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A Novel Antiviral Agent Which Inhibits the Endonuclease of Influenza Viruses

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A novel anti-influenza virus compound, flutimide, was identified in extracts of a recently identified fungal species, *Delitschia confertaspora* (F. Pelaez, J. D. Polishook, M. Valldosera, and J. Guarro, Mycotaxon 50:115–122, 1994). The compound, a substituted 2,6-diketopiperazine, selectively inhibited the cap-dependent transcriptase of influenza A and B viruses and had no effect on the activities of other polymerases. Similar to the 4-substituted 2,4-dioxobutanoic acids, a series of transcriptase inhibitors which we described previously (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), this inhibitor, which is a natural product, affected neither the initiation nor the elongation of influenza virus mRNA synthesis, but it specifically targeted the cap-dependent endonuclease of the transcriptase. Additionally, the compound was inhibitory to the replication of influenza A and B viruses in cell culture. The selective antiviral properties of this compound further demonstrate the utility of influenza virus endonuclease as a target of antiviral agents.

Influenza remains an important medical problem because of recurrent infections in humans of epidemic and pandemic proportions. Annual epidemics account for 10,000 or more excess deaths per year in the United States, and millions of deaths have been attributed globally to periodic pandemics (3, 8). Vaccination with inactivated virus and treatment with amantadine or rimantadine are the current methods of prophylactic and therapeutic treatments. However, both approaches are limited in their clinical usefulness, and there is a need to develop additional vaccines and antiviral agents against influenza virus infection (3, 5).

Although several replicative targets can be considered for chemotherapeutic intervention, primary transcription represents a unique antiviral target for influenza viruses. As an obligatory step in the life cycle, the eight RNA segments of negative polarity which make up the genome are transcribed into positive-sense mRNAs by an associated viral transcriptase. Transcription is initiated by a novel mechanism in which capped and methylated (cap1) RNAs, derived from RNA polymerase II transcripts in the nuclei of infected cells by a virally encoded endonuclease, are used to prime mRNA synthesis. The endonuclease cleaves the cellular transcripts 13 to 15 nucleotides from the 5′ end, and transcription is then initiated by the addition of a G, directed by the penultimate base, C, in the 3′ end of the viral RNA template, onto the 3′ OH terminus of the resulting 13-nucleotide capped primer (9, 14). The initiated cap1 primer, an intermediate in the transcriptase reaction, is then further elongated to produce a full-length mRNA copy of the viral template.

Both the binding of capped RNA and the endonuclease activity are mediated by the PB2 protein, a subunit of the influenza virus transcriptase complex (1, 10, 20, 26). Although it is highly conserved among influenza viruses, the PB2 protein bears minimal homology to the cap binding proteins of eucaryotes (2, 16, 27), and this enzymatic activity has no known cellular counterpart. Only bunyaviruses, which are also segmented, negative-strand RNA viruses, appear to possess a similar activity (7, 12, 15).

The propitious nature of this antiviral target has recently been verified by our finding of a class of highly selective inhibitors for influenza virus transcriptase. The inhibitors, a series of 4-substituted 2,4-dioxobutanoic acids, specifically targeted the endonuclease activity of the transcriptase. These compounds also inhibited the replication of influenza A and B viruses in in vitro cell culture assays and in an in vivo murine challenge model (4).

We now report the finding of an additional inhibitor of influenza virus transcriptase. The compound, flutimide, was identified in fermentation extracts of a new fimicolous loculoascomcyte fungus, *Delitschia confertaspora* (13). This novel natural product, a substituted 2,6-diketopiperazine (6, 21), elicited an inhibitory profile analogous to that of the endonuclease inhibitors mentioned above and was also inhibitory to influenza virus replication in cell culture. Flutimide is the first identified natural product which selectively inhibits the transcriptase of influenza viruses, and similar to the dioxobutanoic acid inhibitors, it demonstrates the unique properties of this antiviral target.

