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Mechanism of action of the antiviral compound MDL 20,610

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Summary

The purpose of this study was to probe the antirhinovirus (RV) mechanism of action of MDL 20,610. Evaluation of the compound's effects on RV RNA synthesis, uncoating of neutral red-sensitized RV, plasma membrane penetration by RV, stabilization of RV against heat (56°C) and low pH (5.0) inactivation, and studies with MDL 20,610-resistant RV mutants indicate that MDL 20,610 binds directly to the RV capsid with subsequent inhibition of acid-mediated virion uncoating.

Antiviral; Flavonoid; Rhinovirus

Introduction

MDL 20,610 [2-(3,4-Dichlorophenyl)-3,4-Dihydro-6-Methylsulfonyl-2*H*-Pyrano[2,3-*b*] [Pyridine] is a potent antirhinovirus compound with a median plaque reduction IC_{50} of 0.03 μg/ml against 32 serotypes evaluated (Kenny et al., 1986a). The compound is also active against human, simian and bovine rotaviruses (CPE IC_{50} of 0.8 to 1.5 μg/ml) but possesses variable enterovirus and paramyxovirus activity. MDL 20,610 is not active (CPE IC_{50} of >5.0 μg/ml) against the orthomyxoviruses, human coronavirus 229E, adenoviruses or herpes simplex virus. In addition, MDL 20,610 has no measurable effect on the macromolecular synthesis or the cloning efficiency of HeLa cells at compound concentrations of up to 20

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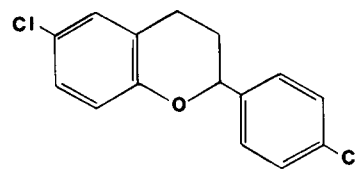
μg/ml. Subsequently, we studied the antiviral mechanism of action of MDL 20,610 and those data are reported here.

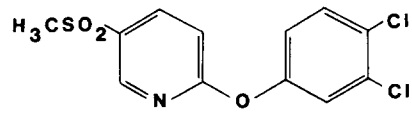
Materials and Methods

Test compounds

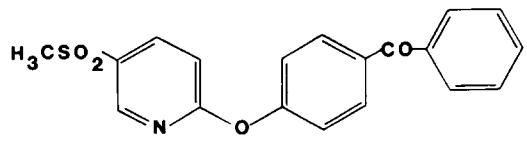
Compounds MDL 20,610 (Fig. 3) and 6-Chloro-2-(4-Chlorophenyl)-3,4-Dihydro-2*H*-1-Benzopyran (BW683C, Fig. 1) were synthesized by published methods (Bargar et al., 1986; Hopkins, 1982). Compound 2-(3,4-Dichlorophenoxy)-5-(Methylsulfonyl)Pyridine (MDL 055, Fig. 1) and 4-[(5-Methylsulfonyl-2-Pyridinyl)oxy]Phenyl)Phenyl Methanone (MDL 26,019, Compound 1 from (Kenny et al., 1986b), Fig. 1) were synthesized by Y.C. Tong and S.G. Wood, Western Division Research Center, The Dow Chemical Company. For assay MDL 20,610 was initially dissolved in acetone at a concentration of 1 mg/50 μl while the remaining compounds were solubilized in dimethyl sulfoxide at a concentration of 1 mg/25 μl. Preliminary studies (data not shown) demonstrated no solvent effect at the final concentrations achieved in the various virus assays.



BW683C



MDL 055



MDL 26,019

Fig. 1. Structures of antiviral compounds BW683C, MDL 055, and MDL 26,019.

Test viruses and reference antisera

Rhinoviruses (RV) 1A and 2 were obtained from B.D. Korant, E.I. duPont deNemours and Co., Inc., Wilmington, DE and experimental stocks were prepared in HeLa cells. Rhinovirus 1A (ATCC VR-1110 AS/GP) and 2 antisera (ATCC VR-1112 AS/GP) and corresponding preimmune control sera (ATCC VR-1110 PI/GP and ATCC VR-1112 PI/GP) were purchased from the American Type Culture Collection (ATCC, Rockville, MD).

