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Effect of chloro-, cyano-, and amidino-substituted flavanoids on enterovirus infection in vitro

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Abstract

Synthetic flavans, isoflavans and isoflavenes substituted with chloro, cyano and amidino groups were tested for their in vitro activity against poliovirus type 2, Coxsackie virus B4, echovirus type 6 and enterovirus 71. Plaque-reduction assays showed that substituted 3-(2*H*)-isoflavenes, carrying a double bond in the oxygenated ring, possess antiviral activity higher than that of the corresponding isoflavans. The most effective compounds were 4′-chloro-6-cyanoflavan and 6-chloro-4′-cyanoflavan. Studies on the mechanism of action of these two compounds suggested an effect on the early stages of viral replication.

Keywords: Enterovirus infection; Flavanoid; RNA synthesis; Protein synthesis

1. Introduction

4′,6-Dichloroflavan (BW863C) (3c) is a highly effective inhibitor of the replication of certain serotypes of rhinovirus (Bauer et al., 1981). Halogenated 3-(2*H*)-isoflavenes and isoflavans were synthesized in order to study their in vitro activity against rhinovirus 1B, in comparison with 4′,6-dichloroflavan (Burali et al., 1987). These compounds, besides

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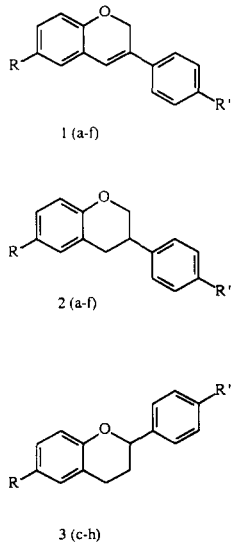

Compound	R	R'
a	H	H
b	Cl	H
c	Cl	Cl
d	CN	H
e	CN	CN
f	$-C(=NH)NH_2 \cdot HCl$	H
g	CN	Cl
h	Cl	CN

Fig. 1. Structural formulae of 3-(2*H*)-isoflavenes (1a–f), isoflavans (2a–f), and flavans (3c–h).

having an effect on rhinoviruses (Burali et al., 1987), exhibited a broader spectrum of antipicornavirus activity than that of 4′,6-dichloroflavan (Bauer et al., 1981; Conti et al., 1988, 1990b; Superti et al., 1989).

The mechanism of action of these substances has been ascribed to an interference with early stages of replication of human rhinovirus 1B (HRV 1B) (Conti et al., 1988), hepatitis A virus (HAV) (Superti et al., 1989) and poliovirus type 2 (Conti et al., 1990b), after viral binding to the cell membrane.

In an attempt to improve the antiviral potency of these compounds, we have synthesized new flavanoids substituted with halo, cyano, and amidino groups (Fig. 1) (Conti et al., 1990a; Desideri et al., 1990). Among these flavanoids, 4′,6-dicyanoflavan (3e) and 4′-chloro,6-cyanoflavan (3g) were more effective against HRV 1B infection than the reference compound BW863C; effective antiviral activity against the same virus was also observed for 6-chloro,4′-cyanoflavan (3h) (Desideri et al., 1990). Investigations carried out to clarify the mechanism of the antiviral activity of 4′,6-dicyanoflavan (3e) suggested that it shares the same behavior of other flavanoids previously described, that is the interference with early events of HRV 1B replication after virus penetration into cells, probably at the uncoating level (Conti et al., 1992).

In this study, the effect of some selected chloro-, cyano- and amidino-isoflavenes, -isoflavans, and -flavans on different enterovirus infections is described. The compounds have been tested against reference strains of poliovirus type 2, Coxsackie virus B4, echovirus type 6, and enterovirus 71. 4′-chloro-6-cyanoflavan (3g) and 6-chloro-4′-cyanoflavan (3h) showed a broad spectrum of activity against all the viruses tested. In this paper, the antiviral activity and the mechanism of action of both compounds are reported.

2. Materials and methods

2.1. Compounds

The substituted 3-(2*H*)-isoflavenes (1a–f), isoflavans (2a–f) and flavans (3d–h) (Fig. 1) were synthesized as previously described (Bauer et al., 1981; Conti et al., 1988; Desideri et al., 1990). Stock solutions of 1 mg/ml in ethanol were stored at 4°C and diluted with tissue culture medium just before use.

WIN 51711 (5-[7-[4-(4,5-dihydro-2-oxazolyl)phenoxy]heptyl]-3-methylisoxazole) was obtained from the Sterling-Winthrop Research Institute (Rensselaer, NY) and was used, as a control, at a final concentration of 1 μg/ml of medium (2.9 μM) (Fox et al., 1986; Zeichardt et al., 1987).

2.2. Cells and viruses

HEp-2 (human epithelioma; originally supplied by B.C. Meyer, Department of Health, Education and Welfare, Rockville, MD, U.S.A.) cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and antibiotics.

Poliovirus type 2 P712 (ATCC VR-1003), Coxsackie virus B4 (ATCC VR-184), echovirus 6 (ATCC VR-1044) and enterovirus type 71 (ATCC VR-784) reference strains were utilized.

