

Efficacy of 2'-Deoxy-2'-Fluororibosides against Influenza A and B Viruses in Ferrets

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Single-dose treatments (5 to 40 mg/kg of body weight given intraperitoneally) of ferrets with 2'-deoxy-2'-fluoroguanosine or its prodrug, 2,6-diamino-purine-2'-fluororiboside, 1 h after infection with influenza A virus significantly inhibited replication of virus in the upper respiratory tract, resulting in amelioration of fever and nasal inflammation. Replication of virus in the lower respiratory tract was also reduced >100-fold, but three doses were required to prevent replication in the lungs. In ferrets infected with influenza B virus, single-dose treatment (40 mg/kg given intraperitoneally) produced a similar but reduced response in comparison with that in ferrets infected with influenza A virus, indicating that dosing was not optimal for this virus.

Recently, a number of 2'-deoxy-2'-fluororibosides have been reported to possess significant inhibitory activities against influenza A and B viruses *in vitro* in cells and human tracheal organ cultures and *in vivo* in mice (9, 10). The most potent, 2'-deoxy-2'-fluoroguanosine (2'-FluorodGuo), was significantly more efficacious than amantadine or ribavirin, as measured by a reduction in the titers of virus in mouse lungs when the drug was given as multiple therapeutic doses (9) or as a single therapeutic dose (10).

On the basis of the encouraging data on drug efficacy in mice, we progressed to studies of efficacy in ferrets, a more comparable model for humans (3, 5, 11, 12). In ferrets, we were able to examine the ability of 2'-FluorodGuo and a prodrug, 2,6-diaminopurine-2'-fluororiboside (2'-FluorodDAP), to inhibit viral replication in the respiratory tract and, consequently, to affect the expression of respiratory and constitutional signs as judged by an influx of leukocytes into the respiratory tract and the production of fever, respectively.

Ferrets were inoculated with influenza A virus, clone 7a (H3N2; virulent), of the reassortant virus A/Puerto Rico/8/34-A/England/939/69 or with influenza virus B/Singapore/222/79 as described previously (11). Animals were treated with the compounds, which were made up as fine suspensions in 1% Triton, by the intraperitoneal route for ease of administration at concentrations of 40, 20, 10, or 5 mg/kg of body weight. To monitor the levels of the compounds in plasma, certain animals were bled by cardiac puncture under halothane anesthesia 15 min after dosing, and the compounds were recovered by solid-phase extraction by using 100-mg Analytichem Bond Elut C18 cartridges, which were washed with distilled water, and the compounds were eluted with 50% (vol/vol) methanol-water. Samples were analyzed by high-performance liquid chromatography by using a SGE glass-lined column packed with 5 μ m ODS-2 and monitored by UV detection at 250 nm. Detailed pharmacokinetic studies have been carried out previously in mice, and 15 min after dosing proved to be the optimal sampling time (10). In both mice and ferrets, the prodrug

2'-FluorodDAP is rapidly converted to 2'-FluorodGuo, such that no prodrug can be detected in the plasma. Monitoring for clinical signs of infection, that is, inflammatory cell counts and determination of pyrexia, was carried out as described previously, as were virus titrations in nasal washes and lung samples (7, 8, 11, 12). Virus titers were assayed by the allantoin-on-shell (egg-bit) or egg techniques (7, 8) or in Madin-Darby canine kidney (MDCK) cells (6).

To estimate immune protection, animals were challenged intranasally under ether anesthesia with 10^6 50% egg infective doses (EID₅₀s) of clone 7a 4 weeks after primary virus inoculation, and the level of protection was assessed as described previously (4).

Effect against influenza A virus reassortant clone 7a. Initial studies in ferrets were designed on the basis of previous studies in mice (9, 10) to determine the minimum number of doses required to limit infection. Ferrets were inoculated with 10^6 EID₅₀s of clone 7a and were divided into groups of four which were treated intraperitoneally with three doses of 40 mg of compound 2'-FluorodGuo per kg at 1, 8, and 24 h post-virus exposure, two doses at 1 and 24 h post-virus exposure, and 1 dose at 1 h post-virus exposure. Treated ferrets were then compared with untreated virus-inoculated controls. Virus titers in nasal washes and lungs, pyrexia, and the nasal inflammatory response were monitored at intervals after infection. Data from the single-dose study are plotted in Fig. 1, and the results calculated as mean level of virus in nasal washes and lungs, and cell and fever indices (determined from areas under individual curves) are shown for all dosing regimes in Table 1.

