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Combination chemotherapy, a potential strategy for reducing the emergence of drug-resistant influenza A variants

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Abstract

Rapid development of resistant influenza variants after amantadine treatment is one of the main drawbacks of M2 blockers. On the other hand, the emergence of variants with low susceptibility to the neuraminidase (NA) inhibitors is limited. In the present study we examined whether combination therapy with two classes of anti-influenza drugs can affect the emergence of resistant variants in vitro. We observed that virus yields of human A/Nanchang/1/99 (H1N1), A/Panama/2007/99 (H3N2), and A/Hong Kong/156/97 (H5N1) viruses in MDCK cells were significantly reduced ($P < 0.005$) when the cells were treated with the combination of amantadine and low doses of oseltamivir carboxylate (≤ 1 μM). After five sequential passages in MDCK cells, the M2 protein of viruses cultivated with amantadine alone mutated at positions V27A and S31N/I. Viruses cultivated with oseltamivir carboxylate (≥ 0.001 μM) possessed mutations in the hemagglutinin (HA) protein. These variants showed reduced efficiency of binding to sialic acid receptors and decreased sensitivity to NA inhibitor in plaque reduction assay. Importantly, no mutations in the HA, NA, and M2 proteins were detected when the drugs were used in combination. Our results suggest that combination chemotherapy with M2 blocker and NA inhibitor reduced the emergence of drug-resistant influenza variants in vitro. This strategy could be an option for the control of influenza virus infection, and combinations with other novel drugs should be explored.

Keywords: Influenza A virus; Oseltamivir; Amantadine; Drug resistance

1. Introduction

Currently, two specific classes of antiviral drugs are available to manage influenza virus infection: the inhibitors of M2 protein, amantadine and its congener rimantadine, and the neuraminidase (NA) inhibitors, zanamivir and oseltamivir (Monto, 2003). These two classes of drugs target different viral proteins and have different mechanisms of action on the replication cycle of the virus. Amantadine and rimantadine inhibit virus replication during the early stage of infection by blocking the ion channel formed by the transmembrane domain of the M2 protein (Hay et al., 1985; Pinto et al., 1992), and NA inhibitors interrupt the established replication cycle by preventing virus release and allowing virus to clump on the cell surface (Madren et al., 1995; Varghese et al., 1992; von Itzstein et al., 1993; Weis et al., 1988).

M2 blockers have an inhibitory effect on different subtypes of influenza A viruses. In addition, these drugs are inexpensive and have good chemical stability and excellent bioavailability (Monto, 2003). However, a major concern about using M2 blockers is their association with rapid emergence of drug resistance and ineffectiveness against influenza B virus infection (Hayden, 1996; Hayden and Hay, 1992). Previous studies showed that naturally occurring amantadine-resistant influenza A viruses are quiet rare (about 0.8% in the general population [Ziegler et al., 1999]). However, it was reported recently that the level of amantadine-resistant variants increased among viruses of the H3 (Bright et al., 2005) and H5 HA subtypes (Ilyushina et al., 2005). Besides, 30–80% of isolates show amantadine resistance after only a few days of M2 blocker therapy in both immunocompetent and immunocompromised patients (Hayden, 1996, 2001; Klimov et al., 1995; Shiraishi et al., 2003). Resistant variants possessed amino acid substitutions at positions 26, 27, 30, 31, and 34 in the target M2 protein (Hay et al., 1985; Pinto et al., 1992). In addition, amantadine-resistant variants showed no

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obvious loss of virulence or transmissibility in animal models or humans, i.e., M2 mutations conferring resistance to amantadine did not compromise the replicative capacity or virulence of the influenza A viruses (Hayden, 1996).

Zanamivir and oseltamivir are potent and specific against all 9 NA subtypes of influenza A and B viruses, and they have minimal adverse effects, as reported in clinical trials (Lew et al., 2000; McKimm-Breschkin, 2000; Monto, 2003; Monto et al., 1999). Unlike M2 blockers, NA inhibitors appear to be associated with a lower frequency of emergence of drug-resistant variants caused by mutations in either the NA or hemagglutinin (HA). The NA substitutions in drug-resistant viruses include amino acid residues 119, 152, 274, 292, and 294 of the enzyme's active centre (Gubareva et al., 1998; Kiso et al., 2004; McKimm-Breschkin, 2000, 2005). To date, viruses with altered susceptibility to NA inhibitors have been recovered from approximately 1% of immunocompetent adult patients (Hayden, 2001; McKimm-Breschkin, 2005), and in only one case was a zanamivir-resistant variant recovered from an immunocompromised patient (Gubareva et al., 1998). However, a high proportion of oseltamivir-resistant mutants (18%) in children was reported recently (Kiso et al., 2004). This finding might be explained in part by the rigorous detection techniques used. Oseltamivir-resistant variants showed reduced infectivity and virulence with low risk of transmission in a ferret model (McKimm-Breschkin, 2000). At the same time, A/Wuhan/359/95-like (H3N2) virus with the E119V NA mutation was recently reported to be transmitted as efficiently as the wild-type virus in ferrets (Herlocher et al., 2004). These observations indicate that NA mutations that confer resistance to NA inhibitors appears to alter the fitness of influenza viruses, but the relevance of oseltamivir-resistant mutations in terms of transmissibility remains uncertain.

Since there are a number of limitations regarding antiviral drugs used individually, there is a need to assess in direct comparative studies the effect of combining the anti-influenza drugs available. Previously, it was shown that the combination of rimantadine hydrochloride and ribavirin exert an additive and, in specific concentrations, synergistic antiviral effect by reducing the virus yield of influenza A/Texas/1/77 (H3N2) and A/USSR/90/77 (H1N1) viruses in MDCK cells (Hayden et al., 1980b). In a mouse model, the antiviral effect of the combined treatment of amantadine and ribavirin was greater than that of either single-drug treatment (Wilson et al., 1980). Zanamivir combined with rimantadine or ribavirin led to more potent effects in vitro when these agents were used in combination (Madren et al., 1995; Smee et al., 2002). Moreover, combined treatment of NA inhibitor and rimantadine produced an additive and synergistic reduction of the extracellular virus yield of human influenza A/New Caledonia/20/99 (H1N1) and A/Panama/2007/99 (H3N2) viruses in MDCK cells (Govorkova et al., 2004).

In the present study, we tested in vitro the hypothesis that combinations of the amantadine and oseltamivir carboxylate can prevent or reduce the emergence of drug-resistant variants. We used H1N1 and H3N2 human influenza virus subtypes that represent antigenically dominant strains that circulated during 1999–2004. We also tested drug combinations on A/Hong Kong/156/97 (H5N1) virus, the influenza subtype that pose a real threat to humans and warrant consideration (Claas et al., 1998; WHO, 2004).

