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Combinations of favipiravir and peramivir for the treatment of pandemic influenza A/California/04/2009 (H1N1) virus infections in mice

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ABSTRACT

Favipiravir, an influenza virus RNA polymerase inhibitor, and peramivir, an influenza virus neuraminidase inhibitor, were evaluated alone and in combination against pandemic influenza A/California/04/2009 (H1N1) virus infections in mice. Infected mice were treated twice daily for 5 d starting 4 h after virus challenge. Favipiravir was 40%, 70%, and 100% protective at 20, 40, and 100 mg/kg/d. Peramivir was 30% protective at 0.5 mg/kg/d, but ineffective at lower doses when used as monotherapy. Combinations of favipiravir and peramivir increased the numbers of survivors by 10–50% when the 0.025, 0.05, and 0.1 mg/kg/d doses of peramivir were combined with 20 mg/kg/d favipiravir and when all doses of peramivir were combined with 40 mg/kg/d favipiravir. Three-dimensional analysis of drug interactions using the MacSynergy method indicates strong synergy for these drug combinations. In addition, an increase in lifespan for groups of mice treated with drug combinations, compared to the most effective monotherapy group, was observed for the 0.025, 0.05, and 0.1 mg/kg/d doses of peramivir combined with favipiravir at the 20 mg dose level. Therefore, the 20 mg/kg/d dose of favipiravir was selected for further combination studies. Increased survival was exhibited when this dose was combined with peramivir doses of 0.1, 0.25 and 0.5 mg/kg/d (1 mg/kg/d of peramivir alone was 100% protective in this experiment). Improved body weight relative to either compound alone was evident using 0.25, 0.5, and 1 mg/kg/d of peramivir. Significant reductions in lung hemorrhage score and lung weight were evident on day 6 post-infection. In addition, virus titers were reduced significantly on day 4 post-infection by combination therapy containing favipiravir combined with peramivir at 0.25 and 0.5 mg/kg/d. These data demonstrate that combinations of favipiravir and peramivir perform better than suboptimal doses of each compound alone for the treatment of influenza virus infections in mice.

1. Introduction

In 2009 a new strain of influenza A (H1N1) emerged in Mexico that soon was transmitted throughout the world (Centers for Disease Control and Prevention, 2009b).

Although the case mortality rate proved to be less than originally feared, the pandemic caused significant morbidity and economic burden (Shrestha et al., 2011). The recent H1N1 pandemic highlights the need for effective antiviral therapy for largely immune-naïve populations. This crisis is over, the population is largely immune to the virus, and the 2009 pandemic virus has now become the new seasonal H1N1 virus undergoing antigenic drift. The world will await the emergence of the next pandemic virus. But the burden of seasonal influenza caused by influenza A (H1N1 and H3N2) and influenza B, will ever be with us (Xue et al., 2010). Additionally, the possibility of avian H5N1, H7N7, and H9N2 viruses adapting more efficiently for infection and spread in humans should not be overlooked (Yen and Webster, 2009).

The pandemic virus that emerged is resistant to the antiviral drugs amantadine and rimantadine (Mossad, 2009), as are the majority of H3N2 viruses (Deyde et al., 2007; Hata et al., 2007; Centers for Disease Control and Prevention, 2009a; Zaraket et al., 2010; Puzelli et al., 2011) and most highly pathogenic H5N1 avian viruses (Cheung et al., 2006). By the end of the 2009–2010 season, almost all (98.9%) of tested 2009 pandemic H1N1 viruses were susceptible to oseltamivir (Fiore et al., 2011). It is fortunate that the 2009 pandemic virus was sensitive to the drug oseltamivir (Hall et al., 2009; Rungrotmongkol et al., 2009), because many seasonal H1N1 viruses in circulation just prior to that time were resistant (Besselaar et al., 2008; Dharan et al., 2009; Meijer et al., 2009).

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One of the most effective means of curtailing the emergence of resistant viruses as well as improving treatment efficacy is to use compounds in combination (Ilyushina et al., 2006). Compounds with different modes of action, given in combination, have proven to be beneficial at improving the outcome of infection. Over the years a number of combination chemotherapy studies have been performed against influenza viruses in mouse models (Govorkova and Webster, 2010). Studies from our laboratory have supported these conclusions (Smee et al., 2002, 2006, 2009, 2010a,b). Combination chemotherapy studies in mice are generally performed using suboptimal doses of the inhibitors so that improvements can be measured. However, in a clinical setting the drugs will be used at their approved doses.

Favipiravir (also referred to as T-705) was first reported in 2002 to be effective against influenza virus infections in cell culture and in mice (Furuta et al., 2002; Sidwell et al., 2007; Kiso et al., 2010). The compound inhibits influenza virus RNA polymerase (Furuta et al., 2005). Recently, favipiravir was shown to inhibit various isolates of the pandemic 2009 H1N1 virus, including some oseltamivir-resistant H275Y viruses, with 50% inhibitory concentrations generally ranging from 0.5 to 6 μM (Sleeman et al., 2010). In that study, there were some viral outliers, such as an A/Illinois/10/2009 isolate that was inhibited at 22.5 μM in a plaque reduction assay. However, the A/Illinois/10/2009 virus had a similar inhibition profile to other isolates when assayed by virus yield reduction. Favipiravir is currently in Phase 3 clinical trials in Japan, while Phase 2 trials in the US are underway. Among the viral neuraminidase inhibitors, peramivir is effective against various influenza virus strains in cell culture at 0.01–1 μM (influenza A viruses) and 0.1–2.3 μM (influenza B viruses) (Smee et al., 2001). It is also effective against influenza virus infections in mice (Sidwell et al., 2001; Bantia et al., 2001, 2006), including the 2009 pandemic virus (Bantia et al., 2011). The poor oral bioavailability of peramivir in humans (Barroso et al., 2005) has hindered its clinical development, and recent studies have investigated its use by intramuscular administration (Bantia et al., 2006, 2011; Boltz et al., 2008; Yun et al., 2008). In Japan, peramivir has been licensed under the trade name Rapiacta®, while in the US, peramivir is currently in Phase 3 clinical trials.

One combination chemotherapy study in mice has been published using favipiravir and oseltamivir to treat infections caused by seasonal H1N1 and H3N2 viruses, and a low-pathogenic avian influenza H5N1 virus (Smee et al., 2010a). In that report, synergistic improvements in survival were achieved using low doses of each inhibitor in combination. In addition, peramivir and ribavirin were combined for treatment of a seasonal H1N1 virus infection (Smee et al., 2002), as were combinations of oseltamivir and peramivir (Smee et al., 2010b). Improvements in survival and in body weight were evident at particular doses. The combination of peramivir and rimantadine was also shown to provide an improved treatment outcome against an amantadine-sensitive influenza A H3N2 virus infection in mice (Bantia et al., 2010).

