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Antipicornavirus Flavone Ro 09-0179

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Ro 09-0179 (4',5-dihydroxy-3,3',7-trimethoxyflavone), isolated from a Chinese medicinal herb, was found to have potent antiviral activity. It selectively inhibited the replication of human picornaviruses, such as rhinoviruses and coxsackieviruses in tissue culture, but not other DNA and RNA viruses. Ro 09-0298 (4',5-diacetyloxy-3,3',7-trimethoxyflavone), an orally active derivative of Ro 09-0179, prevented coxsackievirus (B1) infection in mice. The critical time for the inhibition of rhinovirus replication by Ro 09-0179 was 2 to 4 h after virus adsorption, i.e., in the early stages of virus replication. It markedly inhibited coxsackievirus and rhinovirus RNA synthesis in infected HeLa cells, but not in a cell-free system using the RNA polymerase complex isolated from the infected cells. In the infected cells, the RNA polymerase complex was not formed in the presence of Ro 09-0179. Therefore, it is suggested that Ro 09-0179 interferes with some process of viral replication which occurs between viral uncoating and the initiation of viral RNA synthesis.

Rhinovirus infections are estimated to be associated with at least half of the common cold cases in adults (5). Since the results of serological testing indicated that there are at least 100 types of rhinoviruses, prophylaxis by vaccines has not been practical or effective. Therefore, chemotherapy and chemoprophylaxis against rhinovirus infection deserve serious consideration.

For several years, we have screened microbial and natural products for antirhinovirus activity. Ro 09-0179 (4',5-dihydroxy-3,3',7-trimethoxyflavone) (Fig. 1), the substance of primary interest in this report, was originally isolated from a Chinese medicinal herb, Agastache Folium (*Agastache rugosa* Kuntze) and then synthesized chemically (Y. Suhara, I. Umeda, H. Shirai, and H. Ishitsuka, Abstr. Annu. Meet. Pharm. Soc. Japan 1982, 389). In the present communication, we describe the antiviral activity of Ro 09-0179 and its orally active derivative, Ro 09-0298 (4,5'-diacetyloxy-3,3',7-trimethoxyflavone) in tissue culture and in mice and discuss their mode of action.

MATERIALS AND METHODS

Cells. HeLa (Bristol strain), L929, and MDCK (canine kidney) cells were cultured at 37°C in Eagle minimum essential medium (MEM) containing 10% calf serum, 1% tryptose phosphate broth (TPB), 100 μg of streptomycin sulfate per ml, and 50 U of penicillin G per ml. WI-38 cells (human embryonic lung cells) were cultured with MEM containing 10% fetal calf serum, nonessential amino acids, and the above antibiotics. Maintenance medium for virus infections consisted of MEM, 2% fetal calf serum, 1% TPB, and the antibiotics.

Viruses. All rhinovirus strains excepting type 2 (HGP), which was kindly supplied by R. Kohno, the National Institute of Health, Japan, were purchased from the American Type Culture Collection, Rockville, Md., and were propagated in HeLa and WI-38 cells. Echoviruses (type 7, 11, 12, and 19) and coxsackieviruses (A21 and B1) were obtained from H. Shimojoh, Tokyo University, Tokyo, Japan, and E. Grünberg, Hoffmann-La Roche Inc., Nutley, N.J., respectively, and propagated in WI-38 and HeLa cells. Vesicular stomatitis virus (New Jersey strain) and mengovirus were propagated in L929 cells; vaccinia virus (Rister) and poliovirus type 1 (Sabin) were propagated in HeLa cells; herpes simplex virus type 1 (HF strain) was propagated in both HeLa cells and L929 cells; influenza virus A (NWS) was propagated in MDCK cells; and respiratory syncytial virus was propagated in HEp-2 cells. All viruses were propagated in tissue culture at 37°C except the rhinoviruses, which were propagated at 33°C.

Animals and viral infections. Male ddY mice weighing 17 to 19 g (3 weeks old) were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Japan. Groups of 10 mice were infected with about 10 50% lethal doses (LD_{50}) of coxsackievirus B1 from a stock solution which had been prepared as a homogenate of skeletal musculature of infected suckling mice.