2. Materials and methods

2.1. Compounds

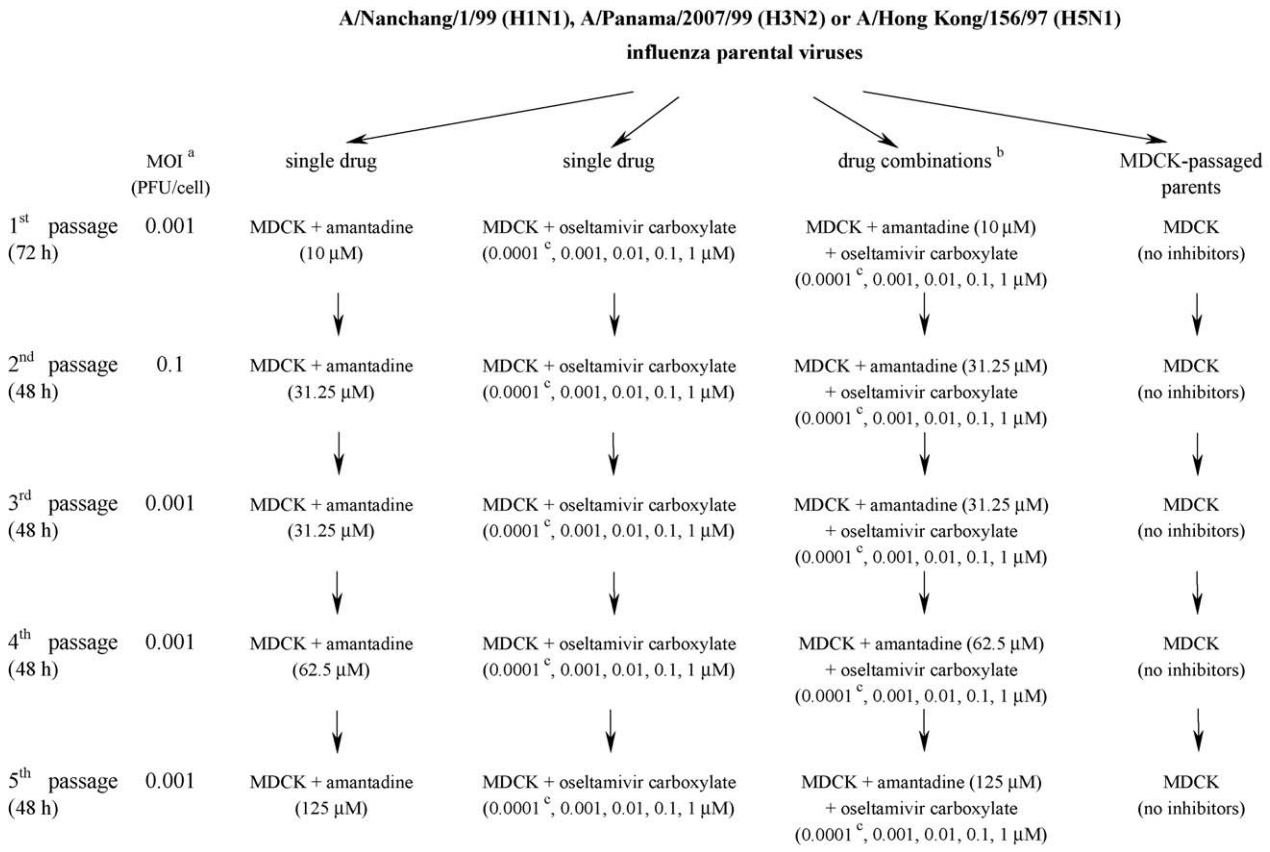
The NA inhibitor oseltamivir carboxylate (the active metabolite of oseltamivir [3*R*,4*R*,5*S*]-4-acetamido-5-amino-3-[1-ethyl propoxy]-1-cyclohexane-1-carboxylic acid) was provided by Hoffmann-La Roche. Amantadine (1-aminoadamantane hydrochloride) was obtained from Sigma–Aldrich, Inc. (St. Louis, MO).

2.2. Viruses and cells

Human influenza A/Nanchang/1/99 (H1N1), A/Panama/2007/99 (H3N2), and A/Hong Kong/156/97 (H5N1) viruses were obtained through the World Health Organization network. Stocks of the viruses were plaque-purified two times in MDCK cells and passaged one time in the allantoic cavity of 10-day-old embryonated chicken eggs at 37 °C for 48 h. The entire HA, NA, and M genes of the virus stocks were sequenced to verify the absence of mutations as compared to the original strains. All experimental work with the H5N1 virus was performed in a biosafety level 3+ laboratory approved for use by the U.S. Department of Agriculture. Madin–Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection (Manassas, VA) and were grown in minimal essential medium (MEM) supplemented with 5% fetal calf serum, 5 mM L-glutamine, sodium bicarbonate, 100 U of penicillin per ml, 100 μg of streptomycin sulfate per ml, and 100 μg of kanamycin sulfate per ml in a humidified atmosphere of 5% CO_2.

2.3. Cultivation of influenza viruses in the presence of amantadine and oseltamivir carboxylate used as a single drug or in combination

The design of the experiments is summarized in Fig. 1. For the first passage MDCK cells were infected with influenza H1N1, H3N2, and H5N1 viruses at multiplicity of infection (MOI) 0.001 PFU/cell and cultivated in infection medium [MEM containing 4% bovine serum albumin, sodium bicarbonate, 100 U of penicillin per ml, 100 μg of streptomycin sulfate per ml, 100 μg of kanamycin sulfate per ml, and 1 μg per ml of L-1-(tosyl-amido-2-phenyl)ethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington Diagnostics, Freehold, NJ)] with amantadine at concentration of 10 μM. The second passage was done at MOI of 0.1 PFU/cell of each virus and the concentration of amantadine was 31.25 μM. Three more sequential passages in MDCK cells were performed at MOI of 0.001 PFU/cell in the presence of increasing concentrations of the compound (31.25, 62.5, and 125 μM, respectively). The high concentrations of amantadine were chosen in order to maintain strong selective pressure of the drug.



[a] Multiplicity of infection.

[b] Two drugs were mixed together for the combination treatment experiments and added 1 h before virus infection.

[c] Concentration of oseltamivir carboxylate used for A/Hong Kong/156/97 (H5N1) strain only.

Fig. 1. Diagram of the passage history of the influenza viruses used in this study.

For passages with oseltamivir carboxylate we used four concentrations of NA inhibitor (0.001, 0.01, 0.1, 1 μM) for H1N1 and H3N2 strains and five drug concentrations for the H5N1 strain (those listed above and 0.0001 μM) (Fig. 1). Each dose of NA inhibitor was used for all five passages in MDCK cells. To study the appearance of drug-resistant viruses in the presence of two drugs, we used the same concentrations of NA inhibitor combined with those doses of amantadine that were applied in single-drug experiment. The parental viruses were passaged five times in MDCK cells without drugs and designated the MDCK-passaged parents (Fig. 1).

2.4. Plaque assay

Plaque assay was performed as described previously (Hayden et al., 1980a) and was used to determine the MOIs, virus yield and diameter of the plaques. Briefly, MDCK cells were inoculated with serial 10-fold dilutions of influenza viruses. After 1 h incubation the cells were overlaid with MEM containing 0.9% agar, 4% bovine serum albumin, 1 μg per ml of L-1-(tosyl-amido-2-phenyl)ethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington Diagnostics, Freehold, NJ). After 3 days of incubation at 37 °C the cells were stained with 0.1% crystal violet in 10% formaldehyde solution.

2.5. Plaque reduction assay

Drug sensitivity of influenza viruses was determined by plaque reduction assay, as described previously (Hayden et al., 1980a). Briefly, the cells were inoculated with virus diluted to give 80–100 plaques per well and then overlaid with infection medium in the presence or absence of a single drug. The results were recorded after 3 days of incubation at 37 °C. Three independent experiments were performed to determine the concentration of compound required to reduce the number/size of plaques by 50%, relative to the number/size of plaques in untreated wells.

2.6. Testing of cytotoxicity

The effect of the drugs on the growth of uninfected MDCK cells in 96-well plates was determined by using a LIVE/DEAD Viability/Cytotoxicity Kit (L-3224) (Molecular Probes Inc., Eugene, OR). Briefly, a confluent monolayer of cells was overlaid with MEM containing amantadine and oseltamivir carboxylate, individually and combined, at each concentration to be tested (Fig. 1). After 48 h of incubation at 37 °C, the plates were stained with calcein AM and ethidium homodimer-1 for 30 min at room temperature. The fluorescence of drug-exposed

and control cell samples was measured by using two sets of filters, according to the manufacturer's protocol. The relative numbers of living and dead cells were expressed in terms of percentages; e.g., the proportion of living cells was expressed as a percentage of the total number of cells.

