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Supplementary webappendix

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Appendices. Summaries of Pre-clinical and Clinical Combination Studies of Influenza Antivirals, Immunomodulators, and Chinese Traditional Medicines

Appendix 1. Pre-clinical and Clinical Studies of Combinations of Influenza Antivirals			
Antiviral combination (reference)	**Model**	**Main findings**	**Comment**
Pre-Clinical Studies			
Rimantadine or amantadine + ribavirin[1] (Hayden F.G. et al., Antimicrob. Agents Chemother. 1980)	**In vitro.** MDCK cells were infected with A/PR/8/34/H1N1, A/USSR/90/77/H1N1, A/New Jersey/76/HSWlNl, A/Texas/1/77/H3N2 or B/Hong Kong/72 virus and incubated for 24h with inhibition-titrated solutions of rimantadine, amantadine and ribavirin, alone or in paired combinations. In certain experiments, repeat drug incubation was performed with repeat harvests at 48h and 72h after virus inoculation. Effect on virus replication was measured through estimation of cytopathic effect and titration of infectivity by plaque assay. Enhancement of antiviral activity was defined as >90% decrease in virus yield at 24h compared to effect of either drug alone.	Enhanced antiviral effect was seen with rimantadine (0·1 to 0·4 μg/ml), + ribavirin against all of the influenza A viruses. The effect varied according to virus strain, drug concentrations and virus inoculum. Amantadine + ribavirin also showed similar enhanced activity. Specific concentrations of rimantadine + ribavirin also reported to demonstrate enhanced effect against influenza B (data were not shown).	Authors state that for certain drug combinations the extent of inhibition of virus multiplication was greater than the additive effects of single agents, suggesting synergy (and not increased cytotoxicity). Ribavirin concentrations were 5-10 times higher than those achievable in blood following oral administration in humans.
Amantadine +	**Ex vivo ferret tracheal rings containing ciliated epithelial cells.** Following dose-	Each drug alone produced a modest delay in virus-induced cytopathic effect,	Rings were pre-treated (for 2h) with agents before being

ribavirin[2] (Burlington D.B. et al., J. Antimicrob. Chemother. 1983)	response experiments with single agents, infected rings were continuously exposed to amantadine + ribavirin at 0·25, 0·5 and 1·0 ml/l. After 2h drug incubation, each ring was inoculated with A/Alaska/6/77 (H3N2) virus at ten times the determined ID_{50}. Controls for drug toxicity were included with the highest concentration experiments. Antiviral effect was assessed by measuring preservation of ciliary activity (controls had 70% of baseline activity at day 28).	whereas the combination synergistically delayed cytopathic effect (75% of rings for the 28-day duration of the experiment at 1·0 mg/l). Peak virus production was suppressed >4 log_{10}-fold by the combination of 1 mg/1 of each drug, significantly greater suppression than seen with single agents.	infected with a relatively small inoculum. Authors state this is a model of prophylaxis of infection.
Rimantadine + oseltamivir, zanamivir, or peramivir[3] (Govorkova E.A. et al., Antimicrob. Agents Chemother. 2004)	**In vitro.** MDCK cells were infected with A/New Caledonia/20/99 (H1N1) virus or A/Panama/2007/99 (H3N2) virus, 30 minutes after incubation with ZNV plus rimantadine, OC plus rimantadine and PMV plus rimantadine, or each drug alone. Concentration of NAIs was 0·0001 to 0·3 µM and 2·5 to 80 µM for rimantadine. Antiviral effects were assessed by virus yield reduction assay and cell-based ELISA. Both extracellular virus and cell-associated virus were assayed.	Reduction of extracellular virus demonstrated additive and synergistic effects with no cytotoxicity for all three combinations. Maximum synergy against A/New Caledonia/20/99 (H1N1) virus infection was observed with <2·5 µM rimantadine paired with low concentrations of NAIs. All combinations reduced the extracellular yield of A/Panama/2007/99 (H3N2) influenza virus synergistically. In contrast, at some drug concentrations (both drugs at a low concentration, or both at a high concentration), the yield	Assays of the cell-associated virus yield may have underestimated the efficacies of the NA inhibitors when used either alone or in combination. The authors state that ZNV-rimantadine and OC-rimantadine interact similarly to inhibit recovery of extracellular H1N1 virus in MDCK cells, but that PMV interacts with rimantadine differently to inhibit extracellular virus.

		of cell-associated virus was inhibited antagonistically. ZNV-rimantadine and OC-rimantadine inhibited recovery of extracellular H1N1 virus in MDCK cells but PMV-rimantadine inhibited extracellular virus.	
Rimantadine + oseltamivir[4] (Leneva I.A. et al., Antiviral Res 2000)	**Murine model.** Mice were challenged with 100 or 5 LD_{50} of an adamantane-susceptible A/Quail/Hong Kong/G1/97(H9N2) virus and given oral oseltamivir 0·01, 0·1, or 1 mg/kg/day and rimantadine 1 or 10 mg/kg/day starting at 4 hours before virus inoculation and continuing for total of 5 days.	When administered singly, oseltamivir and rimantadine were not effective in preventing the death of mice infected with high doses of virus but did delay the time to death. The combination significantly increased the number of survivors and the survival time. Following lower viral inoculum, monotherapy with OP (0·01 mg/kg/day) and rimantadine (1 mg/kg/day) did not prevent weight loss and death of mice but the combination showed dose-related protection.	The two inhibitors were more effective when given in combination than when given as monotherapy across a range of doses. Data on effects on lung titers were not provided.
Amantadine + oseltamivir[5] (Ilyushina N.A. et al., Antiviral Res 2006)	**In vitro** resistance selection. MDCK cells were infected with A/Nanchang/1/99 (H1N1) virus, A/Panama/2007/99 (H3N2), or A/Hong Kong/156/97 (H5N1) virus. MOI was 0·001 PFU/cell for all five passages except the second (0·1 PFU/cell). There were four exposure arms for each strain experiment: amantadine; OC (0·001, 0·01,	Yields of all strains in were significantly reduced ($P < 0·005$) when the cells were treated with the combination of amantadine and low doses of OC (≤1 μM). Sequential passage in the presence of single agents was associated with development of V27A and S31N/l substitutions in M2 with	Replication of all strains was completely blocked by specific concentrations of OC administered in combination with amantadine, acting in an additive and synergistic manner. Sequencing showed that viruses were genetically stable after 5

	0·1, 1 µM, and also 0·0001 µM for H5N1); amantadine (10, 31·25, 31·25, 62·5, 125 µm for the 1st-5th passages, respectively); combination therapy at the concentrations outlined above; no agents (control). Drug sensitivity was determined by plaque reduction assay. Cytotoxicity, sialic acid receptor-binding and NA enzyme inhibition assays were performed. Viral RNA was also isolated, amplified and sequenced.	amantadine and multiple HA mutations with OC (>0·001 uM). Viruses bearing these mutations had reduced efficiency of SA receptor binding and decreased sensitivity to NAI in the plaque reduction assay. Mutations in HA, NA or M2 were not seen with combination therapy.	passages.
Amantadine + oseltamivir[6] Ilyushina N.A. et al., Antivir. Ther. 2007)	**Mouse model.** BALB/c mice were intanasally innoculated with 10 MLD_{50} of one of two recombinant, reverse-engineered A(H5N1)-derived viruses: one amantadine sensitive (S31 in M2) and the other resistant (N31 in M2). 24h prior to inoculation, mice commenced amantadine (1·5, 15 or 30 mg/kg/day) or OP (1 or 10 mg/kg/day) via oral gavage for 5 days, or amantadine (15 or 30 mg/kg/day) with OP (10 mg/kg/day) for 5 days. Survival and weight change were observed. Virus titres in harvested organs were measured by egg inoculation. HA, NA and M genes of viruses from day 9 lungs and brains were	Combination therapy with either dose of amantadine and 10 mg/kg/day of OP provided dose-dependent protection against lethal infection with amantadine-sensitive virus than seen with single agents (60% with 15mg/kg/day and 90% with 30mg/kg/day) compared to controls. With resistant virus, combination therapy produced similar results to OP alone. Mutations in HA, NA and M2 were not seen in with combination therapy, but additionally, mutations did not occur with monotherapy.	The lack of detectable amino acid substitutions in viruses from mice treated with single agents may reflect the relatively short course of treatment, or more limited sequencing.

	sequenced.		
Rimantadine + oseltamivir[7] (Simeonova L. et al., Antiviral Res 2012)	**Mouse model.** Male white mice (ICR line) infected with influenza A/Aichi/2/68 (H3N2) mouse-adapted virus (intranasal inoculationof 10 MLD_{50}). Groups of mice were given OP (0·2 or 0·4 mg/kg/day), rimantadine (5 or 10 mg/kg/day), both agents in combination at variable doses, or placebo. Twice-daily antiviral treatment was commenced either 4h before or 24h following virus inoculation and continued for 5 days. Mice were observed for 14 days and mortality observed, along with lung measurement of virus titre and lung pathology scores in sacrificed animals. Protection index (PI) was calculated using the equation PI = [(PC–1)/PC] ×100, where PC is the coefficient index =% mortality in placebo group /% mortality in the drug-treated group.	In the prophylactic course 5 and 10 mg/kg/day rimantadine with OP 0.2 and 0.4 mg/kg/day (25:1 dose ratio) oseltamivir showed a protection index (PI) of 79·6% and 75%, respectively and a mean survival time (MST) of 13·1 and 12·9 days. By contrast, monotherapy using the same doses was associated with PI values ranging from 0% to 33·3% PI and MST of 8·2 to 10·3 days MST, respectively. Reductions in lung pathology were seen with combination therapy. When given as treatment, higher dosage combination therapy (0·8, 1·6, 3·2 mg/kg OP and 20, 40, 80 mg/kg rimantadine) resulted in PI ranged from 57·6% to 80·5% and the MST was 12·8–13·4 days. Used alone at the same doses the individual compounds' protection varied between 10·7% and 71·8% PI, MST 9·8–12·8 days (8·7 days in PBS control). Compared to vehicle and individual treatment, a decrease in infectious viral titers of up to 1000-fold and other viral pneumonia	Both prophylactic and therapeutic courses of combined oseltamivir and rimantadine had a significant protective effect in this mouse model and optimal dosing strategies were identified.

		parameters were also observed with combination treatment.	
TCAD (amantadine + ribavirin + oseltamivir)[8] (Nguyen J.T. et al., Antimicrob. Agents Chemother. 2009)	**In vitro.** MDCK cells were infected with A/California/04/09(H1N1), A/California/05/09(H1N1) or A/California/10/09(H1N1) virus. An amantadine-resistant V27A mutant of Influenza A/New Caledonia/20/99 (H1N1) virus and an A30T amantadine-resistant variant of A/Duck/1525/81(H5N1) virus were generated by passaging with amantadine. Two OC-resistant mutants were also included: A/Mississippi/3/01(H1N1) [H274Y] and A/Hawaii/21/07(H1N1) virus. Concentrations of amantadine, OC, and ribavirin ranged in 0·5 $\log_{10}$ dilutions from 0·001 to 100 μg/mL. Each drug was tested in triplicate at five or six concentrations in which the highest concentration for each drug was set to approximate the EC_{50} of the drug as a single agent. 50% effective concentration (EC_{50}) and 50% cytotoxic concentration (TC_{50}) was determined for each drug and synergy analysis was	TCAD regimen was synergistic against all three susceptible A(H1N1) viruses over multiple concentrations of all three drugs, at clinically achievable levels. Synergy was greater for TCAD than for double combinations of the component agents. Synergy plots for TCAD showed a concentration-dependent increase in synergy with respect to amantadine. For each component, the EC_{50} was reduced with TCAD compared to the EC_{50} as a single agent, indicative that each drug was active at lower concentrations. TCAD was also highly synergistic against drug-resistant viruses. Amantadine and OC were shown to contribute to the antiviral activity of the TCAD regimen against amantadine- and oseltamivir-resistant viruses, respectively, at concentrations where they had no demonstrable activity when given as single agents.	All three drugs appear to contribute to synergy. The mechanisms by which component agents contribute to synergy remain unclear, however.

