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Triple Combination of Oseltamivir, Amantadine, and Ribavirin Displays Synergistic Activity against Multiple Influenza Virus Strains In Vitro▽

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The recurring emergence of influenza virus strains that are resistant to available antiviral medications has become a global health concern, especially in light of the potential for a new influenza virus pandemic. Currently, virtually all circulating strains of influenza A virus 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 wild-type versions of the predominant circulating seasonal influenza virus strains and A/Duck/MN/1525/81 (H5N1) as a representative of avian influenza virus 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 virus. Importantly, the synergy of the triple combination was 2- to 13-fold greater than the synergy of any double combination depending on the influenza virus 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 virus, allowing the efficient use of the existing drug supplies.

Influenza epidemics are responsible for significant morbidity, mortality, and economic burden annually in the United States, including an estimated 41,000 deaths, more than 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 h of symptom onset (22, 27, 37). However, the therapeutic benefit of these antiviral drugs in cases of severe infection by highly pathogenic avian influenza virus is less clear. In cases of sporadic H5N1 influenza virus 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 virus infections.

The effectiveness of the adamantanes and NAIs has been eroded by emerging viral resistance, both treatment induced and naturally occurring. Resistance to the adamantanes, which block the M2 channel and prevent 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 no longer is 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 to 2009 influenza season, virtually 100% of the characterized A/H3N2 viruses were resistant to amantadine (6). Sporadic resistance to oseltamivir, the most widely used NAI, 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 virus (26, 29, 35) and avian influenza virus (11). Whether treatment induced or naturally occurring, widespread resistance to oseltamivir in A/H1N1 seasonal influenza virus emerged in Europe in early 2008 and now is dominant over large portions of Europe, Asia, North America, and the Southern Hemisphere (43, 51). In the 2009 influenza season, 99.4% of all A/H1N1 viruses isolated from patients in the United States were resistant to oseltamivir (6). As a result, virtually all circulating strains of influenza A virus in the United States currently are 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 being available, the continued use of these drugs as monotherapies may

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result in dual resistance, raising the specter of treatment-induced multidrug-resistant influenza virus. 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, 49).

The emergence of drug-resistant mutants is a significant problem not only for influenza virus but also for other rapidly mutating viruses (7, 30, 42, 48). 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 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 chemicogenetic barrier to drug-resistant mutations (16, 17). With regard to the use of combination therapy for influenza virus, 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 virus in vitro (14, 19, 20, 23, 32, 44) and in animals (13, 25, 31, 44, 47). To date, there are no published studies of the effects of triple antiviral drug combinations for influenza virus.

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 of the viral life cycle, would result in synergistic antiviral activity. In this study, we evaluated the interactions between oseltamivir, amantadine, and ribavirin. To test our hypothesis that these drugs interact synergistically, we explored the 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 virus 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 virus has been demonstrated.

MATERIALS AND METHODS

Antiviral compounds. Oseltamivir carboxylate (the active metabolite of oseltamivir) was obtained from Charnwood Molecular (Loughborough, United Kingdom) through synthesis via the Nboc-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) viruses were provided by the Centers for Disease Control and Prevention (Atlanta, GA). Influenza A/Duck/MN/1525/81 (H5N1) virus was provided by 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, and neuraminidase for each virus were sequenced. Sequencing revealed that for each gene and each virus, there was ≥98% identity to the 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 medium (MEM) containing 5% fetal bovine serum (HyClone Laboratories, Logan, UT). During antiviral evaluations, the serum was removed and the medium was supplemented with gentamicin (50 μg/ml), trypsin (10 U/ml), and EDTA (1 μg/ml).

Cell-based assays. To obtain monotherapy dose-response curves, individual drugs were added to MDCK cells in 96-well microplates (8×10^4 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 μg/ml, and amantadine and ribavirin at 0, 0.001, 0.0032, 0.01, 0.032, 0.1, 0.32, 1, 3.2, 10, 32, and 100 μg/ml. Untreated wells of infected cells (virus controls) and uninfected cells (cell controls) were included on each test plate. At 3 days postinfection, the virus control wells exhibited 100% cytopathology.

For combination studies, each drug was tested in double and triple combinations at six doses (including no drug), in which the high dose for each drug was set above the 50% effective concentration (EC_{50}) for the virus. The doses for influenza H1N1 were the following: oseltamivir carboxylate at 0, 0.0032, 0.01, 0.032, 0.1, and 0.32 μg/ml; amantadine at 0, 0.01, 0.032, 0.1, 0.32, and 1 μg/ml; and ribavirin at 0, 0.032, 0.1, 0.32, 1, and 3.2 μg/ml. The doses for influenza H5N1 were the following: oseltamivir carboxylate at 0, 0.0032, 0.01, 0.032, 0.1, and 0.32 μg/ml; amantadine at 0, 0.01, 0.032, 0.1, 0.32, and 1 μg/ml; and ribavirin at 0, 0.1, 0.32, 1, 3.2, and 10 μg/ml. The doses for influenza H3N2 were the following: oseltamivir carboxylate at 0, 0.001, 0.0032, 0.01, 0.032, and 0.1 μg/ml, and amantadine and ribavirin at 0, 0.032, 0.1, 0.32, 1, and 3.2 μg/ml. Five independent experiments with 13 total replicates were conducted for influenza H1N1 virus; four independent experiments with 12 total replicates were conducted for influenza H5N1 virus; and one experiment with 2 total replicates was conducted for influenza H3N2 virus.