Infection of confluent HEp-2 cell monolayers was carried out at a multiplicity of infection (m.o.i.) of 5 plaque-forming units (PFU) per cell from a purified virus stock (free of defective interfering particles). After viral adsorption (1 h at 37°C), MEM with 2% FCS (maintenance medium) was added. Cells were harvested when the viral cytopathic effect (CPE) was complete (about 18–24 h for poliovirus, 36–72 h for the other viruses) and disrupted by 3 cycles of freezing and thawing. The supernatant was centrifuged at 10,000 × *g* for 20 min to remove cell debris. Titration of viral suspensions was performed by plaque assay on cell monolayers in 35-mm Falcon dishes.

2.3. Virus purification

Monolayers of HEp-2 cells in 175-cm^2 plastic flasks were infected at an m.o.i. of 5 PFU/cell and incubated at 37°C. When CPE was complete, cells were disrupted by 3 cycles of freezing and thawing, and the supernatant was centrifuged at 10,000 × *g* for 20 min to remove cell debris. Virus in the supernatant was spun down at 200,000 × *g* for 3 h at 4°C, resuspended in phosphate-buffered saline (PBS) at pH 7.2, and sedimented again through a 15% sucrose cushion in a Beckman SW41 rotor at 200,000 × *g* for 3 h at 4°C. The viral pellet was resuspended in PBS with the help of an all-glass Dounce homogenizer; CsCl was added up to 1.36 g/ml, and the virus suspension was centrifuged in a fixed-angle Beckmann 65 rotor at 170,000 × *g* at 16°C for 48 h. Fractions containing the viral band were dialyzed against PBS.

2.4. Cytotoxicity of drugs

A total of $1-1.5 \times 10^4$ HEp-2 cells were seeded in 96-well plates in the presence of two-fold increasing concentrations of the compounds and incubated for 3 days at 37°C. Cell morphology was checked daily by microscope examination and cell viability was determined after the third day of incubation using the dye exclusion method (trypan blue). The highest concentration of each drug which did not alter cell morphology (such as swelling, granularity, rounding or floating) over a 3-day period is reported in Table 1 (maximum non-toxic concentration). At the same concentrations the viability of cells were unaffected. The effect of the compounds on cell growth was evaluated for a 3-day period; cells from 4 wells were trypsinized and counted and the mean value which affects cell growth was calculated (Table 1).

Table 1
Maximum non-toxic concentrations of isoflavenes (1a–f), isoflavans (2a–f) and flavans (3d–h) on HEp-2 cells and 50% inhibitory concentrations (IC_{50}) of plaque formation of different enteroviruses

Compound	Maximum non-toxic concentrations (μM) on: [a]		Coxsackie B4 IC_{50} (μM) [b]	Echovirus 6 IC_{50} (μM) [b]	Enterovirus 71 IC_{50} (μM) [b]	Poliovirus type 2 IC_{50} (μM) [b]
	Cell morphology	Cell growth				
BW683C	20	10	2.55 ± 0.38	N.A.	> 10 [c]	N.A.
1a	60	30	1.81 ± 0.19	1.73 ± 0.35	1.66 ± 0.26	1.69 ± 0.47
1b	50	30	1.81 ± 0.22	1.94 ± 0.48	2.79 ± 0.49	3.77 ± 0.86
1c	30	20	7.43 ± 0.44	7.43 ± 0.45	8.18 ± 0.71	15.20 ± 1.99
1d	30	20	4.64 ± 0.49	5.00 ± 0.43	6.22 ± 0.78	17.27 ± 1.79
1e	20	10	7.19 ± 0.89	> 10 [c]	> 10 [c]	> 10 [c]
1f	20	10	7.71 ± 0.65	N.A.	N.A	9.57 ± 0.49
2a	60	30	> 30 [c]	10.00 ± 2.00	6.80 ± 0.63	10.00 ± 1.97
2b	50	20	> 20 [c]	8.27 ± 0.70	> 20 [c]	15.00 ± 1.60
2c	40	10	> 10 [c]	7.86 ± 1.32	8.75 ± 1.10	> 10 [c]
2d	40	20	11.15 ± 0.86	16.92 ± 1.26	> 20 [c]	> 20 [c]
2e	20	10	N.A.	N.A.	N.A.	N.A.
2f	40	30	N.A.	N.A.	N.A.	N.A.
3d	40	20	1.80 ± 0.17	1.74 ± 0.21	1.77 ± 0.30	1.62 ± 0.41
3e	40	20	> 20 [c]	> 20 [c]	> 20 [c]	6.45 ± 0.72
3f	40	30	7.19 ± 0.78	N.A.	14.58 ± 1.39	27.44 ± 1.12
3g	40	30	0.32 ± 0.07	0.48 ± 0.07	0.45 ± 0.05	1.23 ± 0.15
3h	40	30	0.45 ± 0.05	1.20 ± 0.04	1.16 ± 0.15	1.28 ± 0.12

[a] HEp-2 cells were grown for 3 days with or without the compounds at different concentrations. Cytotoxicity was scored by microscope examination of morphological alterations.

[b] Different concentrations of the compounds were added to the inoculum and incorporated into the agar overlay medium of infected cells. Viral plaques were counted 48–72 h after infection. The reported values represent the average of two different experiments each one done in duplicate. N.A., not active up to the maximum non-toxic concentration.

[c] IC_{50} value was higher than the maximum non-toxic concentration.

