Enhancement of Activity Against Influenza Viruses by Combinations of Antiviral Agents

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In an investigation of alternative therapeutic approaches for the treatment of influenza virus infections, the antiviral activities of rimantadine hydrochloride, amantadine hydrochloride, ribavirin, and combinations of these drugs were assessed in vitro. Madin-Darby canine kidney cell monolayers were inoculated with recent isolates of influenza viruses at low multiplicities of infection, and virus titers were determined after 24 h. The combination of rimantadine and ribavirin resulted in an enhanced antiviral effect (a decrease in virus titer of >1.0 log_{10} plaque-forming unit per ml at 24 h relative to the maximal effect of a single drug) against A/USSR/90/77/H1N1, A/Texas/1/77/H3N2, A/New Jersey/76/HSW1N1, and A/PR/834/H0N1 viruses. The degree of inhibition depended on the virus strain used, the drug concentrations, and the virus inoculum. Amantadine and ribavirin showed enhanced activity. Ribavirin in combination with high (50 μg/ml), but not low (1.56 to 25 μg/ml), concentrations of rimantadine showed an enhanced antiviral effect against B/Hong Kong/72 virus. An assay of Madin-Darby canine kidney cell proliferation in the presence of drugs showed that the enhanced inhibitory effect of drug combinations was not due to increased cytotoxicity.

The adamantane compounds amantadine hydrochloride (Symmetrel) and rimantadine hydrochloride have well documented prophylactic and therapeutic activities against uncomplicated human influenza A virus infections (6, 17, 27; L. P. VanVoris, R. B. Betta, F. G. Hayden, W. A. Christman, and R. G. Douglas, Jr., Program Abstr. Intersci. Conf. Antimicrob. Agents, Chemother. 18th, Atlanta, Ga., abstr. no. 483). Anecdotal evidence suggests that high doses of amantadine may have a beneficial effect against influenza viral pneumonia (5). However, no specific therapy of proven value presently exists for treatment of serious influenza virus infections, including primary viral and mixed viral-bacterial pneumonias. Ribavirin (Virazole) has antiviral activity against influenza A and influenza B viruses in vitro (11, 19) and in animal models of influenza, but clinical trials have found little evidence of therapeutic effectiveness (4, 18, 23, 26). In an effort to develop alternative therapeutic approaches to single-drug therapy, the present study was undertaken to determine the antiviral activities of combinations of these drugs in vitro. The results indicated that the combination of rimantadine hydrochloride and ribavirin exerted an enhanced antiviral effect against influenza viruses compared with the effects of single drugs.

MATERIALS AND METHODS

Drugs. Crystalline powders of amantadine hydrochloride and rimantadine hydrochloride were kindly provided by E. I. duPont de Nemours & Co., Inc. Wilmington, Del., and ribavirin was provided by ICN Pharmaceuticals Inc., Covina, Calif. Immediately before each experiment, drugs were solubilized in sterile, double-distilled water, and dilutions were made in cell culture media (see below).

Cell culture. Madin-Darby canine kidney (MDCK) cells (Flow Laboratories, Inc., Rockville, Md.) were passaged weekly with growth medium containing Eagle minimal essential medium, glutamine, 10% heat-inactivated fetal bovine serum, penicillin, and gentamicin.

Viruses. Four influenza A virus strains (A/PR/8/34/H0N1, A/USSR/90/77/H1N1, A/New Jersey/76/HSW1N1, A/Texas/1/77/H3N2) and one influenza B virus strain (B/Hong Kong/72) were used in these studies. Except for the highly passaged A/PR/8/34 strain, these viruses were clinical isolates which were either recovered in the University of Rochester Influenza Surveillance Laboratory or provided by Allan Kendall, Virology Division, Center for Disease Control, Atlanta, Ga. The University of Rochester isolations were made initially in primary rhesus monkey kidney cell monolayers (Microbiological Associates, Bethesda, Md.). Viruses were passaged in embryonated hen eggs (1 to 14 passages), and samples of allantoic fluid were stored at −70°C.

Viral titrations. Viral titrations were performed with modifications of a plaque assay described by Tobita and co-workers (24). Disposable cell culture
plates (24 wells; diameter, 16 mm; Costar, Cambridge, Mass.) were seeded with approximately 5 x 10⁴ MDCK cells in 1.0 ml of growth medium and incubated at 36°C in an humidified atmosphere containing 5% CO₂ for 2 to 3 days, until monolayers became confluent. Triplicate monolayers were washed free of protein-containing media and inoculated with 0.2-ml volumes of virus suspension. Virus dilutions were made in Hanks balanced salt solution (pH 7.2 to 7.4) containing 0.5% gelatin. Plates were incubated at 36°C for 60 min, and then the monolayers were overlaid with 1 ml of 0.6% agarose containing Eagle minimal essential medium and 2 μg of trypsin (Worthington Biochemicals Corp., Freehold, N.J.) per ml. After incubation for 48 h, the monolayers were fixed with 10% Formalin and stained with methylene blue. Virus titers were expressed as log₁₀ plaque-forming units per milliliter.

