ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1992, p. 95–99
0066-4804/92/010095-05$02.00/0

Vol. 36, No. 1

Mechanism of Action of the Antirhinovirus Flavanoid 4′,6-Dicyanoflavan

CINZIA CONTI,[1]* PAOLA TOMAO,[1] DOMENICO GENOVESE,[2] NICOLETTA DESIDERI,[3] MARIA LUISA STEIN,[3] AND NICOLA ORSI[1]

Istituto Pasteur-Fondazione Cenci Bolognetti, Institute of Microbiology,[1] and Department of Pharmaceutical Studies,[3] University of Rome "La Sapienza," and Department of Virology, Istituto Superiore di Sanità,[2] Rome, Italy

Received 12 August 1991/Accepted 4 October 1991

4′,6-Dicyanoflavan (DCF), a new antirhinovirus compound, was shown to inhibit an early event of rhinovirus type 1B replication in HeLa cells. When DCF was present from the beginning of infection or was added no later than the first hour of infection, the compound completely prevented viral RNA and protein synthesis and the virus-induced shutoff of host translation. DCF had no adverse effect either on virus binding to the cell membrane or on virus penetration into cells, whereas it delayed the uncoating kinetics of neutral red-encapsidated rhinovirus. DCF also prevented mild acid or thermal inactivation of virus infectivity, although it reversibly interacted with virions. These results suggest that the stabilizing effect of DCF on virion capsid conformation is responsible for uncoating inhibition.

Flavanoids are among the most potent inhibitors of human rhinovirus (HRV) replication. In the last few years, we have synthesized new anti-HRV flavanoids that are structurally related to the well-known flavanoid 4′,6-dichloroflavan (BW683C) (1). An interesting feature of the synthesized flavanoid compounds is that, besides having an effect on HRV (2), they exhibit an antiviral spectrum that is wider than that of BW683C. They show good antiviral activity against poliovirus type 2 (4), coxsackievirus type B4, echovirus type 6, enterovirus type 71 (8), hepatitis A virus (18), and astrovirus (17). Studies on the mechanism of action indicate that they affect some early process of the virus life cycle (5), similar to the effect of BW683C (20).

Recently, in an attempt to improve their antiviral potency, we synthesized new flavans, isoflavans, and isoflavenes substituted with halogens as well as cyano or amidino residues (3). The cyano residue is a strong electron-withdrawing group known to increase the antipicornavirus activity of substituted phenoxybenzene (13); the amidino group is present in some proteinase inhibitors with antiviral activity (6, 19). Among these drugs, 4′,6-dicyanoflavan (DCF) proved to be more active than the reference molecule BW683C when they were tested under the same experimental conditions (3). DCF had no adverse effect on cell cultures up to a concentration of 40 μM, which is about 1,740 times the 50% inhibitory concentration (IC_{50}) of HRV type 1B plaque formation (IC_{50} = 0.023 μM).

The aim of the present work was to elucidate whether the replacement of chlorine with cyano groups could influence the mechanism of the antiviral action of this type of molecule.

MATERIALS AND METHODS

Cells and virus. The HeLa (Ohio) cell line was routinely grown in Eagle's minimum essential medium (MEM) as described previously (5).

HRV type 1B (HRV 1B) was propagated in HeLa cells as reported previously (5). The virus titer was measured by plaque assay (2).

* Corresponding author.

Radioactively labeled virus was grown in the presence of 10 μCi of 5-[^{3}H]uridine (27 Ci/mmol; Amersham International) per ml and actinomycin D (0.1 μg/ml). The preparation was digested with 1 mg of RNase A per ml (30 min, 33°C) to remove nonencapsidated RNA, and the virus was sedimented at 70,000 × *g* for 2.5 h (4°C). The pellet was taken up in phosphate-buffered saline (PBS; pH 7.4).

Virus yield reduction. Confluent monolayers of HeLa cells in 24-well plates were infected at a multiplicity of infection of 5, in the presence or absence of DCF. After 1 h at 33°C, excess virus was removed and the monolayers were washed twice with PBS. Then, MEM with or without the compound was added. Single-cycle conditions were achieved by incubating the cells at 33°C for 9 h postinfection (p.i.). The cultures were freeze-thawed three times, and the cell debris was removed by low-speed centrifugation. After extraction of the drug with chloroform (5), the supernatants were titrated by plaque assay.

