ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1986, p. 110–116
0066-4804/86/070110-07$02.00/0
Copyright © 1986, American Society for Microbiology
Vol. 30, No. 1

Prevention of Rhinovirus and Poliovirus Uncoating by WIN 51711, a New Antiviral Drug

M. PATRICIA FOX,* MICHAEL J. OTTO, AND MARK A. McKINLAY
Sterling-Winthrop Research Institute, Rensselaer, New York 12144

Received 10 March 1986/Accepted 9 April 1986

WIN 51711, a potent new antipicornavirus drug, has been shown to inhibit an early event in the replication cycle of human poliovirus type 2 and human rhinovirus type 2. WIN 51711 was not virucidal and had no measurable effect on the adsorption of [^{3}H]uridine-labeled virions to cells. When virion penetration of the plasma membrane was determined through loss of sensitivity to neutralizing antisera, WIN 51711 had no effect on poliovirus penetration, but inhibited rhinovirus penetration by 40%. In the presence of WIN 51711, exposure of neutral red-encapsidated virus-infected cells to light at 3 h postinfection resulted in a 3-log reduction in the number of infectious centers, indicating that WIN 51711 maintained the viral RNA in the encapsidated state after penetrating the cell membrane. The inhibition of uncoating by WIN 51711 in the neutral red assay was found to be concentration dependent, with a concentration of 0.03 μg/ml resulting in a 90% inhibition of uncoating. Sucrose gradient sedimentation of lysates from whole cells infected with [^{3}H]uridine-labeled poliovirus showed that poliovirions remained intact in the presence of WIN 51711, but were uncoated in the absence of drug. WIN 51711 also prevented thermal inactivation of poliovirus infectivity, indicating a direct stabilizing effect of this compound on virion capsid conformation.

WIN 51711, 5-[7-[4-(4,5-dihydro-2-oxazolyl)phenoxy]heptyl]-3-methyl-isoxazole, is a potent new antipicornavirus drug (3a). Recently, the inhibitory activity of WIN 51711 against a number of enterovirus and rhinovirus serotypes has been reported (10) with reduction in plaque formation and reduction in virus yield at concentrations below that which inhibits cell growth. In plaque reduction assays, concentrations of WIN 51711 in the range of 0.004 to 0.17 μg/ml caused a 50% reduction in plaque formation for the enteroviruses tested. Generally, the rhinoviruses tested in plaque reduction assays were less sensitive to WIN 51711 than were the enteroviruses. However, the majority of the 33 serotypes which were tested showed a 50% reduction in plaque formation at less than 1 μg of WIN 51711 per ml (2.9 μM). Similar results were reported in virus yield reduction studies (10). WIN 51711 has also been shown to be systemically active in the prevention of paralysis and death in mice infected with human poliovirus type 2 (8) and echovirus-9 (B. A. Steinberg, A. A. Visosky, J. A. Frank, Jr., and M. A. McKinlay, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 433, 1984).

Preliminary data presented here indicate that WIN 51711 exerts its virus-specific activity through the inhibition of an early event in the replication of rhinovirus and poliovirus and that the molecular basis of this mechanism is the inhibition of the virion uncoating process.

MATERIALS AND METHODS

Viruses, cells, and infectivity assays. The MEF strain of poliovirus type 2 was originally obtained from the University of Pittsburgh Medical School. Human rhinovirus type 2 was obtained from the Sterling-Winthrop Research Institute culture collection. Poliovirus and rhinovirus were propagated and titrated on HeLa (Ohio) cell monolayers (Flow Laboratories, Inc., McLean, Va.) and stored as cell-free stocks at −70°C. Cells were grown in medium 199 (Flow), supplemented with 5% Bobby calf serum (BCS) (GIBCO Laboratories, Grand Island, N.Y.) and antibiotics (50 U of penicillin and 50 μg of streptomycin per ml). For virus yield experiments, 30 mM $MgCl_2$ was included in media.

Drug-resistant poliovirus type 2 variants were selected by picking plaques from plates infected with poliovirus in the presence of 1.0 and 0.1 μg of WIN 51711 per ml. The viruses isolated from plaques that developed in the presence of drug were passaged three times in HeLa (Ohio) monolayer cultures in the presence of 1.0 and 0.1 μg/ml and were designated rCl-1.0γ and rCl-0.1γ, respectively. These resistant variant stocks were stored at −70°C after testing for sensitivity to WIN 51711.

Virus titrations were determined by plaque assay on day-old confluent HeLa (Ohio) monolayer cultures in six-well cluster plates (Costar, Cambridge, Mass.). The overlay media contained molten 0.5% SeaKem agarose (FMC Corp., Marine Colloids Div., Rockland, Maine) and 30 mM $MgCl_2$. For rhinovirus, 15 μg of DEAE-dextran per ml was also added. Plates were incubated in a humidified incubator with 2% CO_2 at 33°C for rhinovirus and at 37°C for poliovirus, then fixed with Formalin, and stained with crystal violet.

Isotopes and chemicals. [^{3}H]uridine (specific activity, 25 Ci/mmol for adsorption studies and 37.9 and 43.1 Ci/mmol for sucrose sedimentation studies) and [^{35}S]methionine (specific activity, 1,022 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass. WIN 51711 was solubilized in dimethyl sulfoxide (Me_2SO) and diluted in Me_2SO to 100 times the final test concentrations. A 1:100 dilution of the above drug solutions was made in media to achieve the desired working concentrations. Controls containing no WIN 51711 were adjusted to a final concentration of 1% Me_2SO. Pancreatic RNase A (42 U/mg) was purchased from Calbiochem-Behring, La Jolla, Calif. Ultrapure sucrose was purchased from Bethesda Research Laboratories, Inc.,

* Corresponding author.