Drug studies. The in vitro inhibitory activities of drugs alone and in combinations were determined as follows. Confluent monolayers of MDCK cells grown in 24-well plates as described above were inoculated in quadruplicate with 0.2-ml volumes of virus suspension. Except for experiments to determine the effects of virus inocula the multiplicities of infection ranged from 10⁻² to 10⁻⁴ PFU/monolayer cell. After a 60-min adsorption period at 36°C, Eagle minimal essential media containing trypsin (final concentration, 2 μg/ml) and the appropriate drug dilutions were added to the wells. The drug dilutions were selected to include concentrations that have been found to inhibit influenza virus plaque production in MDCK cell monolayers (10). After incubation at 36°C for 24 h, the supernatants from quadruplicate wells were harvested and pooled. Portions were frozen at −70°C for later titration. In certain experiments the monolayers were refed with freshly prepared, drug-containing medium, and repeat harvests were made at 48 and 72 h after the initial virus inoculation.

The parameters of virus replication tested were estimation of cytopathic effects and titration of infectivity by the plaque assay described above. Enhancement of antiviral activity was considered to be present if a combination of drugs resulted in a more than 90% decrease (>1.0 log₁₀ PFU/ml) in virus yield at 24 h compared with the effect of either drug alone. A synergistic interaction of a combination was defined as a decrease in virus yield at 24 h that was greater than the algebraic sum of the decreases observed with single drugs (13).

Drug cytotoxicity. The effects of single drugs and combinations of drugs on MDCK cells were assessed by observations for cytopathic effects in drug-exposed, uninfected monolayers. MDCK cell proliferation was studied in the presence of single drugs and combinations of drugs. Approximately 2 x 10⁴ MDCK cells in 2.0-ml portions of growth media containing appropriate drug dilutions were placed in disposable plates (diameter, 35 mm; Costar). After incubation for 24, 48, or 72 h, duplicate supernatant fluids were harvested, and adherent monolayer cells were removed by trypsinization with 0.25% trypsin-ethylenediaminetetraacetic acid. Cells were sedimented in the presence of calf serum to stop the action of trypsin and to prevent clumping and then suspended in phosphate-buffered saline for counting. Viability was determined by trypan blue exclusion.

RESULTS

Enhancement of antiviral effect. The combination of rimantadine hydrochloride and ribavirin consistently resulted in an enhanced antiviral effect against representative strains of influenza A viruses compared with the effects of single drugs. The results of a representative assay with A/USSR/90/77/H1N1 virus are shown in Fig. 1. At 24 h after virus inoculation, infected monolayers in the absence of either drug showed cytopathic effects of 1+ to 2+ and yielded virus titers of 5.9 log₁₀ PFU/ml. At 48 h these monolayers and those containing either 1.56 μg of rimantadine per ml or 3.12 μg of ribavirin per ml in the supernatant displayed cytopathic effects of 4+ and contained high titers of infectious virus. In contrast, the combination of rimantadine and ribavirin suppressed all measures of virus replication at 24 h and resulted in an additional decrease in virus yield of 3.4 log₁₀

![Figure 1](http://aac.asm.org/)

**Fig. 1.** Quantity of infectious influenza A/USSR/90/77/H1N1 virus in MDCK monolayers in the presence of trypsin (2 μg/ml) and rimantadine hydrochloride (1.56 μg/ml) (○), ribavirin (3.12 μg/ml) (■), rimantadine hydrochloride and ribavirin (▲), or no drug (●).
PFU/ml compared with the maximal effect of either drug alone. Low titers of infectious virus were present at 48 and 72 h in these wells.

In other experiments with A/USSR/90/77/H1N1, the inhibitory effect of amantadine alone or in combination with ribavirin was similar to the effect observed with the same concentration of rimantadine. Experiments also showed that combinations of rimantadine hydrochloride and amantadine hydrochloride, which are postulated to have the same mechanism of antiviral action (6), and combinations of rimantadine hydrochloride and rifampin, a drug with no recognized activity against influenza viruses (24), did not result in enhanced inhibitory activity compared with the effects of single drugs (data not shown).

