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Activities and Mechanisms of Action of Halogen-Substituted Flavanoids against Poliovirus Type 2 Infection In Vitro

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The effect of some halogen-substituted flavanoids (dichloroflavan, halogenated isoflavans, and isoflavenes) on poliovirus type 2 infection was examined. Only two isoflavenes exhibited a significant inhibitory activity on the virus-induced cytopathic effect and plaque formation. In a single cycle of viral replication, both compounds reduced the viral yield by approximately 90%. The presence of the isoflavenes from the beginning of infection or during the adsorption period only prevented the shutoff of host translation and viral RNA and protein synthesis, suggesting that the drugs blocked an early step of viral replication. Indeed, both isoflavenes were not virucidal, did not protect virus infectivity from heat inactivation, and had no measurable effect on the binding of virus to cells, viral penetration, and uncoating of the viral RNA. In contrast, both compounds significantly reduced the infectivity of free viral RNA. The possibility that compounds interfere with poliovirus replication at a very early stage of translation of the input RNA is discussed.

Halogenated isoflavans and 3(2*H*)-isoflavenes are two series of analogs related to dichloroflavan (2) which possess in vitro antirhinovirus activity, although lower than that of dichloroflavan (4), and anti-hepatitis A virus activity (13).

Studies of the mechanism of action indicated that these compounds interfere with the early stages of rhinovirus (HRV) (6) and hepatitis A virus (HAV) (13) replication without impairing the viral binding to the cell membrane. Dichloroflavan was also seen to possess a strong inhibitory effect on the early events of HRV (14, 15) and HAV (13) multiplication, whereas it was found to be inactive on poliovirus type 1 infection (2). However, the possible effect on other types of poliovirus has not yet been described.

The aim of the present study was to evaluate the effectiveness of dichloroflavan, halogenated isoflavans, and isoflavenes on poliovirus type 2 infection. The compounds were tested on Sabin and wild type 2 (P712), reference strains, and on three type 2 Sabin-like revertants isolated from paralytic patients. The most active drugs were 3(2*H*)-isoflavene (referred to herein as compound 3a) and 6-chloro-3(2*H*)-isoflavene (compound 3b). The mechanism of action of both analogs was examined in more detail to clarify at which level they interfere with picornavirus replication, and in this paper we report that the compounds were able to inhibit the replication cycle initiated by purified viral RNA. Kinetic studies suggest that they may interfere at a very early stage of translation of the input RNA.

MATERIALS AND METHODS

Compounds. The halogenated isoflavans (compounds 4a through d) and 3(2*H*)-isoflavenes (compounds 3a through g) (Fig. 1A) were synthesized as previously described (4); compounds 3b, 4b (7), and 3c (3) were already synthesized by other methods. Stock solutions in ethanol at 1 mg/ml were stored at 4°C before the dilution with tissue culture medium to run the experiments.

Cells and viruses. HEp-2 (human epithelioma; originally supplied by B. C. Meyer, U.S. Department of Health, Education, and Welfare, Rockville, Md.) cell monolayers were grown in Eagle minimum essential medium (MEM) supplemented with 10% fetal calf serum and antibiotics (growth medium).

Poliovirus type 2, Sabin (Behringwerke), and P712 wild-type (ATCC VR-1003) reference strains and three Sabin-like revertants isolated from two patients with paralytic poliomyelitis (one pharyngeal swab [Ia] and two rectal swabs [Ib and II]) (8) were utilized for this study.

Infection of confluent HEp-2 cell monolayers was carried out at a multiplicity of infection (MOI) of 5 PFU per cell. After viral adsorption (30 min at 37°C), MEM with 2% fetal calf serum (maintenance medium) was added. Cells were harvested when the viral cytopathic effect (CPE) was complete (about 18 to 24 h). Titration of viral suspension was performed by plaque assay in monolayers in 35-mm Falcon dishes.

Virus purification and RNA extraction. Monolayers of HEp-2 cells in 175-cm^2 plastic flasks were infected with poliovirus at an MOI of 5 PFU per cell and incubated at 37°C. When CPE was complete (usually within 24 h), cells were disrupted by three cycles of freezing and thawing, and the supernatant was centrifuged at 10,000 × *g* for 20 min to remove the cell debris. Virus in the supernatant was spun down at 200,000 × *g* for 3 h at 4°C, suspended 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 suspended in PBS with an all-glass Dounce homogenizer; CsCl was added up to 1.36 g/ml, and the virus was centrifuged to equilibrium (fixed-angle Beckmann 65 rotor; 170,000 × *g* at 12°C for 48 h). Fractions containing the viral band were dialyzed against PBS. Deproteinization was carried out in 20 mM Tris hydrochloride buffer (pH 7.5) containing 0.1% sodium dodecyl sulfate (SDS) with an equal volume of phenol-chloroform-isoamyl alcohol (50:48:2, vol/vol/vol). Viral RNA was ethanol precipitated overnight at −20°C in the presence of 0.2 M sodium acetate and pelleted by centrifugation (12,000 × *g* at 4°C for 1 h).