Gaithersburg, Md. Cesium chloride was purchased from Sigma Chemical Co., St. Louis, Mo. Nonidet P-40 was obtained from Bethesda Research Laboratories.

Preparation of radiolabeled rhinovirus and poliovirus. Monolayers of HeLa (Ohio) cells in 690-cm^2 roller bottles (Wheaton Scientific Culture Bottles; Wheaton Industries, Millville, N.J.) were infected with rhinovirus or poliovirus at a multiplicity of infection of 1 PFU per cell in 10 ml of medium 199. After incubation for 1 h (at 33°C for rhinovirus; 37°C for poliovirus), 100 ml of medium 199 with 5% BCS was added to each bottle, and incubation was continued for an additional 2 h. Medium was discarded and replaced with 50 ml of medium 199 with 2% BCS and 20 μCi of [^{3}H]uridine per ml. At 18 h postinfection, infected cells were removed from the glass by shaking and decanted into 50-ml centrifuge tubes for centrifugation at 500 × *g* in a CRU-5000 centrifuge (International Equipment Co., Div. Damon Corp., Needham, Mass.) for 10 min. The supernatant was kept on ice; the cell pellet was suspended in 1 ml of phosphate-buffered saline (PBS) and subsequently frozen and thawed twice to release virus. After centrifugation to remove cell debris at 11,950 × *g* in an SS-34 rotor (Ivan Sorvall, Inc., Norwalk, Conn.), the supernatants were pooled and 1.0 mg of RNase A per ml was added for digestion of nonencapsidated viral RNA during a 30-min incubation at room temperature. The virus was pelleted by centrifugation for 90 min at approximately 90,000 × *g* in an SW28.1 rotor at 5°C in an ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.). The pellets were suspended in PBS, and samples were removed to determine radioactivity and PFU.

Radiolabeled poliovirus for sucrose sedimentation studies was grown in HeLa (Ohio) cell monolayers in Falcon flasks (Becton Dickinson Labware, Oxnard, Calif.) (150 cm^2) with a 15-ml overlay of medium 199 containing 1% BCS and 10 μCi of [^{3}H]uridine per ml. The inoculum and overlay also contained WIN 51711 at 1.0 or 0.1 μg/ml, as appropriate, for the resistant variant viruses. The infected cultures were frozen at −70°C at 24 h postinfection, and subsequently cell debris was removed from the thawed cultures by centrifugation at 500 × *g* in a CRU-5000 centrifuge for 15 min at 5°C. The virus was then pelleted as described above.

The viral pellets were suspended in 2 ml of PBS, sonicated to break up aggregates, and treated with RNase A, as described above. The virus was placed atop a 15 to 45% linear sucrose gradient (15 ml) formed by the procedure of Baxter-Gabbard (1). Virus was banded in this gradient by centrifugation at 90,000 × *g* for 3 h at 5°C in a Beckman SW28.1 rotor, and fractions were collected from the bottom.

The fractions containing radioactive virus were identified by plaque assay and by trichloroacetic acid precipitation of radioactivity. The fractions containing radioactive virus were then pooled and adjusted to 450 mg of cesium chloride per ml for further purification by banding in cesium chloride gradients. These gradients were centrifuged at 185,000 × *g* for 20 h at 4°C in a Beckman SW41 Ti rotor. The gradients were fractionated, and virus was located as described for the sucrose gradients.

The [^{35}S]methionine-labeled poliovirus used for cosedimentation was prepared with modifications to the above procedures. At 3 h postinfection, the medium was replaced with Dulbecco minimal essential medium lacking methionine with 1% BCS, to which 10 μCi of [^{35}S]methionine per ml was added. The virus was allowed to replicate for 18 h at 37°C, and cultures were frozen at −70°C. After thawing, cell debris was removed by centrifugation at 10,000 × *g* for 15 min in the SS-34 rotor in a Sorvall centrifuge, and the virus was pelleted from the clarified supernatant and purified by the above precedures.

Virucidal test. A total of 1 ml of virus (containing 7 × 10^6 PFU of poliovirus or 2 × 10^6 PFU of rhinovirus) was incubated at room temperature for 0 or 60 min in medium 199 in the presence of 1% Me_2SO for controls or 100, 10, 1, or 0.1 μg of WIN 51711 per ml in Me_2SO (at 1% final concentration). Infectivity was determined by plaque assay after dilution of the virus below the inhibitory concentration.

Effect of time of addition of WIN 51711 on virus yield from single-round replication. Medium containing 1.0 or 0.3 μg WIN 51711 per ml for rhinovirus or poliovirus, respectively, was added to monolayers of HeLa cells in 25-cm^2 flasks at various times before or after addition of virus. Virus inoculum was removed after 1 h of adsorption, and overlay medium was added, with or without WIN 51711, as indicated. Flasks were incubated for 8 h at 37°C for poliovirus, or at 33°C for 10 h for rhinovirus, and then frozen at −70°C for later titration of total virus yield.

Adsorption of [^{3}H]uridine-labeled virus. Tubes containing 10^6 HeLa cells in a 10-ml suspension were pretreated for 60 min at 37°C with media containing various concentrations of WIN 51711. After pretreatment, cells were centrifuged at approximately 250 × *g* in a CRU-5000 centrifuge for 4 min at room temperature, and the pellets were suspended in 1 ml of radioactive virus. Cells were then infected at a multiplicity of infection of 2 to 5 PFU per cell in the presence of various concentrations of WIN 51711. Cells were incubated for up to 60 min (at 33°C for rhinovirus, at 37°C for poliovirus), and then were immediately centrifuged, as described above, at 4°C, washed twice with ice-cold PBS, and suspended in 0.5 ml of 0.1 M Tris glycine (pH 8.0) plus 1% sodium dodecyl sulfate to solubilize the cells for radioactivity determinations.