DCF was stored as a 0.1% stock solution in ethanol and diluted in culture medium shortly before use.

Time of addition or removal of compound. In order to determine which stage of virus replication is affected by the drug, DCF was added or removed from virus-infected cultures at various times p.i. and the virus yield was determined after a single cycle of virus replication. The infection was synchronized by allowing the virus, at a multiplicity of infection of 5, to attach to the cell surface in the cold (1 h, 0°C) and then raising the temperature to 33°C to permit internalization. At various times p.i. (0, 15, and 30 min and 1, 2, and 3 h), culture medium containing DCF at a concentration of 3.2 μM or not containing DCF was added or removed, and the cells were incubated for 9 h. The virus yield was determined as described above.

Virus inactivation and stabilization. Virus suspensions with or without DCF at different concentrations were incubated at 33°C for 1 h. The virus titer was determined by plaque assay before and after chloroform extraction (5).

The virus was incubated with or without DCF for 1 h at 33°C before mild acid or thermal treatment. For mild acid treatment, the pH of the mixtures was adjusted to 5.0 by adding 0.2 M acetate buffer (pH 5.0). After incubation at 33°C for 30 min, the mixtures were neutralized with 0.85 M

Tris base. For thermal treatment, the mixtures were incubated for 20 min at 56°C (pH 7.2) and then refrigerated on ice. All samples were extracted with chloroform, diluted, and titrated as described above.

Viral RNA synthesis. Cell monolayers were infected (1 h, 33°C) with HRV 1B at a multiplicity of infection of 25 and were then added to MEM containing actinomycin D (2 μg/ml). At the indicated times after infection, 20 μCi of 5-[^{3}H]uridine per ml was added, and the cells were incubated for 1 h. Thereafter, the monolayers were washed three times with PBS and were precipitated with 10% trichloroacetic acid (TCA). TCA was removed and the cells were washed with ethanol. After drying under an infrared lamp, the content of each well was dissolved in 0.02 N NaOH plus 0.1% sodium dodecyl sulfate (SDS). An aliquot was suspended in scintillation liquid to evaluate the radioactivity.

Cellular and viral protein syntheses. Cellular and viral protein syntheses were assayed as described previously (4). Briefly, HeLa cells were incubated in methionine-free medium in the presence of 20 μCi of [^{35}S]methionine (1,176 Ci/mmol; DuPont, New England Nuclear) per ml from hours 8 to 9 p.i. The cells were washed three times with PBS and collected in lysis buffer. Incorporation of [^{35}S]methionine into newly synthesized proteins was determined by precipitation with 10% TCA, filtration through nitrocellulose filters, and counting of the radioactivity by liquid scintillation.

Electrophoresis was performed as described by Laemmli (9). Samples were boiled for 5 min before being applied to 15% SDS-polyacrylamide gels.

Virus binding and internalization. HeLa cell monolayers in four-well plates were washed twice with ice-cold PBS, and [^{3}H]uridine-labeled HRV 1B (7,000 cpm per dish), with or without the compound, was added. Adsorption was allowed to proceed for 2 h. Thereafter, the monolayers were washed five times with cold PBS to remove unbound particles, and TCA-precipitable cell-associated radioactivity was counted after three cycles of freezing-thawing of the cell cultures.

To measure virus entry, [^{3}H]uridine-labeled HRV 1B (7,000 cpm per dish) was incubated with cells for 1 h at 33°C. After washing five times with PBS, the monolayers were incubated for 1 h at 0°C with 5 mM EDTA to remove noninternalized virions (10). After washing five times with cold PBS, the cells were processed as described above.

Neutral red-sensitized virus preparation and measurement of viral uncoating. Photosensitive HRV 1B was propagated in HeLa cells for more than three serial passages in the dark in the presence of 10 μg of neutral red per ml, as described by Mandel (12).