Estimation of MIC_{50}. A suspension of HeLa cells (6×10^4 cells) was mixed with virus (about 5×10^3 PFU), and the mixture was immediately plated into a microtest plate (no. 3040; Falcon Plastics, Oxnard, Calif.), which contained the serially diluted compound to be tested. Viral cytopathic effect was observed microscopically at 2 to 4 days after infection. The concentra-

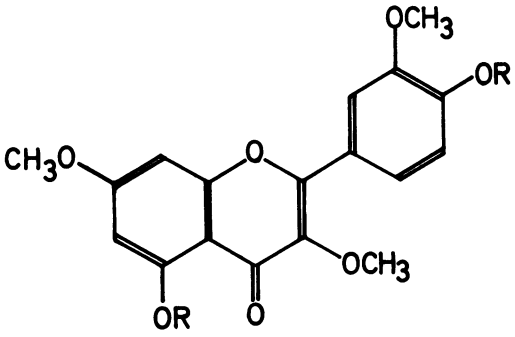

FIG. 1. Structure of Ro 09-0179 and Ro 09-0298. R=H: Ro 09-0179; =CH_3CO: Ro 09-0298.

tion at which the viral cytopathic effect was inhibited 50% as compared with the control was designated the MIC_{50}.

Assay of virus titer. Monolayers of HeLa cells (4×10^5 cells per well) in a tissue culture plate (FB-TS-16-24, Linbro) were inoculated with 0.1 ml of 10-fold serially diluted suspensions of rhinovirus or coxsackievirus in MEM containing 50 μg of DEAE-dextran per ml and 10 mM $MgCl_2$. After 2 h of incubation at 33°C or 1.5 h of incubation at 37°C, the virus suspension was removed, and the cells were overlaid with MEM supplemented with 5% fetal bovine serum, 50 μg of DEAE-dextran per ml, 1% TPB, 10 mM $MgCl_2$, and 0.8% Noble agar. After 2 days of incubation at 37°C or 4 days at 33°C, the cells infected with coxsackievirus or rhinovirus, respectively, were fixed with 10% Formalin in saline and were stained with 0.5% methyl violet. Virus plaques were counted, and the titer was expressed as plaque-forming units per milliliter. In a few experiments, the virus titer was estimated from cytopathogenicity and expressed as 50% tissue culture infective doses per milliliter ($TCID_{50}$).

Cytotoxicity testing. HeLa cells (5×10^4) were cultured on MEM supplemented with 2% fetal calf serum and 1% TPB in the presence or absence of the serially diluted test compound. The 50% cytotoxic dose was expressed as the dose that inhibited growth to 50% of control growth by 2 days after the addition of the compound. The number of cells usually increased three- to fourfold in the control culture.

Preparation of crude RNA polymerase complex. HeLa cell monolayers were infected with rhinovirus type 2 or coxsackievirus B1 at a multiplicity of infection (MOI) of 2 to 20 in the presence of actinomycin D (5 μg/ml). After various times of incubation with or without Ro 09-0179, the cells were removed with a rubber policeman and collected by centrifugation. The cell pellet was frozen at −70°C until used. Approximately 8×10^7 frozen cells were suspended in 2 ml of cold buffer containing 0.05 M Tris-hydrochloride (pH 7.2), 0.002 M $MgCl_2$, and 0.01 M NaCl and then ruptured with a Dounce homogenizer. Unbroken cells and nuclei were removed by centrifugation at $800 \times g$ for 5 min. The supernatant fraction was further centrifuged at $30,000 \times g$ for 20 min, and the pellet was suspended in 0.5 ml of buffer containing 0.05 M Tris-hydrochloride (pH 8.0) and 0.01 M NaCl. This fraction was designated as the RNA polymerase complex, which contained the cytoplasmic membrane and to which template viral RNA and viral RNA polymerase were bound (9).

RNA synthesis by the RNA polymerase complex. The RNA polymerase complex was used for assaying the effects of the antiviral agents on viral RNA synthesis (9). The reaction mixture (0.1 ml) contained 1 μmol of Tris-hydrochloride (pH 8), 0.1 μmol of $MgCl_2$, 0.13 μmol of dithiothreitol, 0.025 μmol of phosphoenol pyruvate, 0.5 μg of pyruvate kinase, 0.025 μmol of ATP, CTP, and UTP (or GTP), 0.25 μCi of [^{3}H]GTP (or UTP), 0.5 μg of actinomycin D, and 0.01 ml of the RNA polymerase complex fraction isolated at various times after infection.

