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Comparative anti-influenza virus activity of 2′-deoxy-2′-fluororibosides in vitro

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Summary

The anti-influenza virus activity of 2′-deoxy-2′-fluoroguanosine was determined in cell culture and in explants of human respiratory epithelium by yield reduction assay. The concentration causing at least 1.0 $\log_{10}$ reduction in influenza A (H3N2) virus yield (EC_{90}) at 24 h was 2.5 μg/ml in primary rhesus monkey kidney and 12 μg/ml in Madin-Darby canine kidney (MDCK) cells, compared to 0.5 μg/ml and 0.9 μg/ml, respectively, for ribavirin. The estimated therapeutic ratios for both compounds were low (<5 to 25) in these cell types. In contrast, the EC_{90} values at 48 h for influenza A and influenza B virus were $\leqslant 0.1$ μg/ml in human respiratory epithelial explants, and concentrations up to 100 μg/ml did not inhibit explant outgrowth. Ribavirin was approximately 50-fold less active in this system and inhibited outgrowth at 10 μg/ml. 2′-deoxy-2′-fluoroguanosine was also approx. 45-fold more potent than the corresponding adenosine and inosine compounds in explant cultures. Partially resistant variants, with approximate 5-fold increases in EC_{50} values, could be selected by serial influenza A virus passage in MDCK cells in the presence of 2′-deoxy-2′-fluoroguanosine, which indicated that its antiviral activity is at least partially virus specific. The exceptional activity of 2′-deoxy-2′-fluoroguanosine in human respiratory epithelial cells against both influenza A and B viruses makes this compound an interesting candidate for further investigation.

Anti-influenza virus; 2′-Deoxy-2′-fluororibosides

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Introduction

The only drugs currently effective in the prevention and treatment of influenza are amantadine hydrochloride and rimantadine hydrochloride. However, the clinical usefulness of both drugs is limited by an antiviral spectrum restricted to influenza A viruses, uncertain therapeutic efficacy in severe influenza, and by the recent findings of recovery of drug-resistant strains in some treated patients associated with apparent transmission to close contacts (Degelau et al., 1992; Hayden et al., 1989; Mast et al., 1992). Ribavirin has been reported to be therapeutically active in uncomplicated influenza after aerosol administration and anecdotally in severe influenza after aerosol or intravenous use (Knight et al., 1987; Ray et al., 1989) but it remains investigational.

In studies to identify new agents active against both influenza A and B viruses, Tisdale et al. (in press) have found that certain analogues of 2′-fluoronucleosides inhibit influenza virus replication in cell culture and in vivo in mouse lung. The most active analog, 2′-deoxy-2′-fluoroguanosine, was found to be inhibitory against a range of influenza A and B viruses at 0.1–3 μM in chick embryo fibroblast (CEF) cells and at 2–22 μM in Madin Darby canine kidney cells (MDCK). It also reduced mouse lung viral titers and increased survival after oral or intraperitoneal administration. The purpose of the current study was to characterize further the comparative antiviral and cytotoxic activities of 2′-deoxy-2′-fluoroguanosine and two related analogs in vitro, including activity in explants of human respiratory epithelium.

Materials and Methods

Compounds

2′-deoxy-2′-fluoroguanosine, 2′-deoxy-2′-fluoroadenosine, and 2′-deoxy-2′-fluoroinosine were provided in powder form by P.M. Raper (Wellcome Foundation Ltd., Kent, UK). Each compound was dissolved in DMSO at a concentration of 10 mg/ml, and aliquots were held frozen at −70°C until diluted in cell culture media just prior to use. Virus controls in each experiment had medium containing 0.1% DMSO, a concentration equal to or greater than that in the test compound dilutions. Ribavirin (ICN Pharmaceuticals, Costa Mesa, CA) and rimantadine hydrochloride (Hoffman-LaRoche Inc., Nutley, NJ) were kindly provided by their manufacturers. Both were dissolved in distilled water at a concentration of 10 mg/ml and held frozen until further diluted in cell culture media.