To examine uncoating kinetics, virus that was mock treated or treated with 3.2 μM DCF (1 h, 33°C) was adsorbed to cell cultures in darkness in the cold (1 h, 0°C) at dilutions suitable for plaque determination. Unadsorbed inoculum was removed by washing the culture three times with cold PBS, and the cells were covered with MEM (2% fetal calf serum) and warmed to 33°C. After the indicated intervals, one set of plates was irradiated for 15 min with a white light (300 W). The other set of cultures, which were kept in the dark, was submitted to identical treatment to serve as a control. After removal of MEM, the cells were overlaid with medium for plaque determinations and were incubated for 72 h at 33°C. The unaltered titer of photosensitive HRV preparations after digestion with 1 mg of RNase A per ml for 30 min at 33°C demonstrated the lack of free viral RNA, which possibly interfered with the results. Results of control experiments with dye-free virus indicated that illumination has no effect on the virus-cell system.

TABLE 1. Effect of DCF on infectivity and effect of heat (56°C) or acid (pH 5) inactivation of HRV 1B

DCF concn (μM)	Reduction in titer ($\log_{10}$ PFU/ml) compared with that of control[a]			
	Virus inactivation (pH 7, 33°C)		Stabilization against inactivation by:	
	Before $CHCl_3$	After $CHCl_3$	pH 5	56°C
0	0.00	0.00	−4.06	−6.21
0.023	0.00	0.00	−4.06	−6.21
0.32	−0.05	0.00	−3.66	−5.06
3.2	−0.05	0.00	−1.16	−3.96

[a] Values are given as differences from the titer of the control virus; the titer of the control virus was 7.46 $\log_{10}$ PFU/ml.

RESULTS

Direct effects of DCF on virus infectivity and stability. Virus infectivity titers were found to be not significantly different in suspensions incubated with or without DCF at different concentrations (Table 1). The doses used (3.2, 0.32, and 0.023 μM) were about 140, 14, and 1 time, respectively, the IC_{50} for HRV 1B in a plaque reduction test (3). The virus titer of DCF-treated samples was completely restored to the original value after extraction of the drug with chloroform.

Table 1 shows that the infectivity of control virus decreased significantly after exposure to either pH 5 or 56°C. No protective effect was observed with the lowest concentration of DCF tested. In the presence of the compound at 0.32 or 3.2 μM, the drop in infectivity was reduced in a concentration-dependent manner.

Dose-response effect of DCF on yield of HRV 1B. Under one-step growth conditions, a concentration of 3.2 μM DCF reduced the virus yield by about 99% (data not shown). The IC_{50} was about 0.18 μM.

When multiple-cycle virus growth (24 h p.i.) was examined under similar conditions, the virus yield in 3.2 μM DCF-treated cells was still reduced by approximately 90% of that in the control. Therefore, the DCF concentration of 3.2 μM was chosen for further experiments.

Effect of time of addition or removal of DCF on yield of HRV 1B. To prevent virus growth by about 80%, DCF had to be added immediately after adsorption (zero time). When addition was delayed to 30 min after infection, a 55% reduction in infectious yield was observed. Addition of the compound later than 1 h had only a minor inhibitory effect on virus infection (data not shown).

Removal of DCF at 30 min p.i. caused only a 25% reduction in virus yield, while at 1 h the inhibition reached about 60%. The reduction was almost irreversible at 2 h p.i. (data not shown).

Effect of DCF on the kinetics of HRV 1B RNA synthesis. Detectable viral RNA incorporation began at 2 h p.i. and reached a maximum at 6 h (Fig. 1). The addition of DCF at the beginning of infection, i.e., together with the virus inoculum, completely prevented viral RNA synthesis. Addition of DCF when the bulk of viral RNA synthesis began (2 h p.i.) did not alter the kinetics of incorporation of [^{3}H]uridine in HRV-infected cells.

In the absence of actinomycin D, DCF had no effect on RNA synthesis in uninfected cells (data not shown).

