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# Comparative Studies on the Modes of Action of the Antirhinovirus Agents Ro 09-0410, Ro 09-0179, RMI-15,731, 4',6-Dichloroflavan, and Enviroxime

Y. NINOMIYA, M. AOYAMA, I. UMEDA, Y. SUHARA, AND H. ISHITSUKA*

*Nippon Roche Research Center, Kajiwara-200, Kamakura City, Kanagawa, Japan*

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**Modes of action of five antirhinovirus agents were compared. Ro 09-0410, 4',6-dichloroflavan, and RMI-15,731 were active preferentially against human rhinovirus. Serotypes of the virus varied in their susceptibility to these three agents, whereas Ro 09-0179 and enviroxime showed activity against all the serotypes of the virus tested to date. Ro 09-0410, RMI-15,731, and 4',6-dichloroflavan inactivated the virus directly, although 4',6-dichloroflavan did so only slightly. Inactivation by 4',6-dichloroflavan and RMI-15,731 was associated with the binding of the agents to the virus, since the infectivity, reduced by exposure to the agents, was restored to the original level by extraction of the agents with chloroform. The binding of [$^3$H]Ro 09-0410 to human rhinovirus type 2 was inhibited by unlabeled Ro 09-0410, 4',6-dichloroflavan, and RMI-15,731 but not by Ro 09-0179 or enviroxime. Furthermore, subtypes resistant to both 4',6-dichloroflavan and RMI-15,731 showed cross-resistance to Ro 09-0410 and vice versa. On the other hand, sublines resistant to these three agents were not cross-resistant to Ro 09-0179 or enviroxime. These results indicate (i) that Ro 09-0410, 4',6-dichloroflavan, and RMI-15,731 exert their activities through the same mode of action, namely, binding to or interaction with some specific site on the viral capsid protein, and (ii) that the binding or interaction sites for these three agents are either the same or very close to each other.**

As reported previously, an antirhinovirus chalcone, Ro 09-0410 (4'-ethoxy-2'-hydroxy-4,6'-dimethoxy chalcone) (Fig. 1) directly inactivated only human rhinoviruses (HRV) (4). It bound to HRV at the specific site on the capsid protein not found on degraded HRV or viruses insensitive to the agent and stabilized the virus particles in such a way that HRV bound to the agent resisted the conformational change in virion size induced by low pH or elevated temperature (56°C) (11). We suggested that Ro 09-0410 interfered with the conformational change occurring during the uncoating process in the cells (11).

Antiviral agents, 4',6-dichloroflavan (2) and RMI-15,731 (1-[5-tetradecyloxy-2-furanyl]-ethanone) (1), seem to have a mode of action against HRV similar to that of Ro 09-0410. These agents are active preferentially against HRV but not against other viruses. Furthermore, RMI-15,731 inactivated HRV directly (1), whereas 4',6-dichloroflavan bound to some extent to HRV (2). On the other hand, enviroxime (LY122772; anti-6-[(hydroxyimino)-phenylmethyl]-1-[(1-methylethyl)sulfonylimidazol-2-amine) (3) and Ro 09-0179 (4',5-dihydroxy-3,3',7-trimethoxyflavone) (5) exhibited different modes of action, possibly inhibiting viral replication at the process of viral RNA synthesis. The structures of these agents are shown in Fig. 1.

In the present study, we examined the antirhinovirus activities of the five agents referred to above and their abilities to interact with HRV type 2. These studies indicate that Ro 09-0410, RMI-15,731, and 4',6-dichloroflavan exert their activity by the same mode of action, binding to or interacting with a specific site on the virus capsid. This has been further confirmed by the fact that HRV (type 2) sublines resistant to 4',6-dichloroflavan and RMI-15,731 were cross-resistant to Ro 09-0410 and vice versa.

* Corresponding author.

