Triple combination of oseltamivir, amantadine, and ribavirin displays synergistic activity against multiple influenza virus strains in vitro

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ABSTRACT

The recurring emergence of influenza strains resistant to available antiviral medications has become a global health concern, especially in light of the potential for a new influenza pandemic. Currently, virtually all circulating strains of influenza A in the United States are resistant to either of the two major classes of anti-influenza drugs (adamantanes and neuraminidase inhibitors). Thus, new therapeutic approaches that can be rapidly deployed and that will address the issue of recurring resistance should be developed. We have tested double and triple combinations of the approved anti-influenza drugs, oseltamivir and amantadine, together with ribavirin against three influenza virus strains using cytopathic effect inhibition assays in MDCK cells. We selected A/New Caledonia/20/99 (H1N1) and A/Sydney/05/97 (H3N2) as representatives of the wildtype versions of the predominant circulating seasonal influenza strains, and A/Duck/MN1525/81 (H5N1) as a representative of avian influenza strains. Dose response curves were generated for all drug combinations, and the degree of drug interaction was quantified using a model that calculates the synergy (or antagonism) between the drugs in double and triple combinations. This report demonstrates that a triple combination of antivirals was highly synergistic against influenza A. Importantly, the synergy of the triple combination was 2- to 13-fold greater than the synergy of any double combination, depending on the influenza subtype. These data support the investigation of a novel combination of oseltamivir, amantadine, and ribavirin as an effective treatment for both seasonal and pandemic influenza, allowing efficient use of the existing drug supplies.
Influenza epidemics are responsible for significant morbidity, mortality, and economic burden annually in the US, including an estimated 41,000 deaths, over 290,000 hospitalizations, and 44 million days of lost productivity (34). Currently two classes of drugs are approved for the treatment of influenza, the adamantanes and the neuraminidase inhibitors (NAIs). When used to treat susceptible seasonal influenza, these antiviral drugs provide a modest benefit by reducing symptoms by approximately 1.5 days in otherwise healthy patients if treatment is initiated within 48 hours of symptom onset (22, 27, 37). However, the therapeutic benefit of these antiviral drugs in cases of severe infection by highly pathogenic avian influenza is less clear. In cases of sporadic H5N1 influenza infection, the data suggest that, while treatment with antivirals may provide some benefit, the mortality rate remains close to 60% (1, 28). Thus, as single agents, influenza drugs do not exhibit sufficient potency to treat severe influenza infections.

The effectiveness of the adamantanes and neuraminidase inhibitors has been eroded by emerging viral resistance, both treatment-induced and naturally occurring. Resistance to the adamantanes, which block the M2 channel and prevents viral uncoating, emerges rapidly in treated patients (21), and resistant strains are transmissible (2). In recent years, the level of resistance to adamantanes has risen to such a high level globally that this drug class is no longer recommended as monotherapy (3, 12). Most recently, resistance to amantadine developed in the majority of A/H3N2 viruses in the United States, such that in the 2008-2009 influenza season, virtually 100% of the characterized A/H3N2 viruses were resistant to amantadine (6). Sporadic resistance to oseltamivir, the most widely used neuraminidase inhibitor, was reported as early as 1999 (8), and the development of drug resistance has been documented with the use of oseltamivir against both seasonal influenza (26, 29, 35) and avian influenza (11). Whether treatment-induced or naturally occurring, widespread resistance to oseltamivir in A/H1N1...
seasonal influenza emerged in Europe in early 2008, and are now dominant over large portions of Europe, Asia, North America, and the Southern Hemisphere (44, 54). In 2009 influenza season, 99.4% of all A/H1N1 viruses isolated from patients in the US were resistant to oseltamivir (6). As a result, virtually all circulating strains of influenza A in the US are currently resistant to either of the two classes of anti-influenza drugs. In light of the widespread resistance patterns among H1N1 and H3N2 subtypes, and with no rapid diagnostic tools to characterize resistance available, the continued use of these drugs as monotherapies may result in dual resistance, raising the specter of treatment-induced multi-drug resistant influenza. In fact, this phenomenon has been documented in severely immunocompromised patients, where the sequential use of NAIs and M2 inhibitors resulted in the generation of viruses resistant to both drugs (26, 52).

The emergence of drug-resistant mutants is a significant problem not only for influenza but also for other rapidly mutating viruses (7, 30, 43, 49). For these viruses, the use of antiviral drugs in combination has proven to be an effective strategy for suppressing the development of drug resistance, resulting in the durability of treatment regimens. For example, it has been known since the mid to late 1990s that of the simultaneous use of three antiviral agents in combination for human immunodeficiency virus (HIV) will block viral replication and decrease the probability of the emergence of resistance, effectively establishing a chemico-genetic barrier to drug-resistant mutations (16, 17).

With regards to the use of combination therapy for influenza, clinical studies have tested the safety and drug interactions of double combinations of available anti-influenza drugs (36), and a number of studies have looked at the effect of double drug combinations for the treatment of influenza \textit{in vitro} (14, 19, 20, 23, 32, 45) and in animals (13, 25, 31, 45, 48). To date, there are no published studies on the effects of triple antiviral drug combinations for influenza.
In order to address dual problems of potency and resistance in treating severe influenza, including avian influenza, we chose to optimize the use of existing antivirals and to determine the effectiveness of triple drug combinations for treating influenza. We hypothesized that a triple combination of drugs with different mechanisms of action, and which act at three different stages at the viral life cycle, could result in synergistic antiviral activity. In this study, we evaluated the interactions between oseltamivir, amantadine, and ribavirin. To test our hypothesis that these drugs might interact synergistically, we explored the \emph{in vitro} antiviral activity and synergism of the single, double, and triple treatments against a panel of influenza A viruses. Our results show that these drugs act synergistically, with triple combinations showing greater synergy than any of the double combinations evaluated. Furthermore, the synergy of the triple combination was maintained across multiple strains representing different influenza A subtypes, including the three major subtypes that currently cause significant morbidity and mortality in humans (H1N1, H3N2, and H5N1). To our knowledge, this is the first time the antiviral activity and synergism of a triple combination of oseltamivir, amantadine and ribavirin for influenza has been demonstrated.
MATERIALS AND METHODS