Incubation was carried out at 34°C for 40 min with or without 1 μg of Ro 09-0179. The reaction was terminated by the addition of 0.5 ml of ice-cold 0.1 M $Na_4P_2O_7$ and 0.5 ml of 50% cold tricholoroacetic acid (TCA). The precipitate was collected on a membrane filter (Millipore Corp., Bedford, Mass.) and washed with 5% cold TCA. The radioactivity of the filter was counted and expressed as disintegrations per minute by a scintillation counter (Intertech model SL-30-multi 8).

Compound. Ro 09-0179 (4',5-dihydroxy-3,3',7-trimethoxyflavone) and Ro 09-0298 (4',5-diacetyloxy-3,3',7-trimethoxyflavone) were synthesized in our laboratory by the method described elsewhere (H. Ishitsuka, H. Shirai, I. Umeda, and Y. Suhara, British patent application 7912610, 1979). Both compounds were dissolved in ethanol before use and diluted with medium. [5,6-^{3}H]UTP (35 Ci/mmol; New England Nuclear Corp., Boston, Mass.), [8-^{3}H]GTP (14 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), and [5-^{3}H]uridine (41.3 Ci/mmol; New England Nuclear) were obtained through the Japan Radioisotope Association. Actinomycin D was purchased from the Nippon Merck Co.

RESULTS

Antiviral spectrum. Ro 09-0179 was active against all picornaviruses except mengovirus. It inhibited virus cytopathic effect caused by all of the serotypes of rhinovirus (20 types), echovirus (type 7, 11, 12, and 19), coxsackievirus (A21 and B1), and poliovirus (type 1 Sabin) included in this study. The MIC_{50} against these picornaviruses ranged from 0.03 to 0.5 μg/ml in HeLa or WI-38 cells. The MIC_{50} and MIC_{90} of 20 types of rhinoviruses were 0.1 and 0.3 μg/ml, respectively. The effective concentrations of Ro 09-0179 were in a similar range when the HeLa cells were infected with rhinoviruses at MOIs of 0.02 to 2.0. In contrast, at a concentration of 10 μg/ml Ro 09-0179 was inactive against influenza virus A (NWS), respiratory syncytial virus, vesicular stomatitis virus, herpes simplex virus (type 1), and vaccinia virus. At a concentration of 100 μg/ml, it was inactive against various strains of bacteria and fungi, such as *Escherichia coli, Pseudomonas aeruginosa, Serratia marcescens, Bacillus subtilis, Sarcina lutea, Staphylococcus aureus, Mycobacterium smegma, Candida albicans, Aspergillus niger, Penicillium chrysogenum, Saccharomyces cerevisiae, Trichoderma viride*, and *Rhizopus arrhizus*.

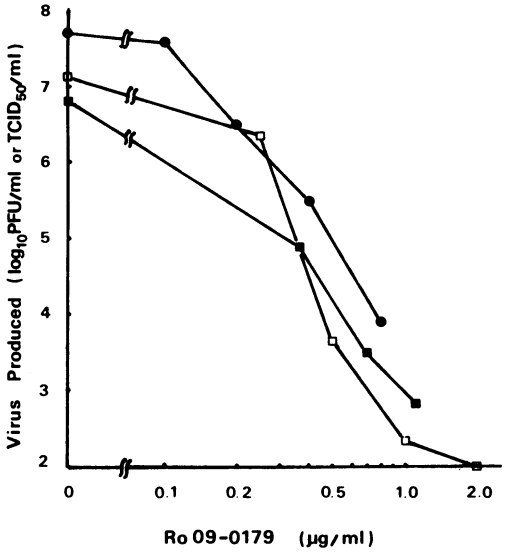

FIG. 2. Inhibition of viral replication by Ro 09-0179. HeLa cells were infected with rhinovirus type 2, rhinovirus 1A, or coxsackievirus B1 at an MOI of 0.1. The cells were then cultured with Ro 09-0179 for 1 or 2 days for rhinovirus- or coxsackievirus-infected cells, respectively. Symbols: ■, rhinovirus type 2; □, rhinovirus 1A; ●, coxsackievirus B1.