## MATERIALS AND METHODS

**Cells and viruses.** HeLa cells (Bristol strain) were grown and used for viral titration by a plaque assay method as described elsewhere (5). HRV and guinea pig anti-HRV type 2 serum (ATCC VR-1112 AS/GP) were purchased from the American Type Culture Collection, Rockville, Md. Sublines of HRV type 2 which are resistant to each of the antivirus agents used in this study were isolated by continuous passage in HeLa cell culture in the presence of sublethal concentrations of the antiviral agents. Briefly, HeLa cells were infected with HRV type 2 in Eagle minimum essential medium containing 2% fetal calf serum, 1% tryptose phosphate broth, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) buffer (pH 7.2), 50 μg of streptomycin sulfate per ml, 50 U of penicillin G per ml, and 0.33, 1, 3, or 9 50% inhibitory concentrations ($IC_{50}$s) of the antiviral agents. After incubation at 33°C for 4 or 5 days, cells showing cytopathogeneic effects at the highest $IC_{50}$ of an agent were harvested and homogenized. This transfer through increasing concentrations of the antiviral agent was repeated several times. Finally, sublines resistant to elevated concentrations of the agents were isolated as single plaques in monolayers of HeLa cells overlaid with Eagle minimum essential medium containing 5% fetal calf serum, 1% tryptose phosphate broth, 10 mM HEPES buffer (pH 7.2), 50 μg of DEAE-dextran per ml, 10 mM $MgCl_2$, 50 μg of streptomycin sulfate per ml, 50 U of penicillin G per ml, 0.8% Noble agar, and the antiviral agents.

**Chemicals.** Ro 09-0410 (4'-ethoxy-2'-hydroxy-4,6'-dimethoxychalcone) and Ro 09-0179 (4',5-dihydroxy-3,3'-7-trimethoxyflavone) used in this report were synthesized as described by M. Fujiu, H. Ishitsuka, and Y. Suhara, U.K. patent 7902907, January 1979, and H. Ishitsuka, H. Shirai, I. Umeda, and Y. Suhara, U.K. patent 7912610, April 1979, respectively. [4-Methoxy-$^3$H]Ro 09-0410 (2 Ci/mmol) was

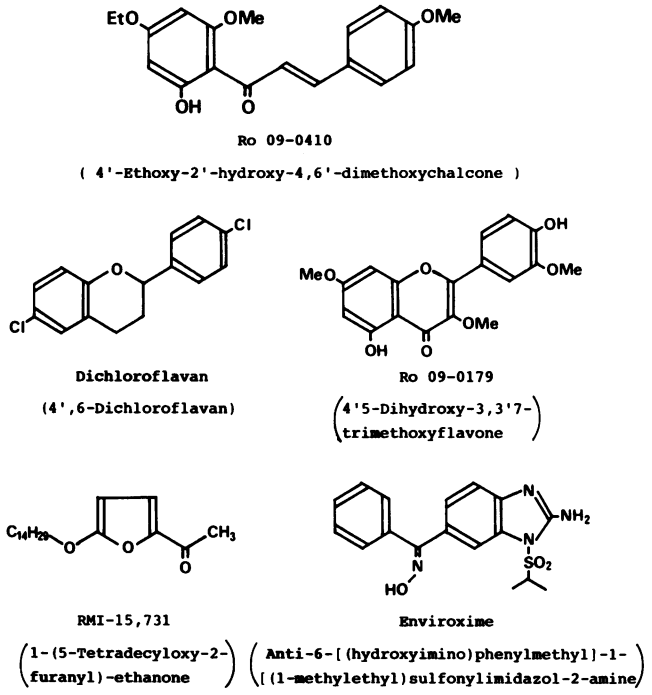


FIG. 1. Chemical structures of antirhinovirus agents.

