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Direct and Specific Inactivation of Rhinovirus by Chalcone Ro 09-0410

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Studies of various analogs related to the antipicornavirus agent, 4′,5-dihydroxy-3,3′,7-trimethoxyflavone (Ro 09-0179), led to the identification of 4′-ethoxy-2′-hydroxy-4,6′-dimethoxychalcone (Ro 09-0410), a new and different type of antiviral agent. Ro 09-0410 had a high activity against rhinoviruses but no activity against other picornaviruses. Of 53 rhinovirus serotypes so far tested, 46 were susceptible to Ro 09-0410 in HeLa cell cultures. The concentration of Ro 09-0410 inhibiting 50% of the types of rhinovirus was about 0.03 μg/ml, whereas the 50% cytotoxic concentration was 30 μg/ml. Ro 09-0410 inactivated rhinoviruses in direct dose-, time-, and temperature-dependent fashion. Since infectivity, reduced by exposure to the agent, completely regained the original level by extraction of the agent with chloroform, the inactivation may be associated with the binding of the agent to some specific site of the rhinovirus capsid.

Ro 09-0179 (4′,5-dihydroxy-3,3′,7-trimethoxyflavone), a potent antipicornavirus agent, was isolated from a Chinese medicinal herb, Agastache Folium (*Agastache rugosa* Kuntze) (3). The discovery of this agent led to the synthesis of a series of derivatives and testing of their antiviral activities in tissue culture. Among hundreds of compounds prepared and evaluated, Ro 09-0410 (4′-ethoxy-2′-hydroxy-4,6′-dimethoxychalcone) (Fig. 1) emerged as an agent exclusively active against rhinoviruses.

The mode of action of Ro 09-0410 differed significantly from that of Ro 09-0179. The latter appeared to inhibit the replication of picornavirus at a stage between uncoating and the initiation of RNA synthesis in infected cells (3). On the other hand, Ro 09-0410 directly inactivated rhinoviruses. Subsequent studies showed that the infectivity of rhinoviruses was reduced by exposure to Ro 09-0410 but was regained completely by the extraction of the agent with chloroform, to which rhinovirus is resistant (1). While bound to the agent, rhinovirus was inactive. In this report, we describe the results of tissue culture studies on the antirhinovirus activity of Ro 09-0410.

(A part of this study was presented elsewhere [H. Ishitsuka, Y. T. Ninomiya, C. Ohsawa, T. Ohiwa, M. Fujiu, I. Umeda, H. Shirai, and Y. Suhara, Abstr. Int. Congr. Chemother. 12th, abstr. no. 318, 1981].)

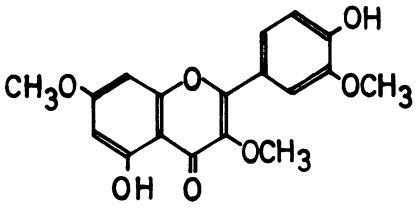
MATERIALS AND METHODS

Cells and viruses. HeLa (Bristol strain) and L-132 (human embryonic lung) 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 (human embryonic lung) cells were cultured with MEM containing 10% fetal calf serum and the antibiotics. Maintenance medium for virus infections consisted of MEM, 2% fetal calf serum, 1% TPB, and the antibiotics.

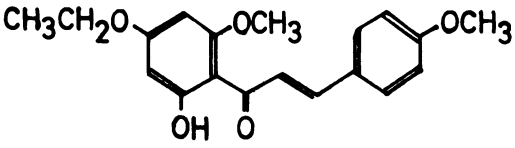
All rhinoviruses used in this study were purchased from the American Type Culture Collection, Rockville, Md., except type 2 (HGP), which was kindly supplied by R. Kohno, the National Institute of Health of Japan. Viruses were propagated in HeLa and WI-38 cells at 33°C. Poliovirus, echoviruses, coxsackieviruses, mengovirus, vesicular stomatitis virus, respiratory syncytial virus, herpes simplex virus, vaccinia virus, and influenza virus used in this study were propagated as described in the accompanying paper (3).

Assay of rhinovirus titer and estimation of MIC_{50}. The rhinovirus titer was assayed, and the 50% minimal inhibitory concentration (MIC_{50}) was estimated as described in the accompanying paper (3). The virus titer was expressed as plaque-forming units per milliliter. The MIC_{50} was expressed as the concentration at which the viral cytopathic effect was inhibited by about 50% as compared with the control.

