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D,L-α-Difluoromethylornithine Inhibits Human Cytomegalovirus Replication

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D,L-α-Difluoromethylornithine (DFMO) is an inhibitor of ornithine decarboxylase, the first enzyme in the polyamine biosynthetic pathway. Exposure of human foreskin fibroblast cells to DFMO before their infection with human strains of cytomegalovirus (CMV) resulted in a reduction in the amount of infectious virus produced. A 3-day exposure to the drug was required to elicit maximal antiviral effect. Cells exposed to DFMO at the time of infection produced normal amounts of infectious virus. Preexposure to the drug for 1, 2, or 3 days before infection resulted in at least 10-, 100-, or 1,000-fold decreases, respectively, in the amount of infectious virus produced. This decrease paralleled the loss of intracellular spermidine and was partially spared by the addition of exogenous putrescine, spermidine, or spermine (10 μM). When added 3 days before infection, DFMO depressed production of herpes simplex virus and simian CMV, as well as wild-type and laboratory prototype strains of human CMV. Although some antiviral effect was observed at a drug concentration of 1 mM, 10 mM gave a stronger effect and was the amount routinely used. At 30 mM DFMO, growth of noninfected cells was slowed but not arrested. Studies to investigate the level at which DFMO interferes with CMV replication showed that DFMO-treated, infected cells (i) exhibit a typical CMV-specific cytopathic effect, (ii) synthesize both viral proteins and viral DNA, (iii) contain at least some capsid forms, and (iv) shed greatly reduced amounts of virus particles into the growth medium. Since CMV virions, like those of herpes simplex virus, contain the polyamines spermidine and spermine, and since DFMO essentially eliminates the pool of intracellular spermidine, the possibility is suggested that this drug may exert its antiviral effect by interfering with virus assembly, perhaps at the level of DNA packaging or capsid envelopment or both.

Cytomegalovirus (CMV) is an enveloped, DNA-containing virus whose nucleic acid is replicated and encapsidated in the cell nucleus. It belongs to the herpesvirus group and is distinguished, among the five members of that group that infect humans, by having the largest genome (i.e., 150×10^6 daltons). This virus is a significant pathogen of humans, causing birth defects and life-threatening complications in immunosuppressed patients and appearing in close association with acquired immune deficiency syndrome and Kaposi's sarcoma (2, 3, 5, 16). Efforts are in progress to develop drugs with antiviral effects on this agent, and several with promise have been described. Perhaps most notable of these to date is phosphonoformate, which selectively interferes with viral DNA synthesis (12, 34). Another drug with antiviral activity (25, 30), D,L-α-difluoromethylornithine (DFMO), has been tested on CMV with mixed results (15, 33) and is the topic of this paper.

DFMO is an analog of the amino acid ornithine and functions as an enzyme-activated, irreversible inhibitor of ornithine decarboxylase (ODC; EC 4.1.1.17), which converts ornithine to 1,4-diaminobutane (i.e., putrescine), the first step in polyamine biosynthesis (20, 23). Putrescine, in turn, is converted to the larger polyamines spermidine and spermine by the sequential addition of an aminopropyl group to one amino terminus (yielding spermidine) and then to the other (yielding spermine). Thus, inhibition of ODC by DFMO blocks the first step in the polyamine biosynthetic pathway and results in a reduction of the intracellular concentrations of these polycations. Infection of cell cultures with herpes simplex virus (HSV) also produces a decrease in ODC activity (9, 10, 21, 22), presumably as a consequence of the virus inhibition of host cell protein synthesis (6, 24, 28). An implication of this observation is that HSV replication requires no more than the amounts of intracellular polyamines available at the time of infection. Consistent with this possibility are reports that inhibitors of putrescine synthesis (i.e., α-methylornithine and DFMO), as well as spermidine and spermine synthesis [i.e., methylglyoxal-bis(guanylhydrazene)], are without effect on HSV replication when added after infection (25, 30, 31, 33). In contrast, ODC activity is markedly stimulated after infection with strains of human cytomegalovirus (HCMV) and continues to increase for 20 to 30 h postinfection (14). This observation, taken together with reports that inhibitors of putrescine, spermidine, and spermine biosynthesis all inhibit production of HCMV (31–33), suggests a tighter coupling between polyamine biosynthesis and HCMV replication and may indicate a requirement for greater amounts of polyamines than are present in the cell at the time of infection.

Although the function(s) of polyamines during herpesvirus replication is not established, a role in assembly is suggested by studies with HSV which have demonstrated that (i) the virion contains both spermidine and spermine in a molar ratio of 1.6 and (ii) these polyamines are compartmentalized in the virion—spermine with the nucleocapsid and spermidine with the envelope (9, 10, 21). If intracellular polyamines play an essential role during virus replication, then their depletion after DFMO treatment would be expected to adversely affect virus production. This expectation is supported by several studies reporting that DFMO inhibits production of infectious HSV (25, 30) as well as CMV (33). In addition, cytochemical evidence has been presented (33) indicating that HCMV DNA synthesis and accumulation is inhibited in DFMO-treated, infected cells. Another study,

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however, concluded that DFMO treatment had little or no effect on either viral DNA synthesis or production of infectious virus (15). With the objective of resolving these apparent discrepancies, we initiated a series of experiments to examine some of the variables that could influence the results of such studies and investigate the level at which DFMO interferes with CMV replication.

Results presented here show that DFMO has a strong antiviral effect on CMV, but only when added to cell cultures before infection. Observations that the drug had little, if any, effect on the synthesis of viral proteins and DNA or on the assembly of some capsid forms are discussed in view of the possibility that DFMO may act by interfering with DNA packaging or nucleocapsid envelopment or both.

(These results were initially presented at the Seventh Herpesvirus Workshop held at Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 31 August to 5 September 1982.)

MATERIALS AND METHODS

Cells and viruses. Human foreskin fibroblast (HFF) cell cultures were prepared as described previously (7) and maintained in Dulbecco modified minimal essential medium (no. 430-2100; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum (Rehatuin F.S.; Reheis Chemical Co., Pheonix, Ariz., or Hyclone; Sterile Systems, Inc., Logan, Utah). Most cell cultures were grown in 6-cm plastic petri dishes (no. 25010; Corning Glass Works, Corning, N.Y.) containing 5 ml of medium and incubated at 37°C in an atmosphere of 5% CO_2–95% air. Cultures used for production of virus containing radiolabeled polyamines were grown in 32-ounce (960 ml) glass bottles, flushed with a mixture of 5% CO_2–95% air, capped tightly, and incubated at 37°C.