Virus penetration studies. The adsorption of rhinovirus and poliovirus to suspended HeLa cells, treatment with specific neutralizing antisera against rhinovirus and poliovirus, and infectious-center assays were performed as described previously (9, 4) with some modification. These modifications included the suspension of cells before adsorption of virus to facilitate washing steps and use of a 60-min treatment with neutralizing antisera which included an initial 30-min incubation at 4°C followed by an additional incubation for 30 min at room temperature.

Rhinovirus type 2 neutralizing antiserum (guinea pig) was used at a 10-fold dilution in PBS, and poliovirus type 2 (horse) was used at a 5-fold dilution in PBS. Both antisera were from the National Institute for Allergy and Infectious Diseases, Bethesda, Md., and were obtained through the American Type Culture Collection, Rockville, Md.

Neutral red-sensitized virus. Neutral red-encapsidated rhinovirus and poliovirus were prepared, handled, and irradiated by the procedures described by Mandel (6). The uncoating assay procedures were essentially those described by McSharry et al. (9).

Briefly, to examine viral uncoating kinetics, confluent HeLa (Ohio) cell monolayers in 25-cm^2 flasks (Corning Glass Works, Corning, N.Y.) were infected in the dark (illuminated by a red safety light) with neutral red virus. At various times postinfection, flasks were irradiated with white light for 10 min, then the infected cells were removed from the flasks, and the number of infectious centers were quantitated. For the zero time point, virus inoculum was irradiated before addition to cells. The number of infectious centers at each time point is representative of the amount of virus no longer light sensitive at these times (i.e., uncoated virus).

To examine the effects of WIN 51711 on rhinovirus and poliovirus uncoating, HeLa (Ohio) cell monolayers were infected in the dark with neutral red virus in the presence and absence of WIN 51711. After 1 h of adsorption and 3 h of additional incubation (at 33°C for rhinovirus, at 37°C for poliovirus) in darkness, one-half of the infected flasks were irradiated with white light for 10 min; the remainder were kept in darkness as controls. Flasks were returned to the dark, and infectious centers were quantitated. An additional experiment on the uncoating of neutral red-encapsidated poliovirus was done by the same procedure with a range of WIN 51711 concentrations.

Sucrose sedimentation analysis of virus from infected cell lysates. Duplicate cultures were infected at a multiplicity of infection of 40 to 100 PFU per cell in the presence and absence of 0.1 μg WIN 51711 per ml. Three types of [^{3}H]uridine-labeled poliovirus were used in these studies: wild type (specific infectivity, 700 PFU/cpm), and two resistant variants, rCl-1.0γ (specific infectivity, 690 PFU/cpm), and rCl-0.1γ (specific infectivity, 1,440 PFU/cpm). The 1-ml inoculum of each virus contained approximately 20,000 cpm. The virus was allowed to adsorb at room temperature (21.5°C) in the presence or absence of drug for 1 h, and then the inoculum was removed, and all cultures were washed once with cold medium 199 (with or without drug) and overlaid with 3 ml of medium 199 (with or without drug) containing 5% BCS. Incubation was continued for 3 h at 37°C to allow uncoating to take place. The cultures were washed twice with 2 ml of cold PBS, and lysates were prepared from each by the addition of 0.5 ml of 1% Nonidet P-40 in PBS. Plates were washed sequentially with 1 ml of PBS, and this wash was added to the 1 ml of lysate for a final sample volume of 2 ml. The samples were frozen at −70°C for later sucrose gradient sedimentation analysis.

Cell lysate samples were thawed and placed atop a 15-ml linear sucrose gradient (15 to 45%) formed by the procedure of Baxter-Gabbard (1), and virus was banded by centrifugation at 90,000 × *g* for 3 h at 5°C in a Beckman SW28.1 rotor. Gradients were fractionated from the bottom, and 3-drop fractions (approximately 0.14 ml) were collected. Samples (0.005 ml) were removed from fractions in the viral band area and frozen at −70°C for later infectivity determinations. [^{35}S]methionine-labeled poliovirus was cosedimented with cell lysates to determine the position of intact virus in the gradients.

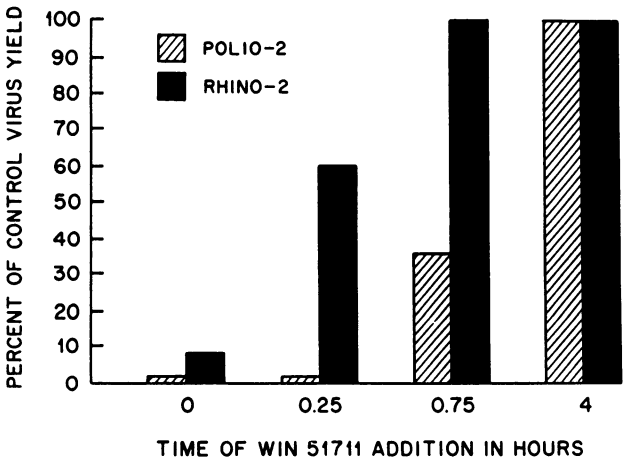

FIG. 1. Effect of time of WIN 51711 addition on virus yield from single-round replication of poliovirus and rhinovirus. Medium containing 1.0 or 0.3 μg of WIN 51711 per ml for rhinovirus or poliovirus, respectively, was added to HeLa cell monolayers at the time of virus inoculation or at various times postinfection. Monolayers were incubated for 8 (poliovirus) or 10 h (rhinovirus) and were then frozen for later titration of total virus yield by plaque assay. Results are plotted as a percentage of the control (no drug) virus yield.