	performed.		
TCAD (amantadine + ribavirin + oseltamivir)[9] Nguyen J. T. et al., PLoS One 2012)	**Mouse model.** BALB/c mice infected with A/Duck/MN/1525/81(H5N1) or mouse-adapted, amantadine-resistant (S31N in M2) A/California/04/09(H1N1) virus. Each mouse received approximately 1×104 $CCID_{50}$ of virus (4× LD_{50}) to achieve 100% lethality. Clinically- relevant dosage of each drug (amantadine 46 mg/kg/day, ribavirin 27 mg/kg/day, OP 25 mg/kg/day) was used alone and in combination for 5 days, commencing 24h after infection for most infections. A 3-fold lower and higher dose of amantadine (15 mg/kg/day and 138 mg/kg/day, respectively) was used alone and in combination for selected experiments. The effect of varying commencement of treatment (4h pre infection, 24, 48 or 72h post infection) was also assessed. Mice were observed for weight loss and death for 21 days. Primary endpoint was survival benefit. Secondary end-point was percentage weight change from baseline (maximum and at day 5).	Treatment with TCAD afforded >90% survival in mice infected with either viruses, whereas treatment with dual and single drug regimens resulted in 0% to 60% survival. When given as mono-therapy, no antiviral activity was seen against amantadine-resistant virus, but amantadine demonstrated dose-dependent protection when given as part of TCAD, even when treatment was commenced up to 72h post infection. With susceptible virus, greatest protection against weight loss was observed in mice treated with TCAD (P<0·001) compared to all double combinations. With resistant virus infection, greatest protection was seen with TCAD, which was significant compared to the amantadine/OP (P= 0·019) and OP/ribavirin (P<0·001) double combinations. With resistant virus infection, the dose response slope for amantadine given in TCAD was significantly greater than the slope when given as a single agent,	Virus titres and emergence of resistant variants were not reported.

		demonstrating synergy.	
TCAD (amantadine + ribavirin + oseltamivir)[10] (Hoopes J.D. et al., PLoS One 2011)	**In vitro** resistance selection**.** MDCK cells were infected with A/Hawaii/31/2007(H1N1)virus and passaged five times (3 days per passage) using three different MOIs (0·1, 0·01, and 0·001) in the presence of fixed concentrations of each drug regimen: amantadine; OC; amantadine + OC; TCAD. Viruses were screened for V27A, A30T and S31N amantadine-resistance substitutions. A similar experiment was conducted with serial passaging in the presence of escalating concentrations of: amantadine; OC; ZNV; ribavirin; amantadine + OC; amantadine + ZNV; TCAD. Passaging was continued until TC_{50} for each drug as a single agent was achieved or ≥25 days had passed. The contribution of each component of TCAD to the suppression of resistance was determined by passaging the virus five times in MDCK cells in the presence of double combinations, with increasing concentrations of a third drug titrated into the double combinations. Virus- induced cytopathic effect was	In the presence of TCAD, there was sustained suppression of drug resistant viruses. With amantadine alone or the amantadine-OC double combination, rapid selection of resistant variants was observed (~100% of the population). Treatment with all three double combinations resulted in 75-100% virus breakthrough (>50% cytopathic effect). Titration of each drug into the appropriate double combination (at levels equivalent to those that are achievable clinically) resulted in the concentration-dependent decrease in virus breakthrough.	Sequencing results demonstrated that multiple mutations were required to escape the effects of all the drugs in the regimen.

	measured by neutral red staining (virus breakthrough was defined as wells having greater than 50% CPE).		
TCAD (amantadine + ribavirin + oseltamivir)[11] (Nguyen J. T. et al., PLoS One 2010)	**In vitro**. MDCK cells infected with three A(H1N1)pdm09 strains as described above. To determine single agent concentration response curves, concentrations of amantadine and ribavirin ranged from 0·001 to 100 μg/mL and OC concentration ranged from 0·000032 to 100 μg/mL. EC_{50} determination, combination agent studies, and a modified synergy analysis were performed. Amantadine, ribavirin and OC were also tested as single agents and in combination against additional amantadine- and OC-resistant viruses: A/Caledonia/20/99(H1N1) [V27A]; A/Duck/1525/81(H5N1) [A30T]; A/Mississippi/3/01(H1N1) [H274Y]; A/Hawaii/21/07(H1N1)	TCAD demonstrated synergistic effect against all three A(H1N1) strains, for all three agents (≥0·1 μg/mL amantadine; ≥0·32 μg/mL ribavirin; ≥0·0032 μg/mL OC). Amantadine <3·2 μg/mL was reported to have no significant antiviral activity as a single agent but contributed to TCAD activity. Synergy for TCAD was greater than synergy for double NAI combinations. Addition of each third agent to a double combination was shown to contribute to synergy. Against OC- and amantadine-resistant viruses, OC and amantadine were shown to contribute to the synergistic effect of TCAD, respectively. No enhanced cytotoxicitty was observed. TCAD did not demonstrate synergistic antiviral effect against the A(H5N1) virus when administered at clinically achievable levels.	The authors state that, with the exception of TCAD for A(H5N1), the concentrations of component agents demonstrating synergy are clinically achievable.

Oseltamivir + zanamivir or peramivir[11] (Nguyen J. T. et al., PLoS One 2010)	**In vitro**. MDCK cell culture infected with A/California/04/09(H1N1) (*CA04*), A/California/05/09(H1N1)(*CA05*), or A/California/10/09(H1N1)(*CA10*) virus. To determine single agent concentration response curves, concentrations of OC, ZNV, and PMV in 0·5 $\log_{10}$ dilutions ranged from 0·000032 to 100 µg/mL. 50% effective concentration (EC_{50}) was determined for each drug. For double NAI studies, each drug was tested in triplicate at 5-6 concentrations in which the highest concentration for each drug was set to approximate the EC_{50} of the drug as a single agent. Synergy analysis was performed.	Synergy volume analysis for ZNV + OC against all three viruses indicated an additive effect. Synergy volumes for ZNV + PMV suggested additivity to moderate antagonism. Synergy plots for either combination against CA05 demonstrated antagonism at *higher* concentrations of ZNV (0·01–0·1 µg/mL) and at variable concentrations of OC or PMV.	In vitro testing of paired NAIs was performed alongside separate evaluation of TCAD in this study. Evaluation of the EC_{50} of first NAI in combination with a fixed concentration of the second NAI also revealed that the antiviral activity of each drug was not enhanced by combination, but data were not shown. Taken together, the results indicated an absence of a synergistic effect and potential for antagonism at high concentrations for ZNV + OC or for ZNV + PMV.
Oseltamivir + peramivir[12] (Smee D. F. et al., Antiviral Res. 2010)	**In vitro and mouse model.** Infected MDCK cells were exposed to OC/PMV at 0·32–100 µM for 3 days. BALB/c mice received intranasal lethal-dose infection, 104·5 $CCID_{50}$/mouse, of A/NWS/33(H1N1) virus. Antagonistic, additive and synergistic effects were assessed using a computer model. 0·05–0·4 mg/kg/day oral OP/IM PMV were given to mice at 12h intervals for	In vitro, additivity with a narrow region of synergy was found In a viral NA assay with combinations of inhibitors at 0·01–10 nM, no significant antagonistic or synergistic interactions were observed across the range of concentrations. In mice, twice daily OP (0·4 mg/kg/day) combined with twice daily PMV (0·1 and 0·2 mg/kg/day) increased survival significantly (80% and 100% protection,	Over all, selected (sub-optimal) dosage combinations produced additive responses.

	5 days, starting 2h before virus challenge	respectively), compared to suboptimal doses of either treatment alone (sum of survivors, 20%).	
Amantadine + oseltamivir or ribavirin[13] (Smee D.F. et al., Antimicrob. Agents Chemother. 2009)	**In vitro and mouse model.** Low pathogenic A/Duck/MN/1525/81(H5N1) virus, passaged three times to increase virulence and an amantadine-resistant (A30T in M2) clone were used to infect MDCK cells and mice. Virus yields from cell culture were determined after 72h. Additive, synergistic, and antagonistic interactions of amantadine, OC and ribavirin were analysed using a computer model. BALB/c mice were intranasally infected (10^4 $CCID_{50}$ amantadine-susceptible virus or 10^5 $CCID_{50}$ of A30T virus). Treatment groups were amantadine, OP, and ribavirin, given alone or sequentially in pairs, and also placebo. Treatments were given by gavage twice-daily for 5 days, starting 4h before infection. Mice were observed for weight loss and death through 21 days. Lung virus titres were determined in sub-groups 72h after infection.	In cell culture, amantadine + OC and amantadine + ribavirin, but not OC + ribavirin, showed synergistic effects over a range of doses against susceptible virus. OC-ribavirin had additive effects against amantadine-resistant virus. Amantadine-containing combinations did not overcome resistance. In mice, combination treatment with amantadine did not provide additional benefit over OP or ribavirin alone. However, OP + ribavirin (25 and 75 mg/kg/day combination) did significantly improve survival. All three combination therapies reduced severity of infection compared to single-agent treatment of susceptible infection.	In mice, amantadine-containing combinations were not of benefit in treating amantadine-resistant virus, but they appeared superior to single-agent therapy in treatment of amantadine-susceptible virus infection.
Ribavirin +	**In vitro and mouse model.** PMV or ribavirin	In cell culture, PMV + ribavirin	Synergistic effects of

peramivir[14] Smee D. F. et al., Chemotherapy 2002)	at 4 x concentration was added alone or combined to MDCK cells. Cells were then infected with 100 x 50% cell culture infectious doses per well of influenza A/NWS/33(H1N1) virus. Cytopathology was assessed at 3 days, when drug-free control wells demonstrated 100% cell destruction. Extracellular virus titres were measured in supernatant using end-point dilution in MDCK cells. BALB/c mice were intranasally infected with approximately 10^4 $CCID_{50}$ of the same virus. Groups of mice received oral ribavirin or PMV alone, or in combination, using different doses of each agent (ribavirin 0-20 mg/kg/day; peramivir 0-1·0 mg/kg/day). All treatments were given twice daily for 5 days, commencing 4h prior to inoculation with virus. Mice were observed through day 21 for death and blood oxygen saturation measurements were also performed. Toxicity controls received the highest doses of component agents, alone and in combination, without virus exposure. Synergistic effects were determined for cell culture and animal studies, using established computer models.	synergistically reduced extracellular virus yield at low concentrations (1·25 µM ribavirin + 0·03 µM and 0·1 µM PMV; 2·5-20 µM ribavirin + 0·03-1 µM PMV). Ribavirin 20 µM alone had a weak antiviral effect, but when combined with 0.03-1 µM PMV, virus became undetectable. In the mouse experiments, ribavirin alone was not protective at 6·25 or 20 mg/kg/day. PMV 1mg/kg/day (but not at other doses) significantly increased survival compared to survival in saline-treated controls (70% survival vs. 20% survival, respectively, $p<0·05$). When given together, all dose combinations resulted in greater survival that when either component agent was given alone. 100% survival was seen with 1 mg/kg/day PMV with both 6.25 and 20 mg/kg/day ribavirin. Although the increases in survival demonstrated statistical significance when compared with ribavirin monotherapy, this was not the case when compared with PMV monotherapy. All dose combinations	combination therapy were demonstrated, with a notable increase in survival compared to monotherapies, especially when using PMV 1·0 mg/kg/day in combination with ribavirin.

		delayed the mean day of death and improved arterial oxygen saturations at day 11. Although data were not shown, the authors state that treatments were non-toxic, as demonstrated by favourable weight change measurements, general observations and survival rates at day 21 in uninfected mice.	
Favipiravir (T-705) + oseltamivir[15] (Smee D. F. et al., Antimicrob Agents Chemother 2010)	**In vitro and mouse model.** Influenza A/NWS/33(H1N1) virus passaged nine times in MDCK cells, influenza A/Victoria/3/75(H3N2) virus passaged in mouse lungs to produce lethality and influenza A/Duck/MN/1525/81(H5N1) virus passaged three times in mice to enhance virulence. Determination of CPE and EC50 for T-705 and OC were determined in MDCK cells. NA inhibition assays were also performed. BALB/c mice were infected intranasally with virus titers of 104.5 to 105.0 $CCID_{50}$ per mouse to achieve 100% lethality. Groups of mice were treated via oral gavage with OP, T-705 or OP + T-705, given twice daily for 5 or 7 days. Treatment commenced 24 h after inoculation	T-705 mono-therapy inhibited viruses in cell culture at 1·4 to 4·3 µM. OC inhibited the three viruses in cells at 3·7, 0·02, and 0·16 µM and in neuraminidase assays at 0·94, 0·46, and 2·31 nM, respectively. In mice infected with H1N1, addition of 20 mg/kg/day T-705 to 0.1 and 0.3 mg/kg/day OP significantly improved survival over OP monotherapy. Effective treatment of H3N2 infection required a higher dose of OP (50 mg/kg/day to achieve 60% protection). T-705 achieved ≥70% protection at 50 to 100 mg/kg/day but was inactive at 25 mg/kg/day. The combination of both agents at 25 mg/kg/day increased survival to 90%.	T-705 + OC showed concentration-related, additive to synergistic effects for influenza A viruses in vitro. Depending on the dose administered and its timing, T-705 + OP improved survival in mice infected with different influenza A viruses compared to individual agents.