The present studies investigated the use of favipiravir and peramivir in combination for treatment of pandemic H1N1 virus infections in mice. Survival, mean day of death, mean body weight, and lung parameters (lung score, lung weight, and virus titers) were evaluated following a lethal virus infection.

2. Materials and methods

2.1. Compounds

Favipiravir and peramivir were provided by Toyama Chemical Co. (Tokyo, Japan) and BioCryst Pharmaceuticals (Birmingham, AL), respectively. Favipiravir was suspended in 0.4% carboxymethylcellulose, and peramivir was dissolved in sterile water. Favipiravir was administered in a 0.1 ml volume by oral gavage, whereas peramivir was injected (0.05 ml) intramuscularly. Routes of administration of each compound were selected based upon known bioavailabilities and preferred routes of administration (Furuta et al., 2002; Barroso et al., 2005; Bantia et al., 2006; Kiso et al., 2010). The placebo was administered both i.m. (sterile water) and p.o. (0.4% carboxymethylcellulose) twice daily for 5 days starting 4 h post-virus exposure.

2.2. Virus

Mouse adapted influenza A/California/04/2009 (H1N1) was received from Elena Govorkova (St. Jude Children's Research Hospital, Memphis, TN). The virus was adapted to replication in the lungs of BALB/c mice by nine sequential passages through mouse lungs (Ilyushina et al., 2010). The received virus had been plaque purified in MDCK cells followed by replication in embryonated chicken eggs and then in MDCK cells. The virus pool was pre-titrated in mice prior to performing these studies to determine an appropriate challenge dose.

2.3. Animal experiment design

Female BALB/c mice (18–20 g, Charles River Laboratories, Wilmington, MA) were anesthetized by intraperitoneal injection of ketamine/xylazine (50/5 mg/kg) followed by challenge infection intranasally with a 90-μl suspension of influenza virus. The challenge inoculation of approximately $10^{5.3}$ cell culture infectious doses ($CCID_{50}$)/mouse equated to three mouse lethal challenge doses (MLD_{50}). The compounds were administered twice daily for 5 d starting 4 h after infection. Ten drug-treated infected mice and 20 placebo-treated controls were observed daily for death through 21 days. Body weights were determined every other day. An additional 10 mice per group were maintained for determining lung infection parameters on days 3 and 6 post-infection. On those days five mice from the placebo and treatment groups were sacrificed, the lungs were weighed and scored for lung hemorrhage on a scale of 0 (normal) to 4 (maximum plum coloration over the entire lung) (Sidwell et al., 2007), and then frozen at −80 °C. At a later date the lungs were homogenized and titrated for the presence of virus by endpoint dilution method (Reed and Muench, 1938) in 96-well microplates (Smee et al., 2009). Virus titers are reported as $\log_{10}$ $CCID_{50}$/g of tissue.

2.4. Statistical methods and drug combination analysis

Kaplan–Meier survival curves were generated and compared by the Mantel–Cox log-rank test followed by pairwise comparison using the Gehan–Breslow–Wilcoxon test. Mean day of death comparisons between drug-treated and placebo groups were made by the two-tailed Mann–Whitney *U*-test. Mean body weights were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Mean lung scores, lung weights, and lung virus titers (on log-transformed values assuming equal variance and normal distribution) were evaluated by one-way ANOVA. Following ANOVA, individual treatment values were compared to placebo controls by Tukey's pair-wise comparison test. Comparative analyses of lung parameters (score and weight) by day post-infection were completed by two-way ANOVA followed by Bonferroni post-tests. All statistical analyses were completed using Prism 5.0d (GraphPad Software, Inc., La Jolla, CA).

Drug–drug interactions were analyzed by the three-dimensional model of Prichard and Shipman (1990), using the MacSynergy II software program at 95% confidence limits. Descriptions of antagonistic, additive, or synergistic interactions using this computer model have been described for data represented as

Table 1
Treatment of an influenza A/California/04/2009 (H1N1) virus infection in mice with the combination of favipiravir plus peramivir. Treatments were administered twice daily for 5 d starting 4 h after virus exposure.

Compound 1 (mg/kg/d)	Compound 2 (mg/kg/d)	Survivors/total	MDD[a] ± SD	Increase in lifespan[b] (d)
Peramivir (0.5)	–	3/10	9.4 ± 1.7	
Peramivir (0.1)	–	0/10	8.8 ± 1.6	
Peramivir (0.05)	–	0/10	7.2 ± 1.4	
Peramivir (0.025)	–	0/10	8.0 ± 1.2	
Peramivir (0.0125)	–	0/10	7.1 ± 0.9	
Favipiravir (100)	–	10/10***	–	
Favipiravir (40)	–	7/10**	11.3 ± 2.5	
Favipiravir (20)	–	4/10	8.0 ± 1.1	
Favipiravir (10)	–	1/10	8.8 ± 1.6	
Favipiravir (5)	–	1/10	7.4 ± 1.2	
Peramivir (0.1)	Favipiravir (40)	9/10***	8.0	0
Peramivir (0.05)	Favipiravir (40)	10/10***	–	0
Peramivir (0.025)	Favipiravir (40)	10/10***	–	0
Peramivir (0.0125)	Favipiravir (40)	9/10***	8.0	0
Peramivir (0.1)	Favipiravir (20)	6/10*	9.3 ± 2.1	0.5
Peramivir (0.05)	Favipiravir (20)	9/10***	9.0	1.0
Peramivir (0.025)	Favipiravir (20)	5/10	8.4 ± 1.1	0.4
Peramivir (0.0125)	Favipiravir (20)	4/10	8.0 ± 2.1	0
Peramivir (0.1)	Favipiravir (10)	4/10	8.3 ± 1.9	0
Peramivir (0.05)	Favipiravir (10)	1/10	7.3 ± 1.2	0
Peramivir (0.025)	Favipiravir (10)	3/10	7.1 ± 1.1	0
Peramivir (0.0125)	Favipiravir (10)	1/10	7.9 ± 1.3	0
Peramivir (0.1)	Favipiravir (5)	0/10	7.5 ± 1.6	0
Peramivir (0.05)	Favipiravir (5)	1/10	6.9 ± 1.3	0
Peramivir (0.025)	Favipiravir (5)	0/10	8.2 ± 1.8	0
Peramivir (0.0125)	Favipiravir (5)	0/10	6.5 ± 1.1	0
Placebo	–	2/20	6.1 ± 1.9	–

[a] Mean day of death for mice that died prior to day 21 post-infection.
[b] Increase in lifespan of mice that died (combination treatment groups compared to monotherapy group that lived the longest time). Example: the MDD for monotherapy peramivir (0.1) is 8.8 and for monotherapy favipiravir (20) is 8.0, but the MDD for the combination of peramivir (0.1) plus favipiravir (20) is 9.3. Therefore, the increase in lifespan is 9.3 − 8.8 = 0.5 d.
* $P < 0.05$ compared to placebo.
** $P < 0.01$ compared to placebo.
*** $P < 0.001$ compared to placebo.