HSV type 1 (HSV-1) was the F prototype strain obtained from Bernard Roizman, University of Chicago, Chicago, Ill.; the sources of HCMV strains AD169, Towne, and 751 and of CMV strain Colburn (simian-like) have been described elsewhere (8). Multiplicities of infection used in these experiments were between 5 and 20. Virus titers were measured by either plaque assay or endpoint dilution. Plaque assays were done by infecting 6-cm petri cultures with serial 10-fold dilutions of the preparation to be tested. After a 1-h period of adsorption, the cell layer was covered with 5 ml of growth medium containing 0.5% agarose. Cultures were incubated for 2 to 3 weeks, after which the plaques (recognized as foci of rounded cells) were counted with the aid of a microscope. Endpoint dilution titrations were done by infecting cell layers, at about 50% confluence in 96-well tissue culture clusters (no. 3596; Costar, Cambridge, Mass.) (200 μl of medium per well), with 20 μl of serial 10-fold dilutions of the sample. The titer of the original material was calculated as the number of plaques observed at the highest dilution showing viral cytopathic effect multiplied by the dilution factor.

Virus purification. Virions were recovered from the media of infected cells by sedimentation in negative viscosity-positive density (glycerol-tartrate) gradients (1, 13, 29). Gradient solutions for preparations to be used in polyamine analyses were buffered with sodium phosphate (0.05 M, pH 7.4) rather than Tris-hydrochloride to avoid potential complications arising from the presence of polyamines in Tris.

Polyamine analyses. Polyamine analyses were done by the method of Seiler and Weichmann (27) as modified by Dion and Herbst (4). Polyamines were extracted from cell and virus pellets with perchloric acid, dansylated (dansyl chloride, no. 21752; Pierce Chemical Co., Rockford, Ill.), and separated from the aqueous reaction mixture with benzene. Dansylated polyamines were resolved by chromatography on 250-μm Silica Gel G plates (no. 5763; E. Merck AG, Darmstadt, Germany) with ethylacetate-cyclohexane (2:3, vol/vol). Plates were developed either three or seven (to resolve DFMO from slightly slower-moving spermine) times with 5 min of drying between each development, sprayed with triethanolamine-isopropanol (1:4, vol/vol), and dried overnight in the dark at reduced pressure. The plates were scanned with a Turner 111 fluorometer, and polyamines were quantified by comparing their peak areas with standard curves determined for each experiment.

The amount of radioactivity in [^{3}H]ornithine (Net 489; New England Nuclear Corp., Boston, Mass.)-radiolabeled polyamines was determined by scraping the appropriate fluorescent spot from the thin-layer plate, combining it with 3 ml of scintillation cocktail (Spectrafluor, diluted with toluene; Amersham Corp., Arlington Heights, Ill.), and measuring the radioactivity with a Searle Analytic ISO-CAP/300 scintillation spectrometer.

Polyacrylamide gel analysis. Proteins were separated in a sodium dodecyl sulfate-containing, 7.5% polyacrylamide gel essentially as described by Laemmli (17). The gel was subsequently stained with Coomassie brilliant blue, and the relative amount of protein in each band was determined from absorbance (540 nm) measurements made with an EC910 scanning densitometer (E-C Apparatus Co., St. Petersburg, Fla.).

DNA analysis. DNA samples were digested to completion with *Bam*HI (Bethesda Research Laboratories, Gaithersburg, Md.) in 50 mM Tris-hydrochloride (pH 8.0)–10 mM $MgCl_2$–50 mM NaCl–10 μg of bovine serum albumin in a volume of 50 μl and subjected to electrophoresis through a 1.0% agarose gel for 15 h at 50 V in a buffer of 0.036 M Tris-hydrochloride–0.03 M NaH_2PO_4–0.001 M EDTA. After electrophoresis, the gel was stained with ethidium bromide (200 μg/ml) and photographed under UV light with Polaroid type 57 film (18).

Electron microscopy. Cells growing in plastic petri dishes were rinsed with phosphate-buffered saline; fixed in 4% glutaraldehyde prepared in 0.1 M cacodylate buffer, pH 7.4 (cacodylate buffer); washed in cacodylate buffer; postfixed in 1% osmium tetroxide prepared in cacodylate buffer; and dehydrated in ethanol. The preparations were then embedded in Epon-Araldite, sectioned, placed on Formvar-carbon grids, stained with uranyl acetate and lead citrate (26), and examined with a Hitachi HU 11F electron microscope.

Protein determination. Protein determinations were done by the procedure of Lowry et al. (19) with bovine serum albumin as the standard.

Chemicals. DFMO (compound RMI 71,782 A) was a gift from Merrell-Dow Pharmaceuticals, Inc., Cincinnati, Ohio. Polyamine standards were obtained from Sigma Chemical Co., St. Louis, Mo. Reagents used in the polyamine analyses were of spectral grade.

RESULTS

Initial experiments in which DFMO was added at the time of or after infection with CMV gave variable results, some showing positive antiviral effect and others not. It became apparent that, when the drug was added at the time of infection, a positive effect was seen only if the multiplicity of infection was very low (e.g., 10^{-2} or 10^{-3}). An explanation of this observation was that the antiviral effect of DFMO required exposure of the cells to the drug for a period of time preceding infection, presumably to deplete intracellular

TABLE 1. Effect of DFMO on intracellular polyamines[a]

Exposure (h) to 10 mM DFMO	Polyamine concn (nmol per 10^6 cells)	
	Spermidine	Spermine
0	1.80	1.10
24	0.50	1.00
48	ND	1.05
72	ND	0.95

[a] Nine HFF petri cultures were started, each containing approximately 2×10^5 cells. Four hours after the addition of the cells, growth medium in two cultures was replaced with medium containing 10 mM DFMO. The medium was similarly replaced in duplicate cultures 24, 48 and 72 h later. Immediately after the last drug addition, one dish of each pair was processed for analysis of intracellular polyamine content, as described in the text. The other set of dishes was tested for virus production as described in the text and summarized in Table 2, experiment 1. ND, None detected.

polyamine pools. To test this possibility, we performed two experiments, as described below (see Tables 1 and 2).