2.5. Plaque-reduction assay

HEp-2 cell monolayers grown in 35-mm dishes were infected with 0.2 ml of virus dilution containing approximately 100 PFU. After 1 h at 37°C, the virus inoculum was removed, and the monolayers were overlaid with 2 ml of MEM containing 2% FCS and 1% agar. Serial dilutions of the test compounds were added to virus inocula and to the overlay medium. After 48 h at 37°C (in a humidified atmosphere of 5% CO_2), the cells were stained with 0.01% neutral red and the plaques were counted.

2.6. Effect on virus yield

Monolayers of HEp-2 cells were infected with virus (10 PFU/cell) in the presence of different concentrations of each compound for 30 min at 37°C; the viral inoculum was washed out and fresh medium with the drugs was added. After 7 h of incubation at 37°C for poliovirus type 2 (P712) and enterovirus 71 and 12 h for echovirus 6 and Coxsackie virus B4, infected cultures were freeze–thawed 3 times. The titer of virus in the clarified supernatants was determined by plaque assay after extraction with chloroform to eliminate the antiviral compound.

2.7. Virucidal test

Viral suspensions (5×10^8 PFU/ml) were incubated with compounds at 20 μM (final concentration) for 1 h at 37°C. Serial 10-fold dilutions of the mixtures were titered by plaque assay.

2.8. Effect of compounds on heat inactivation of virus infectivity

Viral suspensions (5×10^8 PFU/ml) were incubated overnight at 4°C with 3g or 3h (20 μM) or with WIN 51711 (2.9 μM) and then for 30 min at different temperatures. After cooling on ice to stop the reaction, the mixtures were diluted, and titers were determined by plaque assay. WIN 51711 was used as a positive control.

2.9. Determination of viral and cellular protein synthesis

HEp-2 cell monolayers in 35-mm dishes were infected at an m.o.i. of 10 PFU/cell and incubated for 30 min at 37°C. The inoculum was removed and methionine-free medium was added. After 90 min of starvation at 37°C, the medium was replaced with fresh methionine-free medium containing 40 μCi/ml of [^{35}S]methionine (1072 Ci/mmol; Amersham International). After 150 min, the monolayers were washed 3 times with PBS, and the cells were lysed in situ using 0.5 ml of lysis buffer (10 mM Tris hydrochloride (pH 6.8), 2% SDS, 5% β-mercaptoethanol in the presence of 1 μg/ml of pancreatic DNase). Incorporation of [^{35}S]methionine into newly synthesized proteins was determined by precipitation from cell lysates using 10% trichloroacetic acid. The insoluble radioactivity was collected by filtration onto nitrocellulose filters and mea-

sured in a Beckman β-counter. After precipitation of cell lysates (300,000 cpm × sample) with 10 vol. of acetone (20 min at room temperature), proteins were washed twice with acetone, lyophilized, and solubilized in Laemmli buffer. Proteins were then denatured for 3 min at 100°C and immediately placed on ice. Peptides were resolved by electrophoresis in a 15% SDS-polyacrylamide gel in Tris-glycine buffer at 130 V for 16–18 h at 16°C. Standard prestained proteins were used as molecular weight markers. Gels were fixed, dried, and autoradiographed.

2.10. Viral RNA synthesis

HEp-2 cell monolayers in 19-mm dishes were infected at an m.o.i. of 20 PFU/cell with each virus. After the virus adsorption period, the inoculum was removed, the cells washed, and 200 μl of medium supplemented with actinomycin D (2 μg/ml) to inhibit cellular RNA synthesis was added. After 30 min of incubation at 37°C, 2 μCi of [5,6-^{3}H]uridine (27 Ci/mmol; Amersham International) per plate was added and the cells were incubated at 37°C. At different times the medium was removed, and the cells were treated as previously described (Castrillo et al., 1986). Samples of 50 μl were counted in a scintillation spectrometer to measure the incorporation of [5,6-^{3}H]uridine into the TCA-precipitable material.

2.11. RNA transfection

Monolayers of HEp-2 cells in 19-mm plastic dishes were pretreated for 1 h at 37°C with 1.5 mg/ml of DEAE-dextran (MW = 500,000 Da). The cells were transfected with 5 μg per dish of ssRNA, extracted from P712 poliovirus as previously described (Fiore et al., 1987), in the presence (2–20 μM) or absence of drugs for 1 h at 37°C. The inoculum was removed, the cells were washed 3 times with PBS, and maintenance medium was added. After 7 h of incubation at 37°C, the cultures were freeze–thawed, centrifuged at low speed to remove cell debris, and titrated by plaque assay. WIN 51711 was used as a negative control.

3. Results

3.1. Effect of compounds on virus multiplication

The 50% inhibitory concentrations of plaque formation (IC_{50}) by flavanoids have been calculated on a dose–response line by plotting the percent reduction of the number of plaques against the logarithm of the compound concentration.

The reduction of plaque formation was concentration-dependent (data not shown). The IC_{50} of compounds are shown in Table 1. The two chloro-cyanoflavans, 3g and 3h, were the most active substances against all the enteroviruses tested. Among the other flavanoids, 1a, 1b and 3d were also effective, although at higher concentrations. The other drugs showed only a weak effect.