Cells and media

Outgrowths from human adenoid or nasal mucosal epithelium explants were prepared in 24 well plates as previously described (Arruda et al., 1992). Growth media was minimal essential medium with Earle's salts and d-valine (GIBCO-Bethesda Research Laboratories, Grand Island, NY), 10% Nu-Serum

(Collaborative Research, Lexington, MA), and antibiotics (gentamicin 100 μg/ml, vancomycin 20 μg/ml, amphotericin B 1 μg/ml). Explants were used after 12 to 16 days depending on amount of outgrowth. Each experiment used explant material from a single patient, and duplicate or triplicate wells were inoculated for each drug concentration.

Madin-Darby canine kidney (MDCK) cells were passaged weekly using minimal essential media with Earle's salts, 10% bovine (5% fetal plus 5% calf) serum, and antibiotics at the concentrations noted above. Primary rhesus monkey kidney (PRMK) cell monolayers were purchased from Whittaker M.A. Bioproducts (Walkersville, MD) and maintained with minimal essential media with Earle's salts and antibiotics as above. For antiviral studies, the corresponding media without serum were used, and 2 μg/ml trypsin (Worthington Biochemicals, Freehold, NJ) added for the MDCK cells only.

Viruses

Influenza A/Virginia/88(H3N2) (A/Sichuan/2/87-like) was a clinical isolate recovered initially in PRMK cells and passaged four times in MDCK cells. Influenza B/Hong Kong/5/72 was purchased from the American Type Culture Collection (Rockville, MD) and passaged once in allantoic fluid.

Antiviral assays

The susceptibility of influenza A/Virginia/88 was determined by yield reduction assays in triplicate or quadruplicate PRMK and MDCK cell monolayers as previously described (Hayden et al., 1990; Crump et al., 1990). Monolayers of MDCK cells in 24-well plates or of PRMK cells in roller tubes were inoculated with 0.1 ml medium containing approximately 100 $TCID_{50}$ virus and 0.1 ml of 2× drug-containing medium. After a 1–2 h incubation at 37°C, monolayers were washed and refed with 1 ml of drug-containing medium. Depending on the experiment, the final drug concentrations were in 0.5 or 1.0 $\log_{10}$ dilutions (range, 0.01–100 μg/ml) for the 2′-deoxy-2′-fluororibosides and ribavirin and 1.0 μg/ml for rimantadine. After 24 h of incubation at 37°C, the monolayers and supernatants were harvested and frozen at −70°C for later titration.

For the human respiratory epithelium explant studies, the monolayers were processed as above, but the viral inoculum was increased to approximately 1000 $TCID_{50}$ per well (Winther et al., 1990) and supernatants were harvested after 48 h incubation. This time of harvest was selected because prior studies (Winther et al., 1990) and preliminary experiments determined that viral titers increased to a peak at 48–72 h. Viral titers at each tested drug dilution were determined by CPE in 96-well MDCK plates. The drug concentration (EC_{90}) resulting in a reduction of virus yield by at least 1.0 $\log_{10}$ $TCID_{50}$ was determined by median effect plot.

Susceptibility of influenza A/Virginia/88 was also tested in MDCK monolayers by a modified enzyme-linked immunoassay (ELISA) (Hayden et al., 1989; Hayden et al., 1990). Quadruplicate 96-well MDCK monolayers were

overlaid with 0.1 ml of 2 × drug-containing medium for 1 h before addition of an equal volume of virus. After 15–18 h of incubation at 37°C, monolayers were fixed with 80% acetone in PBS at room temperature for 15 min. Plates were washed before addition of 100 μl of a 1:1500 dilution of a pool of two monoclonal antibodies directed to the nucleoprotein of influenza A virus (kindly provided by the Influenza Branch, Centers for Disease Control, Atlanta). After 5 min of room temperature incubation, 100 μl of a 1:2500 dilution of horseradish peroxidase conjugate Protein A (Bio-Rad Laboratories, Richmond, CA) was added prior to a 90 min incubation at 37°C. The substrate added after another wash was 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB) 0.01% in citrate-acetate buffer containing 0.005% H_2O_2. The reaction was stopped with 50 μl of 1 M H_2SO_4 and read at 450 nm. The drug concentration resulting in a reduction of optical density reading by 50% or more compared to control at the optimal virus inoculum (approximately 100 $TCID_{50}$) was determined (EC_{50}).