In untreated animals (Fig. 1), virus peaked at 30 h ($10^{5.5}$ 50% egg-bit infective doses [EBID₅₀s]/ml) and then declined rapidly, with a second small peak of virus shedding occurring 96 h postinoculation (p.i.) ($10^{2.5}$ EBID₅₀s/ml) (Fig. 1A-a). In contrast, in animals treated with one dose of 40 mg of 2'-FluorodGuo per kg at 1 h post-virus exposure, no initial virus peak was detectable but a small peak occurred at 60 h post-virus exposure; this small peak was about 200-fold less ($10^{3.5}$ EBID₅₀s/ml) than the peak in the untreated virus-inoculated animals (Fig. 1A-a). Similar statistically significant reductions in virus titers in nasal washes were seen in animals treated with two or three doses of compound post-virus exposure (Table 1). Thus, infection was considerably delayed

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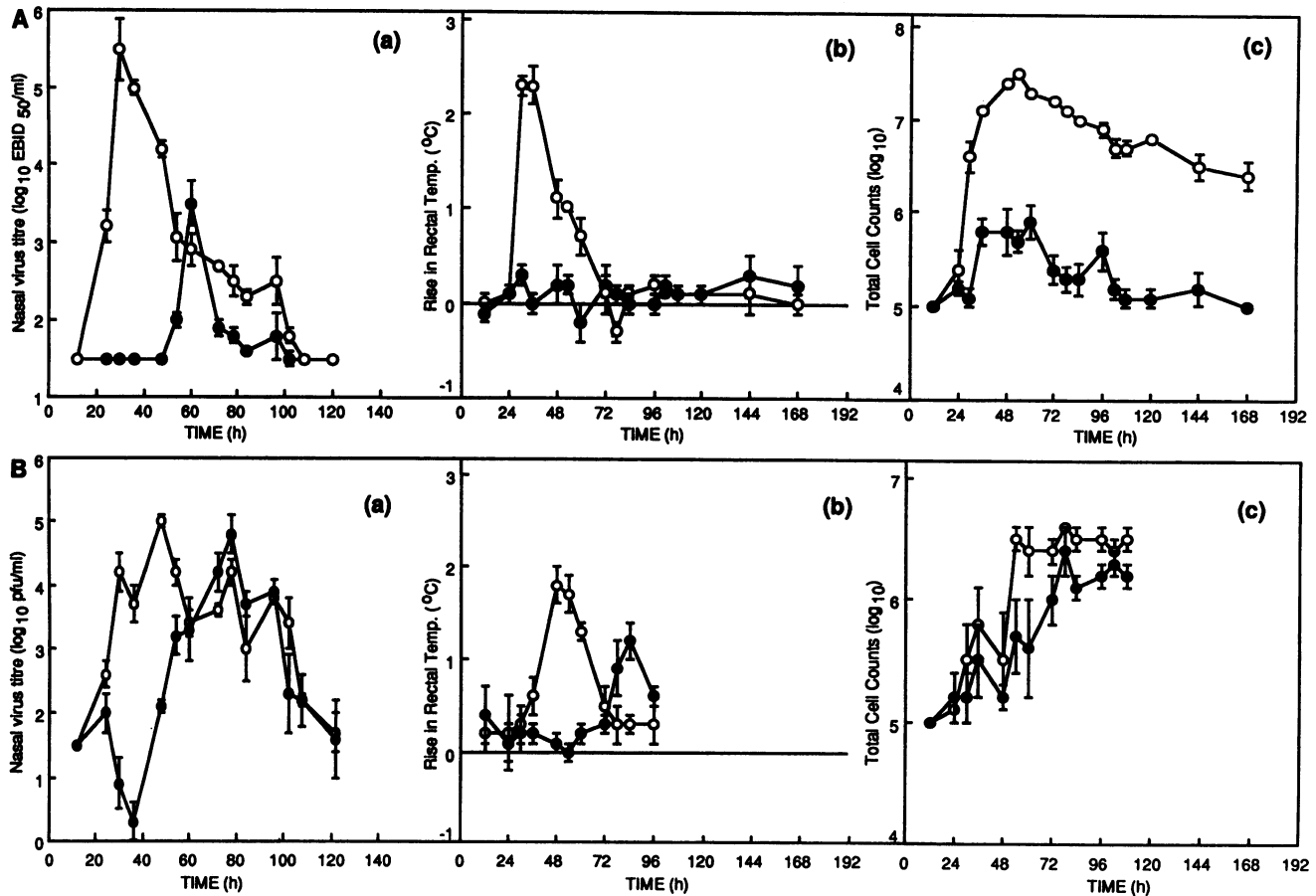