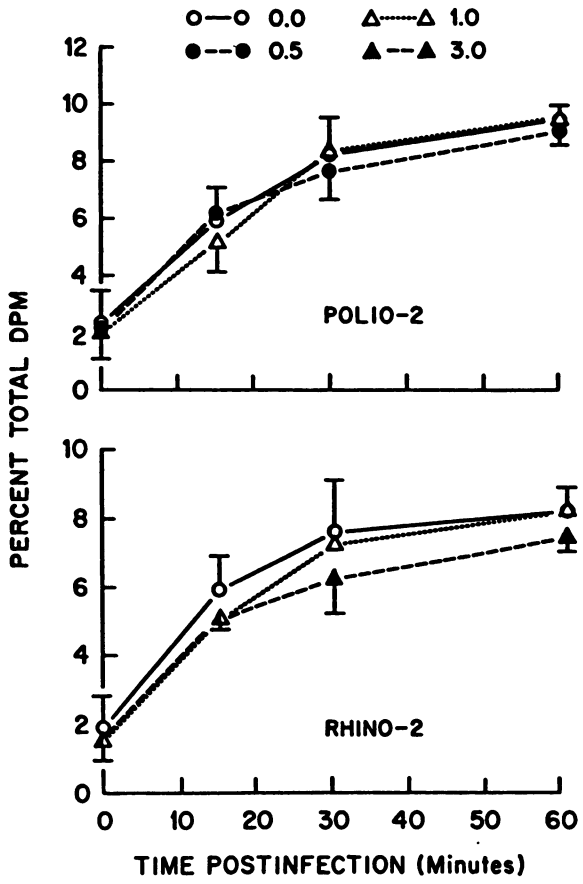

FIG. 2. Adsorption of [^{3}H]uridine-labeled poliovirus and rhinovirus to HeLa cells in the presence or absence of WIN 51711. Cells were incubated with [^{3}H]uridine-labeled virus at a multiplicity of infection of 2 to 5 PFU per cell with or without drug for up to 60 min (at 33°C for rhinovirus; at 37°C for poliovirus) and then were immediately chilled, washed twice, and solubilized to count radioactivity. Concentrations of WIN 51711 are in micrograms per milliliter. Results are plotted as the percentage of total disintegrations per minute (dpm) of virus added.

Thermal inactivation studies. Poliovirus was diluted to approximately 10^6 PFU/ml and divided into portions containing various concentrations of WIN 51711, or 1% Me_2SO for control. The tubes were placed in a shaking water bath at 48°C, and samples were taken at indicated times and placed on ice for titration by plaque assay.

RESULTS

Virucidal test. WIN 51711 did not significantly reduce the titers of rhinovirus or poliovirus after a 60-min incubation at 100 μg/ml, a concentration approximately 100 to 10,000 times the inhibitory concentration in cell culture (data not shown).

Effect of time of addition of WIN 51711 on virus yield from single-round replication. An early event in the replication of rhinovirus and poliovirus was inhibited by WIN 51711 (Fig.

TABLE 1. Effect of WIN 51711 on poliovirus and rhinovirus penetration

Virus	WIN 51711 concn (μg/ml)	Amt of virus penetrated[a] (log infectious centers/ml)				
		+NA			−NA	
		Time zero	30 min	90 min	30 min	90 min
Poliovirus	0	3.04		5.63		5.58
		3.26		5.67		5.59
	0.03	2.96		5.18		5.15
		2.82		5.38		5.34
	0.1	2.70		4.89		5.00
		2.75		4.93		5.15
	0.3	2.64		4.82		4.96
		2.56		4.90		4.99
Rhinovirus	0	3.81	5.54		5.51	
		3.20	5.77		5.63	
	2.0	3.17	5.32		5.60	
		3.77	5.43		5.64	

[a] Penetration was determined by quantitating the number of infectious centers after neutralization by type-specific neutralizing antisera (NA) as described in the text. Data from two experiments are presented for each concentration of drug. Each is the average of duplicate determinations. Infected cultures were exposed to NA at 0, 30, or 90 min after 1 h of adsorption at 4°C.

1). Maximal inhibition of rhinovirus and poliovirus was observed when WIN 51711 was added at the time of virus inoculation. When WIN 51711 was added to rhinovirus-infected cells at 15 min postinfection, the inhibitory effect was already reduced. The step in the replication process inhibited by WIN 51711 appeared to occur at a later time in poliovirus-infected cultures, since maximal inhibition was still observed up to 30 min after infection. A time-dependent loss in drug effect on poliovirus replication occurred when drug was added 30 min postinfection. The inhibitory effect of WIN 51711 on rhinovirus replication was completely lost when the infection was allowed to proceed for 45 min before drug addition.

Effect of WIN 51711 on adsorption. [^{3}H]uridine-labeled rhinovirus and poliovirus adsorbed at the same rate in the presence or absence of WIN 51711, indicating that this drug has no detectable inhibitory effect on adsorption (Fig. 2).

Effect of WIN 51711 on penetration. The effect of WIN 51711 on virion penetration of the host cell membrane (Table 1) was assessed with a method described by Eggers (4) in which the development of resistance to neutralization of virion infectivity by specific antiserum was measured over time. WIN 51711 did not inhibit poliovirus penetration at concentrations up to 0.3 μg/ml, compared with drug-treated controls not exposed to antisera. WIN 51711 reproducibly inhibited rhinovirus penetration at 2 μg/ml by approximately 40% when the titer of the 30-min sample treated with antisera and drug was compared with the drug-treated control which was not treated with antisera.