synthesized from 2',4-dihydroxy-4'-ethoxy-6'-methoxychalcone by methylation in acetone with [*methyl*-$^3$H]dimethylsulfate (4 Ci/mmol, NET 079), purchased from New England Nuclear Corp., Boston, Mass., and purified to a single spot by thin-layer chromatography. We synthesized 4',6-dichloroflavan as described by Bachelor et al, U.K. patent 10251, March 1978. Synthesis of enviroxime (LY122772; anti-6-[(hydroxyimino)phenyl-methyl]-1-[(1-methylethyl)sulfonylimidazol-2-amine) has been described elsewhere (13). RMI-15,731 (MDL-15,731; 1-[5-tetradecyloxy-2-furanyl]-ethanone) (1) was kindly supplied by W. L. Albrecht, Merrell Dow Research Institute, Cincinnati, Oh. Chloroform (analytical grade) used for extraction of the antiviral agents bound to HRV was purchased from Wako Pure Chemicals Co. Ltd., Tokyo, Japan.

**Determination of $IC_{50}$.** The $IC_{50}$ was determined as described previously (5). Briefly, a suspension of HeLa cells ($6 \times 10^4$ cells) was mixed with virus (about $5 \times 10^3$ PFU), and the mixture was immediately plated into a microtest plate which contained the serially diluted compound to be tested. The cells were then cultured at 33°C for 2 to 4 days. Cytopathogeneic effect was observed after staining the residual cells with crystal violet. The $IC_{50}$ was expressed as the concentration at which the cytopathogeneic effect was inhibited by 50% as compared with the control.

**Virus inactivation and recovery by chloroform.** HRV type 2 ($10^5$ to $10^7$ PFU/ml) was incubated with or without antiviral agents (0.5 μg/ml) in Eagle minimum essential medium containing 20 mM HEPES buffer (pH 7.2) at 33°C for 60 min. Thereafter, the virus suspensions were diluted 10-fold serially, and residual virus titer was determined by a plaque assay method (5). For extraction of the antiviral agents, the virus suspension was mixed with an equal volume of cold chloroform, the mixture was shaken vigorously for 1 min at 4 or 33°C and centrifuged at $650 \times g$ for 3 min, and the aqueous supernatant was pooled for titration of its residual infectivity.

**Sucrose gradient centrifugation.** Free [$^3$H]Ro 09-0410 was separated from the bound agent by gradient centrifugation. Linear gradients containing 15 to 35% sucrose in 0.02 M HEPES buffer (pH 7.2)–1 M NaCl–1 mM EDTA–1 mM dithiothreitol were used at 4°C. Gradients of 4.5 ml containing reaction mixture with [$^3$H]Ro 09-0410, HRV type 2, and other antiviral agents were centrifuged for 60 min at 45,000 rpm in a Spinco SW50.1 rotor. The tubes containing the gradients were then punctured at the bottom, and the radioactivity in 4-drop fractions was determined by scintillation counting.

## RESULTS

**Inhibition of viral cytopathogeneic effect by antiviral agents.** The activities of Ro 09-0410, Ro 09-0179, enviroxime, 4',6-dichloroflavan, and RMI-15,731 against various HRV serotypes expressed as $IC_{50}$s are summarized in Table 1. The data show that Ro 09-0179 and enviroxime were active against all types of HRV at concentration under 0.03 to 0.3 and 0.009 to 0.11 μg/ml, respectively. Although highly active against some HRV serotypes, Ro 09-0410, 4',6-dichloroflavan, and RMI-15,731 exhibited low activity against others.

**Direct inactivation of HRV.** The most interesting feature of Ro 09-0410 and RMI-15,731 was their ability to inactivate HRV (1, 4). The capacity of Ro 09-0410 to inactivate the virus correlated well with the $IC_{50}$ of this agent (4). Serotypes of HRV susceptible to the agent in the $IC_{50}$ assay were inactivated by RMI-15,731 as well as by Ro 09-0410 (Table 2). 4',6-Dichloroflavan inactivated HRV type 16 only, although it was highly active against other serotypes by the $IC_{50}$ assay (Table 1). None of the serotypes was inactivated by either Ro 09-0179 or enviroxime (Table 2).