Cellular toxicity

Drug-related inhibition of uninfected cell growth was determined for MDCK and secondary RMK by seeding quadruplicate wells of 24-well plates with approximately 5×10^4 freshly passaged cells and drug-containing growth medium. After 6 days of growth, cells removed with trypsin were stained with trypan blue and counted by hemacytometer. The drug concentrations associated with 50% or greater reductions in cell counts compared to nontreated controls were determined ($CCID_{50}$).

In addition, cellular cytotoxicity for secondary RMK and MDCK was performed using the cellular protein adhering dye sulforhodamine B (SRB) in a microtiter assay (Skehan et al., 1990). Quadruplicate wells of 96-well plates were seeded with approximately $1–2 \times 10^4$ cells in a 0.2 ml volume of growth medium containing 0.5 $\log_{10}$ dilutions of drug. After 6 days of incubation in 35°C CO_2 incubator, plates were stained following previously published guidelines (Skehan et al., 1990). The drug concentrations which reduced optical density readings (492 nm) to less than 50% of untreated controls were considered inhibitory.

The cytostatic effect of drug on human respiratory epithelial tissue was performed by microscopic analysis of outgrowth size after incubation with varying drug concentrations. Epithelial fragments were prepared as previously described and allowed to attach to wells of 24 well plates for 24–48 h in the presence of 0.2 ml growth medium (Arruda et al., 1992). The supernatants were then replaced with 0.2 ml of drug-containing medium. An additional 0.3 ml of drug containing medium was added 2 or 3 days after initial drug exposure to prevent drying of tissue. Quadruplicate fragments in each well were examined under low magnification (40 ×) after 6 days incubation in presence of drug, and the size of outgrowth was determined by counting the total number of fields of growth seen at each drug concentration. Each well was divided into four quadrants of three fields in size. The fraction of outgrowth coverage for each

field was determined by microscopic inspection and the sum of fractions from quadruplicate wells, each containing four fragments, was determined for each experiment.

Passage of virus in presence of 2'-deoxy-2'-fluoroguanosine

To select for the possible development of a drug-resistant strain of virus, influenza A/Virginia/88 (H3N2) was passaged six times in MDCK cells at final drug concentrations of 0 or 32 μg/ml as previously described (Hayden et al., 1990). The drug susceptibilities of the passaged viruses were determined with the ELISA procedure described above.

Results

Activity in non-human cells

Initial studies in PRMK (Fig. 1) and MDCK (Fig. 2) cell monolayers found that 2'-deoxy-2'-fluoroguanosine inhibited the yield (EC_{90}) of a clinical influenza A (H3N2) isolate at concentrations of 2.5 and 12 μg/ml, respectively. Ribavirin tested in parallel was inhibitory at 5-fold lower concentrations in PRMK cells (EC_{90} = 0.5 μg/ml) and at over 10-fold lower concentrations in MDCK (EC_{90} = 0.9 μg/ml). At 10 μg/ml concentrations, ribavirin inhibited the yield of virus by over 5 $\log_{10}$ $TCID_{50}$ in both cell types, whereas the 2'-deoxy-2'-fluoroguanosine reduced yields by less than 2 $\log_{10}$ $TCID_{50}$ (Figs. 1 and 2). Rimantadine at a fixed concentration of 1.0 μg/ml was