NR assay. The extent of viral cytopathology in each well was determined microscopically by inspection and by staining with neutral red (NR) as detailed elsewhere (45). Briefly, the cells were stained with 0.011% NR diluted in MEM to determine cell viability. Two hours later, the plates were processed for the quantification of NR uptake into viable cells. The amount of NR taken up by cells was determined spectrophotometrically.

Virus yield reduction. To validate the 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 as those used for the NR assay. Duplicate wells were pooled, and virus yield, as quantitated by the 50% tissue culture infectious dose ($TCID_{50}$), was determined by titrating samples in MDCK cells in 96-well plates using the endpoint dilution method as previously described (41, 45).

Real-time qPCR. 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 also was analyzed by quantitative real-time PCR (qPCR) using primers directed toward the matrix gene. Methods for qPCR were adapted from Ward et al. (50). 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 GTA 3′) using 12 μl of RNA in a 20-μl 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 C 3′, respectively, and the sequence for the probe was 6-FAM-5′ CGT GCC CAG TGA GC 3′-MGB. A standard curve is necessary to produce accurate copy number estimates for a reaction. The qPCR assay standards were constructed by the ligation of a PCR-amplified product of segment 7 (the matrix gene) into a plasmid vector. The plasmid DNA was amplified in *Escherichia 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 optical densities at 260 and 280 nm. These data were used to calculate the 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

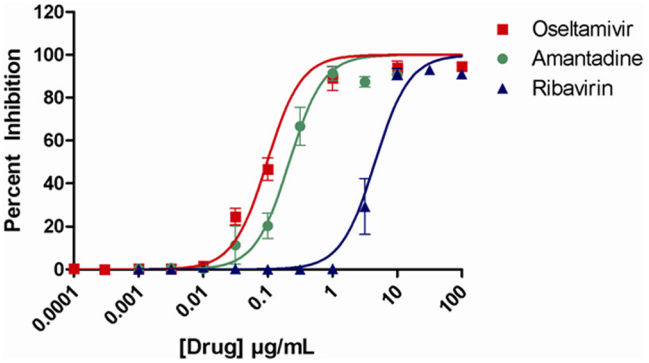

FIG. 1. Inhibition of A/New Caledonia/20/99 (H1N1)-induced cytopathic effect in MDCK cells treated with oseltamivir carboxylate (red squares), amantadine (green circles), and ribavirin (blue triangles) as determined by NR assay. Data are presented as the means from three replicates with standard deviations.

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_{50}. Statistical comparisons between best-fit EC_{50}s for any two curves were performed in Prism using the extra sum-of-squares F test; differences in EC_{50}s 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 in a previous reported 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 gray, 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 blue areas. Conversely, antagonism is represented as red areas. Where indicated, inhibition greater than or less than expected is shown at a level of 95% confidence, which eliminates insignificant deviations from the additive surface.

The synergy volume for each double and triple combination also was 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 of >100 $\mu g/ml^2\%$ for double combinations or >100 $\mu g/ml^3\%$ for triple combinations are considered synergistic. Conversely, combinations with synergy volumes of <−100 $\mu g/ml^2\%$ or $\mu g/ml^3\%$ are considered antagonistic. Synergy volumes between −100 and 100 $\mu g/ml^2\%$ or $\mu g/ml^3\%$ are ambiguous and are not thought to be biologically significant.