	(treatment commenced 2h prior in one experiment). Mice were observed for death through day 21. Additive, synergistic, and antagonistic interactions were measured using a computer model.	OP had no effect against H5N1, but T-705 was 30-70% protective (25 to 100 mg/kg/day); combination therapy only improved survival marginally. Combining ineffective doses of each agent (20 mg/kg/day of T-705 and 10 to 40 mg/kg/day of OP) afforded 60 to 80% protection and improved body weights during infection with H5N1 virus.	
Favipiravir (T-705) + peramivir[16] (Tarbet E. B. et al., Antiviral Res. 2012)	**Mouse model.** BALB/c mice intranasally inoculated with approximately three MLD_{50} of mouse-adapted influenza A/California/04/2009(H1N1) virus. Groups of mice received variable doses of oral (gavage) T-705, IM PMV or both agents given in combination, twice daily for 5 days, starting 4 h after infection. Sub-groups of mice were sacrificed on days 3 and 6 for lung histopathology and virus titres. Antagonistic, additive or synergistic interactions were assessed using a computer model.	T-705 as mono-therapy was 40%, 70%, and 100% protective at 20, 40, and 100 mg/kg/day. IM peramivir was 30% protective at 0.5 mg/kg/d and was ineffective at lower doses. In combination, T-705 + peramivir increased survival to 10-50% according to the doses given (0.025, 0.05, and 0.1 mg/kg/day doses of peramivir were combined with 20 mg/kg/d T-705 and when all doses of PMV were combined with 40 mg/kg/d T-705). Additionally, improvements in body weight were seen, relative to either compound alone. T-705 + PMV at 0.25 and 0.5 mg/kg/day was associated with	Synergy volume analysis (net volume) indicated a strong synergistic interaction for these two antivirals.

		significant reductions in lung viral titres at day 4. Improvements in lung haemorrhage scores were also seen on day 6.	
Favipiravir + oseltamivir, peramivir, or zanamivir[17] (Tarbet E. B. et al., Arch Virol. 2013)	**In vitro.** MDCK cells were infected with 14 different influenza A(H1N1) viruses, including OC-sensitive A/California/09/2009 (H1N1) virus and OC-resistant (H275Y) A/Hong Kong/2369/2009 (H1N1) virus. Susceptibility of infected cells to the antiviral action of favipiravir (T-705) alone or in combination with each of OC, PMV and ZNV was assessed (EC_{50} and EC_{90} values were computed based on the inhibition of virus-induced cytopathic effect). Antiviral concentrations included 0·032, 0·1, 0·32, 1, 3·2, 10, 32 and 100 µM favipiravir, and 0·0032, 0·01, 0·032, 0·1, 0·32 and 1 µM oseltamivir, zanamivir, or peramivir. Sensitivity of viruses to each antiviral agent was measured using IC_{50}. A computer model was used to assess synergy.	With the exception of A/Hong Kong/2369/2009 virus, all viruses were shown to be susceptible to all of the antiviral agents. Dose-response curves showed that A/Hong Kong/2369/2009 was resistant to OC, partially sensitive to PMV, but susceptible to ZNV and T-705. Synergy analysis of drug interactions showed a synergistic effect when T-705 was combined with each of the other three agents in OC-sensitive H1N1-infected cells and an additive effect for each combination against infection with the OC-resistant H1N1 virus.	The authors report that the combination of 3·2 µM T-705 and 1 µM PMV in cells infected with OC-resistant H1N1 virus showed greater than ten-fold higher inhibition of replication than would be expected if the interaction had been simply additive. T-705 + ZNV was not superior to other combinations against the OC-resistant H1N1 virus and the authors speculate that this may be due to antagonism.
Anti-HA monoclonal antibody +	**In vitro and mouse model.** To assess protection against infection, DBA/J2 mice were infected intranasally with the	In vitro neutralization studies demonstrated that 39·29 mAb (and also 81·39 mAb) neutralized all human	Two mAbs, 39·29 and 81·39, demonstrated neutralization against a (non-exhaustive)

oseltamivir[18] (Nakamura G. et al., Cell host Microbe 2013)	minimum LD100 of A/Hong Kong/1/1968(H3N2), A/Port Chalmers/1/1973(H3N2), or A/Aichi/2/1968(H3N2) virus. At 48 or 72h post infection, mice received the identified broadly-neutralising HA mAb, 39·29, intravenously at doses of 900, 300 or 100 µg per mouse. Control mice received an equivalent dose of a mAb against glycoprotein D of herpes simplex virus. Mice were observed for survival and weight loss through day 21. Balb/C mice were also infected with A/PR/8/1934 and treated with one of the following: OP 25mg/kg twice daily for 5 days, commencing 48 or 72h post infection; single IV dose of HA antibody 39·29, 100 µg or 300 µg at 48 or 72 h post infection; combined OP and 39·29. A further animal model of protection against H5N1 was also included. Ferrets were intranasally infected with 1 x 10e3 pfu of A/Vietnam/1203/2004 virus and then received either25mg/kg mAb 39·29, 25 mg/kg mAb 81·39, control IgG or OP 25mg/kg twice-daily for 5 days, 48 or 72h post infection. Ferrets were monitored for	influenza A isolates tested (5 x H1, 1 x H2 and 8 x H3 viruses). In vivo testing of protection afforded by mAb 39·29 in mice infected with 4 different influenza strains revealed that the 900 µg dose afforded 100% protection against death (all strains of influenza). The lower doses were less efficacious in mice infected with A/Hong Kong/1/1968 or A/PR/8/1934 virus (approximately 65% survival and 40-85% survival at day 20, respectively). In mice infected with PR8 virus, OP given for five days and commenced 48-72h post infection protected 37.5% of mice from death (100% mortality was seen in untreated controls). A single dose of 900 µg mAb 39.29 was associated with 87.5% survival and a faster return of lost body mass. In Balb/c mice infected with a high lethal dose of A/PR/8/1934 virus, those that received 100 µg mAb 39·29, control IgG or OP alone all died by day 13, whereas 80% mice that received OP and mAb 39·29 combination therapy survived through day 20. Lost body-	collection of influenza A viruses in vitro and at the highest dose tested, treatment with 39·29 was associated with 100% survival in mice infected with four different influenza A viruses of varying HA subtype. The authors state that differences seen with varying mAb dose and influenza subtype may reflect differences in in vivo viral growth kinetics and difference in host immune responses to different influenza viruses. Furthermore, inhibitory levels in vitro were shown not to correlate with the minimum efficacious dose of mAb in vivo. In a separate experiment involving mice infected with a high-lethal dose of a PR8 virus, survival at day 20 was only seen in mice treated with combined OP + mAb 39·29 (87.5% survival) and not with either treatment given separately. The authors

	mortality and weight loss.	weight was also regained in these survivors. The effectiveness of mAbs 39·29 and 81·39 were also tested in the ferret model of A/Vietnam/1204/2004(H5N1) lethal infection. Ferrets receiving control IgG exhibited 90% mortality, compared with 80% and 90% survival in ferrets that received mAb 39·29 at 48 or 72h post infection, respectively. OP alone was associated with 50% mortality in this model; combination therapy with OP was not assessed in ferrets.	hypothesise that the survival advantage (in this specific model) may be due to synergy of two different modes of action (blocking viral infectivity and disrupting viral budding), or possibly by OP increasing the levels of HA expression on the surface of infected epithelial cells, increasing the potential 'targets' to which the mAb can bind.
Ribavirin + oseltamivir[19] (Smee D.F. et al., Antivir. Chem. Chemother. 2006)	**Mouse model**. Groups of mice were infected intranasally with 10^6 cell culture infectious doses of mouse-passaged A/New Caledonia/20/99(H1N1) or 10^4 cell culture doses of B/Sichuan/379/99 virus. The following twice-daily, 5-day regimens were administered by oral gavage to different groups of mice, commencing 4h prior to inoculation with virus: OP 20mg/kg/day; ribavirin 40 mg/kg/day; OP 20mg/kg/day + ribavirin 40 mg/kg/day. The chose doses were deemed to be non-toxic, based on data from previous experiments. Different	With influenza A virus infection, OP protected 80-100% of mice from death and reduced lung consolidation at 10, 20 and 40mg/kg/day. 90-95% of mice in the placebo group died. Delaying OP treatment by even one day resulted in similar survival rates to those seen in the placebo group. Ribavirin monotherapy also protected from death and reduced lung consolidation scores at 20, 40 and 80 mg/kg/day. In contrast to OP, delays in treatment with ribavirin of 1 to 4 days were still	The authors comment that their results differ from those reported by previous studies because of differences in the antiviral activities of OP and ribavirin against the strains used to infect mice in this study and older strains used in the other studies.

	groups commenced each treatment at different days following inoculation (days 1, 2, 3, 4, 5). A further group received placebo from day 1 post inoculation. Subgroups of mice were sacrificed and lungs were scored for consolidation and virus titre measured in homogenates (50% cell culture infectious dose per 0·1ml, following endpoint dilution in MDCK cells). Survival in each group was recorded. A further, low-dose combination therapy study used lower doses of OP (0, 1·25, 2·5 and 5 mg/kg/day) with lower doses of ribavirin (0, 5, 10, 20mg/kg/day), to assess synergy in mice infected with influenza B and commencing treatment 24h following inoculation.	associated with protection from death (50-80% survival). In combination, OP + ribavirin failed to demonstrate consistent benefit and appeared to be associated with worse survival rates compared to ribavirin monotherapy. Against infection with the influenza B strain, dose-related survival benefits over placebo were seen for both OP monotherapy and ribavirin monotherapy. These survival benefits were seen even when treatment was delayed for up to 4 days, although ribavirin demonstrated greater protection over all (50-80% survival with combination therapy, compared to 30-40% survival with OP). Combination therapy (OP 10 mg/kg/day + ribavirin 40mg/kg/day) was associated with improved survival over the influenza B placebo group but similar to that in the ribavirin monotherapy group. In the low-dose combination therapy study in influenza B-infected mice, synergistic increases in numbers of survivors were seen using 1·25mg/kg/day OP + 5, 10 or	

		20mg/kg/day ribavirin (40%, 60% and 100% survival, respectively). Synergy was not demonstrated with combinations that employed higher doses of OP. A variety of dose combinations were also associated with synergistic improvements in lung scores and lung weights. Lung virus titres were reduced significantly following combination therapy, but a synergistic effect was not evident.	
Ribavirin + oseltamivir[20] (Ilyushina N.A. et al., Antimicrob. Agents Chemother. 2008)	**Mouse model.** NAI-sensitive A/Vietnam/1203/04(H5N1) and A/Turkey/15/06(H5N1) virus were used to infect BALC/c mice (intranasal inoculation, 5 MLD_{50}/ml were ~4 PFU and 20 PFU per mouse, respectively). 4h prior to inoculation, mice were given either ribavirin (37·5, 55, or 75 mg/kg/day) or OP (1, 10, 50, or 100 mg/kg of body weight/day) or both agents together, for eight days. Survival and weight change was observed. Subgroups were sacrificed at day 3 and virus titres were measured in lung, brain and spleen. Soluble immune mediators in day 3 lung homogenates were also quantified by ELISA.	Treatment with either agent alone produced a dose-dependent antiviral effect. Synergy analysis revealed a principally additive effect of combination therapy, occasionally with marginal synergy (ribavirin 37·5 mg/kg/day and OP 1 or 10 mg/kg/day, according to the virus strain used) or possible antagonism, depending on the dose of the agents. The optimal doses were associated with significant inhibition of virus replication in harvested organs, restriction of virus to the respiratory tract and abrogation ($P<0·01$) of pro-inflammatory soluble	Combination therapy had variable positive and negative effects, depending on the virus strain and the concentrations of the component agents.