MATERIALS AND METHODS

Organism. The *Delitschia* Auersw. isolate was obtained from dassie dung collected in Namibia, described and designated *Delitschia confertaspora* by Pelaez et al. (13), and was deposited in the American Type Culture Collection as strain ATCC 74209.

Fermentation. Single-stage seed cultures grown from the vegetative mycelia of a sporulating clone, which were stored in 10% glycerol at −80°C (19), were used to inoculate production medium H (24). All conditions for medium sterilization and fermentation (volumes, seed time, flask size, agitation rate, and incubation temperatures) were those described previously (24). Following a 10-day fermentation, each culture was acidified to pH 2 with phosphoric acid and was extracted with an equal volume of ethyl acetate or methylethyl ketone.

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TABLE 1. Inhibitory activity of flutimide[a]

Assay	Extract activity[b]	Flutimide IC_{50} (μM)[c]	
		Natural product	Synthetic compound
Influenza A virus transcription			
ALMV primed			
880 nt[e]	+	5.1 ± 0.30[d]	5.6 ± 0.10[d]
13 nt	−	>165.0	>333.0
ApG primed	−	>165.0	>333.0
VSV transcription	−	>155.0	>333.0
HeLa RNA polymerase II	−	>50.0	>333.0
HIV reverse transcriptase	−	>165.0	>200.0
Influenza A virus cleavage	+	6.8 ± 0.35[d]	3.4[f]
RNase T1, U2, and A	−	>155.0	>110.0
HIV RNase H	−	155.0	ND[g]

[a] Flutimide was tested in in vitro enzyme assays as described in Materials and Methods.
[b] Activity obtained at 0.1 to 10 times the IC_{50}. +, 50 to 100% inhibition; −, ≤10% inhibition.
[c] IC_{50}s are the geometric means ± standard errors of two determinations unless otherwise noted.
[d] The IC_{50} is the geometric mean ± standard error of three determinations.
[e] nt, nucleotides.
[f] Single determination.
[g] ND, not determined.

Flutimide purification. Flutimide was purified by normal-phase, gel filtration, and reverse-phase high-performance liquid chromatography (HPLC) as described in detailed by Hensons et al. (6). Monitoring of fermentation time courses was conducted by HPLC on a Whatman Partisil-5 octadecaloylsilane-3 column operated at 40°C, and flutimide was eluted with 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid ($k' = 3.7$). Ten-microliter aliquots of methylethyl ketone extracts concentrated 20-fold and reconstituted in methanol were used for these analyses, with UV detection at 275 nm.

Enzyme assays. Reactions for in vitro influenza virus transcription primed with alfalfa mosaic virus, RNA segment 4 (ALMV 4), adenylyl (3′-5′)guanosine (ApG), or synthetic substrates (13, 22, and 70 nucleotides, respectively) with either polymerase cores or purified virions was performed as described previously by Tomassini et al. (25). Large-scale screening was performed as a 75-μl reaction with a Tecan automated dispensing station. Reactions for other polymerases, cleavage, and nuclease assays were also described previously (25).

Chemicals and enzymes. Foscarnet (phosphonoformic acid) was purchased from Aldrich, and 4-*N*-benzenesulfonyl-3-(4-chlorobenzyl)-piperdinyl-substituted dioxobutanoic acid was synthesized as described previously (25). All enzymes and substrates were prepared or purchased as described previously by Tomassini et al. (25).

RESULTS

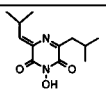
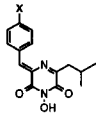
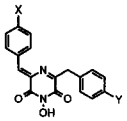
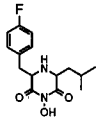
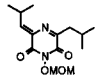
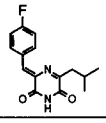
Identification of inhibitory activity in *Delitschia* extracts. An active extract of a fermented culture of the novel fungal species, *D. confertaspora*, was identified by random screening for inhibitors of influenza virus transcription. The inhibitory extract was detected by a cap-dependent transcription assay described previously for the identification of the 4-substituted 2,4-dioxobutanoic acid series of compounds (25). As shown in Table 1, the activity identified in the extract was selective for influenza virus transcription primed with capped ALMV and was not inhibitory in a transcription reaction primed independently of cap with the dinucleotide ApG at a 10-fold higher concentration of extract. Additionally, the inhibitory activity was specific for influenza virus polymerase and had no effect on the polymerase activities of vesicular stomatitis virus (VSV), HeLa RNA polymerase II, or human immunodeficiency virus (HIV).