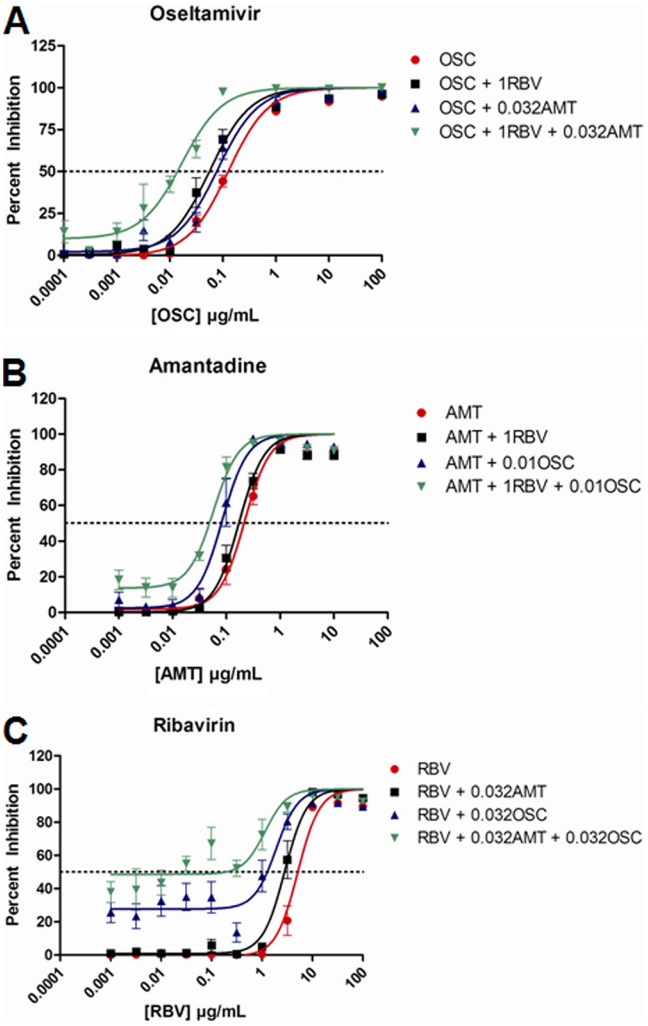

FIG. 2. Inhibition of A/New Caledonia/20/99 (H1N1)-induced cytopathic effect in MDCK cells treated with double and triple combinations of amantadine (AMT), oseltamivir carboxylate (OSC), and ribavirin (RBV) as determined by NR assay. (A) Dose response for OSC alone (red circles), with 1.0 μg/ml RBV (black 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 means from three independent experiments (three replicates each) for each condition with standard deviations.

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 NR. As shown in Fig. 1, an inhibition dose response of influenza H1N1 replication was determined for each drug. The 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 Fig. 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 that of oseltamivir alone,

TABLE 1. EC_{50} 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

Test	EC_{50} (μg/ml)	95% Confidence interval	Fold reduction in EC_{50} compared to that of test:			*P* values compared to results for test:		
			A	B	C	A	B	C
Oseltamivir carboxylate								
Alone (A)	0.14	0.12–0.16						
With 1 μg/ml ribavirin (B)	0.055	0.045–0.067	2.5			<0.0001		
With 0.032 μg/ml amantadine (C)	0.076	0.063–0.092	1.8			0.0002		
With 1 μg/ml ribavirin and 0.032 μg/ml amantadine (D)	0.016	0.011–0.024	8.8	3.4	4.8	<0.0001	<0.0001	<0.0001
Amantadine								
Alone (A)	0.21	0.17–0.25						
With 1 μg/ml ribavirin (B)	0.17	0.14–0.20	1.2			0.14		
With 0.01 μg/ml oseltamivir (C)	0.084	0.067–0.10	2.5			<0.0001		
With 1 μg/ml ribavirin and 0.01 μg/ml oseltamivir (D)	0.058	0.047–0.072	3.6	2.9	1.4	<0.0001	<0.0001	0.012
Ribavirin								
Alone (A)	5.1	4.5–5.9						
With 0.032 μg/ml amantadine (B)	2.8	2.4–3.3	1.8			<0.0001		
With 0.032 μg/ml oseltamivir (C)	1.9	1.3–2.9	2.7			<0.0001		
With 0.032 μg/ml amantadine and 0.032 μg/ml oseltamivir (D)	0.40	0.093–1.7	12.8	7	4.8	<0.0001	0.0002	0.029

indicating that inhibition occurred at lower concentrations of oseltamivir. In the presence of both ribavirin and amantadine at the same concentrations as those used in the double combinations, there was a further leftward shift in the dose-response curve, indicating additional synergy in the triple combination compared to that of double combinations. Figures 2B and C show that, similarly 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}s 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_{50} was reduced in triple combination compared to the EC_{50} in double combinations and as a single agent. For example, the EC_{50} for oseltamivir as monotherapy was reduced by 2.5- or 1.8-fold in double combination with 1 μg/ml ribavirin or 0.032 μg/ml amantadine, respectively. The EC_{50} of oseltamivir carboxylate was reduced by 8.8-fold in combination with both ribavirin and amantadine at the same concentrations. Moreover, the EC_{50} of oseltamivir carboxylate in triple combination showed a 3.4-fold reduction compared to that of oseltamivir carboxylate in double combination with ribavirin and a 4.8-fold reduction compared to that of oseltamivir carboxylate in double combination with amantadine. Likewise, the triple-drug combination reduced the EC_{50} of amantadine and ribavirin by 3.6- and 12.8-fold, respectively, compared to that of monotherapy and 1.4- to 7-fold compared to that of the double combinations. Thus, the activity of each drug was greater in triple combination than in double combination or as a single agent, indicating that each drug was effective at a lower concentration.

Importantly, the data presented here do not represent the maximum reductions in EC_{50}s for the three drugs. Due to the dynamic range of the assay, we were able to obtain precise dose-response curves for each drug only at fixed concentrations of the second and third drugs that were well below their EC_{50}s 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 to quantify synergy across 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 for 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 Fig. 3. Synergy plots showed that amantadine and oseltamivir carboxylate were synergistic against H1N1 replication across wide concentrations of both drugs, whereas double combinations of amantadine plus ribavirin or oseltamivir carboxylate plus ribavirin were largely additive (Fig. 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. At levels greater than 0.1 μg/ml amantadine, the limits of detection for the assay were reached, and thus no