As reported previously, the infectivity of HRV type 2, reduced by exposure to Ro 09-0410, was completely reversible when the agent was extracted with chloroform (11). The infectivity of HRV type 2 was restored by extraction of both RMI-15,731 and Ro 09-0410 (Table 3). Similarly, the infec-

TABLE 1. Antirhinovirus activity of five antiviral agents

| HRV serotype | Antiviral activity ($IC_{50}$ [μg/ml]) of[a]: | | | | |
|---|---|---|---|---|---|
| | Ro 09-0410 | Dichloro-flavan | RMI-15731 | Ro 09-0179 | Enviroxime |
| 1B | 0.44 | 0.016 | 0.6 | 0.15 | 0.074 |
| 2 | 0.0055 | 0.049 | 0.045 | 0.1 | 0.012 |
| 3 | 0.89 | >4 | >0.8 | 0.1 | 0.074 |
| 4 | >4 | >4 | | 0.3 | 0.025 |
| 9 | 0.049 | 0.3 | 0.4 | 0.1 | 0.049 |
| 16 | 0.015 | 0.006 | 0.06 | 0.08 | 0.009 |
| 21 | <0.002 | 0.3 | 0.02 | 0.3 | 0.016 |
| 29 | 0.049 | <0.002 | 0.03 | 0.15 | 0.049 |
| 30 | 0.016 | 0.049 | 0.12 | 0.3 | 0.11 |
| 31 | 1.3 | 0.002 | 0.065 | | 0.049 |
| 35 | 0.049 | >4 | 0.3 | | 0.049 |
| 49 | 0.002 | 0.16 | 0.01 | 0.15 | 0.049 |
| MIC (μg/ml)[b] | 0.023 | 0.049 | 0.062 | 0.12 | 0.032 |
| Cytotoxic concn[c] | 18 | 25 | 21 | 7.5 | 3.6 |

[a] Each $IC_{50}$ was the mean of two to four experiments.
[b] The concentration inhibiting 50% of rhinovirus serotypes tested was expressed as cumulative MIC.
[c] The 50% cytotoxic concentration, at which growth of HeLa cells for 3-day culture periods was inhibited by 50%, was determined.

TABLE 2. Direct inactivation of virus

| HRV serotype | Reduction of infectivity ($log_{10}$ PFU/ml) by[a]: | | | | |
|---|---|---|---|---|---|
| | Ro 09-0410[b] | Dichloro-flavan | RMI-15731 | Ro 09-0179 | Enviroxime |
| 1A | 0.61 | 0.32 | ND[c] | ND | 0.01 |
| 1B | 0.65 | 0.77 | ND | ND | −0.05 |
| 2 | 3.1 | 0.30 | 2.9 | 0.06 | −0.03 |
| 9 | 3.6 | 0.70 | −0.01 | 0.02 | ND |
| 16 | 2.2 | 1.0 | 0.20 | −0.02 | −0.02 |
| 30 | 3.4 | −0.06 | 3.5 | 0.14 | 0.0 |
| 31 | 0.12 | 0.20 | ND | ND | 0.03 |
| 34 | 3.1 | 0.1 | 2.3 | −0.01 | 0.08 |

[a] Each value is the mean of two to four experiments.
[b] Each drug was tested at a concentration of 0.5 μg/ml.
[c] ND, Not done.

tivity of HRV type 16 was restored by extraction of these agents and 4′,6-dichloroflavan. The fact that the extraction of Ro 09-0410 from HRV type 16 required a temperature of 33°C suggests that this agent may bind more tightly to HRV type 16 than to the others.

