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Anti-Influenza Virus Activities of 4-Substituted 2,4-Dioxobutanoic Acid Inhibitors

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We previously identified a series of compounds which specifically inhibited the transcription of influenza A and B viruses (J. Tomassini, H. Selnick, M. E. Davies, M. E. Armstrong, J. Baldwin, M. Bourgeois, J. Hastings, D. Hazuda, J. Lewis, W. McClements, G. Ponticello, E. Radzilowski, G. Smith, A. Tebben, and A. Wolfe, Antimicrob. Agents Chemother. 38:2827–2837, 1994). The compounds, 4-substituted 2,4-dioxobutanoic acids, selectively targeted the cap-dependent endonuclease activity of the transcriptase complex. Additionally, several of these compounds effectively inhibited the replication of influenza virus but not other viruses in cell culture assays. Here, we report on the anti-influenza virus activities of other potent derivatives of the series evaluated in both in vitro and in vivo infectivity assays. These compounds inhibited the replication of influenza virus in yield reduction assays, with 50% inhibitory concentrations ranging from 0.18 to 0.71 μM. These 50% inhibitory concentrations were similar to those observed for inhibition of in vitro transcription (0.32 to 0.54 μM). One selected compound also elicited a dose-dependent inhibition of influenza virus replication in mice following an upper respiratory tract challenge. These studies demonstrate the antiviral efficacy of this inhibitor class and thereby establish the utility of influenza virus endonuclease as a chemotherapeutic target.

The transcription of influenza virus mRNA, an essential activity of the virus, has been considered a target for the development of chemotherapeutic agents (5, 10). A novel feature of influenza virus transcription is the initiation of mRNA synthesis by capped and methylated (cap1) RNA primers which are cleaved from host RNA polymerase II transcripts by a virally encoded endonuclease (12). Both the binding and the endonucleolytic cleavage of capped mRNAs are mediated by the PB2 polymerase protein (2, 7, 8, 18, 20), a subunit of the multimeric transcriptase complex which is highly conserved among influenza viruses (3, 24). With the exception of a similar activity identified in bunyaviruses (4, 11, 14), the cap-dependent endonuclease appears to be unique to influenza viruses and also to have no known cellular counterpart (16).

Consistent with the notion that endonucleolytic cleavage is a distinctive property of the influenza virus transcriptase, we previously identified a class of cap-dependent transcriptase inhibitors, 4-substituted 2,4-dioxobutanoic acids, through a random screening effort (19). The compounds, cyclic or multicyclic structures containing a 2,4-dioxobutanoic acid side chain, were highly selective for the polymerases of influenza A and B viruses and were found to specifically target the endonuclease function of the transcriptase, with 50% inhibitory concentrations (IC_{50}s) ranging from 0.2 to 20 μM. Piperidine-substituted dioxobutanoic acids were found to be the most potent class of derivatives, and further modification of the piperidine nitrogen by alkyl and benzyl substitution yielded basic compounds which exhibited antiviral activity in cell culture. This antiviral activity was observed in the absence of apparent cytotoxicity and without an effect on the replication of other viruses (19).

The in vitro transcription and replicative properties of one such compound, L-735,882 (Table 1; compound 4), a benzyl-substituted piperidine, were previously described in detail (19). The compound inhibited in vitro transcription, with an IC_{50} of 1.1 μM, which is similar to the IC_{50} (1.6 μM) observed in the virus yield assay (Table 1). We have extended those studies and now report on the characterization of additional derivatives of this inhibitor series which were efficacious both in cell culture assays and in a murine challenge model for influenza virus.

In vitro transcription and replicative activities. Additional derivatives synthesized within this structural class, including potent compounds 1 to 3, elicited an antiviral effect in cell culture. Comparative analysis of compounds 1 to 3 with compound 4 revealed that the potencies of these compounds in cell culture were similar to those observed in in vitro transcription (Table 1). These compounds exhibited IC_{50}s ranging from 0.3 to 0.5 μm in in vitro transcriptions assays, with similar IC_{50}s, 0.2 to 0.7 μm, in the virus yield assay (Table 1). As previously reported for compound 4, the antiviral effects of compounds 1 to 3 in cell culture were also observed in the absence of cytotoxicity. None of the compounds had an effect on the proliferation of MDCK cells, as measured by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt)] colorimetric assay, when they were tested at concentrations of up to 100 μM, as shown in Fig. 1 for compound 2.