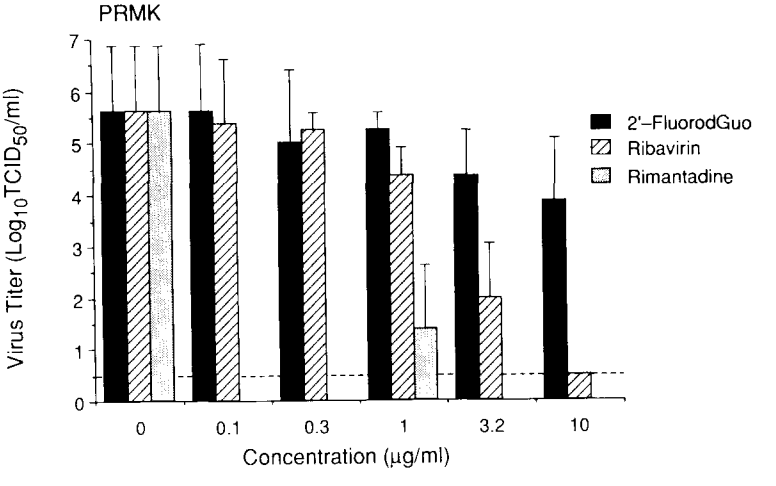

Fig. 1. Inhibition of influenza A/Virginia/88/H3N2 virus replication in primary rhesus monkey kidney cell monolayers at 24 h. The results represent the mean ± S.D. values of two experiments performed in triplicate monolayers. Titers at 24 h in the virus controls were 4.75 and 6.50 $\log_{10}$ $TCID_{50}$/ml. The dash line indicates the lower limit of detectability of the assay (0.5 $TCID_{50}$/ml). The EC_{90} values were 2.5 μg/ml for 2'-deoxy-2'-fluoroguanosine and 0.5 μg/ml for ribavirin.

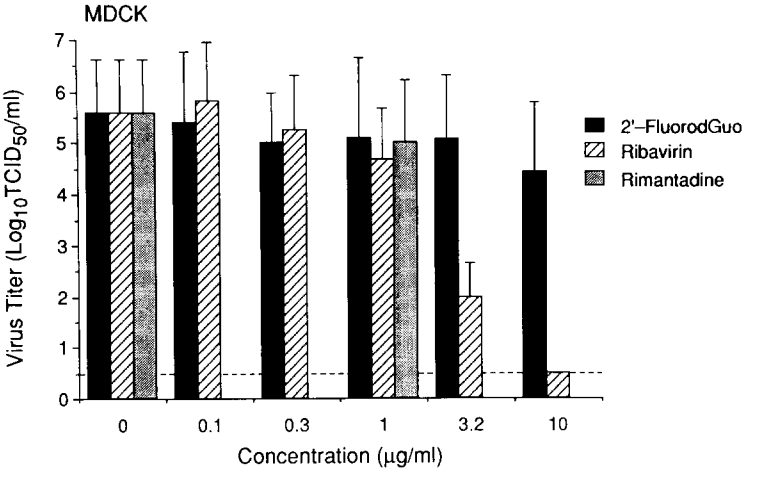

Fig. 2. Inhibition of influenza A/Virginia/88/H3N2 replication in Madin Darby canine kidney cell monolayers at 24 h. The results represent the mean ± S.D. values of three experiments. See legend to Fig. 1. The EC_{90} values were 11.9 μg/ml for 2'-deoxy-2'-fluoroguanosine and 0.9 μg/ml for ribavirin.

very active in PRMK cells (Fig. 1) but inhibited yield by less than 1 log_{10} in MDCK (Fig. 2). The lack of potent antiviral activity of 2'-deoxy-2'-fluoroguanosine in MDCK cells was also observed for the same influenza strain by ELISA. The EC_{50} values for 2'-deoxy-2'-fluoroguanosine in two assays averaged 13.5 μg/ml (data not shown), similar to the EC_{90} in the yield reduction assay.

Cytotoxicity based on cytostatic effects was found at 2'-deoxy-2'-fluoroguanosine concentrations of 8.0 μg/ml and above for both cell types (Table 1). Ribavirin tested in parallel had anti-proliferative effects at approximately 6–12-fold lower concentrations in MDCK and 3–5-fold lower in secondary RMK cells. The values determined in the microtiter SRB method were substantially higher for both drugs than those determined by direct cell counts (Table 1). The therapeutic ratios ($CCID_{50}/EC_{90}$) for both compounds were less than 5 in

TABLE 1

Antiproliferative effects of anti-influenza compounds in Madin Darby canine kidney and secondary rhesus monkey kidney cells

Cell type	Method	$CCID_{50}$ (μg/ml)	
		2'-Deoxy-2'-fluoroguanosine	Ribavirin
MDCK	Cell count	8.5 (7.7–9.2)	0.7
	SRB	17.7 (11.2–21.4)	2.8 (1.2–4.4)
Secondary	Cell count	8.0	1.6
RMK	SRB	61.4 (37.0–74.5)	11.7 (5.7–18.0)

The mean (range) results of 1–3 individual assays performed in quadruplicate are listed. See Methods for description of the assays. Counts in controls found that the number of cells increased 4-fold or greater during the 6 day incubation period.