Cell culture

HeLa cells (GIBCO, Grand Island, NY) were grown and maintained at 36°C in Corning 75 cm^2 tissue culture flasks (Scientific Products, McGaw Park, IL) using Minimal Essential Medium with Earle's salts (EMEM, GIBCO) supplemented with 50 μg/ml penicillin G, 50 μg/ml streptomycin sulfate and 100 μg/ml neomycin sulfate (1% PSN, GIBCO). Heat-inactivated fetal bovine serum (HIFBS, JR. Scientific, Inc., Woodland, CA), 7–10%, was used for cell growth (growth medium) and the concentration reduced to 1–2% for cell maintenance (maintenance medium). For virus assay HeLa cells were seeded in 96-well, flat-bottom plates (Falcon) at a concentration of 2×10^5 cells/ml in a 0.1 ml growth medium per well; in 24-well plates (Costar, Cambridge, MA) at a concentration of 1.0×10^5 cells/ml in 1 ml growth medium per well; or in 6-well plates (Costar) at a concentration of 1.3×10^5 cells/ml in 2.0 ml growth medium. All plates were then incubated at 36°C in a humidified CO_2 (5% CO_2, 95% air) incubator for 2 to 3 days to allow for monolayer formation.

Virus assay

Virus plaque and cytopathic effect endpoint assays were performed in HeLa cells as previously described (Kenny et al., 1985; Torney et al., 1982).

Effect of MDL 20,610 treatment time on virus yield from RV 2-infected HeLa cells

HeLa cells in 24-well microtiter plates were treated with 0.1 μg MDL 20,610 in 1.0 ml maintenance medium for 1, 2 or 4 h before virus adsorption. Cultures with compound-free maintenance medium were also included for use as no compound controls and for the study of postinfection compound effects. The concentration of MDL 20,610 employed was in 50-fold excess of the IC_{50} for RV 2. For virus challenge, the medium was removed and each monolayer inoculated with 0.1 ml RV 2 (MOI 20) in maintenance medium containing 0.1 μg MDL 20,610/ml (continuation of pretreatment series and initiation of postinfection treatment series) or in compound-free maintenance medium (virus control). After an adsorption period of 1 h at 36°C the excess inoculum was removed, the monolayers washed once with compound-free maintenance medium, and the cultures refed with 1.0 ml maintenance medium containing 0.1 μg MDL 20,610 or compound-free maintenance medium (virus controls). In this manner cultures were treated with MDL 20,610 at −4, −2, −1, 0, 1 and 4 h relative to initiation of RV 2 infection. All variables were tested in quadruplicate. After 20-h incubation at 36°C the cultures were frozen at −50°C. To measure virus yields sample cultures were thawed; the cells

scraped into the culture fluid; and the samples from each replicate pooled. After 2 additional freeze-thaw cycles the samples were clarified by centrifugation in the cold at 800 × *g* for 10 min. The resultant supernates were assayed by the plaque reduction method.

Mechanism of compound action

The effect of MDL 20,610 on RV 1A (actinomycin D-resistant) RNA synthesis was measured indirectly by determining the incorporation of [5-^{3}H]uridine into a trichloroacetic acid (TCA)-insoluble cell fraction. Virion uncoating was monitored using neutral red photosensitized RV 1A. Detailed descriptions of these techniques have been published previously (Kenny et al., 1985; Kuchler, 1977; Torney et al., 1982). The effect of MDL 20,610 on RV 1A penetration was examined by the method of Eggers et al. (1977). Briefly, the growth medium was aspirated from HeLa cell cultures in 6-well plates and the monolayers washed once with 3.0 ml Hanks' Balanced Salt Solution (HBSS). Three cultures were used for each variable. After washing, 0.1 ml (52–54 PFU) virus was added to each monolayer and the cultures placed at 4°C for 25 min to allow for virus adsorption and to minimize virus penetration. At the end of this time all cultures, except the 0 time set (see Table 2), were placed at 37°C. The 0 time cultures were treated with 0.1 ml HBSS, optimally-diluted RV 1A antiserum, 2 μg/ml MDL 20,610 or a mixture of antibody and MDL 20,610. After 30 min at room temperature the supernatant fluids were aspirated and 2.0 ml agar overlay added. The same procedure was repeated for the virus pre-treated cultures incubated at 37°C for 30, 60 and 90 min. After agar overlay was added all cultures were incubated at 35°C for 3 days to allow for plaque development. To test the direct virucidal activity of MDL 20,610, RV 1A was incubated at 35–37°C for 4 h both in the presence and in the absence of 25 μg compound/ml. Surviving virus was assayed by the CPE endpoint assay. To determine the effect of MDL 20,610 on RV 2 pH stability, 475 μl stock virus ($10^{6.8}$ $TCID_{50}$/0.1 ml) was added to a series of test tubes containing 500 μl of a 0.2 M acetic acid/0.2 M sodium acetate buffer solution mixed to give a final pH of 5.0 (Tisdale and Selway, 1984). The compound was then added as a 25 μl aliquot of a 1.0 mg/ml stock suspension to give a final concentration of 25 μg/ml. Control tubes for each pH value received 25 μl maintenance medium. Sufficient test and control tubes were prepared so that samples could be assayed after 0, 15, 30, 45 and 60 min incubation in a 37°C water bath. At each designated time interval test and control samples were immediately diluted 10-fold with ice cold maintenance medium and further 10-fold dilutions prepared in cold maintenance medium for CPE endpoint assay. To determine the effect of MDL 20,610 on RV 2 heat stability at pH 7.0, 975 μl stock virus ($10^{6.8}$ $TCID_{50}$/0.1 ml) was added to a series of test tubes followed by the addition of 25 μl of a 1.0 mg/ml stock compound suspension or maintenance medium. For each test sufficient tubes were prepared so that samples could be assayed after 0, 5, 10, 15 and 20 min incubation in a 56°C water bath. At the designated time intervals samples were diluted and assayed for infectivity as described above.