Virus inactivation. Rhinovirus (10^5 to 10^7 PFU/ml) was incubated with or without the test compound in MEM containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) buffer (pH 7.4) at 33°C. Thereafter, the virus suspension was diluted 10-



Ro09-0179



Ro09-0410

FIG. 1. Structures of Ro 09-0179 and Ro 09-0410.

fold serially and assayed for remaining virus in HeLa cells, where the concentration of the test compound should be less than 10 ng/ml so as not to interfere with the titration. For the extraction of Ro 09-0410 from the virus exposed to the agent, the virus suspension was mixed with an equal volume of chloroform, and the mixture was shaken vigorously for 1 min at room temperature. The mixture was then centrifuged at 900 × *g* for 3 min, and the aqueous solution was removed for the purpose of titration.

Cytotoxicity. A suspension of HeLa cells (5×10^4) was plated into a multiplate (Nunc no. 168357), which contained MEM, 2% fetal calf serum, 2% TPB, and the twofold serially diluted compound to be tested. After 2 days of incubation at 37°C in a CO_2 (5%) incubator, the number of viable cells was counted. In the control culture, the number of HeLa cells increased three- to fourfold. The 50% cytotoxic dose was expressed as the concentration at which cell growth was inhibited by 50% as compared with the control culture.

Chemicals. Ro 09-0410 (4'-ethoxy-2'-hydroxy-4,6'-dimethoxychalcone) was synthesized in our laboratory by a method described elsewhere (M. Fujiu, Y. Suhara, and H. Ishitsuka, British patent application 7902907). The compound was dissolved in ethanol (1 mg/ml) for antiviral testing and in dimethoxysulfoxide (10 mg/ml) for cytotoxicity testing and diluted with medium before use. Actinomycin D was purchased from Nippon Merck. [5-^{3}H]uridine (41.3 Ci/mmol) was obtained from New England Nuclear Corp., Boston, Mass.

RESULTS

Specific activity to rhinovirus infection. The MIC_{50}s of Ro 09-0410 against rhinoviruses (53 types) were measured in HeLa cell cultures. The MIC_{50}s ranged from 0.001 to more than 10 μg/ml. Ro 09-0410 was highly active against many types of rhinoviruses, although rhinovirus types varied widely in susceptibility to the agent. The MIC_{50}s of several types, such as type 11, 18, 21, 49, and 51, were lower than 0.003 μg/ml, whereas those of several other types, such as type 4, 5, 45, 48, and 52 were higher than 3 μg/ml. Of 53 types so far tested, 46 types (87%) were susceptible under 3 μg/ml. The MIC_{50} and MIC_{90} of the susceptible types of rhinoviruses were about 0.03 and 0.4 μg/ml, respectively. Similar results were observed in other tissue culture systems using human embryonic lung cells, L-132 and WI-38. On the other hand, Ro 09-0410 was well tolerated by HeLa cells. The 50% cytotoxic dose, at which the growth of HeLa cells was inhibited for 2 days by 50%, was 30 μg/ml so that the antiviral activity was selective by a factor (the cytotoxic dose/MIC_{50}) of about 1,000 (10 to 10,000).

In contrast, Ro 09-0410 was inactive against poliovirus (type 1), coxsackievirus (type A21 and B1), echovirus (type 7, 11, 12, and 19), mengovirus, influenza virus A (NWS), respiratory syncytial virus, vesicular stomatitis virus, vaccinia virus (Rister), and herpes simplex virus (type 1, HF) at concentrations up to 3 μg/ml.

Inhibition of viral replication. The effects of Ro 09-0410 on the yields of several types of rhinovirus were studied. Various doses of Ro 09-0410 were added at zero time of virus infections, and total yields of the viruses replicated during 2- or 3-day periods of the culture were assayed. At concentrations of Ro 09-0410 under 0.3 μg/ml, there were 10^3 to 10^4 reductions in virus yield (Fig. 2).

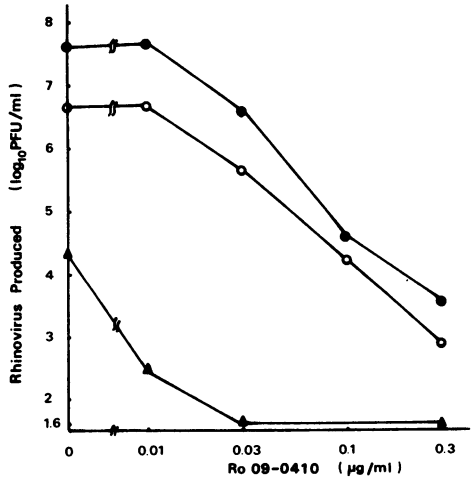


FIG. 2. Yield reduction of rhinovirus by Ro 09-0410. A suspension of HeLa cells (4×10^5) was mixed with rhinovirus (2×10^4 PFU) and was immediately plated onto a tissue culture plate, containing various doses of Ro 09-0410. After 2 to 3 days of incubation at 33°C, rhinovirus replicated in the culture was titrated. Symbols: ●, rhinovirus type 2; ○, rhinovirus type 30; ▲, rhinovirus type 21.