Protocol for upper respiratory tract challenge in the mouse. The selective therapeutic index observed in cell culture for these inhibitors encouraged us to further characterize the compounds in an in vivo mouse challenge model. The efficacies of influenza virus vaccine and antiviral agents have routinely been evaluated in the mouse pneumonia model, in which the virus inoculum is delivered directly into the lung of an anesthetized animal (6). This causes a lower respiratory tract infection which can result in a fatal pneumonitis, depending on the severity of the infection. For preliminary testing of these compounds, an alternate model was chosen. In that model, the inoculum virus was administered intranasally to unanesthetized mice. This type

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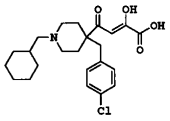
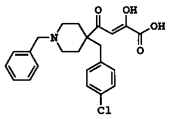
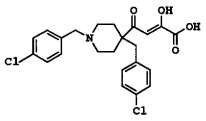
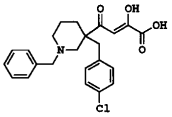
TABLE 1. In vitro activities of selected dioxobutanoic acids

Compound	Structure	IC_{50} (μM)[a] Transcription[b]	Virus Yield[c]
1		0.32 ± 0.01[d]	0.71
2		0.43 ± 0.02	0.35[e]
3		0.54 ± 0.01	0.18
4 (L-735,882)		1.10 ± 0.2	1.60

[a] Compounds were tested in an influenza virus in vitro transcription assay with polymerase cores purified from influenza virus (A/PR/8/34) and primed with cap1 alfalfa mosaic virus RNA in a virus yield assay with influenza virus (A/PR/8/34) as described previously (19).

[b] IC_{50}s are geometric means ± standard errors of at least three determinations.

[c] IC_{50}s were determined by testing all compounds within the same assay.

[d] Mean of the previously reported IC_{50} (0.33 ± 0.02 μM) (19) and an additional determination.

[e] The IC_{50} is the mean of two determinations.

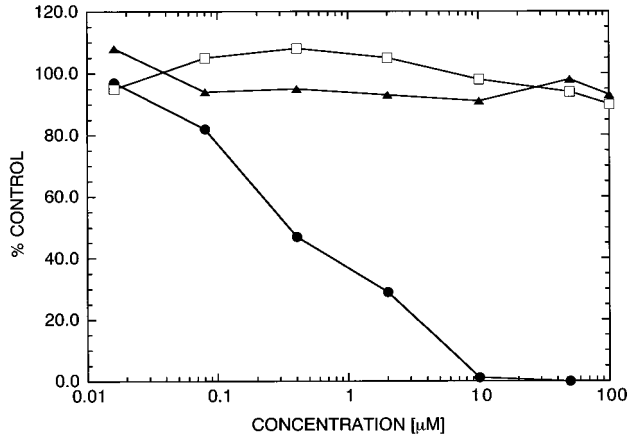


FIG. 1. Effect of compound 2 on virus yield and cell proliferation assays in MDCK cells. Compound 2 was tested at the indicated concentrations in the virus yield and MTS cell proliferation assays on MDCK cells as described previously (19). Briefly, compound was added at the indicated concentrations 30 min prior to infection of confluent monolayers of MDCK cells grown in 96-well plates with 50 PFU of A/PR/8/34 virus per well, and at 24 h postinfection, the virus titer in supernatants was quantitated by plaque titration on MDCK cells. Cytotoxicity was evaluated by the Cell Titer 96 AQueous nonradioactive cell proliferation assay (Promega, Madison, Wis.) at 24 and 48 h following treatment of 50% confluent MDCK cell monolayers with the indicated concentrations of flutimide. ●, percentage of virus control titer obtained in virus yield assay from cell supernatants measured by plaquing in duplicate. The percentage of solvent-treated control optical density obtained from duplicate samples in the MTS proliferation assay at 24 h (□) and at 48 h (▲) is also shown. Solvent-treated control cell monolayers contained 1.2×10^4 cells per well at 0 h, 2.9×10^4 cells per well at 24 h, and 5.9×10^4 cells per well at 48 h.

of challenge results in an upper respiratory tract infection, similar to the pathogenesis of human influenza, in which virus replication initiates in the nasal epithelium and then progresses to the lung (13). The outcome of infection can be quantitatively assessed by measurement of virus present in nasal washes and/or lung homogenates, and additionally, multiple doses of compound can easily be delivered to the site of infection in an awake animal by intranasal administration. These combined attributes therefore provided the most discriminating model in which to initially evaluate the antiviral potentials of the dioxobutanoic acids.