2.7. NA enzyme inhibition assay

NA activity was determined by the method described by Potier et al. (1979). Briefly, viruses and various concentrations of oseltamivir carboxylate were preincubated for 30 min at 37 °C before the addition of the substrate 2′-(4-methylumbelliferyl)-D-*N*-acetylneuraminic acid (MUN, Sigma, UK). After 1 h, the reaction was terminated by adding 14 mM NaOH and fluorescence was quantitated in a Perkin-Elmer fluorimeter (model LS50B) with an excitation wavelength of 360 nm, an emission wavelength of 448 nm. The IC_{50}s were defined as the concentration of NA inhibitor necessary to reduce the NA activity by 50% relative to in a reaction mixture containing virus but no inhibitor.

2.8. Assay of virus binding to sialic acid-containing substrates

The synthesis of polyvalent synthetic sialoglycoconjugates [3′- and 6′-sialylglycopolymers obtained by conjugation of a 1-*N*-glycyl derivative of 3′-sialyllactose (p3′SL), 6′-sialyllactose (p6′SL), or 6′-sialyllactosamine with poly(4-phenylacrylate) (p6′SLN)] was described earlier (Bovin et al., 1993). The binding of human influenza viruses to fetuin was performed in a direct solid-phase assay using the immobilized virus and horseradish peroxidase (HRP)-conjugated fetuin, as described previously (Gambaryan and Matrosovich, 1992). The affinity of viruses toward p3′SL, p6′SL, and p6′SLN was measured in a competitive assay based on the inhibition of binding of the HRP-labeled fetuin (Matrosovich et al., 1993). The dissociation constants (K_d) were determined as sialic acid (Neu5Ac) concentration at the point $A_{max}/2$ of Scatchard plots.

2.9. RNA isolation, PCR amplification and sequencing

Viral RNA was isolated from virus-containing allantoic fluid using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Reverse transcription of viral RNA and subsequent PCR was performed using primers specific for the HA, NA, and M gene segments, as described previously (Hoffmann et al., 2001). The sequencing reaction was performed by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital. The DNA template was sequenced by using rhodamine or dRhodamine dye terminator cycle-sequencing Ready Reaction kits with AmpliTaqDNA polymerase FS (Perkin-Elmer, Applied Biosystems, Inc., Foster City, CA) and synthetic oligonucleotides. Samples were analyzed in a Perkin-Elmer Applied Biosystems DNA sequencer (model 373 or 377). DNA sequences were completed and edited by using the Lasergene sequence analysis software package (DNASTAR, Madison, WI).

2.10. Statistical analysis

Data points (at least triplicates) are presented as the arithmetic means ± standard errors of the mean. Experimental data were analyzed by Student's *t*-test for significant differences. *P*-values were subject to a significance level of 5%.

3. Results

3.1. Inhibitory activities of antiviral drugs on influenza A virus infection in MDCK cells

To evaluate the susceptibility of influenza A/Nanchang/1/99 (H1N1), A/Panama/2007/99 (H3N2), and A/Hong Kong/156/97 (H5N1) viruses to amantadine and oseltamivir carboxylate, we determined reduction of virus yield in the presence of the drugs by plaque assay in MDCK cells (Fig. 2). Single-agent amantadine at concentration of 10 μM resulted in ∼90% reduction in virus replication and at concentration of 100 μM inhibited virus

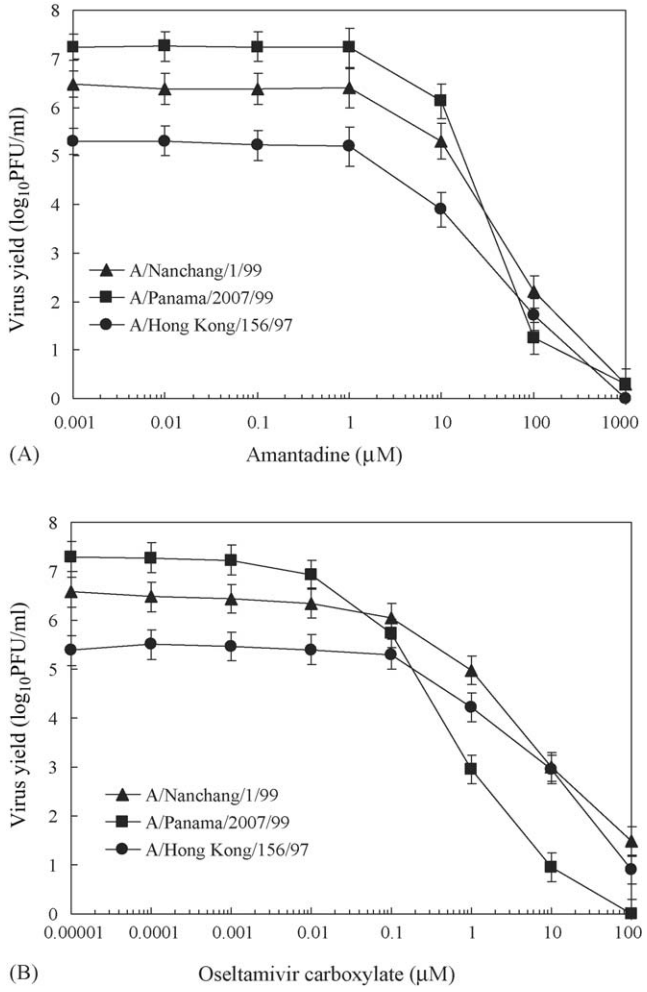


Fig. 2. Reduction of influenza virus yield by amantadine (A) and oseltamivir carboxylate (B) in MDCK cells. Cells were infected with either H1N1, H3N2 or H5N1 viruses at MOI of 0.001 PFU/cell and after 1 h of adsorption overlaid with infection medium in the presence or absence of a single drug. Virus yield was determined in culture supernatants 72 h after infection by plaque assay. Values are the means (log_{10}PFU/ml) ± S.D. for three independent experiments.

yield of all tested strains from $>10^5$ to $\sim10^2$ PFU/ml (Fig. 2A). Treatment with 1 μM oseltamivir carboxylate alone reduced virus yield of A/Nanchang/1/99 and A/Hong Kong/156/97 viruses by 90%; treatments as low as 10 μM inhibited virus yield of influenza A/Panama/2007/99 virus from $>10^7$ to ∼10 PFU/ml in MDCK cells (Fig. 2B). Based on these data, we selected a wide range of concentrations of both agents to obtain variants with stable drug-resistance phenotypes (Fig. 1). Increasing amantadine concentrations (10, 31.25, 62.5, and 125 μM) reduced the parental virus yields by 90% to ∼100%, and those of oseltamivir carboxylate (0.0001, 0.001, 0.01, 0.1, and 1 μM) reduced virus yield by 0% to ∼90% (Fig. 2).