Effect of WIN 51711 on uncoating. Exposure of cells infected with neutral red-encapsidated poliovirus to white light in the presence of 0.3 μg of WIN 51711 per ml at 3 h postinfection resulted in a 3-log loss of infectivity as compared with untreated controls (Table 2). Similar results were obtained with rhinovirus-infected cells in the presence of 2 μg of WIN 51711 per ml, although the magnitude of the reduction in infectivity was lower (2 log) because of the lower initial titer of neutral red rhinovirus used in these experiments.

A dose-dependent inhibition of uncoating of poliovirus as measured by photosensitivity of neutral red virus in the presence of 0 to 0.3 μg WIN 51711 per ml is shown in Fig. 3. The WIN 51711 concentrations effective in preventing uncoating were similar to the concentrations previously shown to inhibit virus replication as measured by virus yield (10).

When rates of uncoating of neutral red-encapsidated rhinovirus and poliovirus were compared by measuring the rate of loss of sensitivity to photoinactivation, rhinovirus was observed to uncoat at a faster rate than poliovirus (Fig. 4). At the 30-min time point, only about 5% of the poliovirus had become photoresistant (uncoated), compared with 45% of the rhinovirus. By 60 min, about 40% of the poliovirus was uncoated, compared with 70% of the rhinovirus.

Sucrose gradient sedimentation of virus from infected cell lysates. When [^{3}H]uridine-labeled wild-type poliovirus-infected cell lysates from cultures infected in the presence of 0.1 μg of WIN 51711 per ml were sedimented in sucrose gradients, a major peak of radioactivity cosedimenting with [^{35}S]methionine-labeled marker poliovirus was observed (Fig. 5A). This radioactivity peak was reduced when lysates from cultures infected in the same manner in the absence of WIN 51711 were examined, indicating that uncoating had occurred in the absence of drug (Fig. 5A).

Quantitation of virus in samples from the gradients indicated that the quantity of infectious virus obtained from cell lysates which were infected in the presence of WIN 51711 was much greater than that recovered in samples infected in the absence of drug (Fig. 6A). In the presence of WIN 51711, the specific infectivity of the wild-type poliovirus was 500 PFU/cpm, which is 71% of that present in the original inoculum.

In contrast to the results obtained with wild-type poliovirus, sucrose gradient analysis showed that the variant virus, rCl-1.0γ, uncoated in the presence as well as in the absence of 0.1 μg of WIN 51711 per ml (Fig. 5C). The resistant variant, rCl-0.1γ, uncoated to a partial extent in the presence of 0.1 μg of WIN 51711 per ml, compared with the absence of drug (Fig. 5B). The infectivity results for the resistant variants correlated well with the radioactivity re-

TABLE 2. Effect of WIN 51711 on uncoating of poliovirus and rhinovirus[a]

Virus	WIN 51711 concn (μg/ml)	Light irradiation	No. of infectious centers/ml[b]
Poliovirus	0	−	$2.2 \times 10^6 \pm 0.25 \times 10^6$
		+	$6.8 \times 10^5 \pm 0.85 \times 10^5$
	0.3	−	$5.0 \times 10^5 \pm 0.3 \times 10^5$
		+	$5.9 \times 10^2 \pm 0.6 \times 10^2$
Rhinovirus	0	−	$1.2 \times 10^4 \pm 0.1 \times 10^4$
		+	$9.5 \times 10^3 \pm 1.0 \times 10^3$
	2.0	−	$4.6 \times 10^3 \pm 0.75 \times 10^3$
		+	$1.1 \times 10^2 \pm 0.5 \times 10^2$

[a] See text for description of methods.
[b] Data represent the mean of four determinations ± standard deviation.

sults (Fig. 6B and C). In the presence of WIN 51711, the specific infectivities of the rCl-1.0γ and the rCl-0.1γ variants were 24 and 57%, respectively, of that present in the original inocula.

Effect of WIN 51711 on thermal inactivation of poliovirus. The infectivity of the untreated poliovirus control was rapidly inactivated at 48°C with a reduction in titer of 2 log in 5 min and 3 log in 20 min. At concentrations of 0.03 and 0.17 μg/ml, WIN 51711 protected poliovirus against thermal inactivation. Following 40 min of exposure at 48°C, the titer in the untreated controls was reduced by 3 log, compared with only 1.0 and 0.5 log titer reduction in virus exposed to 0.03 and 0.17 μg of WIN 51711 per ml, respectively.

DISCUSSION

The data presented here show that WIN 51711 inhibits an early event in the replication cycle of two representative picornaviruses, rhinovirus and poliovirus. WIN 51711 was not virucidal, since exposure of rhinovirus and poliovirus to 100 to 10,000 times the reported effective concentration had no effect on virion infectivity (10). WIN 51711 also had no effect on the adsorption of rhinovirus and poliovirus to host cells as measured by the kinetics of [^{3}H]uridine-labeled virus bound to cells in the presence or absence of drug.

WIN 51711 had no effect on poliovirus penetration at 0.3 μg/ml, a concentration which inhibited uncoating by greater than 99.9% (Fig. 3) and caused a 99.9% reduction in virus yield (10). The dose-dependent reduction in infectious centers observed in cultures not exposed to antisera may be caused by inefficient removal of drug from cells, resulting in a partial inhibition of uncoating. However, WIN 51711 did inhibit rhinovirus penetration. At 2 μg/ml, the concentration which resulted in a 90% reduction in the yield of rhinovirus (10), WIN 51711 inhibited penetration by 40%.

