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Combination of peramivir and rimantadine demonstrate synergistic antiviral effects in sub-lethal influenza A (H3N2) virus mouse model

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ABSTRACT

Efficacy of combination of the intramuscularly administered neuraminidase (NA) inhibitor, peramivir, and the orally administered M2 ion channel blocker, rimantadine was evaluated in mouse influenza A/Victoria/3/75 (H3N2) model. Mice were challenged with a sub-lethal virus dose (0–40% mortality in placebo group) and changes in body weights were analyzed by three-dimensional effect analysis to assess mode of drug interactions.

Compounds were administered in a 5-day treatment course starting 1 h before viral inoculation. The peramivir and rimantadine doses ranged from 0.3–3 mg/kg/d and 5–30 mg/kg/d, respectively. The maximum mean weight loss of 5.19 g was observed in the vehicle-infected group on day 10. In the 1 and 3 mg/kg/d peramivir monotherapy groups, the weight losses were 4.3 and 3.55 g, respectively. In the rimantadine monotherapy group, the weight losses were 3.43, 2.1, and 1.64 g for the 5, 10, and 30 mg/kg/d groups, respectively. Combination of 1 mg/kg/d peramivir with 5 and 10 mg/kg/d rimantadine produced weight losses of 1.69 and 0.69 ($p < 0.05$ vs. vehicle and individual agent), respectively, whereas the combination of 3.0 mg/kg/d peramivir with 10 and 30 mg/kg/d rimantadine did not show any weight loss ($p < 0.05$ vs. vehicle and individual agent). The three-dimensional analysis of the weight loss for the majority of the drug combinations of peramivir and rimantadine tested demonstrated synergistic antiviral effects.

1. Introduction

Two classes of drugs are approved for prophylaxis and treatment of influenza: M2 ion channel blockers (amantadine and its derivative rimantadine) and neuraminidase inhibitors (oseltamivir and zanamivir). Amantadine and rimantadine block the ion channel of the M2 protein of influenza A virus, and inhibit viral replication at an early stage by preventing the uncoating of the virus and blocking the entry into the nucleus of the cell (Wang et al., 1993; Belshe et al., 1988). Neuraminidase inhibitors interrupt at a late stage by inhibiting the release of virions from infected cells. They also cause aggregation of the released virion which is then less able to penetrate mucus secretions and infect other cells (Mendel et al., 1998). Thus, the two classes of available anti-influenza drugs act by different mechanisms and at different stages of the virus replication cycle.

Generally, compounds with different modes of action are predicted to act synergistically against viral infections (Ilyushina et al., 2008; Smee et al., 2010). Antiviral drugs that target different viral proteins and have different mechanisms of action when acting synergistically can provide several advantages over single agent treatment—greater potency, superior efficacy, and reduction of drug dosages. In addition, application of antivirals with different modes of action, which do not possess cross resistance, is a potential approach for counteracting the emergence of a resistant mutant (Ison et al., 2003). The Centers for Disease Control and Prevention (CDC) has made specific recommendations for the use of influenza antiviral medications in the setting of oseltamivir resistance among circulating influenza A (H1N1) viruses for the 2008–09 Influenza season. Depending upon the strain of the virus, the combination treatment with oseltamivir and rimantadine was considered as an acceptable alternative and might be necessary for patients that cannot receive zanamivir (CDC HEALTH ADVISORY Distributed via Health Alert Network Friday, December 19, 2008, 11:50 EST (11:50 AM EST) CDCHAN-00279-2008-12-19-ADV-N). Oseltamivir and zanamivir were recommended by CDC for the treatment of patients infected with 2009 pandemic influenza virus.

Peramivir is a specific and potent inhibitor of influenza NA and has demonstrated *in vitro* activity that is comparable to or better than oseltamivir carboxylate and zanamivir (Babu et al., 2000; Bantia et al., 2001; Drusano et al., 2001; Govorkova et al., 2001; Sidwell et al., 2001; Smee et al., 2002). Peramivir is active when administered intramuscularly (IM) and intravenously (IV) in

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both prophylaxis and treatment of mouse influenza virus infections (Bantia et al., 2006; Boltz et al., 2008; Kobayashi et al., 2009). Intravenous administration of peramivir has been evaluated in clinical trials for seasonal influenza and in the treatment of influenza in hospitalized patients (Kohno et al., 2009; Ison et al., 2009).