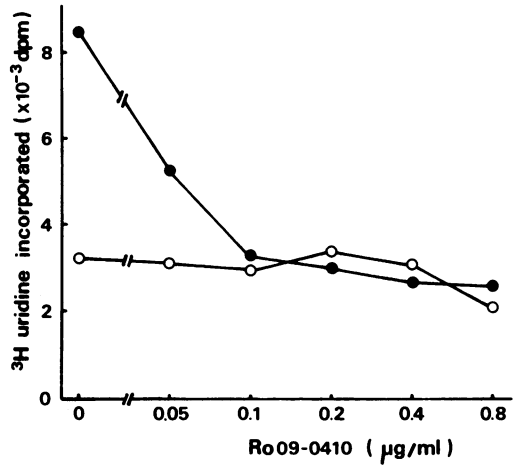


FIG. 3. Inhibition of viral RNA synthesis by Ro 09-0410. Monolayers of HeLa cells (4×10^5) were mixed with or without rhinovirus type 2 (8×10^5 PFU) and incubated immediately with actinomycin D (5 μg/ml) and various doses of Ro 09-0410 at 33°C for 10 h. [5-^{3}H]uridine (1 μCi/ml) was added during the last 5 h of incubation. Viral RNA synthesized in the cells was expressed by the radioactivity in cold TCA insolubles. Symbols: ●, infected cells; ○, uninfected cells.

The inhibition of viral replication by Ro 09-0410 was also reflected by the synthesis of viral RNA in cells infected with rhinovirus type 2 (Fig. 3). The infected cells incorporated [^{3}H]uridine into cold trichloroacetic acid (TCA) insolubles in the presence of actinomycin D, whereas in the presence of Ro 09-0410 at doses over 0.1 μg/ml, the incorporation was completely inhibited. On the other hand, incorporation into noninfected cells was not affected by 0.8 μg of Ro 09-0410 per ml, the highest concentration used in this experiment.

Virus inactivation. In an effort to elucidate the antiviral mechanism of Ro 09-0410, suspensions of rhinovirus type 2 were mixed with this agent at various concentrations and incubated for 1 h at 33°C. Thereafter, each mixture, as well as the control virus suspension containing no Ro 09-0410, was diluted with MEM and titrated for residual infectivity in HeLa cells. Ro 09-0410 (0.05 μg/ml) reduced the infectivity by a factor of 10^2 to 10^3, whereas a subline of rhinovirus type 2 (NR2-1) resistant to the agent (MIC_{50}, 1 ~ 3 μg/ml) was inactivated only slightly even at 0.5 μg/ml (Fig. 4). Virus inactivation was time and temperature dependent (Fig. 5 and 6), rapid at 33°C and relatively insignificant at 4°C.

Ro 09-0410 did not affect all virus particles. More than 1/1,000 of rhinovirus type 2 particles survived even at high doses or with incubation for longer periods. However, all 12 virus clones which survived exposure to this agent at a concentration of 0.5 μg/ml for 1 h at 33°C showed the same susceptibility to the agent

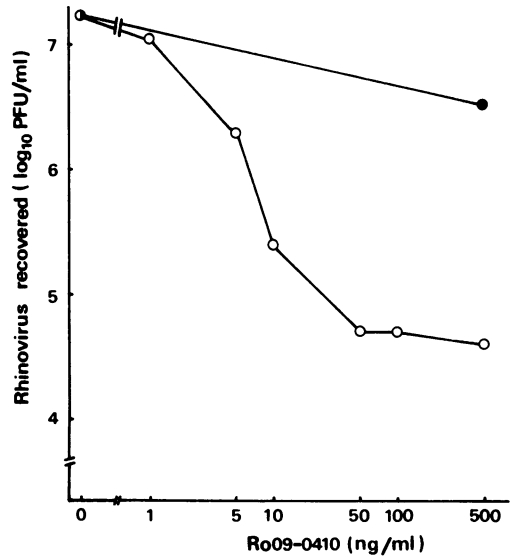


FIG. 4. Dose-dependent virus inactivation. Rhinovirus type 2 (1.4×10^7 PFU/ml) and its subline NR2-1 (1.3×10^7 PFU/ml), resistant to Ro 09-0410, were incubated with various concentrations of Ro 09-0410 at 33°C for 2 h. Symbols: ○, rhinovirus type 2; ●, rhinovirus type 2 NR2-1.