WIN 51711 was shown to have a potent inhibitory effect on the uncoating of rhinovirus and poliovirus. In the uncoating assay, the infectivity of neutral red-encapsidated rhinovirus and poliovirus in cells infected in the presence of WIN 51711 was inactivated by exposure to light at 3 h after infection. In addition, the concentrations of WIN 51711 which inhibited uncoating were in the same range as those resulting in a reduction in virus yield (10). The marked inhibitory effects of WIN 51711 on the uncoating of neutral red-encapsidated rhinovirus and poliovirus, and the fact that WIN 51711 reduces virus yield at the same concentrations that affect uncoating, provide evidence that the uncoating step is the major site of activity of this compound. Furthermore, data from the time of addition of WIN 51711 studies showed that although the compound was inhibitory at an early time for both viruses, this inhibitory effect was lost at an earlier time for rhinovirus than for poliovirus. These data are consistent with the faster uncoating rate observed for rhinovirus compared with poliovirus.

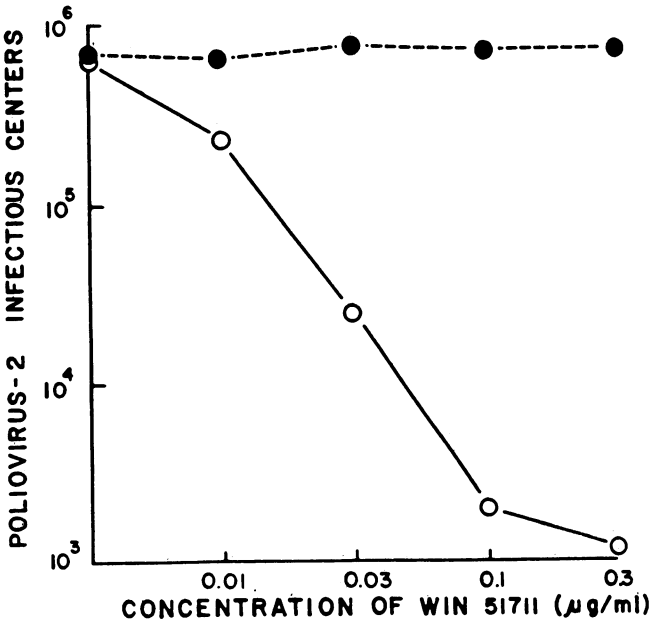

FIG. 3. Effect of various concentrations of WIN 51711 on the uncoating of poliovirus. For experimental procedures, see the text. Each point represents the mean of four determinations of the number of infectious centers. Symbols: O——O, light-irradiated samples; ● - - - ●, dark controls.

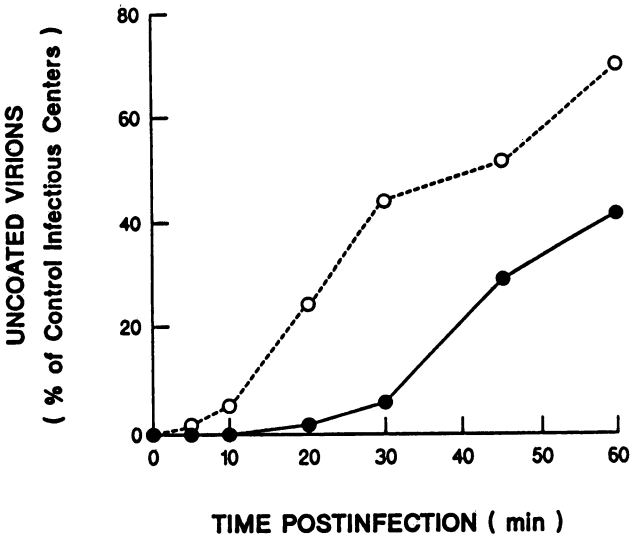

FIG. 4. Kinetics of rhinovirus and poliovirus uncoating. For experimental procedures, see the text. The results are plotted as the percentage of control unirradiated infectious centers (uncoated virions). Symbols: rhinovirus, O - - - - O; poliovirus, ●——●.

WIN 51711 prevented thermal inactivation of poliovirus infectivity. This result suggests that the direct interaction between WIN 51711 and the virion results in a stabilization of the virion structural conformation. This stabilization may be the explanation for the inhibition of the uncoating process.

Additional support for uncoating as the major site of action of WIN 51711 was provided by studies on sucrose gradient sedimentation of lysates from cells infected with [^{3}H]uridine-labeled poliovirus which indicated that the majority of the poliovirus remained intact and infectious in the presence of WIN 51711. A single major [^{3}H]uridine-labeled viral peak was observed in these gradients. Additional evidence that this virus was intact was provided by the observation that a [^{35}S]methionine-labeled marker poliovirus cosedimented with the [^{3}H]uridine-labeled virus band. In the absence of drug, the virus band was diminished in size with radioactive counts occurring in other areas of the gradient correlating with the release of viral RNA from uncoated virus.

The determination of the specific infectivity of the wild-type poliovirus in the presence of WIN 51711 showed that it was 71% of that in the original inoculum. This reduction in

