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RMI 15,731 (1-[5-Tetradecyloxy-2-furanyl]-ethanone), a New Antirhinovirus Compound

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RMI 15,731 (1-[5-tetradecyloxy-2-furanyl]-ethanone) is a new antiviral compound with activity specific for rhinoviruses. Virus synthesis in R-HeLa cells was susceptible to low concentrations (0.25 μg/ml) of the compound. RMI 15,731 was effective if added for 1 h before infection and removed or if added as late as 6 h postinfection. Low concentrations of the compound directly inactivated infectious rhinovirus in a time- and temperature-dependent fashion. RMI 15,731 appears to be a promising new antirhinovirus compound for clinical evaluation since it (i) blocks intracellular virus synthesis (without cellular toxicity), and (ii) directly inactivates virions. The last property may be important in limiting virus shedding from an infected host.

Rhinoviruses are the most frequent etiological agents of the common cold syndrome in adults (1). There are over 100 different serotypes, and a useful vaccine against these agents seems unlikely in the near future. A compound with a broad antirhinovirus spectrum would be useful in an epidemiological situation. Treatment of individuals with an antiviral compound before infection, just after virus exposure, or during the incubation period could prevent other contacts from contracting the disease. An effective antirhinovirus substance might also decrease the severity and length of symptomatology in infected individuals if administered in the course of the disease.

RMI 15,731 (1-[5-tetradecyloxy-2-furanyl]-ethanone) is a rhinovirus-specific antiviral compound synthesized at the Merrell Research Center (Fig. 1). The in vitro activity of this compound is the subject of this report. RMI 15,731 is effective in inhibiting rhinovirus replication when present before or after virus infection of cell cultures.

MATERIALS AND METHODS

Cells. Rhinovirus-sensitive HeLa cells (R-HeLa) were obtained from Schering Laboratories, Kenilworth, N.J. A human foreskin fibroblast line was originally obtained from T. C. Merigan, Stanford University, Stanford, Calif. Both cell types were propagated in Eagle basal medium (BME) supplemented with 10% fetal calf serum.

Virus. Rhinovirus type 39 (RV 39) was used for most of the experiments described. Other serotypes were tested for susceptibility to the compound by J. M. Gwaltney, Jr., University of Virginia School of Medicine, Charlottesville, as indicated below. Stock virus was prepared by infecting R-HeLa cells at an input multiplicity of approximately 0.1 plaque-forming unit (PFU) per cell. Virus was adsorbed from a small volume for 90 min at 35°C. Cell sheets were washed with BME, and fresh medium was added. Incubation was at 35°C for 20 to 24 h.

Virus assays. Rhinovirus titers were determined by the plaque method on R-HeLa cell sheets (80% confluent) contained in 35-mm petri dishes. Each assay was performed in duplicate. A 0.1-ml amount of virus was adsorbed to the cell layer for 90 min at 35°C; 4 ml of overlay medium (0.5% Methocel in BME) containing added magnesium (2) was used on each dish after the adsorption period. Infected cells were incubated for 48 h at 35°C before fixation with Formalin and crystal violet staining.

Measurement of cytotoxicity. The toxic effect of RMI 15,731 was assessed by two methods: (i) measurement of cellular protein concentration by the Lowry method as modified by Oyama and Eagle (3) (cell sheets were solubilized in 0.4 N NaOH after washing with saline [3]); and (ii) measurement of total cell number per culture after trypsinization. In all cases duplicate cultures were examined in each experiment at each concentration of compound.

Measurement of RMI 15,731 binding to cells. Cell cultures in 35-mm petri dishes were exposed to different concentrations of *carbonyl*-^{14}C-labeled RMI 15,731 in 1 ml of BME at 35°C. At various times the medium was removed from duplicate cultures, and the cell sheets were washed three times with phosphate-buffered saline, pH 7.2. The cells in each culture were dissolved in 1 ml of hyamine hydroxide (Sigma Chemical Co., St. Louis, Mo.) and counted by liquid scintillation spectrometry in a toluene-based fluid (4.2 g of 2,5-diphenyloxazole and 52 mg of phenyloxazolylphenyloxazolyl-phenyl per liter).

Chemicals. RMI 15,731 is a water-insoluble powder. This compound homogenized into aqueous media was an effective antirhinovirus compound. We found,

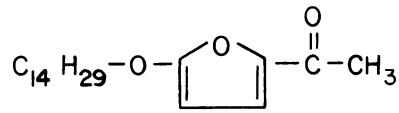

FIG. 1. *Structure of RMI 15,731.*

however, that more consistent results were obtained if RMI 15,731 was dissolved initially in ethanol and subsequently diluted in BME. *carbonyl*-^{14}C-labeled RMI 15,731 (0.43 μCi/ml) was prepared by New England Nuclear Corp., Boston, Mass.