Reduction of intracellular polyamine levels by DFMO. In the first experiment, the length of time required for DFMO to reduce intracellular polyamine pools in HFF cells was examined. Two sets of cultures were treated in parallel with 10 mM DFMO (Table 1). After an appropriate incubation period with the drug, one set was assayed for intracellular polyamine content. The other set was infected with HCMV 751 and subsequently assayed for the production of infectious progeny as described below. Results of the polyamine analysis (Table 1) show that the amount of spermidine was reduced by more than 70% in cultures that had been incubated in DFMO for 24 h and to levels below the sensitivity of our assay by 48 h, whereas the amount of spermine remained essentially unchanged, an observation reported by others (20). Putrescine, present in nontreated cell cultures in amounts too low to be measured accurately, was not detected in any of the DFMO-treated cultures. We found that, under the conditions used in these studies, drug-treated HFF cells retained a normal appearance, continued to grow slowly (even in the presence of 30 mM DFMO), and could be subcultured and regrown to confluence at least twice (data not shown).

DFMO reduces CMV yields. HCMV-infected cultures that had been pretreated with DFMO for 1, 2, or 3 days before infection, as part of the experiment described above, were incubated for 2 weeks and then tested for the presence of infectious virus. An infected culture treated with DFMO immediately before assay and a nontreated, infected culture were tested as controls. All infected cultures exhibited extensive cytopathic effect, and those treated with drug were not readily distinguished from nontreated cultures in this respect. Results of the infectivity determinations (Table 2, experiment 1) show that there was no reduction in virus yield when DFMO was added at the time of infection and only a slight reduction (4-fold) with a 1-day preincubation, but a 4,000-fold reduction occurred after 2 or 3 days of pretreatment with the drug. The experiment was repeated with another HCMV strain (Table 2, experiment 2). Again, virus yields were reduced to a much greater extent (1,000-fold) in cultures that had been treated with DFMO 3 days before infection than in those treated for shorter periods. In contrast to the preceding experiment, however, virus titers were significantly lower after 3 days of pretreatment than after 2 days. We have not investigated the effect of longer preincubations.

TABLE 2. Effect of DFMO pretreatment on virus titer

Expt[a]	Length of pretreatment (h)	Infectious virus produced (PFU/ml)	Reduction factor
1	No drug	2×10^7	
	0	2×10^7	
	24	5×10^6	4
	48	5×10^3	4,000
	72	5×10^3	4,000
2	No drug	5×10^5	
	24	5×10^4	10
	48	5×10^3	100
	72	5×10^2	1,000

[a] HCMV 751 was used in experiment 1, and HCMV AD169 was used in experiment 2.

TABLE 3. Effect of DFMO concentration on virus titer

DFMO concn (mM)	Infectious virus[a] produced (PFU/ml)	Reduction factor
No drug	10^8	
1	10^4	10^4
5	10^2	10^6
10	10^2	10^6
30	ND	10^8

[a] HCMV AD169. ND, None detected.

To determine what effect increasing or decreasing the concentration of drug would have on its antiviral activity, cultures were incubated in 1, 5, 10, or 30 mM DFMO for 3 days and then infected with HCMV. The amount of virus produced was assayed 14 days after infection (Table 3). A greater antiviral effect was seen at 30 mM DFMO than at 5 or 10 mM, but even at the lowest concentration tested (i.e., 1 mM), there was a 10,000-fold reduction in virus titer. The unusually strong antiviral effect observed in this experiment (e.g., 10^6 reduction at 10 mM DFMO) may correlate with the fact that the starting cell density was lower than usual (i.e., 25% confluence versus 50 to 75%). For reasons of economy and convention, we have chosen to use 10 mM as a standard concentration.

Other herpesviruses inhibited by DFMO. For comparative purposes, we also tested the antiviral effect of DMFO on CMV Colburn (simian-like isolate) and HSV-1. This was done as described above by pretreating HFF cultures with 10 mM DFMO for 3 days before infection and then assaying samples of the medium for infectious virus 1 week after infection. Results (Table 4) show that the drug reduced yields by at least 95% for all three viruses but had a more potent effect on the HCMV strain than on CMV Colburn or HSV-1. We have not further studied the apparent difference in sensitivity of these three viruses to the effects of DFMO.

Antiviral effect reduced by exogenous polyamines. Initial experiments in which we tried to reverse the antiviral effect of DFMO by adding polyamines 4 days after infection were not successful. We then attempted to reduce the antiviral effect on CMV by supplying polyamines concomitantly with

TABLE 4. DFMO inhibits both CMV and HSV

DFMO pretreatment	Infectious virus produced (PFU/ml)		
	Wild type[a]	Colburn[b]	HSV-1[c]
No drug	5×10^6	5×10^7	2.5×10^7
72	2.5×10^2	2.5×10^6	5×10^5

[a] HCMV 751; reduction factor, 20,000-fold.
[b] Simian-like isolate; reduction factor, 20-fold.
[c] Reduction factor, 50-fold.

the drug. This was done by incubating cultures for 3 days in the presence of 10 mM DFMO; 10 μM polyamines, i.e., putrescine, spermidine, or spermine; 10 mM DFMO in addition to 10 μM polyamines; or no drug. Pretreated cells were then infected with HCMV, and the titer of progeny virus was determined by plaque assay. The results of this experiment (Table 5) show that DFMO alone reduced virus titer by 1,000-fold. The presence of putrescine during pretreatment with DFMO increased the amount of virus by 50-fold, to 5% of the control value. Addition of either spermidine or spermine along with the drug increased virus yield 80-fold (i.e., 8% of control) above that of the culture containing DFMO alone. The presence of 10 μM putrescine, spermidine, or spermine alone was without detectable effect on virus yield. The influence of these drug combinations on the types of particles produced by the infected cells is shown in Fig. 1 and described below.

In a similar experiment in which intracellular polyamine levels were measured, it was found that spermine was the most abundant species present and that its relative concentration among the different cultures did not vary appreciably (i.e., ≤20%; data not shown). These data also showed that the spermidine concentration in infected, nontreated cultures was approximately one-third that of spermine.

