate of the respective virus taxonomic group, which is very high in picorna-viruses and moderate in influenza viruses. For the time being, the mechanisms of preserving the influenza virus drug-resistant mutants in the human population have not been clarified. It seems that expectations for their elimination (‘washing away’) as a result of antigenic drift can not be justified.

The combined application of antivirals with different mode of action, which do not possess cross-resistance, is a potential approach for counteracting the emergence of resistance mutants. An increase in selectivity ratio, a decrease in side effects and some economic benefits (lessening of production amounts required and production costs, and lower prices) are among the beneficial consequences of the introduction of efficient synergistic combinations. As a result, combination effects of anti-flu agents were tested. Thus, investigations were carried out on the combination effect of amantadine with ribavirin, a non-specific viral inhibitor (Galegov et al., 1977; Wilson et al., 1980; Hayden et al., 1980, 1984; Hayden, 1986), and with some biological response modifiers: human interferon-α (Hayden et al., 1984), protease inhibitor aprotonin (Zhirmov, 1987) and the antioxidant ionol (4-methyl-2,6-ditertbutylphenol) (Galabov et al., 1994b).

The first study on the combination of specific influenza virus replication inhibitors used rimantadine plus mopyridone (which targeting M1 protein of influenza A virus) and showed a synergistic effect (Galabov et al., 1991, 1994a). It was followed by investigations on the effect of combinations with neuraminidase inhibitors. Thus, zanamivir in combination with rimantadine, ribavirin and 2′-desoxy-2′-fluoroguanosine displayed an additive effect against influenza A virus in MDCK cells (Madren et al., 1995). Peramivir and ribavirin produce an additive to synergistic effect against influenza A virus in vitro and in experimental influenza virus A (H1N1) infection in mice (Smee et al., 2002). Govorkova et al. (2004) demonstrated in MDCK cells additive to synergistic effect of combinations consisting of neuraminidase inhibitors (zanamivir, oseltamivir carboxilate, peramivir) and rimantadine against influenza A virus (H1N1 and H3N2 variants). The highest synergistic effect was found against H1N1 (New Caledonia) and H3N2 (Panama) viruses by measuring the extracellular virus. The investigations of Leneva et al. (2000) on the effect of oseltamivir in combination with rimantadine in experimental infection with the avian flu virus A(H9N2) in mice, which displayed a marked beneficial effect, merited a more special interest.

These studies serve as a basis for us to explore the combination effect of rimantadine and oseltamivir in vivo with an experimental infection of influenza virus A (H3N2) in albino mice by multi-parametric analysis of virus-induced processes in the lungs.

Materials and methods

Compounds

Rimantadine hydrochloride was provided by the All-Union Institute of Flu and Respiratory Viral Infections, St. Petersburg, Russia, and oseltamivir phosphate (the ethyl ester prodrug of oseltamivir) was purchased from F. Hoffmann-La Roche Ltd (Basel, Switzerland).

Viruses

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

Cells

MDCK cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were grown in Dulbecco MEM (Gibco BRL, Paisley, Scotland, UK) supplemented with 10% fetal bovine serum (Gibco BRL), 3.7 mg/ml sodium bicarbonate, 10 mM HEPES buffer (AppliChem GmbH, Darmstadt, Germany), 100 IU/ml of penicillin, 100 µg/ml of streptomycin and 50 µg/ml of gentamicin in a 5% CO₂ incubator HERA cell 150 (Heraeus, Hanau, Germany).

Mice

Male white mice of the ICR line weighing 8–12 g (obtained from the Laboratory Animals Farm of the Bulgarian Academy of Sciences, Slivnitzia, Bulgaria) were used.