RESULTS

Inhibition of rhinovirus synthesis. Table 1 indicates the susceptibilities of several rhinoviruses to RMI 15,731. A concentration of 0.5 μg/ml (1.5×10^{-6} M) was sufficient to suppress RV 39 and RV 56 by more than 99% (99.68 ± 0.19%; five experiments).

In contrast, RMI 15,731 was inactive when tested against vaccinia virus, vesicular stomatitis virus, influenza virus A2, encephalomyocarditis virus, Semliki Forest virus, *Herpesvirus hominis* type 1, and poliovirus type 2. The growth of both gram-positive and gram-negative bacteria (*Staphylococcus aureus, Streptococcus pyogenes, Streptococcus faecalis, Salmonella schottmuelleri, Proteus mirabilis, Pseudomonas aeruginosa*) was unaffected by the compound. *Candida albicans* and *Cryptococcus neoformans* were likewise not inhibited. RMI 15,731, therefore, appears to have a high degree of specificity toward rhinoviruses.

A number of different human rhinovirus types were tested for susceptibility to RMI 15,731. Altogether 52 serotypes (types 1A through 25, 39, 56 through 81) were examined; of these, 40 (77%) were susceptible to RMI 15,731. One untyped clinical isolate was also inhibited by the compound. RV 39 was used in the rest of the experiments reported here.

Cellular toxicity. RMI 15,731 was nontoxic to cultured cells at concentrations far in excess of those required to inhibit virus synthesis. The effect of the compound on HeLa cells, as judged by cell number or protein concentration, is shown in Table 2. Sparse cultures of cells in 35-mm petri dishes were exposed to RMI 15,731 in 2 ml of BME for 24 h before protein determination or cell counts. A concentration of more than 25 μg of compound per ml was required to demonstrate toxicity. This represents 100 times the amount required to inhibit virus synthesis by more than 90%.

Binding of RMI 15,731 to cells. The binding of *carbonyl*-^{14}C-labeled RMI 15,731 to R-HeLa cells is shown in Fig. 2. Bound compound is defined here as the compound which could not be removed by the washing procedures used. Incorporation or uptake of RMI 15,731 by cells may be a component of the binding measurements. Binding of RMI 15,731 to cells was rapid. The amount of compound bound was proportional to the concentration in the medium. The labeled compound was bound at the same rate to uninfected and rhinovirus-infected R-HeLa cells (data not shown).

Prophylactic efficacy of RMI 15,731. The rapid rate of compound binding suggested that treatment of cells for short time periods before infection might be sufficient to establish an antiviral state. To test this idea, cell cultures were pretreated for different times with RMI 15,731 before RV 39 challenge (1 PFU/cell). Cell cultures were washed twice with BME before rhi-

TABLE 1. *Inhibition of rhinovirus replication by RMI 15,731*

Virus[a]	Host cell	Concn of RMI 15,731 (μg/ml)	PFU/ml	% of untreated
RV 39	R-Hela	0	5.20×10^7	100
		0.25	6.40×10^5	1.2
		0.5	1.00×10^5	0.2
		1.0	2.60×10^4	0.05
RV 39	HFF[b]	0	1.29×10^6	100
		0.25	1.50×10^4	1.2
		0.5	5.00×10^2	0.04
		1.0	5.00×10^2	0.04
RV 23	R-HeLa	0	3.30×10^6	100
		0.5	4.80×10^4	1.4
		1.0	2.00×10^4	0.6
RV 56	R-HeLa	0	2.70×10^6	100
		0.25	1.40×10^4	0.5
		0.5	4.50×10^3	0.16

[a] Virus was adsorbed to cells for 1 h before addition of BME containing the indicated concentrations of RMI 15,731. Total virus was determined on pooled duplicate cultures at 20 to 24 h after challenge.

[b] HFF, Human foreskin fibroblast.

TABLE 2. *Toxicity of RMI 15, 731 for R-HeLa cells*

Concn of RMI 15,731 (μg/ml)	No. of cells (% of control)[a]		Cell protein (% of control)[a]	
	Expt 1	Expt 2	Expt 1	Expt 2
0.5	96	100	100	98
1	93	100	98	96
5	100	100	95	100
10	95	97	97	100
25	80	90	91	91
50	11	7	71	74

[a] Cell cultures were incubated with RMI 15,731 for 24 h before determination of cell number and cellular protein concentration as described in the text. The average of duplicate determinations in each experiment is given.