Effect of strain variation. Table 1 shows the results of individual assays with a combination of 1.56 μg of rimantadine hydrochloride per ml and 3.12 μg of ribavirin per ml against a variety of influenza viruses. This combination consistently resulted in an enhanced antiviral effect, in that an additional reduction of a least 1.0 log₁₀ PFU/ml beyond the decrease observed with either drug alone was observed for all of the influenza A viruses studied. This combination of rimantadine and ribavirin resulted in a significantly greater reduction in 24-h virus titers than either single drug alone for both A/Texas/1/77/H3N2 (P < 0.05, paired t test) and A/USSR/90/77/H1N1 (P < 0.02). The mean additional decrement in virus titers beyond the sum of single-drug effects was 1.4 log₁₀ PFU/ml for A/USSR/90/77/H1N1, but only 0.4 log₁₀ PFU/ml for A/Texas/1/77/H3N2 (P > 0.1, t test for unpaired samples). A/PR/8/34/H0N1, which is known to be relatively rimantadine resistant (9, 15) and which was not inhibited by rimantadine alone at this concentration, was inhibited to a greater extent by the combination than by single drugs. In contrast, influenza B/Hong Kong/72 virus, which is highly resistant to rimantadine (10), was not inhibited by this combination beyond the inhibitory effect of ribavirin alone. Other experiments showed that rimantadine in concentrations of 50 but not 25 μg/ml in combination with 3.12 or 6.25 μg of ribavirin per ml resulted in an enhanced antiviral effect against B/Hong Kong/72 (data not shown).

Effect of varying drug concentrations. The concentrations of each drug in the supernatants influenced the inhibitory effects of single drugs or combinations. Table 2 lists the decreases in A/USSR/90/77/H1N1 virus titers at 24 h compared with infected control wells, as observed when the rimantadine concentration was varied from 0 to 1.56 μg/ml and the concentration of ribavirin was held constant. The values listed are the means of two experiments. Rimantadine alone at 0.1 μg/ml resulted in a decrease in virus yield of 2.5 log₁₀ PFU/ml, but increasing concentrations of rimantadine over a 15-fold range did not increase this degree of inhibition. The additional decrease in virus yield observed with the addition of ribavirin at a fixed dose was less than 0.6 log₁₀ PFU/ml at the lower rimantadine concentrations, but increased to over 1.8 logs at the highest concentration tested.

Table 3 shows the effect of varying ribavirin concentrations from 0 to 6.25 μg/ml while holding the rimantadine concentration at 1.56 μg/ml for A/Texas/1/77/H3N2. The values listed are the means of three experiments. In contrast to rimantadine, ribavirin alone demonstrated a dose-response inhibition of virus yield at 24 h that ranged from a reduction of 0.9 log₁₀ PFU/ml at 1.56 μg/ml to a reduction of 3.5 log₁₀ PFU/ml at 6.25 μg/ml. The additional decrease in virus yield observed with the addition of rimantadine at 1.56 μg/ml was 0.4 log₁₀ PFU/ml or

Table 1. Inhibition of influenza virus multiplication in MDCK cell monolayers by rimantadine hydrochloride and ribavirin

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Decrease in virus titer at 24 h (log₁₀ PFU/ml) with:</th>
<th>Effect of combination (log₁₀ PFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rimantadine (1.56 μg/ml)</td>
<td>Ribavirin (3.12 μg/ml)</td>
</tr>
<tr>
<td>A/Texas/1/77/H3N2</td>
<td>1.7 ± 0.7</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>A/USSR/90/77/H1N1</td>
<td>2.4 ± 0.5</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td>A/New Jersey/76/HSW1N1</td>
<td>1.6</td>
<td>3.2</td>
</tr>
<tr>
<td>A/PR/8/34/H0N1</td>
<td>-0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>B/Hong Kong/72</td>
<td>0.1</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* Decrease in virus titer relative to infected control monolayers, expressed as mean ± standard deviation or as the results of individual experiments.

Additional decrement in virus titers at 24 h beyond the algebraic sum of the decreases observed with either single drug.

P < 0.02 by the paired t test (combination versus maximal effect of either single drug).

P < 0.05 by the paired t test (combination versus maximal effect of either single drug).
less for all concentrations tested.