Antiviral compounds. Oseltamivir carboxylate (the active metabolite of oseltamivir) was obtained from Charnwood Molecular (Loughborough, U.K.) through synthesis via the N\textsubscript{Boc} protected acid from oseltamivir phosphate. Amantadine was obtained from Spectrum Chemical Manufacturing Corp. (Gardena, CA). Ribavirin was purchased from Sigma-Aldrich (St. Louis, MO).

Influenza virus. Influenza A/New Caledonia/20/99 (H1N1) and A/Sydney/05/97 (H3N2) virus were provided by the Centers for Disease Control and Prevention (Atlanta, GA). Influenza A/duck/MN/1525/81 (H5N1) was provided by Dr. Robert Webster at St. Jude’s Medical Center (Memphis, TN). The viruses were passaged in Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection, Manassas, VA) to create working stocks, which were used for the antiviral assays. Additionally, the genes for the matrix protein 2 (M2), hemagglutinin (HA), and neuraminidase (NA) for each virus were sequenced. Sequencing revealed that for each gene and each virus, there was $\geq 98\%$ identity with published sequences, and no mutations were identified that are known to confer resistance to either oseltamivir or amantadine.

Cells and growth media. Virus was grown in MDCK cells. Cells were passaged in minimal essential media (MEM) containing 5\% fetal bovine serum (Hyclone Laboratories, Logan, UT). During antiviral evaluations, the serum was removed and the media was supplemented with gentamicin (50 $\mu$g/ml), trypsin (10 units/ml) and EDTA (1 $\mu$g/ml).

Cell-based assays. To obtain monotherapy dose-response curves, individual drugs were added to MDCK cells in 96-well microplates (8 x 10\textsuperscript{4} cells/well) using three wells for each concentration used. The compounds were added at the following concentrations: oseltamivir carboxylate at 0, 0.000032, 0.0001, 0.00032, 0.001, 0.0032, 0.01, 0.032, 0.1, 1.0, 10.0 and 100 $\mu$g/ml; amantadine and ribavirin at 0, 0.001, 0.0032, 0.01, 0.032, 0.1, 0.32, 1, 3.2, 10, 32 and 100 $\mu$g/ml. Untreated wells of infected cells...
(virus controls) and uninfected cells (cell controls) were included on each test plate. At three days post-infection, the virus control wells exhibited 100% cytopathology.

For combination studies, each drug was tested in double and triple combinations at 6 doses (including no drug), in which the high dose for each drug was set to be above the 50% effective concentration (EC\textsubscript{50}) for the virus. The doses for influenza H1N1 were: oseltamivir carboxylate at 0, 0.0032, 0.01, 0.032, 0.1, 0.32 µg/ml; amantadine at 0, 0.01, 0.032, 0.1, 0.32, 1 µg/ml; and ribavirin at 0, 0.032, 0.1, 0.32, 1, 3.2 µg/ml. The doses for influenza H5N1 were: oseltamivir carboxylate at 0, 0.0032, 0.01, 0.032, 0.1, 0.32, 1 µg/ml; and ribavirin at 0, 0.01, 0.032, 0.1, 0.32, 1, 3.2 µg/ml. The doses for influenza H3N2 were: oseltamivir carboxylate at 0, 0.001, 0.0032, 0.01, 0.032, 0.1 µg/ml; and amantadine and ribavirin at 0, 0.032, 0.1, 0.32, 1, 3.2 µg/ml. Five independent experiments with 13 total replicates were conducted for influenza H1N1; four independent experiments with 12 total replicates were conducted for influenza H5N1; and one experiment with 2 total replicates was conducted for the influenza H3N2.

**Neutral Red assay.** The extent of viral cytopathology in each well was determined microscopically by inspection and by staining with neutral red (NR) as detailed elsewhere (46). Briefly, the cells were stained with 0.011% NR diluted in MEM to determine cell viability. Two hours later the plates were processed for quantification of NR uptake into viable cells. The amount of NR taken up by cells was determined spectrophotometrically.