Effect of DCF on HRV 1B-induced shutoff of host translation and viral protein synthesis. After infection with HRV 1B, [^{35}S]methionine incorporation into the proteins of HeLa

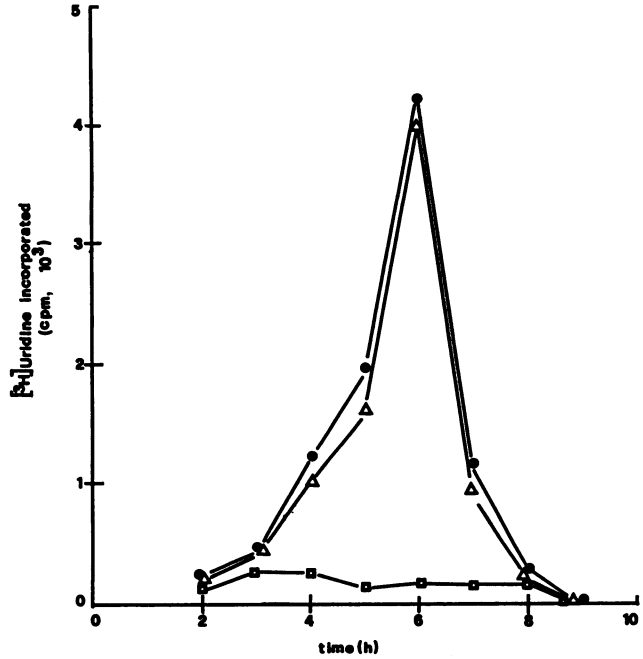

FIG. 1. Effect of DCF on HRV 1B RNA synthesis by determination of incorporation of [^{3}H]uridine into the TCA-insoluble fraction of virus-infected cells (2×10^6 cells) in the presence of actinomycin D. ●, untreated cells; □, cells treated with DCF from the beginning of infection; △, cells treated with DCF from 2 h p.i. Each point represents the average of two separate experiments. Cell controls were included for each reading, and the cell background activity was deducted for each reading.

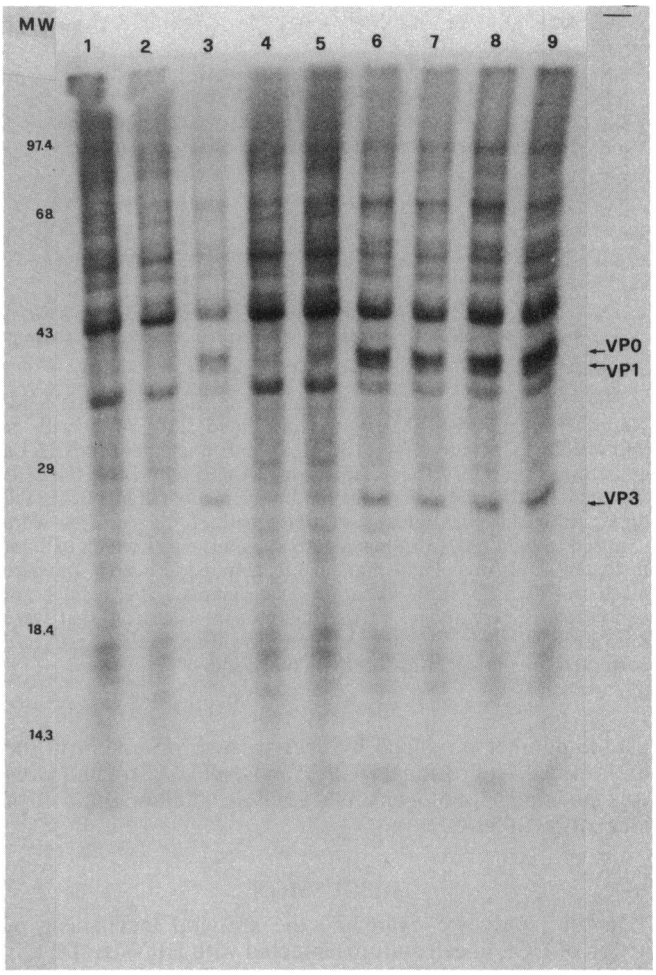

FIG. 2. Analysis by SDS-polyacrylamide gel electrophoresis of the proteins synthesized in HRV 1B-infected HeLa cells. Cells were labeled with 20 μCi of [^{35}S]methionine per ml from 8 to 9 h p.i. and were processed as described in the text. Lanes: 1, uninfected, untreated cells; 2, uninfected, drug-treated cells; 3, infected, untreated cells; 4, infected cells treated with DCF from the beginning of infection; 5, infected cells treated with DCF during virus adsorption only (1 h, 33°C); 6, infected cells treated with DCF after virus adsorption; 7 to 9, infected cells treated with DCF from 1, 2, and 3 h after infection, respectively. Molecular weights are given on the left (in thousands), and viral proteins (VPs) are indicated on the right.

cells decreased significantly (about 75%). The addition of DCF at the beginning of infection prevented almost completely the decrease of cellular protein synthesis. A similar effect was observed when the compound was present only during the viral adsorption period (1 h, 33°C). Addition of the drug 1 h p.i. or later had a negligible effect on shutoff of cellular protein synthesis (data not shown).