Cytotoxicity of compounds. An average of 2 × 10^4 HEp-2

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cells in growth medium were seeded in 96-well plates in the presence of increasing concentrations of the compounds and incubated for 3 days at 37°C. Treated cells were inspected daily to detect changes in cell morphology. Cells of five wells were trypsinized and counted, and the mean value was calculated.

Effect of compounds on the viral CPE. HEp-2 cells (1.5×10^5) in maintenance medium were added to each strain of poliovirus to obtain an MOI of 5 PFU per cell and seeded in 96-well plates containing varied concentrations of each compound. The appearance of viral CPE in drug-treated and untreated cells after a period of 24 to 72 h at 37°C was monitored under a phase-contrast microscope.

Plaque reduction assay. HEp-2 cell monolayers grown on 35-mm dishes were infected with 0.2 ml of virus dilution containing approximately 100 PFU. After adsorption (1 h at 37°C), the excess virus was removed, and the monolayers were overlaid with 2 ml of MEM containing 2% fetal calf serum, 1% agar, and the test compounds. The incubation was performed at 37°C in a humidified atmosphere of 5% CO_2 in air. After 48 h, the cells were stained with 0.01% neutral red, and the plaques were counted.

Effect on virus yield. Monolayers of HEp-2 cells were infected with poliovirus type 2 (P712) (10 PFU per cell) in the presence of each compound for 30 min at 37°C. After the viral inoculum was washed out, fresh medium with the drugs was added. After 7 h of incubation at 37°C, infected cultures were freeze-thawed three times, and the titer of virus in the clarified supernatants was determined by plaque assay after extraction with chloroform to eliminate the antiviral compound.

Determination of viral and cellular protein synthesis. HEp-2 cell monolayers in 35-mm dishes were infected at an MOI of 10 PFU per cell with poliovirus type 2 (P712) and incubated for 30 min at 37°C. The inoculum was removed, and methionine-free medium was added. After 90 min of incubation at 37°C, the medium was replaced with fresh methionine-free medium containing 40 μCi/ml of [^{35}S]methionine (1,072 Ci/mmol; Amersham International). At the times indicated in the figure legends, monolayers were washed three times with PBS, and the cells were lysed with 0.5 ml of lysis buffer (10 mM Tris hydrochloride [pH 6.8], 2% SDS, 5% beta-mercaptoethanol) in the presence of pancreatic DNase (1 μg/ml). Samples of the lysates were adsorbed on 3 MM Whatman filter paper and precipitated (15 min at 0°C) in 10% trichloroacetic acid (TCA). The filter was then boiled for 30 min in 5% TCA, washed with 5% TCA, ethanol, and acetone, and air dried. The radioactivity was measured by scintillation counting in a Beckman beta counter.

After precipitation of cell lysates with 10 volumes of acetone (20 min, room temperature), proteins were washed twice with acetone, lyophilized, and solubilized in 50 mM Tris hydrochloride (pH 6.8)–8.5% beta-mercaptoethanol–3.5% SDS–8 M urea–0.025% bromophenol blue. Proteins were then denatured for 3 min at 100°C and immediately placed on ice. Peptides were resolved by SDS-polyacrylamide gel electrophoresis in 15% acrylamide. Electrophoresis was performed in Tris-glycine buffer at 130 V for 16 to 18 h at 16°C. Prestained protein standards were used for determination of molecular weights. The gels were fixed with methanol-acetic acid, dried, and autoradiographed.

Viral RNA synthesis. HEp-2 cell monolayers in 35-mm dishes were infected at an MOI of 25 PFU per cell with poliovirus type 2 (P712). After the adsorption period (30 min at 37°C), the inoculum was removed, and 500 μl of maintenance medium was added. At the same time, actinomycin D (2 μg/ml) was added to inhibit cellular RNA synthesis. At different times after infection, 5 μCi of [5,6-^{3}H]uridine (27 Ci/mmol; Amersham International) per plate was added, and the cells were incubated for 1 h at 37°C. The medium was then removed, and the cells were treated as described by Castrillo et al. (5). Samples of 50 μl were counted in a scintillation spectrometer to measure the incorporation of uridine into TCA-precipitable material.

Radiolabeling of virions. Confluent HEp-2 cell monolayers in 175-cm^2 plastic flasks were infected with poliovirus type 2 (P712) (10 PFU per cell) in the presence of 2 μg of actinomycin D per ml. After adsorption (1 h, 37°C), the viral inoculum was removed, and fresh medium containing 2 μg of actinomycin D per ml and 25 μCi of [5,6-^{3}H]uridine per ml was added. When the CPE appeared (about 18 h postinfection), cells were disrupted by three cycles of freezing and thawing, and the medium was clarified by low-speed centrifugation. The supernatant was then spun down at 200,000 × *g* for 3 h at 4°C in an SW35 rotor (Beckman Instruments). The viral pellet was suspended in 10 mM Tris hydrochloride (pH 7.5) and homogenized with a Dounce homogenizer.

Virus attachment. To measure virus binding, HEp-2 cell monolayers were incubated for 1 h at 4°C with 0.2 ml of [5,6-^{3}H]uridine-labeled P712 poliovirus (6,000 cpm per dish) in the presence or absence of test compounds at 20 μM. Cells were then washed three times with cold PBS and lysed with lysis buffer. After precipitation with cold TCA, the total radioactivity associated with cells was measured.