**Competition of the antiviral agents with Ro 09-0410 for the Ro 09-0410 binding site on HRV type 2.** As shown previously, [$^3$H]Ro 09-0410 binds to the native virion of HRV type 2 at a specific capsid site (11). In this study we evaluated the capacities of RMI-15,731 and 4′,6-dichloroflavan to compete with Ro 09-0410 for binding at this site. HRV type 2 was exposed to [$^3$H]Ro 09-0410 at a concentration of 0.013 μg/ml. After incubation at 33°C for 60 min, the amount of [$^3$H]Ro 09-0410 bound to the virus was assayed by centrifugation through a linear sucrose gradient. A distinct peak of [$^3$H]Ro 09-0410 was observed at the position expected for the virus particles (11), indicating that Ro 09-0410 binds to the virus (Fig. 2A). When HRV type 2 was incubated with [$^3$H]Ro 09-0410 in the presence of an excess amount of unlabeled Ro 09-0410 at a ratio of 1:38, the binding of the radioactive compound was inhibited (Fig. 2B). Binding was also inhibited by 4′,6-dichloroflavan (0.5 μg/ml) and to a lesser extent by RMI-15,731 (0.5 μg/ml), at which the molar ratios of [$^3$H]Ro 09-0410 to these agents were 1:32 and 1:37, respectively (Fig. 2C and D). These results suggest that either the binding sites for these three agents on HRV type 2 are very similar, differences in chemical structure not withstanding, or the binding site for Ro 09-0410 is changed topologically by interaction with the other two agents. As for Ro 09-0179 and enviroxime, these agents did not compete for the binding of [$^3$H]Ro 09-0410 (Fig. 2E and F), indicating that their modes of action are completely different from those of the other three.

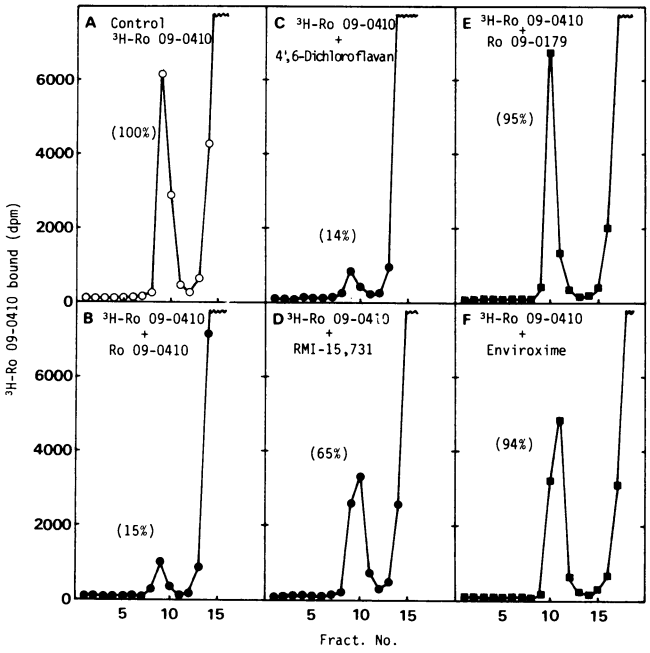


FIG. 2. Inhibition of binding of [$^3$H]Ro 09-0410 to HRV by antirhinovirus agents. HRV type 2 ($10^8$ PFU/ml) was incubated with [$^3$H]Ro 09-0410 (0.013 μg/ml, 2.0 mCi/μmol) or [$^3$H]Ro 09-0410 and the unlabeled antirhinovirus agent described in the figure (0.5 μg/ml) at 33°C for 60 min in Eagle essential medium containing 30 mM HEPES (pH 7.2). The mixture was subjected to centrifugation in a sucrose gradient. The radioactivity in fractions was determined by a scintillation counter. The amount of [$^3$H]Ro 09-0410 bound to HRV type 2 was expressed as a percentage of the control ([$^3$H]Ro 09-0410 alone) in parentheses.

