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Anti-influenza virus activity of the neuraminidase inhibitor 4-guanidino-Neu5Ac2en in cell culture and in human respiratory epithelium

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Abstract

The anti-influenza activity of the neuraminidase inhibitor 4-guanidino-Neu5Ac2en (4-G-NAc) was determined in Madin-Darby canine kidney (MDCK) cells by yield reduction and ELISA and in explants of human respiratory epithelium by yield reduction. In MDCK cells, 50% inhibitory concentrations (EC_{50}) averaged 0.5 μg/ml for influenza A/Virginia/88(H3N2) and 0.04 μg/ml for A/Texas/36/91(H1N1) by ELISA, and < 0.01 μg/ml for influenza A/Virginia by yield reduction. In human adenoid explants, concentrations causing at least 1.0 $\log_{10}$ $TCID_{50}$/ml decrease in yield (EC_{90}) at 48 h were < 0.01 μg/ml for A(H1N1) and A(H3N2) viruses and 0.25 μg/ml for B/Hong Kong/5/72. 100 μg/ml 4-G-NAc did not inhibit outgrowth of human adenoid epithelium at 6 days, whereas ribavirin 10 μg/ml reduced outgrowth by $> 50\%$. 4-G-NAc is a potent and selective inhibitor of clinical isolates of influenza A and B viruses in human respiratory epithelium.

Keywords: 4-guanidino-Neu5Ac2en; Respiratory epithelium; Influenza virus; (Human)

1. Introduction

The neuraminidase, or sialidase, of influenza viruses is involved in a release of progeny virus from the surface of infected cells and is also thought to promote virus movement through respiratory tract mucus (Palese et al., 1976; Colman and Ward, 1985). Active or passive neuraminidase-specific immunization is effective at reducing

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virus replication and disease expression in animal models of influenza (Webster et al., 1988), and neuraminidase vaccine has been shown to permit infection but partially prevent disease in experimental and naturally occurring infections of humans (Beutner et al., 1979; Couch et al., 1974). Although this enzyme represents a suitable target for antiviral therapy, earlier studies with a non-selective neuraminidase inhibitor, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (Neu5Ac2en), found no beneficial effects in experimental murine influenza (Palese and Schulman, 1977), although more recent studies found some antiviral activity after topical administration in mice (von Itzstein et al., 1993).

Characterization of crystal structure of the neuraminidase has found it to be a tetramer of identical subunits with the enzyme active site present as a deep cavity on the protein surface (Colman et al., 1983). This site is lined entirely by amino acids that are conserved in neuraminidases of all strains of influenza A and B viruses which have been characterized to date, whereas variable amino acids are found at antigenically-changing areas encircling the active site (von Itzstein et al., 1993). Based on this crystal structure, a potent and selective neuraminidase inhibitor, the 4-guanidino analog of Neu5Ac2en, has been recently described (von Itzstein et al., 1993). In comparison to Neu5Ac2en, 4-guanidino-Neu5Ac2en was approximately 100-fold less active against human lysosomal sialidase (von Itzstein et al., 1993) and over 1000-fold more potent in inhibiting influenza virus A and B sialidase activities (Woods et al., 1993). By plaque assay in MDCK cells, 4-guanidino-Neu5Ac2en concentrations of 0.02–16 μM (0.007–5.3 μg/ml) were inhibitory for a broad range of clinical isolates of influenza A and B viruses, including those resistant to amantadine and rimantadine (Woods et al., 1993). When administered intranasally to mice infected with influenza A virus, 4-guanidino-Neu5Ac2en had antiviral effects at weight-adjusted doses which were approximately 100- and 1000-fold lower than those for amantadine and ribavirin, respectively (von Itzstein et al., 1993). It was also found to be highly active when given intranasally in ferrets, with minimal effective doses approximately 1000-fold lower than those of amantadine and ribavirin. In contrast, it was approximately 4000-fold less active when given intraperitoneally as compared to intranasally in mice. The current studies were undertaken to characterize further the antiviral and cytotoxic activities of 4-guanidino-Neu5Ac2en in human respiratory epithelium in vitro.

2. Materials and methods

2.1. Compounds

4-guanidino-Neu5Ac2en (GR121167X, GG167) was provided as a white crystalline powder (Glaxo Group Research, Greenford, UK). Rimantadine hydrochloride (Hoffman-LaRoche, Nutley, NJ) and ribavirin (ICN Pharmaceuticals, Costa Mesa, CA), were kindly provided by their respective manufacturers. All compounds were dissolved in distilled water at a concentration of 10 mg/ml, and aliquots were held frozen at −20°C until further diluted in cell culture media.