Viral penetration and uncoating. HEp-2 cell monolayers in 35-mm petri dishes were infected (1 h, 37°C) with 6,000 cpm of [5,6-^{3}H]uridine-labeled poliovirus (P712) per dish in the presence or absence of compounds (20 μM). Cells were then washed three times with PBS and treated with 0.4 ml of proteinase K (250 μg/ml) for 10 min at 4°C to remove noninternalized virions (1). To determine the amount of radioactivity inside the cells, monolayers were collected by scraping and washed three times with PBS. After three cycles of freezing and thawing, portions of each sample were precipitated in 10% TCA and counted.

The same samples of cell lysate were used to do uncoating experiments; samples were treated with 25 μg of RNase A (Sigma Chemical Co.) per ml for 30 min at 37°C and subsequently precipitated in 10% TCA to measure RNase-resistant radioactivity.

Virucidal test. Poliovirus type 2 (P712) (5×10^8 PFU/ml) was preincubated with the compounds at 20 μM (final concentration) for 1 h at 37°C. After 10-fold serial dilutions, the titers of the mixtures were determined by plaque assay.

Effect of compounds on heat inactivation of virus infectivity. Poliovirus type 2 (P712) (5×10^8 PFU/ml) was incubated for 1 h at 37°C with the compounds (20 μM) and then for 20 min at 56°C. After cooling on ice to stop the reaction, the mixtures were diluted, and titers were determined by plaque assay.

Infectious RNA assay. Monolayers of HEp-2 cells in 19-mm plastic dishes were pretreated for 1 h at 37°C with 1.5 mg of DEAE-dextran (molecular weight, 500,000) per ml in PBS to which calcium and magnesium had been added. The cells were infected with RNA extracted from P712 poliovirus (5 μg per dish) in the presence or absence of drugs for 1 h at 37°C. The inoculum was removed, the cells were washed three times with PBS, and maintenance medium was added. After 7 h of incubation at 37°C, the cultures were freeze-thawed three times and centrifuged at low speed to remove cell debris, and titers were determined by plaque assay in HEp-2 cells.

TABLE 1. Maximum nontoxic concentration and action of dichloroflavan (DCF), 3(2*H*)-isoflavenes (compounds 3a through g), and isoflavans (compounds 4a through d) on poliovirus type 2 plaque formation in HEp-2 cells

Compound	Maximum nontoxic concn (μM)[a] affecting:		% Plaque reduction[b]				
	Cell morphology	Cell growth	Sabin	Wild type	Ia	Ib	II
DCF	20	10	5	5	31	10	15
3a	60	30	100	100	100	42	93
3b	50	30	100	95	100	85	96
3c	40	20	20	0	70	15	20
3d	30	20	48	42	55	5	10
3e	40	10	23	0	10	0	0
3f	40	10	50	40	47	0	10
3g	20	20	5	26	48	29	4
4a	60	30	47	45	70	15	36
4b	50	20	44	30	63	19	43
4c	40	10	0	0	20	0	0
4d	40	20	20	0	0	0	8

[a] HEp-2 cells (2×10^4) were grown for 3 days in the presence or absence of compounds at different concentrations. Cytotoxicity was scored by microscope examination of morphological alterations and by counting the cell number in drug-treated and untreated wells.

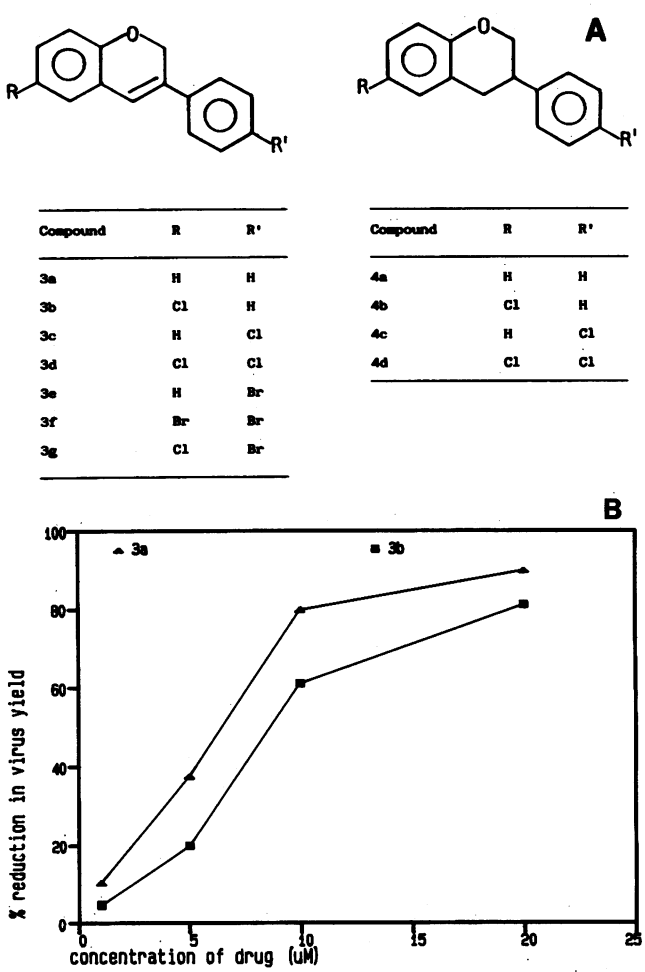
[b] Compounds at 10 μM were incorporated into the agar overlay medium of infected cells. Viral plaques were counted 36 to 48 h after infection. Approximately 100 plaques were counted in untreated infected control cultures. The values reported represent the mean of percentages of plaque reduction obtained in three different experiments, using at least three wells for each drug.