TABLE 3. Recovery of the virus infectivity by chloroform extraction

| Expt and treatment (μg/ml) | Virus titer ($log_{10}$ PFU/ml) | | |
|---|---|---|---|
| | Before $CHCl_3$ extraction | After $CHCl_3$ extraction at: | |
| | | 4°C | 33°C |
| Expt 1, HRV type 2 | | | |
| Control | 7.4 | 7.3 | |
| Ro 09-0410 (0.5) | 4.2 | 7.5 | |
| 4′,6-Dichloroflavan (10) | 7.4 | 7.3 | |
| RMI-15731 (10) | 4.4 | 7.3 | |
| Expt 2, HRV type 2 | | | |
| Control | 7.7 | 7.7 | |
| Ro 09-0410 (0.5) | 4.6 | 7.6 | |
| 4′,6-Dichloroflavan (10) | 7.7 | 7.3 | |
| RMI-15731 (1) | 5.3 | 7.3 | |
| Expt 3, HRV type 16 | | | |
| Control | 6.3 | 6.4 | 6.5 |
| Ro 09-0410 (0.5) | <3.0 | <3.0 | 5.3 |
| 4′,6-Dichloroflavan (10) | 4.8 | 5.7 | 6.1 |
| RMI-15731 (1) | 5.2 | 6.4 | 6.3 |
| Expt 4, HRV type 16 | | | |
| Control | 6.4 | 6.5 | |
| Ro 09-0410 (0.5) | <3.0 | 3.1 | 5.9 |
| 4′,6-Dichloroflavan (10) | 3.0 | 6.4 | |
| RMI-15731 (10) | 4.3 | 6.0 | |

**Studies on cross-resistance of HRV type 2 sublines resistant to the individual test agents.** Subtypes of HRV type 2, NR2-410, NR2-DCF, and NR2-RMI, which are resistant to Ro 09-0410, 4′,6-dichloroflavan, or RMI-15,731, were acquired by serial passages of the virus in the presence of sublethal concentrations of these agents. These sublines were 9 to 370 times less susceptible to the agents than the original subtypes, whereas their antigenicity detected by neutralizing antibody remained unchanged (Table 4). Except for NR2-RMI and NR2-DCF, sublines of HRV resistant to Ro 09-0410, 4′,6-dichloroflavan, or RMI-15,731 were resistant to the other two agents. This supports the suggestion that the modes of action of these three agents are essentially the same. On the other hand, Ro 09-0179 and enviroxime were still active against the resistant sublines, indicating that their

TABLE 4. Cross-resistance of HRV type 2 subtypes to antiviral agents

| HRV subtype resistant to antiviral agent | Susceptibility ($IC_{50}$ [μg/ml])[a] | | | | | Neutralization factor[c] |
|---|---|---|---|---|---|---|
| | Ro 09-0410 | RMI-15,731 | 4',6-Dichloroflavan | Ro 09-0179 | Enviroxime | |
| Original strain HRV type 2 | 0.0038 | 0.053 | 0.053 | 0.093 | 0.011 | 13,920 |
| NR2-410[b] | 1.4 (370) | 0.22 (4.2) | >2 (>38) | 0.14 (1.5) | 0.0083 (0.75) | 13,440 |
| NR2-RMI[b] | 0.088 (23) | 0.47 (8.9) | 0.094 (1.8) | 0.099 (1.1) | 0.014 (1.3) | 15,360 |
| NR2-DCF[b] | 0.088 (23) | 0.053 (1.0) | >2 (>38) | 0.14 (1.5) | 0.016 (1.5) | 14,080 |

[a] The degree of increase of the drug resistance as compared with the susceptibility of the original strain was expressed as fold increase in the parenthesis.
[b] NR2-410, NR2-RMI, and NR2-DCF are sublines resistant to Ro 09-0410, RMI-15, 731, and 4',6-dichloroflavan, respectively.
[c] The reciprocal of the dilution of anti-HVR type 2 serum required for neutralization of the infectivity of each HRV type 2 subline.

modes of action are different from those of Ro 09-0410, RMI-15,731, and 4',6-dichloroflavan.