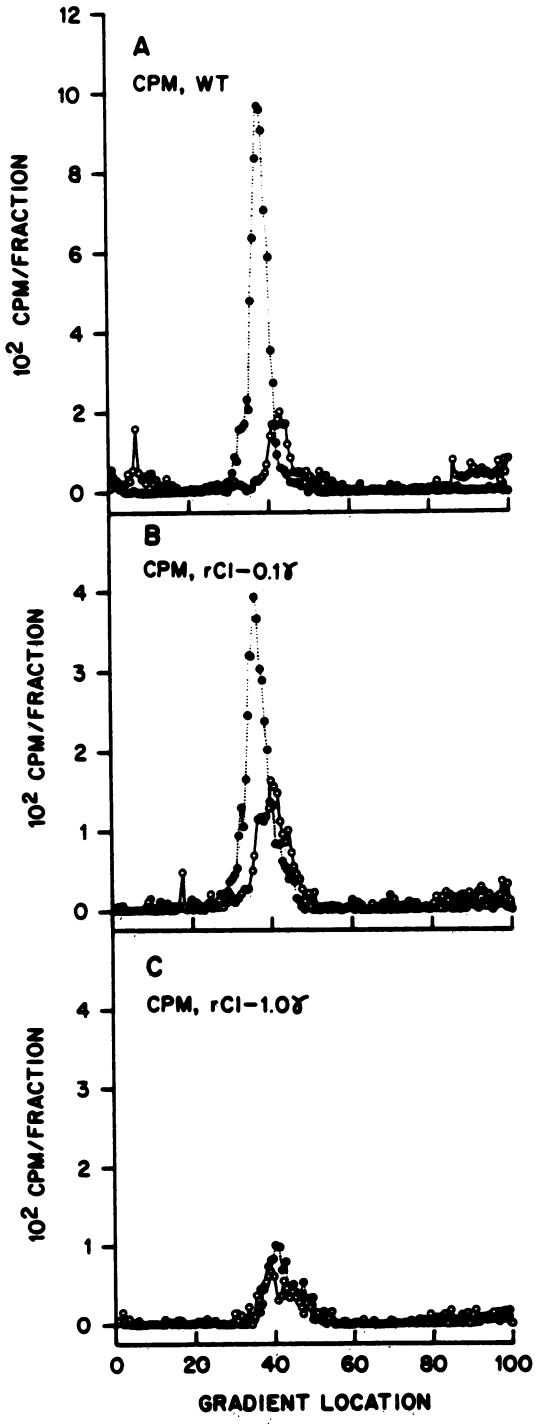

FIG. 5. Sucrose gradient sedimentation of lysates from cells infected with [^{3}H]uridine-labeled poliovirus in the presence and absence of 0.1 μg of WIN 51711 per ml. Gradients were collected from the bottom (0) in 3-drop fractions. The results of radioactivity determinations are plotted as 10^2 cpm per fraction with the fraction number normalized to 100% for gradient location. Each panel contains the data for one type of virus (wild-type, A; RCl-0.1γ, B; rCl-1.0γ, C) in the presence (● - - - - - - ●) or absence (○——○) of drug.

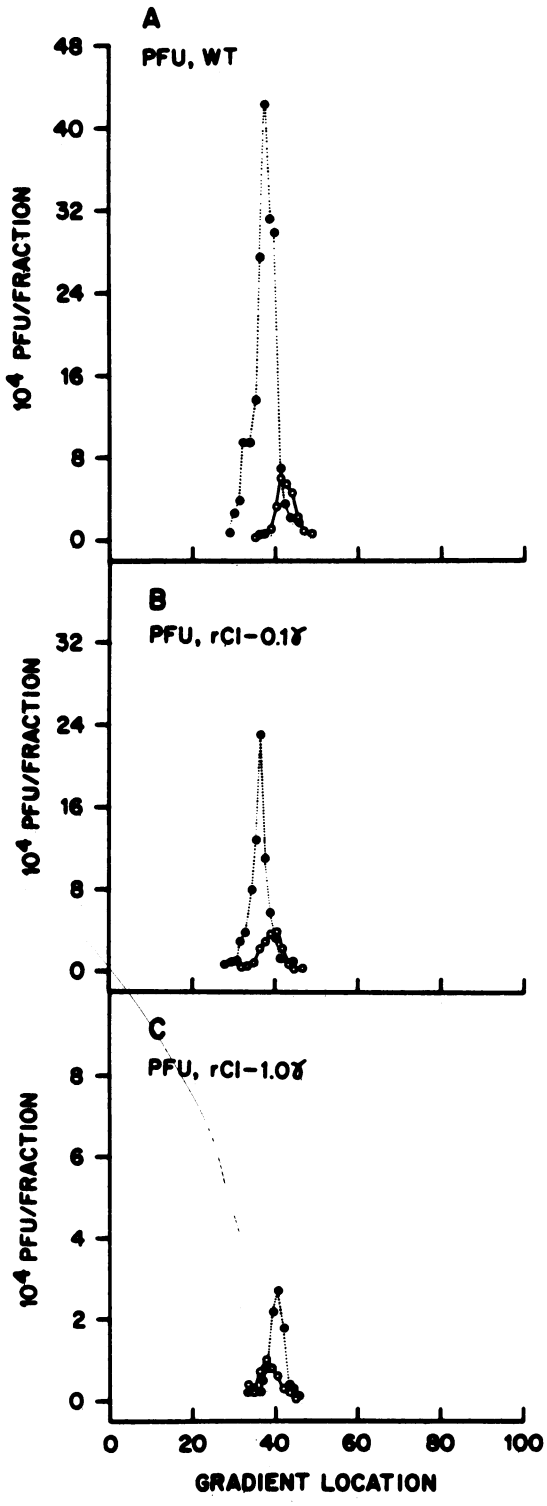

FIG. 6. Sucrose gradient sedimentation of lysates from cells infected with [^{3}H]uridine-labeled poliovirus in the presence and absence of 0.1 μg WIN 51711 per ml. The infectivity results for the experiment shown in Fig. 5 are plotted as 10^4 PFU per fraction. Each panel contains the data for one type of virus (wild-type, A; rCl-0.1γ, B; rCl-1.0γ, C) in the presence (● - - - - - - ●) or absence (○——○) of drug.

specific infectivity of 29% indicates that a small portion of the virus recovered was no longer infectious, although sedimenting with intact virions. However, the variability in the assay procedures may also be a factor in the apparent loss of specific infectivity. These results support the conclusion that the majority of the virus has not uncoated in the presence of WIN 51711.