3.2. Biological characterization of selected variants

To investigate the effect of amantadine, oseltamivir carboxylate, and their combination, we cultured the H1N1, H3N2, and H5N1 strains in MDCK cells in the presence of the drugs and determined biological characteristics of the viruses after five sequential passages. Viruses cultivated in the presence of each drug individually had the same virus yield as MDCK-passaged parental strains, indicating that some resistant variants occurred (Table 1). Viruses cultivated in the presence of both drugs showed three different patterns of viral yield changes that depended on the dose of the NA inhibitor. First, no virus was detected in culture supernatants when amantadine was paired with oseltamivir carboxylate, but the dose of NA inhibitor required varied between the strains. Specifically, when combined with amantadine, 1 μM NA inhibitor blocked infection of the H1N1 strain, ≥0.1 μM NA inhibitor was needed for the H3N2 strain, and ≥0.01 μM NA inhibitor was needed for the H5N1 strain. Inhibition of virus yield was shown at different passages depending on the virus and the dose of the NA inhibitor used. Second, amantadine combined with the lowest doses of NA inhibitor (≤0.01, ≤0.001, and ≤0.0001 μM for H1N1, H3N2, and H5N1 viruses, respectively) did not influence virus yield. Third, the combination of amantadine and oseltamivir carboxylate (0.1, 0.01, and 0.001 μM for H1N1, H3N2, and H5N1 strains, respectively) resulted in ≥100-fold suppression of extracellular virus yield ($P<0.005$; Table 1).

Plaque morphology of H1N1, H3N2, and H5N1 variants selected after five sequential passages in vitro in the presence of antiviral drugs showed variations. Viruses cultivated with NA inhibitor (≥0.1 and ≥0.01 μM for H1N1 and H3N2 viruses, respectively) produced plaques that were three to five times larger in diameter than those of MDCK-passaged parents (Table 1). Variants of A/Hong Kong/156/97 virus cultured in vitro with ≥0.001 μM NA inhibitor had a moderate, although statistically significant increase in plaque size ($P<0.003$). In contrast, we did not detect changes in plaque size of variants cultured with amantadine alone or in combination with the NA inhibitor (Table 1).

3.3. Susceptibility of selected variants to antiviral drugs

We tested the susceptibility to amantadine of H1N1, H3N2, and H5N1 variants cultivated with single-agent by plaque reduction assay. MDCK-passaged parents that lacked the drug pressure were used as sensitive controls (Table 2). The latter exhibited ∼100- to 200-fold higher susceptibility to amantadine than did those cultured with amantadine alone. Conversely, the M2 blocker (≤100 μM) failed to inhibit the replication of H1N1, H3N2, and H5N1 variants selected during sequential passages with amantadine. Hence, the design of our experiments

Table 1
Effect of amantadine–oseltamivir carboxylate combinations on virus yield and plaque diameter of influenza H1N1, H3N2 and H5N1 variants

Amantadine (μM)	Oseltamivir carboxylate (μM)	Influenza viruses used for combination treatment[a]					
		A/Nanchang/1/99 (H1N1)		A/Panama/2007/99 (H3N2)		A/Hong Kong/156/97 (H5N1)	
		Virus yield ($\log_{10}$PFU/ml)	Plaque diameter ± S.D. ($\times10^{-1}$ mm)	Virus yield ($\log_{10}$PFU/ml)	Plaque diameter ± S.D. ($\times10^{-1}$ mm)	Virus yield ($\log_{10}$PFU/ml)	Plaque diameter ± S.D. ($\times10^{-1}$ mm)
–[b]	–	6.5	3.3 ± 0.6	7.2	7.5 ± 1.1	5.3	1.8 ± 0.3
125[c]	–	6.8	3.3 ± 0.6	7.0	6.9 ± 1.3	5.5	1.9 ± 0.2
–	1	6.6	16.5 ± 1.1*	7.1	21.2 ± 2.0*	5.3	3.3 ± 0.3*
–	0.1	6.3	15.7 ± 0.9*	7.6	19.3 ± 1.1*	5.8	3.2 ± 0.4*
–	0.01	6.2	3.6 ± 0.5	7.0	18.9 ± 1.5*	5.9	3.1 ± 0.3*
–	0.001	6.2	3.2 ± 0.8	7.3	7.5 ± 1.3	5.5	3.4 ± 0.2*
–	0.0001	NT[d]	NT	NT	NT	5.3	1.9 ± 0.2
125	1	<[e]	<	<	<	<	<
125	0.1	4.5*	4.1 ± 0.6	<	<	<	<
125	0.01	6.8	3.5 ± 0.5	2.3*	7.7 ± 1.5	<	<
125	0.001	6.7	3.8 ± 0.6	7.0	6.9 ± 1.0	3.9*	1.8 ± 0.2
125	0.0001	NT	NT	NT	NT	5.5	2.1 ± 0.2

[a] Virus yield and plaque diameter were determined in the culture supernatants of MDCK cells after five sequential passages of either H1N1, H3N2 or H5N1 viruses in the presence of amantadine, oseltamivir carboxylate used individually or in combinations.
[b] –, drug was not administered; wild-type virus.
[c] Concentration of amantadine used in the fifth passage. Concentrations used in the previous passages are presented in Fig. 1.
[d] NT, not tested.
[e] <, below level of detection (<0.1 $\log_{10}$PFU/ml).
* $P<0.005$ analyzed by Student's *t*-test compared to MDCK-passaged parental virus.

Table 2
Effect of amantadine–oseltamivir carboxylate combinations on susceptibility of influenza variants to antiviral drugs

Amantadine (μM)	Oseltamivir carboxylate (μM)	Influenza viruses used for combination treatment[a]								
		A/Nanchang/1/99 (H1N1)			A/Panama/2007/99 (H3N2)			A/Hong Kong/156/97 (H5N1)		
		Plaque reduction assay[b] (μM)		NA inhibition assay (nM, mean $IC_{50} \pm$ S.D.)[c]	Plaque reduction assay (μM)		NA inhibition assay (nM, mean $IC_{50} \pm$ S.D.)	Plaque reduction assay (μM)		NA inhibition assay (nM, mean $IC_{50} \pm$ S.D.)
		Amantadine	Oseltamivir carboxylate	Oseltamivir carboxylate	Amantadine	Oseltamivir carboxylate	Oseltamivir carboxylate	Amantadine	Oseltamivir carboxylate	Oseltamivir carboxylate
–[d]	–	0.55 ± 0.07	0.01	3.3 ± 0.4	0.56 ± 0.03	0.001	0.67 ± 0.08	0.95 ± 0.05	0.01	3.3 ± 0.4
125[e]	–	>100	NT	NT	>100	NT	NT	>100	NT	NT
–	1	NT[f]	0.1*	3.1 ± 0.3	NT	0.1*	0.66 ± 0.05	NT	≥10*	3.4 ± 0.4
–	0.1	NT	0.1*	3.4 ± 0.4	NT	1*	0.68 ± 0.03	NT	10*	2.9 ± 0.6
–	0.01	NT	0.01	2.8 ± 0.2	NT	0.01*	0.71 ± 0.06	NT	≥10*	2.7 ± 0.5
–	0.001	NT	0.01	3.6 ± 0.5	NT	0.001	0.66 ± 0.05	NT	1*	3.1 ± 0.3
–	0.0001	NT	NT	NT	NT	NT	NT	NT	0.01	3.5 ± 0.6
125	1	<[g]	<	<	<	<	<	<	<	<
125	0.1	0.58 ± 0.03*	0.01	2.9 ± 0.3	<	<	<	<	<	<
125	0.01	>100	0.01	3.1 ± 0.2	0.63 ± 0.08*	0.001	0.69 ± 0.08	<	<	<
125	0.001	>100	0.01	3.0 ± 0.6	>100	0.001	0.72 ± 0.13	0.90 ± 0.11*	0.01	3.2 ± 0.3
125	0.0001	NT	NT	NT	NT	NT	NT	>100	0.01	2.6 ± 0.5

[a] Sensitivity of H1N1, H3N2 and H5N1 influenza viruses to amantadine and oseltamivir carboxylate was determined after five sequential passages in MDCK cells in the presence of the drugs.