DFMO reduces amount of extracellular particles. Since extensive viral cytopathic effect was observed, even in the presence of 30 mM DFMO, it was apparent that at least some virus products were being made. As a first step in attempting to determine how DFMO exerts its antiviral effect on HCMV, we tested the extracellular medium for the presence of virus particles. Media samples from the experiment described above were processed and subjected to rate-velocity centrifugation in glycerol-tartrate gradients, and absorbance (280 nm) profiles were recorded as the gradients were collected (as described in Materials and Methods). Figure 1 shows two types of extracellular virus particles recovered from HFF cells normally infected with HCMV AD169. These are referred to as noninfectious enveloped particles (NIEPs [13]) and virions. In the context of this study it should be emphasized that NIEPs and virions are nearly indistinguishable in protein composition and architecture but differ in that NIEPs contain no DNA. Medium from the culture treated with DFMO contained neither type of particle (Fig. 1, lower left). However, when putrescine, spermidine, or spermine was added together with DFMO, a virion peak was observed. Furthermore, in the presence of DFMO, exogenous polyamines altered the relative amounts of NIEPs and virions. Putrescine had a stronger sparing effect on NIEPs than virions, whereas the opposite was true for spermidine and spermine. Comparison of the infectivities of these preparations (Table 5) with the amount of virions present in each (i.e., absorbance; Fig. 1) indicates that

TABLE 5. DFMO effect reduced by exogenous polyamines

Treatment[a]	Virus[b] titer (PFU/ml)	Protein (mg per dish)	Virus titer per mg of protein	% of nontreated
None	1.5×10^7	3.6	4.2×10^6	100
DFMO	1.0×10^4	2.3	4.4×10^3	0.1
DFMO + Put.	5.7×10^5	2.6	2.2×10^5	5.2
DFMO + Spd.	6.5×10^5	1.9	3.4×10^5	8.1
DFMO + Sp.	5.5×10^5	1.5	3.7×10^5	8.8

[a] Put., Putrescine; Spd., spermidine; Sp., spermine.
[b] HCMV AD169.

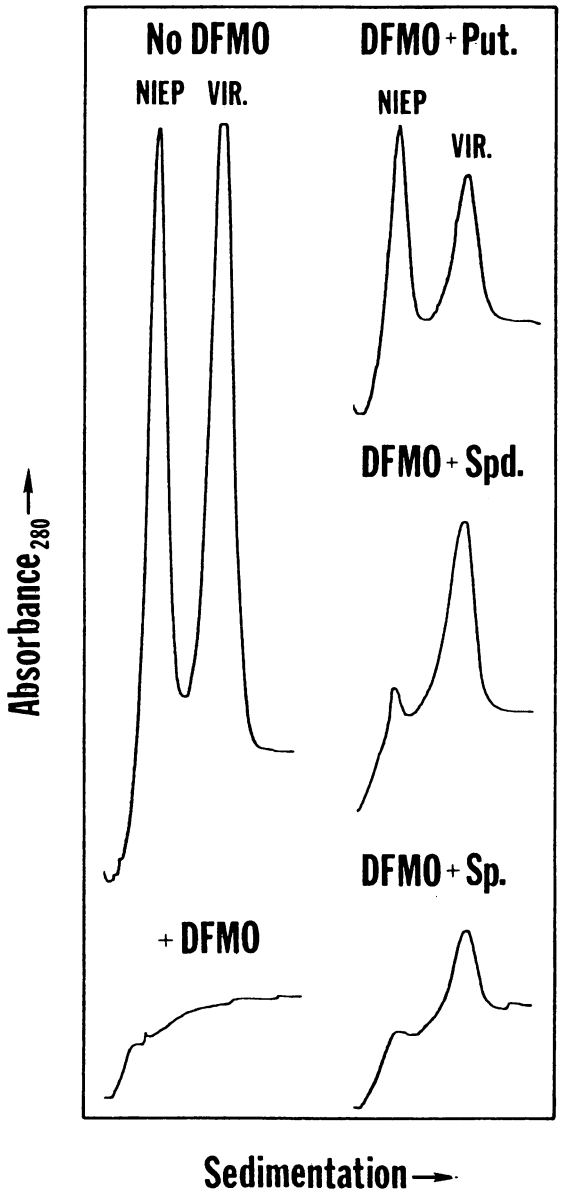

FIG. 1. Effect of DFMO on NIEP and virion production by HCMV-infected cells. HFF cells were incubated for 3 days with 10 mM DFMO or with 10 mM DFMO supplemented with either 10 μM putrescine, spermidine, or spermine and then infected with HCMV AD169. Fourteen days after infection, media were collected from the cultures, clarified by centrifugation at 1,500 × *g* at 4°C for 10 min, layered above glycerol-tartrate gradients, and subjected to centrifugation at 40,000 rpm at 4°C for 20 min in a Beckman SW41 rotor. The gradients were monitored at 280 nm as they were displaced from the tubes with a model 185 density gradient fractionator and a model UA-5 absorbance monitor (ISCO, Lincoln, Neb.). Full-scale absorbance for all gradients was 0.2 optical density units. The positions of NIEPs and virions (VIR.) are indicated. The profiles shown are from infected cells that had been preincubated and continued in the absence of drug (No DFMO), in the presence of DFMO alone (+DFMO), in the presence of DFMO and putrescine (DFMO + Put.), in the presence of DFMO and spermidine (DFMO + Spd.), or in the presence of DFMO and spermine (DFMO + Sp.). Shown here is a collage prepared from photocopies of the recordings.