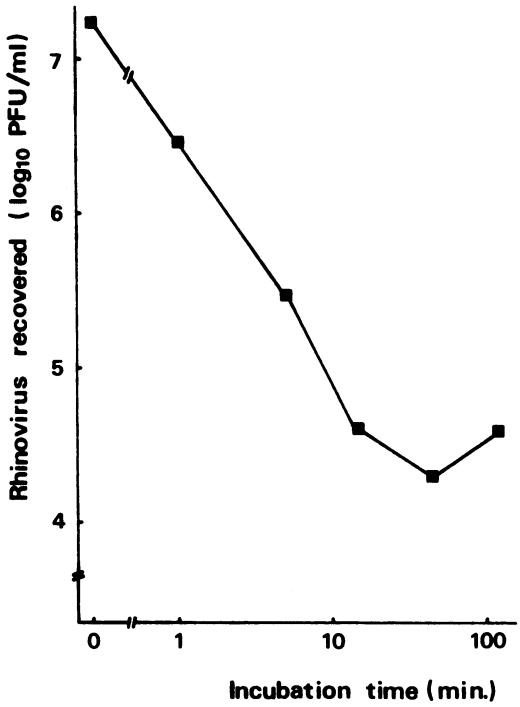


FIG. 5. Time-dependent virus inactivation. Rhinovirus type 2 (1.4×10^7 PFU/ml) was incubated with Ro 09-0410 (0.5 μg/ml) at 33°C for various times.

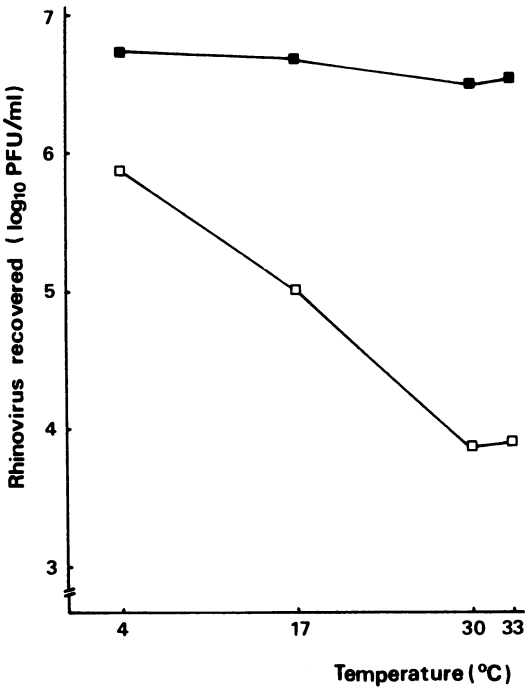


FIG. 6. Temperature-dependent virus inactivation. Rhinovirus type 2 (1.4×10^7 PFU/ml) was incubated with or without Ro 09-0410 (0.5 μg/ml) at various temperatures for 2 h. Symbols: □, with Ro 09-0410; ■, without Ro 09-0410.

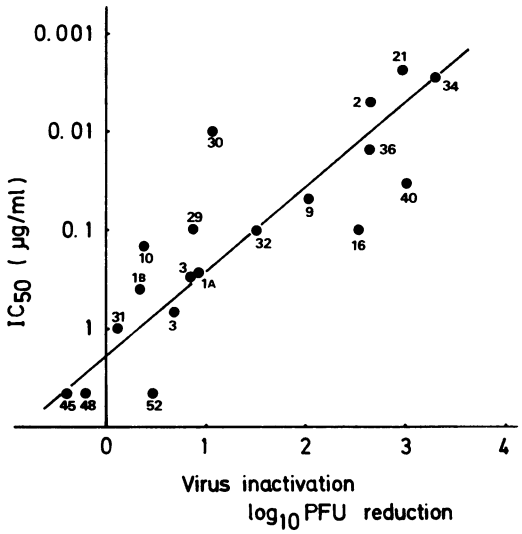


FIG. 7. Comparison of susceptibility of rhinoviruses to Ro 09-0410 as determined by direct inactivation and by inhibition of viral replication in cell culture. For direct virus inactivation, 19 types of rhinovirus ($1.2 \times 10^4 \sim 2 \times 10^7$ PFU/ml) were incubated with or without Ro 09-0410 (0.5 μg/ml) at 33°C for 1 h. The susceptibility was expressed as the difference in infectivity ($\log_{10}$ PFU) between the untreated virus and that exposed to the agent.