## DISCUSSION

The present study shows that antiviral agents, Ro 09-0410, 4',6-dichloroflavan, and RMI-15,731, which are active exclusively against HRV, exert the activity through the same mode of action, whereas Ro 09-0179 and enviroxime, which are active against most picornaviruses, have different modes of action. The former group was suggested to bind to or interact with similar sites on the capsid and make the virus inactive.

A possible obstacle to acceptance of the conclusion that Ro 09-0410, RMI-15,731, and 4',6-dichloroflavan have the same mode of action is that these three agents differ significantly with respect to their activities against susceptible subtypes of HRV (Table 1). Conformation of the binding site on the capsid may be slightly different among HRV serotypes. The conformation on some serotypes may favor one agent, but that on other serotypes may favor the other agents. Another obstacle to acceptance of the conclusion is that the virus-inactivating activity of 4',6-dichloroflavan is not associated with the activity measured by the $IC_{50}$ assay (Tables 1 and 2). A possible explanation is that binding of either Ro 09-0410 or RMI-15,731 occurs irreversibly in an aqueous solution, whereas 4',6-dichloroflavan interacts somewhat reversibly with HRV type 2 but irreversibly with HRV type 16. In the $IC_{50}$ assay, the agents are present in culture medium throughout the assay. In the assay for virus inactivation, HRV ($10^6$ to $10^7$ PFU/ml) exposed to the agents (0.5, 1, or 10 μg/ml) was diluted to about a $10^{-3}$ to $10^{-5}$ level to avoid interference of the agents with titration of the residual virus. Therefore, agents which inactivate HRV through reversible interaction, could not show activity in this assay.

Studies on competition of the antiviral agents with Ro 09-0410 for the Ro 09-0410 binding site suggest that the site for Ro 09-0410 is at least partly common to that for RMI-15,731 and 4',6-dichloroflavan. It is also possible that the binding of RMI-15,731 and 4',6-dichloroflavan causes topological alteration of the capsid proteins to an extent that Ro 09-0410 can no longer bind to the virus. However, this is unlikely. The HRV sublines resistant to 4',6-dichloroflavan and RMI-15,731 were cross-resistant to Ro 09-0410 and vice versa, indicating that targets of these agents are the same capsid protein.

Instability of HRV at a pH below 6 or at 56°C is the most common characteristic that distinguishes it from other human picornaviruses (10, 12). Acid and heating at 56°C caused alteration in size, conformation, and antigenicity of the virus (6, 7, 9). The same type of alteration was observed in infected cells during the uncoating process (8). Since Ro 09-0410 that bound to the virus prevented it from alteration caused by treatment at pH 5 or 56°C, it was suggested that Ro 09-0410 stabilizes the virus particles in such a way that the virus does not proceed to the uncoating process (11). RMI-15,731 and 4',6-dichloroflavan may exert antiviral activities by a similar mechanism, and the capsid protein bound to these agents must be important for triggering the uncoating process in the replication of HRV. Further details remain to be investigated.

The activity of Ro 09-0179 and enviroxime was not associated with the capsid protein, to which the other three agents were bound. As reported previously (5), Ro 09-0179 interfered with replication of HRV type 2 at the process after uncoating and inhibited formation of the RNA polymerase complex, a machinery for viral RNA synthesis. Enviroxime also showed similar activity (C. Y. E. Wu, L. D. Nelson, B. R. Warren, and D. C. Delong, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, S128, p. 234). We tried to isolate sublines resistant to Ro 09-0179 and enviroxime but had no success. The sites of viral proteins with which Ro 09-0179 and enviroxime associate may be important for virus replication to an extent that amino acid sequences of the sites are genetically conservative and that mutation at these sites is lethal. Any other possible explanation is that these agents act on cellular functions required for virus replication.

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