TABLE 2

Inhibition of influenza A/Virginia/88/H3N2 replication in explants of human respiratory epithelium by 2′-deoxy-2′-fluoroguanosine

Compound (μg/ml)	No. experiments	Reduction in titer ($\log_{10}$ $TCID_{50}$/ml)	EC_{90} (μg/ml)
2′-Deoxy-2′-fluoroguanosine			
0.01	2	1.38 ± 0.88	<0.01
0.1	4	⩾2.07 ± 1.14	
1.0	6	⩾2.77 ± 0.97	
10.0	5	⩾2.75 ± 0.58	
Ribavirin			
10.0	3	⩾3.08 ± 0.72	
Rimantadine			
1.0	4	0.25 ± 0.50	>1.0

Results expressed as mean ± S.D. of 2 or 3 pooled supernatants for each drug concentration in separate experiments. Depending on the availability of explant material, not all drug concentrations could be tested in each experiment. The lower limit of detection was 0.5 $\log_{10}$ $TCID_{50}$/ml. The titers at 48 h in the virus controls ranged from 2.75–4.50 $\log_{10}$ $TCID_{50}$/ml (mean ± S.D., 3.50 ± 0.75 $\log_{10}$ $TCID_{50}$/ml).

MDCK cell by both the cell counting and SRB endpoints. In RMK cells, the therapeutic ratio was less than 5 by cell counting for both compounds. By the SRB method, the ratio was 25 for 2′-deoxy-2′-fluoroguanosine and 23 for ribavirin.

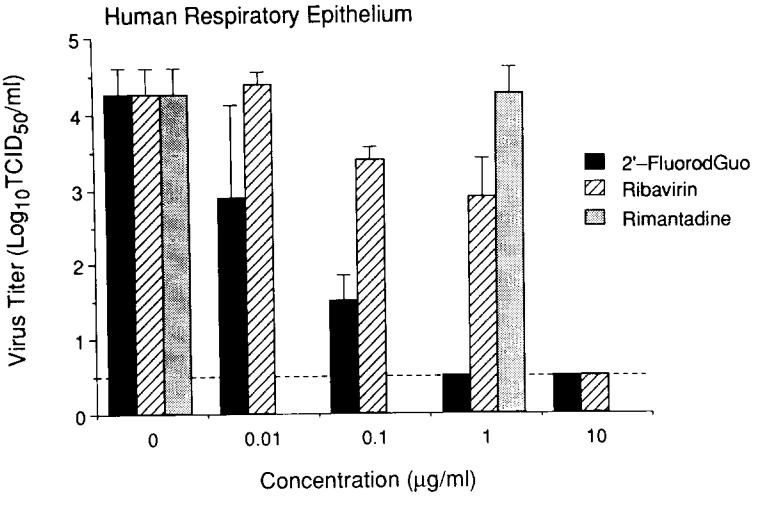

Fig. 3. Comparative inhibition of influenza A/Virginia/88/H3N2 replication in explants of human respiratory epithelium by 2′-deoxy-2′-fluoroguanosine and ribavirin at 48 h. The results represent the mean ± S.D. of two or three pooled supernatants for each drug concentration in two experiments. Each experiment used epithelium samples from a single donor. The EC_{90} values were <0.01 μg/ml for 2′-deoxy-2′-fluoroguanosine and 0.5 μg/ml for ribavirin.

TABLE 3

Growth of human respiratory epithelium after 6 days incubation with plain or drug-containing medium

Compound (μg/ml)	No. experiments	Outgrowth index[a]	Percent of control
2′-deoxy-2′-fluoroguanosine (μg/ml)			
1.0	5	5.6 ± 2.9	97%
3.2	5	7.8 ± 3.7	139%
10	5	6.1 ± 3.9	105%
32	5	6.5 ± 2.1	112%
100	4	6.9 ± 2.2	119%
Ribavirin (μg/ml)			
10	4	3.1 ± 2.9	53%
100	4	1.9 ± 2.5	34%

[a]Mean ± S.D. total number of microscopic fields of outgrowth from explant fragments in 4 wells per drug dilution (4 fragments per well) observed under 40 × magnification. See Methods section for experimental details.