2.2. Cells, media, and viruses

Outgrowths from human adenoid or nasal mucosal epithelium explants were prepared in 24-well plates as previously described (Winther et al., 1990; Arruda et al., 1992; Rollins et al., 1993). Madin-Darby canine kidney (MDCK) were passaged twice weekly using media and methods previously described (Rollins et al., 1993).

Influenza A/Virginia/88(H3N2) (A/Sichuan/2/87-like) was a clinical isolate initially recovered in primary rhesus monkey kidney cells and passaged four times in MDCK cells. Influenza B/Hong Kong/5/72 was purchased from American Type Culture Collection (Rockville, MD) and passaged once in embryonated hen's eggs. Influenza A/Texas/36/91(H1N1) was a clinical isolate initially recovered in primary chick kidney cells and passaged four times in embryonated hen's eggs. A pool was kindly provided by L. Potash, Program Resources (Rockville, MD) for use in human challenge studies.

2.3. Antiviral and cytotoxicity assays

Susceptibility of influenza A and B viruses was determined by previously described yield reduction assays in explants of human respiratory epithelium (Winther et al., 1990; Rollins et al., 1993) with minor modifications. Briefly, drug-containing media was added 15 min prior to virus inoculation and both were removed 2 h later. Following three washes, the explants were replenished with drug-containing medium. The multiplicity of infection (MOI) was estimated to be approximately 0.01–0.1 $TCID_{50}$ per cell. Supernatant fluids were harvested at 24 h and replaced with fresh medium containing the same drug concentration. The final supernatant harvest was done at 48 h after virus inoculation. Titers were determined in microtiter plates of MDCK cells by hemadsorption. The drug concentration (EC_{90}) resulting in a reduction of virus yield of at least 1.0 $\log_{10}$ $TCID_{50}$/ml was determined with dose-effect analysis software (Biosoft, Cambridge, UK).

Susceptibility of influenza A viruses was also tested in MDCK monolayers by a modified enzyme-linked immunoassay (ELISA) as previously described (Rollins et al., 1993). The concentration causing a 50% reduction in optical density compared to control (EC_{50}) was determined. A modified yield reduction assay was also used to assess activity in MDCK monolayers. Quadruplicate monolayers were inoculated with 100–320 $TCID_{50}$/monolayer (MOI, approximately 0.001–0.01) and incubated overnight in the presence of plain or drug-containing medium. Supernatant fluids (1.0 ml/well) were harvested, pooled, and titered in MDCK cells. Cell-associated virus was determined by adding 1.0 ml of medium to wells, scraping off the monolayers and subjecting the harvests to two freeze-thaw cycles ($-70°C$) prior to titration.

The cytostatic effect of compounds on outgrowth of human epithelial tissue was preformed in uninfected fragments over a 6-day period of drug exposure as previously described (Arruda et al., 1992; Rollins et al., 1993). The number of fields of outgrowth from explant fragments in four wells per drug dilution containing four fragments per well was determined under low magnification ($40\times$) after 6 days incubation in the presence of plain or drug-containing medium. Toxicity for growing MDCK cells over 6

Table 1
Inhibition of influenza A/Virginia/88(H3N2) virus yield in MDCK monolayers after overnight incubation

Compound	Concentration (μg/ml)	Reduction in virus yield compared to control ($\log_{10}$ $TCID_{50}$/ml)	
		Supernatant	Cells
5-guanidino-	0.01	1.2	1.1
-Neu5Ac2en	0.1	3.6	2.1
	1.0	$\geq$ 5.5	2.5
	10	$\geq$ 5.5	2.4
Ribavirin	10	2.8	2.1

Results represent mean values of three experiments for supernatants and two experiments for cells. The virus yield in the controls for experiments were 6.5, 6.5 and 5.75 $\log_{10}$ $TCID_{50}$/ml for supernatants and 7.0 and 6.5 for cells. The virus used in these studies was passaged eight times by endpoint dilution in MDCK monolayers.

days was performed using the cellular protein dye sulforhodamine B in a microtiter assay as described previously (Skehan et al., 1990; Rollins et al., 1993).