RESULTS

Cytotoxicity and effect of compounds on viral plaque formation and CPE. The cytotoxicity of dichloroflavan and of the halogenated isoflavans (compounds 4a through d) and isoflavenes (compounds 3a through f) for HEp-2 cells was determined by measuring the effects produced on cell morphology and cell growth. The highest amounts of each drug that did not cause any alteration of cell morphology, such as swelling, granularity, rounding, or floating, over a 3-day period are reported in Table 1. At the indicated concentrations the viability of cells, as monitored by neutral red uptake, was unaffected. The maximum concentrations of each substance which did not affect the cell growth rate over a 3-day exposure period were 2 to 4 times lower than those previously noticed by morphological examination of cells (Table 1).

The ability of dichloroflavan, halogenated isoflavans, and isoflavenes to inhibit multiple cycles of virus replication was studied by measuring the effects produced on the formation of plaques and on the development of the viral CPE in HEp-2 cells.

To investigate the influence on virus plaque formation, the drugs at 10 μM were incorporated into the agar overlay of infected cultures. Compounds 3a and 3b were the most active compounds, reducing the plaque formation by all poliovirus strains tested (Table 1). The inhibition by both compounds was dose dependent. The 50% inhibitory concentrations varied from 2 to 3.6 μM, as determined by plotting the percentage of plaque number reduction obtained in drug-treated infected cells versus compound concentrations. Among the other drugs, dichloroflavan was one of the least active compounds, showing only a weak effect (31% plaque reduction) against one of the three Sabin-like strains tested.



Compound	R	R'
3a	H	H
3b	Cl	H
3c	H	Cl
3d	Cl	Cl
3e	H	Br
3f	Br	Br
3g	Cl	Br

Compound	R	R'
4a	H	H
4b	Cl	H
4c	H	Cl
4d	Cl	Cl

FIG. 1. (A) Structural formulae of 3(2*H*)-isoflavenes (compounds 3a through g) and isoflavans (compounds 4a through d). (B) Effect of compounds 3a and 3b on wild-type poliovirus yield from single-round replication. Drugs at the indicated concentrations were added at the time of the virus inoculation and maintained throughout the incubation (8 h at 37°C). Infected cultures were freeze-thawed thrice, extracted with chloroform to remove the drugs, and diluted, and titers were determined by plaque assay.

At the same 10 μM concentration, all isoflavans, dichloroflavan, and most isoflavenes did not interfere with the appearance of viral CPE. Instead, only compounds 3a and 3b were effective against all viral strains tested (data not shown).

Effect of compounds 3a and 3b on the yield of virus from a single round of replication. The effect of compounds 3a and 3b on the replication of the wild-type poliovirus type 2 strain was determined under conditions of a single growth cycle (see Materials and Methods). The inhibition was dose dependent; at 20 μM both drugs were effective in reducing the virus yield by about 90% (Fig. 1B). The 50% inhibitory concentrations were 6.4 and 8.3 μM for compounds 3a and 3b, respectively.

Effect of compounds 3a and 3b on poliovirus-induced shutoff of host translation and viral protein synthesis. To clarify the mechanism of virus growth inhibition, we analyzed the effect of compounds 3a and 3b on the virus-induced shutoff of host translation and on viral protein synthesis in HEp-2 cell cultures. At 5 h after infection with wild-type poliovirus, [^{35}S]methionine incorporation into proteins of HEp-2 cells