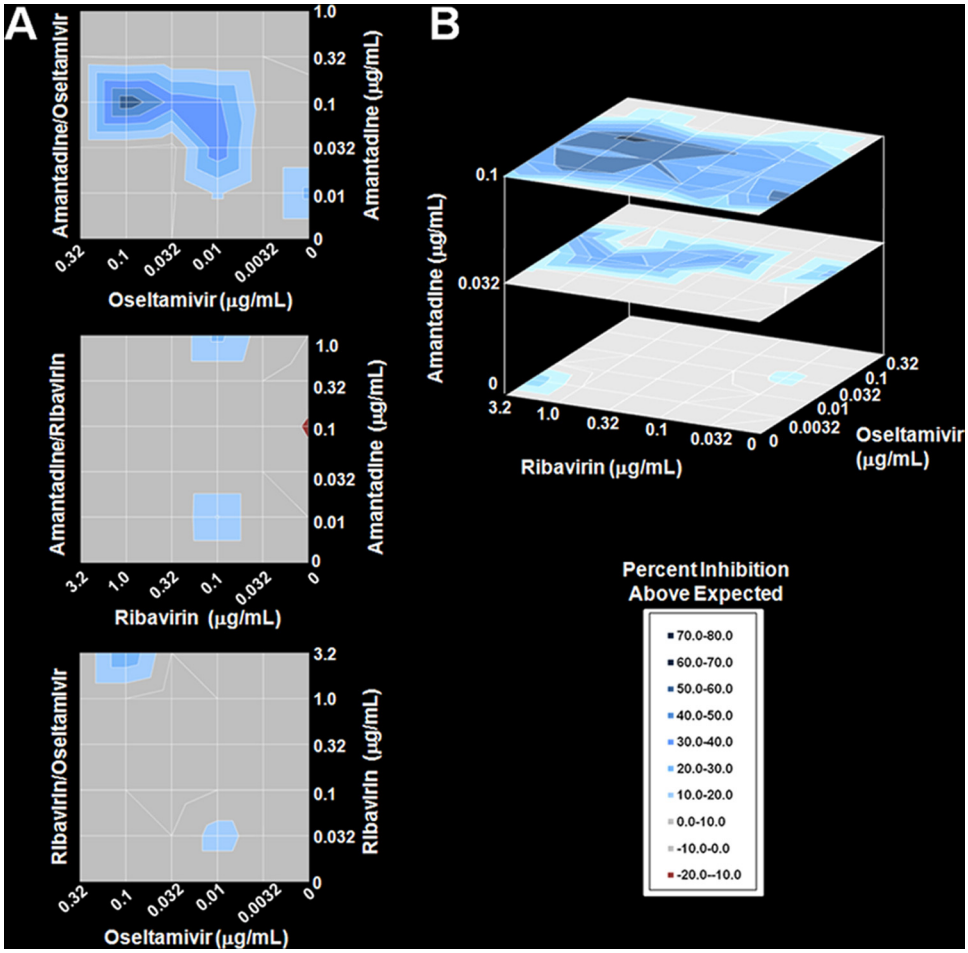

FIG. 3. Synergistic inhibition of A/New Caledonia/20/99 (H1N1) replication as determined by NR assay in MDCK cells. Calculated additive interactions were subtracted from the experimentally determined inhibition to reveal regions of synergy (inhibition above the expected level) or antagonism (inhibition below the expected level). Values were derived from mean triplicate data and are 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 the expected level. (A) Double combinations of amantadine and oseltamivir carboxylate (top), amantadine and ribavirin (middle), and 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 (0.21 μg/ml) (Table 1) were excluded due to the efficacy of amantadine alone, which precluded the assessment of synergy.

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 to 0.1 μg/ml amantadine and 0.0032 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 for 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 concentrations 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 of 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 the 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 combinations is ro-