To establish the protocol for the model, the time course of influenza virus infection in mice during a 48-h time period was evaluated following intranasal inoculation with a mouse-adapted strain, strain A/HK/68, and repeated administration of compound diluent, as outlined in Fig. 2. Groups of six animals per time point were inoculated with 100 PFU of virus, and the amount of virus present in nasal wash fluids obtained by tracheal lavage and in lung homogenates of mice was assayed by hemagglutination of cell supernatants subsequent to growth and amplification in MDCK cells.

As early as at 10 min and at 2 h postinfection, virus was not detected in the nasopharyngeal tract. A low level of virus, however, was first detected at 7 h postinfection in both nasal washes and lung homogenates, thus indicating that the virus inoculum had been rapidly absorbed. The mean virus titer detected in nasal washes at 7 h was 0.6 ± 0.6 $\log_{10}$ 50% tissue culture infective dose ($TCID_{50}$)/ml, and at 24 h it was 5.1 ± 0.3 $\log_{10}$ $TCID_{50}$/ml, which then increased by only 1.0 log to 6.1 ± 0.3 $\log_{10}$ $TCID_{50}$s/ml at 48 h. In contrast, the titer in lung homogenates was 0.4 ± 0.4 $\log_{10}$ $TCID_{50}$/ml at 7 h and 3.0 ± 0.6 $\log_{10}$ $TCID_{50}$s/ml at 24 h, and it then increased 2.6 logs to 5.6 ± 0.3 $\log_{10}$ $TCID_{50}$s/ml at 48 h. Thus, virus replication in the nasopharyngeal tract appeared to peak at 24 to 48 h, whereas the replication of virus in lungs increased nearly 400-fold during the period from 24 to 48 h, peaking at ≥48 h. This finding was similar to those of previous studies (9, 25) in which influenza virus replicated first in the upper respiratory tract and then spread to the lung during an upper respiratory tract

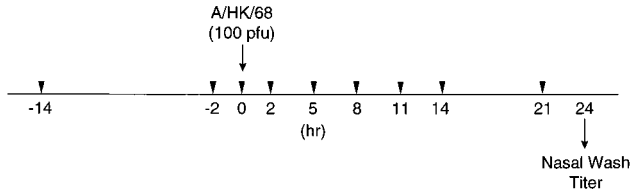


FIG. 2. Protocol for evaluation of antiviral efficacy in mouse challenge model. Compound was administered at levels of 1.0, 3.5, and 9.6 mg/kg of animal body weight to 10-week-old, female BALB/c mice by intranasal instillation in 50 μl of 40 mM sodium phosphate (pH 7.9). Compound was delivered to awake animals at 14 and 2 h prechallenge, the time of challenge, and the indicated times postchallenge (▼). While they were awake, the animals were infected intranasally with 100 PFU of A/HK/68 (21) in 20 μl of infection medium (Dulbecco's modified Eagle medium containing 1% bovine serum albumin, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 50 U of penicillin, and 50 μg of streptomycin per ml). At 24 h postinfection, the nasopharynges were lavaged by injection of 0.5 ml of infection medium containing 1% bovine serum albumin into the trachea. Nasal wash fluid was collected from the nares and was stored frozen at −70°C. Virus titer in nasal washes was determined by endpoint infectivity assay on MDCK cells quantitated by hemagglutination of virus in cell supernatants, as follows. A 50-μl volume of serial 10-fold dilutions of nasal washes was incubated for 60 min on confluent monolayers of MDCK cells growing in 96-well plates and was then overlaid with 150 μl of infection medium. At 48 h postinfection, 50 μl of the supernatants was assayed by hemagglutination with 1% guinea pig erythrocytes for 60 min at 4°C (1).

infection in mice. These results further indicated that a sufficient virus titer was obtained at 24 h postinfection in nasopharyngeal tracts following inoculation of the upper respiratory tract of mice with influenza virus, thereby enabling a 24-h evaluation of antiviral efficacy by using nasal washes in this model.

Of the more potent compounds (compounds 1 to 3) described in Table 1, compound 2 was found to be the most soluble (3.4 mg/ml) in aqueous buffer (pH 7.9) and was therefore selected for evaluation in a mouse challenge model for influenza virus. Prior to testing, the clearance of compound from the nasopharyngeal tract following intranasal instillation was considered because nasal washes would be analyzed for infectivity in this model. Nasal washes collected from mice at 1 h postadministration of 3.5 mg of compound per kg of body weight were not inhibitory to influenza virus infection of MDCK cells, and the amount of virus growth was compared to that observed in the presence of nasal washes obtained from mock-infected mice (data not shown). This indicated that the compound was adsorbed rapidly into the nasopharyngeal tract and that the nasal washes did not interfere with the infectivity assay.