**Effects of inoculum size.** Table 4 shows the effect of progressively higher virus inputs in this test system. The values listed are the means of two experiments. The lowest multiplicity of infection tested (10^4 PFU/cell) was similar to that used in the experiments described above, and the combination of 1.56 μg of rimantadine per ml and 3.12 μg of ribavirin per ml resulted in an additional reduction in virus yield at 24 h of 1.3 log_{10} PFU/ml compared with the sum of the effects observed with each drug alone. With 100-fold-greater virus inoculum, the inhibitory effects of single drugs, particularly rimantadine, and of the combination decreased sharply but were still present. At a virus input equivalent to 1 PFU/cell, antiviral activity, as defined by an inhibition of virus yield of at least 1 log_{10}, was not apparent. Whether increasing drug concentrations could overcome this inoculum effect was not studied.

**Drug cytotoxicity.** High rimantadine concentrations (50 μg/ml), alone and in combination with ribavirin, resulted in slight visible cytotoxic effects. No obvious cytopathic effects were apparent at the highest ribavirin concentration (12.5 μg/ml) studied. As Table 5 shows, MDCK cell proliferation at 48 and 72 h decreased in the presence of ribavirin in a dose-

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**TABLE 2. Inhibition of A/USSR/90/77/H1N1 by combinations of ribavirin (3.12 μg/ml) and various concentrations of rimantadine hydrochloride**

<table>
<thead>
<tr>
<th>Rimantadine concn (μg/ml)</th>
<th>Decrease in virus titer (log_{10} PFU/ml)*</th>
<th>Rimantadine and ribavirin (3.12 μg/ml)</th>
<th>Additional effect of combination®</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>2.5</td>
<td>3.6</td>
<td>-0.3</td>
</tr>
<tr>
<td>0.39</td>
<td>2.7</td>
<td>4.6</td>
<td>0.6</td>
</tr>
<tr>
<td>1.56</td>
<td>2.6</td>
<td>≥5.8</td>
<td>≥1.8</td>
</tr>
</tbody>
</table>

* Decrease in 24-h virus titers relative to infected control wells (mean of two experiments).

® Additional decrement in virus yield with drug combination beyond the algebraic sum of the decreases observed with either single drug.

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**TABLE 3. Inhibition of A/Texas/1/77/H3N2 by combinations of rimantadine hydrochloride (1.56 μg/ml) and various concentrations of ribavirin**

<table>
<thead>
<tr>
<th>Ribavirin concn (μg/ml)</th>
<th>Ribavirin alone</th>
<th>Ribavirin and rimantadine (1.56 μg/ml)</th>
<th>Additional effect of combination®</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>1.56</td>
<td>0.9</td>
<td>3.0</td>
<td>0.3</td>
</tr>
<tr>
<td>3.12</td>
<td>1.7</td>
<td>3.8</td>
<td>0.4</td>
</tr>
<tr>
<td>6.25</td>
<td>3.5</td>
<td>4.7</td>
<td>≤ -0.2</td>
</tr>
</tbody>
</table>

* Decrease in 24-h virus titers relative to infected control wells (mean of three experiments).

® Additional decrement in virus yield with drug combination beyond the algebraic sum of the decreases observed with either single drug.

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**TABLE 4. Effect of inoculum size on inhibition of A/USSR/90/77/H1N1 by combinations of rimantadine hydrochloride (1.56 μg/ml) and ribavirin (3.12 μg/ml)**

<table>
<thead>
<tr>
<th>Multiplicity of infection (PFU/cell)</th>
<th>Ribavirin (3.12 μg/ml)</th>
<th>Rimantadine (1.56 μg/ml)</th>
<th>Both drugs</th>
<th>Additional effect of combination®</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^{-4}</td>
<td>1.7</td>
<td>2.8</td>
<td>5.8</td>
<td>1.3</td>
</tr>
<tr>
<td>10^{-2}</td>
<td>0.8</td>
<td>0.7</td>
<td>1.8</td>
<td>0.3</td>
</tr>
<tr>
<td>10^0</td>
<td>0.2</td>
<td>0.3</td>
<td>0.5</td>
<td>0.1</td>
</tr>
</tbody>
</table>

* Decrease in 24-h virus titers relative to infected control wells (mean of two experiments).

® Additional decrement in virus yield with drug combinations beyond the algebraic sum of the decreases observed with either single drug.