HRV 1B protein synthesis in the presence of DCF was analyzed by SDS-polyacrylamide gel electrophoresis. Figure 2 shows that a complete inhibition of viral protein synthesis was observed when the compound was present either throughout the entire incubation time or during the first hour of infection (33°C) only. The appearance of viral proteins was unaffected when DCF was added to cells at different times p.i. Protein synthesis in uninfected cells incubated with DCF for 9 h at 33°C appeared to be normal.

Effect of DCF on adsorption and internalization of HRV 1B. Virus binding to HeLa cells was not modified by the presence of the compound, as measured by two procedures. In measuring the yield of infectious virus after a single growth cycle in cells exposed to the drug only during the attachment step (1 h) at 0°C, no differences were found in the yields between DCF-treated (7.7×10^5 PFU/ml) and control (7.9×10^5 PFU/ml) cells. Also, in counting the amount of radioactive virus bound to cells after 2 h of contact at 0°C in the presence (total TCA-precipitable count [mean ± standard deviation], 981 ± 85) or absence (total TCA-precipitable count, 897 ± 79) of DCF, no differences were observed.

Similarly, the compound did not appear to affect internalization (1 h, 33°C) of [^{3}H]uridine-labeled HRV (total TCA-precipitable counts [mean ± standard deviation], for controls, 299 ± 32; for DCF-treated cells, 310 ± 28).

Effect of DCF on uncoating of HRV 1B. Neutral red-labeled HRV 1B preparations preincubated with or without DCF were tested for their ability to uncoat at different times after being inoculated into the cells. The complete lack of plaque development in cultures exposed to light immediately after adsorption (0°C, 1 h), before uncoating could occur, demonstrated the photosensitivity of the HRV preparation used (Fig. 3). The number of infective virus centers which had become photoresistant in cultures infected with mock-treated virus reached nearly 80% after incubation at 33°C for 1 h. In contrast, in DCF-treated virus-infected cultures exposed to light, the number of light-resistant virus centers increased to only 27% after 1 h. These results were observed, in addition to the very small reduction (<10%) of the

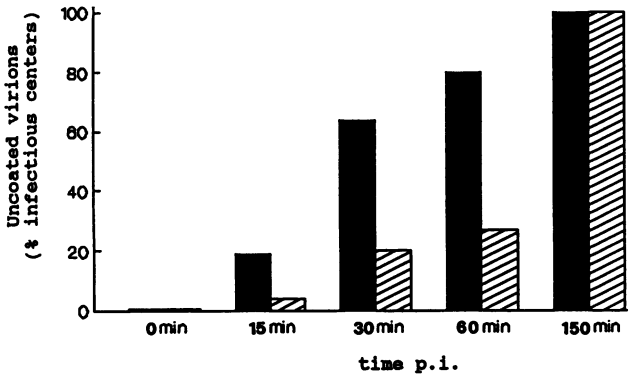

FIG. 3. Effect of DCF on the uncoating kinetics of HRV 1B. Neutral red-labeled HRV 1B was incubated (1 h, 33°C) with or without DCF, serially 10-fold diluted, and inoculated onto HeLa cells for 1 h at 0°C. After washing three times with PBS, the cells were added to drug-free medium and were incubated at 33°C in the dark. After different lengths of time, half of the cultures were exposed to light (300 W), thus inactivating any virus which had not uncoated. The virus plaque number was counted 72 h later. Data are means of two to three separate experiments. The results are expressed as percentages of the inhibitor-free controls that were unexposed to light. Values reported for drug-treated virus are corrected for the effect of residual compound.

plaque number produced by drug-treated virus kept in the dark, because of residual DCF. Almost all DCF-treated virus was no longer photosensitive after an additional 1.5 h of incubation at 33°C.