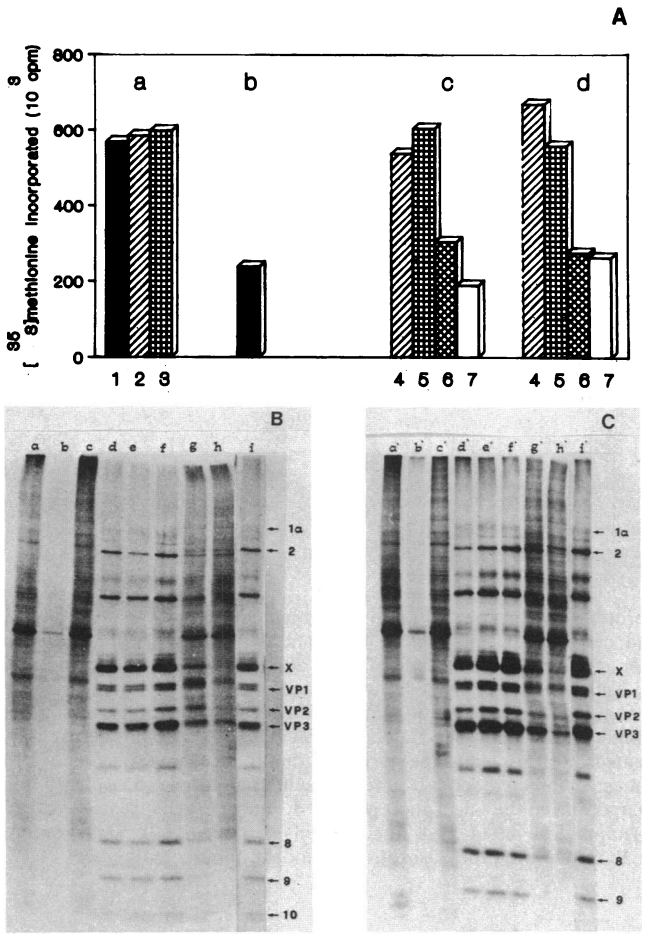


FIG. 2. Effect of compounds 3a and 3b on the shutoff of protein synthesis in P712 (wild-type strain)-infected cells (A) and analysis by SDS-polyacrylamide gel electrophoresis of the dose increment of compound 3a (B) or 3b (C) on the synthesis of viral proteins. (A) Values represent the averages of three separate experiments. Compounds were present at 20 μM. (a) Uninfected cells: 1, untreated; 2, treated with 3a; 3, treated with 3b. (b) Infected cells, untreated. (c and d) 3a and 3b, respectively, were present as follows: 4, during viral adsorption only (30 min at 37°C); 5, during viral adsorption and in the following incubation time (5 h at 37°C); 6, only in the incubation time after adsorption (5 h at 37°C); 7, from 30 min after adsorption until 5 h. (B and C) Cells were labeled with [^{35}S] methionine, and the proteins were analyzed as described in Materials and Methods at 5 h postinfection. Lanes: a and a', uninfected cells; b and b', uninfected cells treated with compound at 10 μM; c and c', uninfected cells treated with compound at 20 μM; d and d', infected cells treated with compound at 0.1 μM; e and e', infected cells treated with compound at 0.5 μM; f and f', infected cells treated with compound at 2 μM; g and g', infected cells treated with compound at 10 μM; h and h', infected cells treated with compound at 20 μM; i and i', untreated infected cells.

decreased significantly (by 60%) (Fig. 2A). The addition of compounds 3a and 3b at 20 μM from the beginning of infection prevented the shutoff of cellular protein synthesis. The same inhibitory effect was observed when compounds were present only during the viral adsorption period (30 min, 37°C). The addition of the inhibitors 30 min after adsorption or later had a negligible effect on the shutoff.

Poliovirus protein synthesis in the presence of different concentrations of compounds 3a and 3b was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2B and C). The labeling of the viral proteins was normal when drugs were present up to 2 μM (Fig. 2, lanes d, d', e, e', f, and f'), whereas it was partially reduced at 10 μM (lanes g and g'). At higher doses of both substances (20 μM), neither viral synthesis nor precursor accumulation of the same viral proteins was discerned (lanes h and h'). Under the same conditions, none of the reagents appeared to impair the cellular protein synthesis (lanes b and b' and c and c') in uninfected cells. The 20 μM concentration was then used in further experiments.

Complete inhibition of viral protein synthesis was observed when compounds 3a and 3b were added at the time of virus inoculation and maintained throughout the entire incubation period (6 h, 37°C) (Fig. 3A and B, lanes d and d'). The inhibitory effect was completely lost when compounds were added immediately after virus adsorption (0 time) or later (lanes e, e', f, f', g, g', h, h', i, and i'). The presence of the compounds during the viral adsorption period only (30 min, 37°C) was also sufficient to entirely suppress viral protein synthesis (lanes c and c'), indicating that their action was effected in the early stage(s) of infection.

Poliovirus protein synthesis was also fully prevented when cell cultures were pretreated with compounds (20 μM, 30 min, 37°C) before infection (Fig. 3C).

Effect of compounds 3a and 3b on poliovirus RNA synthesis. To study the effect of compounds 3a and 3b on viral RNA synthesis, the kinetics of [5,6-^{3}H]uridine incorporation in the presence of actinomycin D to prevent cellular RNA synthesis was measured in infected and mock-infected cells. When 20 μM compound 3a or 3b was added at the beginning of infection, the viral RNA synthesis was significantly reduced (by 50 to 60% at the third hour after infection) (Fig. 4A and B). The inhibitory action is considered to be dose dependent, since no effect was observed at the 2 μM concentration (Fig. 4A and B). When the compounds were added after virus adsorption or later, no inhibitory effect was observed (Fig. 4C). Neither compound exerted any inhibition on RNA synthesis in uninfected cells in the absence of actinomycin D (data not shown).

Effect of compounds 3a and 3b on the early events of poliovirus infection. The experiments so far reported tended to indicate that compounds 3a and 3b interfered at a very early stage of the poliovirus infectious cycle. Then we analyzed the capacity of both drugs to interfere with viral attachment, penetration, and uncoating. [^{3}H]uridine-labeled poliovirus adsorbed to the same extent either in the presence or in the absence of drugs (Table 2), thus indicating that these compounds had no detectable inhibitory effect on adsorption.