[b] Sensitivity of viruses to amantadine and oseltamivir carboxylate in plaque reduction assay. We determined the dose of amantadine required to give a 50% reduction in plaque numbers and the dose of oseltamivir carboxylate required to reduce the plaque size by 50% disregarding the number of plaques.

[c] Sensitivity of viruses to oseltamivir carboxylate in NA enzyme inhibition assay. IC_{50} was defined as the concentration of NA inhibitor necessary to reduce the NA activity by 50% relative to in a reaction mixture containing virus but no inhibitor.

[d] –, drug was not administered; wild-type virus.

[e] Concentration of amantadine used in the fifth passage. Concentrations used in the previous passages are presented in Fig. 1.

[f] NT, not tested.

[g] <, below level of detection (<0.1 $\log_{10}$PFU/ml).

* $P < 0.05$ analyzed by Student's *t*-test compared to MDCK-passaged parental virus.

allowed us to generate amantadine-resistant variants in vitro (Table 2).

To determine whether NA inhibitor-resistant variants occurred during multiple passages in MDCK cells, we examined the sensitivity of H1N1, H3N2, and H5N1 influenza viruses cultivated with oseltamivir carboxylate in a plaque reduction assay (Table 2). Plaque size and number were inhibited at different concentrations of the drug; furthermore, two of the H5N1 variants generally produced more plaques in the presence of the NA inhibitor than in its absence (data not shown). There was apparent dose dependence, seen as an increase in plaque number with increasing drug concentrations. We calculated the dose of oseltamivir carboxylate required to reduce the plaque size by 50% disregarding the number of plaques (Table 2) (Blick et al., 1998; McKimm-Breschkin, 2000). Our results showed that H1N1, H3N2, and H5N1 influenza viruses cultivated with NA inhibitor (≥0.1, ≥0.01, and ≥0.001 μM, respectively) produced large plaques in the absence of drug. Their resistant phenotype was confirmed by the fact that they were approximately 10- to 10^3-fold less sensitive to the NA inhibitor than were MDCK-passaged parental strains (Table 2).

Furthermore, influenza viruses, which had been through five passages with oseltamivir carboxylate alone or in combination with amantadine were tested in an enzyme inhibition assay against the NA inhibitor. MDCK-passaged parental strains were used for comparison. No significant difference in sensitivity to oseltamivir carboxylate was detected (Table 2).

On the other hand, viruses cultured with drug combinations developed three patterns of drug susceptibility that were dependent on the dose of oseltamivir carboxylate. First, replication of influenza viruses was below level of detection after five passages in MDCK cells in the presence of amantadine combined with oseltamivir carboxylate (1 μM for H1N1, ≥0.1 μM for H3N2, and ≥0.01 μM for H5N1 viruses) (Table 2). Second, combination therapy resulted in the appearance of amantadine-resistant variants when the M2 blocker was paired with lower concentrations of the NA inhibitor (i.e., ≤0.01 μM for H1N1, ≤0.001 μM for H3N2, and ≤0.0001 μM for H5N1 viruses) (Table 2). Finally, because the selected variants exhibited the same enhancement in susceptibility to M2 blocker as sensitive MDCK-passaged parents, the drug-resistant variants did not occur when amantadine was combined with 0.1 μM oseltamivir carboxylate for H1N1 virus, 0.01 μM for H3N2 virus, and 0.001 μM for H5N1 virus (Table 2). It seems plausible that the ability of antiviral drugs to generate resistant variants could be decreased, and possibly suppressed, by certain combinations of the two compounds.

3.4. Sequence analysis of selected variants

To monitor the emergence of resistant variants under selection pressure of two drugs, we determined the sequence of the entire HA, NA, and M genes after five passages in the presence of different concentrations of each drug. No mutations occurred in the surface glycoproteins or M proteins when the viruses were cultured in vitro in the absence of the two drugs (Table 3). Analysis of the NA protein from all variants revealed no differences in sequence as compared with initial viruses (data not shown). However, amino acid substitutions in the M2 and HA proteins were observed in the majority of tested variants (Table 3).

The sequence of influenza A viruses passaged in the presence of amantadine alone, in two independent experiments, differed from that of the initial strains by the single mutations in the M2 protein: V27A and S31N/I (Table 3). Single-agent oseltamivir carboxylate at certain concentrations (≥0.1, ≥0.01,

Table 3
Sequence analysis of H1N1, H3N2 and H5N1 variants selected in the presence of antiviral drugs in MDCK cells

Amantadine (μM)	Oseltamivir carboxylate (μM)	Influenza viruses used for combination treatment[a] (amino acid substitutions)					
		A/Nanchang/1/99 (H1N1)		A/Panama/2007/99 (H3N2)		A/Hong Kong/156/97 (H5N1)	
		HA[b]	M2	HA	M2	HA	M2
–[c]	–	[e]					
125[d]	–		V27A		S31I		S31N
–	1	N187D		A131T, K160N		K222I	
–	0.1	N187D		K160E		S227I	
–	0.01			K158E		K222I	
–	0.001					H183N	
–	0.0001	NT[f]	NT	NT	NT		
125	1	<[g]	<	<	<	<	<
125	0.1			<	<	<	<
125	0.01		A30T			<	<
125	0.001		V27A		A30V		
125	0.0001	NT	NT	NT	NT		V27A

[a] Sequence analysis was performed with culture supernatants of MDCK cells after five sequential passages of either H1N1, H3N2 or H5N1 viruses in the presence of amantadine, oseltamivir carboxylate used individually or in combinations.
[b] Amino acid numbering is based on H3 HA (Nobusawa et al., 1991).
[c] –, drug was not administered; wild-type virus.
[d] Concentration of amantadine used in the fifth passage. Concentrations used in the previous passages are presented in Fig. 1.
[e] No mutations were detected.
[f] NT, not tested.
[g] <, below level of detection (<0.1 $\log_{10}$PFU/ml).

Table 4
Affinity of H1N1, H3N2 and H5N1 variants for sialyl substrates

Virus	Amino acid substitutions in HA protein[a]	Affinity for substrate[b]			
		Fetuin	p6′SL	p6′SLN	p3′SL
A/Nanchang/1/99 (H1N1)	[c]	0.09 ± 0.01	5.42 ± 0.17	3.09 ± 0.16	>100
	N187D	0.39 ± 0.07	22.11 ± 0.44	14.31 ± 0.28	>100
A/Panama/2007/99 (H3N2)		0.11 ± 0.03	0.49 ± 0.08	0.51 ± 0.04	>100
	A131T, K160N	0.64 ± 0.04	11.15 ± 0.71	13.51 ± 0.83	>100
	K160E	0.72 ± 0.11	4.41 ± 0.34	3.94 ± 0.29	>100
	K158E	0.58 ± 0.13	4.04 ± 0.26	4.23 ± 0.48	>100
A/Hong Kong/156/97 (H5N1)		0.06 ± 0.01	>100	>100	0.13 ± 0.01
	K222I	0.39 ± 0.03	>100	>100	0.89 ± 0.02
	S227I	0.47 ± 0.09	>100	>100	1.15 ± 0.03
	H183N	0.58 ± 0.11	>100	>100	1.19 ± 0.04

[a] Amino acid numbering is based on H3 HA (Nobusawa et al., 1991).
[b] K_d = mean ± S.E. × $t_{\alpha,n-1}$ (μM sialic acid), where t_α is Student's coefficient with probability $\alpha = 0.95$, from four independent experiments.
[c] HA sequence is identical to MDCK-passaged parents.

and ≥0.001 μM for H1N1, H3N2, and H5N1 viruses, respectively) selected the following amino acid changes in the HA protein: A131T, K158E, K160N/E, H183N, N187D, K222I, S227I (in H3 numbering here and in the whole text [Nobusawa et al., 1991]) inside or near the receptor-binding site (Table 3).