Determination of lung virus titres

Three mice from each experimental group were sacrificed at various days after infection. Under aseptic 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 2,000 rpm for 10 min, the supernatants were diluted in 10-fold steps, and the virus titres were determined in MDCK cells in 96-well plastic microplates (Cellstar, Frickenhausen, Germany) following the endpoint dilution design (Reed and Muench, 1938). Twenty-four hour cell monolayers were inoculated with
100 µl per well by 60 min adsorption at room temperature (four wells per dilution). Maintenance medium (200 µl per well) contained Dulbecco MEM with 0.5 % fetal bovine serum, 3 µg/ml trypsin (Gibco BRL), 3.7 mg/ml sodium bicarbonate, 10 mM HEPES buffer (AppliChem) and antibiotics. Titres of infectious virus were presented as log10 of 50% cell culture infectious doses (CCID50)/ml by recording the virus cytopathic effect (CPE) following 48–72 h of incubation at 37°C.

**In vivo virus assay**

Groups of eight mice per dilution of the consecutive 10-fold dilutions of the stock virus in PBS were infected via intranasal inoculation of 0.05 ml/mouse. Following the endpoint dilution procedure, the virus titres were evaluated in log10 of 50% cell culture infectious doses (CCID50)/ml by recording the virus cytopathic effect (CPE) following 48–72 h of incubation at 37°C.

**General procedure for in vivo antiviral experiments**

Mice were anaesthetized by inhalation of ether and were inoculated intranasally with 0.05 ml/mouse of diluted virus, containing 10 or 20 MLD50. Treatment course with the tested compound, administered twice a day (separately or in combination), started 4 h before virus inoculation and lasted 5 days. Each experimental group consisted of 11–24 mice per drug dosage (15–35 mice in the placebo group). Three to five mice were used in toxicity control groups as normal controls. Parameters for evaluation of antiviral activity such as protection index (PI) were based on cumulative mortality rate and the measurement of the mean survival time (MST), and were determined through 14 days post-infection.

In certain experiments, an additional three infected mice were used at each dosage. They were sacrificed at days 3, 5 or 7 of infection, their body weight was measured, and their lungs were removed and weighed. As a subsequent step, the lung consolidation score was estimated following the range from 0 (normal lungs) to 4 (maximal consolidation). The lung consolidation parameter represents the percentage of the lung with characteristic plum-like colouration. Simultaneously, the lung index was evaluated by determining the lung weight/body weight ratio.

**Combination activity effect**

The character of the combined antiviral effect 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). A checkerboard design of the experiments was made with a series of daily doses of each partner in the combinations. Experiments were done in duplicate. The experimental data were evaluated by the MacSynergy™ II program (Prichard *et al.*, 1992). Theoretical additive interactions were calculated from the dose–response curves for each drug tested individually. 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, that is, peaks above the

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**Figure 1.** Combination effect of orally applied rimantadine and oseltamivir on experimental infection in albino mice with 20 MLD50 of influenza virus A/Aichi/2/68 (H3N2), as demonstrated using MacSynergy™ computerized three-dimensional program.

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Data are at 95% confidence intervals. MLD50, 50% mouse lethal dose.
horizontal plane at 0% inhibition, indicated synergy; negative values, that is, depression in the plane indicated antagonism. These values are shown on the ordinate in Figure 1.

Statistical analysis
The survival time included the period from the day of virus inoculation until the day before the animal's death. The mortality was recorded until day 14. PI was evaluated by the equation $PI=(PC−1/PC)×100$, where PC is the protection coefficient (that is, % mortality in placebo group/% mortality in the drug-treated group). Fisher’s exact test was applied to compare mouse survival rate between the experimental groups. Differences both in MST and in lung virus titres between groups were compared using a two-tailed

### Table 1. Effect of combinations of orally administered rimantadine and oseltamivir on survival of albino mice infected with influenza virus A/Aichi/2/68 (H3N2) at 20 MLD$_{50}$