The action of both isoflavenes on the penetration of [^{3}H]uridine-labeled poliovirus into HEp-2 cell cytoplasm was studied by measuring the TCA-precipitable labeled material in cell lysates. To remove noninternalized virus, infected cultures were treated with proteinase K before lysis. None of compounds significantly interfered with viral internalization (Table 2).

The effect of drugs on viral uncoating was measured as the remaining TCA-precipitable materials after treatment of cell lysates with RNase to digest free viral RNA. Neither compound had an effect on the viral uncoating (Table 2).

Effect of compounds 3a and 3b on poliovirus infectious titer and heat inactivation of virus infectivity. At the inhibitory concentration in cell cultures (20 μM), compounds 3a and 3b neither reduced the poliovirus infectious titer, even after a 60-min incubation at 37°C, nor stabilized the virus infectivity against heat inactivation at 56°C for 20 min (data not shown).

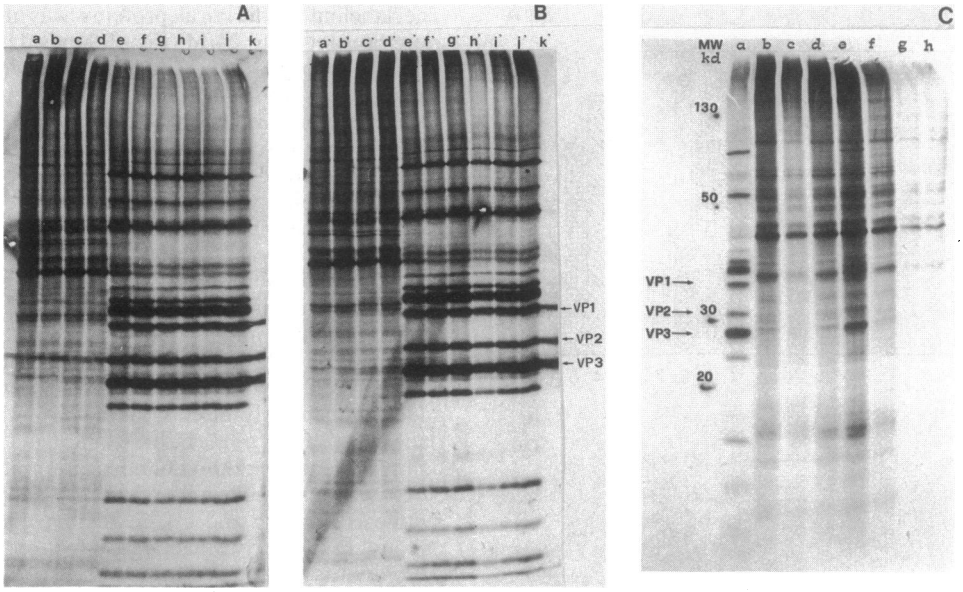


FIG. 3. Analysis by SDS-polyacrylamide gel electrophoresis of the proteins synthesized in poliovirus-infected cells added with 20 μM compound 3a (A) or 3b (B) at different times postinfection (A, B) and before virus infection (C). (A and B) Cells were labeled with [^{35}S]methionine, and the proteins were analyzed as described in Materials and Methods. The time of virus addition was considered as −30 min, and 0 time was taken as when the virus was removed. Lanes a and a', Uninfected cells; b and b', uninfected cells treated with compound; c and c', compound present during the adsorption period only; d and d', compound added at −30 min; e and e', compound added at 0 time; f and f', compound added at +30 min; g and g', compound added at +60 min; h and h', compound added at +90 min; i and i', compound added at +120 min; j and j', P712 poliovirus-infected cells; k and k', viral proteins from P712 wild-type purified virus. (C) Cells were labeled with [^{35}S]methionine, and the proteins were analyzed at 5 h postinfection. Lanes: a, infected cells; b, infected cells treated with 3a only 30 min before infection; c, infected cells treated with 3a 30 min before infection and during the adsorption period; d, infected cells treated with 3a only during the adsorption period; e, infected cells treated with 3b only 30 min before infection; f, infected cells treated with 3b 30 min before infection and during the adsorption period; g, infected cells treated with 3b only during the adsorption period; h, uninfected cells.

Effect of compounds 3a and 3b on free viral RNA. Since compounds 3a and 3b did not appear to act on the uncoating of the viral genome, it was of interest to determine whether the compounds would affect the infectivity of free viral RNA under similar conditions.

Monolayers of HEp-2 cells in 35-mm plastic dishes were treated with DEAE-dextran and infected with phenol-extracted poliovirus RNA in the presence or absence of different concentrations of compounds 3a and 3b; the viral yield was measured as described in Materials and Methods. At a 20 μM concentration, compounds 3a and 3b significantly reduced the viral replication (by about 80%), whereas no inhibition was observed at a 2 μM concentration for either drug (Table 2).