As determined by microscopic examination, the compound was well tolerated by HeLa and WI-38 cells over a 5-day exposure; however, at concentrations over 10 μg/ml, cells appeared somewhat thinner than controls. The 50% cytotoxic dose was about 15 μg/ml in a 2-day culture.

These results indicate that Ro 09-0179 has a high degree of specificity for picornaviruses, although it did not inhibit the growth of mengovirus.

Yield reduction of picornaviruses. The effect of Ro 09-0179 on the yield of several types of picornaviruses was studied. Ro 09-0179 was added at 1 h after the inoculation of virus, and total yields of the viruses replicating in the cultures were assayed 2 or 3 days later. Ro 09-0179 reduced the yields of rhinoviruses type 1A and 2 and coxsackievirus B1 by 10^4 to 10^5 at a concentration of about 1 μg/ml in a HeLa cell culture (Fig. 2). Ro 09-0179 also effected a yield reduction of four serotypes of Echoviruses in WI-38 cell cultures by 10^2 to 10^4 at concentrations of 0.5 and 2 μg/ml without any cytotoxicity (Table 1).

TABLE 1. Reduction of yield of echovirus from Ro 09-0179-treated cells in culture[a]

Ro 09-0179 added (μg/ml)	Reduction of yield (log_{10}) of echovirus type:			
	7	11	12	19
0.5	2.8	2.5	>3.6	3.1
2.0	3.7	3.3	>3.6	3.6

[a] Monolayers of WI-38 cells were infected with four serotypes of echovirus (MOI, 0.2 to 20) for 1 h and then cultured with Ro 09-0179 at a concentration of 0.5 or 2 μg/ml for 10 h. After the cells and culture fluids were frozen and thawed twice, the total virus content of the culture was assayed in WI-38 cell cultures.

In vivo antiviral activity. The diacetyl derivative of Ro 09-0179, Ro 09-0298, was examined for its activity against lethal infections of mice with coxsackievirus B1. Mice were infected intraperitoneally with about 10 LD_{50} of coxsackievirus B1, and then Ro 09-0298 was administered orally twice daily in nine doses. All of the control mice died within 4 days after the infection, whereas Ro 09-0298 administered orally increased the survival rate significantly to 30, 40, and 50% at doses of 10, 20, and 40 mg/kg, respectively.

In coxsackievirus infection in mice, there was a viremia from the early stage of the infection to death. Ro 09-0298 was then examined for its activity against the viremia caused by coxsackievirus B1 (Table 2). Ro 09-0298 administered orally prevented viremia for 2 days after infection, although some of the treated mice developed viremia on day 3 and died later.

Stages susceptible to Ro 09-0179 during virus replication. Antiviral activity with respect to the time of addition of the agent after virus adsorption was measured in one-step growth curve experiments to determine what stages of virus replication were susceptible to inhibition by Ro 09-0179. HeLa cells were infected with rhinovirus type 2 at an MOI of 2 for 1 h at 33°C and then cultured with Ro 09-0179 (1 μg/ml) at different intervals. After 13 h, the total virus content of

TABLE 2. Inhibition of viremia caused by coxsackievirus B1[a]

Treatment	Virus titer (log_{10} PFU/ml of blood) at day after infection:		
	1	2	3
None	5.7	6.5	7.0
	6.2	6.7	6.3
	6.4	7.3	6.7
Ro 09-0298 (10 mg/kg)	<4	<4	<4
	<4	<4	6
	<4	<4	6.7
Ro 09-0298 (20 mg/kg)	<4	<4	<4
	<4	<4	<4
	<4	<4	5.7

[a] Groups of three mice were infected intraperitoneally with about 10 LD_{50} of coxsackievirus B1 and were treated orally four times with Ro 09-0298 at 1, 2, 5, and 19 h after infection. Fifteen microliters of blood was collected from the *fundus oculi* of each mouse 1, 2, and 3 days after infection for assaying its virus content.