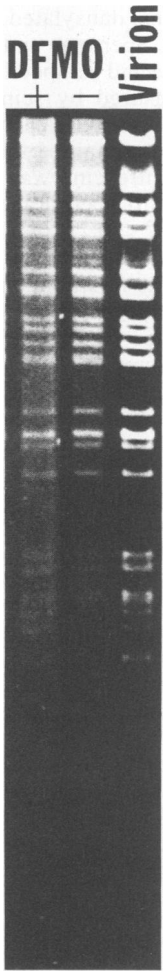

FIG. 2. Cells infected in the presence of DFMO produce viral DNA. HFF cells were pretreated with DFMO for 3 days or not treated and then were infected (in presence or absence of drug, respectively) with HCMV 751. Infected cell DNA was extracted 6 days postinfection and cleaved with *Bam*HI restriction endonuclease. Such preparations from cells infected in the presence (+) or absence (−) of DFMO were subjected to electrophoresis in a 1% agarose gel for 15 h at 50 V. A sample of strain 751 virion DNA was compared in an adjacent channel. Shown here is a photograph of the resulting gel, stained with ethidium bromide.

cultures treated with spermine produced approximately the amount of virions expected from the infectivity measurement (i.e., about 10% the amount of non-drug-treated control). The cultures treated with putrescine or spermidine, however, produced about five- and fourfold more particles, respectively, than was predicted from their infectivities.

Viral DNA made in DFMO-treated cells. We next looked for intracellular evidence of virus replication and began by testing for viral DNA synthesis. This was done by preparing DNA from cultures infected with HCMV 751 in the presence or absence of drug, cleaving the DNA with *Bam*HI restriction endonuclease, and separating the resulting fragments in a 1% agarose slab gel (as described in Materials and Methods). A photograph of the resulting gel, stained with ethidium bromide (Fig. 2), demonstrates that viral DNA was present in both DFMO-treated and nontreated cultures. An adjacent channel containing DNA from purified HCMV 751 virions, cleaved in parallel with the cellular preparations, shows that the bands visualized are viral. Although we are unable to make precise quantitative comparisons between these preparations, it is clear that, in the DFMO-treated cultures, viral DNA is replicated and accumulates in substantial amounts (e.g., ≥50% normal). On the basis of results of similar experiments in which the infected cell DNA was labeled with $^{32}P_i$ (data not shown), we are confident that the bands shown here represent newly synthesized DNA rather than input molecules introduced by infection.

Viral proteins made in DFMO-treated cells. To determine whether viral proteins were also being synthesized in DFMO-treated cells, we compared drug-treated and nontreated, infected cultures by gel electrophoresis. HCMV strains Towne and 751 were both used. Seven days after infection, the cells were collected and processed for analysis by polyacrylamide gel electrophoresis (Fig. 3). A photograph of the resulting gel after staining with Coomassie brilliant blue is shown in Fig. 3. Only the nuclear fractions, which contain most of the CMV protein mass (8), are shown. All of the infected-cell-specific proteins detected in the normally infected cells were also present in the DFMO-treated cells. Among these, the major capsid protein, 140,000-kilodalton protein, matrix protein, DNA-binding protein, and assembly protein are the most prominent. This figure also demonstrates that exposure to 10 mM DFMO does not appreciably alter the pattern of nuclear proteins in noninfected cells. Although there is some variability among experiments, densitometric measurements made from such comparative gels indicate that, relative to the amount of actin or major capsid protein present, preparations from DFMO-treated, infected cells contain more matrix protein than do those from nontreated, infected cells. Expression and accumulation of these viral proteins was the same in cells treated with 30 mM DFMO (data not shown).

Some virus particles are assembled in DFMO-treated cells. Since viral DNA and proteins were both present in DFMO-treated cells, we became interested in the possibility that this drug may exert its inhibitory effect at the level of virus assembly. This possibility was explored by using an electron microscope to examine thin sections of drug-treated and nontreated, HCMV-infected cells. Electron micrographs prepared from these cultures (e.g., Fig. 4 and 5) showed that DFMO-treated and nontreated cultures contained a comparable number of particles. Among those observed in nontreated cells were intranuclear capsids that were either empty or partially filled with densely stained material (e.g., DNA) or that contained an apparent internal structure that was variable in configuration but often looked like a concentric ring about 15 nm thick or a string of six symmetrically distributed beads, each 10 to 15 nm in diameter (see Fig. 5). In addition, enveloped capsids containing a densely stained center were frequently observed in the cytoplasm (e.g., Fig. 4, "Virion"; Fig. 5E). The particles present in DFMO-treated cells appeared more uniform in structure. Few particles with darkly stained centers were seen (Fig. 4B); the number of enveloped particles in the cytoplasm was reduced, and those that were observed did not have densely stained centers (e.g., Fig. 5F). Although these general observations were characteristic of the cells examined, no attempt was made to quantify the various particle types since they were heterogeneous in appearance and we could not assess with any certainty whether internal capsid structures represented DNA or protein or both. In the course of this study, numerous cells were examined. The structures shown in Fig. 4 and 5 are representative of those observed and were present in the majority of cells.

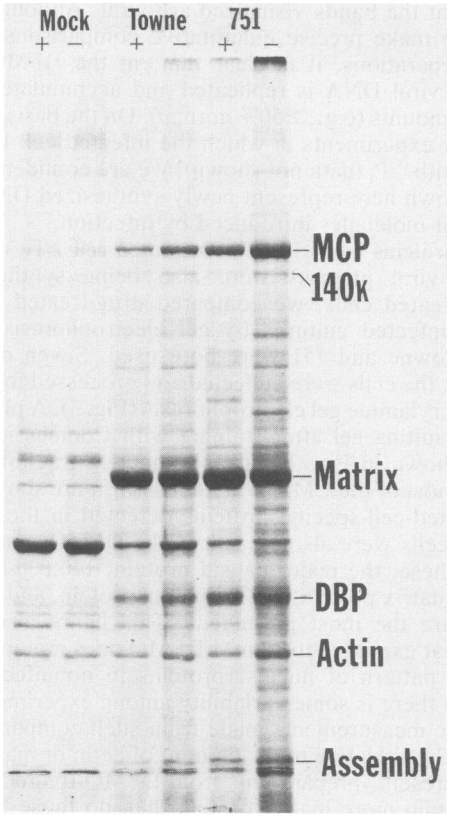

FIG. 3. Proteins present in DFMO-treated, HCMV-infected HFF cells. HFF cells were incubated for 3 days in the presence (+) or absence (−) of DFMO. One culture each was then infected with HCMV (Towne or 751) or was left uninfected (Mock). Seven days after infection, the cells were scraped from the petri dishes into the culture medium, collected by centrifugation at 1,500 × *g* at 4°C for 10 min, disrupted with Nonidet P-40 (0.5% in 0.15 M NaCl–0.04 M sodium phosphate buffer, pH 7.4; 0°C, 10 min), and separated into nuclear pellet and cytoplasmic supernatant fractions by centrifugation as described above, and each fraction was solubilized in preparation for analysis by gel electrophoresis. Aliquots of the resulting Nonidet P-40 nuclear fractions were subjected to electrophoresis in a sodium dodecyl sulfate-containing, 7.5% polyacrylamide gel. Shown here is a photograph of the Coomassie brilliant blue-stained gel after it had been dried onto a piece of filter paper. Five HCMV proteins and the cellular protein (Actin) are indicated in the right-hand margin. CMV protein designations (molecular weight) (8): MCP, major capsid protein (153,000); 140K (140,000); Matrix, matrix-like protein (69,000); DBP, DNA-binding protein (53,000); Assembly, assembly protein (35,000).