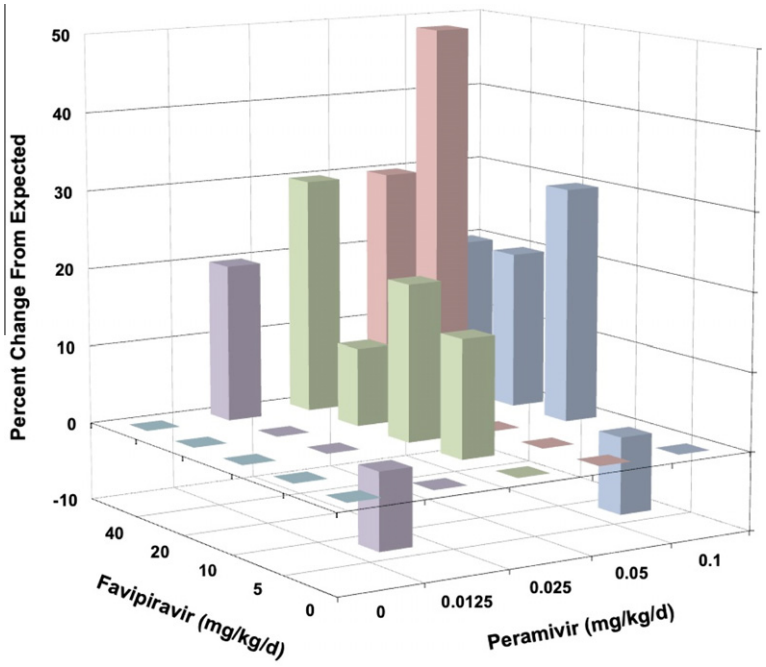

Fig. 1. Three-dimensional (MacSynergy™ II) plot for the interaction of favipiravir and peramivir based upon the mouse survival data presented in Table 1. The net volume of synergy for this plot was 225 (indicative of strong synergy).

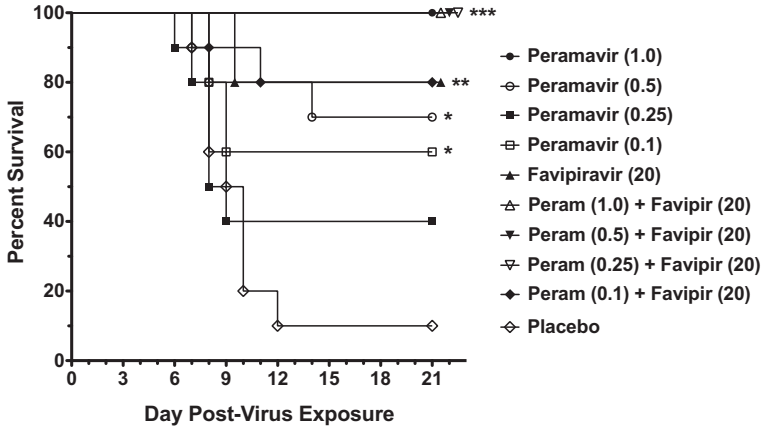

Fig. 2. Effects of a favipiravir (favipir) at 20 mg/kg/d combined with peramivir (peram) at 0.1, 0.25, 0.5, or 1.0 mg/kg/d for treatment of an influenza A/California/04/2009 (H1N1) virus infection in mice. Oral treatments were administered twice daily for 5 d starting 4 h after virus exposure ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$ compared to placebo).

percentages (Ilyushina et al., 2008). Briefly, 0–25, 25–50, 50–100, and >100 μm^2 unit % calculated values in either a positive or negative direction using MacSynergy software are defined as insignificant synergy or antagonism (indifference), minor synergy or antagonism, moderate synergy or antagonism, or strong synergy or antagonism, respectively. The reported values represent the calculated volume of synergy minus the volume of antagonism for each set of data. Three-dimensional analyses were made using MacSynergy™ II software (Prichard and Shipman, 1990).

3. Results

3.1. Antiviral combination study to evaluate survival and antiviral drug synergy

Survival results following combination treatment using peramivir and favipiravir for a lethal infection with influenza H1N1 virus are reported in Table 1. The concentrations of peramivir used in this study appear to be suboptimal because even the highest dose,