Selection of MDL 20,610-resistant RV 2 mutants

To prepare starting material an isolated area of CPE with surrounding cells was removed from a 75 cm^2 HeLa cell flask culture infected with RV 2 (10 $TCID_{50}$). This harvest was subjected to 3 freeze-thaw cycles and then passed twice in HeLa cells to obtain a large pool of parent virus designated as RV 2p. The virus identity was confirmed by neutralization with hyperimmune reference serum. For isolation of MDL 20,610-resistant mutants, HeLa cell monolayers in 6-well plates were pretreated for 3 h with 0, 0.25, 1.0 or 4.0 μg compound in 1.0 ml maintenance medium. The monolayers were then challenged with RV 2p (MOI 13) in compound-containing maintenance medium, the monolayers overlayed with 1.0 ml plaquing medium containing 0, 0.25, 1.0 or 4.0 μg MDL 20,610, and the cultures incubated at 33°C in a humidified CO_2 incubator. A simultaneous plaque titration of the challenge virus under compound-free conditions was also performed. After 48 to 96 hours isolated plaques were picked from appropriate test plates and the monolayers stained for enumeration of the remaining plaques. These plaque counts were used to determine the frequency of MDL 20,610 resistant mutants in the RV 2p population using the formula:

$$F_r = \frac{\text{Mean } PC_{rx}}{\text{Mean } PC_{vc}}$$

where F_r is the frequency of resistant mutants, PC_{rx} is the plaque count in the compound-treated cultures and PC_{vc} is the plaque count in the control cultures. Stocks of resistant mutants isolated from sampled plaques were prepared by 2 passages in noncompound-treated HeLa cells and their identity verified by neutralization with hyperimmune RV 2 antiserum.

Results

Effect of MDL 20,610 pretreatment on RV 2 yield from infected HeLa cells

A preliminary study (Fig. 2) in which MDL 20,610 treatment of HeLa cell monolayers was begun at various time intervals before or after a high MOI RV 2 challenge showed that compound treatment must be initiated before or at the time of virus challenge to inhibit viral replication. These data infer that MDL 20,610 acts on free virions or at some early stage in the virus replicative cycle.

Effect of MDL 20,610 on RV 1A RNA synthesis

Fig. 3 shows that as little as 0.19 to 0.39 μg MDL 20,610/ml inhibits viral (actinomycin D-resistant) RNA synthesis and that 90% inhibition occurs at a compound concentration of 3.12 μg/ml.

Effect of MDL 20,610 on the uncoating of RV 1A

Table 1 summarizes the effect of 0.5 μg MDL 20,610/ml on the uncoating of photosensitized RV 1A. For each experiment, one set of cultures (conditions A