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**TABLE 5. MDCK cell proliferation in the presence of rimantadine hydrochloride and ribavirin**

<table>
<thead>
<tr>
<th>Drug(s)</th>
<th>No. of viable monolayer cells per ml (×10^5) at the following times after planting:*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>None</td>
<td>2.0</td>
</tr>
<tr>
<td>Rimantadine (1.56 μg/ml)</td>
<td>2.4</td>
</tr>
<tr>
<td>Rimantadine (3.12 μg/ml)</td>
<td>2.3</td>
</tr>
<tr>
<td>Ribavirin (3.12 μg/ml)</td>
<td>1.7</td>
</tr>
<tr>
<td>Ribavirin (6.25 μg/ml)</td>
<td>1.3</td>
</tr>
<tr>
<td>Ribavirin (12.5 μg/ml)</td>
<td>2.0</td>
</tr>
<tr>
<td>Rimantadine (1.56 μg/ml) + ribavirin (3.12 μg/ml)</td>
<td>1.4</td>
</tr>
<tr>
<td>Rimantadine (1.56 μg/ml) + ribavirin (6.25 μg/ml)</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Values represent means of two monolayers grown in 35-mm-diameter plates. Viability was determined by trypan blue exclusion.
related manner. The number of viable monolayer cells increased 10-fold over 72 h in non-drug-exposed plates. The number of cells increased only threefold in monolayers exposed to 3.12 μg of ribavirin per ml and did not increase in those exposed to 6.25 μg of ribavirin per ml. The addition of 1.56 μg of rimantadine per ml did not further depress MDCK cell proliferation.

**DISCUSSION**

We found that the combination of rimantadine hydrochloride and ribavirin had greater antiviral activity against influenza A viruses in vitro than either drug alone. Previously, Galegov et al. reported an increased antiviral effect with rimantadine hydrochloride and ribavirin in vitro experiments with an avian influenza A virus and in mouse protection experiments with influenza A2/Frunze virus (7). The present study extended this observation to include contemporary human strains and multiple serotypes of influenza A viruses. In addition, an enhanced antiviral effect was demonstrated against the relatively rimantadine-resistant A/PR/8/H0N1 virus and, with high rimantadine concentrations, against an influenza B virus. The present study also showed that combinations of a related drug, amantadine hydrochloride, and ribavirin had enhanced antiviral effects against influenza A viruses.

The degree of inhibition from the drug combinations depended not only on the virus strain, but also on the virus inoculum. The inhibitory effects of single drugs or combinations were overcome at higher multiplicities of infection. An inoculum effect has been recognized previously in drug susceptibility tests with amantadine and rimantadine (7, 9). Galegov et al. found that the combination of rimantadine hydrochloride and ribavirin continued to demonstrate an enhanced antiviral effect despite progressively higher multiplicities of infection, although eight- to 10-fold-higher ribavirin concentrations were used in their study than in the present one.

The antiviral effects of ribavirin and rimantadine combinations were also dose dependent. In contrast to the findings of Galegov et al., ribavirin alone at concentrations of 12.5 and 6.25 μg/ml resulted in marked inhibition of influenza A virus replication. This single drug activity masked any potential augmentation of antiviral effects with the addition of rimantadine. In contrast, rimantadine alone showed a relatively constant inhibitory effect over a broad concentration range. The addition of ribavirin at a fixed concentration resulted in substantially greater antiviral activity in combination with high rimantadine concentrations compared with low ones.

The mechanism of enhancement of antiviral activity due to drug combinations was not determined in the present study. For certain drug combinations the extent of inhibition of virus multiplication was greater than the additive effects of the single drugs, and this finding suggested a synergistic interaction (13). Although rimantadine and ribavirin appear to affect different stages of influenza virus replication, the antiviral mechanisms of these drugs alone have not been fully elucidated (18, 19, 21, 22). Another possible mechanism of enhanced antiviral activity with combinations of drugs may be inhibition of the selection and multiplication of drug-resistant viruses. Exposure of influenza A viruses in vitro has been shown to select drug-resistant viruses (3, 20). However, preliminary studies with the present assay methods have not detected a substantial shift toward drug resistance after exposure to single drugs.

The enhanced antiviral effects of the combinations were probably not secondary to increased cytotoxicity. The ribavirin concentrations used in this study inhibit ribonucleic acid synthesis in uninfected MDCK cells (2). Previous studies have shown an inhibitory effect on mammalian cell proliferation at higher concentrations (11, 14; J. F. Hruska, personal communication), but we observed inhibition of MDCK cell proliferation at concentrations of from 3.12 to 12.5 μg/ml. However, the combination of ribavirin and rimantadine did not result in an additional anti-proliferative effect.

The clinical significance of these observations remains to be determined. Enhanced antiviral effects against influenza A viruses were demonstrated with rimantadine concentrations of 0.1 to 0.4 μg/ml, which are well within the range of achievable blood levels after oral administration (1, 9). In contrast, the ribavirin concentrations used in the present study are approximately 5- to 10-fold higher than those reported after oral administration in human subjects (12). However, these results do provide impetus for further investigations of drug combinations for antiviral therapy in other models of influenza infection.

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