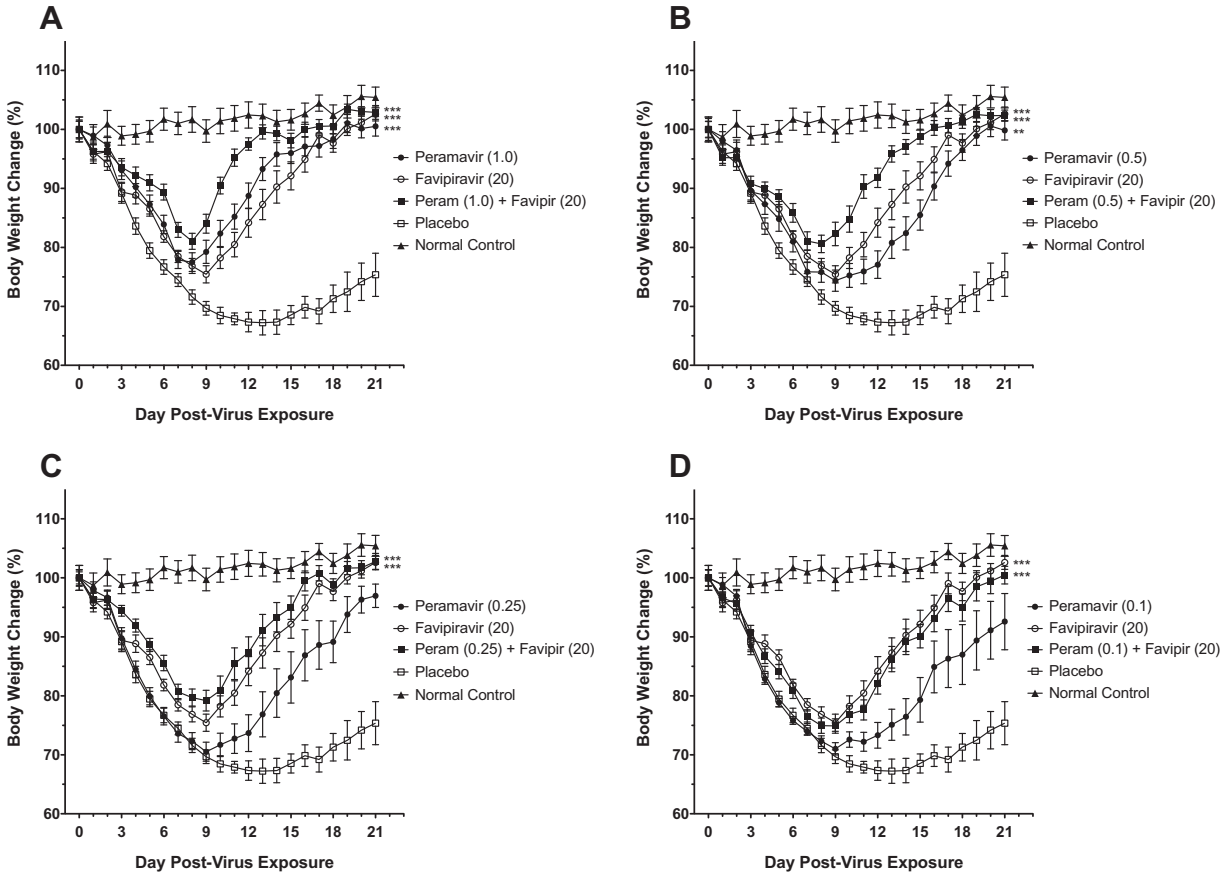

Fig. 3. Body weight changes as percent of initial body weight during treatment of an influenza A/California/04/2009 (H1N1) virus infection in mice with combinations of favipiravir (favipir) and peramivir (param). Oral treatments were administered twice daily for 5 d starting 4 h after virus exposure. The data accompany those of Fig. 2 ($^{**}P < 0.01$, $^{***}P < 0.0001$ compared to placebo).

0.5 mg/kg/d, produced only 30% protection following monotherapy. Monotherapy with favipiravir provided 40%, 70%, and 100% protection against lethal infection following administration of 20, 40, and 100 mg/kg/d, respectively. Nine groups treated with favipiravir (10, 20, and 40 mg/kg/d) combined with peramivir (0.0125, 0.025, 0.05, and 0.1 mg/kg/d) gave additive results in which the number of survivors following combination treatment was greater than monotherapy alone. A three-dimensional (MacSynergy™ II) plot of mouse survival following combination drug treatment is presented in Fig. 1. The net synergy volume for the results was 225, which has been defined as strong synergy (Ilyushina et al., 2008). Mean day of death determinations are also shown in Table 1. The mean day of death increased for all monotherapy and combination therapy groups when compared to placebo controls. However, the greatest increase was observed for combinations containing favipiravirat 20 mg/kg/d and peramivir at 0.025, 0.05, and 0.1 mg/kg/d. In addition, an increase in lifespan for groups treated with drug combinations compared to the most effective monotherapy group is shown in Table 1. The greatest effects following combination therapy were observed for favipiravir at the 20 mg dose level.

3.2. Antiviral combination study to evaluate the use of higher drug doses on survival and lung hemorrhage, weight, and virus titers

Additional studies included the use of higher doses of peramivir for combination therapy with favipiravir. A second experiment was completed using low dose (20 mg/kg/d) favipiravir combined with peramivir at 0.1, 0.25, 0.5, and 1.0 mg/kg/d for evaluation of influenza virus infection parameters. Fig. 2 shows the Kaplan–Meier survival curves for each treatment group. All mice survived infection in the treatment group receiving 1.0 mg/kg peramivir and in the groups receiving combination treatment with favipiravir plus peramivir at 0.25, 0.5, and 1.0 mg/kg/d. Some mortality was observed in all other treatment groups. However, monotherapy with favipiravir (20 mg/kg/d), or peramivir at 0.1 and 0.5 mg/kg/d produced statistically significant survival results compared to placebo. The combinations of favipiravir plus peramivir at 0.25 or 0.5 mg/kg/d increased survival to 100%, whereas survival from monotherapy with peramivir at 0.25 or 0.5 mg/kg/d, or favipiravir at 20 mg/kg/d was 40%, 70%, and 80% in Kaplan–Meier survival curves, respectively.

Body weight changes as percent of initial body weight for groups receiving combination therapy or monotherapy with favipiravir or peramivir at various doses beginning at 4 h after virus exposure are shown in Fig. 3. Body weights dropped during the infection in all treatment groups, reaching a nadir on day 7–9. However, the extent of weight loss at nadir was not as great and/or the rebound of weight was more rapid in the drug combination groups receiving combination treatment with favipiravir plus peramivir at 0.25, 0.5, and 1.0 mg/kg/d. The combination containing the 0.1 mg/kg/d dose of peramivir showed equivalent protection to the favipiravir monotherapy group.

The effects of treatment on lung hemorrhage score, observed as tissue discoloration and considered an indicator of gross pathology, is shown in Fig. 4. Tissue discoloration became apparent on day 4, although none of the treatment groups showed a significant difference from placebo controls (Fig. 4A). Significant differences in lung scores were observed on day 6 for the monotherapy groups treated with favipiravir, or peramivir (0.5 and 1.0 mg/kg/d), and in the treatment groups receiving combination therapy at peramivir doses of 0.25, 0.5, and 1.0 mg/kg/d when combined with favipiravir (Fig. 4B).

Lung weights, which increase over time as a result of infection, are presented in Fig. 5. None of the treatment groups were significantly different from placebo controls on day 4 (Fig. 5A). However, significant differences in lung weight were observed on day 6 for the monotherapy groups treated with peramivir (0.25 and 1.0 mg/kg), and the combination therapy groups containing 0.25, 0.5, and 1.0 mg/kg/d peramivir plus favipiravir (Fig. 5B).

Fig. 6 shows the results of virus titer determinations from lung tissues. Significant differences in virus titers were observed for the treatment groups receiving 0.25 or 0.5 mg/kg/d peramivir in combination with favipiravir on day 4 (Fig. 6A). It is not known why the combination containing the highest dose of peramivir was not efficacious in reducing the virus titer. On day 6 virus titers declined in all groups, so significant differences from controls were not observed on that day.

4. Discussion

These studies are the first, to our knowledge, to evaluate the effects of combination antiviral drug therapy using favipiravir and peramivir to treat an infection with pandemic H1N1 influenza virus. The animal studies presented here demonstrated that the combination of favipiravir and peramivir at suboptimal doses for each could provide significant improvements in survival and body weight. The estimated improvement in survival for the combination was about 10–50% compared to the use of favipiravir alone, based upon the 20 and 40 mg/kg/d doses used as monotherapy (Table 1 and Fig. 2). The effect of the combination treatments on