3. Results

3.1. Activity in MDCK cells

Initial studies in MDCK cell monolayers found that the EC_{50} values of 4-guanidino-Neu5Ac2en were < 1 μg/ml for two clinical isolates of influenza A virus by ELISA. The EC_{50} value was approximately 10-fold lower for the influenza A/Texas/36/91(H1N1) strain (0.04 μg/ml) than for the A/Virginia/88(H3N2) strain (0.52 μg/ml) despite comparable viral replication based on the optical density values in the virus controls (data not shown). In these assays, ribavirin completely suppressed influenza A virus ribonucleoprotein antigen expression at 10 μg/ml, whereas rimantadine at a fixed concentration of 0.1 μg/ml inhibited RNP expression by 68% compared to control for influenza A(H3N2) but only 28% for influenza A(H1N1) under these test conditions.

In yield reduction assays, low concentrations of 0.01 μg/ml inhibited virus replication by $\geq$ 1.0 $\log_{10}$ for the A/Virginia/88 strain (Table 1). Concentrations of 1.0 μg/ml and above completely inhibited recovery of virus in supernatant fluids and appeared more inhibitory than ribavirin 10 μg/ml tested in parallel. Cell-associated virus recovery was reduced to a smaller extent and remained detectable at all concentrations tested.

The highest concentration of 4-guanidino-Neu5Ac2en tested, 100 μg/ml, did not inhibit MDCK proliferation over 6 days, whereas the mean $\pm$ S.D. 50% cytostatic concentration of ribavirin was 7.0 ± 2.2 μg/ml when tested in parallel (data not shown). In comparison to an EC_{90} value of 0.01 μg/ml by yield reduction assay, the therapeutic index for 4-guanidino-Neu5Ac2en was at least 10^4 in MDCK.

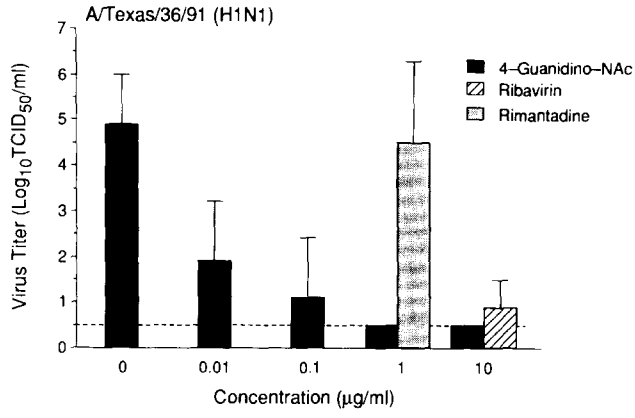

Fig. 1. Inhibition of influenza A/Texas/36/91(H1N1) replication in explants of human respiratory epithelium by 4-guanidino-Neu5Ac2en, ribavirin, and rimantadine. The results represent the mean ± S.D. of two or three pooled supernatants for each drug concentration in four separate experiments. Each experiment used samples from a single donor. The dashed line indicates the lower limit of detectability of the assay. The calculated EC_{90} values were < 0.01 μg/ml at 24 and 48 h.

3.2. Activity in human respiratory epithelium

For both influenza A viruses, virus yields at 24 and 48 h were reduced more than 1.0 $\log_{10}$ $TCID_{50}$/ml by 4-guanidino-Neu5Ac2en at the lowest concentrations tested, so that the EC_{90} values for both viruses were < 0.01 μg/ml at both time points. For influenza A/Texas/36/91(H1N1), the reduction in titer compared to control at 48 h averaged 3.0 $\log_{10}$ at this concentration, and inhibition was complete at 1 μg/ml (Fig. 1). Ribavirin 10 μg/ml was also highly inhibitory, but rimantadine 1 μg/ml did not significantly reduce virus yields (Fig. 1).

For influenza A/Virginia/88(H3N2), the concentration-related decreases in virus yields were more gradual (Fig. 2). Some residual virus replication was detectable at concentrations of 10 μg/ml, although this concentration did reduce virus yields by over 4.0 $\log_{10}$. Ribavirin 10 μg/ml was also highly inhibitory, whereas rimantadine 1 μg/ml reduced virus titers less than 1.0 $\log_{10}$ at 48 h (Fig. 2). When human respiratory epithelial explant cultures were infected with influenza A(H3N2) virus for 24 h before the addition of study compounds, delayed addition of 4-guanidino-Neu5Ac2en at 1 or 10 μg/ml reduced virus yields by over 1.0 $\log_{10}$ (Fig. 3). However, the antiviral effect was much smaller than observed in the experiments in which drug was added before exposure of monolayers to virus (Fig. 2). Similarly, delayed addition of ribavirin 10 μg/ml showed much less inhibitory activity (Fig. 3) than addition prior to virus inoculation (Fig. 2). Compared to the corresponding virus control, the observed titer reduction was over 3.0 $\log_{10}$ less than that found with pretreatment.