Peramivir was shown to interact favorably with ribavirin to reduce influenza A virus infection in cell culture and mice (Smee et al., 2002). In this study we have evaluated the efficacy of peramivir given intramuscularly combined with rimantadine given orally against influenza A/Victoria/3/75 (H3N2) virus infection in a mouse model. To determine the mode of interaction between the two anti-influenza drugs, mortality and viral titers have been generally used (Ilyushina et al., 2008; Smee et al., 2009). Sometimes because of high variability in viral titers and mortality, it is difficult to show drug combination effects *in vivo* (Smee et al., 2009, 2010). Hence, in this study, a sub-lethal dose of virus was used for infection and the impact of combination treatment on the weight loss parameter was chosen to characterize the mode of interaction between the two anti-influenza drugs, peramivir and rimantadine. To our knowledge this is the first report that uses a weight loss parameter to assess the interaction between the two anti-influenza drugs.

2. Materials and methods

2.1. Viruses

The influenza A virus used in this study was obtained from Dr. Robert Sidwell, Utah State University, Logan, Utah (Influenza A/Victoria/3/75; H3N2), grown in cell culture, and was adapted to mice.

2.2. Mice

Specific pathogen-free female BALB/c mice 6–8 weeks old (16–20 g) were obtained from Charles Rivers Laboratories (Raleigh, North Carolina). Mice were permitted an acclimation period of greater than 48 h (prior to inoculation) during which time the animals were observed for signs of disease and/or physical abnormalities. Mice were maintained on rodent diet from Harlan Teklad and tap water from the laboratory animal research center of BioCryst Pharmaceuticals, Inc. The study was conducted in accordance with the current facility's Standard Operating Procedures (SOPs). The study was conducted in compliance with the Animal Welfare Act (9 CFR Parts 1, 2 and 3).

2.3. Compounds and reagents

Peramivir was synthesized by BioCryst Pharmaceuticals, Inc. (Birmingham, AL). Rimantadine was purchased from Sigma–Aldrich (St. Louis, MO). Each compound was prepared in sterile phosphate-buffered saline (PBS) for *in vivo* experiments. Isoflurane (5%) in oxygen was administered for anesthesia.

2.4. General procedure for in vivo antiviral experiments

Female BALB/c mice were anesthetized with isoflurane and exposed to 50 μL of 10-fold serial dilutions of A/Victoria/3/75 (H3N2) influenza virus in PBS by intranasal instillation. An approximately 20% mouse lethal dose of virus was calculated after a 14 day observation period. In three separate experiments, the 20% mouse lethal dose of virus gave a consistent weight loss of about 5 g by day 9–10 post virus inoculation. Groups of 9–15 mice were anesthetized with isoflurane and given an approximately 20% mouse lethal dose of virus. Drug was administered 1 h before viral inoculation. Each infected, drug-treated group contained 9–10 mice and the saline-treated group contained 10–15 mice. In the single agent study, intramuscular (IM) treatment with peramivir began 1 h before virus exposure and continued once daily (qd) for 5 days. Oral treatment of rimantadine also began 1 hr prior to viral inoculation and dosing continued twice a day (bid) for 5 days. Handling animals during infection and dosing can cause stress which can impact the immune system and may alter the course of the disease. To alleviate this concern, all the animals were handled in a similar manner, i.e., peramivir-treated animals were dosed orally with vehicle bid for 5 days and the rimantadine-treated animals were dosed IM with vehicle qd for 5 days. Control group animals were dosed with vehicle IM qd and orally bid for 5 days. In the combination study, both rimantadine (orally, bid) and peramivir (IM, qd) were administered 1 h prior to virus inoculation and dosing continued for 5 days. Parameters for evaluation of antiviral activity included weight loss, reduction in mortality and/or increase in mean day to death (MDD) determined through 16–21 days post virus inoculation (p.i.).