HCMV virions contain polyamines. Finally, it was of interest to determine whether HCMV virions contain polyamines. Since it has not yet been possible for us to measure the polyamine content of HCMV virions directly (e.g., dansylation-fluorometry or high-pressure liquid chromatography), we have used a radiolabeling approach in these initial experiments. This was done by radiolabeling HFF cells with [^{3}H]ornithine (5 μCi/ml) for 24 h and then infecting the cultures with HCMV strain AD169 or 751 or HSV-1. Radiolabel remained in the culture medium throughout the infection. When strong cytopathic effect was observed (i.e., 7 to 10 days for HCMV and 2 to 3 days for HSV-1), virus particles were collected from the maintenance media by rate-velocity sedimentation in glycerol-tartrate gradients and concentrated by pelleting. Polyamines were acid extracted from the pelleted virions, dansylated, and separated by thin-layer chromatography. Spermine and spermidine spots were located by fluorescence and scraped from the plate, and their radioactivity was measured by scintillation spectrometry. All experiments were done at least twice (Table 6). HCMV AD169 and 751 virions contained the polyamines spermine and spermidine. No putrescine was detected. The ratio of radiolabeled spermine to spermidine was calculated to be approximately 2.3 (2.17 ± 0.27 for strain AD169 and 2.54 ± 0.22 for strain 751).

This ratio of spermine to spermidine is much higher than that previously determined for HSV virions by radiolabeling (i.e., 0.3; see reference 9, Table 3). We considered the possibility that this discrepancy may be due to experimental differences between the two studies, such as cell type (i.e., HFF versus HEp-2 cells), radiolabeling conditions (i.e., continuous versus 18-h preinfection only), or virus recovery regimen (i.e., glycerol-tartrate gradients and extracellular virus versus dextran gradients and intracellular virus). This was tested by analyzing HSV virions grown and purified in the same way that we prepared HCMV virions. Results of two such experiments (Table 6) show that the HSV spermine/spermidine ratio was the same as cited above (i.e., 0.3) and, therefore, not significantly affected by the different experimental conditions of this study.

We note, without further comment, first, that the HCMV polyamine ratio, calculated here from measurements of radioactivity, is predicted to be a minimal estimate since the specific activity of spermine is slower to increase than that of spermidine. Second, the absolute ratio of spermine to spermidine in HSV virions is approximately 0.6 when measured directly (9, 21), a value still three- to four-fold lower than the theoretically underestimated ratio of approximately 2.3 determined for HCMV in the present experiments.

DISCUSSION

In this report we have presented evidence that DFMO, an enzyme-activated inhibitor of the polyamine biosynthetic enzyme ODC, exhibits a strong inhibitory effect on the production of infectious CMV by HFF cells. Although this general conclusion is in agreement with an earlier report by Tyms and Williamson (33), specific details differ significantly and warrant further discussion.

First, with respect to the time of drug addition, we found that, when sufficiently high multiplicities of infection were used to ensure infection of every cell, a 2- to 3-day preincubation of the cell culture with DFMO was necessary to

TABLE 6. Virions of HCMV contain polyamines

Strain and expt no.	Radioactivity (cpm)[a]			
	Spermine	Spermidine	Ratio	Mean ± SEM
HCMV AD169				2.17 ± 0.27
1	2,634	1,413	1.86	
2[b]	5,441	2,579	2.11	
3	3,980	1,574	2.53	
HCMV 751				2.54 ± 0.22
1	1,956	708	2.76	
2[b]	14,714	6,352	2.32	
HSV-1				0.34 ± 0.04
1	865	2,208	0.39	
2[b]	5,166	17,141	0.30	

[a] Radiolabel was supplied to cells as [^{3}H]ornithine.
[b] Done together as part of the same experiment.