3.2. Effect of compounds 3g and 3h on the yield of virus from a single cycle of replication

To define the mechanism of action of the most active compounds against picornaviruses, we first examined the effect of compounds 3g and 3h on the replication of each virus after a single growth cycle (see Materials and methods). The inhibition of virus growth was concentration-dependent; at 20 μM, both drugs were effective in reducing the virus yield by 50–90% (Fig. 2). The 50% inhibitory concentrations are shown in Table 2.

3.3. Effect of compounds 3g and 3h on the enterovirus-induced shut-off of host translation and viral protein synthesis

The effect of compounds 3g and 3h on the virus-induced shut-off of host translation and on viral protein synthesis was investigated. After infection with each virus, [^{35}S]methionine incorporation into proteins of HEp-2 cells decreased significantly (Fig. 3). The presence of compounds during the entire infectious cycle, or during the first

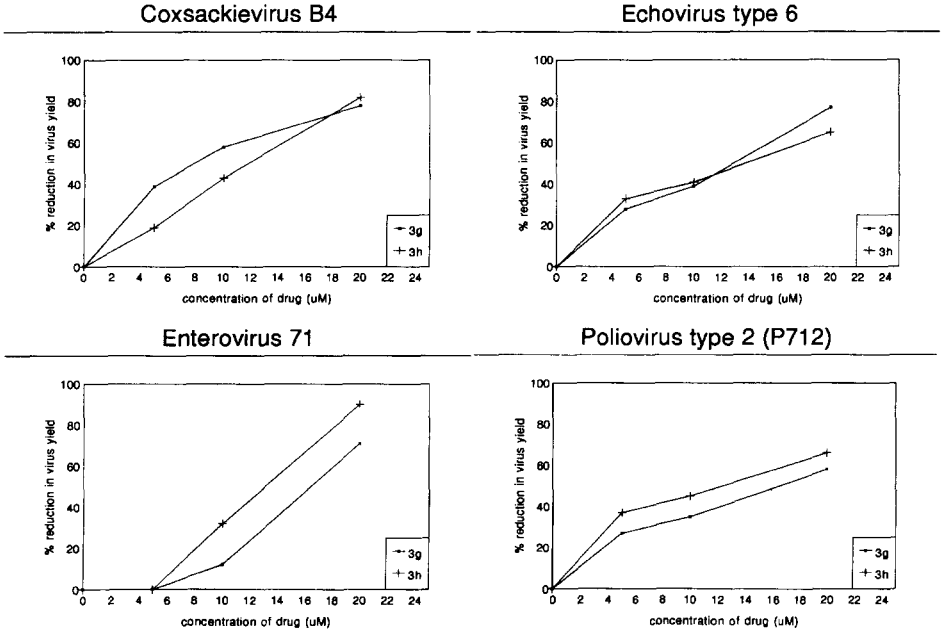

Fig. 2. Effect of compounds 3g and 3h on virus yield from single-cycle replication. Drugs at the indicated concentrations were added at the time of the virus inoculation (m.o.i. of 10 PFU/cell) and maintained throughout the incubation. Infected cultures were freeze–thawed 3 times, extracted with chloroform to remove the drugs and diluted. Titers were determined by plaque assay. The yields of the untreated control viruses after a single-cycle replication are: 8.57×10^6 PFU/ml for Coxsackie virus B4; 1.54×10^7 PFU/ml for echovirus 6; 7.16×10^6 PFU/ml for enterovirus 71; and 1.26×10^9 PFU/ml for poliovirus type 2.

Table 2
The 50% inhibitory concentrations of compounds 3g and 3h on enterovirus yield after a single cycle of virus replication

Virus	Compound	Concentration (μM)
Coxsackie virus	3g	7.9
	3h	11.8
Echovirus type 6	3g	12.7
	3h	13.5
Enterovirus 71	3g	16.2
	3h	12.9
Poliovirus type 2	3g	16.7
	3h	12.5

Drugs at different concentrations were added at the time of virus inoculation and maintained throughout the incubation. Infected cultures were freeze–thawed 3 times, extracted with chloroform to remove the drugs and diluted. Titers were determined by plaque assay.

hour of infection only, did not interfere with the shut-off of protein synthesis in virus-infected cells. Under the same experimental conditions, the overall [^{35}S]protein synthesis in uninfected cells was unaffected by the presence of the compounds. Data from SDS–PAGE electrophoresis (Fig. 4) showed, on the contrary, that both drugs completely suppressed viral protein synthesis when present either during the entire cycle of infection or during the adsorption time only (1 h at 37°C) (data not shown).

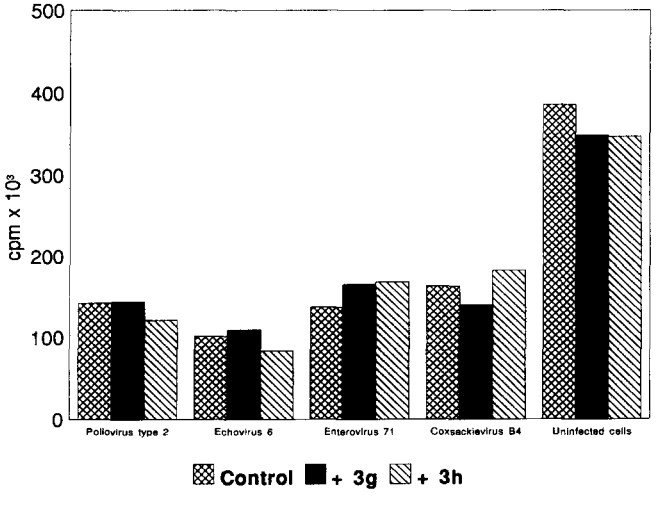

Fig. 3. Effect of compounds 3g and 3h (20 μM) on the shut-off of protein synthesis in virus-infected cells. Cells were labeled with [^{35}S]methionine, and the incorporation of labeled methionine was analyzed as described in Materials and methods at 5 h postinfection. Values represent the averages of 3 separate experiments.