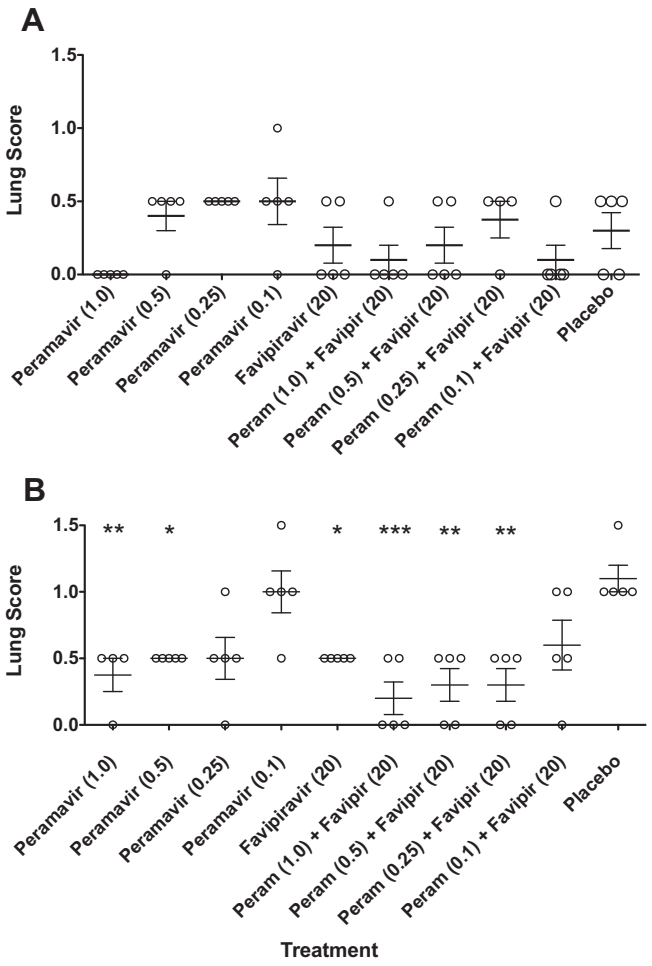

Fig. 4. Mean lung hemorrhage scores on day 4 (A) and day 6 (B) of an influenza A/California/04/2009 (H1N1) virus infection in mice treated with combinations of favipiravir (favipir) and peramivir (peram). Oral treatments were administered twice daily for 5 d starting 4 h after virus exposure. The data accompany those of Fig. 2 ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$ compared to placebo).

body weight indicated that the mice did not get as ill as mice treated with only one compound, and they recovered sooner from the infection.

The pandemic H1N1 virus appears to replicate more readily in the lungs and lower airways than seasonal H1N1 and H3N2 viruses, but lacks many of the mutations associated with the higher pathogenicity observed in other influenza viruses (Garten et al., 2009; Itoh et al., 2009; Bautista et al., 2009). Combination treatment did not have as obvious of a beneficial effect compared to monotherapy on lung parameters as it did on mortality and weight loss. On day 4, statistically significant differences in lung virus titers were observed in the treatment groups receiving 0.25 and 0.5 mg/kg/d peramivir combined with favipiravir, when compared to placebo. Although, lung virus titer differences by day 6 were less pronounced. Lung hemorrhage and lung weight were more severe on day 6 compared to day 4 with minimal differences observed between treated and placebo groups on day 4. However, a slight trend in the data indicates an earlier reduction in virus titer and improvement in lung score and weight at lower doses of peramivir when combined with favipiravir. In a previous report we demonstrated that treatment with amantadine plus oseltamivir, amantadine plus ribavirin, and oseltamivir plus ribavirin reduced lung viral titers and were more protective from death than placebo-treated mice (Smee et al., 2009). However, the viral titer data in that report was not conclusive in showing that drug combination treatment was more inhibitory than monotherapy. We have yet to understand what virological or immunological factors are altered (or when they are altered) during treatment that allow for greater survival and a shortened course of morbidity.

An important advantage to drug combination treatment that is difficult to assess in vivo is the suppression of emergence of drug-resistant viruses. It is evident from cell culture studies that drug-resistant influenza viruses are less likely to emerge with combination chemotherapy (Ilyushina et al., 2006). Recovery of amantadine-resistant viruses during treatment of immunocompetent mice has been reported (Oxford et al., 1970), as has the recovery of oseltamivir-resistant viruses from immunodeficient mice (Ison et al., 2006). We have been unsuccessful in producing favipiravir-resistant influenza viruses by extensive cell culture passage (data not published), which may reflect rigid specificity of the amino acid sequence of the viral RNA polymerase. Future studies will

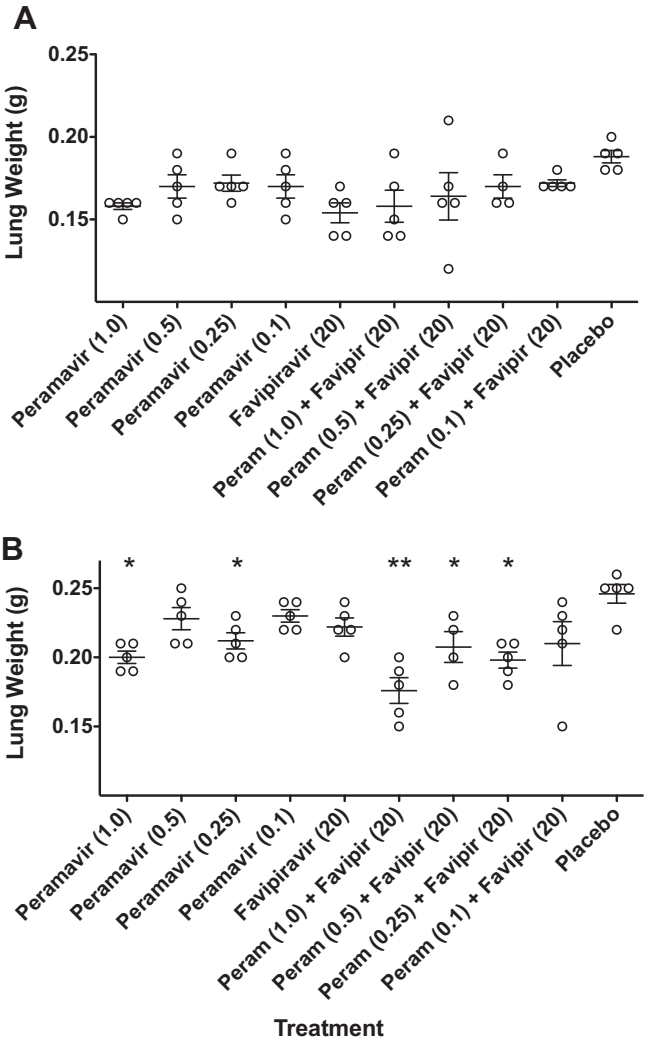

Fig. 5. Mean lung weights on day 4 (A) and day 6 (B) of an influenza A/California/04/2009 (H1N1) virus infection in mice treated with combinations of favipiravir (favipir) and peramivir (peram). Oral treatments were administered twice daily for 5 d starting 4 h after virus exposure. The data accompany those of Fig. 2 (*$P < 0.05$, **$P < 0.01$ compared to placebo).