	A separate, uninfected group was given the highest dose of each drug for toxicity monitoring. Theoretical additive interactions were calculated from the dose-response curves for each drug used individually. Volumes of the peaks of dose-response curved were calculated and used to quantify the volume of synergy (or antagonism) produced.	immune mediators (note mediator induction by A/Vietnam/1203/04 (H5N1) on day 3 was negligible). Over all, higher doses of agents were needed for the protection of mice against A/Turkey/15/06 virus than for the protection of mice against A/Vietnam/1203/04 virus.	
Tizoxanide + NAIs[21] (Belardo G. et al., IDSA Annual Meeting 2011)	**In vitro.** The effects of nitazoxanide (NTZ) activity and that of its active metabolite, tizoxanide (TIZ) were investigated in MDCK cells after infection (5 HAU/105 cells) with the following viruses: A/Puerto Rico/8/34(H1N1) (PR8), A/Wisconsin/33(H1N1), A/Firenze/7/03(H3N2), amantadine-resistant A/Parma/06/07(H3N2), OC-resistant A/Parma/24/09(H1N1), and low pathogenicity avian A//Ck/Italy/9097/97(H5N1), A/Goose/Italy/296246/03(H1N1) and A/Turkey/Italy/RA5563/99(H7N1), as well as B/Parma/3/04/RA5563/99. Virus titres were determined by hemagglutinin titration and infectivity assay, and cell viability by MTT	For TIZ, EC_{50} values ranging from 0.3 - 1.5 µg/ml were recorded with all 8 strains. In A(PR/8)-infected cells, EC_{50} values for OC and ZNV alone were 11·2 µM and 2·1 µM, respectively. When combined with NTZ 1.0 µg/ml, there was a 3- and 7-fold increase in potency versus OC and ZNV, respectively.	Enhanced antiviral activity was observed *in vitro* with TIZ and NAIs in limited testing.

	assay. Effects of combining NTZ with OC or ZNV were assessed using isobologram analysis.		
AVI-7100 + oseltamivir[22] (Iversen P. L. et al., ICAAC 2011)	**Ferret model.** Ferrets were infected intranasally with 5 x 10^5 pfu OC-resistant A/Hong Kong/2369/09(H1N1) virus. Different groups were treated daily with: OP 10 mg/kg/day orally; OP 30mg/kg intraperitoneally; OP 3 mg/kg intranasally; OP 10mg/kg + AVI-7100 10 mg/kg intraperitoneally. Controls received saline and a "scrambled" phosphorodiamidate morpholino oligomer (PMO). Ferrets were observed and nasal-wash viral loads were measured at days 1, 3, 5 and 7 post inoculation. Viral load in BAL was determined on day 8. Histological changes were assessed in harvested lungs.	Compared to controls and OP-monotherapy groups, AVI-7100-treated ferrets demonstrated reduced sneezing, nasal discharge, and respiratory distress. The cumulative viral load in nasal wash from OP and saline control groups was $TCID_{50}$ of 7·6 ± 3·0 (n=14) and 6·1 ± 3.2 (n=14), respectively. Cumulative viral load was reduced to TCID50 3·4 ± 1·2 (n=16) in the 30mg/kg i.p. and 3·5 ± 1.4 (n=8) in the 10mg/kg i.p. AVI-7100 groups ($p < 0.05$ for both treated vs. both controls. In ferrets treated with AVI-7100 + OP, cumulative viral load was reduced further to 2·0 ± 0·8 (n=8; $p < 0·05$ vs. both controls)	Possible additional antiviral effect for an OC-resistant virus when OP used in combination with AVI-7100 but confirmatory studies needed.
MBX2329 or MBX2546 + oseltamivir or amantadine[23]	**In vitro.** Two small molecule compounds that bind to HA and inhibit HA-mediated viral entry (MBX2329 and MBX2546) were tested alone and in combination with OC or amantadine. Drugs at different dilutions	Mean synergies (μM^2 ± SD) were: 331 ± 112 for MBX2329 + OC; 7·8 for MBX2329 + amantadine; 36 ± 2.8 for MBX2546 + OC; 0 for MBX2546 + amantadine. No drug-related	Strong synergy of viral inhibition was observed when either agent was combined with OC, but not when combined with amantadine. The authors

(Basu A. et al., J Virology 2014	alone and in combination were added to MDCK cells infected with A/California/10/2009 (H1N1) virus at a low multiplicity of infection. Cell viability was determined at 72h using neutral red. Calculated additive effects were subtracted from observed effects to identify areas where inhibition was greater than predicted. Synergy plots were created and synergy analysis was performed.	cytotoxicity was observed.	propose that synergy resulted from a balance between early inhibition of HA-mediated virus entry and later NA-mediated egress. The virus used is OC-sensitive but amantadine-resistant.
Clinical Studies			
Oseltamivir + IV zanamivir[24] (Pukrittayakamee S. et al., Antimicrob. Agents Chemother. 2011)	**Human PK study.** Sixteen healthy Thai adults, open label, four-period, randomized two-sequence crossover study; variable-dose ZNV by IV injection or infusion alone or with fixed-dose oral OP; serial blood sampling up to 72h.	No significant difference in maximum plasma concentrations (AUC) of OP or its metabolite when OC was given alone or in combination with ZNV. Maximum plasma concentrations of ZNV were 10% (95% confidence interval, 7 to 12%) higher when ZNV was infused concurrently with oral OP than with infusions before or after oral OP. Two mild adverse events (AEs), following OMP only (abnormal LFTs).	No clinically significant PK interaction identified and no serious adverse events.
Oseltamivir + IV peramivir or oral	**Human PK study.** Sixteen healthy adults in USA, randomized, open-label, crossover	Assessment of the 90% confidence interval for the geometric mean ratio of	No clinically significant PK interaction identified and no

rimantadine[25] (Atiee G. et al., J. Clin. Pharmacol. 2012)	study; 1 dose of IV PMV 600mg, 1 dose of oral OP 75 mg or rimantadine 100 mg, or PMV + OP or rimantadine; serial blood sampling up to 120h).	PMV and OC or rimantadine PK parameters showed no effect of OP or rimantadine on the PK of PMV and no effect of PMV on the PK of OC or rimantadine. No significant AEs; headache in 5-10% of any treatment arm.	serious adverse events.
TCAD (amantadine + ribavirin + oseltamivir)[26] (Seo S. et al., Antivir. Ther. 2013)	**Healthy control PK study and prospective pilot study in influenza-infected patients.** The PK study was performed as a randomized, open-label, crossover, single-dose study in 42 healthy adults. Three groups of 14 people were enrolled to compare single oral doses of amantadine, OP and ribavirin alone and in combination as TCAD. Each received two treatments in cross-over. Amantadine and OP were administered thrice-daily to maintain trough plasma concentrations at or above the target EC_{50}. Serial blood samples were collected and volunteers were observed for adverse events and side-effects. Following the PK study, a prospective pilot study of TCAD therapy (amantadine 75 mg, OP 50 mg and ribavirin 200 mg, thrice daily for 10 days) in influenza-infected patients	Single-dose PKs of individual components of TCAD were not altered when they were given together in the first 24h. PK parameters were also similar between combination therapy and mono-therapy with each component. In the pilot study of immunocompromised patients, six received TCAD and one received OP mono-therapy. One patient was infected with OC-resistant A(H1N1), two had amantadine-resistant A(H3N2) and two had amantadine-resistant A(H1N1)pdm09 virus infections; virus subtype could not be determined in the remaining two patients. No serious adverse events were reported. All but one of the TCAD patients completed the ten-day course of treatment. Six	The small sample size of the pilot study meant that comprehensive anti-viral efficacy analyses were not possible. Only one patient was randomized to receive OP-monotherapy, making comparison difficult. However, in this immunocompromised population, TCAD appeared to be relatively well-tolerated and PK values were comparable to those for monotherapy with component agents.

	undergoing chemotherapy or haematopoietic cell transplantation was performed. The randomized comparison group was OP 50 mg thrice daily for ten days. All patients had confirmed influenza A infection and onsets of illness ≤ 5 days prior to diagnosis. Patients had serial nasopaharyngeal samples for viral loads and resistance detection, and were observed for adverse events and blood PK measurements were performed.	adverse events were reported to have occurred in three patients, but these were judged not to be related to the study drugs and. gastrointestinal and neurological symptoms were not reported. Viral load was decreased after TCAD therapy in four patients with detectable virus at baseline, even in those infected with amantadine- or OC-resistant viruses. Viral load reductions of 1·6-3·9 log10 RNA copies/ml were seen in three patients receiving TCAD. H275Y-mediated OC resistance evolved in the patient who received OP mono-therapy; sequence analysis of NA and M2 genes did not identify new resistance substitutions at any time point in those who received TCAD.	
Convalescent plasma + NAI[27] (Zhou B. et al., N. Eng. J. Med. 2007)	**Case report.** Single adult patient in Shenzhen, China, with confirmed HPAI H5N1 virus infection.	The patient presented after 4 days of symptoms and had lung infiltrates on a chest radiograph. OP 150mg was commenced on day 9 of illness. Convalescent plasma had been obtained from another patient with confirmed HPAI H5N1 virus infection	If the decrease in viral RNA can be attributed to the addition of convalescent plasma therapy, then the response was extremely rapid. The neutralizing antibody titres were reportedly maintained, and so

		(neutralizing antibody titre 1:80 dilution). This plasma was given to the patient, (3 x 200ml transfusions, commencing day 12 of illness) because of rising viral RNA loads in tracheal aspirates despite OP therapy. Within the first 12h of plasma therapy, viral load was reduced by a factor of ~12 (from $1{\cdot}68\times10^5$ to $1{\cdot}42\times10^4$ copies/ml). By 32 hours, viral RNA was undetectable and OP was stopped on day 13 of illness. Eventually the patient was discharged. Assays of neutralizing antibodies against A/chicken/Hong Kong/282/2006, a virus closely related to A/Shenzhen/406H/2006, revealed that the patient initially had undetectable antibody titres, which steadily rose to between 1:40 and 1:80 by day 17 of illness.	may be due to the patient's own humoral immune response and not only plasma therapy. It was reported that subsequent analysis of the infecting viruses of donor and recipient revealed 99% genetic homology in hemagglutinin genes.
Amantadine + inhaled ribavirin[28] (Kirshon, B. et al., J. Reprod. Med.	**Case report**. 34 year old woman at 33 weeks' gestation was hospitalised on day 3 of illness with presumed influenza, which was initially treated symptomatically. The patient developed type 1 respiratory failure, with bilateral lower lobe infiltrates seen on	Serial arterial blood gas analysis suggested that the rapidly progressive type 1 respiratory failure improved following cesarean section and therapy with ribavirin and amantadine. The patient was extubated the day	It is impossible to determine the role of amantadine and ribavirin in the observed clinical improvement. The dose and frequency of nebulized ribavirin

1988)	the chest radiograph. Empiric therapy with erythromycin and a third-generation cephalosporin was commenced on day 2 of admission but was stopped quickly when bacterial infection was not identified (precise duration not stated). The patient continued to deteriorate and nebulized ribavirin was commenced. A caesarean section was performed and oral amantadine 100 mg twice-daily was also commenced at that time. Ribavirin and amantadine were given for 5 days.	following the caesarean section and was discharged at day 7 following delivery (asymptomatic, no supplemental oxygen). The infant had mild hyaline membrane disease but was extubated on the fourth day of life and discharged four weeks later. At one year of age, the infant was reported to have a normal physical examination. The authors state that a "throat culture" taken before the caesarean section was positive for A(H3N2) virus.	were not stated.
Oseltamivir + IV zanamivir[29] (Fraaij P.L. et al., J. Inf. Dis. 2011)	**Retrospective observational study.** Multi-centre IV ZNV use in 19 intensive care patients in the Netherlands with confirmed A(H1N1)pdm09 virus infection and prior OP treatment. All had received IV ZNV for >48h, had no evidence of ZNV resistance at baseline and had serial virological samples available for RT-PCR and attempted culture. Antiviral response was determined by differences in viral load between a sample collected closest to day of starting IV ZNV and follow-up samples.	Thirteen patients met the inclusion criteria. All were pre-treated with oral/NG OP (variable dose) for a median of 5 days. 3 patients then received IV ZNV monotherapy and 10 received IV ZNV + OP. In 6 of 13 patients with a sustained reduction of the viral load (≥1 log10 vp/mL for at least 10 days), the median time to start IV ZNV was 9 days (range, 4–11 days) compared with 14 days (range, 6–21 days) in 7 patients without viral load reduction (P=0·052). VL did not influence mortality. 4 patients had	When added late to oral OP, IV ZNV appears to have limited effectiveness in this small cohort of critically-ill patients. Concurrent administration of OP may antagonise antiviral effects of IV zanamivir.