3.4. Effect of compounds 3g and 3h on viral RNA synthesis

To study the effect of compounds 3g and 3h on viral RNA synthesis, the kinetics of [5,6-^{3}H]uridine incorporation was measured in infected and not infected cells; actinomycin D was added to prevent cellular RNA synthesis. When 20 μM of compounds 3g or 3h were added at the beginning of infection, the viral RNA synthesis was reduced by about 50% (Fig. 5). The compounds did not exert any inhibition on RNA synthesis in uninfected cells in the absence of actinomycin D (data not shown).

3.5. Effect of compounds 3g and 3h on viral infectious titer and heat inactivation of virus infectivity

At the concentration inhibiting virus replication (20 μM), both compounds 3g and 3h did not reduce the infectious titer of all the viruses tested after an incubation of 60 min at 37°C (data not shown).

At the same concentration, compounds 3g and 3h did not protect viral infectivity against heat inactivation at the temperatures of 37, 42, and 48°C (Fig. 6). WIN 51711, a well known inhibitor of picornavirus uncoating (Fox et al., 1986; Zeichardt et al., 1987; Rombaut et al., 1991), was used as a positive control. At 48°C, WIN 51711 fully protected (from 1 to 3 log) the infectivity of all the viruses tested. Therefore, our compounds, in contrast to the WIN 51711 known to stabilize picornavirus capsid conformation, did not act on the virus particle itself.

3.6. Effect of compounds 3g and 3h on the infectivity of free viral RNA

Monolayers of HEp-2 cells were treated with DEAE-dextran and then transfected with purified poliovirus ssRNA in the presence or absence of compounds 3g and 3h. At a concentration of 20 μM, both compounds were able to reduce viral replication by about 60% (Table 3), as determined by plaque assay after one cycle of virus multiplication. At a concentration of 2 μM, they were found to be completely ineffective (Table 3). Under the same experimental conditions, the WIN 51711 did not affect the infectivity of the transfected RNA (Table 3).

4. Discussion

In this study we examined the antiviral activity of halogen-, cyano- and amidino-substituted flavans, isoflavans and isoflavenes against different enteroviruses (poliovirus type 2, Coxsackie virus B4, echovirus type 6 and enterovirus 71). The plaque-reduction assays indicate that these compounds are able to inhibit virus multiplication in vitro. These data are consistent with the inhibitory activity previously described against HRV 1B, HAV, poliovirus type 2 and astrovirus replication in vitro (Burali et al., 1987; Conti et al., 1988, 1990a, 1990b, 1992; Superti et al., 1989, 1990; Desideri et al., 1990). The reference compound BW683C was only slightly active against Coxsackie virus B4 and enterovirus 71, confirming its restricted antiviral spectrum (Bauer et al., 1981). 4′,6-Di-