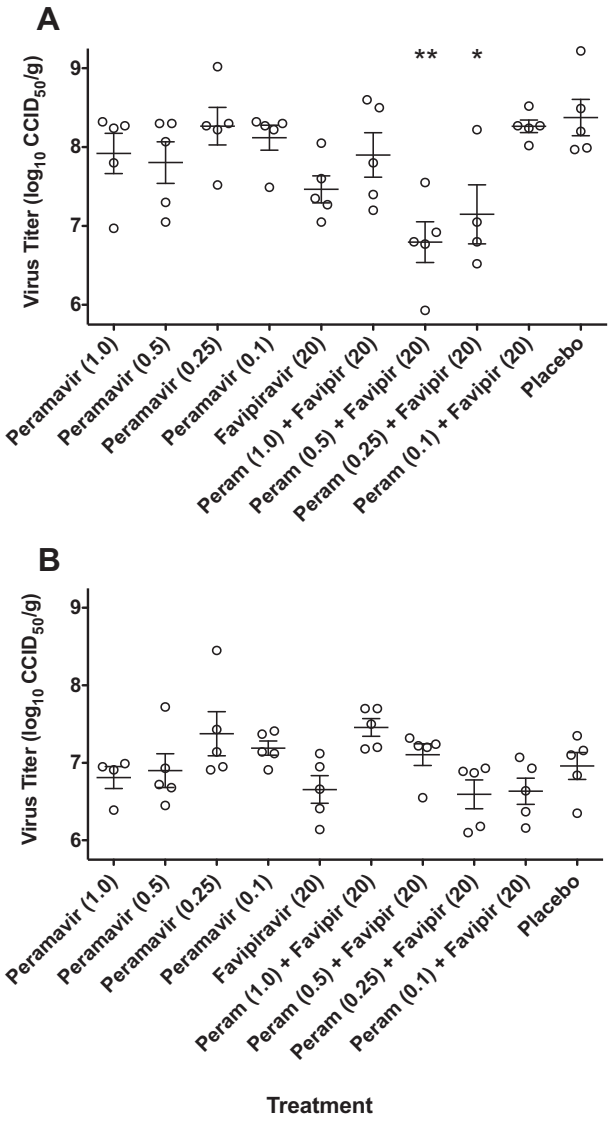

Fig. 6. Mean lung virus titers on day 4 (A) and day 6 (B) of an influenza A/California/04/2009 (H1N1) virus infection in mice treated with combinations of favipiravir (favipir) and peramivir (peram). Oral treatments were administered twice daily for 5 d starting 4 h after virus exposure. The data accompany those of Fig. 2 (*$P < 0.05$, **$P < 0.01$ compared to placebo).

evaluate combination treatment with favipiravir plus a neuraminidase inhibitor against an oseltamivir-resistant influenza virus infection.

The doses of favipiravir and peramivir used in these studies to treat pandemic influenza A H1N1 virus infections in mice provided a strong synergistic effect over monotherapy. Therefore, such a treatment, or combination treatment of favipiravir with other similarly acting neuraminidase inhibitors, may make a difference between survival and death in hospitalized patients that are critically ill. Thus, such treatments should be considered in these circumstances.

In addition, the recent pandemic highlights the need for better antiviral therapies for influenza virus infections. These therapies could include new formulations of conventional antiviral drugs, earlier intervention in hospitalized patients, and/or development of novel treatment strategies. One novel therapeutic approach includes treatment with anti-inflammatory compounds. This approach, based on the observation that uncontrolled viral replication and hyperactivated cytokine and chemokine responses contribute to disease manifestation, lends itself to a treatment strategy involving the use of inhibitors of inflammation (McAuley et al., 2010). Recently, combination therapy using anti-inflammatory compounds was evaluated in mice. In this study, it was shown that "two classes of drugs, peroxisome proliferator-activated receptor-gamma agonists and AMP-activated protein kinase agonists, provided protection against infection with highly pathogenic and pandemic strains of influenza virus" (Moseley et al., 2010). Therefore, future strategies for combination therapy could include dual or triple combinations containing influenza virus-specific drugs, or combinations of antivirals and novel agents, like anti-inflammatory compounds.

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References

Bantia, S., Parker, C.D., Ananth, S.L., Horn, L.L., Andries, K., Chand, P., Kotian, P.L., Dehghani, A., El-Kattan, Y., Lin, T., Hutchison, T.L., Montgomery, J.A., Kellog, D.L., Babu, Y.S., 2001. Comparison of the anti-influenza virus activity of RWJ-270201 with those of oseltamivir and zanamivir. Antimicrob. Agents Chemother. 45, 1162–1167.

Bantia, S., Arnold, C.S., Parker, C.D., Upshaw, R., Chand, P., 2006. Anti-influenza virus activity of peramivir in mice with single intramuscular injection. Antiviral Res. 69, 39–45.

Bantia, S., Kellogg, D., Parker, C.D., Babu, Y.S., 2010. Combination of peramivir and rimantadine demonstrate synergistic antiviral effects in sub-lethal influenza A (H3N2) virus mouse model. Antiviral Res. 88, 276–280.

Bantia, S., Kellogg, D., Parker, C., Upshaw, R., Ilyushina, N.A., Babu, Y.S., 2011. A single intramuscular injection of neuraminidase inhibitor peramivir demonstrates antiviral activity against novel pandemic A/California/04/2009 (H1N1) influenza virus infection in mice. Antiviral Res. 90, 17–21.

Barroso, L., Treanor, J., Gubareva, L., Hayden, F.G., 2005. Efficacy and tolerability of the oral neuraminidase inhibitor peramivir in experimental human influenza: randomized, controlled trials for prophylaxis and treatment. Antiviral Ther. 10, 901–910.

Bautista, E., Chotpitayasunondh, T., Gao, Z., Harper, S.A., Shaw, M., Uyeki, T.M., Zaki, S.R., Hayden, F.G., Hui, D.S., Kettner, J.D., Kumar, A., Lim, M., Shindo, N., Penn, C., Nicholson, K.G., 2009. Clinical aspects of pandemic 2009 influenza A (H1N1) virus infection. N. Engl. J. Med. 362, 1708–1719.

Besselaar, T.G., Naidoo, D., Buys, A., Gregory, V., McAnerney, J., Manamela, J.M., Blumberg, L., Schoub, B.D., 2008. Widespread oseltamivir resistance in influenza A viruses (H1N1), South Africa. Emerg. Infect. Dis. 14, 1809–1810.

Boltz, D.A., Ilyushina, N.A., Arnold, C.S., Babu, Y.S., Webster, R.G., Govorkova, E.A., 2008. Intramuscularly administered neuraminidase inhibitor peramivir is effective against lethal H5N1 influenza virus in mice. Antiviral Res. 80, 150–157.

Centers for Disease Control and Prevention (CDC), 2009a. Update: drug susceptibility of swine origin influenza A (H1N1) viruses, April 2009. MMWR Morb. Mortal. Wkly Rep. 58, 433–435.