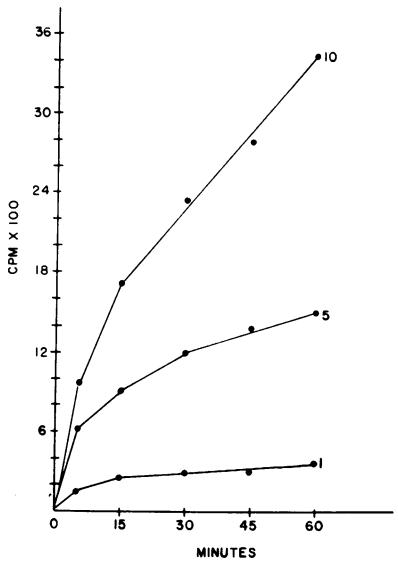

FIG. 2. *Binding of carbonyl-^{14}C-labeled RMI 15,731 to R-HeLa cells. The concentrations of compound (in micrograms per milliliter) are indicated on each curve.*

novirus infection. Viral yields were assayed at 24 h postinfection. Pretreatment of cells for 1 h with compound reduced virus synthesis by nearly 90% (Table 3). Five hours of contact with RMI 15,731 rendered cells refractory to virus replication; i.e., complete inhibition of virus synthesis occurred. Other experiments (data not shown) demonstrated that pretreatment with the compound for as long as 18 h did not affect virus attachment to cells. RMI 15,731 was, therefore, an effective antiviral compound when administered to cells before infection.

Therapeutic efficacy of RMI 15,731. The effect of RMI 15,731 on established rhinovirus infections was also examined. R-HeLa cell cultures were infected with RV 39 (1 PFU/cell), and, after washing with BME, replicate cultures received medium containing 1 μg of compound per ml at various times after infection. Viral yields were assayed at 18 h postinfection. Progeny virus was first detected between 5 and 6 h postinfection under the conditions used. It is apparent from the data (Table 4) that compound added as late as 6 h postinfection was able to suppress the infectious virus yield.

Direct inactivation of RV 39. The effectiveness of RMI 15,731 when added late in the infectious cycle suggested that the compound might be capable of directly inactivating rhinovirus particles at concentrations well below those toxic for R-HeLa cells. Figure 3 shows the results of an experiment in which RV 39 suspensions were mixed with different concentrations of RMI 15,731 and held at 37°C for 1 h. The virus-compound mixtures were diluted and assayed for infectious virus. RMI 15,731 was capable of directly inactivating RV 39. The inactivation curve displays multihit features. Direct

TABLE 3. *Inhibition of RV 39 replication by pretreatment of cells with RMI 15,731*

Length of pretreatment (h)[a]	Virus yield (PFU/ml)	% of untreated
0	1.47×10^7	100
1	1.90×10^6	13
2	5.80×10^5	3.9
3	1.90×10^5	1.3
4	1.50×10^5	1.0
5	7.50×10^4	0.5
6	7.50×10^4	0.5

[a] Cell cultures were pretreated for the indicated times with RMI 15,731 (5 μg/ml) before infection with RV 39.

TABLE 4. *Antiviral effect of RMI 15,731 added after virus infection*

Time of addition (h postinfection)[a]	Virus yield (PFU/ml)	% of untreated
1	1.40×10^4	0.06
2	1.30×10^4	0.06
3	1.00×10^4	0.05
4	1.25×10^4	0.06
5	1.40×10^4	0.06
6	2.15×10^4	0.10
No compound	2.05×10^7	100

[a] RMI 15,731 (1 μg/ml) was added to the culture medium at the indicated times after the initiation of virus infection. Virus was adsorbed for 1 h. The end of the adsorption period was considered as 1 h postinfection.

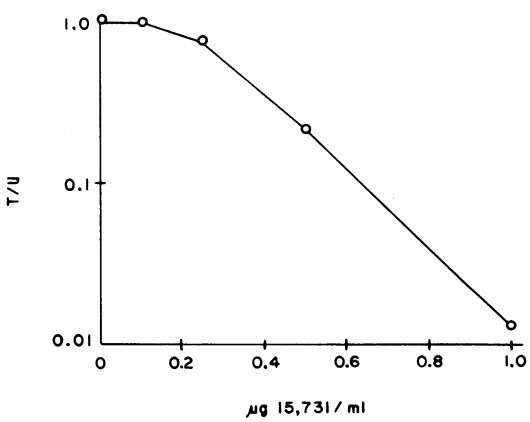

FIG. 3. *Direct inactivation of RV 39 by RMI 15,731. The ratio of PFU from compound-treated virus suspensions (T) to untreated virus suspensions (U) is plotted versus the concentration of RMI 15,731.*

inactivation of RV 39 with higher concentrations of compound gave virus half-lives of 5, 3, and 2 min for 5, 10, and 20 μg of RMI 15,731 per ml, respectively.

