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Antirhinovirus Compound 44 081 R.P. Inhibits Virus Uncoating

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44 081 R.P., or 2-[(1,5,10,10*a*-tetrahydro-3*H*-thiazolo[3,4-*b*]isoquinolin-3-ylidine)amino]-4-thiazole acetic acid, is a compound which selectively inhibits rhinovirus in cell cultures. The compound, which was unable to inactivate infectivity of virions, appeared to act prior to RNA and protein synthesis without affecting adsorption or penetration of virus in MRC_5 cells. In contrast, it protected intracellular [5-^{3}H]uridine-labeled virions against the effects of RNase treatment, indicating that inhibition of virus uncoating is the mode of action of 44 081 R.P.

As reported previously (13), 44 081 R.P., or 2-[(1,5,10,10*a*-tetrahydro-3*H*-thiazolo[3,4-*b*]isoquinolin-3-ylidene)amino]-4-thiazole acetic acid (molecular weight, 345), is a compound which displays selective antirhinovirus properties in cell cultures. Of the 69 rhinovirus samples (12 known serotypes and 57 clinical isolates) that were studied, 48 (70%) were inhibited at nontoxic concentrations (≤125 μg/ml). 44 081 R.P. appeared to inhibit some early process of rhinovirus replication because under conditions of a single cell growth cycle, the greatest inhibitory effect was noted when the compound was added to cells simultaneously with the infecting virus. No direct virucidal activity was observed. The mechanism of action appeared also to involve some cellular functions because, contrary to that which occurred in MRC_5 cells, primary monkey kidney cells, and fetal tonsil fibroblasts, rhinovirus growth in the Ohio line of HeLa cells was not inhibited. In this report we present additional observations on the mode of action of 44 081 R.P., which indicate that the compound inhibits virus uncoating.

MATERIALS AND METHODS

Compound. Compound 44 081 R.P. was synthesized as described previously (European Patent Application 30,198, filed by Rhône-Poulenc Ind. [Chem. Abstr. **96**:6715r, January 1982]). Stock solutions of the compound, as sodium salt, were first diluted in phosphate-buffered saline (PBS) and subsequently in tissue culture medium.

Cell cultures. Human embryonic fibroblasts (MRC_5, supplied by Bio-Mérieux) were grown in basal Eagle medium (with Earle salts) supplemented with 5% newborn calf serum–benzylpenicillin (40 IU/ml)–streptomycin sulfate (40 μg/ml).

Protein synthesis in RV-1B-infected cells. Cell monolayers in Falcon multiwell plates were infected for 1 h at 37°C with rhinovirus type 1B (RV-1B) at 20 PFU per cell in the presence or absence of 44 081 R.P. (30 μg/ml). Cells were labeled at different times postinfection with 50 μCi of [^{35}S]methionine (800 Ci/mmol) per ml for 1 h. They were then lysed in 0.1 N NaOH–1% sodium dodecyl sulfate (SDS), and macromolecules were precipitated in trichloroacetic acid (TCA) at 10%. Radioactivity was collected on glass fiber filters as described previously (1, 2).

For gel electrophoresis experiments, labeled cells (1-h pulse at 5 h postinfection) were washed with PBS and lysed in sample buffer (1). SDS-polyacrylamide gel electrophoresis, fluorography, and autoradiography were performed as described previously (1).

Virus adsorption. Virus adsorption was measured with both rhinovirus type 3 (RV-3) (unlabeled) and RV-1B ([5-^{3}H]uridine labeled); the two serotypes were sensitive to 44 081 R.P. (13).

RV-3. Confluent MRC_5 cells in petri dishes (3.5-cm diameter; Becton Dickinson Labware, Oxnard, Calif.) were incubated with 1.6×10^6 PFU of RV-3 at 4°C in the absence or presence of 44 081 R.P. (30 μg/ml). At different times cells were washed twice with PBS and lysed by two cycles of freezing and thawing. The number of infectious particles associated with the monolayers was determined by titration in HeLa Ohio cells (13).