FIG. 1. Effect of 2'-deoxy-2'-fluororibosides on influenza A and B viruses. Groups of four ferrets inoculated with influenza A virus clone 7a (A) or influenza B virus B/Singapore (B) were treated with a single dose of 40 mg of 2'-FluorodGuo (A) or 2'-FluorodDAP (B) per kg at 1 h after virus inoculation. Mean virus titers in nasal washes (a), mean rise in rectal temperatures (b), and mean cell counts (c) were monitored in control untreated (○) and treated (●) animals.

and reduced in treated animals compared with that in untreated animals.

Constitutional signs represented by febrile responses were also suppressed in treated, virus-inoculated animals. In untreated, inoculated animals, the febrile response lasted approximately 30 h, from 30 to 60 h p.i., with a peak mean temperature rise of 2.3°C (Fig. 1A-b). Animals treated with one, two, or three doses of 40 mg of 2'-FluorodGuo per kg showed little

or no febrile response (Fig. 1A-b; Table 1). However, animals treated with three, but not fewer, doses of compound became subdued for 48 h after the third injection, which was associated with a transient hypothermia. This apparent toxicity for ferrets is atypical since no clinical effects were observed in rats or dogs that had been dosed intravenously at the same level in 2-week studies (unpublished data).

Respiratory signs, as indicated by the number of inflamma-

TABLE 1. Effect of number of doses on efficacy of 2'-FluorodGuo in ferrets infected with clone 7a influenza virus

Treatment ^a	Mean virus peak in nasal washes (log ₁₀ EBID ₅₀ /ml)	Mean virus index in nasal washes ^b	P value ^c	Mean rise in rectal temp (°C)	Mean fever index ^b	P value ^c	Mean cell peak (log ₁₀)	Mean cell index ^b	P value ^c	Mean virus peak in lung (log ₁₀ EID ₅₀)	Mean virus index in lungs ^b	P value ^c
Untreated	5.9 (0.2) ^d	129.1 (2.6)		2.3 (0.2)	30.7 (2.1)		7.4 (0.1)	252.3 (14.0)		6.1 (0.1)	387.6 (44.1)	
One dose	3.5 (0.3)	26.3 (3.5)	<0.001	0.3 (0.1)	0 (0)	<0.001	5.9 (0.2)	49.7 (7.5)	<0.001	4.3 (0.4)	178.8 (30.5)	<0.05
Two doses	3.0 (0.1)	32.7 (3.5)	<0.001	0.6 (0.2)	0.31 (0.31)	<0.001	5.4 (0.1)	20.3 (5.7)	<0.001	4.5 (0.0)	148.8 (5.1)	<0.02
Three doses	3.6 (0.1)	58.7 (3.3)	<0.001	0.4 (0.3)	0.19 (0.19)	<0.001	5.9 (0.3)	68.4 (14.5)	<0.001	2.3 (0.1)	18 (3.4)	<0.01

^a Animals were infected with influenza virus and remained untreated or were treated with one, two, or three doses of 2'-FluorodGuo (40 mg/kg) at 1 h, 1 and 24 h, or 1, 8, and 24 h post-virus exposure, respectively.

^b Mean of the mathematically calculated areas under the curves for individual animals.

^c The statistical significance of differences in virus in nasal washes, fever, cell, and virus in lung indices was tested by the Student *t* test.

^d Standard errors of the means are given in parentheses.

TABLE 2. Effect of timing of dose on efficacy of 2'-FluorodDAP in ferrets infected with clone 7a influenza virus