DISCUSSION

In this study we examined the antiviral mechanism of action of DCF in cell cultures infected with HRV 1B. DCF is the most active anti-HRV 1B compound in a series of cyano- or amidino-substituted flavans, isoflavans, and isoflavenes synthesized by us (3).

Analysis of virus growth in drug-treated cells suggested that DCF acts on the early stages of the HRV 1B replicative cycle. Experiments on viral RNA and protein syntheses confirmed the early effect of the compound, since the presence of DCF from the beginning of infection suppressed synthesis of both viral RNA and viral proteins. However, the lack of inhibition observed when the compound was added after adsorption indicated that DCF has no direct effect on HRV 1B RNA or protein synthesis. DCF completely reversed the virus-induced shutoff of host translation when it was present either during the entire cycle of infection or during the adsorption period only. Inhibition of host cell protein synthesis after picornavirus infection is an early event, and translation of parental viral RNA is a prerequisite for inhibition to occur (11). Therefore, the compound could act on early events that occur prior to translation of the RNA of the infecting virus.

A more detailed investigation carried out during the early steps of infection indicated that prevention of virus binding to the cell membrane is not the basis for HRV inhibition. Furthermore, the amount of internalized [^{3}H]uridine-labeled virions was virtually the same in untreated and drug-treated cultures, indicating that DCF affects viral processes distinct from attachment and penetration into cells. Indeed, the experimental results obtained with neutral red-sensitized HRV 1B treated with DCF indicate that the site of inhibition is located at the uncoating level. It is interesting that the mode of action of capsid-binding compounds can vary with the particular HRV serotype and, possibly, with receptor grouping. There is good evidence that compounds that inhibit the uncoating of minor serotypes, such as HRV 1B, are able to prevent adsorption of major serotypes (15).

It is known that uncoating of picornaviruses requires the participation of cell components (16). The main disadvantage of the experimental conditions that we used to measure the uncoating was that they allowed only the study of the effect of DCF bound to virus. In fact, the continuous presence of the compound until the time of exposure to light entirely blocked plaque formation of both exposed and unexposed cultures (data not shown). Therefore, this experimental design is not suitable for evaluation of the requirement of cellular functions in the HRV uncoating process. This leaves open the possibility that DCF could interact with some cellular targets.

Soon after HRV particles enter the cells, the proteins of the capsid begin to undergo conformational changes (uncoating) similar to those produced by acidification or thermal treatment of virions. Although DCF did not inactivate virus infectivity, it stabilized the virion structural conformation against mild acid or thermal degradation. These data indicate that the compound interacts directly with the virions and that binding is probably important to the inhibitory action, although it was largely reversible by dilution.

DCF, like chalcone (14), BW683C (21), disoxaril (7), and other antiviral agents, probably acts by binding directly with viral capsid proteins, making HRV resistant to uncoating. Unlike DCF, BW683C has been found to be inactive against other viruses (1). The different inductive effect and/or the capacity for bond formation between chlorine and the cyano group could result in a stronger interaction with the amino acid residues present at the same or different hydrophobic sites on the viral capsid proteins and could explain the broader antiviral spectrum of DCF compared with that of BW683C. However, more detailed knowledge of the molecular aspects is necessary to clarify how DCF interacts with the virus.