		H275Y OC-resistant virus. Late switch to IV ZNV was associated with sustained viral load reductions in 3 of 3 given ZNV monotherapy but in only 3 of 10 given OP + ZNV.	
Oseltamivir + inhaled zanamivir[30] (Petersen E. et al., Scand. J. Infec. Dis. 2011)	**Retrospective observational study.** Twenty-one adult intensive care patients in Denmark with confirmed A(H1N1)pdm09 virus infection. Nasogastric OP 75 mg; aqueous solution of ZNV 25 mg × 4 administered by a nebulizer in the inspiratory ventilator tube. If there were signs of GI malabsorption, oral OP was changed to IV ZNV 600 mg twice-daily alone. Antiviral treatment was continued until the patient had two tracheal aspirates negative for A(H1N1)pdm09 virus. One patient started OP the day before admission, 2 started 2 days before, and 1 patient started OP 75 mg twice daily 6 days before admission. Primary outcomes were survival at discharge from ICU, 90-day survival, days to clearance of virus, and days on ECMO or mechanical ventilation.	Ninety-day mortality was 28·5%, and 75% remained virus RNA positive after 7 days of antivirals. Surviving patients were virus -positive for an average of 12 days (range, 3-25 days), from admission and start of treatment, whereas fatal cases remained positive for an average of 9 days (3-16 days). All patients with a lag-phase of antiviral treatment of 4–7 days excreted virus for more than 10 days. One patient had H275Y OC-resistant virus, detected retrospectively after subsequent negative RT-PCR results had been received.	Long duration of viral RNA-positivity in spite of combined therapy. However, average day of illness at admission was 6·6 days. All patients with ARDS received methylprednisolone 2 mg/kg/day IV from day 7 after the ARDS was diagnosed, which may have delayed viral clearance. Bacterial complications were noted in a number of patients.
TCAD (amantadine	**Retrospective cohort analysis.** Cohort of	Twenty-four patients received TCAD	Virological outcomes (shedding,

+ ribavirin + oseltamivir)[31] (Kim W. Y. et al., Antimicrob. Agents Chemother. 2011)	245 critically-ill adults in South Korea with confirmed A(H1N1)pdm09 virus infection. Data from 127 mechanically-ventilated patients were analysed. During the 2009-10 pandemic, TCAD (150 mg OP twice-daily, 100 mg amantadine twice daily, and 300 mg ribavirin three times daily) was included in treatment protocols for patients with severe influenza. Dose adjustments were made for renal or hepatic failure or if patients were of advanced age. The primary outcome was death. Possible treatment-related adverse events were also assessed, along with changes in liver enzymes and serum creatinine (baseline, days 3, 7 and 14).	and 103 received OP mono-therapy. Mean day of illness was approximately 5 days when starting treatment (both groups). The 14-day mortality was 17% in the TCAD group and 35% in the OP group (P = 0.08), and the 90-day mortality was 46% in the TCAD group and 59% in the OP group (P = 0.23). Haemolytic anaemia, neurological events and hepatic toxicity relating to treatment were not seen with TCAD. The median duration of ribavirin treatment was 7 (range 2 to 24) days; 25% received ribavirin treatment for longer than 14 days. The mean dose of ribavirin was less than 600 mg/day. Seven cases in the OP group had severe liver enzyme elevation and three had fulminant liver failure, although it could not be determined whether this was related to treatment. Adjuvant corticosteroids were administered to approximately half the patients in each group.	resistance) were not assessed and a relatively small number of TCAD patients were compared to OP-treated patients. In this retrospective observational study, TCAD appeared to be well tolerated, with treatment outcomes no worse and perhaps better than OP monotherapy. OP dose and antiviral duration were not associated with increased or decreased survival by linear regression analysis.
TCAD (amantadine + ribavirin +	**Retrospective observational study**. Thirty-seven adults from Republic of Korea with	Deaths occurred in two (25%) patients given OP mono-therapy and two (33%)	Due to the study design, conclusions cannot be made

oseltamivir)[32] (Kang S. et al., Jpn. J. Infect. Dis., 2013)	confirmed A(H1N1)pdm09 virus infection were identified. Fourteen patients were described as having serious illness and were studied further. All had pneumonia and nine had ARDS, of which seven required intensive care. Eight patients received OP 150 mg twice daily and six patients received TCAD (150 mg oseltamivir twice a day, 100 mg amantadine twice a day, and 300 mg ribavirin three times a day). Mortality was assessed at 14 and 90 days. Viral clearance was also assessed, defined as non-detectable virus RNA in respiratory secretions after five days of therapy. Resistance to OC was assessed by sequencing for those who died before completing five days of treatment or those with evidence of prolonged viral shedding (not defined).	patients given TCAD. Viral clearance was observed in nine patients (75%). Persistent viral shedding was reported in three patients (reported as 17% of the OP mono-therapy group and 33% of the TCAD group). Resistance to OC was not detected in samples from patients who met the criteria for resistance screening. One patient developed haemolytic anaemia on day 3 of TCAD therapy (treatment was stopped).	about the effects of TCAD on mortality or viral shedding, following comparison with outcomes in those who received OC mono-therapy. Haemolytic anaemia was reported in one of six patients who received TCAD.
Convalescent plasma + NAI[33] (Hung I. F. et al., Clin. Infect. Dis. 2011)	**Prospective cohort study.** Ninety-three adult patients in Hong Kong with critical illness caused by A(H1N1)pdm09 infection, of which twenty received convalescent plasma. Treatment and control groups were matched by age, sex, and disease severity scores. Patients received 500 ml	In addition to plasma therapy, all 20 patients in the treatment group received OP, 42% received inhaled ZNV and 10% received IV ZNV. Crude mortality in the treatment group was significantly lower than in the non-plasma-treatment group (20.0% vs	In this non-randomized study, patients that received plasma in addition to antivirals had a greater number of risk factors associated with disease severity, including a lower lymphocyte count and generally more

	convalescent plasma with a neutralizing antibody titre of ≥1:160 over four hours on median day 2 of ICU admission (IQR 1-2·5 days). The plasma had been harvested by apheresis from patients recovering from A(H1N1)pdm09 infection. Clinical outcome was compared to that of patients who declined plasma treatment as the untreated controls. Antiviral treatment including oralOP, IV PMV, or IV/inhaled ZNV was recorded. Serial respiratory samples were obtained for viral load measurement and a panel of plasma cytokines/chemokines was also measured.	54.8%; p = 0.01). Multivariate analysis showed that plasma treatment was associated with reduced mortality (OR 0·20; 95% CI 0·06-0·69; p=0·011). In 44 patients with available serial viral loads and cytokine/chemokine results, plasma therapy was associated with significantly lower day 3, 5, and 7 viral loads, compared with the control group (p <0·05). Shortly after VL decreased, reductions in IL-6, IL-10 and TNF-α decreased and the levels were lower in the treatment group than the control group (p <0·5). Radiological consolidation was reported to have improved and the respiratory tract viral load decreased by >3 log10 copies/mL within 48 h after plasma therapy.	severe symptoms at presentation. Obesity was also more common than in the control group (NAIs without plasma therapy). However, the treatment group still had lower mortality and plasma therapy was reportedly well- tolerated. Similar proportions in each group received other supportive therapies, including N-acetylcysteine, corticosteroids and ECMO.
Oseltamivir + inhaled zanamivir[34] (Escuret V. et al., Antivir. Res. 2012)	**Phase II clinical trial.** Adult outpatients without chronic diseases but with ILI for <42h, with laboratory-confirmed A(H1N1)pdm09 virus infection. 24 patients were appropriate for analysis; 12 randomized to oral OP 75 mg twice daily and 12 randomized to OP + inhaled ZNV 10 mg twice daily for 5 days. Serial nasal	Mean viral load decreased at a rate of approximately 1 log10 cgeq/µl per day, regardless of the allocated treatment group, with no significant different in time to resolution of symptoms. All treatments well tolerated and oseltamivir resistance was not	In terms of anti-viral effectiveness, the sample size was too small to be informative.

	washes and nasal swabs with quantitative RT-PCR. Assessment of antiviral efficacy, resolution of symptoms, tolerability and prevention of OC resistance (H275Y).	detected.	
Amantadine + oseltamivir[35] Morrison D. et al., PLoS One 2007)	**Randomized open-label crossover clinical trial.** Eighteen healthy adult subjects consisting of six groups of three. Each group received three different treatments over three periods: amantadine 100mg; OP 75mg; amantadine 100 mg + OP 75 mg. All medications were oral and given twice-daily for 5 days, with wash-out periods between switches. Primary endpoint was to characterise plasma PK. Secondary objective was safety and tolerability.	Giving OP with ribavirin had no clinically important effect on the PK of either agent. Eight mild adverse events were reported, two with amantadine alone and three with OP alone or in combination.	No evidence of increase in adverse events with combination therapy, but the trial was not powered for adverse event endpoints.
Oseltamivir + inhaled zanamivir[36] (Duval X. et al., PLoS Med 2010)	**Randomized placebo-controlled trial.** Five hundred and forty-one adult outpatients with ILI duration <36h and laboratory-confirmed influenza, mainly H3N2; Four dose groups: oral OP 75 mg twice daily plus inhaled ZNV 10 mg twice-daily, either agent alone, or placebo, The virological primary end-point was proportion of patients with	In an ITT analysis of 447 with RT-PCR confirmed influenza A, the primary virological endpoint was achieved in 46% OP + ZNV, 59% OP + placebo and 34% in ZNV + placebo. Mean day 0 to day 2 viral load decrease was 2.14, 2·49, and 1·68 log10 cgeq/µl, respectively, and median time to	OP + ZNV was less effective than OP alone and not significantly more effective than ZNV alone.

	nasal influenza RNA <200 cgeq/µl at day 2; clinical endpoint was time to alleviation of symptoms until d14.	alleviation of symptoms was 4·0, 3·0, and 4·0 days, respectively. Nausea and/or vomiting were seen more frequently in the OP + ZNV arm.	
Oseltamivir + inhaled zanamivir[37] (Carrat F. et al., Antivir. Ther. 2012)	**Household transmission study.** Pre-specified post hoc sub-group analysis of study outlined above[36]. 543 household contacts of 267 index patients, of which 466 had follow-up assessment. Rate of secondary illness (fever with cough within 7 days from randomization of index patients) was assessed, comparing arms in index cases.	Secondary illness reported in 12·5% contacts with no significant difference between index treatment arms (P=0·07). However, for index cases who commenced treatment <24h after onset, secondary illness was lower in contacts of index cases who received OP + ZNV (4%) than in OP (17%, p=0·014) or ZNV (15%, p=0·031).	Secondary influenza illness was not laboratory-confirmed in contacts. Authors hypothesis that apparent benefit may be due to a more rapid onset of antiviral activity of OP + ZNV commenced <24h from onset of symptoms in index cases, although there are no PK or virological data to support this.
Rimantadine + inhaled zanamivir[38] Ison M. G., Antivir. Ther. 2003)	**Double-blinded RCT.** Study to assess tolerability and efficacy of nebulized ZNV (16mg four times daily for 5 days) in combination with oral rimantadine (100mg twice daily for five days), compared to rimantadine with nebulized saline control, in 41 hospitalised adults with confirmed seasonal influenza infection (predominantly influenza A). Primary end-point was absence of pharyngeal influenza viral shedding on day 3 of treatment. Secondary	Inhaled ZNV + rimantadine did not cause decline in peak expiratory flow rates in 20 treated patients, compared to the 21 who received inhaled saline. 3 patients who received combination therapy experienced serious adverse events, but only one was thought to be drug-related (retrosternal burning with dyspnoea, possibly due to inhaled ZNV). SAE frequencies did not differ significantly between the two groups.	The study was terminated early because the regulatory approval of ZNV made further enrolment untenable. Several potentially favourable (but non-significant) trends were seen, but the study was under-powered.