<table>
<thead>
<tr>
<th>Oseltamivir, mg/kg/day</th>
<th>Rimantadine, mg/kg/day</th>
<th>Survivors/total, n*</th>
<th>Mortality, %</th>
<th>MST $\pm$SE, days$^i$</th>
<th>PC</th>
<th>PI, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>2.5</td>
<td>6/24</td>
<td>75.0</td>
<td>8.3 $\pm$0.73$^i$</td>
<td>1.18</td>
<td>15.3</td>
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<td>0.05</td>
<td>5.0</td>
<td>11/23$^i$</td>
<td>52.2</td>
<td>9.5 $\pm$0.82$^i$</td>
<td>1.7</td>
<td>41.2</td>
</tr>
<tr>
<td>0.05</td>
<td>7.5</td>
<td>11/24$^i$</td>
<td>54.2</td>
<td>9.9 $\pm$0.89$^i$</td>
<td>1.63</td>
<td>38.7</td>
</tr>
<tr>
<td>0.05</td>
<td>–</td>
<td>4/24</td>
<td>83.3</td>
<td>8.3 $\pm$0.69$^i$</td>
<td>1.06</td>
<td>5.7</td>
</tr>
<tr>
<td>0.1</td>
<td>2.5</td>
<td>6/23</td>
<td>73.9</td>
<td>8.9 $\pm$0.7$^i$</td>
<td>1.2</td>
<td>16.7</td>
</tr>
<tr>
<td>0.1</td>
<td>5.0</td>
<td>10/24$^i$</td>
<td>58.3</td>
<td>9.9 $\pm$0.8$^i$</td>
<td>1.52</td>
<td>34.2</td>
</tr>
<tr>
<td>0.1</td>
<td>7.5</td>
<td>11/23$^i$</td>
<td>52.2</td>
<td>10.0 $\pm$0.8$^i$</td>
<td>1.7</td>
<td>41.2</td>
</tr>
<tr>
<td>0.1</td>
<td>–</td>
<td>3/24</td>
<td>87.5</td>
<td>7.9 $\pm$0.6</td>
<td>1.01</td>
<td>1.0</td>
</tr>
<tr>
<td>0.2</td>
<td>2.5</td>
<td>10/23$^i$</td>
<td>56.5</td>
<td>10.3 $\pm$0.8$^i$</td>
<td>1.57</td>
<td>36.3</td>
</tr>
<tr>
<td>0.2</td>
<td>5.0</td>
<td>10/24$^i$</td>
<td>58.3</td>
<td>9.7 $\pm$0.8$^i$</td>
<td>1.52</td>
<td>34.2</td>
</tr>
<tr>
<td>0.2</td>
<td>7.5</td>
<td>10/24$^i$</td>
<td>58.3</td>
<td>10.3 $\pm$0.8</td>
<td>1.52</td>
<td>34.2</td>
</tr>
<tr>
<td>–</td>
<td>2.5</td>
<td>3/24</td>
<td>87.5</td>
<td>7.1 $\pm$0.8</td>
<td>1.01</td>
<td>1.0</td>
</tr>
<tr>
<td>–</td>
<td>5.0</td>
<td>2/24</td>
<td>91.7</td>
<td>7.2 $\pm$0.5</td>
<td>0.97</td>
<td>0</td>
</tr>
<tr>
<td>–</td>
<td>7.5</td>
<td>9/24$^i$</td>
<td>62.5</td>
<td>9.0 $\pm$0.8$^i$</td>
<td>1.42</td>
<td>29.6</td>
</tr>
<tr>
<td>Placebo (PBS)</td>
<td>–</td>
<td>4/35</td>
<td>88.6</td>
<td>6.4 $\pm$0.5</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Fisher’s exact test. $^i$Student’s t-test. $^p<0.05$, $^p<0.01$ or $^p<0.001$ compared to placebo control group. MLD$_{50}$, 50% mouse lethal dose; MST, mean survival time; PC, protection coefficient; PI, protection index; SE, standard error.