Centers for Disease Control and Prevention (CDC), 2009b. Outbreak of swine-origin influenza A (H1N1) virus infection – Mexico, March–April 2009. MMWR Morb. Mortal. Wkly Rep. 58, 467–470.

Cheung, C.L., Rayner, J.M., Smith, G.J., Wang, P., Naipospos, T.S., Zhang, J., Yuen, K.Y., Webster, R.G., Peiris, J.S., Guan, Y., Chen, H., 2006. Distribution of amantadine-resistant H5N1 avian influenza variants in Asia. J. Infect. Dis. 193, 1626–1629.

Dharan, N.J., Gubareva, L.V., Meyer, J.J., Okomo-Adhiambo, M., McClinton, R.C., Marshall, S.A., St. George, K., Epperson, S., Brammer, L., Klimov, A.I., Bresee, J.S., Fry, A.M.Oseltamivir-Resistance Working Group, 2009. Infections with oseltamivir-resistant influenza A (H1N1) virus in the United States. JAMA 301, 1034–1041.

Deyde, V.M., Xu, X., Bright, R.A., Shaw, M., Smith, C.B., Zhang, Y., Shu, Y., Gubareva, L.V., Cox, N.J., Klimov, A.I., 2007. Surveillance of resistance to adamantanes among influenza A(H3N2) and A(H1N1) viruses isolated worldwide. J. Infect. Dis. 196, 249–257.

Fiore, A.E., Fry, A., Shay, D., Gubareva, L., Bresee, J.S., Uyeki, T.M., 2011. Antiviral agents for the treatment and chemoprophylaxis of influenza – recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm. Rep. 60, 1–24.

Furuta, Y., Takahashi, K., Fukuda, Y., Kuno, M., Kamiyama, T., Kozaki, K., Nomura, N., Egawa, H., Minami, S., Watanabe, Y., Narita, H., Shiraki, K., 2002. In vitro and in vivo activities of anti-influenza virus compound T-705. Antimicrob. Agents Chemother. 46, 977–981.

Furuta, Y., Takahashi, K., Kuno-Maekawa, M., Sangawa, H., Uehara, S., Kozaki, K., Nomura, N., Egawa, H., Shiraki, K., 2005. Mechanism of action of T-705 against influenza virus. Antimicrob. Agents Chemother. 49, 981–986.

Garten, R.J., Davis, C.T., Russell, C.A., Shu, B., Lindstrom, S., Balish, A., Sessions, W.M., Xu, X., Skepner, E., Deyde, V., Okomo-Adhiambo, M., Gubareva, L., Barnes, J., Smith, C.B., Emery, S.L., Hillman, M.J., Rivailler, P., Smagala, J., de Graaf, M., Burke, D.F., Fouchier, R.A., Pappas, C., Alpuche-Aranda, C.M., López-Gatell, H., Olivera, H., López, I., Myers, C.A., Faix, D., Blair, P.J., Yu, C., Keene, K.M., Dotson Jr, P.D., Boxrud, D., Sambol, A.R., Abid, S.H., St George, K., Bannerman, T., Moore, A.L., Stringer, D.J., Blevins, P., Demmler-Harrison, G.J., Ginsberg, M., Kriner, P., Waterman, S., Smole, S., Guevara, H.F., Belongia, E.A., Clark, P.A., Beatrice, S.T., Donis, R., Katz, J., Finelli, L., Bridges, C.B., Shaw, M., Jernigan, D.B., Uyeki, T.M., Smith, D.J., Klimov, A.I., Cox, N.J., 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science 325, 197–201.

Govorkova, E.A., Webster, R.G., 2010. Combination chemotherapy for influenza. Viruses 2, 1510–1529.

Hall, R.J., Peacey, M.P., Ralston, J.C., Bocacao, J., Ziki, M., Gunn, W., Quirk, A., Huang, Q.S., 2009. Pandemic influenza A(H1N1) viruses currently circulating in New Zealand are sensitive to oseltamivir. Euro. Surveill. 14, 19282.

Hata, M., Tsuzuki, M., Goto, Y., Kumagai, N., Harada, M., Hashimoto, M., Tanaka, S., Sakae, K., Kimura, T., Minagawa, H., Miyazaki, Y., 2007. High frequency of amantadine-resistant influenza A (H3N2) viruses in the 2005–2006 season and rapid detection of amantadine-resistant influenza A (H3N2) viruses by MAMA-PCR. Jpn. J. Infect. Dis. 60, 202–204.

Ilyushina, N.A., Bovin, N.V., Webster, R.G., Govorkova, E.A., 2006. Combination chemotherapy, a potential strategy for reducing the emergence of drug-resistant influenza A variants. Antiviral Res. 70, 121–131.

Ilyushina, N.A., Hay, A., Yilmaz, N., Boon, A.C., Webster, R.G., Govorkova, E.A., 2008. Oseltamivir–ribavirin combination therapy for highly pathogenic H5N1 influenza virus infection in mice. Antimicrob. Agents Chemother. 52, 3889–3897.

Ilyushina, N.A., Khalenkov, A.M., Seiler, J.P., Forrest, H.L., Bovin, N.V., Marjuki, H., Barman, S., Webster, R.G., Webby, R.J., 2010. Adaptation of pandemic H1N1 influenza viruses in mice. J. Virol. 84, 8607–8616.

Ison, M.G., Mishin, V.P., Braciale, T.J., Hayden, F.G., Gubareva, L.V., 2006. Comparative activities of oseltamivir and A-322278 in immunocompetent and immunocompromised murine models of influenza virus infection. J. Infect. Dis. 193, 765–772.

Itoh, Y., Shinya, K., Kiso, M., Watanabe, T., Sakoda, Y., Hatta, M., Muramoto, Y., Tamura, D., Sakai-Tagawa, Y., Noda, T., Sakabe, S., Imai, M., Hatta, Y., Watanabe, S., Li, C., Yamada, S., Fujii, K., Murakami, S., Imai, H., Kakugawa, S., Ito, M., Takano, R., Iwatsuki-Horimoto, K., Shimojima, M., Horimoto, T., Goto, H., Takahashi, K., Makino, A., Ishigaki, H., Nakayama, M., Okamatsu, M., Takahashi, K., Warshauer, D., Shult, P.A., Saito, R., Suzuki, H., Furuta, Y., Yamashita, M., Mitamura, K., Nakano, K., Nakamura, M., Brockman-Schneider, R., Mitamura, H., Yamazaki, M., Sugaya, N., Suresh, M., Ozawa, M., Neumann, G., Gern, J., Kida, H., Ogasawara, K., Kawaoka, Y., 2009. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. Nature 460, 1021–1025.

Kiso, M., Takahashi, K., Sakai-Tagawa, Y., Shinya, K., Sakabe, S., Le, Q.M., Ozawa, M., Furuta, Y., Kawaoka, Y., 2010. T-705 (favipiravir) activity against lethal H5N1 influenza A viruses. Proc. Natl. Acad. Sci. USA 107, 882–887.