Activity in human respiratory epithelium

The human respiratory epithelium explant experiments produced remarkably different results. Virus yield at 48 h was reduced more than 1.0 $\log_{10}$ $TCID_{50}$ at 2′-deoxy-2′-fluoroguanosine concentrations as low as 0.01 μg for A/Virginia/88/H3N2 (Table 2). In a direct comparison in tissue explants obtained from the same donors (Fig. 3), the EC_{90} value for 2′-deoxy-2′-fluoroguanosine (<0.01 μg/ml) was over 50-fold lower than that for ribavirin (0.5 μg/ml). Rimantadine 1.0 μg/ml was not active in these experiments (Table 2). In addition, separate studies of influenza B/Hong Kong virus found that the EC_{90} of 2′-deoxy-2′-fluoroguanosine averaged 0.1 μg/ml in three experiments (data not shown).

No cytostatic effects were observed at 2′-deoxy-2′-fluoroguanosine concentrations up to 100 μg/ml (Table 3), whereas ribavirin reduced outgrowth at 10 μg/ml. In comparison to the 2′-fluoroguanosine concentrations effective in reducing the yield of influenza A and B viruses (≤0.1 μg/ml), the therapeutic ratio in the respiratory epithelium explants was at least 1000. The corresponding therapeutic ratio for ribavirin was estimated to be approximately 20.

Comparative activity of 2′-fluororibosides

The relative antiviral activities of 2′-deoxy-2′-fluoroguanosine, adenosine, and inosine were assessed in parallel in human respiratory epithelial cultures (Fig. 4). Similar to earlier experiments (Table 2, Fig. 3), the guanosine analog produced nearly 2.0 $\log_{10}$ $TCID_{50}$ reductions in virus yield at 0.1 μg/ml. In contrast the adenosine and inosine analogs were inactive at this concentration and had EC_{90} values that were 45-fold or higher than that of the guanosine analog (Fig. 4).

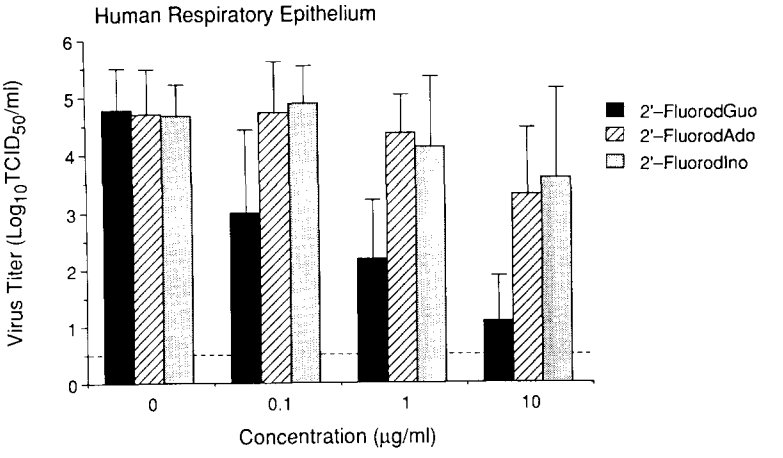

Fig. 4. Comparative inhibition of influenza A/Virginia/88/H3N2 replication in explants of human respiratory epithelium by 2'-deoxy-2'-fluororiboside analogs. The results are expressed as mean ± S.D. of two or three pooled supernatants for each drug concentration in 5–7 separate experiments. The EC_{90} values were <0.1 μg/ml for 2'-deoxy-2'-fluoroguanosine, 4.7 μg/ml for 2'-deoxy-2'-fluoroadenosine, and 4.5 μg/ml for 2'-deoxy-2'-fluoroinosine.

Selection of resistant virus

Virus passed in MDCK in the presence of 32 μg/ml of drug had an apparent increase in titer at the third passage. When compared to the EC_{50} of virus passaged in plain medium (12.7 and 14.2 μg/ml), the values observed for passage 5 and 6 virus grown in the presence of drug were 67 and 63 μg/ml, respectively.

Discussion

These studies found that the anti-influenza virus activity of 2'-deoxy-2'-fluoroguanosine was highly dependent on the type of cell culture in which the compound was tested. Although it inhibited influenza A virus replication in PRMK and to a lesser extent MDCK cells, 2'-deoxy-2'-fluoroguanosine was exceptionally active in human respiratory epithelium explant cultures. In contrast, ribavirin showed comparable activity in all three cell types. Consequently, in PRMK and especially MDCK cells, 2'-deoxy-2'-fluoroguanosine was less active than ribavirin, which gave EC_{90} corresponding closely to those observed in earlier studies (Hayden et al., 1984; Hayden et al., 1990), but 2'-deoxy-2'-fluoroguanosine was active at lower concentrations than ribavirin in the human respiratory epithelium explants. It was also much more active than two other 2'-fluororibosides examined in this test system.