### Table 2. Effect of combinations of orally administered rimantadine (5 and 7.5 mg/kg) and oseltamivir (0.05 mg/kg) on survival in influenza virus A/Aichi/2/68 (H3N2) infected mice

<table>
<thead>
<tr>
<th>Viral dose</th>
<th>Oseltamivir, mg/kg/day</th>
<th>Rimantadine, mg/kg/day</th>
<th>Survivors/total, n*</th>
<th>Mortality, %</th>
<th>MST $\pm$SE, days$^i$</th>
<th>PC</th>
<th>PI, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 MLD$_{50}$</td>
<td>0.05</td>
<td>5.0</td>
<td>8/11$^i$</td>
<td>27.3</td>
<td>12.6 $\pm$0.73$^i$</td>
<td>2.93</td>
<td>65.9</td>
</tr>
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<td>0.05</td>
<td>7.5</td>
<td>6/11$^i$</td>
<td>45.5</td>
<td>10.7 $\pm$1.2$^i$</td>
<td>1.75</td>
<td>42.9</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>–</td>
<td>0/11</td>
<td>100</td>
<td>5.3 $\pm$0.8</td>
<td>0.79</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>5.0</td>
<td>1/11</td>
<td>90.9</td>
<td>7.6 $\pm$0.9</td>
<td>0.91</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>7.5</td>
<td>4/11</td>
<td>63.6</td>
<td>9.2 $\pm$1.2</td>
<td>1.23</td>
<td>18.7</td>
<td></td>
</tr>
<tr>
<td>Placebo (PBS)</td>
<td>–</td>
<td>4/19</td>
<td>78.9</td>
<td>6.8 $\pm$0.9</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>10 MLD$_{50}$</td>
<td>0.05</td>
<td>5.0</td>
<td>6/11$^i$</td>
<td>45.5</td>
<td>11.4 $\pm$1.0$^i$</td>
<td>1.75</td>
<td>42.9</td>
</tr>
<tr>
<td>0.05</td>
<td>7.5</td>
<td>9/10$^i$</td>
<td>10</td>
<td>13.2 $\pm$0.9$^i$</td>
<td>0.79</td>
<td>87.3</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>–</td>
<td>0/11</td>
<td>100</td>
<td>6.0 $\pm$0.3</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>5.0</td>
<td>1/11</td>
<td>90.9</td>
<td>7.3 $\pm$0.8</td>
<td>0.91</td>
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</tr>
<tr>
<td>0.1</td>
<td>7.5</td>
<td>4/11</td>
<td>63.6</td>
<td>9.5 $\pm$1.1</td>
<td>1.23</td>
<td>18.7</td>
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*Fisher’s exact test. $^i$Student’s t-test. $^p<0.05$, $^p<0.01$ or $^p<0.001$ compared to placebo control group. MLD$_{50}$, 50% mouse lethal dose; MST, mean survival time; PBS, phosphate buffer solution; PC, protection coefficient; PI, protection index; SE, standard error.
Student’s t-test. P-values <0.05 were estimated as statistically significant. Mann–Whitney rank sum test was applied in the lung consolidation score analysis.

**Results**

**Selection of the optimal combination doses of rimantadine and oseltamivir**

Initially, a series of experiments were performed in order to find out the most effective combinations of daily doses of the compounds. Mice were infected with 20 MLD$_{50}$ and treatment courses lasted 5 days from the day of virus inoculation. Three daily doses of each compound were applied. They were selected on the basis of literary data in order to be equal or lower than the minimum effective doses versus influenza virus A (H3N2) infection in mice. These were 10 mg/kg of rimantadine, as found against several A(H3N2) strains, including A/Aichi/2/68 (H3N2) (Ilyenko et al., 1972; Galabov et al., 1991), and 1 mg/kg of oseltamivir as registered against A/Victoria/3/75(H3N2) and A/Shandong/09/93(H3N2) (Sidwell et al., 1998). The results summarized in Table 1 demonstrate a marked protective effect of the combi-
nations of 0.05, 0.1 or 0.2 mg/kg oseltamivir and 5 or 7.5 mg/kg rimantadine. PI values varied between 34–41%, the MST being prolonged by 3.1–3.9 days. Doses of 0.2 mg/kg oseltamivir plus 2.5 mg/kg rimantadine also showed an effect. The individual administration of the compounds at these doses and the remaining combinations did not significantly influence the course of infection checked by the cumulative mortality rate. Some effect of rimantadine 7.5 mg/kg (PI=29.6% and lengthening of MST by 2.6 days) compared to the placebo group was only registered.