Isolation of the inhibitory component from extracts. Following the identification of selective transcriptase inhibition by *Delitschia* culture extracts, the inhibitory component was purified and characterized. The active component was isolated via a sequence of normal-phase, gel filtration, and reverse-phase HPLC steps as described in detail by Hensens et al. (6). The activity profile elicited by the purified component was similar to that elicited by the original active extract. The component specifically inhibited cap-dependent transcription, with a 50% inhibitory concentration (IC_{50}) of 5.1 μM (Table 1), and it had no effect on influenza virus transcription primed with ApG or in other polymerase assays.

The structure of the active component (compound 1; Table 2) was determined by nuclear magnetic resonance analysis to be a substituted 2,6-diketopiperazine (6) which was later confirmed by total synthesis (21). Again, the activity of the synthetic compound paralleled those of both the initial extract and the purified component (Table 1) in selectively inhibiting cap-dependent transcription. The synthetic compound had an IC_{50} of 5.6 μM in ALMV-primed transcription and had no effect in ApG-primed transcription or other polymerase assays when it was tested at a concentration 50-fold above the IC_{50}. This inhibitory activity was conserved for the transcriptases of several strains of influenza A and B viruses (Table 3).

Mode-of-action studies. Further characterization of flutimide in assays which uncoupled the reactions of influenza virus transcription revealed that flutimide is an inhibitor of cap-dependent cleavage. The compound inhibited the cleavage of capped RNA by influenza virus endonuclease, with an IC_{50} of 6.8 μM, and it had no effect on the other nucleases tested (Fig. 1; Table 1). This mode of action was confirmed in transcription reactions primed with capped, synthetic RNA substrates derived from the 5′-end sequence of ALMV segment 4 RNA. Flutimide inhibited transcription primed only with capped

TABLE 2. Structures and inhibitory potencies of selected compounds[a]

Compound	Structure		IC_{50} [μM][b]
1, Flutimide			5.5 ± 0.15[c]
2		X = H	4.8 ± 0.04
3		X = F	0.9 ± 0.10
4		X = OMe	0.8 ± 0.09
		X Y	
5		OMe H	2.8 ± 0.06
6		F H	3.5 ± 0.04
7		H F	6.5 ± 0.05
8			85[d]
9			>355[d]
10			>365[d]

[a] Compounds were tested by in vitro transcription primed with cap1 ALMV with purified polymerase cores from influenza A/PR/8/34 virus.
[b] IC_{50}s are the geometric means ± standard errors of two determinations unless otherwise noted.
[c] The IC_{50} is the geometric mean ± standard error of three determinations.
[d] IC_{50}s are the average of two determinations.

TABLE 3. Inhibition of influenza A and B virus transcription[a]

Virus	IC_{50} (μM) for transcription[b]
A/PR/8/34 (H1N1)	4.3
A/Japan/305/57 (H2N2)	5.5
A/Port Chalmers/1/73 (H3N2)	3.5
A/WSN/33 (H1N1)	4.3
B/Hong Kong/5/72	3.8

[a] Flutimide was tested in in vitro transcription assays with various influenza viruses as described in Material and Methods.
[b] Single determination for all viruses tested within the same assay.

primers which undergo endonucleolytic processing and had no effect on transcription primed with a 13-nucleotide capped substrate representing the cleavage product of ALMV RNA, an intermediate in the transcription reaction which can be directly elongated (Table 1).