**Virus yield reduction.** To validate cytopathic effect as a measure of synergistic activity, the amount of H3N2 infectious virus produced in the presence of inhibitors was quantified from the supernatants from the same wells used for the NR assay. Duplicate wells were pooled and virus yield, as quantitated by the 50% tissue culture infectious dose (TCID\textsubscript{50}), was determined by titrating samples in MDCK cells in 96-well plates using the endpoint dilution method as previously described (42, 46).
Quantitative real-time PCR. To validate cytopathic effects as a measure of synergistic activity, the amount of H3N2 viral RNA in the supernatants of each well used for NR assays was also analyzed by quantitative real-time PCR (qPCR) using primers directed towards the matrix gene. Methods for qPCR were adapted from Ward et al. (53). Briefly, viral RNA was extracted from 140 μL of supernatant using the QIAamp Viral RNA mini kit (Qiagen, Valencia, CA) and eluted into 40 μL of AVE buffer. The RNA was reverse transcribed using the Qiagen Omniscript RT kit using a primer directed to the matrix gene (5’TCT AAC CGA GGT CGA AAC GTA3’) using 12 μL of RNA in 20 μL of reaction volume. Real-time PCR was performed using 2 μL of template in a volume of 10 μL of the Applied Biosystems (Foster City, CA) Taqman Universal Master mix and run on the real-time PCR 7900HT system. Sequences for the forward and reverse primers were 5’AAG ACC AAT YCT GTC ACC TCT GA3’ and 5’CAA AGC GTC TAC GCT GCA GTC C3’, respectively, and the sequence for the probe was 5’FAM CGT GCC CAG TGA GC3’. A standard curve is necessary to produce accurate copy number estimates in a reaction. The qPCR assay standards were constructed by ligation of a PCR amplified product of segment 7 (matrix gene) into a plasmid vector. The plasmid DNA was amplified in E. coli strain TOP10 and purified using a Qiagen plasmid purification kit. The plasmid insert sequence was confirmed with sequencing in both directions using six different primers. The concentration and purity of the plasmid DNA was calculated by measuring the OD260/280. These data were used to calculate target copy number in the standard. Our standard curves were created using 10-fold dilutions from $10^9$ to $10^1$ target copies/reaction and had a typical $R^2$ of 0.99 (data not shown). Each real time analysis is run with at least four standards. The assay is able to detect <10 target copies/reaction and is able to quantify accurately down to 100 target copies/reaction.

EC$_{50}$ and synergy calculations. EC$_{50}$ calculations were made by normalizing the NR data for each well against the virus control data, which was assumed to represent 100% virus infection.
Normalized data were plotted as percent infected cells versus compound concentration. The data points were then fitted using four-parameter curve fitting in Graphpad Prism (Graphpad Software, La Jolla, CA) to derive the EC\textsubscript{50}. Statistical comparisons between best-fit EC\textsubscript{50} values for any two curves were performed in Prism using the extra sum-of-squares F test; differences in EC\textsubscript{50} values between two curves with a P-value of <0.05 were considered significant.

Synergy was calculated using the MacSynergy II software developed by Prichard and Shipman, which was modified to accommodate a three-drug combination (38) and is similar to that reported previously describing this approach (39). The theoretical additive interactions were calculated from the concentration-response curves of each drug as a single agent. This calculated additive surface was then subtracted from the observed, experimental surface to reveal regions that deviate from the calculated additive effects. Purely additive interactions are represented as areas in grey, indicating that they do not differ from the calculated additive effects. Synergistic interactions result in greater inhibition than the calculated inhibition, and are represented as areas in blue. Conversely, antagonism is represented as areas in red. Where indicated, synergy volumes are shown at a level of 95% confidence, which eliminates insignificant deviations from the additive surface.

Synergy volume for each double and triple combination was also calculated, which represents the sum of the synergy or antagonism across all concentrations of a combination. Synergy volumes are presented as a quantitative measure of the overall interaction of the drugs within a combination. As determined by cytopathic effect (NR assay), synergy volumes >100 µg/ml\textsuperscript{2%} for double combinations or >100 µg/ml\textsuperscript{3%} for triple combinations are considered to be strongly synergistic. Conversely, combinations with synergy volumes < -100 µg/ml\textsuperscript{2%} or µg/ml\textsuperscript{3%} are considered to be antagonistic. Synergy volumes between -100 and 100 µg/ml\textsuperscript{2%} or µg/ml\textsuperscript{3%} are ambiguous and are not thought to be biologically significant.
RESULTS

Antiviral activity of oseltamivir carboxylate, amantadine, and ribavirin alone and in combination. We initially selected the H1N1 influenza virus subtype to perform our synergy analysis. The sensitivity of A/New Caledonia/20/99 (H1N1) influenza virus replication to oseltamivir carboxylate, amantadine, and ribavirin treatment was determined by measuring the inhibition of virus-induced cytopathic effect in MDCK cells as determined by staining by Neutral Red (NR). As shown in Figure 1, an inhibition dose-response of influenza H1N1 replication was determined for each drug. The 50% effective concentration (EC$_{50}$) for oseltamivir carboxylate, amantadine, and ribavirin as single agents were determined to be 0.14 µg/ml (0.49 µM), 0.21 µg/ml (1.1 µM), and 5.1 µg/ml (20.4 µM), respectively.

We next tested the activity of each drug in the presence of fixed concentrations of the second and third drugs against influenza H1N1 replication at a range of concentrations where the assay response was still linear. As shown in Figure 2A, the potency of oseltamivir carboxylate was enhanced by the presence of either ribavirin at 1 µg/ml or amantadine at 0.032 µg/ml, as demonstrated by the shift of the dose-response curves to the left compared to oseltamivir alone, indicative that inhibition occurred at lower concentrations of oseltamivir. In the presence of both ribavirin and amantadine at the same concentrations used in the double combinations, there was a further leftward shift in the dose-response curve, indicative of additional synergy in the triple combination compared to double combinations. Figures 2B and 2C show that, similar to oseltamivir carboxylate, the potencies of amantadine and ribavirin were enhanced modestly in the presence of a second drug, and were further enhanced in the triple combination.

The EC$_{50}$ values for the inhibition of influenza H1N1 replication for oseltamivir carboxylate, amantadine, and ribavirin as single agents and in double and triple combinations are summarized in...
Table 1. For each drug, the EC\textsubscript{50} was reduced in triple combination compared to the EC\textsubscript{50} in double combinations and as a single agent. For example, the EC\textsubscript{50} for oseltamivir as monotherapy was reduced by 2.5-fold or 1.8-fold in double combination with 1 µg/ml ribavirin or 0.032 µg/ml amantadine, respectively. By comparison, the EC\textsubscript{50} of oseltamivir carboxylate was reduced by 8.8-fold in combination with both ribavirin and amantadine at the same concentrations. Moreover, the EC\textsubscript{50} of oseltamivir carboxylate in triple combination showed a 3.4-fold reduction as compared to oseltamivir carboxylate in double combination with ribavirin, and a 4.8-fold reduction as compared to oseltamivir carboxylate in double combination with amantadine. Likewise, the triple drug combination reduced the EC\textsubscript{50} of amantadine and ribavirin by 3.6-fold and 12.8-fold, respectively, compared to monotherapy; and 1.4- to 7-fold compared to the double combinations. Thus, the activity of each drug was greater in triple combination than in double combination or as a single agent, indicative that each drug was effective at a lower concentration.