DISCUSSION

In previous reports (4, 13), we demonstrated that the halogenated isoflavans and 3(2*H*)-isoflavenes under study exhibited a significant inhibitory effect against rhinovirus 1B and hepatitis A virus infection in cell cultures. Results presented here indicate that the activity of some of these drugs extends also to poliovirus type 2 infection in vitro, although at higher doses the drugs inhibit both plaque formation and viral CPE. The related compound dichloroflavan was inactive on poliovirus type 2 replication (with the exception of one of the retromutant strains, which exhibited marginal susceptibility). This is in agreement with data found for poliovirus type 1 (2). Thus, the studied compounds exhibit an expanded antiviral spectrum with respect to dichloroflavan.

TABLE 2. Effect of compounds 3a and 3b on [^{3}H]uridine-labeled poliovirus attachment, penetration, and uncoating and effect of different concentrations of both compounds on the infectivity of purified poliovirus RNA

Compound (μM)	Attachment[a] (cpm)	Penetration[b] (cpm)	Uncoating[c] (cpm)	Infectivity of purified RNA[d] (virus yield)
None	1,428	1,187	708	2.80×10^5 (0)
3a (2)	ND[e]	ND	ND	2.68×10^5 (4)
3a (20)	1,504	1,318	722	5.20×10^3 (98)
3b (2)	ND	ND	ND	2.75×10^5 (2)
3b (20)	1,328	1,164	664	7.90×10^4 (78)

[a] Total TCA-precipitable material of lysates prepared from cells infected with 6,000 cpm of [^{3}H]uridine-labeled poliovirus for 1 h at 4°C.

[b] Total TCA-precipitable material of lysates obtained from cells infected for 1 h at 37°C with [^{3}H]uridine-labeled poliovirus (6,000 cpm) and treated with proteinase K (10 min, 4°C) before lysis.

[c] RNase-resistant TCA-precipitable material of cells lysates prepared as described in footnote *b*. Values represent the averages of the counts of at least three dishes for each sample.

[d] HEp-2 cells were pretreated with DEAE-dextran before infection with purified poliovirus RNA in the presence or absence of compound 3a or 3b. After cells were washed three times with PBS, maintenance medium was added, and the infected cultures were incubated for 7 h at 37°C. Virus yield was determined by plaque assay. Values are the mean of two different experiments. In parenthesis is reported the percent reduction of virus yield by comparison with the control value.

[e] ND, Not done.