The patterns of selected mutations observed after combination antiviral treatment differed from that observed after single drug use. Amantadine combined with the concentrations of NA inhibitor (1 μM for H1N1, ≥0.1 μM for H3N2, and ≥0.01 μM for H5N1 virus) completely blocked virus replication (Table 3). Amantadine used in combination with the concentrations of NA inhibitor (≤0.01 μM for H1N1, ≤0.001 μM for H3N2, and ≤0.0001 μM for H5N1 virus) selected mutations within the transmembrane domain of M2 protein (V27A, A30T/V) (Table 3). However, the combination of amantadine with 0.1 μM oseltamivir carboxylate for H1N1 virus, 0.01 μM for H3N2 virus, and 0.001 μM for H5N1 virus did not select any amino acid changes in the HA, NA or M proteins.

3.5. Affinity of selected variants cultivated in the presence of oseltamivir carboxylate to sialic acid substrates

We measured the affinity of A/Nanchang/1/99, A/Panama/2007/99, and A/Hong Kong/156/97 variants selected after five sequential passages with oseltamivir carboxylate toward high molecular weight sialic acid substrates, both natural (fetuin) and synthetic (p3′SL, p6′SL, p6′SLN) (Table 4). Because most human influenza viruses display Sia2-6Gal-containing receptor-binding specificity (Matrosovich et al., 1999), the affinity to the 3′-substrate was negligible in the H1N1 and H3N2 variants carrying the mutations in the HA protein. The affinity of the latter to synthetic 6′-substrates and fetuin was approximately four to nine times less than that of the MDCK-passaged parents. Furthermore, influenza A/Panama/2007/99 (H3N2) strain with two mutations in the HA, A131T and K160N, showed a 25-fold decrease in its affinity to both 6′-substrates and did not distinguish p6′-SL from p6′-SLN, which has been proposed to be more similar to the human receptor than is 6′SL (Eisen et al., 1997) (Table 4). The A/Hong Kong/156/97 virus bound the receptors that are typical of avian but not human viruses (Matrosovich et al., 1999). As expected, the latter exhibited an expressed affinity toward 3′- and an inability to bind sialyl receptors with a 2′–6′ link between the sialic acid residue and galactose. All H5N1 variants with HA amino acid substitutions had a reduced affinity to fetuin and p3′SL (Table 4). In the present study, we demonstrated that the increase in resistance to oseltamivir carboxylate of at least some H1N1, H3N2, and H5N1 variants resulted from their low affinity to sialic acid receptors independent of their receptor-binding specificities (Table 4).

3.6. Cytotoxicity

Oseltamivir carboxylate used as a single agent caused no cytotoxicity in MDCK cells at the range of concentrations tested. Amantadine caused some cytotoxicity (~5–7% reduction of live cells) at the highest concentration tested (125 μM), whether used alone or in combination with the NA inhibitor. The combined chemotherapy did not enhance drug cytotoxicity in MDCK cells.

4. Discussion

In the present study we tested the hypothesis that treatment of influenza with compounds acting by different mechanisms may lead to more potent effects and benefits, such as reduced emergence of drug resistance. A few previous studies demonstrated that the combination of two drugs produced synergistic interactions in vitro and in vivo with no enhancement of cellular toxicity (Govorkova et al., 2004; Hayden et al., 1980b; Stephenson and Nicholson, 2001; Wilson et al., 1980), but effect of drug combinations on the emergence of resistance was not studied. We tested our concept by analysis of amino acid substitutions in HA, NA, and M proteins, virus yields, plaque morphology, drug sensitivity, and affinity to sialic acid receptors of human influenza H1N1, H3N2, and H5N1 viruses

cultured five sequential passages in MDCK cells in the presence of amantadine or oseltamivir carboxylate used individually or in combination.

Our results demonstrated that after influenza viruses were cultivated in MDCK cells in the presence of amantadine alone, the variants with amino acid changes in transmembrane domain of M2 protein at positions V27A and S31N/I emerged. This phenomenon was observed for the viruses of all three HA subtypes tested. Despite the fact that high concentrations of amantadine were used in our studies, we could surmise that strong selection pressure of the drug resulted in selection of amantadine-resistant variants. Previously, it was reported that mutations that confer resistance to amantadine are located at one of five amino acid positions (residues 26, 27, 30, 31, and 34) of the M2 protein (Hay et al., 1985; Pinto et al., 1992). The phenotypes of all identified variants were characterized by decreased drug sensitivity when plaqued in the presence of amantadine, demonstrating resistance 100-fold or greater than that of the MDCK-passaged parental viruses of different HA subtypes. Furthermore, our data have shown that the S31I substitution in the M2 protein of the H3N2 amantadine-resistant variant is associated with the same level of drug resistance as other observed mutations (Hay et al., 1985; Pinto et al., 1992). This finding is in agreement with the fact that the genetic basis for amantadine resistance appears to be single changes at certain positions in the M2 protein, but for different HA subtypes it could appear with different pattern of selection, that is, selection of different amino acid substitutions at the same positions. In addition, there was almost no impact of the different M2 mutations on viral phenotypic characteristics, or, at least, the effect was too low to be revealed as statistically significant.

Five passages with oseltamivir carboxylate alone resulted in the emergence of H1N1, H3N2, and H5N1 influenza viruses with reduced sensitivity to the compound in plaque reduction assay. We did not detect significant differences in the sensitivity to NA inhibitor in the NA enzyme inhibition assay for all tested viruses and no sequence changes in the conserved sites of the NA. However, we did detect changes in amino acids of the HA protein at residues 131, 158, 160, 183, 187, 222, and 227. These amino acids were located at either the HA receptor-binding site (positions 158, 160, 183), at the edge of the HA receptor-binding pocket (residues 131, 222 and 227), or next to this pocket (position 187) (Weis et al., 1988). In previous studies, a single amino acid change, L226Q, in the left edge of the receptor-binding pocket was shown to be responsible for altering the specificity of the HA from an Sia2-6Gal to Sia2-3Gal linkage (Rogers et al., 1983), as well as altering the host range of the virus. The T155Y substitution was associated with an altered affinity for a substrate with a methyl group replaced by a hydroxymethyl group (*N*-glycolyl neuraminic acid), whereas the T155A mutation caused both a decrease in the affinity of the H1 HA for sialic acid and resistance to NA inhibitor (McKimm-Breschkin et al., 1996). Amino acid substitutions S165N, S186F, and K222T occurred in the same position as in the present study and conferred high-level resistance to zanamivir (Blick et al., 1998; McKimm-Breschkin, 2000). Thus, mutations in H1, H3, and H5 HA proteins located inside or in the vicinity of the receptor-binding site could be expected to affect the affinity or specificity of the HA binding (Blick et al., 1998; Gubareva et al., 1996; McKimm-Breschkin, 2000; McKimm-Breschkin et al., 1996); that was assessed in vitro on the basis of the affinity of mutant viruses toward sialic acid substrates. All mutants, independent of their preferences to the linkage between sialic acid and galactose, showed reduced efficiency of virus binding to sialic acid substrates. Our data provided additional information that compensating mutations that result in weak binding of HA appear to circumvent inhibition of the NA by allowing the virus to be released from cells with less dependence on the NA function (Kaverin et al., 2000; McKimm-Breschkin, 2000). Notably, nearly all HA amino acid substitutions determined in the present study, except A131T and S227I, were found to be charged and thus could increase the negative charge of the HA molecule. This increase in negative charge may lead to an electrostatic repulsion between the virus particle and the macromolecules on the cell surface surrounding the sialic receptor, thereby reducing the efficiency of virus binding (Kaverin et al., 2000).