Importantly, the data presented here do not represent the maximum reductions in EC\textsubscript{50} values for the three drugs. Due to the dynamic range of the assay, we were only able to obtain precise dose-response curves for each drug at fixed concentrations of the second and third drugs which were well below their EC\textsubscript{50} values, and well below concentrations where maximum synergy occurred (see below). At higher concentrations, the antiviral activity of the second and third drug contributed significantly to the inhibition, which decreased the linear range of the assay and reduced the accuracy of the curve-fitting. A comprehensive assessment of the interaction of two or three drugs in combination requires the evaluation of multiple concentrations of each drug in order to quantify synergy over the entire dosing range.

**Synergy of double and triple combinations.** We next assessed the interactions of the drugs in double combinations and in triple combination over multiple concentrations of each drug using the NR
assay. The data are presented as contour plots, in which regions where inhibition is greater (synergy) or less (antagonism) than expected are identified by subtracting the observed inhibition from the theoretical additive inhibition. Synergy plots of double and triple combinations against influenza H1N1 replication are shown in Figure 3. Synergy plots showed that amantadine and oseltamivir carboxylate were synergistic against H1N1 replication over wide concentrations of both drugs, whereas double combinations of amantadine plus ribavirin or oseltamivir carboxylate plus ribavirin were largely additive (Figure 3). For amantadine and oseltamivir carboxylate, synergy was observed within the concentration range of 0.01 to 0.32 µg/ml for amantadine and 0.01 to 0.32 µg/ml for oseltamivir carboxylate. Synergy plots for the triple drug combination showed a dose-dependent increase in synergy with respect to amantadine, with maximal synergy occurring at 0.1 µg/ml amantadine (top plane). At this concentration, synergy was observed at all concentrations of oseltamivir carboxylate and ribavirin tested. Above 0.1 µg/ml amantadine, the limits of detection for the assay were reached, and thus no additional increase in efficacy could be determined. No significant antagonism was observed.

We repeated these studies against two additional influenza virus subtypes, H5N1 and H3N2, to assess the spectrum of this antiviral activity. Figure 4 shows the synergy plots obtained for A/Duck/MN/1525/81 (H5N1). Amantadine and oseltamivir carboxylate were synergistic from 0.01 µg/ml to 0.1 µg/ml amantadine and 0.0032 µg/ml to 0.32 µg/ml oseltamivir carboxylate. For this subtype, as observed with influenza H1N1, little synergy was observed between amantadine and ribavirin. Synergy was observed for the oseltamivir carboxylate and ribavirin double combination over most of the ribavirin concentrations and at higher oseltamivir carboxylate concentrations (between 0.032 and 0.1 µg/ml). For the triple combination, as with influenza H1N1, the synergy increased with increasing amantadine concentration up to 0.32 µg/ml.
Figure 5 shows double and triple synergy plots obtained against A/Sydney/05/97 (H3N2).

Amantadine and oseltamivir carboxylate were synergistic against H3N2 in the range of 0.032 to 1 µg/ml and 0.0032 to 0.1 µg/ml for these drugs, respectively. Synergy was observed between amantadine and ribavirin within the concentration ranges 0.032 to 3.2 µg/ml for amantadine and 0.1 to 3.2 µg/ml for ribavirin. No synergy was observed between oseltamivir carboxylate and ribavirin. As was observed for influenza H1N1 and H5N1, the triple drug combination was highly synergistic against H3N2 virus subtype, and synergy was dose-dependent with respect to amantadine. Maximal synergy occurred at 0.32 µg/ml amantadine, above which synergy was not measurable due to the contribution from amantadine. At the amantadine concentration where maximum synergy occurred, synergy was observed at all concentrations of oseltamivir carboxylate and ribavirin. Results from H1N1, H5N1 and H3N2 strains taken together suggest that the synergy observed with the three drug combination is robust, with synergy being observed in all influenza A strains tested to date.

Additionally, no cytotoxicity was observed for the drug combinations. The 50% cytotoxic concentrations (TC_{50}) was >32 µg/ml for amantadine as a single agent, and >100 µg/ml for oseltamivir carboxylate and ribavirin. Within the concentration ranges tested for the combination studies (1 µg/ml or less for amantadine, 0.32 µg/ml or less for oseltamivir carboxylate, and 10 µg/ml or less for ribavirin), no cytotoxicity was observed for any double combination or the triple combination (data not shown).

**Comparison of synergy for double and triple combinations across subtypes.** Synergy volumes, the cumulative synergy and antagonism across all doses for a drug combination, for the double and triple drug combinations as determined by the NR assay against all three influenza virus subtypes are provided in Table 2. Synergy volumes are presented as the mean between experiments with standard deviation for the H1N1 and H5N1 viruses, and between replicates with standard deviation for the H3N2
virus. For the H1N1 and H5N1 viruses, the average variation in synergy volume between replicates within an experiment was 25%. Data for the triple combination at amantadine concentrations above the EC$_{50}$ (0.21 µg/ml for H1N1, Table 1) were excluded due to the efficacy of amantadine alone, which precluded the assessment of synergy. Synergy for the double combinations varied significantly depending on the drugs in the combination and the virus subtype. For instance, amantadine plus oseltamivir carboxylate was synergistic against all three subtypes, whereas the amantadine plus ribavirin was synergistic against influenza H3N2, but not H1N1 or H5N1. Finally, oseltamivir carboxylate plus ribavirin was not synergistic against any subtype.