RV-1B. For the preparation of [^{3}H]uridine-labeled virus, the following procedure was used. Confluent MRC_5 cell monolayers (in 250-ml bottles; Becton Dickinson Labware) were infected with RV-1B at 0.5 PFU per cell in the presence of 0.4 μg of actinomycin D per ml. Later (4 h) the viral inoculum was removed, and fresh medium containing actinomycin D and 100 μCi of [5-^{3}H]uridine (60 Ci/mmol) per ml was added. At 20 h postinfection, when a cytopathic effect was observed, cells were disrupted by two cycles of freezing and thawing. The extract was centrifuged at 4°C at 10,000 × *g* for 10 min in an IEC centrifuge (International Equipment Co., Needham Heights, Mass.). The pellet was discarded, and the supernatant was further centrifuged at 100,000 × *g* for 2 h in a SW41 rotor (Beckman Instruments, Inc., Fullerton, Calif.). The virus (approximately 60,000 to 90,000 TCA-precipitable cpm per bottle) was recovered from the pellet by mild sonication.

To carry out adsorption experiments, confluent MRC_5 cells in Falcon petri dishes were incubated at 4°C with 0.2 ml of [5-^{3}H]uridine-labeled RV-1B (6,000 cpm per dish)–0.4 μg of actinomycin D per ml to avoid RNA synthesis in the presence of free tritiated uridine that was eventually present in the virus inoculum. At different times postinfection cells were washed three times with ice-cold PBS and lysed in 0.1 N NaOH–1% SDS. Radioactive viral RNA was precipitated with 10% TCA and collected on filters.

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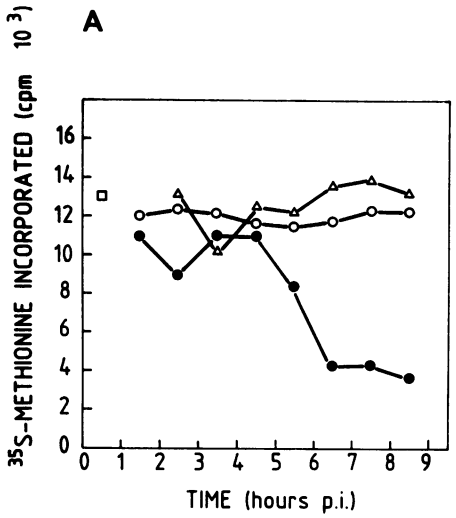

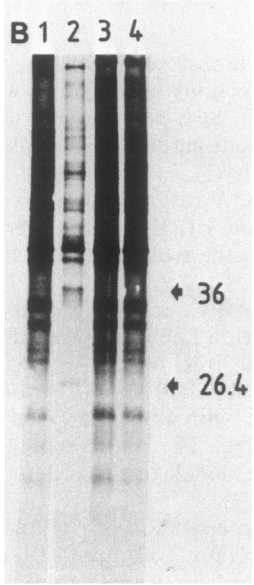

FIG. 1. Effect of 44 081 R.P. on protein synthesis in RV-1B-infected MRC_5 cells. (A) Time course of [^{35}S]methionine incorporation in RV-1B-infected cells. Symbols: □, uninfected, untreated cells; ●, infected, untreated cells; ○, infected cells treated with 44 081 R.P. (30 μg/ml) from the beginning of infection; △, infected cells treated with 44 081 R.P. during the first hour of infection only. (B) SDS-polyacrylamide gel electrophoresis of labeled proteins at 5 h postinfection; lane 1, uninfected cells; lane 2, infected, untreated cells; lane 3, infected cells treated with 44 081 R.P. (30 μg/ml) from the beginning of infection; lane 4, infected cells treated with 44 081 R.P. (30 μg/ml) during the first hour of infection (adsorption period only).

Virus penetration and uncoating. [5-^{3}H]uridine-labeled virus was added to confluent MRC_5 cultures (in 3.5-cm-diameter petri dishes) and incubated for 1 h at 37°C (unless otherwise specified). Cells were then washed twice in PBS and treated with 0.4 ml of proteinase K (250 μg/ml) for 10 min at 4°C, to remove noninternalized virions.

Cells were then collected by scraping and washed three times by low-speed centrifugation. The pellet was sonicated and either immediately precipitated in TCA (as described in the legend to Fig. 1) to determine the amount of radioactivity inside cells or incubated with 50 μg of RNase A per ml for 30 min at 37°C and then precipitated in TCA to assess the radioactivity that was RNase resistant.