An earlier report also found that the concentrations of 2'-deoxy-2'-fluoroguanosine inhibiting plaque formation in CEF cells were about 10- to 20-fold lower than those inhibiting plaque formation in MDCK cells and that

this difference in antiviral activity was correlated with similar differences in the intracellular triphosphate levels of 2′-deoxy-2′-fluoroguanosine (Tisdale et al., in press). Our findings confirm these cell type-related differences in antiviral activity but differ somewhat from those of Tisdale et al. in regard to the cytotoxicity of the compound in MDCK cells. Whereas the therapeutic ratio of 2′-deoxy-2′-fluoroguanosine was estimated to be 20–200 (Tisdale et al., in press), our results indicated a much lower degree of selectivity in the MDCK cells used in our studies.

The most important finding of our studies was that 2′-deoxy-2′-fluoroguanosine inhibited both influenza A/H3N2 and influenza B virus replication in human respiratory epithelium cultures at low concentrations ($\leqslant 0.1$ μg/ml) and did not cause apparent cytotoxicity in the explants at much higher concentrations. Tisdale et al. (in press) also found that low concentrations (2 μM, approximately 0.5 μg/ml) partially inhibited the replication of an influenza A (H3N2) virus in human embryonic trachea culture. It is unclear why the compound appears to exert selective activity in human respiratory epithelium, in contrast to the findings in non-human cells. Studies in MDCK and CEF cells found that intracellular triphosphate levels were similar in infected and uninfected cells (Tisdale et al., in press). Metabolic studies have not been performed in human respiratory epithelial cells. Perhaps differences in intracellular disposition could account for the observed differences in biologic effects in different cell types.

In addition to differences in cell type, differences in assay methods could explain some of the cell dependent differences in activity of 2′-deoxy-2′-fluoroguanosine. Unlike the studies in MDCK and PRMK cells, in which the harvests included both cells and supernatants, only the supernatants were titrated in the human respiratory epithelium experiments, so that intracellular virus was not assayed. Furthermore, the duration of incubation was shorter in our MDCK and PRMK studies (24 h) than in the human respiratory epithelium explants (48 h), although increasing duration of incubation generally leads to diminishing antiviral effects in yield reduction assays with influenza viruses (Hayden et al., 1980; Hayden et al., 1984). However, the antiviral activity of ribavirin was not greatly different among the cell systems tested, indirect evidence suggesting that the selective activity of 2′-deoxy-2′-fluoroguanosine in respiratory epithelial cell cultures was not simply due to methodologic differences. The reasons for rimantadine's inconsistent activity are not clear, but variable antiviral activity has been observed previously in yield reduction assays with rimantadine in MDCK and PRMK cells (Hayden et al., 1980; Hayden et al., 1984). In such assays, rimantadine's inhibitory effect is dependent on the virus subtype, virus inoculum, and duration of incubation, so that inhibition is often not demonstrable by 48 hours after infection of cell culture.

The relatively low activity of the 2′-fluoroguanosine analog in MDCK cells made selection of a resistant variant difficult. However, our finding of an approximate 5-fold increase in EC_{50} values by ELISA after 5–6 passes

indicated selection of a partially resistant variant and confirmed the results of similar studies performed in CEF cells (Tisdale et al., in press). Despite the more potent activity of the compound in CEF cells, Tisdale et al., were not successful in selecting highly resistant virus and speculated that this could indicate targeting of an essential function for virus growth. In any case, the development of resistance suggests that the antiviral activity of 2′-deoxy-2′-fluoroguanosine is at least partially virus-specific. Genetic studies and further biologic characterization would be useful to determine the mechanism, stability, and pathogenetic consequences of resistance. Even with these questions unanswered, the exceptional activity of 2′-deoxy-2′-fluoroguanosine in human respiratory epithelial cells against both influenza A and B viruses makes it an interesting candidate for further investigation.

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