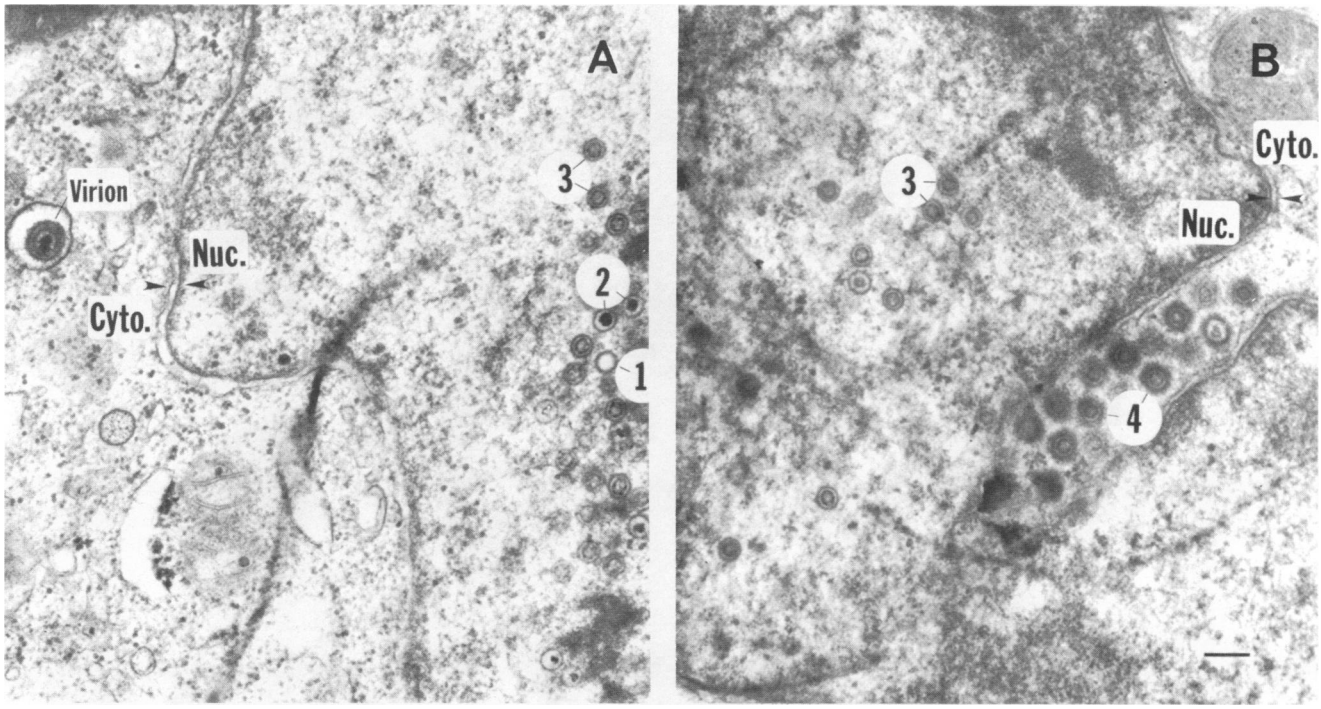

FIG. 4. DFMO-treated, HCMV-infected cells contain virus particles. HFF cell cultures, either nontreated or grown continuously in the presence of 10 mM DFMO added 3 days before infection, were infected with HCMV AD169. Nine days later, the cell layers were rinsed with phosphate-buffered saline, fixed, scraped from the petri dish, and processed for thin sectioning and analysis by electron microscopy as described in the text. Shown here are representative thin sections of cells infected in the absence (A) or presence (B) of DFMO. Capsids that appear empty (1), partially filled with dense-staining material (2), and contain internal beadlike structures (3) were seen in the nucleus (Nuc.) of cultures lacking DFMO; enveloped particles with a densely stained core (Virion) were present in the cytoplasm (Cyto.). Capsids with a beaded internal structure (3) were present in the nucleus of DFMO-treated cultures, and empty, apparently coated particles (4) were seen within cytoplasmic invaginations into the nucleus. Arrows indicate nuclear membrane. Bar, 200 nm. Representative capsid forms from such cells are shown in greater detail in Fig. 5.

confer the antiviral effect. This observation is consistent with the earlier findings of Tuomi et al. (30), who showed that the inhibitory effect of DFMO on Semliki Forest virus (enveloped, RNA-containing virion) and HSV-2 occurred only when infection followed a 3-day incubation with the drug. In light of the consistency of these results from studies in which different cells (i.e., human or monkey) and viruses (i.e., HCMV, Semliki Forest virus, and HSV-2) were used, it is not clear why in some studies DFMO and D,L-α-methylornithine produced an antiviral effect without preincubation (31, 33) whereas in others DFMO failed to confer an antiviral effect even with a 3-day preincubation (15). It seems unlikely that these differences were due to the cells or virus, since all studies with HCMV used diploid human fibroblast cells and all but one used CMV AD169. A more plausible explanation, therefore, may be differences in the culture conditions (e.g., cell density, serum composition, and concentration), which we also believe to be responsible for variability in the extent of the antiviral effect observed in the present studies. Since the antiviral effect correlated with depletion of the intracellular spermidine pool, it would be informative to know how this value changed in the other studies.

Second, experiments to investigate the level at which DFMO interferes with the production of infectious HCMV showed that the drug did not spare the cells from typical viral cytopathic effect, nor did it block either viral DNA or protein synthesis. Further, since at least empty capsids (i.e., partially or nonstained center) were present in the drug-treated cells (Fig. 4), it is apparent that the level of inhibition is not capsid assembly per se. We did find, however, that the drug treatment greatly reduced the number of mature particles (e.g., enveloped with densely stained core) both within the cell and released into the medium. These observations are more compatible with the antiviral effect of DFMO being directed against a late event in the virus replication cycle, such as DNA packaging or capsid envelopment or both, than with an early event, as suggested elsewhere (33).

Since CMV virions, like those of HSV (9, 10, 21), contain the polyamines spermidine and spermine (Table 6) and since DFMO essentially eliminates the intracellular pool of spermidine (Table 1), the possibility that this drug exerts its antiviral effect by interfering with virus assembly is mechanistically attractive. If polyamines serve as counterions to neutralize the electronegativity of virion constituents during assembly, then at least two steps in the maturation pathway might be particularly sensitive to a decrease in their availability. The first is DNA packaging, in which nucleotide phosphates would require neutralization to permit encapsidation, and the second is nucleocapsid envelopment, during which phosphate charges of the tegument proteins coating the capsid (W. Gibson and A. Irmiere, *in CMV: Pathogenesis and Prevention of Human Infection*. March of Dimes Birth Defects, original article series, vol. 19, in press), envelope phospholipids (W. Gibson, unpublished observations), or other electronegative virion constituents may require neutralization.

If the inhibitory effect of DFMO were on DNA packaging alone, then one would not expect the production of particles