RESULTS

We have shown previously (13) that the greatest inhibition of virus growth or viral RNA synthesis occurred when 44 081 R.P. was added to MRC_5 cells simultaneously with the virus. Such observations were confirmed by a new set of experiments (Fig. 1A and B) in which protein synthesis was examined. [^{35}S]methionine incorporation into proteins decreased significantly in MRC_5 cells 5 to 6 h postinfection with RV-1B (Fig. 1A). However, by adding 44 081 R.P. (30 μg/ml) at the beginning of infection, the shutdown of protein synthesis was fully prevented. It should be noted that, as indicated in our previous report (13), the compound was devoid of cytotoxicity at this concentration. In fact, for the first 4 h postinfection, protein synthesis in cells treated with 44 081 R.P. was virtually the same as that in uninfected and untreated cells. It was sufficient for 44 081 R.P. to be present only during the adsorption period to prevent inhibition of protein synthesis (Fig. 1A). Even though the compound was removed by washing after viral adsorption, its antiviral effect persisted for at least 8 h. This finding strongly supports our previous hypothesis about the mechanism of action of 44 081 R.P., namely, that the compound acts early during the rhinovirus replication cycle.

Similar results were obtained by analysis by polyacrylamide gel electrophoresis of lysates of cells which were pulse-labeled with [^{35}S]methionine between 5 and 6 h postinfection (Fig. 1B). The electrophoretic profiles of mock-infected cells and of infected cells are shown in lanes 1 and 2, respectively, of Fig. 1B. In the presence of the compound, the switch-off of cellular protein synthesis and the appearance of viral proteins (e.g., 36 and 26 kilodaltons, as shown in lane 2 [Fig. 1B]) were completely prevented. This effect was obtained in cultures treated with 44 081 R.P., either throughout the incubation period (Fig. 1B, lane 3) or during viral adsorption only (Fig. 1B, lane 4).

Such observations prompted us to examine in more detail the early events of virus replication, namely, adsorption, penetration, and uncoating. In Fig. 2 is shown the adsorption of two 44 081 R.P.-sensitive rhinovirus serotypes, RV-3 and RV-1B, toward which the compound displays MICs (13) of 3 and 0.15 μg/ml, respectively. Neither the number of infectious RV-3 particles (Fig. 2A) nor the amount of radioactive ([^{3}H]uridine-labeled) RV-1B (Fig. 2B) associated with cells as a function of time was reduced by 44 081 R.P., even at a high concentration (30 μg/ml) of the compound. Therefore, virus adsorption does not appear to be affected by the compound.

To study virus entry, we first treated MRC_5 cells, infected with [5-^{3}H]uridine-labeled virus, with proteinase K which efficiently removes noninternalized virions (1), and then determined the amount of cell-associated radioactivity. Virus penetration was temperature dependent, being greater at 37°C than at 4°C (Table 1, experiment 1). 44 081 R.P. (Table 1, experiments 1, 2, and 3) did not reduce the amount of TCA-precipitable radioactivity inside the cells, indicating that the compound does not affect virus entry.

The next step examined was virus decapsidation. This was accomplished by taking advantage of the fact that the genomic viral RNA is protected by its capsid against RNase A attack but becomes RNase sensitive once the virion enters the cell and decapsidates. The radioactivity inside MRC_5 cells, which is resistant to RNase, was greater in 44 081