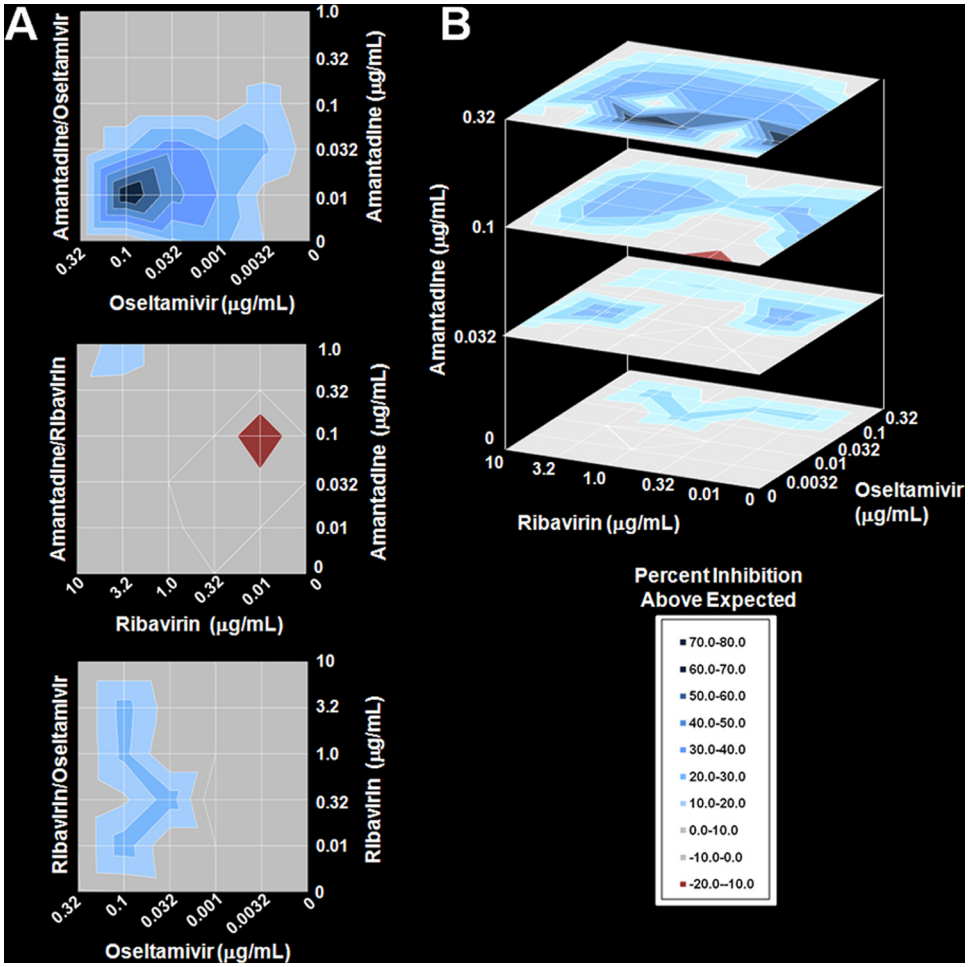

FIG. 4. Synergistic inhibition of A/Duck/MN/1525/81 (H5N1) replication as determined by NR assay in MDCK cells. Values were derived from mean triplicate data and are presented at 95% confidence. This experiment was repeated four times with similar results. Areas in blue indicate doses of each drug that 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), and 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 (0.54 μg/ml; data not shown) were excluded due to the efficacy of amantadine alone, which precluded the assessment of synergy.

bust, with synergy being observed in all influenza A virus strains tested to date.

Additionally, no cytotoxicity was observed for the drug combinations. The 50% cytotoxic concentration (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 deviations for the H1N1 and H5N1 viruses and between replicates with standard deviations 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 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 1,000 $\mu g/ml^3\%$ against all three subtypes. Figure 6 compares the synergy volumes for double combina-

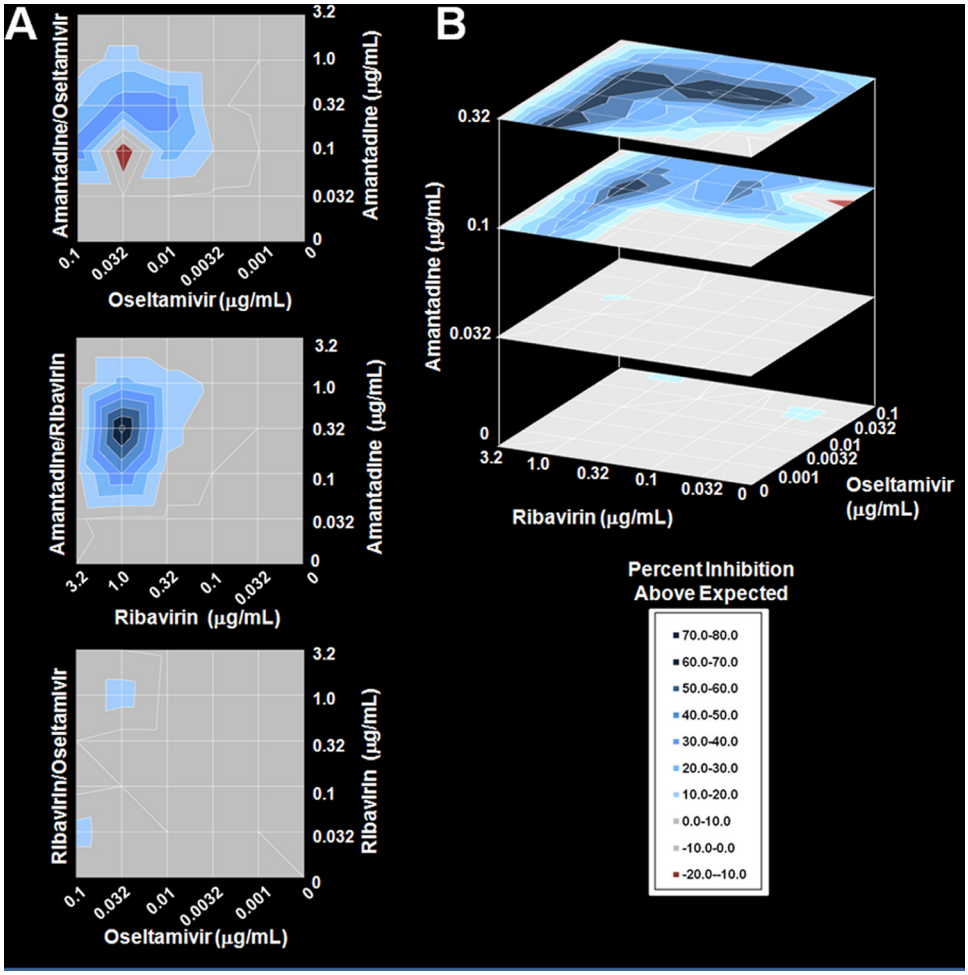

FIG. 5. Synergistic inhibition of A/Sydney/05/97 (H3N2) replication as determined by NR assay in MDCK cells. Values were derived from mean duplicate data. Areas in blue indicate doses of each drug that are synergistic, gray areas indicate doses that are additive, and red areas indicate doses that are antagonistic. (A) Double combinations of amantadine and oseltamivir carboxylate (top), amantadine and ribavirin (middle), and 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.