As can be seen, combinations of oseltamivir at the lowest dose used (0.05 mg/kg) and rimantadine at 5 or 7.5 mg/kg deserve a special attention with the highest protective effect.

Estimation of the character of the combination effect
The experimental data shown in Table 1 were included in the Prichard and Shipman model for evaluating the character of the combination effect. The final result of the analysis is presented in Figure 1. A well-drawn zone of synergism is seen in the following combinations: 0.05 mg/kg oseltamivir + 5.0 or 7.5 mg/kg rimantadine, 0.1 mg/kg oseltamivir + 5.0 mg/kg rimantadine, and 0.2 mg/kg oseltamivir + 2.5 or 5.0 mg/kg rimantadine.

Effect of selected synergistic combinations on the mortality rate and MST
Further experiments on the efficacy of the synergistic combination selected as a result of the screening evaluation of the combined effect, as presented in Table 1, were carried out on mice infected with 10 or 20 MLD_{50} of influenza A (H3N2) virus. The results obtained are shown in Table 2 and Figures 2A and 2B. The very close activity of the combinations of the daily dose of 0.05 mg/kg oseltamivir and rimantadine at doses 5 and 7.5 mg/kg was very apparent – there were no significant differences observed between the activities against influenza virus A infection induced by 10 and 20 MLD_{50}. This is expressed by protection index values reaching 66%–87%, and a lengthening of MST by 3.9–6.9 days.

Effect of the synergistic combination of oseltamivir 0.05 mg/kg and rimantadine 5 mg/kg on lung parameters
The combination of the lowest oseltamivir dose of 0.05 mg/kg plus the lower dose of 5 mg/kg rimantadine was selected for the further experiments. The oseltamivir dose was 200× and 20× lower than the compound’s optimal and minimal effective daily dose of 10 and 1 mg/kg, respectively (Sidwell et al., 1998), and the rimantadine dose was 8–16× lower than the compound’s optimal effective dose.

The titres of influenza virus A/Aichi/2/68 (H3N2) in the lungs of infected mice (10 MLD_{50}) subjected to a 5-day combined treatment course of 0.05 mg/kg of oseltamivir and 5 mg/kg of rimantadine daily, and with the two compounds administered individually were recorded within the first 4 days post virus inoculation. Figure 3 illustrates the summarized data of three experiments. When compared with the placebo group, a sharp decrease of the infectious virus content was found in the group of mice treated with the synergistic combination: by 1 log_{10} CCID_{50} at 24 h, by 2 log_{10} at 36 h, and by 2.8 log_{10} at 48 and 60 h (the virus titre peak in the placebo group). The lung virus content curve in the combination treated group had a sloping character, increasing gradually and reaching its highest level at 84 h, marking a 24 h delay as compared to the placebo group. The results clearly show that the individual treatment courses of 0.05 mg/kg of
synergistic combination of rimantadine and oseltamivir versus flu in mice

Table 3. Effect of the orally administered combination of rimantadine (5 mg/kg) and oseltamivir (0.05 mg/kg) on influenza A/Aichi/2/68 (H3N2) virus infected mice: lung parameters