In parallel experiments with WIN 51711-resistant variant viruses, the virus resistant to 1 μg of WIN 51711 per ml, rCl-1.0γ, uncoated similary in both the presence and absence of drug, which indicated that WIN 51711 was not able to prevent uncoating of the resistant virus. The virus resistant to 0.1 μg of WIN 51711 per ml, rCl-0.1γ, showed partial uncoating in the presence of 0.1 μg of WIN 51711 per ml when compared with the gradient of the untreated cell lysate. The results of the infectivity determinations for the resistant variants correlated well with the radioactivity results.

A consequence of viral uncoating is a reduction in the number of infectious particles and a corresponding reduction in the specific infectivity of the virus. Since the resistant variants were observed to uncoat in the presence of WIN 51711, the specific infectivities of the rCl-1.0γ and the rCl-0.1γ variants were reduced when compared with those of the original inocula. The specific infectivities of the rCl-1.0γ and the rCl-0.1γ variants were 24 and 57%, respectively, of those in the original inocula. The difference in the percentages obtained for the two resistant variants indicates the difference between the two variants in their relative resistance to WIN 51711.

The inhibition of poliovirus and rhinovirus uncoating by WIN 51711 is similar to the effects of arildone on the uncoating of poliovirus (9). It has been suggested that arildone may interact directly with the viral capsid to stabilize it or to prevent changes which are required for uncoating. Additional studies (3) supplied further evidence that arildone interacts directly with the poliovirus capsid to stabilize the virion in vitro against heat and alkaline treatment, thus preventing the loss of VP_4 capsid polypeptide and release of viral RNA from the capsid.

The loss of VP_4 polypeptide has been shown to correlate with a loss of viral infectivity (2, 5). In studies presented here, rhinovirus and poliovirus adsorbed and penetrated in the presence of WIN 51711 and were found to be infectious and, therefore, still had the VP_4 polypeptide. These results indicated that the loss of VP_4 polypeptide was not a prerequisite to viral adsorption and penetration. This supports evidence supplied by Mandel (6) in a series of studies with neutral red-encapsidated poliovirus and neutralizing antisera which indicated that virus penetrated and then uncoated intracellularly. A situation similar to that observed with WIN 51711 was described by Rosenwirth and Eggers (4, 11) where in the presence of the compound rhodanine, infectious echovirus-12 containing VP_4 polypeptide was found at 4 h postinfection. Rhodanine stabilized echovirus-12 so that modification of the virion was impaired but penetration of intact virus occurred. These authors concluded that modification of the virus to noninfectious particles was not a necessary precondition for penetration.

In 1976, Mandel (7) reported that antibody bound to the poliovirus capsid resulted in an alteration that consequently stabilized the conformation of the virion, making it resistant to uncoating. WIN 51711 might interact with rhinovirus and poliovirus to prevent the preliminary alteration or conformational change that is required for uncoating in a manner in some way analogous to that which Mandel observed with the stabilization of poliovirus by bound antibody with concurrent resistance to uncoating. WIN 51711 interferes with the loss of the VP_4 polypeptide by a mechanism which remains to be elucidated with a resultant stabilization of the virion conformation so that the virus is maintained in a coated state. Further studies are in progress to determine at the molecular level how WIN 51711 interacts with the viral capsid to stabilize virion conformation and thus prevent loss of the VP_4 polypeptide and uncoating.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of W. Shave and the editorial and technical assistance of L. Brennan in the preparation of this manuscript.

LITERATURE CITED

1. **Baxter-Gabbard, K. L.** 1972. A simple method for the large-scale preparation of sucrose gradients. FEBS Lett. **20**:117–119.
2. **Breindl, M.** 1971. The structure of heated poliovirus particles. J. Gen. Virol. **11**:147–156.
3. **Caliguiri, L. A., J. J. McSharry, and G. W. Lawrence.** 1980. Effect of arildone on modifications of poliovirus *in vitro*. Virology **105**:86–93.

3a. **Diana, G. D., M. A. McKinlay, M. J. Otto, V. Akullian, and C. Oglesby.** 1985. [[(4,5-Dihydro-2-oxazolyl)phenoxy]alkyl] isoxazoles. Inhibitors of picornavirus uncoating. J. Med. Chem. **28**:1906–1910.

4. **Eggers, H. J.** 1977. Selective inhibition of uncoating of echovirus 12 by rhodanine. Virology **78**:241–252.
5. **Lonberg-Holm, K., and B. D. Korant.** 1972. Early interaction of rhinoviruses with host cells. J. Virol. **9**:29–40.
6. **Mandel, B.** 1967. The relationship between penetration and uncoating of poliovirus in HeLa cells. Virology **31**:702–712.
7. **Mandel, B.** 1976. Neutralization of poliovirus: a hypothesis to explain the mechanism and the one-hit character of the neutralization reaction. Virology **69**:500–510.
8. **McKinlay, M. A., and B. A. Steinberg.** 1986. Oral efficacy of WIN 51711 in mice infected with human poliovirus. Antimicrob. Agents Chemother. **29**:30–32.
9. **McSharry, J. J., L. A. Caliguiri, and H. J. Eggers.** 1979. Inhibition of uncoating of poliovirus by arildone, a new antiviral drug. Virology **97**:307–315.
10. **Otto, M. J., M. P. Fox, M. J. Fancher, M. F. Kuhrt, G. D. Diana, and M. A. McKinlay.** 1985. In vitro activity of WIN 51711, a new broad-spectrum antipicornavirus drug. Antimicrob. Agents Chemother. **27**:883–886.
11. **Rosenwirth, B., and H. J. Eggers.** 1979. Early processes of echovirus 12-infection: elution, penetration, and uncoating under the influence of rhodanine. Virology **97**:241–255.