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]

Synergy volume	H1N1	H5N1	H3N2
Double combinations ($\mu g/ml^2\%$)			
Amantadine/oseltamivir	452 ± 351	543 ± 166	297 ± 40
Amantadine/ribavirin	207 ± 131	89 ± 46	375 ± 92
Ribavirin/oseltamivir	140 ± 100	218 ± 204	95 ± 2
Triple combinations ($\mu g/ml^3\%$)			
0.032 μg/ml amantadine	690 ± 516	480 ± 85	127 ± 17
0.1 μg/ml amantadine	1137 ± 495	930 ± 249	1175 ± 6
0.32 μg/ml amantadine		919 ± 196	1596 ± 183

[a] Synergy volumes are presented as the means 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 of >100 $\mu g/ml^2\%$ for double combinations or >100 $\mu g/ml^3\%$ for triple combinations are considered to be synergistic. Conversely, combinations with synergy volumes of <-100 $\mu g/ml^2\%$ or $\mu g/ml^3\%$ are considered to be antagonistic. Synergy volumes between -100 and 100 $\mu g/ml^2\%$ or $\mu g/ml^3\%$ are additive.

tions and the triple combination at 0.1 μg/ml amantadine against all three subtypes. For 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} of amantadine against the H3N2 strain. At 0.1 μg/ml amantadine, the synergy for the 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, the viral titer was determined by $TCID_{50}$ assay, the

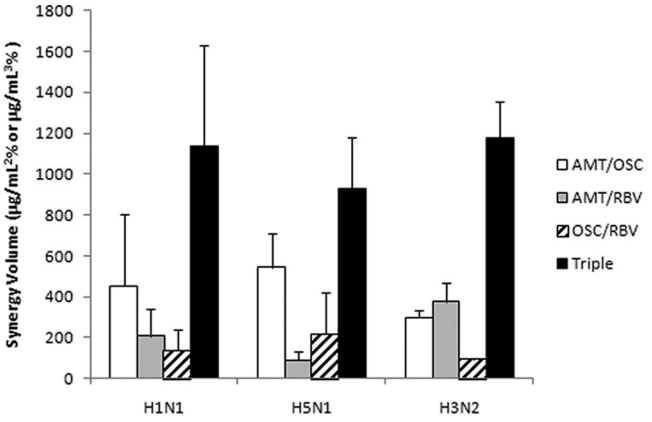

FIG. 6. Plot of synergy volume for double combinations and the triple combination at 0.1 μg/ml amantadine (AMT) against influenza H1N1, H5N1, and H3N2 as determined by NR assay. Amantadine plus oseltamivir carboxylate (OSC) (white bars), amantadine plus ribavirin (RBV) (gray bars), oseltamivir carboxylate plus ribavirin (hatched bars), and the triple combination (black bars) are shown. Five independent experiments with 13 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 2 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.

genome copy number was measured by qPCR, and synergy (i.e., the reduction in viral load above the expected reduction) was calculated in the same manner as that described above. Synergy plots for the double and triple combinations as determined by $TCID_{50}$ and qPCR are provided in Fig. 7 and 8, respectively. This analysis confirmed the results from the NR assay and showed that viral titer and genome copy number were synergistically reduced by the three-drug combination. Synergy calculations using both $TCID_{50}$ and qPCR endpoints revealed that the double combinations of amantadine plus oseltamivir carboxylate and amantadine plus ribavirin 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 0.32 μg/ml amantadine, similarly to the NR assay. For the $TCID_{50}$ assay, synergy was observed at similar concentration ranges of each drug in double and triple combinations, as seen with the NR assay (Fig. 7). For the qPCR assay, the synergy was more modest than that seen with the NR or $TCID_{50}$ assay, 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 (Fig. 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, $TCID_{50}$, and qPCR. The percent inhibition above the expected value as determined by the NR assay was converted to a $\log_{10}$ scale in order for the synergy volume to be expressed in the same units as synergy volumes determined by $TCID_{50}$ and qPCR assays. 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-drug combination therapy in the treatment of influenza is supported by the demonstrated superiority of triple-drug combination therapy over single- and double-drug 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 was highly effective at suppressing the 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, acts synergistically and provides a much higher level of antiviral activity than single- or double-drug combination therapies. Oseltamivir interferes with the viral neuraminidase activity, blocking the release of new virions from an infected cell (15, 33). Amantadine blocks the M2 ion channel, which inhibits the 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, 46); amantadine plus oseltamivir (23, 46); rimantadine plus ribavirin (20, 32); rimantadine plus zanamivir, oseltamivir, or peramivir (14); ribavirin plus peramivir (44); and ribavirin plus oseltamivir (46). The antiviral activity of double drug combinations also has been evaluated in animal studies for the combinations of oseltamivir plus rimantadine (13, 31), oseltamivir plus amantadine (25, 46), ribavirin plus peramivir (45), ribavirin plus oseltamivir (24, 46, 47), and ribavirin plus amantadine (46).