Treatment ^a	Mean virus peak in nasal washes (log ₁₀ EBID ₅₀ /ml)	Mean virus index ^b	P value ^c	Mean cell peak (log ₁₀)	Mean cell index ^b	P value ^c	Mean rise in rectal temp (°C)	Mean fever index ^b	P value ^c
Untreated	6.3 (0.2) ^d	173.3 (4.9)		7.8 (0.1)	209.9 (3.1)		3.2 (0.2)	34.8 (2.1)	
1 h	3.5 (0.3)	26.3 (3.5)	<0.001	5.9 (0.2)	49.7 (7.5)	<0.001	0.3 (0.1)	0 (0)	<0.001
4 h (2'-FluorodGuo)	2.5 (0.2)	40.2 (3.3)	<0.001	5.9 (0.1)	36.6 (3.1)	<0.001	1.5 (0.1)	7.95 (2.4)	<0.01
4 h	3.0 (0.1)	46.0 (3.0)	<0.001	5.9 (0.1)	33.4 (5.2)	<0.001	1.7 (0.4)	25.1 (15.0)	>0.05
12 h	4.9 (0.3)	101.4 (10.8)	<0.01	6.4 (0.3)	71.7 (1.7)	<0.001	1.7 (0.1)	18.2 (5.2)	>0.05
24 h	6.2 (0.1)	165.0 (5.6)	>0.05	7.7 (0.1)	216.2 (2.7)	>0.05	2.9 (0.2)	39.6 (5.3)	>0.05

^a Animals were infected with influenza virus and remained untreated or were treated with one dose of 2'-FluorodDAP (40 mg/kg) at 1, 4, 12, or 24 h post-virus exposure, respectively. One group was treated with 2'-FluorodGuo for comparison.

^b Mean of the mathematically calculated areas under the curves for individual animals.

^c The statistical significance of differences in virus in nasal washes, cell, and fever indices were tested by the Student *t* test.

^d Standard errors of the mean are given in parentheses.

tory cells in nasal washes, were also considerably reduced (Fig. 1A-c). In untreated, virus-inoculated animals, the inflammatory cellular response started at about 24 h p.i., reached a maximum of 10^{7.5} cells at 54 h after inoculation, and remained approximately 100-fold above preinfection levels up to 168 h p.i. (Fig. 1A-c). Virus-inoculated animals treated with three, two, or one dose of 2'-FluorodGuo showed a significantly reduced response (Fig. 1A-c; Table 1).

Virus (10^{4.7} EID₅₀s) was detected in the lungs of untreated virus-infected animals at 72 h p.i.; the titer rose to a peak of 10^{6.1} EID₅₀s at 120 h p.i. and declined rapidly thereafter, barely reaching detectable levels (10^{2.6} EID₅₀s) by 192 h p.i. Peak virus titers were reduced about 100-fold in animals treated with one and two doses of 40 mg of 2'-FluorodGuo per kg and were barely detectable in animals treated with three doses (Table 1). Viruses present in the lungs of treated animals had susceptibilities to 2'-FluorodGuo similar to that of the inoculum virus when assayed by plaque reduction in MDCK cells.

The levels of the compound in plasma for six individual virus-inoculated ferrets at 15 min after injection of the compound ranged from 19.0 to 76.5 μM (mean, 43.2 ± 9.1 μM), which is well above the 50% inhibitory concentration of 6.9 μM 2'-FluorodGuo for clone 7a as determined in MDCK cells (9).

Despite the reduced level of replication in both the upper and lower respiratory tracts of the virus-inoculated treated animals, the hemagglutination-inhibition antibody titers determined by standard methods (2) were similar to those in controls (mean, 560 ± 80 in comparison with 240 ± 80, 560 ± 80, and 480 ± 92 in animals treated with three, two, or one dose, respectively), and animals were protected against subsequent challenge.

From this study it was found that one dose given 1 h p.i. was sufficient to limit virus replication and inflammation in the upper respiratory tract and eliminate fever. Therefore, in subsequent studies, single-dose treatment regimens were used.

Effect of time of dosing. To determine the latest that the compound could be given but still control the infection, groups of virus-inoculated ferrets were injected with 40 mg of the prodrug 2'-FluorodDAP per kg at 4, 12, or 24 h post-virus exposure. Although previous work with mice indicated that the prodrug and 2'-FluorodGuo were interchangeable, a group of virus-inoculated ferrets was also injected with 40 mg of 2'-FluorodGuo per kg at 4 h post-virus exposure for comparison.

