Comparative Activity of Amantadine and Ribavirin Against Influenza Virus In Vitro: Possible Clinical Relevance

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The activities of amantadine and ribavirin against influenza A viruses were compared against low-multiplicity (plaque inhibition) and high-multiplicity (protein synthesis inhibition) infections. Our results suggest that the predictive value of in vitro data for the clinic may be improved by consideration of tests against a high-multiplicity infection.

Inhibition of virus replication, particularly virus plaque formation, in cell cultures is widely used as a preliminary screening method for detecting anti-influenza agents that may have a role in the treatment of clinical infections. Recent findings, however, suggest that this test may have some deficiencies in determining the relative clinical efficacies of amantadine and ribavirin. Although amantadine has generally proved more potent in reducing plaque formation than has ribavirin (2, 7, 23), a recent randomized trial in humans suggests that the results of ribavirin aerosol treatment are distinctly superior, particularly in the suppression of virus shedding, to those obtained with amantadine in an earlier, comparable study (10). Furthermore, others have found that results obtained by measuring the effect of amantadine on hemagglutinin yield after in vitro infection at a high multiplicity do not correlate with inhibition of plaque formation (14, 20). We therefore examined the comparative effectiveness of amantadine and ribavirin against isolates of all three subtypes of human influenza A virus in Madin-Darby canine kidney (MDCK) cells, using both high- and low-multiplicity infections.

MDCK cells were obtained from Flow Laboratories Ltd., Irvine, Scotland, and passaged as described previously (4). Amantadine-susceptible, plaque-purified influenza A/Singapore/1/57 (H3N2) (1) was obtained from G. Appleyard, Wellcome Research Laboratories, Beckenham, England, and used at the first passage in this laboratory. Influenza strains A/Hong Kong/1/68 (H3N2), A/Singapore/4/57 (H3N2), A/Port Chalmers/1/73 (H3N2), and A/NWS (H1N1) were obtained from the National Institute for Medical Research, Mill Hill, London. Virus stocks were grown in fertile hen eggs; allantoic fluids were stored at −70°C.

Amantadine hydrochloride was obtained from Sigma Ltd., Poole, England; ribavirin was obtained from ICN Pharmaceuticals Inc., Life Sciences Group, Cleveland, Ohio; and [35S]-methionine (>600 Ci/mmol) was obtained from Amersham International plc, Amersham, England.

The effects of amantadine and ribavirin on influenza virus plaque formation were determined by incorporation of the compounds into the agar overlay (after virus inoculation) at a range of concentrations (Table 1).

The activities of these agents were also measured against a high-multiplicity infection. MDCK cell cultures were incubated for 16 h in the presence of amantadine or ribavirin (both 100 µg/ml) at 37°C and then inoculated with virus at 5 PFU per cell. Virus was adsorbed for 1 h at 35°C in the presence of test compounds. After a further 5 h of incubation at 35°C in serum-free medium containing the test compounds, the cultures were pulse-labeled for 20 min by replacing the medium with phosphate-buffered saline containing [35S]methionine (10 µCi/ml). After labeling, the cells were rinsed in ice-cold phosphate-buffered saline and lysed in electrophoresis sample buffer (11). Electrophoresis on 13% polyacrylamide slab gels and fluorography were carried out as described previously (3). Ribavirin effectively inhibited the production of viral proteins in all strains tested (Fig. 1), showing results in good agreement with previous findings (3, 15–17). Preliminary experiments (data not shown) indicated that amantadine was much less active than ribavirin. Amantadine had little or no effect at a concentration of 100 µg/ml, except for a partial inhibition in the case of A/Singapore/4/57 virus (Fig. 1). The relative activity of these agents was confirmed by observation of virus yield inhibition (data not shown). Other reports have shown that inhibition of influenza protein synthesis by amanta-
dine (18, 21) required concentrations which greatly exceed the 50% inhibitory concentrations (IC50s) published for most influenza viruses (1, 2, 7, 12, 14).

The results of this study show that both amantadine and ribavirin inhibit plaque formation by influenza A viruses (Table 1). The IC50s of ribavirin found in this study are slightly higher than those previously reported by this labora-

tory (4), although they fall into the range reported by others (2, 7, 17). Similarly, the IC50s found for amantadine in this study are consistent with those obtained in previous reports (1, 2, 7, 12, 14). The relative activity of these agents agrees well with results obtained in earlier reports (2, 7, 23, 25). The data thus suggest that amantadine (IC50s of 0.2 and <1.0 μg/ml for H2N2 and H3N2 viruses, respectively) may be more potent than ribavirin (IC50s of 3.6 to 8.5 μg/ml), unless the virus is amantadine resistant (as in the case of A/NWS).