In contrast, the triple combination produced strong synergy across all subtypes, with maximal synergy volumes approximating or exceeding 1000 µg/ml against all three subtypes. Figure 6 compares the synergy volumes for double combinations and the triple combination at 0.1 µg/ml amantadine against all three subtypes. For the influenza H1N1 and H5N1, the maximal synergy occurred at 0.1 µg/ml amantadine, while the maximal synergy occurred at 0.32 µg/ml amantadine for influenza H3N2, reflecting the slightly increased EC$_{50}$ value of amantadine against the H3N2 strain. At 0.1 µg/ml amantadine, the synergy for triple combination was up to 2- to 13-fold greater than the synergy for any double combination, depending on the subtype. Thus, the triple combination was highly synergistic against strains from all three influenza A subtypes, and the synergy of the triple combination was greater than the synergy for all double combinations.

**Comparison of synergy using multiple endpoints.** We further evaluated the interactions of the drug combination against the H3N2 strain by quantitating synergy using more direct endpoints of viral replication such as viral titer and RNA copy number. Aliquots of supernatants from the NR assay were retained and viral titer was determined by TCID$_{50}$ assay and genome copy number was measured by qPCR, and synergy (reduction in viral load above expected) was calculated in the same manner.
Synergy plots for the double and triple combinations as determined by TCID\textsubscript{50} and qPCR are provided in Figures 7 and 8, respectively. This analysis confirmed the results from the NR assay and showed that viral titer and genome copy number were synergistically inhibited by the three drug combination. Synergy calculations using both TCID\textsubscript{50} and qPCR endpoints revealed that the amantadine plus oseltamivir carboxylate and amantadine plus ribavirin double combinations were synergistic, whereas the ribavirin plus oseltamivir carboxylate double combination was additive. For the triple combination, synergy increased as a function of amantadine concentration, starting at 0.1 µg/ml and reaching a maximum at to 0.32 µg/ml amantadine, similar to the NR assay. For the TCID\textsubscript{50} assay, synergy was observed at similar concentration ranges of each drug in double and triple combinations as seen with the NR assay (Figure 7). For the qPCR assay, the synergy was more modest than seen with NR or TCID\textsubscript{50} assays, particularly for double combinations, but the regions of synergy for double and triple combinations coincided with the regions of synergy determined by the other assays (Figure 8). Thus, the pattern of synergy for double and triple combinations was consistent when assessed by different endpoint measures.

Figure 9 provides a comparison of the synergy volumes for the double combinations and the triple combination at the concentration of amantadine that produced the maximum synergy (0.32 µg/ml) against influenza H3N2 as determined by NR, TCID\textsubscript{50}, and qPCR. The percent inhibition above expected as determined by the NR assay was converted to \( \log_{10} \) scale in order for the synergy volume to be expressed in the same units as synergy volumes determined by TCID\textsubscript{50} and qPCR assays. The synergy volumes as determined by all three endpoints show similar trends, with the amantadine plus oseltamivir carboxylate and amantadine plus ribavirin double combinations having greater synergy volumes than the oseltamivir carboxylate plus ribavirin double combination. Importantly, all three
endpoints show that the synergy volume of the triple combination was significantly greater than the synergy volume of any double combination.
DISCUSSION

The pharmacological rationale for the use of triple combination therapy in the treatment of influenza is supported by the demonstrated superiority of triple combination therapy over single and double combination therapy against HIV infection. With HIV infection, maintaining the plasma viral load below detectable levels is associated with the durability of antiviral effect and sustained virologic response (40). Previous studies have shown that a combination of three drugs given simultaneously for the treatment of HIV were highly effective at suppressing viral load and preventing the emergence of resistance (16, 17).

In influenza, and particularly avian influenza, high viral load and prolonged viral shedding is associated with poor outcome and the emergence of resistance (10, 11, 26). In the study reported here, we tested the hypothesis that a triple combination of drugs, each with a different mechanism of action, might act synergistically and provide a much higher level of antiviral activity than single or double combination therapies. Oseltamivir interferes with the viral neuraminidase activity, blocking the release of new virions from an infected cell (15, 33). Amantadine inhibits the M2 ion channel, which inhibits fusion of the viral envelope and the endosome membrane, as well as viral uncoating and disassembly (18). Ribavirin interferes with viral replication, although its precise mechanism of action remains to be determined (4, 5, 9).

Previously, the response of double combinations has been explored. The in vitro studies evaluating double combinations for influenza have included: amantadine plus ribavirin (20, 47); amantadine plus oseltamivir (23, 47); rimantadine plus ribavirin (20, 32); rimantadine plus zanamivir, oseltamivir, or peramivir (14); and ribavirin plus peramivir (45), and ribavirin plus oseltamivir (47). The antiviral activity of double drug combinations has also been evaluated in animal studies for the
combinations of oseltamivir plus rimantadine (13, 31), oseltamivir plus amantadine (25, 47), ribavirin plus peramivir (45), ribavirin plus oseltamivir (24, 47, 48), and ribavirin plus amantadine (47).