REFERENCES

1. **Bauer, D. J., J. W. T. Selway, J. F. Batchelor, M. Tisdale, I. C. Caldwell, and D. A. B. Young.** 1981. 4',6-Dichloroflavan (BW683C), a new anti-rhinovirus compound. Nature (London) **292**:369–370.
2. **Burali, C., N. Desideri, M. L. Stein, C. Conti, and N. Orsi.** 1987. Synthesis and anti-rhinovirus activity of halogen-substituted isoflavenes and isoflavans. Eur. J. Med. Chem. **22**:119–123.
3. **Conti, C., N. Desideri, N. Orsi, I. Sestili, and M. L. Stein.** 1990. Synthesis and antirhinovirus activity of cyano and amidino substituted flavanoids. Eur. J. Med. Chem. **25**:725–730.
4. **Conti, C., D. Genovese, R. Santoro, M. L. Stein, N. Orsi, and L. Fiore.** 1990. Activities and mechanisms of action of halogen-substituted flavanoids against poliovirus type 2 infection in vitro. Antimicrob. Agents Chemother. **34**:460–466.
5. **Conti, C., N. Orsi, and M. L. Stein.** 1988. Effect of isoflavans and isoflavenes on rhinovirus 1B and its replication in HeLa cells. Antiviral Res. **10**:117–127.
6. **Dubovi, E. J., J. D. Geratz, S. R. Shaver, and R. R. Tidwell.** 1981. Inhibition of respiratory syncytial virus-host cell interactions by mono- and diamidines. Antimicrob. Agents Chemother. **19**:649–656.
7. **Fox, M. P., M. J. Otto, and M. A. McKinlay.** 1986. Prevention of rhinovirus and poliovirus uncoating by WIN 51711, a new antiviral drug. Antimicrob. Agents Chemother. **30**:110–116.
8. **Genovese, D., C. Conti, L. Fiore, R. Santoro, N. Desideri, M. L. Stein, and N. Orsi.** 1990. Azione di flavanoidi sull'infezione *in vitro* da Enterovirus, p. 61. Proceedings of the XXIII National Congress of the Italian Society of Microbiology, Palermo, Italy,

26 to 30 November 1990.
9. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
10. **Lonberg-Holm, K.** 1975. The effect of concanavalin A on the early events of infection by rhinovirus type 2 and poliovirus type 2. J. Gen. Virol. **28**:313–327.
11. **Lucas-Lenard, J.** 1979. Inhibition of cellular protein synthesis after virus infection, p. 73–100. *In* R. Perez Bercoff (ed.), The molecular biology of picornaviruses. Plenum Publishing Corp., New York.
12. **Mandel, B.** 1967. The relationship between penetration and uncoating of poliovirus in HeLa cells. Virology **31**:702–712.
13. **Markley, L. D., Y. C. Tong, J. K. Dulworth, D. L. Steward, C. T. Goralski, H. Johnston, S. T. Wood, A. P. Vinogradoff, and T. M. Bargar.** 1986. Antipicornavirus activity of substituted phenoxybenzenes and phenoxypyridines. J. Med. Chem. **29**: 427–433.
14. **Ninomiya, Y., C. Ohsawa, M. Aoyama, I. Umeda, Y. Suhara, and H. Ishitsuka.** 1984. Antivirus agent Ro-09-0410 binds to rhinovirus specifically and stabilizes the virus conformation. Virology **134**:269–276.
15. **Pevear, D. C., M. J. Fancher, P. J. Felock, M. G. Rossmann, M. S. Miller, G. Diana, A. M. Treasurywala, M. A. McKinlay, and F. J. Dutko.** 1989. Conformational change in the floor of the human rhinovirus canyon blocks adsorption to HeLa cell receptors. J. Virol. **63**:2002–2007.
16. **Roesing, T. G., P. A. Toselli, and R. L. Crowell.** 1975. Elution and uncoating of coxsackievirus B3 by isolated HeLa cell plasma membranes. J. Virol. **15**:654–667.
17. **Superti, F., L. Seganti, N. Orsi, N. Desideri, M. L. Stein, A. Tinari, M. L. Marziano, and G. Donelli.** 1990. *In vitro* effect of synthetic flavanoids on astrovirus infection. Antiviral Res. **13**:201–208.
18. **Superti, F., L. Seganti, N. Orsi, M. Divizia, R. Gabrieli, A. Paná, and M. L. Stein.** 1989. Effect of isoflavans and isoflavenes on the infection of Frp/3 cells by hepatitis A virus. Antiviral Res. **11**:247–254.
19. **Tidwell, R. R., J. D. Geratz, and E. J. Dubovi.** 1983. Aromatic amidines: comparison of their ability to block respiratory syncytial virus induced cell fusion and to inhibit plasmin, urokinase, thrombin, and trypsin. J. Med. Chem. **26**:294–298.
20. **Tisdale, M., and J. W. T. Selway.** 1983. Inhibition of an early stage of rhinovirus replication by dichloroflavan (BW683C). J. Gen. Virol. **64**:795–803.
21. **Tisdale, M., and J. W. T. Selway.** 1984. Effect of dichloroflavan (BW683C) on the stability and uncoating of rhinovirus type 1B. J. Antimicrob. Chemother. **14**:97–105.