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

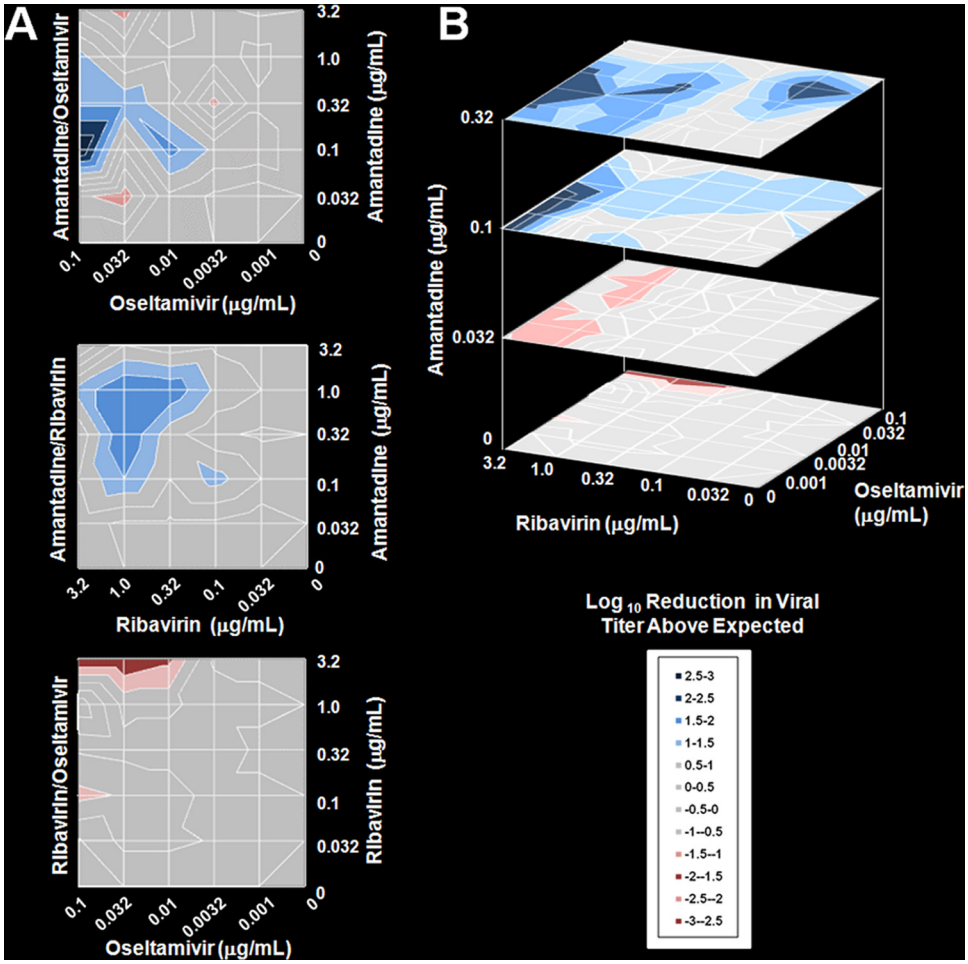

FIG. 7. Synergistic inhibition of A/Sydney/05/97 (H3N2) replication as determined by $TCID_{50}$ assay in MDCK cells. Values were derived from pooled replicate wells. Areas in blue indicate doses of each drug that are synergistic, gray areas indicate doses that are additive, and red areas indicate doses that are antagonistic. (A) Double combinations of amantadine and oseltamivir carboxylate (top), amantadine and ribavirin (middle), and 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.

in another study (32). Finally, both Govorkova et al. (14) and Smee et al. (47) 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 the literature, whether due to the specific drugs in the combinations or to the 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) (46). Similarly 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 in which 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 virus 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 to 1,596 $\mu g/ml^3\%$ at 0.1 μg/ml or higher amantadine) against all three subtypes and was greater than that of the double combinations (Fig. 6). Depending on the specific double combination and influenza subtype, the synergy volume of the triple combination was 2- to 13-fold greater than