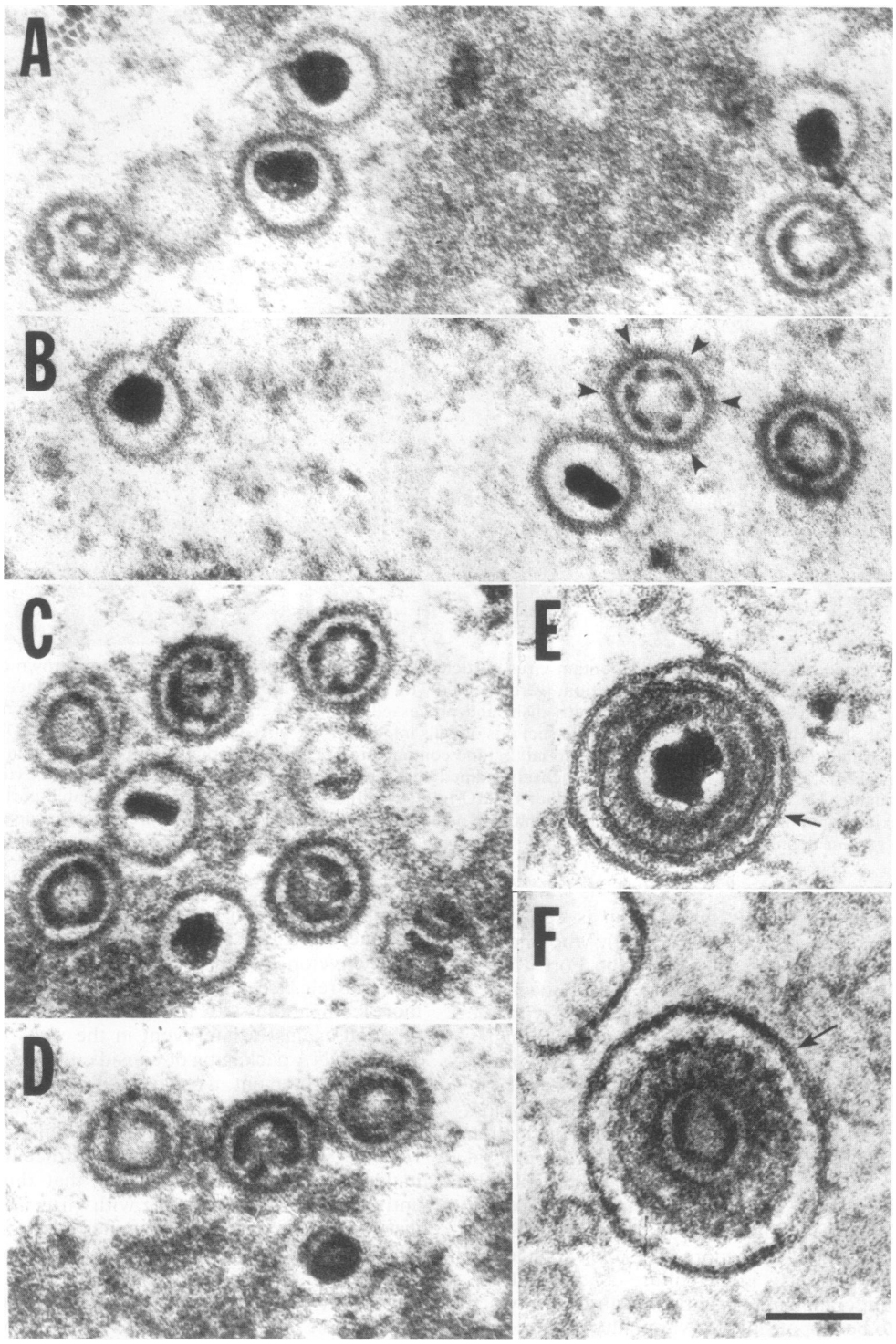

FIG. 5. Intranuclear HCMV capsid forms and enveloped cytoplasmic particles present in DFMO-treated and nontreated HFF cells. Shown here are high-magnification pictures of virus particles in nontreated (A and B) and DFMO-treated (C and D) cells, prepared as described in the legend to Fig. 4. (A) Three particles with densely stained centers of the type designated 2 in Fig. 4, one with a ring-shaped internal structure of the type designated 3 in Fig. 4 (lower right-hand corner), one with less clearly organized internal material (lower left-hand corner), and adjacent to it, an empty capsid of the type designated 1 in Fig. 4. (B) Particle containing an internal structure, beaded in appearance. Arrows indicate coincidence of beads with capsid vertices. Many of the particles in drug-treated cells contained ring-shaped internal structures of the type designated 3 in Fig. 4, but other organizations were also observed (C and D). (E) Enveloped capsid with a densely stained center (e.g., virion) within a cytoplasmic tubule (arrow indicates tubule membrane). (F) Enveloped particle with a weakly stained center (e.g., NIEP [13]) also within a cytoplasmic tubule (see arrow). All micrographs are the same magnification; bar, ca. 100 nm.

lacking DNA to be interrupted. This prediction is supported by experiments in which we have shown that the production of virion-like NIEPs, which contain all of the virion proteins but no DNA, is unaffected by inhibitors of DNA synthesis that abolish virion production (Gibson and Irmiere, in press). Our finding that DFMO treatment blocks the production of NIEPs as well as virions (Fig. 1) is, therefore, taken as evidence for an additional, or different, level of inhibition, such as envelopment. In this connection, we have been interested in determining whether one of the putative assembly intermediates (e.g., intranuclear A and B capsids or cytoplasmic C capsids [7]) may accumulate in DFMO-treated, infected cells, thereby providing an indication of the step blocked. Since recovery of such particles from non-treated, HCMV-infected cells has proven difficult, these experiments are being done with strain CMV Colburn, previously shown (7) to produce both intracellular and extracellular particles in good yield during a normal infection. It is anticipated that DFMO will be useful in studying aspects of virus replication and assembly that are dependent upon cellular polyamines and are not otherwise directly accessible.

Although DFMO inhibits the growth of CMV (Tables 2 and 4; 33), HSV (Table 4; 30), and Semliki Forest virus (30) in cell culture, it remains to be determined whether it will be of value as an antiviral agent in humans. The need for preexposure and high concentrations to achieve an antiviral effect with DFMO (in vitro), coupled with its generalized suppression of cell proliferation, would tend to argue against this possibility. Despite these potential limitations, there are features of this drug that make its clinical evaluation as an antiviral agent seem attractive. First, DFMO is well tolerated in humans (11). Second, since DFMO acts on a host cell protein rather than on one encoded by the viral genome, the probability of a drug-resistant variant or mutant virus emerging is expected to be low. Third, unlike many chemotherapeutic agents used to inhibit DNA viruses, DFMO does not interfere directly with DNA replication. Thus, concerns are reduced about it promoting DNA replication-mediated mutagenesis, transformation, or integration of viral DNA into the host chromosome. Fourth, although DFMO inhibits the production of infectious virus and thereby the spread of infection, it does not spare the initially infected cell from death. Therefore, the possibility of perpetuating the virus in a drug-mediated latent form is reduced. Finally, because DFMO alters the intracellular environment by inhibiting a cellular enzyme, it is expected to interfere with a broad spectrum of viruses that contain cellular polyamines or otherwise depend upon their availability during replication.

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