	virological end-points were duration and quantity of viral shedding in respiratory samples and emergence of drug-resistant virus. Secondary clinical end-points included durations of fever, supplemental oxygen use and hospitalization; time to recovery of normal oxygen saturations; severity of cough; frequency of complications; time to usual daily activities.	No significant differences were observed in the proportion of patients shedding virus by treatment day 3 (57% ZNV plus rimantadine, 67% placebo plus rimantadine), or in the durations of hospitalization and supplemental oxygen use. 94% of patients receiving combination therapy had no cough or only mild cough by day 3, compared 55% of those who received rimantadine monotherapy (p=0·01). Two rimantadine-resistant viruses emerged during rimantadine monotherapy; ZNV resistance was not detected.	
Convalescent plasma + NAI[39] (Hung I. F. et al., Chest 2013)	**Double-blind randomized controlled trial.** Thirty-five adult patients in Hong Kong with A(H1N1)pdm09 virus infection were randomized to receive hyperimmune intravenous immunoglobulin (H-IVIG, 17 patients) obtained from patients who had recovered from A(H1N1)pdm09 virus infection, or IVIG obtained prior to 2009 (18 patients). Clinical outcome and adverse effects were compared and viral loads were measured in serial respiratory tract samples. A panel of cytokines in serum was	The median interval from symptom onset to ICU admission was 3 days (IQR 1-5 days). Demographic and clinical characteristics were similar for the two groups at recruitment. Antiviral therapy was reported not to have differed between the two groups. All 34 patients received OP 75mg twice-daily and 3 patients also received inhaled ZNV. Univariate analysis showed no difference in mortality, length of ICU and hospital stay between the two	These findings suggest that early administration (<5 days of illness) of polyclonal neutralising antibodies, in combination with neuraminidase inhibitor therapy, was beneficial and is worthy of further study.

	also measured. Log-rank test was used to evaluate the overall survival over a period of 21 days after treatment	groups. A subgroup multivariate analysis of patients who received H-IVIG/IVIG treatment within 5 days of symptom onset (n=22), showed that only H-IVIG treatment independently reduced mortality (0% vs.40%) (Odds ratio [OR] 0·14, 95% confidence interval [CI], 0·02-0·92; p=0·04). The log-rank test also showed that earlier H-IVIG treatment was associated with significantly better survival than IVIG treatment over a period of 21 days after treatment (p=0·02). Viral load on day 5 (3·3 vs. 4·67 $\log_{10}$ copies/mL; p=0.04), and day 7 (undetectable vs. 4·53 $\log_{10}$ copies/mL; p=0·02) after treatment was significantly lower in the H-IVIG than the IVIG group, becoming undetectable by day 6 of treatment. Initial serum levels of IL-10, IL-1ra and MIP-1α were significantly higher in the H-IVIG group but fell to similar levels by day 2 after treatment, compared to the IVIG group. There was no significant difference in the cytokine profile between the 22 patients who	

		commenced H-IVIG up to day 5 of illness, compared to those who received early IVIG.	

Appendices. Summaries of Pre-clinical and Clinical Combination Studies of Influenza Antivirals, Immunomodulators, and Chinese Traditional Medicines

Appendix 2. Pre-clinical and Clinical Studies of Combinations of Influenza Antivirals and Immunomodulators				
Immunomodulator (reference)	**Antiviral in combination**	**Model**	**Main findings**	**Comment**
Pre-Clinical Studies				
Type I interferon[40] (Lavrov et al, Nature 1968)	Amantadine	**In vitro.** Cell culture model using chick embryo cells. A laboratory-adapted strain of influenza A/ WSN was used at MOI=0.001 PFU/cell. The endpoints were viral titre and haemagglutinins at 1, 24, 48, and 72h after inoculation.	Mixed type 1 interferons appeared to have an additive effect in reducing viral replication *in vitro.* Interferon alone resulted in a 2-log decrease in viral titre; addition of both agents 1hr after inoculation almost completely ablated viral replication at 72h.	The combination was not effective when administered after viral replication was underway (at 24hr). Inhibition was consistent with an additive effect.
Interferon-α2[41] (Hayden et al, Antimicrob Agents Chemother, 1984)	Rimantadine (1μg/ml); ribavirin (0.3 μg/ml or 1.5μg/ml)	**In vitro.** Cell culture model using rhesus monkey kidney (RMK) cells. Clinical isolates of influenza A/Aichi/68/H3N2 and A/England/80/H1N1 and a laboratory-adapted strain of iB/Lee/40 were used. Lyophilised human interferon α2 (2,000 or	Interferon-α2 alone caused a dose-dependent decrease in viral titer (max 2-log decrease). The addition of interferon-α2 to rimantadine, ribavirin, or both agents reduced viral replication. With all three agents, no replication was seen.	Inhibition was consistent with an additive or synergistic effect in different experiments with both antivirals.

		10,000 IU/ml) was used in addition to either rimantadine or ribavirin, or both.		
Type I interferons[42] (D'Agostini et al, Immunopharmacol 1996)	Amantadine 20mg/kg	**Mouse model.** Challenge with a laboratory-adapted influenza A/PR/8/34/H1N1 virus in young (4 week old) mice. The primary endpoint was survival. A mixture of α and β interferons was used. Treatment with amantadine began concurrently with infection.	Interferon alone did not significantly affect survival (20% untreated vs. 25% treated). In combination with amantadine, it provided no additional survival benefit (survival 30% with amantadine only, 30% with combination therapy). There was no reduction in lung infectious viral titre. Interestingly, the combination of amantadine, interferons and thymosin improved survival to 60% (see below).	This is the only whole animal study to follow a comparable protocol to Lavrov et al's cell culture study. [1] The results do not support a synergistic or additive effect *in vivo.* However, interferon was required to produce a survival benefit with thymosin (see below).
Thymosin α1[42] (D'Agostini et al, Immunopharmacol 1996)	Amantadine and interferon	**Mouse model.** (see above) Thymosin α1 200μg/kg was administered by intraperitoneal injection.	Thymosin alone did not affect survival (20%). Survival was unchanged in combination with interferon (25%) or amantadine(30%), but all three agents together improved survival (60%) and decreased lung	Neither interferon nor thymosin alone had any effect on viral titre, but the combination of all three appeared to have a marked effect. Since antiviral treatment began concurrently with inoculation, this model

			infectious viral titre (3-log reduction).	has limited clinical relevance.
Triamcinolone[43] (Ottolini et al, Pediatr Pulmon 2003)	Intranasal zanamivir, oral oseltamivir, or intranasal pooled convalescent serum.	**Cotton rat model.** Challenge with non-adapted A(H3N2) virus (10^7 $TCID_{50}$) followed 3 days later with nasal lavage administration of triamcinolone with or without other therapies.	Reduced pulmonary histopathology with topical triamcinolone at 4 or 16 mg/kg, an effect that was not enhanced by NAI or antibody. Suppression of IFN-gamma levels observed in all combinations where 4 or 16 mg/kg of triamcinolone was used.	Viral replication was not prolonged by corticosteroid therapy but very short duration in this model.
N-acetylcysteine (NAC)[44] (Ghezzi et al, Int J Immunopathol Pharmacol 2004)	Ribavirin 100mg/kg by intraperitoneal injection.	**Mouse model.** **C**hallenge with A/PR/8/34/ H1N1 virus. A single daily dose of N-acetylcysteine 1000mg/kg was used, beginning from 4 hours after inoculation. The primary endpoint was survival at 14 days following infection.	The dose of NAC was chosen to be too low to be effective alone. There was improved survival following the addition of NAC to ribavirin (ribavirin alone: 58%; combination: 92%), compatible with a synergistic effect. No viral titres were reported.	Mice were treated with NAC from 4h post-infection in a dose that was titrated down to avoid completely protecting mice with NAC alone. Survival with NAC alone was 25% vs 17% in controls. No data on delayed treatment effect were provided.
N-acetylcysteine (NAC)[45] (Garozzo A et al, Int J	Oseltamivir 1mg/kg/day (two divided doses) for 5 days.	**Mouse model.** **C**hallenge with a laboratory-adapted A/PR/8/34/H1N1 virus. A single daily dose of	Improved survival following the addition of NAC to oseltamivir (oseltamivir alone: 60%, combination: 100%).	Survival with NAC alone was better than control (20% vs 0%), but since treatment with antivirals and NAC began *before*

Immunopathol Pharmacol 2007)		N-acetylcysteine 1000mg/kg was used, beginning from 4 hours before inoculation. The primary endpoint was survival at 21 days following infection.	This was compatible with a synergistic effect; no viral titres were reported.	inoculation with virus, the clinical relevance of this observation is limited. This study elucidates a mechanism of injury, but not a therapeutically-relevant treatment effect.
Surfactant[46] (Fukushi et al. PLoS One 2012)	Laninamivir (50µl, 267µg/ml)	**Mouse model.** Challenge with a laboratory-adapted A/PR/8/H1N1 virus in which the virus was grown in the lungs of mice. Study mice were then inoculated with extract from the lungs of infected mice. Surfactant (45µl 20mg/ml) was administered from 3 days after infection.	There was improved survival with combination therapy (40%) compared with laninamivir monotherapy (0%). Results for surfactant treatment alone were not presented.	No effect was detected on viral titre or inflammatory cytokines. A very high dose of virus (3741 x MLD_{50}) was used in order to ensure mortality in the laninamivir-only group.
Protectin (PD1/PDX)[47] (Morita et al, Cell, 2013)	Peramivir (10mg/kg intravenously)	**Mouse model.** **C**hallenge with a laboratory-adapted A/PR/8/34/H1N1 virus in moderate dose (500 $TCID_{50}$). Treatment with both peramivir and PD1/PDX was intitiated 2 days after	PD1/PDX alone improved survival compared to controls (23% vs 0%). In combination with peramivir, survival was 100%, compatible with a synergistic effect.	This experiment used a clinically-relevant therapeutic timepoint (2 days following infection) for treatment with both agents. Readers should note that it has been proposed that the biological

		infection.		activities attributed to PD1 in this study are in fact attributable to an isomer, PDX. [9]
Sphingosine analog (AAL-R)[48] (Walsh et al Proc Natl Acad Sci U S A 2011)	Oseltamivir 5mg/kg/day	**Mouse model.** Intranasal **c**hallenge with clinical isolates of A(H1N1)pdm09 viruses. The treatment group received an S1P receptor agonist prodrug and analog of FTY720/finglimod, AAL-R (0.2mg/kg) via the intra-tracheal route 1hr after infection. Antiviral treatment with oseltamivir started one day after infection. The endpoint was survival at 12 days.	AAL-R alone, given 1hr after infection, improved survival to 82% compared with 21% in controls and 50% in oseltamivir-treated. In combination with oseltamivir, survival was 96%. The additional benefit of oseltamivir was not statistically significant. AAL-R treatment also significantly reduced inflammatory cytokines in bronchoalveolar lavage fluid (including IFN-α, IL-6, and CXCL10).	AAL-R treatment was initiated 1 hour after infection; hence this study provides mechanistic insight but little clinical relevance. This is consistent with the effect of anti-inflammatory treatments operating through the same pathway[11] in other animal models of severe systemic inflammation. However the effects seen from early treatment with anti-inflammatory drugs in systemic sepsis have generally been much smaller, or absent, when treatment is given at a therapeutically relevant timepoint[12].
Celecoxib and mesalazine[49] (Zheng BJ, Proc Natl	Zanamivir (3mg every 12hr intraperitoneal)	**Mouse model.** **C**hallenge with clinical isolates of highly pathogenic influenza	Each agent trended towards benefit as dual therapy (20% survival for celecoxib+ zanamivir, 20%	This experiment used a clinically-relevant therapeutic timepoint (2 days following infection)