Since rhinoviruses are acid labile (4), the possibility that RMI 15,731 was acting by decreasing the pH of the incubation medium was considered. Measurement of the pH of the compound-containing medium did not show a decrease which might account for the inactivation.

The effect of temperature on the inactivation of RV 39 by 1 μg of RMI 15,731 per ml is presented in Table 5. Virus-compound mixtures were incubated at the indicated temperatures for 1 h before infectivity assays. RMI 15,731 was incapable of inactivating RV 39 at low temperature.

Two types of experiments indicated that the direct action of the compound on the virus was not merely clumping, an effect which could explain the decrease in virus titer. First, sonication of virus-compound mixtures after incubation failed to increase the virus titer. Second, ultraviolet inactivation of virus-compound mixtures revealed single-hit kinetics of inactivation of the survivors rather than the multihit kinetics expected from clumped virions (Fig. 4).

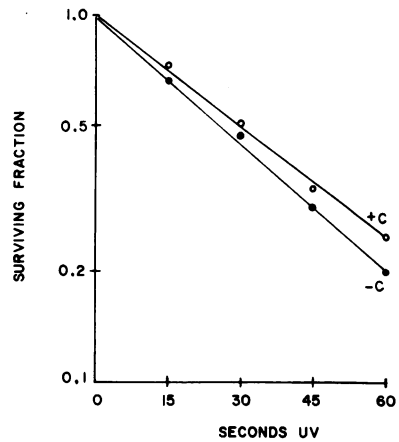

FIG. 4. *Ultraviolet (UV) inactivation of RV 39 with or without RMI 15,731 treatment. Virus suspensions were incubated for 1 h in the presence or absence of RMI 15,731 (C) (1 μg/ml) followed by irradiation with ultraviolet light (distance, 20 cm) for the times indicated. Symbols: ○, RMI 15,731-treated virus; ●, untreated virus.*

DISCUSSION

Addition of RMI 15,731 to rhinovirus-infected cell cultures resulted in a depression of virus yield. This inhibition could be achieved with low concentrations of compound. The compound was also effective prophylactically. Exposure of cells to RMI 15,731 for short time periods before infection reduced the amount of virus synthesized. It appears that RMI 15,731 was taken up by cells and exerted an antiviral effect during the rhinovirus replicative cycle. The limited virus specificity of RMI 15,731 and our inability to detect interferon in compound-treated cultures (unpublished data) argue against interferon as the mechanism of rhinovirus inhibition.

The fact that RMI 15,731 was effective late in the virus replicative cycle suggested a direct effect on virions. Direct inactivation of rhinovirus was demonstrated at compound concentrations which were nontoxic to R-HeLa cells. Although preliminary experiments indicate that RMI 15,731 can block certain intracellular stages in the synthesis and assembly of rhinoviruses, we are inclined to believe that the bulk of virus inhibition results from a direct interaction between the compound and the virus capsid. The effect appears to be specific since a number of related and unrelated viruses were not affected by high concentrations of RMI 15,731.

Since rhinoviruses are harbored within infected cells for extended time periods after synthesis (4), we suggest that RMI 15,731 binds to or is taken up by cells and subsequently inactivates both intracellular virions, comprising the bulk of the progeny (90% or more), and extracellular virions recently liberated. The activity of RMI 15,731 appears to be unique among antiviral compounds in that it is capable of directly inactivating virions at nontoxic concentrations.

The extreme specificity of RMI 15,731 for rhinoviruses suggests that the compound may be relatively nontoxic in vivo. Preliminary data indicate that this is the case. RMI 15,731 was not noticeably toxic at 250 mg/kg when given subcutaneously to mice once a day for 3 days.

The direct action of RMI 15,731 on rhinovirions suggests that this compound might be useful in clinical situations to prevent long-term virus shedding from infected individuals. Such an effect would help terminate spread of the disease.

TABLE 5. *Effect of temperature on the direct inactivation of RV 39 by RMI 15,731*

Temperature (°C)	Virus titer (PFU/ml)		% of untreated
	Untreated	+RMI 15,731	
4	2.55×10^6	3.40×10^6	100
23	2.90×10^6	1.83×10^6	63
32	2.20×10^6	4.35×10^5	19.7
37	2.15×10^6	2.55×10^4	1.1

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