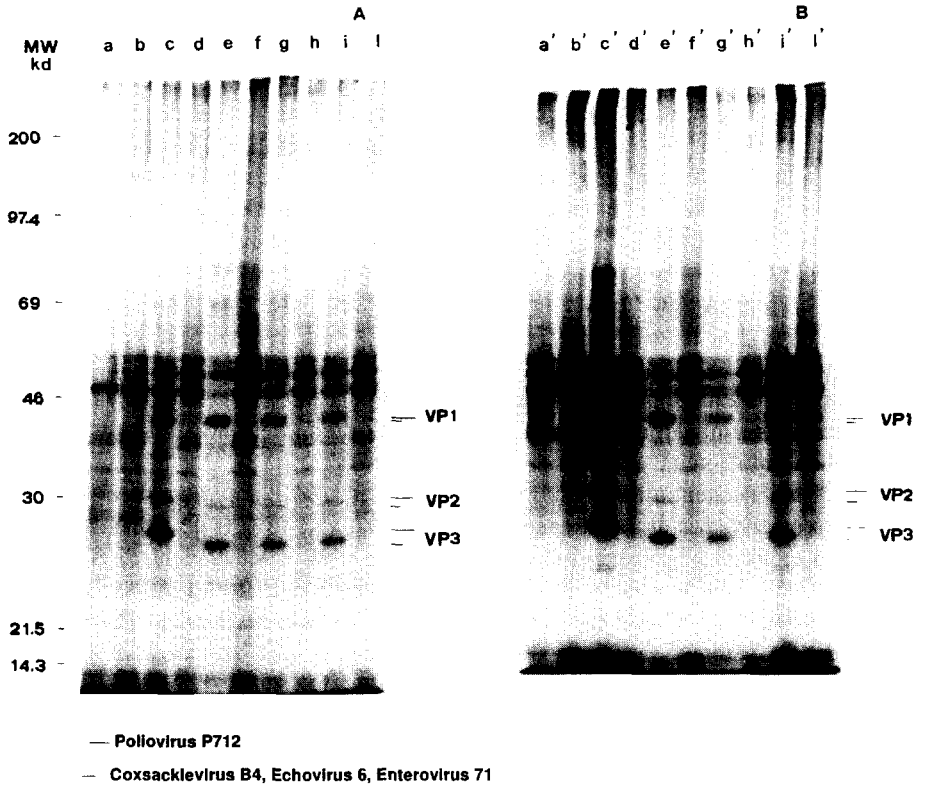

Fig. 4. Analysis by SDS–PAGE of the effect of compounds 3g (A) and 3h (B) on the synthesis of viral proteins. Cells were labeled with [^{35}S]methionine, and the proteins were analyzed as described in Materials and methods at 5 h postinfection. Compounds were present at 20 μM from the beginning of infection. Lanes: a and a', uninfected cells; b and b', uninfected cells treated with the compound; c and c', poliovirus P712-infected cells; d and d', poliovirus P712-infected cells treated with the compound; e and e', echovirus 6-infected cells; f and f', echovirus 6-infected cells treated with the compound; g and g', enterovirus 71-infected cells; h and h', enterovirus 71-infected cells treated with the compound; i and i', Coxsackie virus B4-infected cells; l and l', Coxsackie virus B4-infected cells treated with the compound.

cyanoflavan (3e) behaved in a similar manner, its antiviral activity being confined only against rhinovirus (Conti et al., 1992). The comparison of the data obtained with isoflavenes (1a–f) and the corresponding isoflavans (2a–f) suggests that the anti-enterovirus activity is favored by the planarity of the molecule; in fact, the isoflavenes, with the presence of a double bond in the oxygenated ring, were always more effective. An opposite susceptibility has been reported for HRV 1B (Conti et al., 1988) and HAV infection (Superti et al., 1989). All the amidino flavanoids (1f, 2f and 3f) were only slightly active against enterovirus replication; they were also the least effective compounds regarding HRV 1B infection (Conti et al., 1990a). Among all the drugs tested, 4′-chloro-6-cyanoflavans (3g) and 6-chloro-4′-cyanoflavans (3h) were the most potent inhibitors of enterovirus infection. 3-(2*H*)-isoflavene (1a), 6-chloro-3-(2*H*)-isoflavene

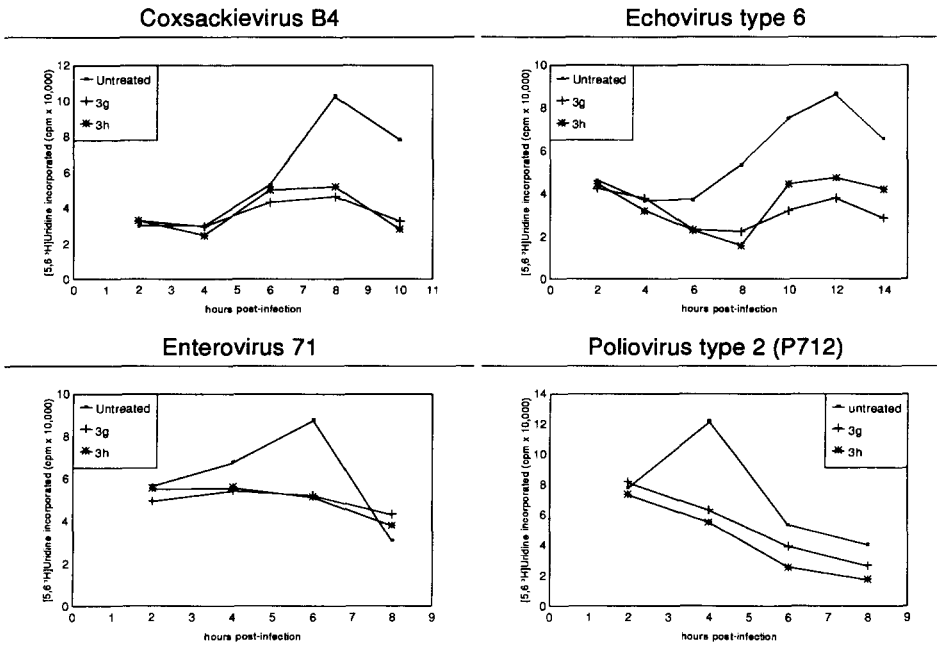

Fig. 5. Effect of 3g and 3h at 20 μM concentration on enterovirus RNA synthesis in HEp-2 cells. Analysis of RNA synthesis was performed as described in Materials and methods. Actinomycin D (2 μg/ml) was added after the viral adsorption period (30 min at 37°C) and maintained until the end of the incubation. Each point represents the average of 3 separate experiments.

(1b) and 6-cyanoflavan (3d) were also effective, although to a minor extent. These findings differ from those reported for HRV infection. The antiviral activity of compounds 3g and 3h was also confirmed by virus yield experiments, under single growth cycle conditions.

At 20 μM concentrations, compounds 3g and 3h did not reduce the viral infectious titer after an incubation of 1 h at 37°C, and did not protect the viral infectivity against heat inactivation at the different temperatures considered. This is in contrast with the results obtained with the capsid binder WIN 51711 which fully protects the virus infectivity from heat inactivation up to 48°C; therefore, both flavanoids may be considered to not act on the virus particle itself.