As described above, animals treated with 2'-FluorodGuo at 1 h post-virus exposure shed only low levels of virus, and both peak virus titers and total virus shed (virus index) were significantly reduced (*P* < 0.001) in comparison with those values for untreated controls (Table 2). Virus levels were also

significantly reduced when compound, both 2'-FluorodDAP and 2'-FluorodGuo, was given at 4 h post-virus exposure, but 2'-FluorodDAP was less effective when it was given at 12 h post-virus exposure, although virus levels were still significantly reduced (*P* < 0.01), and it was too late to influence the course of the infection when it was given at 24 h post-virus exposure (Table 2). The effects on the nasal inflammatory cell response were similar to those on nasal virus levels, but fever was observed in all groups given compound later than 1 h, and in most cases this fever was not significantly less than that in control animals (Table 2). Considering the high level of virus challenge (10⁶ EID₅₀s) required to produce consistent infection in ferrets, these results are encouraging and indicate that these inhibitors have considerable therapeutic potential. In humans 0.6 to 50% tissue culture infective doses 3.0 are sufficient for infection (1).

Effect of dose. To ascertain the minimum effective dose, groups of infected ferrets were inoculated with single doses of 20, 10, or 5 mg of 2'-FluorodDAP per kg at 1 h post-virus exposure. Control virus-inoculated, untreated animals showed responses similar to those in the animals in the experiments described above, and all three dose levels significantly reduced virus replication, nasal inflammatory responses, and fever (Table 3). While the magnitudes of these responses did not differ significantly from those observed in ferrets treated with 40 mg/kg, virus was detectable over longer periods of time as the dose of compound was reduced.

Levels of 2'-FluorodGuo in plasma were 5.1 and 8.2, 16.9 and 18.4, and 40.8 and 42.4 μM, respectively, for plasma from each of two virus-inoculated animals taken 15 min after injection with doses of 5, 10, and 20 mg of compound per kg.

Effect against influenza virus B/Singapore/222/79. To determine whether 2'-FluorodDAP was effective against influenza B virus, four animals were inoculated with 10⁶ EID₅₀s of B/Singapore/222/79, and another group of four inoculated ferrets was treated with the compound 1 h later. Virus-inoculated, untreated animals started shedding virus within 24 h of inoculation, which peaked at 10⁵ PFU/ml at 48 h p.i. but which remained at relatively high levels (between 10^{3.0} and 10^{4.0} PFU/ml) until 104 h p.i., when the levels declined rapidly (Fig. 1B-a). Virus levels in treated, virus-inoculated animals were considerably reduced initially but peaked at levels (10^{4.8} PFU/ml) similar to those in untreated animals at 78 h post-virus exposure before declining (Fig. 1B-a); the overall virus index for treated animals was significantly reduced in comparison with those for untreated animals (*P* < 0.01). Fever and nasal inflammation were also significantly reduced (Fig. 1B-b and B-c). However, the efficacy of 2'-FluorodDAP was not as great against influenza B virus as it was against influenza A virus,

TABLE 3. Effect of dosage on efficacy of 2'-FluorodDAP in ferrets infected with clone 7a influenza virus

Treatment ^a	Mean virus peak in nasal washes (log ₁₀ EBID ₅₀ /ml)	Mean virus index ^b	P value ^c	Mean cell peak (log ₁₀)	Mean cell index ^b	P value ^c	Mean rise in rectal temp (°C)	Mean fever index ^b	P value ^c
Untreated	6.3 (0.2) ^d	148.3 (15.4)		7.5 (0)	168.9 (6.3)		2.2 (0.3)	17.3 (3.7)	
20 mg/kg	2.4 (0.1)	26.5 (2.9)	<0.001	6.0 (0.1)	54.8 (5.7)	<0.001	0.6 (0.1)	0 (0)	<0.01
10 mg/kg	2.6 (0.1)	29.2 (2.9)	<0.001	5.9 (0.2)	57.0 (6.5)	<0.001	0.5 (0.1)	0.08 (0.08)	<0.01
5 mg/kg	2.9 (0.2)	31.8 (2.4)	<0.001	6.2 (0.1)	65.6 (3.7)	<0.001	0.5 (0.2)	0.89 (0.84)	<0.01

^a Animals were infected with influenza virus and remained untreated or were treated with 20, 10, or 5 mg/kg at 1 h post-virus exposure, respectively.

^b Mean of the mathematically calculated areas under the curves for individual animals.

^c The statistical significance of differences in virus in nasal washes cell, and fever indices were tested by the Student *t* test.

^d Standard errors of the mean are given in parentheses.

despite similar efficacy in cell culture (9). This suggests that more than one dose is required to obtain an optimum effect against influenza B virus.

Thus, in conclusion, 2'-FluorodGuo and 2'-FluorodDAP are effective against influenza A and B virus infections in ferrets by reducing viral replication, which significantly reduces respiratory and constitutional signs of infection.

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