McAuley, J.L., Chipuk, J.E., Boyd, K.L., Van De Velde, N., Green, D.R., McCullers, J.A., 2010. PB1-F2 proteins from H5N1 and 20 century pandemic influenza viruses cause immunopathology. PLoS Pathog. 6, e1001014.

Meijer, A., Lackenby, A., Hungnes, O., Lina, B., van der Werf, S., Schweiger, B., Opp, M., Paget, J., van de Kassteele, J., Hay, A., Zambon, M.European Influenza Surveillance Scheme, 2009. Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007–08 season. Emerg. Infect. Dis. 15, 552–560.

Moseley, C.E., Webster, R.G., Aldridge, J.R., 2010. Peroxisome proliferator-activated receptor and AMP-activated protein kinase agonists protect against lethal influenza virus challenge in mice. Influenza Other Respir. Viruses 4, 307–311.

Mossad, S.B., 2009. The resurgence of swine-origin influenza A (H1N1). Cleve. Clin. J. Med. 76, 337–343.

Oxford, J.S., Logan, I.S., Potter, C.W., 1970. In vivo selection of an influenza A2 strain resistant to amantadine. Nature 226, 82–83.

Prichard, M.N., Shipman Jr., C., 1990. A three-dimensional model to analyze drug–drug interactions. Antiviral Res. 14, 181–205.

Puzelli, S., Facchini, M., Di Martino, A., Fabiani, C., Lackenby, A., Zambon, M., Donatelli, I., 2011. Evaluation of the antiviral drug susceptibility of influenza viruses in Italy from 2004/05 to 2009/10 epidemics and from the recent 2009 pandemic. Antiviral Res. 90, 205–212.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27, 493–498.

Rungrotmongkol, T., Intharathep, P., Malaisree, M., Nunthaboot, N., Kaiyawet, N., Sompornpisut, P., Payungporn, S., Poovorawan, Y., Hannongbua, S., 2009. Susceptibility of antiviral drugs against 2009 influenza A (H1N1) virus. Biochem. Biophys. Res. Commun. 385, 390–394.

Shrestha, S.S., Swerdlow, D.L., Borse, R.H., Prabhu, V.S., Finelli, L., Atkins, C.Y., Owusu-Edusei, K., Bell, B., Mead, P.S., Biggerstaff, M., Brammer, L., Davidson, H., Jernigan, D., Jhung, M.A., Kamimoto, L.A., Merlin, T.L., Nowell, M., Redd, S.C., Reed, C., Schuchat, A., Meltzer, M.I., 2011. Estimating the burden of 2009 pandemic influenza A (H1N1) in the United States (April 2009–April 2010). Clin Infect Dis. 52 (Suppl. 1), S75–S82.

Sidwell, R.W., Smee, D.F., Huffman, J.H., Barnard, D.L., Bailey, K.W., Morrey, J.D., Babu, Y.S., 2001. In vivo influenza virus-inhibitory effects of the cyclopentane neuraminidase inhibitor RJW-270201. Antimicrob. Agents Chemother. 45, 749–757.

Sidwell, R.W., Barnard, D.L., Day, C.W., Smee, D.F., Bailey, K.W., Wong, M.H., Morrey, J.D., Furuta, Y., 2007. Efficacy of orally administered T-705 on lethal avian influenza A (H5N1) virus infections in mice. Antimicrob. Agents Chemother. 51, 845–851.

Sleeman, K., Mishin, V.P., Deyde, V.M., Furuta, Y., Klimov, A.I., Gubareva, L.V., 2010. In vitro antiviral activity of favipiravir (T-705) against drug-resistant influenza and 2009 A(H1N1) viruses. Antimicrob. Agents Chemother. 54, 2517–2524.

Smee, D.F., Huffman, J.H., Morrison, A.C., Barnard, D.L., Sidwell, R.W., 2001. Cyclopentane neuraminidase inhibitors with potent in vitro anti-influenza virus activities. Antimicrob. Agents Chemother. 45, 743–748.

Smee, D.F., Bailey, K.W., Morrison, A.C., Sidwell, R.W., 2002. Combination treatment of influenza A virus infections in cell culture and in mice with the cyclopentane neuraminidase inhibitor RWJ-270201 and ribavirin. Chemotherapy 48, 88–93.

Smee, D.F., Wong, M.H., Bailey, K.W., Sidwell, R.W., 2006. Activities of oseltamivir and ribavirin used alone and in combination against infections in mice with recent isolates of influenza A (H1N1) and B viruses. Antiviral Chem. Chemother. 17, 185–192.

Smee, D.F., Hurst, B.L., Wong, M.H., Bailey, K.W., Morrey, J.D., 2009. Effects of double combinations of amantadine, oseltamivir, and ribavirin on influenza A (H5N1) virus infections in cell culture and in mice. Antimicrob. Agents Chemother. 53, 2120–2128.

Smee, D.F., Hurst, B.L., Wong, M.H., Bailey, K.W., Tarbet, E.B., Morrey, J.D., Furuta, Y., 2010a. Effects of the combination of favipiravir (T-705) and oseltamivir on influenza A virus infections in mice. Antimicrob. Agents Chemother. 54, 126–133.

Smee, D.F., Hurst, B.L., Wong, M.-H., Tarbet, E.B., Babu, Y.S., Klumpp, K., Morrey, J.D., 2010b. Combinations of oseltamivir and peramivir for the treatment of influenza A (H1N1) virus infections in cell culture and in mice. Antiviral Res. 88, 38–44.

Xue, Y., Kristiansen, I.S., de Blasio, B.F., 2010. Modeling the cost of influenza: the impact of missing costs of unreported complications and sick leave. BMC Public Health 10, 724.

Yen, H.L., Webster, R.G., 2009. Pandemic influenza as a current threat. Curr. Top. Microbiol. Immunol. 333, 3–24.

Yun, N.E., Linde, N.S., Zacks, M.A., Barr, I.G., Hurt, A.C., Smith, J.N., Dziuba, N., Holbrook, M.R., Zhang, L., Kilpatrick, J.M., Arnold, C.S., Paessler, S., 2008. Injectable peramivir mitigates disease and promotes survival in ferrets and mice infected with the highly virulent influenza virus, A/Vietnam/1203/04 (H5N1). Virology 374, 198–209.

Zaraket, H., Saito, R., Wakim, R., Tabet, C., Medlej, F., Reda, M., Baranovich, T., Suzuki, Y., Dapat, C., Caperig-Dapat, I., Dbaibo, G.S., Suzuki, H., 2010. Antiviral drug susceptibilities of seasonal human influenza viruses in Lebanon, 2008–09 season. J. Med. Virol. 82 (7), 1224–1228.