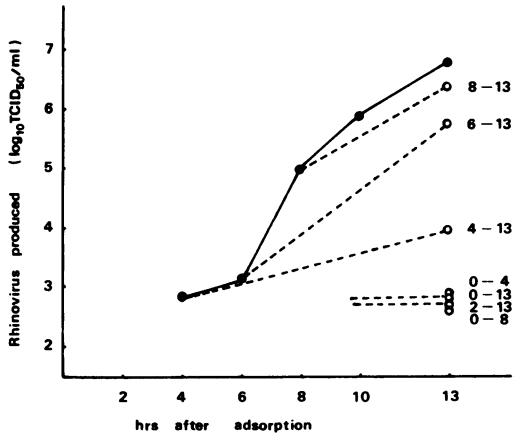

FIG. 3. Effect of discontinuous treatment with Ro 09-0179 on rhinovirus replication. HeLa cells (4×10^5) were infected with rhinovirus type 2 at an MOI of 2 for 1 h. Then the cells were washed and further cultured for 13 h. Ro 09-0179 (1 μg/ml) was added at the time indicated on the figure.

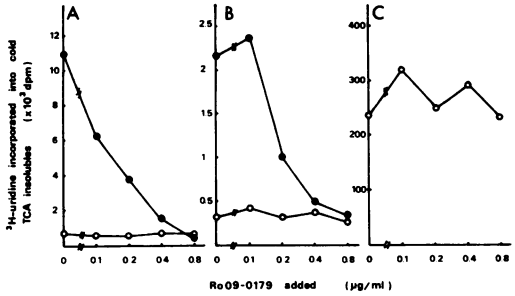

FIG. 5. Effect of Ro 09-0179 on RNA synthesis. (A and B) HeLa cells (4×10^5) were infected with coxsackievirus B1 (MOI, 20) or rhinovirus type 2 (MOI, 2). The incorporation of uridine was carried out as described in the legend to Fig. 4 for 4 or 10 h for coxsackievirus- (A) or rhinovirus (B)-infected cells, respectively. Then, cold TCA insolubles in the cells were counted for radioactivity. (C) HeLa cells were incubated with Ro 09-0179 in the presence of [5-^{3}H]uridine (1 μCi/ml) for 4 h at 37°C. Then, cold TCA insolubles in the cells were counted for radioactivity. Symbols: ●, infected cell; ○, uninfected cell.

the cultures was determined. The progeny virus began to appear at about 6 to 8 h and reached a peak level (10^7 $TCID_{50}$/ml) at 13 h after virus adsorption, whereas viral replication was completely inhibited by the presence of Ro 09-0179 during the incubation (Fig. 3). This complete inhibition was observed when the agent was added within 2 h after virus adsorption. Although the inhibition was still evident when the agent was added at 4 h, little effect was observed when the agent was added at 6 h after virus adsorption. On the other hand, when the infected cells were treated with Ro 09-0179 during the first 4 h of the culture, virus production was also inhibited completely. These results suggest that the critical stage for the inhibition of rhinovirus replication by Ro 09-0179 is 2 to 4 h after virus adsorption and that the inhibitory action is irreversible.

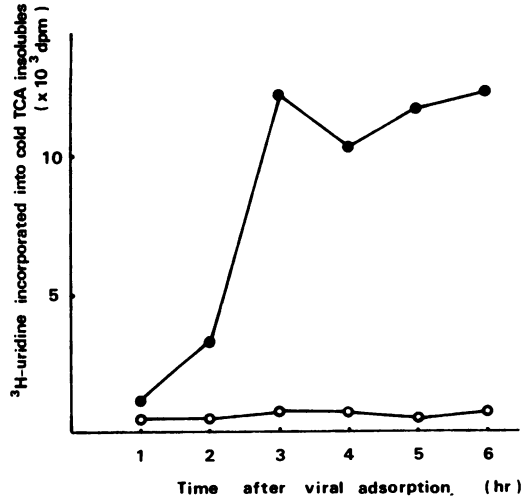

FIG. 4. Inhibition of coxsackievirus RNA synthesis. Monolayers of HeLa cells (4×10^5) were infected with coxsackievirus B1 at an MOI of 20 for 1 h. Then the cells were washed and incubated with or without 1 μg of Ro 09-0179 per ml in the presence of actinomycin D (5 μg/ml) and [5-^{3}H]uridine (1 μCi/ml). After various times of incubation, the cells were harvested, and cold TCA insolubles were counted for radioactivity. Symbols: ○, without Ro 09-0179; ●, with Ro 09-0179.