The highly selective nature of flutimide was further demonstrated by comparison with the activity profiles of several elongation inhibitors such as foscarnet, a pyrophosphate analog known to inhibit several polymerases (22), and polyphenolic- and lignin-type compounds which we encountered as being inhibitory to influenza virus transcriptase in our random screening effort. In contrast to flutimide, these elongation inhibitors were inhibitory to VSV and HeLa RNA polymerases at similar concentrations which inhibited influenza transcriptase. These inhibitors also characteristically inhibited both cap-primed and ApG-primed influenza virus transcription (data not shown). As summarized in Table 4, inhibitors of the elongation type were inhibitory to both influenza virus transcription and transcription initiation reactions (addition of the GTP of the first nucleotide onto the cleaved RNA) when they were primed with ApG, the capped 13-nucleotide intermediate, or capped primers which undergo endonucleolytic processing (22, 70, and 880 nucleotides, respectively), whereas flutimide and the 4-*N*-benzenesulfonyl - 3 - (4-chlorobenzyl)piperdinyl-substituted dioxobutanoic acid specifically inhibited transcription and initiation primed only with the capped substrates which required cleavage prior to elongation. Consistent with these observations, the

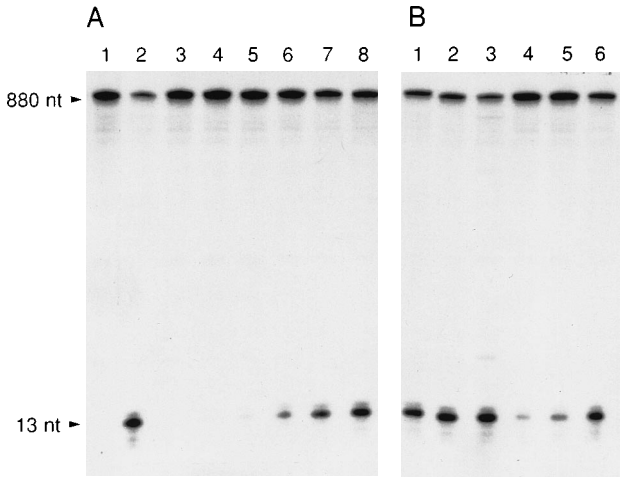


FIG. 1. Effects of various inhibitors on influenza virus cleavage activity. Cleavage of ^{32}P-labeled ALMV substrate with polymerase cores was determined in the presence of inhibitors as described in Materials and Methods. (A) Lane 1, ALMV substrate minus enzyme; lanes 2 to 8, 0, 500, 100, 20, 5, 1, and 0.2 μM flutimide, respectively. (B) Lanes 1 to 3, 500, 100, and 20 μM foscarnet, respectively; lanes 4 to 6, 1.0, 0.2, and 0.04 μM 4-*N*-benzenesulfonyl-3-(4-chlorobenzyl)piperdinyl-substituted dioxobutanoic acid, respectively.

TABLE 4. Effect of inhibitors on initiation, elongation, and cleavage[a]

Activity assay	Effect of inhibitor type[b]	
	Elongation[c]	Cleavage[d]
Influenza virus cleavage	−	+
Influenza A virus transcription		
ALMV primed		
880 nt[e]	+	+
22 nt	+	+
70 nt	+	+
13 nt	+	−
ApG primed	+	−
Influenza virus initiation, ALMV primed		
880 nt	+	+
13 nt	+	−

[a] Inhibitors were tested in various activity assays as described in Materials and Methods at concentrations ranging from 1 to 100 times the IC_{50}.
[b] +, 50 to 100% inhibition; −, ≤10% inhibition.
[c] Foscarnet, lignins, and polyphenolic-type compounds.
[d] Flutimide and 4-substituted 2,4-dioxobutanoic acids.
[e] nt, nucleotides.

elongation inhibitor foscarnet (Fig. 1B, lanes 1 to 3, and Table 4) had no effect on the endonucleolytic cleavage of ^{32}P-capped RNA substrate, while flutimide and the dioxobutanoic acid inhibited cleavage, with IC_{50}s comparable to those obtained in transcription (Fig. 1A, lanes 2 to 8, for flutimide and Fig. 1B, lanes 4 to 6, for the dioxobutanoic acid; Table 4). The IC_{50}s determined for the indicated dioxobutanoic acid inhibitor were 0.23 and 0.6 μM in the cap-primed transcription and cleavage assays, respectively.