Acad Sci U S A 2008)		A(H5N1) virus (1000 LD_{50}). Mice were treated with celecoxib (2mg/day by intraperitoneal injection) or mesalazine (1mg/day by intraperitoneal injection), vehicle, or both treatments.	survival for mesalazine+ zanamivir). Triple therapy with celecoxib, mesalazine and zanamivir improved survival to 50%.	for treatment with both agents. There was a clear signal for incremental benefit with the addition of each agent.
4 commercially-available inhibitors of MEK1/MEK2 (PD-0325901, AZD-6244, AZD-8330, and RDEA-119)[50] (Haasbach E Antiviral Research, 2013)	Oseltamivir (0.01µM to 10 µM)	**In vitro.** Cell culture model using A549 cells, with viral replication as the endpoint. A clinical isolate of A/Regensburg/D6/09 (H1N1) virus was used.	There was evidence of synergism at optimal concentrations for all 4 MEK1/2 inhibitors.	All four inhibitors were chosen because they have already been through pre-clinical investigations for treatment of cancer.
Simvastatin[51] (Belser, et al Virology 2013)	Oseltamivir (50mg/kg)	**Mouse model.** **C**hallenge with clinical isolates of A/Chicken/Korea/Gimje/08 (H5N1) and A/Mexico/4482/09 (H1N1) viruses. Mice were treated with simvastatin 10mg/kg/day from 3 days *before* infection, and with oseltamivir	Addition of simvastatin did not improve weight loss, viral titre, or survival in oseltamivir-treated mice infected with either strain.	This result is consistent with another murine model study of statins tested alone in influenza[52]

		from 1 day before infection.		
Clinical Studies				
Any statin[53] (Vandermeer et al, J Infect Dis 2012)	Antiviral use was not protocolised as part of this observational study. Patients received standard care in 10 US states (antivirals were used in 276/1013 cases within 48h of symptom onset).	**Observational study.** Including 1013 hospitalised influenza A cases treated with statins for another reason. A logistic regression model was constructed to control for numerous other variables known or perceived to alter disease outcome.	Multivariate analysis found statin use was associated with reduced mortality (adjusted OR for death = 0.59, 95%CI 0.38-0.92, in patients receiving statins). Individuals who were treated with statins were more likely to have chronic heart and lung disease, and hence were more likely to have been vaccinated against influenza. Vaccination was included in the multivariate model.	Similar results have been reported for other acute severe infections; RCT data are awaited. Most statin recipients were receiving long-term administration, and this study did not address the effect of initiating statin use at the time of hospitalization for influenza-related complications.
Any corticosteroid, macrolide or statin[54] (Viasus et al, J Infect 2011)	Antiviral use was not protocolised as part of this observational study: clinicians chose from oseltamivir 150mg/day orally,	**Observational study.** This study included 234 patients with A(H1N1)pdm09 admitted to participating hospitals during the prospective recruitment period in 2009. 37 hospitalised cases were treated	There was no detectable effect on composite endpoint of ICU admission/death from any of the interventions considered in a multivariate model.	The odds ratio for steroids alone was not reported; for immunomodulatory therapies as a group (corticosteroids, macrolides or statins) OR for death = 0.75 (95%CI, 0.2-1.9)

	oseltamivir 300mg/day orally, or zanamivir 600mg/day intravenously. Patients received standard therapy at 13 hospitals in Spain (including early NAI in 8/37 cases)	with corticosteroids (dose > 300mg/day hydrocortisone or equivalent), 11 were receiving statins from before admission, and 31 patients were treated with any macrolide.		
Any corticosteroid[55] (Brun-Buisson et al, Am J Respir Crit Care Med 2011)	Antiviral use was not protocolised as part of this observational study, but the authors report that almost all patients received antiviral treatment. Patients received standard therapy in participating	**Observational study.** Out of 208 ICU patients in a national ARDS registry, 83 patients with ARDS and A(H1N1)pdm09 infection were treated with corticosteroids, with a median dose of 270mg/day of hydrocortisone or equivalent.	Increased risk of death was found in patients receiving steroids. The propensity-score adjusted hazard ratio was 2.4 (95%CI 1.5-5.4).	Patients from a large nationwide ARDS registry were included. A subgroup analysis of patients treated early in the course of illness (<3 days in ICU) with corticosteroids showed an even greater increased risk of death in the treatment group.

	French hospitals during the recruitment period.			
Any corticosteroid[56] (Martin-Loeches et al, Intensive Care Med 2011)	Antiviral use was not protocolised as part of this observational study, but the report suggests that all patients received oseltamivir. Patients received standard care in the treating hospital. Hospitals in 33 countries on 3 continents contributed cases.	**Observational study.** Of 220 ICU patients with confirmed or probable A(H1N1)pdm09 illness included in this prospective study, 126 were treated with corticosteroids. The dose of corticosteroids was not reported.	No effect on mortality (adjusted HR 1.3; 95%CI, 0.7-2.4) was found. Steroids use was associated with increased risk of hospital-acquired pneumonia (adjusted OR 2.2; 95%CI, 1.0-4.8). The crude mortality figures were substantially higher in the corticosterioid-treated group.	Data are from the European H1N1 registry, but from a very diverse group of contributing sites including hospitals in Eastern and Western Europe, South America and the Middle East and Asia.
Any corticosteroid[57] (Xi et al, BMC Infect Dis 2010)	80% of patients received oseltamivir but only 12% of patients received oseltamivir	**Observational study.** 155 hospitalised patients with A(H1N1)pdm09 illness were included, of whom 52 were treated with corticosteroids.	There was no significant effect on mortality (OR for death in corticosteroid group 2.7; 95%CI, 0.99-1.6)	A subgroup comparison between "low dose" corticosteroids (<80mg/day methylprednisolone or equivalent) and high dose (>80mg/day

	within 48hrs of symptom onset.			methylprednisolone) showed a trend towards higher rate of bacterial pneumonia.
Any corticosteroid[58] (Kim et al, Am J Respir Crit Care Med 2011)	Oseltamivir 150mg/day (31% of patients), oseltamivir 300mg/day (46%), triple combination therapy with oseltamivir, amantadine, and ribavirin (16%) or combinations with two of these drugs (8%). Antiviral therapy was started within 48h of symptom onset in 46% of patients. Patients received standard care in one of 28	**Observational study**. Of 245 hospitalised patients with influenza A(H1N1)pdm09 in this study, 107 were treated with corticosteroids.	After matching cases and controls using a propensity score, there was an increased risk of death among patients receiving corticosteroids (adjusted OR for death 2.2; 95%CI, 1.03-4.71. There was also increased risk of bacterial and fungal infections.	As with the other observational studies described here, some caution is warranted in the interpretation of the results because of the risk of hidden confounding. However the results of clinical studies of corticosteroid treatment in influenza seem to consistently show no benefit, and many significant harm.

	Korean hospitals.			
Any corticosteroid[59] (Linko et al, Acta Anaesthesiol Scand 2011)	Oseltamivir in 96% (dose and duration not specified) at median 4 (IQR 2–6) days from symptom onset. Methylpredniso-lone in 46 (highest daily mean±SD,1.1± 0.6 mg/kg), hydrocortisone in 10 (highest daily mean±SD, 214±66 mg), and 12 patients received both.	**Observational study.** Prospective study of 132 ICU patients (78% ventilatory support, 49% ARDS) with influenza A(H1N1)pdm09 infection. Corticosteroids administered to 72 (55%) patients.	Crude hospital mortality was not significantly different in patients given corticosteroids compared to those not: 8 of 72 (11%, 95% CI 4–19%) vs. 2 of 60 (3%, 95% CI 0–8%), respectively. Increased numbers of positive blood cultures (7/59 vs 2/39) and other positive bacterial cultures (33/59 vs 5/39; P<0.001) in corticosteroid group than in the non-corticosteroid group,	Corticosteroid recipients were more severely ill and more likely to have ARDS. Low power to detect mortality difference.
Any corticosteroid[60] (Diaz et al, J Infection 2012)	NAI therapy in 100%.	**Observational study.** Prospective, multi-centre study of 372 patients with the diagnosis of primary A(H1N1)pdm09 viral pneumonia performed in 148 Spanish ICUs (70% mechanically ventilated). Corticosteroids given	Overall mortality (N= 66) did not differ between patients treated with corticosteroids and those who were not (18.4% vs 17.4%). After adjustments for illness severity and potential confounding factors, the use of corticosteroid therapy was not significantly	No evidence that corticosteroid use in critically ill patients with primary influenza viral pneumonia improved survival.

		to 136 (36.6%) patients .	associated with mortality (HR =1.06, 95% CI, 0.63 - 1.83)	
Any corticosteroid[61] (Kudo et al, PLoS ONE 2012)	NAI therapy in 100% at median 2 days (range, 0–7) from symptoms onset. Oseltamivir (usual dose, 150 mg/day for 5 days in adults), inhaled ZNV (20 mg/day for 5 days) or both used. Systemic MP (1.0–1.5 mg/kg given 2–4 times/day, in subjects under 15 years of age, and 40–80 mg given 2–4 times/day in those over 15 years old) used.	**Observational study.** Retrospective analysis group of 89 children and adults hospitalized with influenza A(H1N1)pdm09 infection (65% pneumonia, 44% wheezing, 52% supplemental oxygen, none ventilated). Systemic corticosteroids were used in 93.3% of pneumonia with wheezing patients, 77.8% of wheezing illness patients, and 64.3% of pneumonia without wheezing patients (P<0.001).	No significant differences between the corticosteroid and non-steroid groups in hours to fever alleviation from the initiation of antiviral agents and hospitalization duration (median, 7 days). Bacterial co-infection was found in 52% of corticosteroid group and 25% of the no steroid group at the time of admission (p=0.093).	Heterogeneous patient population with non-critical illness. Unclear whether corticosteroid use associated with benefit or any adverse effects.
Sirolimus[62] (Wang et al, Crit Care	Oseltamivir 75mg twice daily and	**Clinical trial.** Small randomised-controlled trial. There were 19	After correcting for multiple comparisons, there were no significant	Although the trend is interesting, this trial was underpowered, no

Med 2014)	prednisolone 20mg/day.	ICU patients in each group, all of whom had severe respiratory failure secondary to A(H1N1)pdm09 influenza.	differences in any outcome variable between the treatment and control groups (weaning from mechanical ventilation, duration of ventilator support, requirement for extracorporeal membrane oxygenation, mortality). 7 days after beginning treatment, organ dysfunction (SOFA) scores and day 7 viral RNA detection rates were lower in the sirolimus-treated group.	primary endpoint was used, corticosteroids were routinely administered, and there was a trend (p=0.06) towards worse oxygenation in the control group at recruitment. For these reasons no firm conclusions can be drawn about the efficacy of sirolimus from this report alone.
Any macrolide[63] (Martin-Loeches et al, Intens Care Med 2013)	148 recruiting hospitals in Spain. Three antiviral regimens were used: oseltamivir 150mg/day orally, oseltamivir 300mg/day orally, or zanamivir 600mg/day	**Observational study**. 733 ICU patients with confirmed influenza A(H1N1)pdm09 and respiratory failure were included, of whom 190 received treatment with any macrolide.	Propensity scores calculated from a multivariate model were used to estimate treatment effects. There was no improvement in survival among patients treated with macrolides.	The authors point out that this study could not assess the effect of early treatment with macrolides.

	intravenously.			
Clarithromycin[64] (Ishii et al, J Infect 2012)	Oseltamivir, inhaled zanamivir, or inhaled lanamivir were used.	**Observational study**. Open-label, non-randomised trial in 141 outpatients with influenza A, of whom 74 received clarithromycin and all received a NAI.	There was no statistically significant effect on any clinical endpoint.	No primary endpoint was reported. This study included a patient cohort with primarily mild disease.
Clarithromycin[65] at 5.0–7.5 mg/kg body weight (Shinahara et al, PLoS ONE 2013)	Oseltamivir or inhaled zanamivir	**Observational study.** In this retrospective, non-randomized case series, 195 children (mean $\pm$ SD, 5.9 $\pm$ 3.3 years) with influenza A in 2008/2009 season were given 1 of 5 regimens: oseltamivir +/- clarithro, zanamivir+/- clarithro, or no treatment.	The titers of early mucosal S-IgA and systemic IgG influenza-specific ELISA antibodies appeared to be reduced by NAI therapy alone compared to no treatment. Co-administration of claritro with an NAI (N=30) was associated with improved antibody responses. Other clinical outcomes or viral shedding data were not described.	The proportions of children treated in the previous year with oseltamivir or zanamivir alone who developed infection in the 2009–2010 season (predominant A(H1N1)pdm09) were significantly higher than in untreated; the infection rates were intermediate in the clarithro + NAI groups.
N-acetylcysteine[66] (Lai et al, *Ann Intern Med.* 2010)	Standard care, including oseltamivir (initially 75mg twice daily, then 150mg twice daily), empirical	**Case report.** One patient with fulminant viral pneumonitis and multi-organ failure. The report states that the patient had no predisposing co-morbid conditions but	The primary finding reported is that clinical improvement, including resolution of pyrexia and inflammatory markers, appeared to be concurrent with the initiation of NAC infusion.	This single case report of NAC treatment in severe influenza cannot provide evidence of efficacy. However it is a very useful demonstration of the safe use of the drug in critical influenza ,and

	antibiotics (including clarithromycin) and hydrocortisone ("physiologic doses") for septic shock.	does not describe the length of the prodromal illness. although tests for bacterial infection were negative, the presence of bacterial co-infection cannot be excluded.	Once the infusion was stopped, the pyrexia returned. After the NAC infusion was restarted, there was a further improvement in clinical condition with resolution of pyrexia.	this information may be of value in the design of a future clinical trial.