In aggregate, the body of published work indicates that the synergy of double combinations varied and was dependent on the drug combination, drug dose, experimental design and virus strain. For instance, the combination of rimantadine (another M2 inhibitor) and oseltamivir was synergistic in vitro and in vivo against influenza H1N1 and H3N2 (13, 14), but the combination of amantadine and oseltamivir was not synergistic in vivo against influenza H5N1 (25). In addition, the rimantadine/ribavirin combination was found to be synergistic against influenza H1N1 and H3N2 strains in one study (20), but was found to be additive against different H1N1 and H3N2 strains in another study (32). Finally, both Govorkova et al. (14) and Smee et al. (48) found that oseltamivir in combination with ribavirin was antagonistic, additive, or synergistic in mouse models, depending on the virus strain and/or the dose of the drugs. The variability in synergy for double combinations reported in literature, whether due to the specific drugs in the combinations or to virus strain, is consistent with the in vitro data we present here for double combinations. For instance, while we found that amantadine plus oseltamivir was uniformly synergistic against all three subtypes, amantadine plus ribavirin was synergistic for H3N2 but additive for H1N1 and H5N1, and ribavirin plus oseltamivir was additive for all three subtypes (Table 2). Recently, Smee et al. tested double combinations of amantadine plus oseltamivir, amantadine plus ribavirin, and ribavirin plus oseltamivir in vitro on the same H5N1 virus that was used in this study (A/Duck/MN/1525/81)(47). Similar to the data presented here, the authors found that amantadine plus oseltamivir was synergistic and that ribavirin plus oseltamivir was additive. However, the authors did find that amantadine plus ribavirin was synergistic, as opposed to our data that showed that this combination was additive. These differences may be attributable to the different doses
of each drug used in the studies, different virus stocks, different multiplicities of infection, and/or
different cell stocks.

In contrast to double combinations where synergy was variable, our data clearly demonstrate for
the first time that the triple combination of oseltamivir, amantadine, and ribavirin was uniformly
synergistic against multiple influenza A subtypes (H1N1, H3N2, and H5N1). Using the same
experimental design and dosing ranges, and also the same cell and virus stocks, we can directly compare
the synergy between double and triple combinations. We found that the synergy volume of the triple
antiviral drug combination was consistently high (930-1596 µg/ml% at 0.1 µg/ml or higher amantadine)
against all three subtypes, and was greater compared to the double combinations (Figure 6). Depending
on the specific double combination and influenza subtype, the synergy volume of the triple combination
was 2- to 13-fold greater than the synergy volume of any double combination. Synergy resulted in the
increase in the antiviral activity of each drug in the triple combination compared to its activity in double
combinations or as single agents, as demonstrated by the reduction in the EC50 (Figure 3 and Table 1).

For the H3N2 virus, the superior synergy of the triple combination compared to double
combinations was confirmed using multiple assays which measured different endpoints, including
inhibition of cytopathic effect, reduction in live viral titer, and reduction in viral RNA copies (Figure 9).
All three assays revealed that the synergy volume of the triple combinations was at least 3-fold greater
than the synergy of any double combinations. Minor variations in synergy volumes between the
different assays may be due to the dynamic range of each assay (2 log10 for NR versus 5 to 6 log10 for
TCID50 and qPCR) or the specific endpoint (infectivity for NR and TCID50 versus total RNA for qPCR).
Importantly, the synergy between the three antiviral drugs is observed at concentrations that are
achievable in plasma in humans. Synergy of the triple combination occurred at concentrations of 0.032 –
1 µg/ml for amantadine, 0.001 – 0.32 µg/ml for oseltamivir, and 0.032 – 3.2 µg/ml for ribavirin; these
concentrations are below the steady-state plasma concentration (Css) for the recommended doses of
amantadine (Css=0.43 µg/ml), oseltamivir carboxylate (Css=0.3 µg/ml) and ribavirin (Css=2.2 µg/ml)
(based on data from product labels (41, 50, 51)). This has important implications for the clinical use of
triple antiviral drug combination therapy in humans, where markedly improved antiviral effects might be
expected at doses already known to be achievable and safe, or possibly at reduced doses. In addition, we
observed no significant antagonism in the in vitro model system for any of the drug combinations at any
of the doses evaluated, suggesting that the triple combination is unlikely to lead to reduced efficacy over
monotherapy or double combinations in the clinic.

Our data strongly support the development of a triple antiviral drug combination of oseltamivir,
amantadine, and ribavirin for treatment of both severe seasonal and avian influenza. Further studies are
underway to demonstrate the efficacy of the triple combination in in vivo models of influenza infection,
to evaluate the effectiveness of the triple combination in the treatment of drug resistant influenza strains,
to assess the effect of the triple combination on the emergence of resistance, and to understand the
mechanism of the synergy of the triple combination.
We would like to thank Brett Hurst, Benjamin Christensen, Kelly Sheff, and Erin Kelley for technical assistance, Linda Wuestehube for editorial assistance, Kristin Porter for statistical analysis, and Richard Whitley and Arnold Monto for critical review of the manuscript.
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FIGURE LEGENDS

Figure 1. Inhibition of A/New Caledonia/20/99 (H1N1) induced cytopathic effect in MDCK cells treated with oseltamivir carboxylate (red squares), amantadine (green circles), and ribavirin (blue triangles) as determined by NR assay. Data are presented as the mean of three replicates with standard deviations.

Figure 2. Inhibition of A/New Caledonia/20/99 (H1N1) induced cytopathic effect in MDCK cells treated with double and triple combinations of amantadine (AMT), oseltamivir carboxylate (OSC) and ribavirin (RBV) as determined by NR assay. (A) Dose response for OSC alone (red circles), with 1.0 µg/ml RBV (black squares), with 0.032 µg/mL AMT (blue triangles), and with 1.0 µg/ml RBV plus 0.032 µg/ml AMT (green triangles). (B) Dose response for AMT alone (red circles), with 1.0 µg/ml RBV (black squares), with 0.01 µg/ml OSC (blue triangles), and with 1.0 µg/ml RBV plus 0.01 µg/ml OSC (green triangles). (C) Dose response for RBV alone (red circles), with 0.032 µg/ml AMT (black squares), with 0.032 µg/ml OSC (blue triangles), and with 0.032 µg/ml AMT plus 0.032 µg/ml OSC (green triangles). Data are presented as the mean of three independent experiments (three replicates each) for each condition with standard deviations.