The mechanism of action of the most active compounds indicate that compounds 3g and 3h did not interfere with the enterovirus induced shut-off of host translation, although they inhibited viral RNA and protein synthesis. The absence of precursors of viral proteins in the electrophoretic pattern of infected cells indicates that both 3g and 3h prevented viral protein synthesis. Kinetic studies on the viral protein synthesis strongly support these data since the compounds were found to exert the same effect only when present during the first hour of infection. These results differ from those previously described for isoflavenes 1a and 1b on poliovirus type 2 infection, where compounds inhibited both poliovirus induced shut-off of host translation and viral protein synthesis

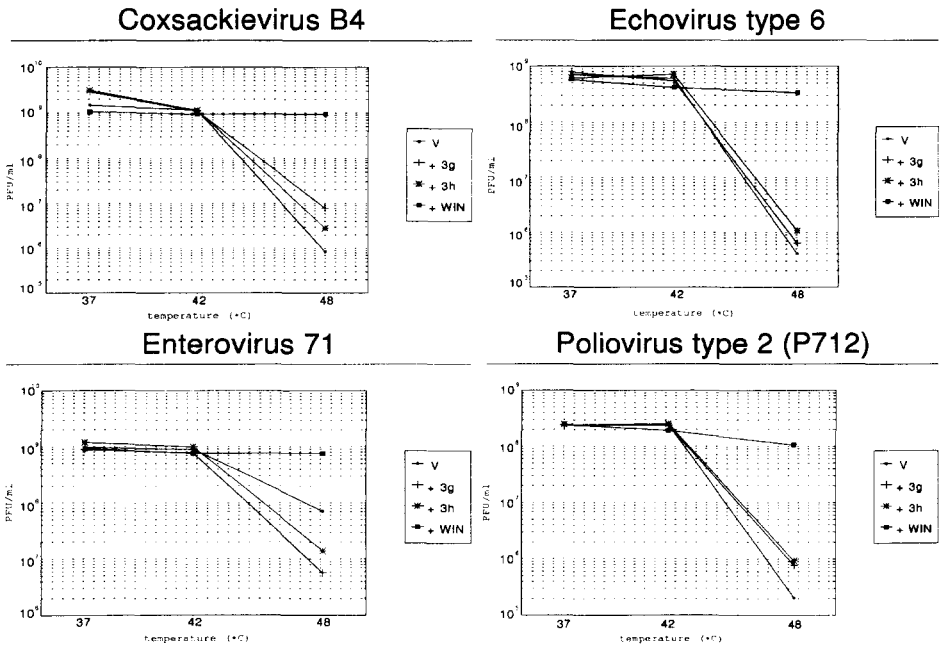

Fig. 6. Effect of 3g, 3h and WIN 51711 on heat inactivation of enterovirus infectivity. Viral suspensions (5×10^8 PFU/ml each) were incubated with the compounds as described in Materials and methods and titers were determined by plaque assay. V, mock-treated virus control; WIN, WIN 51711.

(Conti et al., 1990b). However, when compounds 3g and 3h were tested for their inhibiting effect on viral RNA infectivity (poliovirus type 2 only), they were found to be active, although to a lesser extent than isoflavenes 1a and 1b (Conti et al., 1990b). The different behavior of compounds 3g and 3h with respect to 1a and 1b could be dependent on several factors, such as: (1) different molecular shape and planarity of

Table 3
Effect of flavans 3g and 3h on the infectivity of purified poliovirus RNA

Compound (μM)	RNA infectivity
None	8.30×10^4 (0)
3g (2)	8.22×10^4 (1)
3g (20)	3.51×10^4 (58)
3h (2)	8.26×10^4 (1)
3h (20)	3.23×10^4 (61)
WIN 51711	8.65×10^4 (0)

HEp-2 cells were transfected with purified poliovirus RNA in the presence or absence of compounds 3g or 3h. Virus yield was determined by plaque assay after one cycle of virus replication. Values are the mean of two different experiments. The percent reduction of virus yield by comparison with the control value is reported in parentheses.

flavans in comparison with isoflavenes; and (2) different bulk, lipophilicity, location and electronic effect of the substituents in the rings.

Although we cannot exclude an effect of compounds 3g and 3h on the very early stages of viral infection (attachment, penetration and uncoating), the reduction of the infectivity observed after transfection of free viral RNA and the absence of protection of virus infectivity from heat inactivation, strongly suggest that the compounds could act at some stage between viral uncoating and protein synthesis. Other flavanoids (Ro 09-0179, 3-methylquercetin) have been reported to act on picornavirus infection; they do not interfere with the viral uncoating, but a stage between RNA transcription and RNA replication, which occurs later in time with respect to our compounds, seems to be involved (Castrillo et al., 1986; Ishitsuka et al., 1982).

It is known that the ability of poliovirus to cause a strong shut-off of host protein synthesis facilitates the access of viral RNA to the host translational machinery by preventing competition from host mRNA (Sonenberg, 1988). The shut-off of host protein synthesis is caused by the dissociation of the cellular mRNA from the ribosomes (Penman and Summers, 1965), which consequently can bind to the viral RNA. This process is probably due to the poliovirus-mediated inactivation of some cellular factors involved in the binding of the ribosomes to the cap structure of cellular mRNAs (Bonneau and Sonenberg, 1987; Etchison et al., 1982, 1984). The finding that compounds 3g and 3h, inhibit the viral protein synthesis without impairing the shut-off of host translation suggests that a direct interference with viral translation could be involved.