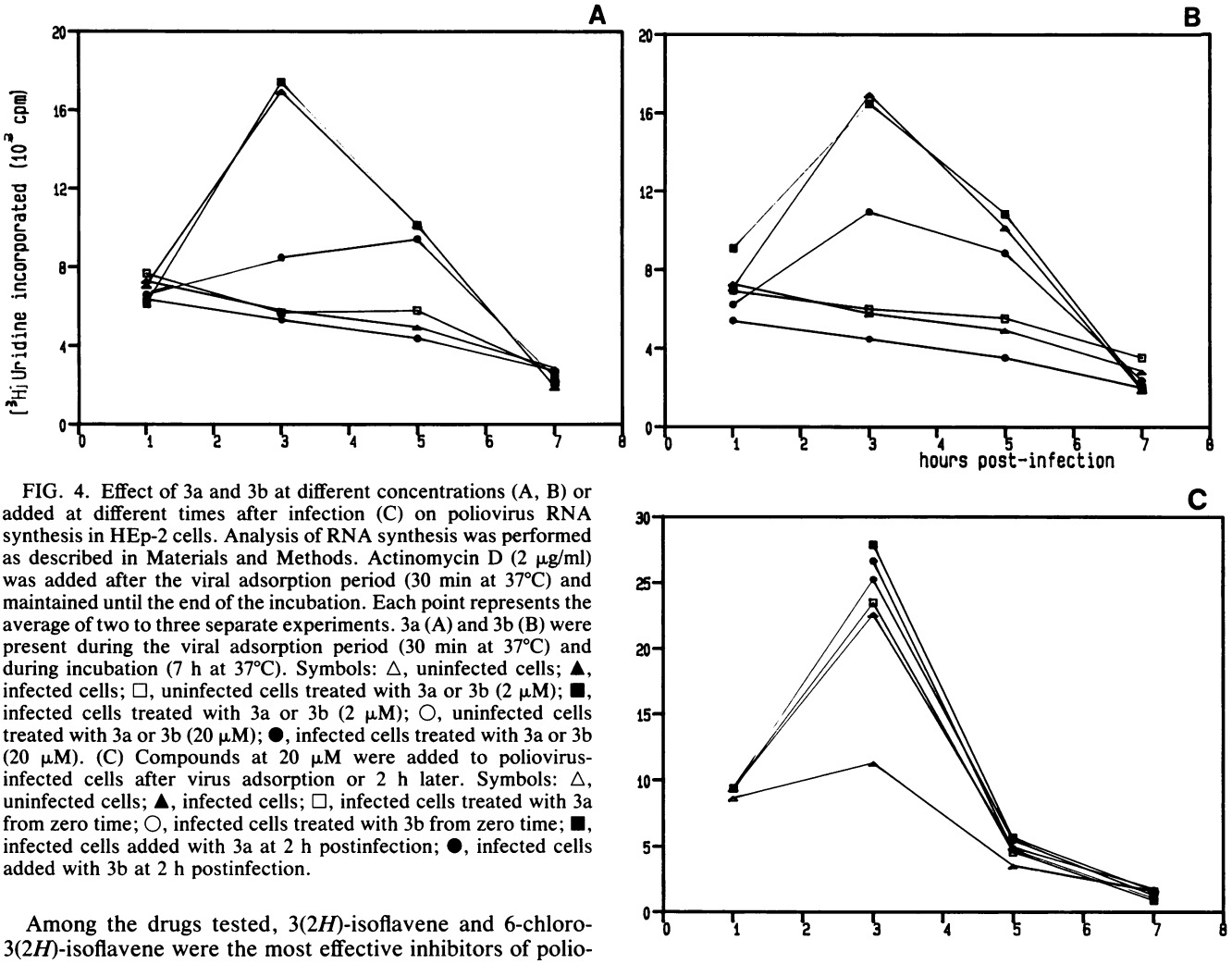


FIG. 4. Effect of 3a and 3b at different concentrations (A, B) or added at different times after infection (C) on poliovirus RNA synthesis in HEp-2 cells. Analysis of RNA synthesis was performed as described in Materials and Methods. Actinomycin D (2 μg/ml) was added after the viral adsorption period (30 min at 37°C) and maintained until the end of the incubation. Each point represents the average of two to three separate experiments. 3a (A) and 3b (B) were present during the viral adsorption period (30 min at 37°C) and during incubation (7 h at 37°C). Symbols: △, uninfected cells; ▲, infected cells; □, uninfected cells treated with 3a or 3b (2 μM); ■, infected cells treated with 3a or 3b (2 μM); ○, uninfected cells treated with 3a or 3b (20 μM); ●, infected cells treated with 3a or 3b (20 μM). (C) Compounds at 20 μM were added to poliovirus-infected cells after virus adsorption or 2 h later. Symbols: △, uninfected cells; ▲, infected cells; □, infected cells treated with 3a from zero time; ○, infected cells treated with 3b from zero time; ■, infected cells added with 3a at 2 h postinfection; ●, infected cells added with 3b at 2 h postinfection.

Among the drugs tested, 3(2*H*)-isoflavene and 6-chloro-3(2*H*)-isoflavene were the most effective inhibitors of poliovirus type 2 infection. These findings are in contrast with those reported for infection with rhinovirus (6) and hepatitis A virus (13), which exhibited a greater susceptibility to the action of isoflavans. Therefore, the planarity of the molecule of isoflavenes seems to be in direct correlation with the antiviral activity. The finding that compound 3b exhibited an antipoliovirus effect similar to that of compound 3a indicates that the presence of the chlorine atom in the 6 position also favored the antiviral activity. The halogen in position 6 enhances, with its inductive effect, the conjugation of the pyrane with the benzene ring and therefore the planarity of the molecule. Both compounds reduced the virus yield in a dose-dependent manner under a single cycle of virus replication and completely suppressed the poliovirus-induced shutoff of host translation when present either throughout the entire incubation time or during the adsorption period only. These results suggest that the substances might act on early stages of infection.

At the same concentration, these compounds inhibited viral RNA and protein synthesis. Kinetic studies indicated that the inhibitory effect depended on the presence of drugs during the adsorption period. The absence of precursors and viral proteins in the electrophoretic pattern of virus-infected cells strongly suggest that compounds 3a and 3b prevent viral proteins synthesis. Interference with the proteolytic processing of the viral polyprotein, therefore, looks like a rather unlikely possibility. Furthermore the prevention of shutoff of cellular protein synthesis in the presence of compounds 3a and 3b is consistent with the hypothesis that the translation of parental viral RNA is also prevented (9, 11, 12).

Our results strongly suggest that an early step of the viral replication cycle may be selectively inhibited. Nevertheless, compounds 3a and 3b had no noticeable effect on viral attachment, penetration, and uncoating. Furthermore, both compounds reduced the infectivity of phenol-extracted viral RNA as effectively as they inhibited the replication of whole virions, and they did not protect the virus infectivity from heat inactivation, indicating that compounds could probably act immediately after the uncoating step. However, it cannot be conclusively excluded that uncoating of the virus genome is a possible target of compound(s) under study. As already suggested by Tisdale and Selway (15), the measurement of uncoating as RNase sensitivity of the isotopically labeled viral nucleic acid might be too insensitive because of the large number of uninfectious particles, which, entering the cells through an abortive pathway, may obscure the final result (10). Taking into account that HRV and poliovirus infections are more susceptible to isoflavans and isoflavenes, respectively, we cannot rule out the involvement of different mechanisms of action for the antiviral activity of these two classes of compounds.

An interesting feature of these compounds is their capac-

ity to protect cell cultures from poliovirus infection when added to the medium before viral adsorption. This property may be due to the ability of the compounds to insert themselves into the cell membrane because of their lipophilic nature. However, as previously reported, their action is not mediated by interferon production (6). This characteristic makes these substances suitable for further in vivo studies.

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