Figure 3. Synergistic inhibition of A/New Caledonia/20/99 (H1N1) replication as determined by NR assay in MDCK cells. Calculated additive interactions were subtracted from the experimentally determined inhibition to reveal regions of synergy (inhibition above expected) or antagonism (inhibition below expected). Values were derived from mean triplicate data and presented at 95% confidence. This experiment was repeated five times with similar results. Areas in blue indicate doses of each drug that are synergistic, gray areas indicate doses that are additive, and red areas indicate doses that are
antagonistic. The intensity of the color (blue or red) corresponds to the percent inhibition above or below expected. (A) Double combinations of amantadine and oseltamivir carboxylate (top); amantadine and ribavirin (middle); ribavirin and oseltamivir carboxylate (bottom). Concentrations of each drug are indicated on the axes. (B) Triple combinations of oseltamivir carboxylate, amantadine, and ribavirin. Concentrations of each drug are indicated on the axes, with each plane representing a different concentration of amantadine. Data for the triple combination at amantadine concentrations above the EC\textsubscript{50} of amantadine (0.21 µg/ml, Table 1) were excluded due to the efficacy of amantadine alone, which precluded the assessment of synergy.

Figure 4. Synergistic inhibition of A/duck/MN/1525/81 (H5N1) replication as determined by NR assay in MDCK cells. Values were derived from mean triplicate data and presented at 95% confidence. This experiment was repeated four times with similar results. Areas in blue indicate doses of each drug that are synergistic, gray areas indicate doses that are additive, and red areas indicate doses that are antagonistic. (A) Double combinations of amantadine and oseltamivir carboxylate (top); amantadine and ribavirin (middle); ribavirin and oseltamivir carboxylate (bottom). Concentrations of each drug are indicated on the axes. (B) Triple combinations of oseltamivir carboxylate, amantadine, and ribavirin. Concentrations of each drug are indicated on the axes, with each plane representing a different concentration of amantadine. Data for the triple combination at amantadine concentrations above the EC\textsubscript{50} of amantadine (0.54 µg/ml, data not shown) were excluded due to the efficacy of amantadine alone, which precluded the assessment of synergy.
Figure 5. Synergistic inhibition of A/Sydney/05/97 (H3N2) replication as determined by NR assay in MDCK cells. Values were derived from mean duplicate data. Areas in blue indicate doses of each drug that are synergistic, gray areas indicate doses that are additive, and red areas indicate doses that are antagonistic. (A) Double combinations of amantadine and oseltamivir carboxylate (top); amantadine and ribavirin (middle); ribavirin and oseltamivir carboxylate (bottom). Concentrations of each drug are indicated on the axes. (B) Triple combinations of oseltamivir carboxylate, amantadine, and ribavirin. Concentrations of each drug are indicated on the axes, with each plane representing a different concentration of amantadine. Data for the triple combination at amantadine concentrations above the EC_{50} of amantadine (0.72 µg/ml, data not shown) were excluded due to the efficacy of amantadine alone, which precluded the assessment of synergy.

Figure 6. Plot of synergy volume for double combinations and the triple combination at 0.1 µg/ml amantadine against influenza H1N1, H5N1, and H3N2 as determined by NR assay. Amantadine plus oseltamivir carboxylate (white bars), amantadine plus ribavirin (grey bars), oseltamivir carboxylate plus ribavirin (hatched bars), and triple combination (black bars). Five independent experiments with thirteen total replicates were conducted for the H1N1 virus, four independent experiments with 12 total replicates were conducted for the H5N1 virus, and one experiment with two replicates was conducted for the H3N2 virus. Synergy volumes are presented as the mean between experiments with standard deviations for the H1N1 and H5N1 viruses, and the mean between replicates with standard deviations for the H3N2 virus.

Figure 7. Synergistic inhibition of A/Sydney/05/97 (H3N2) replication as determined by TCID_{50} assay in MDCK cells. Values were derived from pooled replicate wells. Areas in blue indicate doses of each
drug that are synergistic, gray areas indicate doses that are additive, and red areas indicate doses that are antagonistic.  (A) Double combinations of amantadine and oseltamivir carboxylate (top); amantadine and ribavirin (middle); ribavirin and oseltamivir carboxylate (bottom). Concentrations of each drug are indicated on the axes.  (B) Triple combinations of oseltamivir carboxylate, amantadine, and ribavirin. Concentrations of each drug are indicated on the axes, with each plane representing a different concentration of amantadine. Data for the triple combination at amantadine concentrations above the EC$_{50}$ of amantadine (0.72 µg/ml, data not shown) were excluded due to the efficacy of amantadine alone, which precluded the assessment of synergy.

Figure 8. Synergistic inhibition of A/Sydney/05/97 (H3N2) replication as determined by qPCR assay. Values were derived from mean duplicate data. Areas in blue indicate doses of each drug that are synergistic, gray areas indicate doses that are additive, and red areas indicate doses that are antagonistic.  (A) Double combinations of amantadine and oseltamivir carboxylate (top); amantadine and ribavirin (middle); ribavirin and oseltamivir carboxylate (bottom). Concentrations of each drug are indicated on the axes.  (B) Triple combinations of oseltamivir carboxylate, amantadine, and ribavirin. Concentrations of each drug are indicated on the axes, with each plane representing a different concentration of amantadine. Data for the triple combination at amantadine concentrations above the EC$_{50}$ of amantadine (0.72 µg/ml, data not shown) were excluded due to the efficacy of amantadine alone, which precluded the assessment of synergy.