In our examination of other phenotypic characteristics (e.g., extracellular virus yield and plaque size) of human influenza viruses cultivated in vitro with NA inhibitor alone, we were not able to detect any significant effect of the HA mutations on virus yield. In contrast, the HA mutations were crucial for viral plaque diameter. These mutations in H1N1 and H3N2 influenza viruses caused an increase in plaque size; thus, an altered HA–NA balance was indicated (McKimm-Breschkin, 2000). Furthermore, two variants of influenza A/Hong Kong/156/97 (H5N1) virus with amino acid substitution K222I in HA protein demonstrated fewer plaques in the absence of NA inhibitor (data not shown), i.e., they exhibited drug-dependent phenotype that can be explained by the crucial mutation leading to functional mismatch between HA and NA as well (Gubareva et al., 1996; Kaverin et al., 2000; McKimm-Breschkin, 2000; McKimm-Breschkin et al., 1996). The decrease in HA affinity of these H5N1 variants could be too great and cause a deleterious effect on the fitness of the virus. Hence, the H5N1 mutants might have difficulty absorbing to cells before the NA cleaves off the target receptors. Therefore, these H5N1 mutants may infect cells more efficiently if the NA is not inhibited by the drug. Taken together, the results of our single-agent experiments suggest that HA mutations associated with the resistance to NA inhibitor compromised the viral fitness of influenza viruses.

The results of our combined chemotherapy experiments revealed three patterns of antiviral effects. (1) Replication of the influenza virus was completely blocked when the concentrations of the NA inhibitor (1, $\geq$0.1, and $\geq$0.01 μM for H1N1, H3N2, and H5N1 viruses, respectively) were combined with amantadine. Taking into account that similar results were obtained in tests of human influenza viruses of three different HA subtypes, we could surmise that two drugs caused a greater antiviral effect than did each drug alone (Scholtissek et al., 1998). The compounds were found to interact both additively and synergistically (Govorkova et al., 2004). (2) Combination therapy selected variants with mutations within the transmembrane domain of M2 protein at residues 27 and 30. This finding is consistent with

the idea that oseltamivir carboxylate at low concentrations did not have any effect, and therefore both drugs caused the same antiviral effect as single drug amantadine. (3) No amino acid substitutions in the HA, NA, or M proteins were detected when the combination of amantadine with relatively low doses of NA inhibitor was used. Moreover, influenza viruses were genetically stable after five subsequent passages in MDCK cells and had reduced extracellular virus yield ($P < 0.005$) as compared to that of MDCK-passaged strains. According to our data, one could speculate that antivirals that react, in our case, with both targets, the M2 ion-channel activity and the HA/NA function, might have enhanced antiviral activity that reduces probability to develop resistance to one of the anti-influenza agents. To escape such a compound, both targets would have to be mutated and some point mutations in two proteins might accumulate simultaneously (Scholtissek et al., 1998), but such an event is likely to be less frequent among mutational events in virus genome, even of RNA viruses. Hence, NA inhibitors and their derivatives that interfere with HA or NA function might prevent or delay amantadine resistance, if they are used in mixture with amantadine. Combination treatment may inhibit the selection and multiplication of drug-resistant viruses by decreasing the number of replication cycles. Thus, this treatment may reduce the quantity of mutated virus particles. In the present study, the results proved true for H1N1 and H3N2 viruses, which are the currently circulating strains, and for the more pathogenic H5N1 virus. Notably, the emergence of amantadine-resistant variants of A/Hong Kong/156/97 in vitro was delayed by concentrations of oseltamivir carboxylate as low as 0.001 μM.

Although we did not studied the effect of combination therapy in vivo, we suggest that in vitro assays could not be completely reliable to predict effectiveness of combination therapy in clinical studies (Ward et al., 2005). Therefore, additional animal experiments and clinical trials should be conducted in order to define dose requirements and dose-response relationships between antiviral agents. Further studies in animals are underway with the doses of the drugs relevant to those used in humans. These results will help to provide specific recommendations about appropriate usage of combined treatment based on evidence rather than extrapolation.

In conclusion, influenza is a globally important contagion that continues to cause an unacceptable number of deaths and substantial economic losses worldwide. Although up until now vaccination strategies constitute the mainstay of influenza control and prevention, the efficacy of this approach may be limited by a poor match between the vaccine and circulating strain, poor response of elderly patients, and the 6-month minimum time required to produce specific vaccine against a newly emergent pandemic virus (Stephenson and Nicholson, 2001). Therefore, both classes of antiviral agents serve important roles in the management of influenza. The present paper clearly indicates that combination therapy provides an advantage over single-agent therapy, such as reduction in the emergence of drug resistance. Together, the findings suggest that with the development of new drugs and optimized combinations, the combined treatment may provide an effective basis for prophylaxis and therapy of influenza virus infection.

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References

Blick, T.J., Sahasrabudhe, A., McDonald, M., Owens, I.J., Morley, P.J., Fenton, R.J., McKimm-Breschkin, J.L., 1998. The interaction of neuraminidase and hemagglutinin mutations in influenza virus in resistance to 4-Guanidino-Neu5Av2en. Virology 246, 95–103.

Bovin, N.V., Korchagina, E.Yu., Zemlyanukhina, T.V., Byramova, N.E., Galanina, O.E., Zemlyakov, A.E., Ivanov, A.E., Zubov, A.E., Mochalova, L.V., 1993. Synthesis of polymeric neoglycoconjugates based on N-substituted polyacrylamides. Glycoconj. J. 10, 142–151.

Bright, R.A., Medina, M., Xu, X., Perez-Oronoz, G., Wallis, T.R., Davis, X.M., Povinelli, L., Cox, N.J., Klimov, A.I., 2005. Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. Lancet 366, 1175–1181.

Claas, E.C., Osterhaus, A.D.M.E., Van Beek, R., De Long, J.C., Rimmelzwaan, G.F., Senne, D.A., Krauss, S., Shortridge, K.F., Webster, R.G., 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 351, 472–477.

Eisen, M.B., Sabesan, S., Skehel, J.J., Wiley, D.C., 1997. Binding of the influenza A virus to cell-surface receptors: structures of five hemagglutinin–sialyloligosaccharide complexes determined by X-ray crystrallography. Virology 232, 19–31.

Gambaryan, A.S., Matrosovich, M.N., 1992. A solid-phase enzyme-linked assay for influenza virus receptor-binding activity. J. Virol. Meth. 39, 111–123.

Gubareva, L.V., Bethell, R., Hart, G.J., Murti, K.G., Penn, C.R., Webster, R.G., 1996. Characterization of mutants of influenza A virus selected with the neuraminidase inhibitor 4-Guanidino-Neu5Ac2en. J. Virol. 70, 1818–1827.

Gubareva, L.V., Matrosovich, M.N., Brenner, M.K., Bethell, R.C., Webster, R.G., 1998. Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus. J. Infect. Dis. 178, 1257–1262.

Govorkova, E.A., Fang, H.B., Tan, M., Webster, R.G., 2004. Neuraminidase inhibitor-rimantadine combinations exert additive and synergistic anti-influenza virus effects in MDCK cells. Antimicrob. Agents Chemother. 48, 4855–4863.

Hay, A.J., Wolstenholm, A.J., Skehel, J.J., Smith, M.H., 1985. The molecular basis of the specific anti-influenza action of amantadine. EMBO J. 4, 3021–3024.

Hayden, F.G., Cote, K.M., Douglas, R.G., 1980a. Plaque inhibition assay for drug susceptibility testing of influenza viruses. Antimicrob. Agents Chemother. 17, 865–870.