Viral RNA synthesis in the cells. The effect of Ro 09-0179 on viral RNA synthesis was examined. Viral RNA synthesis in the infected cells was monitored by the incorporation of [5-^{3}H]uridine into cold TCA insolubles in the presence of actinomycin D. Cells infected with coxsackievirus B1 incorporated [5-^{3}H]uridine during 3 h after virus adsorption, whereas incorporation was completely inhibited by the addition of Ro 09-0179 to the culture medium (Fig. 4). Figure 5 shows the dose-response effect of Ro 09-0179 on RNA synthesis. Ro 09-0179 completely inhibited the RNA synthesis of both rhinovirus and coxsackievirus at doses of 0.4 to 0.8 μg/ml, whereas it did not show any inhibitory effects on cellular RNA synthesis at these same concentrations (Fig. 5C).

Viral RNA synthesis by RNA polymerase complex. Ro 09-0179 was further examined for its effect on viral RNA synthesis in a cell-free system using the RNA polymerase complex, which appears in cells infected with picornaviruses and contains the virus template RNA and

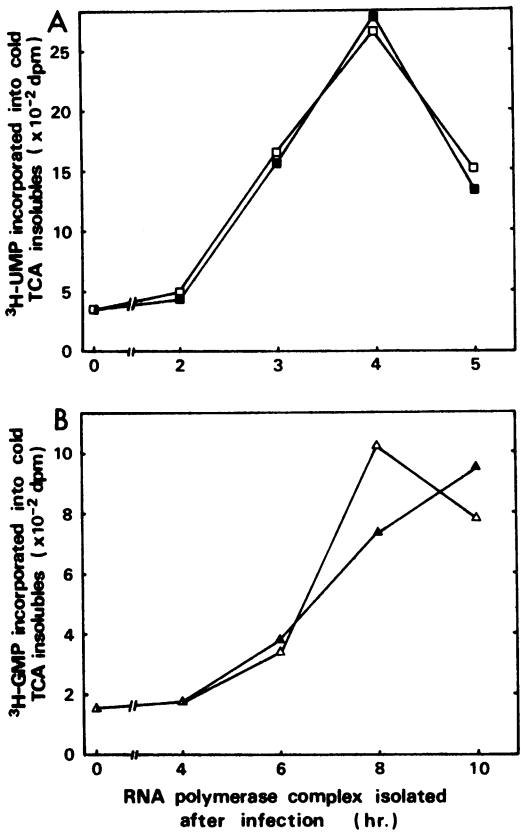

FIG. 6. Inhibition by Ro 09-0179 of viral RNA synthesis by the RNA polymerase complex. The RNA polymerase complex was isolated from HeLa cells infected either with rhinovirus type 2 (B) or coxsackievirus B1 (A) after various times of incubation. RNA synthesis by these RNA polymerase complexes was carried out in the presence or absence of 1 μg of Ro 09-0179 per ml as described in the text. (A) Presence (□) or absence (■) of Ro 09-0179; (B) presence (△) or absence (▲) of Ro 09-0179.

RNA polymerase (2). Figure 6 illustrates the complex-dependent viral RNA synthesis in the presence or absence of Ro 09-0179. Viral RNA synthesis by the complex isolated from infected cells peaked at 3 to 5 h and 6 to 10 h after infection with coxsackievirus B1 and rhinovirus type 2, respectively. Ro 09-0179 did not show any inhibitory activity when the activities of the complexes isolated at various times after infection were compared.

In contrast to the above observation, Ro 09-0179 prevented infected cells from forming the RNA polymerase complex (Table 3). In these experiments, the infected HeLa cells were cultured with or without Ro 09-0179, and the fraction of the RNA polymerase complex was isolated at 4 h after coxsackievirus infection or 10 h after rhinovirus infection. The fraction of RNA polymerase complex isolated from control cultures synthesized viral RNA, whereas that isolated from the cells treated with Ro 09-0179 did not have such activity (Table 3).