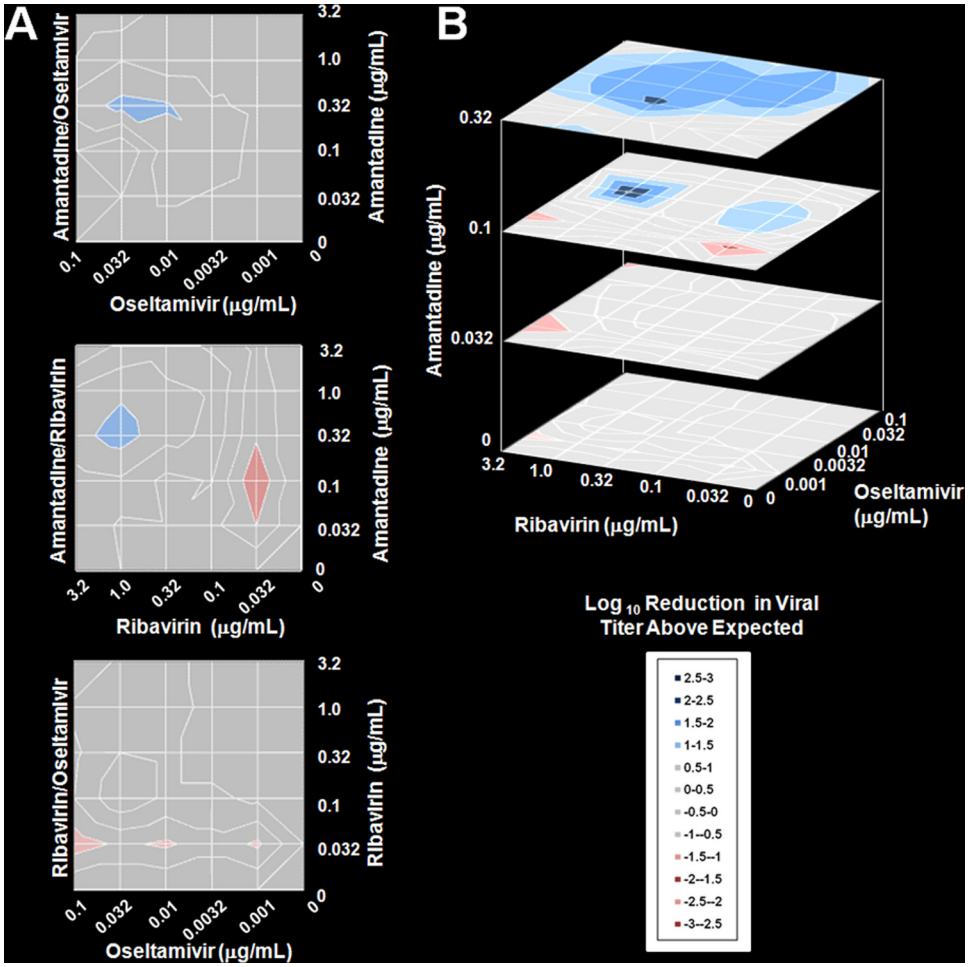

FIG. 8. Synergistic inhibition of A/Sydney/05/97 (H3N2) replication as determined by qPCR assay. Values were derived from mean duplicate data. Areas in blue indicate doses of each drug that are synergistic, gray areas indicate doses that are additive, and red areas indicate doses that are antagonistic. (A) Double combinations of amantadine and oseltamivir carboxylate (top), amantadine and ribavirin (middle), and 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.

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 EC_{50} (Fig. 3 and Table 1).

For the H3N2 virus, the superior synergy of the triple combination compared to that of double combinations was confirmed using multiple assays that measured different endpoints, including the inhibition of cytopathic effect, a reduction in the live viral titer, and a reduction in viral RNA copies (Fig. 9). All three assays revealed that the synergy volume of the triple combinations was at least threefold greater than the synergy of any double combination. Minor variations in synergy volumes between the different assays may be due to the dynamic range of each assay (2 $\log_{10}$ for NR and 5 to 6 $\log_{10}$ for $TCID_{50}$ and qPCR) or the specific endpoint (infectivity for NR and $TCID_{50}$ compared to total RNA for qPCR).

Importantly, the synergy between the three antiviral drugs is observed at concentrations that are achievable in plasma in humans. The synergy of the triple combination occurred at concentrations of 0.032 to 1 μg/ml for amantadine, 0.001 to 0.32 μg/ml for oseltamivir, and 0.032 to 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 [www.drugs.com/pro/rebetol.html, www.drugs.com/pro/symmetrel.html, and www.drugs.com/pro/tamiflu.html]). 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 anti-

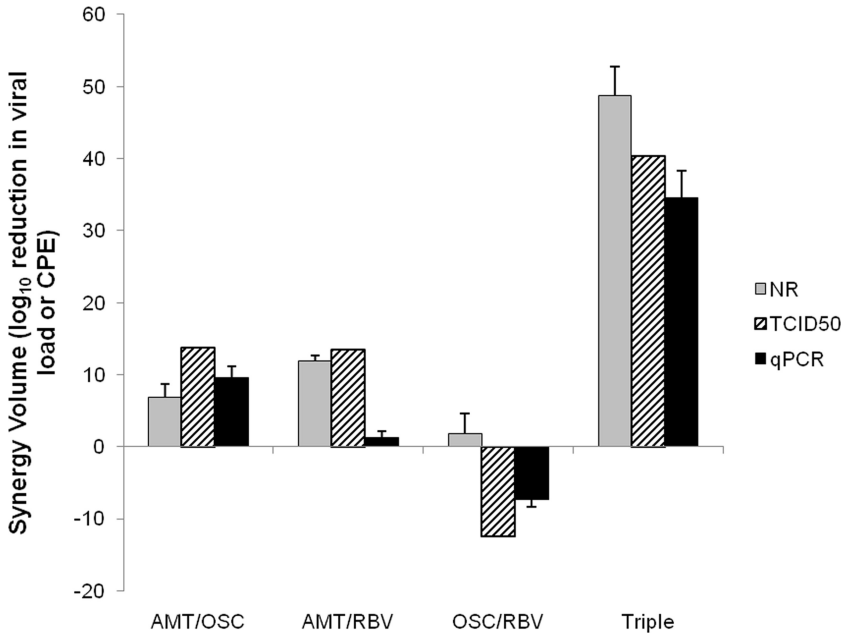

FIG. 9. Plot of synergy volume for double combinations and the triple combination at 0.32 μg/ml amantadine (AMT) against influenza H3N2 as determined by NR, $TCID_{50}$, and qPCR assays. OSC, oseltamivir carboxylate; RBV, ribavirin; CPE, cytopathic effect. The percent inhibition above the expected level as determined by the NR assay was converted to a $\log_{10}$ 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 means of duplicates with standard deviation for NR and qPCR and from pooled duplicate samples for $TCID_{50}$.

viral drug combination of oseltamivir, amantadine, and ribavirin for treatment of both severe seasonal and avian influenza. Further studies are under way 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.

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