Figure 9. Plot of synergy volume for double combinations and the triple combination at 0.32 µg/ml amantadine against influenza H3N2 as determined by NR, TCID$_{50}$, and qPCR assays. The percent inhibition above expected as determined by the NR assay was converted to log$_{10}$ scale in order for the
synergy volume to be expressed in the same units as synergy volumes determined by TCID$_{50}$ and qPCR assays. Data are presented as the mean of duplicates with standard deviation for NR and qPCR, and from pooled duplicate samples for TCID$_{50}$. 
TABLE 1. The inhibitory concentration (EC50) of oseltamivir carboxylate, amantadine, and ribavirin as single agents and in double and triple combinations against A/New Caledonia/20/99 (H1N1) as determined by NR assay.

<table>
<thead>
<tr>
<th>Test Article</th>
<th>EC50 (µg/ml)</th>
<th>95% Confidence interval</th>
<th>Fold Reduction in EC50 Compared to</th>
<th>P-values Compared to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µg/ml)</td>
<td></td>
<td>(A)</td>
<td>(B)</td>
</tr>
<tr>
<td><strong>Oseltamivir carboxylate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(A) alone</td>
<td>0.14</td>
<td>0.12 – 0.16</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>(B) with 1 µg/ml ribavirin</td>
<td>0.055</td>
<td>0.045 – 0.067</td>
<td>2.5</td>
<td>--</td>
</tr>
<tr>
<td>(C) with 0.032 µg/ml amantadine</td>
<td>0.076</td>
<td>0.063 – 0.092</td>
<td>1.8</td>
<td>--</td>
</tr>
<tr>
<td>(D) with 1 µg/ml ribavirin and 0.032 µg/ml amantadine</td>
<td>0.016</td>
<td>0.011 – 0.024</td>
<td>8.8</td>
<td>3.4</td>
</tr>
<tr>
<td><strong>Amantadine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(A) alone</td>
<td>0.21</td>
<td>0.17 – 0.25</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>(B) with 1 µg/ml ribavirin</td>
<td>0.17</td>
<td>0.14 – 0.20</td>
<td>1.2</td>
<td>--</td>
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<tr>
<td>(C) with 0.01 µg/ml oseltamivir</td>
<td>0.084</td>
<td>0.067 – 0.10</td>
<td>2.5</td>
<td>--</td>
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<tr>
<td>(D) with 1 µg/ml ribavirin and 0.01 µg/ml oseltamivir</td>
<td>0.058</td>
<td>0.047 – 0.072</td>
<td>3.6</td>
<td>2.9</td>
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<tr>
<td><strong>Ribavirin</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>(A) alone</td>
<td>5.1</td>
<td>4.5 – 5.9</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>(B) with 0.032 µg/ml amantadine</td>
<td>2.8</td>
<td>2.4 – 3.3</td>
<td>1.8</td>
<td>--</td>
</tr>
<tr>
<td>(C) with 0.032 µg/ml oseltamivir</td>
<td>1.9</td>
<td>1.3 – 2.9</td>
<td>2.7</td>
<td>--</td>
</tr>
<tr>
<td>(D) with 0.032 µg/ml amantadine and 0.032 µg/ml oseltamivir</td>
<td>0.40</td>
<td>0.093 – 1.7</td>
<td>12.8</td>
<td>7</td>
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</table>
TABLE 2. Synergy volumes for double and triple combinations of oseltamivir carboxylate, amantadine, and ribavirin for influenza H1N1, H5N1, and H3N2 as determined by NR assay\(^a\).

<table>
<thead>
<tr>
<th></th>
<th>H1N1</th>
<th>H5N1</th>
<th>H3N2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Synergy Volumes for Double Combinations (µg/ml(^2%)</strong>)**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amantadine/Oseltamivir</td>
<td>452 ± 351</td>
<td>543 ± 166</td>
<td>297 ± 40</td>
</tr>
<tr>
<td>Amantadine/Ribavirin</td>
<td>207 ± 131</td>
<td>89 ± 46</td>
<td>375 ± 92</td>
</tr>
<tr>
<td>Ribavirin/Oseltamivir</td>
<td>140 ± 100</td>
<td>218 ± 204</td>
<td>95 ± 2</td>
</tr>
<tr>
<td><strong>Synergy Volumes for Triple Combinations (µg/ml(^3%)</strong>)**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.032 µg/ml Amantadine</td>
<td>690 ± 516</td>
<td>480 ± 85</td>
<td>127 ± 17</td>
</tr>
<tr>
<td>0.1 µg/ml Amantadine</td>
<td>1137 ± 495</td>
<td>930 ± 249</td>
<td>1175 ± 6</td>
</tr>
<tr>
<td>0.32 µg/ml Amantadine</td>
<td>---</td>
<td>919 ± 196</td>
<td>1596 ± 183</td>
</tr>
</tbody>
</table>

\(^a\)Synergy volumes are presented as the mean between experiments with standard deviations for the H1N1 and H5N1 viruses, and between replicates with standard deviations for the H3N2 virus. Combinations with synergy volumes >100 µg/ml\(^2\%) for double combinations or > 100 µg/ml\(^3\%) for triple combinations are considered to be synergistic. Conversely, combinations with synergy volumes < -100 µg/ml\(^2\%) or µg/ml\(^3\%) are considered to be antagonistic. Synergy volumes between -100 and 100 µg/ml\(^2\%) or µg/ml\(^3\%) are additive.