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Mean mouse weight, g</th>
<th>Mean lung weight, g</th>
<th>Lung index, %</th>
<th>Lung consolidation score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oseltamivir 0.05 mg/kg</td>
<td>12.0 ±0.21†</td>
<td>0.159 ±0.002</td>
<td>1.32</td>
<td>1.15 ±0.08†</td>
</tr>
<tr>
<td>+ rimantadine 5 mg/kg</td>
<td>13.27 ±0.24‡</td>
<td>0.193 ±0.004†</td>
<td>1.45</td>
<td>3.13 ±0.11</td>
</tr>
<tr>
<td>Oseltamivir 0.05 mg/kg</td>
<td>10.7 ±0.17</td>
<td>0.189 ±0.003§</td>
<td>2.14</td>
<td>3.25 ±0.1</td>
</tr>
<tr>
<td>Rimantadine 5 mg/kg</td>
<td>10.2 ±0.21</td>
<td>0.217 ±0.004</td>
<td>2.14</td>
<td>3.25 ±0.1</td>
</tr>
<tr>
<td>Placebo (PBS)</td>
<td>11.33 ±0.18</td>
<td>0.09 ±0.019</td>
<td>0.8</td>
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<td>Uninfected and untreated</td>
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</tbody>
</table>

*Lung index – the ratio of lung weight/body weight. †Lung consolidation score – ranges from 0 (normal lungs) to 4 (maximal consolidation). ‡P < 0.001 compared to placebo control group (Student’s t-test for comparing mouse body and lung weight and Mann–Whitney rank sum test for consolidation score comparison).PBS, phosphate buffer solution; SS, standard error.

The two compounds daily doses in the combinations used in our studies were quite far below the optimal effective doses for influenza A virus infection in mice, that is, 10 mg/kg and 40–80 mg/kg for oseltamivir and rimantadine, respectively. Individually administered doses of oseltamivir and rimantadine, 0.05 mg and 5 mg/kg, respectively, were without any effect, but when applied in combination, they ensured to 66–87% survival of the treated animals. Evidently, this activity was based on the pronounced reduction of the infectious virus content in the lungs by approximately 3 log10 after 60 h post virus inoculation, the time peak of virus lung titre.

The advantages of the use of this combination as an anti-flu agent could be summarized as follows: (i) increased selectivity; (ii) the potential to decrease drug-resistance development towards the two compounds in the combination (which is based on the reduced probability of the appearance of viral mutants with double drug-resistance, a phenomenon not observed in flu viruses at the present); and (iii) such a process could be considered as reduced to a minimum when compounds in combinations are used at very low doses, and that is the case with our selected anti-flu combination. It is well known that the so-called ‘pressure of the dose’ (use of antiviral agents at high doses) is a strongly favourable factor for accelerated development of drug-resistance.

The potential of a combined rimantadine–oseltamivir preparation deserves special attention in view of the increase of drug-resistant influenza A virus isolates, particularly expressed towards M2 inhibitors (Bright et al., 2005), and to a lesser but gradually increasing degree towards NA inhibitors (de Jong et al., 2005).

Discussion

Our study unambiguously shows that when applied in combination rimantadine hydrochloride and oseltamivir, representatives of the two classes influenza A virus replication inhibitors, viral structural proteins M2 and NA ligands, respectively, manifested a pronounced synergistic effect in experimental infection with influenza virus A(H3N2) in albino mice. Previous investigation on this combination also demonstrated a beneficial effect on influenza A virus avian variant H9N2, the character of the combination effect had not been assessed (Leneva et al., 2004) by measuring extracellular virus yields in MDCK cells.

In view of the dominant role of the H3N2 variant of influenza A virus as a causative agent of flu epidemics, as well as the flu seasonal incidence rate on a global scale for the whole period after 1968, these studies deserve particular interest. This is especially valid at the present time in view of the threatening menace of flu pandemic, which necessitates the broadening of the arsenal of agents for urgent prophylaxis and chemotherapy of flu.

References


Leneva IA, Roberts N, Govorkova EA, Goloubzeva OG & Webster RG (2000) The neuraminidase inhibitor GS4104 (oseltamivir phosphate) is efficacious against A/Hong Kong/156/97 (H5N1) and A/Hong Kong/1074/99 (H9N2) influenza virus. *Antiviral Research* **48**:101–115.