The relevance of the IC50s of amantadine and ribavirin to clinical utility (7) is unclear, however, particularly in terms of reduction in detectable influenza virus. Reports on the antiviral activity of orally administered ribavirin are not consistent. Thus, ribavirin had a significant effect in an experimental A/Victoria/3/75 (H3N2) infection (13) and in a natural outbreak of A/England/42/72 (H3N2) (19) but was ineffective against naturally occurring A/Brazil/11/78 (H3N1) (22). Ribavirin was also ineffective in an induced A/Dunedin/73 (H3N2) infection (5). Prophylactic oral administration of amantadine results in fewer isolations of virus; however, therapeutic administration has minimal or no effect (6). In a comparative evaluation of the prophylactic capacity of these agents against an induced A/University of Maryland/2/74 (H3N2) infection, orally administered amantadine significantly reduced virus isolation, whereas ribavirin was without effect (5).

**TABLE 1. Comparative activity of anti-influenza agents in a plaque inhibition assay**

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>IC_{50} (μg/ml) of:</th>
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<tbody>
<tr>
<td></td>
<td>Amantadine</td>
</tr>
<tr>
<td>A/NWS (N_{1}N_{1})</td>
<td>&gt;30 (4)</td>
</tr>
<tr>
<td>A/Singapore/1/57 (H_{2}N_{2})</td>
<td>0.2 ± 0.01 (2)</td>
</tr>
<tr>
<td>A/Singapore/4/57 (H_{2}N_{2})</td>
<td>0.2 ± 0.02 (2)</td>
</tr>
<tr>
<td>A/Hong Kong/1/68 (H_{2}N_{2})</td>
<td>0.5 ± 0.2 (2)</td>
</tr>
<tr>
<td>A/Port Chalmers/1/73 (H_{3}N_{2})</td>
<td>0.7 ± 0.2 (2)</td>
</tr>
</tbody>
</table>

* Plaque assays were performed on MDCK cell monolayers with an overlay containing serum-free medium supplemented with 2 μg of trypsin per ml (24). Cultures were incubated at 35°C for 36 h before fixing and staining. Results are expressed as mean ± standard error; the number of independent tests is shown in parentheses. The IC50 was defined as the concentration of compound causing a 50% reduction of virus plaque number compared with control untreated cultures.

**FIG. 1. Inhibition of influenza virus protein synthesis by amantadine or ribavirin.** MDCK cells were treated with 100 μg of amantadine or ribavirin per ml before being infected at 5 PFU per cell as described in the text. Cultures were then incubated in the presence of the test compound before being labeled with [35S]methionine. Electrophoresis and fluorography were carried out as described in the text. Cell cultures were uninfected (U), virus infected and amantadine treated (Am), virus-infected and ribavirin treated (Rib), or virus infected and left untreated (VC). P, polymerase-associated protein; HA, hemagglutinin; NP, nucleoprotein; M/NS, matrix-nonstructural protein.
More recently, there have been therapeutic trials of these agents delivered by aerosol. In a study of a naturally occurring A/USSR/77 (H1N1) infection, amantadine reached a concentration of >20 μg/ml in respiratory secretions. Nevertheless, the frequency of virus isolation was not reduced, and there was only a marginal effect on the quantity of virus shed, even though the virus had an amantadine IC50 of <0.1 to 0.6 μg/ml (8). In contrast, aerosol delivery of ribavirin effected a significant reduction in virus shedding in an outbreak of A/England/333/80 (H1N1) (10). The results of this trial were regarded as distinctly superior to those of an earlier, comparable study of oral amantadine by the same group (9).

Although the inception of an influenza virus infection in vivo is likely to require only a relatively few infectious particles and is therefore probably akin to infection at a low multiplicity in vitro, cells in the respiratory tract may well be infected at a high multiplicity once the infecting virus has completed a few cycles of replication. Data obtained in vitro with a high-multiplicity infection may therefore have direct relevance to this in vivo situation. In our comparative experiments (Fig. 1), ribavirin was invariably more effective than amantadine. The greater effectiveness of ribavirin against a high-multiplicity infection in vitro does appear to relate well to recent clinical findings (8, 10).

In conclusion, our results suggest that the use of IC50s determined in vitro for amantadine and ribavirin may alone give an oversimplified impression of the relative clinical potential of these agents. The predictive value of in vitro data may be improved by consideration of additional tests against a high-multiplicity infection.

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LITERATURE CITED