Structure-activity relationship studies. The inhibitory activities of several chemically synthesized analogs of the compound were evaluated in influenza virus transcription, and it was found that both the *N*-hydroxy and olefin groups were required for activity (Table 2). Blocking of the *N*-hydroxy group (compound 9) or its removal (compound 10) resulted in greater than a 70-fold decrease in activity. The results obtained with the tetrahydro analog of compound 3 (compound 8) indicated that either or both of the olefin groups was required for activity. The most potent analogs were the *para*-fluorophenyl- and *para*-methoxyphenyl-substituted diketopiperazines (compounds 3 and 4).

Antiviral activity. Flutimide and the more potent analogs (compounds 3 and 4) were tested in an influenza virus cell culture assay. Although compounds 3 and 4 were more potent in the in vitro transcription assay, these compounds exhibited apparent cytotoxicity at concentrations of ≥10 μM in MDCK cells as measured by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt)] proliferation assay. However, compound 1 was not cytotoxic during a 48-h growth period (Fig. 2), and consequently, cell culture studies were pursued with this compound. Compound 1 was found to inhibit influenza virus infection of MDCK cells, with an IC_{50} of 5.9 μM in the virus yield assay and no apparent cytotoxic effect on MDCK cells at concentrations of up to 100 μm (Fig. 2). There was also no effect on the replication of VSV when the compound was tested in the virus yield assay at concentrations of ≥50 μM (data not shown).

DISCUSSION

Several inhibitors of influenza virus transcriptase have been described, including heavy metal-chelating compounds, pyro-

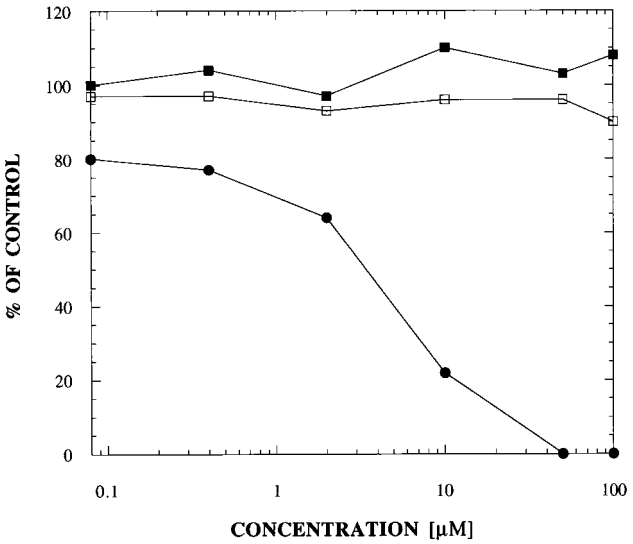


FIG. 2. Effect of flutimide on influenza virus yield and cell proliferation in MDCK cells. Flutimide was tested in the virus yield and MTS cell proliferation assays as described previously (25). Briefly, compound was added at the indicated concentrations 30 min prior to infection of confluent monolayers of MDCK cells grown in 96-well plates with 50 PFU of A/PR/8/34 virus per well. At 24 h, the virus titer in the supernatants was quantitated by plaque titration on MDCK cells. Cytotoxicity was determined at 24 and 48 h following treatment of 50% confluent MDCK cell monolayers with the indicated concentrations of flutimide by using the Cell Titer 96 AQueous nonradioactive cell proliferation assay (Promega, Madison, Wis.). ●, percentage of virus control titer obtained in cell supernatants at 24 h postinfection measured by plaquing in duplicate; percentage of solvent-treated control optical density obtained at 24 h (□) and 48 h (■) from duplicate samples. The average IC_{50} was obtained from two experiments.