Because both the compound and virus would be administered intranasally to mice in this model, the effect of the compound on neutralization of virus infectivity was also determined. Studies showed that preincubation of virus with compound 2 at 250 μM for 30 min at 37°C had no effect on the infectivity of the mouse challenge virus, measured by plaquing on MDCK cells following a 10,000-fold dilution of the compound (data not shown). Additionally, in time course of addition studies, compound 4 was found to be effective in plaque inhibition assays when it was added up to 4 h postinfection (100 μM; ≥70% inhibition) (data not shown). These studies indicated that the compound exerted its effect on a virus replicative step after entry into the animal, and thus, antiviral efficacy could be evaluated by intranasal administration.

The potent neuraminidase inhibitor 4-guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetyl neuraminic acid (4-guanidino-neu5ac2en), which was previously shown to have antiviral efficacy in mice (15, 22), was chosen to serve as a control compound in the challenge experiments. The 4-guanidino-neu5ac2en compound synthesized by Scheigetz et al. (17) was similarly absorbed into the nasopharyngeal tract and had no effect on the infectivity of the virus (data not shown).

Antiviral efficacy in mice. The antiviral effect of compound 2 was then evaluated in the influenza virus challenge model. The compound was instilled intranasally into mice by the multiple-dosing regimen indicated in Fig. 2, which included two compound doses prechallenge, one dose at the time of challenge, and continued dosing postchallenge throughout a 24-h period. Quantitation of the virus titer in nasal washes showed that the compound inhibited the replication of influenza virus in a dose-dependent manner when the compound was delivered at 0.1, 3.5, and 9.6 mg/kg of animal body weight (Table 2). A 3.9-log reduction ($P < 0.0001$) in virus titer was observed at 9.6 mg/ml, the highest level of soluble compound which could be delivered in the given instillation medium. No effect on the physical state of the mice or on body weight was observed when the compound was administered by multiple dosing during a 48-h period (data not shown). Note that the neuraminidase inhibitor, tested in parallel in this model, elicited a 4.3-log ($P < 0.00001$) reduction in titer at 3.5 mg/kg of animal body weight, whereas compound 2 caused a 3.3-log ($P < 0.002$) reduction at this dose. The neuraminidase compound has been reported to have IC_{50}s of 0.6 to 7.9 nM for various influenza virus sialidases in in vitro neuraminidase assays, with IC_{50}s ranging from 0.005 to 16 μM in influenza virus plaque inhibition assays (23). The greater potency observed for the neuraminidase compound compared with those of the transcriptase inhibitors in in vitro assays and the extracellular nature of the neuraminidase target may explain the differential efficacy observed in our in vivo analysis.

TABLE 2. Effect of compound 2 on influenza virus titers in nasal washes of mice

Compound	Dose (mg/kg)[a]	Mean virus titer (log_{10} $TCID_{50}$/ml)[b]		Reduction in virus titer	
		Expt 1	Expt 2	Log_{10}	*P* value[c]
Compound 2	0.0	4.28 (0.3)[d]	4.21 (0.3)	NA[e]	NA
	1.0	ND[f]	2.41 (0.5)	1.8	<0.05
	3.5	0.99 (0.5)	0.98 (0.5)	3.3[g]	<0.002
	9.6	ND	0.35 (0.4)	3.9	<0.0001
4-Guanidino-neu5ac2en	3.5	0.0 (0.0)	ND	4.3	<0.00001

[a] Animals were dosed and challenged at the indicated levels as described in the legend to Fig. 2.
[b] Virus titers in nasal washes were determined at 24 h postinfection as described in the legend to Fig. 2 and are expressed as the mean value for eight animals.
[c] Student *t* test.
[d] Standard errors of the means are given in parentheses.
[e] NA, not available.
[f] ND, not determined.
[g] The average log_{10} reduction of experiments 1 and 2.

We found that the 4-substituted 2,4-dioxobutanoic acids were effective inhibitors of influenza virus replication in both in vitro cell culture replication assays and an in vivo mouse challenge model. These compounds represent the first class of highly selective inhibitors identified to date for the cap-dependent endonuclease of influenza viruses. The in vivo efficacy demonstrated for this inhibitor class thereby establishes the utility of this unique viral enzyme as a target of intervention in the pursuit of anti-influenza virus agents. Given the exceptionally selective activity exhibited by this anti-influenza virus agent, an investigation of its full antiviral potential is warranted, including studies of drug-resistant viruses and an evaluation of its therapeutic efficacy.

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