2.5. Statistical analysis and synergy determination

The data were analyzed by Sigma Plot (Windows Version 10.0, SPSS, Chicago, IL) and Sigma Stat (Windows Version 3.5, Jandel Corporation, San Rafael, CA). The *t*-test was used to evaluate differences in mean day to death. One way analysis of variance (ANOVA) was performed to evaluate differences in weight loss. Kaplan-Meier Survival analysis (log rank test) was applied to survival number differences. A latin square design of the experiment was made with a series of daily doses of each drug in combination.

The experimental data were evaluated by the three dimensional analysis using Mac Synergy II™ software program (Prichard and Shipman, 1990). For the three dimensional analysis, the weight loss in each animal in each group was converted to decrease in percent of weight loss compared to the control group using the following formula:

$$\left(1 - \frac{\%\ \text{wt loss in treated group}}{\%\ \text{wt loss in control group}}\right) \times 100$$

Using the percent weight loss, the software calculates the theoretical additive interactions from the dose–response curves of the individual drugs. The calculated additive surface, which represents the predicted additive interactions, is then subtracted from the experimental surface to reveal regions of greater (synergy) or less (antagonism)-than-expected interactions.

3. Results

3.1. Influenza A mouse model

In the mouse influenza model, viral infection leads to loss of body weight and mortality and this decrease in body weight correlates with pulmonary viral titer and pulmonary lesion scores (Johansson et al., 1993). Therefore, the efficacy of IM administered peramivir and orally administered rimantadine as single agents and in combination, were evaluated on the basis of the weight loss, mean day to death and survival rate measured for 16–21 days post infection for treated, infected animals relative to untreated, infected (control) animals. The dose of the virus used for infection in these studies was sub-lethal and showed maximum weight loss in the vehicle-treated infected mice by day 9–10.

In the first experiment, a single agent dose ranging study was performed to provide guidance for the combination study. The dose for IM peramivir, qd for 5 days, ranged from 1 to 30 mg/kg/d, whereas the doses of oral rimantadine ranged from 10 to 300 mg/kg/d administered bid for 5 days. There were 10 mice per group and 2 mice were used in the control, vehicle-treated uninfected group. No deaths were observed in any of the groups.

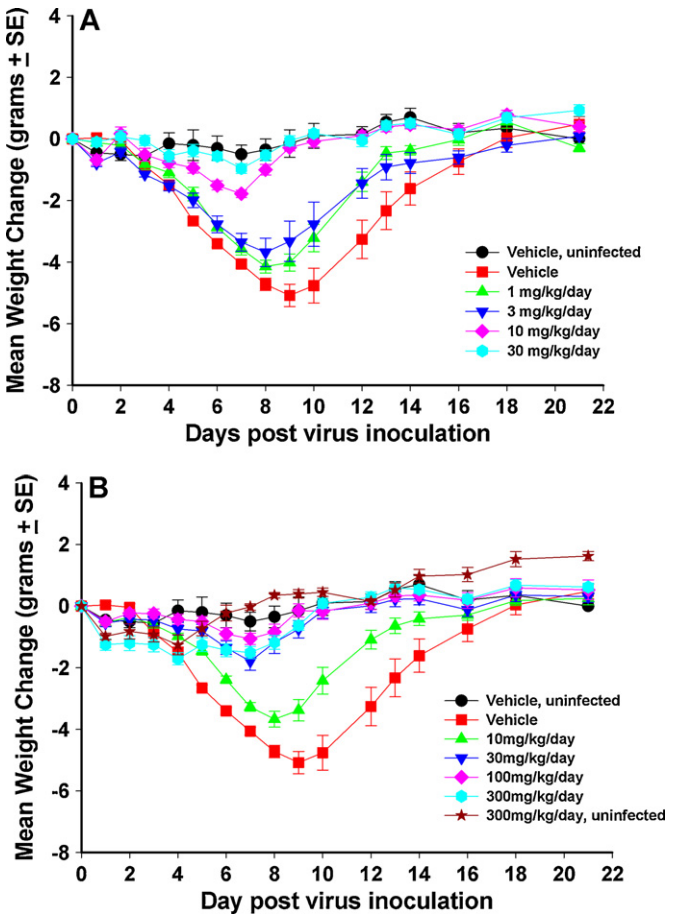

Fig. 1. Effect of peramivir and rimantadine on weight loss of influenza A-infected mice. Mice were infected with mouse-adapted Influenza A/Victoria/3/75 (H3N2) virus. The number of mice in each infected group was 10; number of mice in uninfected groups ranged from 2 to 5 mice per group. (A) Peramivir administered by IM injection, qd × 5 days and (B) rimantadine administered orally bid × 5 days. Dosing began 1 h prior to viral inoculation.