Further studies are necessary to better define the mechanism of action of these substances. The interesting features of these compounds make them suitable candidates for studies on the replication cycle of enteroviruses.

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References

Bauer, D.J., Selway, J.W.T., Batchelor, J.F., Tisdale, M., Caldwell, I.C. and Young, D.A.B. (1981) 4′,6-Dichloroflavan (BW683C), a new anti-rhinovirus compound. Nature 292, 369–370.

Bonneau, A.M. and Sonenberg, N. (1987) Proteolysis of the p220 component of the cap-binding protein complex is not sufficient for complete inhibition of host cell protein synthesis after poliovirus infection. J. Virol. 61, 986–991.

Burali, C., Desideri, N., Stein, M.L., Conti, C. and Orsi, N. (1987) Synthesis and anti-rhinovirus activity of halogen- substituted isoflavenes and isoflavans. Eur. J. Med. Chem. 22, 119–123.

Castrillo, J.L., Vanden Berghe, D. and Carrasco L. (1986) 3-Methylquercetin is a potent and selective inhibitor of poliovirus RNA synthesis. Virology 152, 219–227.

Conti, C., Orsi, N. and Stein M.L. (1988) Effect of isoflavans and isoflavenes on rhinovirus 1B and its replication in HeLa cells. Antiviral Res. 10, 117–127.

Conti, C., Desideri, N., Orsi, N., Sestili, I. and Stein, M.L. (1990a) Synthesis and anti-rhinovirus activity of cyano and amidino substituted flavanoids. Eur. J. Med. Chem. 25, 725–730.

Conti, C., Genovese, D., Santoro, R., Stein, M.L., Orsi, N. and Fiore, L. (1990b) Activities and mechanisms of action of halogen-substituted flavanoids against poliovirus type 2 infection in vitro. Antimicrob. Agents Chemother. 34, 460–466.

Conti, C., Tomao, P., Genovese, D., Desideri, N., Stein, M.L. and Orsi, N. (1992) Mechanism of action of antirhinovirus flavanoid 4′,6-dicyanoflavan. Antimicrob. Agents Chemother. 36, 95–99.

Desideri, N., Sestili, I., Stein, M.L., Conti, C., Tomao, P. and Orsi, N. (1990) Synthesis and anti-rhinovirus 1B activity of oxazolinylflavans. Antiv. Chem. Chemother. 1, 307–312.

Etchison, D., Milburn, S.C., Edery, I., Sonenberg, N. and Hershey, J.W.B. (1982) Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000 dalton polypeptide associated with eukaryotic initiation factor 3 and a cap binding protein complex. J. Biol. Chem. 257, 14806–14810.

Etchison, D., Hansen, J., Ehrenfeld, E., Edery, I., Sonenberg, N., Milburn, S.C. and Hershey, J.W.B. (1984) Demonstration in vitro that eucaryotic initiation factor 3 is active but that a cap-binding protein complex is inactive in poliovirus infected HeLa cells. J. Virol. 51, 832–837.

Fiore, L., Pierangeli, A., Lombardi, F., Santoro, R., Crainic, R., Venuti, A. and Perez-Bercoff, R. (1987) Antigenic and biochemical characterization of poliovirus type 2 isolated from two cases of paralytic disease. Intervirology 27, 196–204.

Fox, M.P., Otto, M.J. and McKinlay M.A. (1986) Prevention of rhinovirus and poliovirus uncoating by WIN 51711, a new antiviral drug. Antimicrob. Agents Chemother. 30, 110–116.

Ishitsuka, H., Ohsawa, C., Ohiwa, T., Umeda, I. and Suhara, Y. (1982) Antipicornavirus flavone Ro 09-0179. Antimicrob. Agents Chemother. 22, 611–616.

Penman, S. and Summers, D.F. (1965) Effects on host cell metabolism following synchronous infection with poliovirus. Virology 27, 614–620.

Rombaut, B., Andries, K. and Boeyé, A. (1991) A comparison of WIN 51711 and R 78206 as stabilizers of poliovirus virions and procapsids. J. Gen. Virol. 72, 2153–2157.

Sonenberg, N. (1988) Cap-binding proteins of eukariotic messenger RNA: functions in initiation and control of translation. Prog. Nucleic Acid Res. Mol. Biol. 35, 173–207.

Superti, F., Seganti, L., Orsi, N., Divizia, M., Gabrieli, R., Panà, A. and Stein, M.L. (1989) Effect of isoflavans and isoflavenes on the infection of Frp/3 cells by hepatitis A virus. Antiviral Res. 11, 247–254.

Superti, F., Seganti, L., Orsi, N., Desideri, N., Stein, M.L., Tinari, A., Marziano M.L. and Donelli G. (1990) In vitro effect of synthetic flavanoids on astrovirus infection. Antiviral Res. 13, 201–208.

Zeichardt, H., Otto, M.J., McKinlay, M.A., Willingmann, P. and Habermehl, K.O. (1987) Inhibition of poliovirus uncoating by disoxaril (WIN 51711). Virology 160, 281–285.