Influenza B/Hong Kong/5/72 grew less well in the human respiratory epithelial system, and control viral titers averaged 1.5–2.4 $\log_{10}$ lower than those for the influenza A viruses. Reductions in virus yield were evident at higher 4-guanidino-Neu5Ac2en

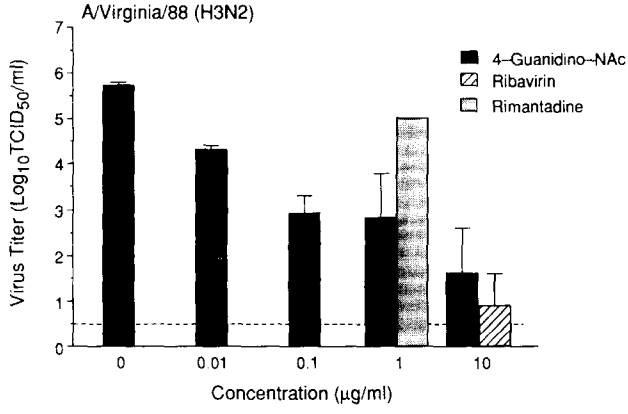

Fig. 2. Inhibition of influenza A/Virginia/88(H3N2) replication in explants of human respiratory epithelium by 4-guanidino-Neu5Ac2en, ribavirin, and rimantadine. The results represent the mean ± S.D. of pooled supernatants in three experiments, except for rimantadine which was tested in only one assay. See legend to Fig. 1. The EC_{90} values were < 0.01 μg/ml at 24 and 48 h.

concentrations tested (Fig. 4) and the calculated EC90 values were 0.54 μg/ml and 0.25 μg/ml at 24 and 48 h, respectively.

No cytostatic effects on outgrowth of cells from human respiratory epithelium explants was observed at 4-guanidino-Neu5Ac2en concentrations up to 100 μg/ml (Table 2). When tested in parallel, ribavirin at 10 and 100 μg/ml significantly inhibited outgrowth. Compared to EC_{90} values of < 0.01 μg/ml for the two influenza A viruses tested, the therapeutic index of 4-guanidino-Neu5Ac2en was over 10^4 in human respiratory epithelium.

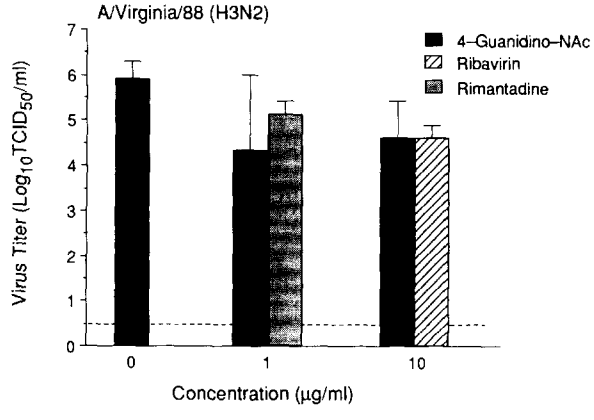

Fig. 3. Inhibition of influenza A/Virginia/88(H3N2) replication in explants of human adenoid epithelium with delayed administration of 4-guanidino-Neu5Ac2en, ribavirin, and rimantadine. Compounds were added at 24 h after initial viral infection, at which time the supernatant viral titers averaged 3.7–3.9 $\log_{10}$ $TCID_{50}$/ml in the different drug-containing wells. Supernatant harvests were made at 48 h after initial viral infection, and the results are expressed as the mean ± S.D. values of four experiments. See legend to Fig. 1.

4. Discussion

Previous studies have shown that 4-guanidino-Neu5Ac2en is a potent and selective inhibitor of influenza virus replication in cell culture and, when administered topically, in animal models (von Itzstein et al., 1993; Woods et al., 1993). The results of our studies confirm the antiviral activity of this compound against clinical isolates of influenza virus replication in MDCK cells and demonstrate that this activity extends to human respiratory epithelium in vitro. High therapeutic indices, exceeding 10^4 for two influenza A viruses, were observed in yield reduction assays in explants of human respiratory epithelium.