Peramivir and rimantadine showed a dose response relationship for the outcome parameter of weight loss; the time course of observed weight change is displayed in Fig. 1. The maximum mean weight loss of 5.08 g was observed in the vehicle-treated infected group on day 9. In the 1, 3, 10, and 30 mg/kg/d peramivir-treated groups, the weight losses observed on day 9 were 4.01, 3.32, 0.28 and 0.06 g, respectively. In the rimantadine-treated mice, the observed weight losses on day 9 were 3.38, 0.75, 0.14 and 0.63 g for the 10, 30, 100 and 300 mg/kg/d groups, respectively. In general, a lower dose resulted in greater weight loss when compared with a higher dose for both drugs used. The optimal effective doses (no significant weight loss) for peramivir and rimantadine as single agents in this Influenza A/Victoria/3/75 (H3N2) virus mouse model were 10 mg/kg/d and 30 mg/kg/d, respectively.

Based on the results from the single agent study, a combination study with a 4X4 latin square design was planned. Appropriate doses were used such that it was possible to assess drug interaction. The doses of peramivir and rimantadine ranged from 0.3 to 3 mg/kg/d and 5 to 30 mg/kg/d, respectively. There were 9–10 animals in each treatment group and 15 mice in the infected, vehicle-treated control group. Table 1 presents the mean weight change on day 10 (maximum weight loss observed in the vehicle-treated group) for all the groups. The average baseline weights for the mice in all the groups were very similar (<0.5 g difference between various groups). Significant differences in weight loss were noted between the combination treated groups versus the single agent and vehicle treatment groups (Table 1, shaded area). The maximum mean weight loss of 5.19 g was observed in the vehicle-treated infected group on day 10. In the 1 and 3 mg/kg/d peramivir monotherapy treatment groups, the weight loss observed on day 10 were 4.3 and 3.55 g, respectively. In the rimantadine monotherapy treatment group, the weight losses on day 10 were 3.43, 2.1, and 1.64 g for the 5, 10, and 30 mg/kg/d groups, respectively. The combination of 1 mg/kg/d peramivir with 5, 10 and 30 mg/kg/d rimantadine produced weight losses of 1.69, 0.69 and 0.41 g, respectively, and the combination of 3.0 mg/kg/d peramivir with 10 and 30 mg/kg/d rimantadine did not show any weight loss. Consistent with the previous experiment, both rimantadine and peramivir groups as single agents, generally demonstrated dose-related decreases in weight loss.

The weight loss in each animal in each group was converted to decrease in percent of weight loss compared to the control group. The data for decrease in percentage of weight loss for each group compared to control was analyzed using the MacSynergy II^{TM} software program. The final result of the analysis is shown in Fig. 2. Positive values, that is, peaks above the horizontal plane indicate synergy and negative values, i.e., depression in the plane, indicates antagonism. The combinations of 1 mg/kg/d peramivir with 5, 10 and 30 mg/kg/d rimantadine and 3 mg/kg/d peramivir with 5, 10 and 30 mg/kg/d rimantadine (Fig. 2) demonstrated synergy. The volume of synergy was equal to 136 μM^2 unit % which is considered to be strongly synergistic (Ilyushina et al., 2008). Mean day to death and survival data are presented in Table 2. Although a sub lethal dose of virus was used, 40% of the mice died in the vehicle group in this study. In addition to the 60% survival observed in the vehicle group, the majority of the treatment groups had

Table 1
Effect of combinations of peramivir and rimantadine on weight loss in influenza A/Victoria/3/75 (H3N2) virus-infected mice.