phosphate analogs (e.g., foscarnet), and the nucleoside analog 2′-deoxy-2′-fluororibosides (11, 22, 23). However, these compounds inhibit the elongation of RNA by influenza virus transcriptase and other polymerases as well. We recently identified a highly selective class of transcriptase inhibitors for influenza A and B viruses. The compounds, 4-substituted 2,4-dioxobutanoic acids, targeted the cap-dependent endonuclease activity of the transcriptase complex and were also efficacious in both cell culture replication assays and a mouse challenge model for influenza viruses (4, 25).

This report describes the identification of yet another selective inhibitor for influenza virus transcriptase. The inhibitor, flutimide, a natural product, was identified by random screening in fermentation extracts of a novel fungal species, *D. confertospora* (13). Similar to the dioxobutanoic acid inhibitors, the compound inhibited neither initiation nor elongation reactions of transcription, but it specifically targeted the endonuclease function of the transcriptase. Flutimide also inhibited the replication of influenza virus in cell culture studies with a potency comparable to that observed in in vitro transcription.

Although they are similar in their inhibitory activities, flutimide and the dioxobutanoic acids differ structurally. The structure of flutimide was determined to be a substituted 2,6-diketopiperazine. The *N*-hydroxy and olefin groups were shown to be required for activity, and an increase in potency was obtained by aromatic substitution of the isopropyl side chains. However, these more potent analogs were found to be cytotoxic in cell culture. Structural analysis of the dioxobutanoic acid inhibitors, a series of 2,4-dioxobutanoic acid derivatives containing cyclic or multicyclic substituents at the 4 position, indicated that the dioxobutanoic acid side chain was required for inhibitory activity. Potency within this chemical class was derived by lipophilic substitution, and in particular, piperidine substitutions were the most potent. Cytotoxicity has not been observed with this class of compounds.

The inhibitory profiles observed for both flutimide and the dioxobutanoic acids were distinct from the activities of other transcriptase inhibitors, such as foscarnet, which inhibited both the initiation and elongation of influenza virus mRNA synthesis but had no effect on the cleavage of the capped substrate. Flutimide and the dioxobutanoic acids represent a new inhibitor type for influenza virus transcriptase. The mechanistic differences observed between the two inhibitor groups may be attributed to the interaction with differential forms of the polymerase complex which are involved in accomplishing the various activities of the transcriptase complex. This includes the discrete steps of mRNA transcription, capped primer recognition and cleavage, and initiation of RNA polymerization and subsequent elongation of mRNA, as well as the associated activities required for the replication of viral RNA. Further studies are required to precisely define the mechanistic roles of flutimide and the dioxobutanoic acids in endonuclease inhibition.

The natural product flutimide is the first selective inhibitor identified for influenza virus transcription to date. Another inhibitor of influenza virus transcription that was a natural product was identified previously (18). It was a lignin-like substance from pine cone extracts which inhibited influenza virus transcription primed by the dinucleotide ApG (18). Although this lignin-type inhibitor exhibited anti-influenza virus activity in cell culture, the inhibitor also affected the replication of other viruses. Furthermore, lignins are also known to possess antitumor, antiviral, and immunopotentiating activities induced by a variety of mechanisms (17).

Apart from the dioxobutanoic acid inhibitors, flutimide is the only other specific inhibitor of the transcriptases of influenza A and B viruses that has been identified. Flutimide represents an additional structural entity within a novel class of influenza virus transcriptase inhibitors, the exceptional selectivity of which is attributed to the inhibition of the cap-dependent endonuclease, an activity unique to influenza virus. The identification of this inhibitor further substantiates the potential utility of influenza virus endonuclease as a target of antiviral agents.

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