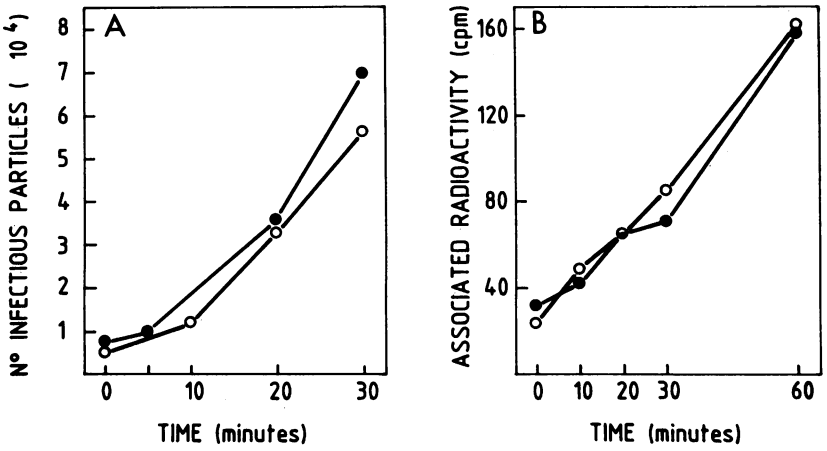

FIG. 2. Effect of 44 081 R.P. on rhinovirus adsorption to MRC_5 cells. (A) Adsorption of RV-3. Symbols: ○, without 44 081 R.P.; ●, with 44 081 R.P. (30 μg/ml) (each value represents the average of two determinations). (B) Adsorption of [5-^{3}H]uridine-labeled RV-1B. Symbols: ○, without 44 081 R.P.; ●, with 44 081 R.P. (30 μg/ml).

R.P.-treated cultures than in the untreated ones (Table 1, experiments 2 and 3). In other words, 44 081 R.P. appears to inhibit virus decapsidation. It should be noted that because of the lack of activity of the compound in HeLa cells, we found no evidence of an inhibitory effect on RV-1B decapsidation in this cell line (data not shown).

DISCUSSION

44 081 R.P. is a synthetic compound which selectively inhibits rhinovirus multiplication in cell cultures at concentrations far below the cytotoxic concentration (250 μg/ml). As indicated previously (13), the compound acts on early events of the virus cycle. In this report we further analyzed its mode of action, namely, its effect on adsorption, penetration, and uncoating. Adsorption of two sensitive rhinovirus serotypes (RV-1B and RV-3) was not affected by the compound. Furthermore, the amount of internalized [5-^{3}H]uridine-labeled virions was virtually the same in untreated and drug-treated cultures, indicating that virus penetration is not affected by 44 081 R.P. In contrast, the uncoating step, as determined by the amount of cell-penetrated viral RNA which became RNase sensitive, was remarkably inhibited.

The mechanism of action of several antipicornavirus compounds appears to involve binding to viral particles and their subsequent stabilization. These include compounds such as arildone, rhodanine, chloroquine, 2-thiouracil, and Ro 09-410 (3, 4, 6) which affect uncoating. Direct binding to virions has also been demonstrated for compounds such as 4-6-dichloroflavane and RMI 15 731 (2, 4, 8, 11) which do not inhibit uncoating. As radiolabeled 44 081 R.P. was not available, we could not test whether the inhibitory effect on rhinovirus uncoating was the result of its binding to virions.

In addition to virion capsid proteins, uncoating of picornaviruses requires the participation of specific cell membrane constituents (10). This leaves open the possibility that 44 081 R.P. interacts with some cellular targets. This hypothesis is suggested by the facts that (i) 44 081 R.P. binds to or enters into cells (0.84 and 1.5 μg of compound were found to be associated with 2×10^7 MRC_5 cells in 2 and 5 h, respectively; J. Dow, personal communication); (ii) MRC_5 cells pretreated with the compound acquire a state of resistance against further rhinovirus challenge (13); and (iii) growth of RV-1B and RV-3 (13) and decapsidation of RB-1B (data not shown) in HeLa cells were not inhibited by 44 081 R.P. The early events (those occurring before viral RNA and protein synthesis) of rhinovirus replication appear to be vulnerable and suitable targets for chemotherapy (2–6, 8, 12). Unfortunately, either poor or negative clinical results were obtained with some of these compounds, including 44 081 R.P. (7, 9, 13). Studies such as the one reported here on new antiviral agents and on their mode of action should contribute to the design of compounds to be successfully used in the control of rhinovirus infection in humans.

TABLE 1. Effect of 44 081 R.P. on the entry and decapsidation of RV-1B virus

Expt	Antiviral treatment (temp [°C])	TCA-precitable counts (cpm) with the following RNase treatments[a]:	
		None	50 μg/ml
1	None	127 ± 23	
	None (37)	224 ± 31	
	44 081 R.P. (37)	232 ± 27	
2	None (37)	509 ± 48	250 ± 61
	44 081 R.P. (37)	499 ± 54	519 ± 66
3	None (37)	779 ± 43	188 ± 19
	44 081 R.P. (37)	731 ± 12	492 ± 36

[a] Values represent the average of the counts per minute of three dishes ± standard deviation.

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