(MIC_{50}, 0.003 ~ 0.01 μg/ml) as the original virus. This indicates that the surviving virus is not a subpopulation of resistant viruses existing in the original virus preparation.

Susceptibility of rhinoviruses to Ro 09-0410 detected by inhibition of viral replication and by direct virus inactivation. To determine whether Ro 09-0410 inhibits viral replication in cell culture through the direct inactivation of rhinovirus, susceptibility to the agent detected by the virus inactivation was compared with that detected by the inhibition of viral replication in cell culture, represented as MIC_{50} (μg/ml). In Fig. 7, the susceptibilities of 19 types of rhinovirus measured by the two detection methods are plotted, showing a rather high degree of correlation between MIC_{50}s and log inactivation values. Those viruses most susceptible to the agent in the antiviral test were also most susceptible in the virus inactivation. Ro 09-0410 may inactivate intact rhinoviruses in cell culture at a step before uncoating, leading to the inhibition of further processes of viral replication.

Binding of Ro 09-0410 to rhinovirus. Since Ro 09-0410 is chemically stable and nonreactive, it is unlikely that it destroys rhinovirus via a classical chemical reaction. Ro 09-0410 may bind to the virus and make it inactive. To investigate this possibility, rhinovirus type 2 exposed to Ro 09-0410 was treated with chloroform, in which the agent is freely soluble and to which rhinovirus is resistant. The exposure of rhinovirus to 0.5 μg of Ro 09-0410 per ml for 1 h at 33°C reduced the infectivity from $10^{6.9}$ to $10^{<4.0}$ PFU/ml (Table 1, experiment 1). Howev-

TABLE 1. Recovery of infectivity of rhinovirus exposed to Ro 09-0410 by treatment with chloroform[a]

Incubation with:	Mean ± SD virus titer ($\log_{10}$ PFU/ml)	
	No treatment	Treated with $CHCl_3$
Expt 1		
None	6.94 ± 0.11	7.11 ± 0.13
Ro 09-0410	<4.0	6.95 ± 0.13
Expt 2		
None	7.58 ± 0.05	7.62 ± 0.05
Ro 09-0410	4.88 ± 0.05	7.45 ± 0.07
Expt 3		
None	7.28 ± 0.07	7.21 ± 0.11
Ro 09-0410	4.67 ± 0.05	7.13 ± 0.09

[a] Rhinovirus type 2 (1.4×10^7 PFU/ml) was incubated with or without Ro 09-0410 (0.5 μg/ml) at 33°C for 2 h in MEM. A part of the virus suspension was mixed with chloroform and then shaken. Thereafter, an aqueous solution was pooled for its titration.

er, the infectivity of the virus exposed to the agent regained the original level after the extraction of the agent with chloroform. Rhinovirus, while bound to the agent, was inactive. On the other hand, the complex thus formed appeared to be stable. The infectivity of rhinovirus type 2, which was exposed to the agent and isolated by sucrose gradient centrifugation, was not regained at all even after incubation at 33°C for 23 h, although it was regained by chloroform extraction.

DISCUSSION

The present work showed that Ro 09-0410 is highly and specifically active against rhinoviruses. The activity against 46 of 53 types of rhinoviruses was demonstrable at concentrations as low as 0.003 to 3 μg/ml. Ro 09-0410 was completely inactive against other viruses. It also inactivated rhinovirus directly.

The direct inactivation of rhinoviruses by low concentration of Ro 09-0410 suggests that these viruses have a capacity for binding this agent on the capsid not common to other picornaviruses. The structure of rhinovirus is known to be slightly different from that of other picornaviruses, such as enteroviruses and cardioviruses, and the instability at mildly acidic pH (below 6) is the most common characteristic property of this virus (2, 4–6).

Chemotherapeutic indices of Ro 09-0410 in tissue cultures against several types of rhinovirus were greater than 10^4. In toxicological studies Ro 09-0410 produced no adverse effects when administered to rodents by either the intraperitoneal or oral route at a dose of 1 g/kg daily for 2 weeks (personal observation). Further evaluations should be carried out in humans or nonhuman primates, the only subjects susceptible to infection with rhinoviruses. Pharmacokinetic studies of Ro 09-0410 and its orally active prodrugs are under way in rats and in humans.

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