As described previously (Woods et al., 1993), our results also indicated strain-dependent inhibition of viral replication by 4-guanidino-NeuAc2en. In MDCK cells, the compound was approximately 10-fold more active by ELISA against a clinical isolate of influenza A(H1N1) than against an A(H3N2) strain. The H3N2 subtype virus was also inhibited in yield reduction assays in MDCK cells, whereas the relatively modest effect observed on inhibition of cell-associated virus is consistent with the role of neuraminidase in helping with release of virus from infected cells. Both influenza A subtypes grew readily in explants of human respiratory epithelium and were inhibited by at least 1.0 $\log_{10}$ $TCID_{50}$/ml at low concentrations. However, corresponding to the differences observed in MDCK cells, the degree of inhibition at higher concentrations (1 or 10 μg/ml) was greater for the A(H1N1) subtype virus than for the A(H3N2) one. The influenza B virus grew relatively poorly in the explant system and required higher 4-guanidino-NeuAc2en concentrations for inhibition. Although earlier studies have found no clear effect of passage history (egg versus cell culture) on in vitro susceptibility determined by plaque assay (Woods et al., 1993), it remains possible that apparent susceptibility differences may relate in part to passage effects.

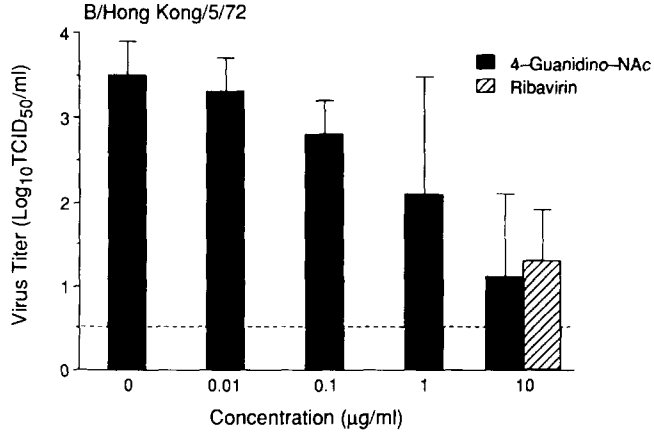

Fig. 4. Inhibition of influenza B/Hong Kong/5/72 replication in explants of human respiratory epithelium. The results represent the mean ± S.D. values of three experiments. See legend to Fig. 1. The EC_{90} values were 0.54 and 0.25 μg/ml at 24 and 48 h, respectively.

Table 2
Effect of 4-guanidino-Neu5Ac2en and ribavirin on outgrowth of cells in uninfected human adenoid epithelial explants

Compound (μg/ml)	Outgrowth index	Percent of control
0	1.7 ± 1.0	100%
4-guanidino-Neu5Ac2en		
10	1.6 ± 1.0	96%
32	1.9 ± 1.1	113%
100	1.9 ± 2.2	111%
Ribavirin		
10	0.6 ± 0.7	37%
100	0.1 ± 0.2	7%

Values shown as mean ± S.D. number of fields of outgrowth from explant fragments in four wells per drug dilution (four fragments per well) observed under low magnification (40×) for nine separate adenoid specimens. Both concentrations of ribavirin significantly inhibited outgrowth ($P < 0.02$, t-test).

The relatively weak inhibitory activity of rimantadine against the influenza A(H3N2) confirms our earlier observations with rimantadine in this assay system (Rollins et al., 1993). The current studies also found that this lack of significant inhibition extended to an A(H1N1) subtype virus. These findings correlate with earlier studies in cell culture based assays which found that an increasing duration of incubation generally diminishes antiviral effects with amantadine or rimantadine in the yield reduction assays (Hayden et al., 1980; Hayden et al., 1984). In addition, ribavirin's potent antiviral activity at a fixed concentration of 10 μg/ml in the human respiratory epithelium explant system corresponds with our previous observations (Rollins et al., 1993).

We also found that delayed administration of 4-guanidino-NeuAc2en or ribavirin, after virus replication was well established, was associated with inhibition of virus replication. However, the 48 h virus titers were reduced by only 1.3 $\log_{10}$ at 10 μg/ml concentrations for either compound, compared to over 4.0 $\log_{10}$ reductions when the compounds were present throughout the period of virus exposure. The possible clinical significance of such observations with respect to treatment of established infections remains to be determined. In summary, the potent and selective activity of 4-guanidino-NeuAc2en in human respiratory epithelial explants against both influenza A and B viruses make it a promising candidate for clinical study.

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