Mean weight change at day 10* (g ± SEM)				
Rimantadine	Peramivir (P) (mg/kg/d)			
(R) (mg/kg/d)	0.0	0.3	1.0	3.0
0.0	−5.19 ± 0.16	−2.6 ± 0.75[a]	−4.3 ± 0.42[a]	−3.55 ± 0.35[a]
5.0	−3.43 ± 0.55[a]	−1.97 ± 0.47[a,b]	−1.69 ± 0.63[a,c]	−1.31 ± 0.34[a,c]
10.0	−2.1 ± 0.37[a]	−1.25 ± 0.55[a,d]	−0.69 ± 0.25[a,c]	0.05 ± 0.22[a,c]
30.0	−1.64 ± 0.54[a]	−1.52 ± 0.42[a]	−0.41 ± 0.22[a,e]	0.25 ± 0.14[a,c]

There were 9–10 animals in each treatment group and 15 mice in the infected, vehicle-treated group. Peramivir was administered by IM injection, qd; Rimantadine was administered orally, bid. Dosing began 1 h prior to inoculation with virus for 5 days. Shaded area represents synergistic anti-viral effects.

[a] $p < 0.05$ vs. vehicle (0P/0R).
[b] $p < 0.05$ vs. 0P/5R.
[c] $p < 0.05$ vs. either compound used alone.
[d] $p < 0.05$ vs. 0.3P/0R.
[e] $p < 0.05$ vs. 1P/0R.
* Maximum weight loss in the vehicle group with no death.

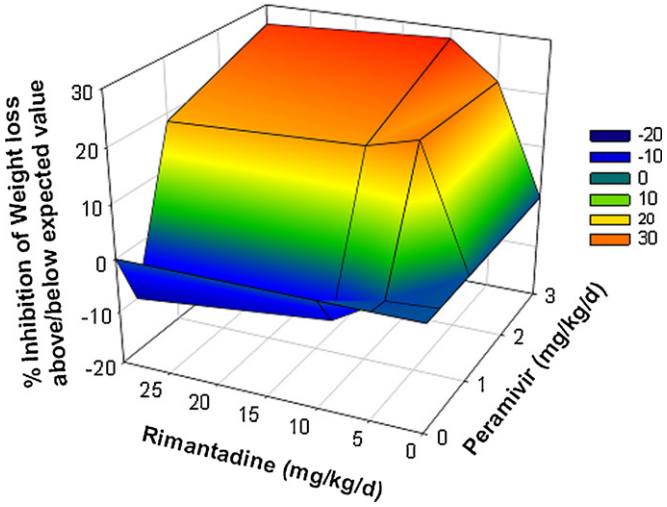

Fig. 2. Three dimensional synergy plot of interactions of peramivir and rimantadine in the influenza A/Victoria/3/75 (H3N2) mouse model. The graph was derived from the weight loss data shown in Table 1. The percent decrease in weight loss compared to the control group was determined as shown in Section 2. The volume of synergy (or antagonism) was calculated using MacSynergy II™ software program (Prichard and Shipman, 1990). The volume of synergy was found to be 136 μM^2 unit % (95% confidence interval = 260–12) and the antagonism was calculated as −16.85 (95% confidence interval = not significant). 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 (Ilyushina et al., 2008).

100% survival, except for the groups that received monotherapy with 5 mg/kg/d rimantadine (0 mg/kg/d peramivir) and 0.3 and 1.0 mg/kg/d peramivir (0 mg/kg/d rimantadine) which had 80% and 90% survival, respectively.

4. Discussion

Our results demonstrate that when applied in combination, rimantadine and peramivir, representatives of two classes of anti-influenza agents (M2 ion channel and neuraminidase, respectively), show a synergistic antiviral effect in experimental infections with influenza A (H3N2) virus in mice. The daily doses of peramivir and rimantadine used in combination in our studies were below the optimal effective doses for influenza A virus infection in mice. Individually administered doses at 3 and 10 mg/kg/d for peramivir and rimantadine, respectively, did show decreases in weight loss (3.5 g and 2.1 g, respectively) compared to the vehicle-treated group (5.2 g), but the combination of peramivir and rimantadine at 3 and 10 mg/kg/d, respectively, completely prevented weight loss in these mice.

To characterize the mode of interactions between the two anti-influenza drugs, we performed the three dimensional effect analysis of weight changes in different treatment groups following the sub-lethal infection of mice with influenza A (H3N2) virus. In the three dimensional effect analysis, 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. Positive values, that is, peaks above the horizontal plane indicate synergy and negative values, i.e., depression in the plane, indicates antagonism. Mouse infection studies were performed with the dose of the virus that consistently gave maximum weight loss (about 5 g) without any death in the vehicle-treated infected mice by day 9–10.