Appendices. Summaries of Pre-clinical and Clinical Combination Studies of Influenza Antivirals, Immunomodulators, and Chinese Traditional Medicines

Appendix 3. Clinical Studies of Combinations of Influenza Antivirals and Chinese Traditional Medicines			
Combination (reference)	**Model**	**Main findings**	**Comment**
Oseltamivir + Baicalin[67] (Su Y. et al., Chinese Journal of Hospital Pharmacy 2014)	Clinical trial in severe influenza A (H1N1). According to a stratified randomization method, the control group was given oseltamivir (n=33), the experimental group was given oral Baicalin and oseltamivir (n=30). Clinical symptoms, laboratory parameters and cellular immune functions were monitored.	Gender and age were comparable between groups, but baseline clinical symptoms and complications, including pneumonia were not compared. The combination was significantly better than oseltamivir monotherapy in improvement of clinical symptoms, chest radiography, and markers of cellular immune function and laboratory indicators ($P<0.05$). The duration of viral shedding was significantly shorter in the experimental group compared with control group ($p<0.05$).	The authors reported that Baicalin combined with antivirals can significantly improve the clinical course and immunity of patients with severe influenza A(H1N1) influenza. Definitions of clinical improvement and chest radiography/CT scan changes were not clearly described, nor were data on changes of LDH, CK, CRP and CD8 counts.. No adverse effects data were reported.

Oseltamivir + Lianhuaqingwen[68] (Jiang T. J. et al., Chinese General Practice 2010)	**Clinical trial** A total of 325 ambulatory patients with influenza A (H1N1) was randomly divided into four groups: 95 received oseltamivir as group A, 97 took oseltamivir and Lianhuaqingwen capsule as group B, 90 were treated with oseltamivir and Banlangen particle as group C and 43 with Lianhuaqingwen capsule as group D. The clinical and virological outcomes on the third and fifth day among the four groups were compared.	Gender and age were comparable between groups, but there was significant differences of fever among groups ($p=0.04$). The rates of body temperature normalization, clinical symptoms resolution, and viral clearance in groups A, B, C were higher than those in group D ($p<0.05$). These parameters were similar in group A compared to groups B and C ($p>0.05$). The efficacy tended to be better in group B with oseltamivir and Lianhuaqingwen on third and fifth day, but the difference was not significant ($p>0.05$).	The study confirmed that early use of oseltamivir can shorten the duration of symptoms and viral shedding. Combination therapy with Lianhuaqingwen and oseltamivir appears to warrant further study but Banlangen did not improve efficacy. No randomization procedures and blinding were described in the paper. No adverse effects data were reported.

Appendices. Summaries of Pre-clinical and Clinical Combination Studies of Influenza Antivirals, Immunomodulators, and Chinese Traditional Medicines

Oseltamivir + GegenjiejiTang[69] (Tan . H. et al., Journal of Guangzhou University of Traditional Chinese Medicine 2010)	**Clinical trial** Hospitalized patients with laboratory confirmed influenza A (H1N1) were divided into four groups: oral GegenjiejiTang (n=29), oseltamivir (n=43), oseltamivir + GegenjiejiTang (n=42) and placebo (n=15). The duration of fever and viral shedding, length of stay in hospital were compared between the groups.	Gender and age were comparable between groups, but baseline clinical symptoms and complications, including pneumonia were not compared. The length of stay in hospital and duration of viral shedding was shorter in oseltamivir group compared with placebo group ($p<0.05$). The resolution of fever was better in combination group ($p<0.05$) compared with single therapy and placebo ($p<0.05$). No significant differences in reducing the duration of viral shedding were found ($p>0.05$).	The authors reported that combination therapy with GegenjiejiTang and oseltamivir might fasten fever recovery, but no difference in reducing duration of viral shedding. Randomization procedures (unbalanced enrollment), inclusion and exclusion criteria, blinding methods, and the method of virological monitoring were not clearly described. No adverse effects data were reported.
Ribavirin + Xiyanping[70] (Li Sh. Zh. et al., Journal Of Practical Traditional Chinese Internal Medicine. 2012)	**Clinical trial** A total of 102 hospitalized cases were randomized by coin toss into two groups: Xiyanping infusion (n=54) and the control group given intravenous ribavirin (n=48). The efficacy was defined as fever recovery after 72 h therapy.	Resolution of fever was faster in Xiyanping group compared with ribavirin group ($p<0.01$).	The authors concluded that combination therapy with Chinese medicine and ribavirin is better than ribavirin alone in fever recovery, but they did not enroll any patients with combination therapy. The conclusions were only based on the comparison between Chinese medicine and ribavirin as single therapy. No adverse effects data were reported. .

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Oseltamivir + Banlangen[71] (Tu B. et al., Med J Chin PAPF 2013)	**Clinical trial** A total of 235 inpatients with influenza A(H1N1) infection were divided into two groups: oseltamivir + oral Banlangen (n=128) and control group, oseltamivir alone (n=107). Fever duration, clinical symptoms, and length of stay were compared between both groups.	The two groups were comparable in baseline age, gender, symptoms and WBC ($p>0.05$). Resolution of fever, cough, sputum, sore throat was better in combination group ($p<0.05$). Duration of hospitalization was significantly shorter in combination group (mean ± SD days ,5.2 ± 3.8 vs 7.9-5.4 ds, $P<0.05$). .	The authors concluded that combination therapy with Banlangen and oseltamivir is better than oseltamivir alone. Randomization procedures and blinding methods were not reported in the text. No adverse effects data were reported.
Oseltamivir + Tanreqing [72] (Xie Y. et al., China Modern Doctor. 2010)	**Clinical trial** A total of 87 inpatients with influenza A(H1N1) infections were divided into two groups: oseltamivir + intravenous Tanreqing (n=44) and oseltamivir alone(n=43). Clinical efficacy was compared between the groups.	No comparisons of baseline age, gender and symptoms were done between groups. Clinical efficacy was better in combination group ($p<0.01$). No side effects were reported in both groups. No data of length of stay was reported.	The authors concluded that combination therapy with Tanreqing and oseltamivir is better than oseltamivir alone. The definition of clinical efficacy is unclear in the text. In addition to limited number of cases, randomization procedures and blinding methods were not described.

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Oseltamivir + Tanreqing[73] (Qian J. et al., Journal of Jilin Medicine 2011)	**Clinical trial** A total of 54 inpatients with influenza A(H1N1) infections were divided into two groups: oseltamivir + iv Tanreqing (n=25) and oseltamivir alone (n=29). Clinical efficacy and duration of hospitalization were compered.	Age, gender and symptoms were comparable between groups (P>0.05). Clinical efficacy and length of stay in hospital (mean + SD days, 7.2 ± 1.9 vs 8.2 ± 1.7 was better in combination group ($p<0.05$). Adverse effects were similar between groups.	The authors reported that combination therapy with Tanreqing and oseltamivir is better than oseltamivir alone. In addition to the limited number of cases, randomization procedures and blinding methods were not described.
Oseltamivir + Tanreqing[74] (Li G. et al., JETCM 2010)	**Clinical trial** A total of 110 inpatients with influenza A(H1N1) were randomly divided into the oseltamivir alone group (n=55 patients) and the experimental group (n=55 patients) that received oseltamivir and Tanreqing injection once daily. Treatment lasted 7 to 14 days. Overall effects, key symptoms and physical signs and the radiologic changes were compared.	No comparisons of baseline age, gender and symptoms were done between groups. Overall effectiveness of the combination group was superior to that of the control group. Improvements were also noted in clinical symptoms and radiologic changes.	The authors concluded that combination therapy with Tanreqing and oseltamivir is better than oseltamivir alone. The definitions of clinical efficacy, randomization procedures, blinding methods were not clearly described. No adverse effects data were reported.

Oseltamivir + Maingshigan/Yinqiaosan[75] (Wang C. et al., Ann Intern Med 2011)	**Clinical trial** A multicenter, prospective non-blinded, randomized controlled trial compared the efficacy and safety of oseltamivir, Maxingshigan/Yinqiaosan, and the combination of both in treating 410 adult outpatients with A(H1N1)pdm09 infection. Primary outcome was time to fever resolution. Secondary outcomes included symptom scores and viral shedding based on real time RT-PCR. (ClinicalTrials.gov number: NCT00935194)	Compared to control (26.0h, 95% confidence interval [CI] 24.0 to 33.0h), the estimated median time to fever resolution was significantly reduced by 34% in oseltamivir (95% CI 20% to 46%, p<0.001), by 37% in Maxingshigan/ Yinqiaosan (95% CI 23% to 49%, p<0.001), and by 47% in osletamivir plus Maxingshigan/ Yinqiaosan (95% CI 35% to 56%, p<0.001). Oseltamivir+ Maxingshigan/Yinqiaosan exhibited a borderline statistically significant reduction in time to fever resolution compared to oseltamivir (19% , 95% CI 0.3% to 34%, p=0.05). There was no difference in the decline of symptom scores comparing any intervention to control (p=0.38). Two patients given Maxingshigan/Yinqiaosan reported nausea and vomiting.	The authors reported that both oseltamivir and Maxingshigan/ Yinqiaosan alone and in combination reduced time to fever resolution in patients with H1N1 virus infection. Combination exhibited a borderline statistically significant reduction in time to fever resolution compared to oseltamivir alone. The limitations include that the study subjects were young and had mild A(H1N1) infection. Missing data prohibited definitive conclusions about effects on viral shedding.

Abbreviations: OC, oseltamivir carboxylate; OP, oseltamivir phosphate; PMV, peramivir; ZNV, zanamivir; $CCID_{50}$, median cell culture infectious dose; $LD_{50,}$ median lethal dose; MLD_{50}, mouse median lethal dose; $EC_{50,}$ 50% effective concentration; $TC_{50,}$ 50% cytotoxic concentration; IM, intramuscular; IV, intravenous; IQR, interquartile range; RT-PCR, reverse transcriptase-polymerase chain reaction

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Appendices. Summaries of Pre-clinical and Clinical Combination Studies of Influenza Antivirals, Immunomodulators, and Chinese Traditional Medicines

The following studies were identified during our literature search but were not included in the table summarising combinations of influenza antivirals (appendix 1), because they did not assess standardised compounds, and/or were not published in English, and/or were not available in English:

Serkedjieva J. Combined antiinfluenza virus activity of Flos verbasci infusion and amantadine derivatives. Phytother Res. 2000 Nov;14(7):571-4.

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Haidari M, Ali M, Ward Casscells S 3rd, Madjid M. Pomegranate (Punica granatum) purified polyphenol extract inhibits influenza virus and has a synergistic effect with oseltamivir. Phytomedicine. 2009 Dec;16(12):1127-36. doi: 10.1016/j.phymed.2009.06.002. Epub 2009 Jul 7.

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Grishchenko S, Lavrukhina L, Ketiladze E, Krylov V, Ershov, F. Rezul'taty kompleksnoi terapii bol'nykh grippom, oslozhennnym pnevmoniei, s ispol'zovaniem levamizola. [Results of combined therapy using levamisole for patients with influenza complicated by pneumonia]. [Russian]. Voprosy Virusologii. 29(2):175-9, 1984 Mar-Apr.

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