Hayden, F.G., Douglas, R.G., Simons, R., 1980b. Enhancement of activity against influenza viruses by combinations of antiviral agents. Antimicrob. Agents Chemother. 18, 536–541.

Hayden, F.G., Hay, A.J., 1992. Emergence and transmission of influenza A viruses resistant to amantadine and rimantadine. Curr. Top. Microbiol. Immunol. 176, 119–130.

Hayden, F.G., 1996. Amantadine and rimantadine—clinical aspects. In: Richman, D.D. (Ed.), Antiviral Drug Resistance. Wiley, New York, pp. 59–77.

Hayden, F.G., 2001. Prospectives on antiviral use during pandemic influenza. Phil. Trans. R. Soc. London 356, 1877–1884.

Herlocher, M.L., Truscon, R., Elias, S., Yen, H.L., Roberts, N.A., Ohmit, S.E., Monto, A.S., 2004. Influenza viruses resistant to the antiviral drug oseltamivir: transmission studies in ferrets. J. Infect. Dis. 190, 1627–1630.

Hoffmann, E., Stech, J., Guan, Y., Webster, R.G., Perez, D.R., 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. 146, 2275–2289.

Ilyushina, N.A., Govorkova, E.A., Webster, R.G., 2005. Detection of amantadine-resistant variants among avian influenza viruses isolated in North America and Asia. Virology 341, 102–106.

Kaverin, N.V., Matrosovich, M.N., Gambaryan, A.S., Rudneva, I.A., Shilov, A.S., Varich, N.L., Makarova, N.V., Kropotkina, E.A., Sinitsin, B.V., 2000. Intergenic HA–NA interactions in influenza A virus: postreassortment substitutions of charged amino acid in the hemagglutinin of different subtypes. Virus Res. 66, 123–129.

Kiso, M., Mitamura, K., Sakai-Tagawa, Y., Shiraishi, K., Kawakami, C., Kimura, K., Hayden, F.G., Sugaya, N., Kawaoka, Y., 2004. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. Lancet 364, 759–765.

Klimov, A.I., Rocha, E., Hayden, F.G., Shult, P.A., Roumillat, L.F., Cox, N.J., 1995. Prolonged shedding of amantadine-resistant influenza A viruses by immunodeficient patients: detection by polymerase chain reaction-restriction analysis. J. Infect. Dis. 172, 1352–1355.

Lew, W., Chen, X., Kim, C.U., 2000. Discovery and development of GS4104 (oseltamivir): an orally active influenza neuraminidase inhibitor. Curr. Med. Chem. 7, 663–672.

Madren, L.K., Shipman, C., Hayden, F.G., 1995. In vitro inhibitory effects of combinations of anti-influenza agents. Antivir. Chem. Chemother. 6, 109–113.

Matrosovich, M.N., Gambaryan, A.S., Tuzikov, A.B., Byramova, N.A., Mochalova, L.V., Golbraikh, A.A., Shenderovich, M.D., Finne, J., Bovin, N.V., 1993. Probing of the receptor-binding sites of the H1 and H3 influenza virus hemagglutinins by synthetic and natural sialosides. Virology 196, 111–123.

Matrosovich, M.N., Zhou, N., Kawaoka, Y., Webster, R.G., 1999. The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. J. Virol. 73, 1146–1155.

McKimm-Breschkin, J.L., Blick, T.J., Sahasrabudhe, A., Tiong, T., Marshall, D., Hart, G.J., Bethell, R.C., Penn, C.R., 1996. Generation and characterization of variants of NWS/G70C influenza virus after in vitro passage in 4-amino-Neu5Ac2en and 4-guanidino-Neu5Ac2en. Antimicrob. Agents Chemother. 40, 40–46.

McKimm-Breschkin, J.L., 2000. Resistance of influenza viruses to neraminidase inhibitors—a review. Antivir. Res. 47, 1–17.

McKimm-Breschkin, J.L., 2005. Management of influenza virus infections with neuraminidase inhibitors: detection, incidence, and implications of drug resistance. Treat. Respir. Med. 4, 107–116.

Monto, A.S., Fleming, D.M., Henry, D., de Groot, R., Makela, M., Klein, T., Elliott, M., Keene, O.N., Man, C.Y., 1999. Efficacy and safety of the neuraminidase inhibitor zanamivir in the treatment of influenza A and B virus infections. J. Infect. Dis. 180, 254–261.

Monto, A.S., 2003. The role of antivirals in the control of influenza. Vaccine 21, 1796–1800.

Nobusawa, E., Aoyama, T., Kato, H., Suzuki, Y., Tateno, Y., Nakajima, K., 1991. Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. Virology 182, 475–485.

Pinto, L.H., Holsinger, L.J., Lamb, L.A., 1992. Influenza virus M2 protein has ion channel activity. Cell 69, 517–528.

Potier, M., Mameli, L., Belislem, M., Dallaire, L., Melanxon, S.B., 1979. Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl-D-*N*-acetylneuraminate) substrate. Anal. Biochem. 94, 287–296.

Rogers, G.N., Paulson, J.C., Daniels, R.S., Skehel, J.J., Wilson, I.A., Wiley, D.C., 1983. Single amino acid substitutions in influenza hemagglutinin change receptor binding specificity. Nature 304, 76–78.

Scholtissek, C., Quack, G., Klenk, H.-D., Webster, R.G., 1998. How to overcome resistance of influenza A viruses against adamantane derivatives? Antivir. Res. 37, 83–95.

Shiraishi, K., Mitamura, K., Sakai-Tagawa, Y., Goto, H., Sugaya, N., Kawaoka, Y., 2003. High frequency of resistant viruses harboring different mutations in amantadine-treated children with influenza. J. Infect. Dis. 188, 57–61.

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

Stephenson, I., Nicholson, K.G., 2001. Influenza: vaccination and treatment. Eur. Respir. J. 17, 1282–1293.

Varghese, J.H., McKimm-Breschkin, J.L., Caldwell, J.B., Kortt, A.A., Colman, P.M., 1992. The structure of the complex between influenza virus neuraminidase and sialic acid, the viral receptor. Proteins 14, 327–332.

von Itzstein, M., Wu, W.-Y., Kok, G.K., Pegg, M.S., Dyason, J.C., Jin, B., Van Phan, T., Smithe, M.L., White, H.F., Oliver, S.W., Colman, P.M., Varghese, J.N., Ryan, D.M., Woods, J.M., Bethell, R.C., Hotham, V.J., Cameron, J.M., Penn, C.R., 1993. Rational design of potent sialidase-based inhibitors of influenza virus protection. Nature 363, 418–423.

Ward, P., Small, I., Smith, J., Suter, P., Dutkowski, R., 2005. Oseltamivir (Tamiflu) and its potential for use in the event of an influenza pandemic. J. Antimicrob. Chemother. 55, 5–21.

Weis, W., Brown, J.H., Cusack, S., Paulson, J.C., Skehel, J.J., Wiley, D.C., 1988. Structure of the influenza virus hemagglutinin complexed with its receptor, sialic acid. Nature 333, 426–431.

Wilson, S.Z., Knight, V., Wyde, P.R., Drake, S., Couch, R.B., 1980. Amantadine and ribavirin aerosol treatment of influenza A and B infection in mice. Antimicrob. Agents Chemother. 17, 642–648.

World Health Organization, 2004. Avian influenza A (H5N1). Weekly Epidemiol. Rev. 79, 65–70.

Ziegler, T., Hemphill, M.L., Ziegler, M.L., Perez-Oronoz, G., Klimov, A.I., Hampson, A.W., Regnery, H.L., Cox, N.J., 1999. Low incidence of rimantadine resistance in field isolates of influenza A viruses. J. Infect. Dis. 180, 935–939.