Combination of oseltamivir (neuraminidase inhibitor) and rimantadine has shown synergistic effects in the experimental infection with influenza virus in albino mice (Galabov et al., 2006). The parameters used in this study to characterize the interaction between the two drugs were viral titers and survival. Generally, mortality and viral titer are used to evaluate the drug interactions in the combination treatment. Sometimes because of high variability in viral titer and mortality, it is difficult to show drug combination effects *in vivo* (Smee et al., 2009, 2010). Hence, in our study, we used a sub-lethal dose of virus for infection and used the weight loss parameter to characterize the interaction between the two drugs, peramivir and rimantadine. Combination of peramivir and rimantadine in the mouse model demonstrated a highly synergistic antiviral effect with a volume of synergy equal to 136 μM^2 unit % (Fig. 2; >100 μM^2 unit % considered to be highly synergistic) (Ilyushina et al., 2008). The synergism was observed in the majority of the drug combinations of peramivir and rimantadine tested. This study provides a model to characterize the mode of interaction of the two drugs using weight loss parameters in mice with a sub-lethal virus infection dose. Although the virus used in the current study is an H3N2 virus (sensitive to rimantadine) and the currently circulating H3N2 viruses are resistant to rimantadine, these studies support the use of the combination of an NA inhibitor, peramivir, and M2 ion channel blocker, rimantadine, for

Table 2
Effect of combinations of peramivir and rimantadine on survival and mean day to death in influenza A/Victoria/3/75 (H3N2)-infected mice.

Compound peramivir[a] (mg/kg/d)	Compound rimantadine[b] (mg/kg/d)	Mean day to death ±SEM	Survival/total (% survival)
0 uninfected	0	–	5/5 (100%)
0	0	11.5 ± 0.34	9/15 (60%)
	5	7.5 ± 3.5	8/10 (80%)
	10	–	10/10 (100%)*
	30	–	10/10 (100%)*
0.3	0	11 ± 0.0	9/10 (90%)
	5	–	10/10 (100%)*
	10	–	10/10 (100%)*
	30	–	10/10 (100%)*
1	0	12 ± 0.0	9/10 (90%)
	5	–	10/10 (100%)*
	10	–	10/10 (100%)*
	30	–	10/10 (100%)*
3	0	–	10/10 (100%)*
	5	–	10/10 (100%)*
	10	–	10/10 (100%)*
	30	–	9/9 (100%)*

[a] Peramivir was administered by IM injection, qd for 5 days.
[b] Rimantadine was administered orally, bid for 5 days. Treatments began 1 h prior to virus inoculation.
* $p < 0.03$ vs. vehicle infected (peramivir (0 mg/kg) + rimantadine (0 mg/kg)).

the treatment of adamantine- and NA inhibitor-sensitive influenza virus infections.

The generation of influenza resistant strains to M2 ion channel blockers and neuraminidase inhibitors has been noted in the last few years (Correia et al., 2010; Ujike et al., 2010). Emergence of resistant variants with M2 ion channel blockers is more common than with neuraminidase inhibitors. We have not evaluated the emergence of resistant variants in the current study with combination treatment. However, *in vitro* studies suggest that combination chemotherapy with M2 blocker and NA inhibitor reduced the emergence of drug-resistant influenza variants (Ilyushina et al., 2006). Furthermore, in the clinic analysis of 41 patients treated with either rimantadine or combination of rimantadine and zanamivir, showed that the combination of zanamivir and rimantadine group did not lead to the emergence of resistant variants, while rimantadine monotherapy group showed emergence of two rimantadine-resistant variants (Ison et al., 2003).

In summary, peramivir in combination with rimantadine which are representative of the two classes of influenza A virus replication inhibitors of NA and viral structural protein M2, respectively, demonstrate a synergistic anti-viral effect in experimental infections with influenza virus in mice. The weight loss parameter could be used, in a mouse model with a sub-lethal dose of virus for infection, to characterize the mode of interaction between the two drugs. These data support exploring the combination of peramivir and rimantadine for the treatment of adamantine- and peramivir-sensitive influenza in the clinic.

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