DISCUSSION

The present studies showed that a natural flavonoid, Ro 09-0179, has antiviral activity without showing cytotoxicity in tissue culture against all of the serotypes of rhinovirus, echovirus, coxsackievirus, and polioviruses, but not against other RNA and DNA viruses, indicating a high degree of specificity for picornaviruses. Like other antipicornavirus agents, such as guanidine and 2-(α-hydroxybenzyl)-benzimidazole (HBB) (8), Ro 09-0179 was inactive against mengovirus, one of the picornaviruses. These agents may recognize a difference in the fine structure or mechanism of replication among picornaviruses.

Unlike Ro 09-0179, its diacetyl derivative (Ro 09-0298), administered orally, protected mice from viremia and lethal infections with coxsackievirus B1. Both compounds had the same activity in tissue culture, but Ro 09-0179 was poorly absorbed from the intestinal tract, and its antiviral activity in mice was marginal (personal observation). In preliminary toxicological studies, Ro 09-0298 administered to mice orally at daily

TABLE 3. Inhibition of the formation of the RNA polymerase complex by Ro 09-0179 in infected cells[a]

Reaction mixture	RNA synthesis (dpm) by the RNA polymerase complex prepared from cells:		
	Not infected	Infected	Infected and treated with Ro 09-0179
Rhinovirus type 2 infection			
Complete	264	1,109	194
Substrate (−CTP, −UTP)	257	502	221
Coxsackievirus B1 infection			
Complete	107	1,021	418
Substrate (−CTP, −UTP)	105	580	176

[a] Monolayers of HeLa cells (8×10^7) were infected either with coxsackievirus (MOI of 20) or by rhinovirus HGP (MOI of 2) for 60 min. Then the cells were cultured with or without 1 μg or Ro 09-0179 per ml in the presence of 5 μg of actinomycin D per ml for 10 or 4 h for rhinovirus- or coxsackievirus-infected cells, respectively. Thereafter, the RNA polymerase complexes were prepared from the cells, and their ability to synthesize RNA was assayed.

doses of 1 g/kg for 1 week did not elicit toxic symptoms.

Several flavone derivatives are known to have virucidal activity against enveloped viruses, including herpes virus, but not nonenveloped viruses (4). Although the data are not shown here, Ro 09-0179 inactivated herpes simplex virus type 1 at a quite high concentration (30 μg/ml), toxic for the growth of HeLa cells. However, Ro 09-0179 did not show any virucidal activity against the nonenveloped viruses, such as rhinoviruses and coxsackieviruses, even at concentrations 30 to 100 times higher than the MIC_{50} for this agent against these particular viruses. Ro 09-0179 must, therefore, exert its activity through a mechanism other than direct inactivation of picornaviruses. In this respect, Ro 09-0179 differs from compounds such as 2,4'-dichloroflavone (3) and 4'-ethoxy-2'-hydroxy-4,6-dimethoxychalcone (Ro 09-0410) (6). These are exclusively active against rhinoviruses but through different mechanisms than Ro 09-0179. Ro 09-0410 binds to rhinovirus and makes it inactive (6).

Studies on the time of the addition of Ro 09-0179 to rhinovirus-infected cell cultures suggest that the agent might inhibit some process of viral replication in the middle stage of the latent period in an irreversible fashion (Fig. 3). In the process, Ro 09-0179 inhibited viral RNA synthesis in the cell culture. Further studies on RNA synthesis with the RNA polymerase complex revealed that Ro 09-0179 might not inhibit viral RNA chain elongation. Since the RNA polymerase complex was not formed in the presence of the agent in infected cells, it must inhibit viral replication before the initiation of viral RNA synthesis. Therefore, all of the molecular events which subsequently follow the formation of the RNA polymerase complex might be inhibited. Similar explanations for the antiviral activities of guanidine and HBB have been offered (1). Events related to the susceptible stage may include uncoating, the synthesis of viral precursor protein, the cleavage of the protein, and the initiation of viral RNA synthesis. The least probable mechanism of Ro 09-0179 is that the agent might prevent viral uncoating, because it could inhibit viral replication even when added at 4 h after virus adsorption. The most characteristic properties of the replication of picornaviruses, against which Ro 09-0179 is selectively active, are the participation of the RNA polymerase complex and the viral genome protein (7). The antiviral mechanism may be involved in the function or formation of these processes. Further details remain to be investigated.

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