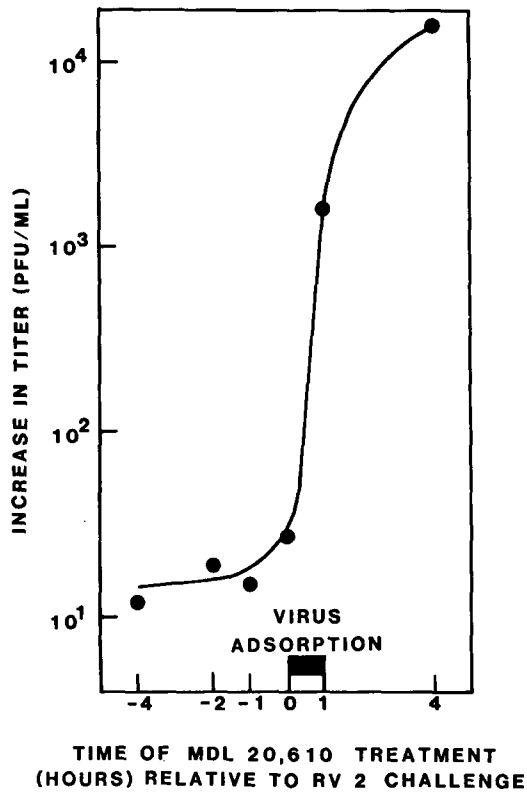


Fig. 2. Effect of MDL 20,610 treatment time on virus yield from RV 2-infected HeLa cells. Each point represents the mean of 4 replicates.

and B) was exposed to light within 5–10 min after addition of virus. A second set (conditions C and D) was exposed to light 3 h after virus challenge. The third set (conditions E through H) received no light exposure. All monolayers, except the compound (condition G) and cell (condition H) controls were thoroughly washed 3 h after virus challenge, agar overlay medium added, and the plaques allowed to develop. The photosensitivity of the RV 1A used in these studies is shown by the lack of plaque development in those cultures where light exposure was begun immediately after virus addition before uncoating could occur (conditions A and B). The amount of virus uncoated within 3-h inoculation in untreated virus control cultures (condition D) was found to be 71.4 to 88.9% of the amount generated in cultures never exposed to light (condition F). A 100% reduction in plaque development was observed in unexposed cultures maintained on 0.5 μg MDL 20,610/ml throughout the duration of the test (condition G). In these experiments not all of the test compound could be removed by washing as shown in a comparison of conditions E and F (residual compound effect). Nonetheless, MDL 20,610 reduced RV 1A plaques by 81.3 to 85.7% in treated cultures exposed to light 3 h after inoculation when compared to untreated culture (conditions C vs. D).

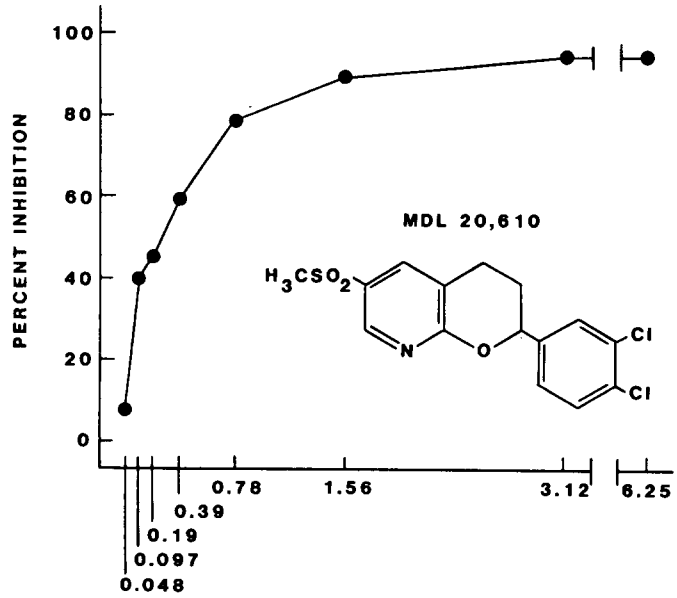


Fig. 3. Effect of MDL 20,610 on RV 1A RNA synthesis in infected HeLa cells. Each point represents the mean of 2 experiments, 3 replicates per experiment.

TABLE 1

Effect of MDL 20,610 on the uncoating of neutral red photosensitized RV 1A

Test condition	Infected HeLa cells exposed to light (H Post-Infection)	Compound added[a]	Av. No. of plaques[b]		Percent plaque reduction	
			Exp. 1	Exp. 2	Exp. 1	Exp. 2
A	0	Yes	0	0	–	–
B	0	No	0	0	–	–
C	3	Yes	1(5.3)[c]	0.3(2.1)[c]	93.8(81.3)	97.0(85.7)
D	3	No	16	10	–	–
E	No light	Yes	6	3	66.6	78.6
F	No light	No	18	14	–	–
G	No light	Yes (retained)	0	0	100	100
H	No light (no virus)	No	0	0	–	–

[a] MDL 20,610 present at 0.5 μg/ml through periods of virus adsorption and light inactivation. All plates were then washed three times (except conditions G and H) and agar overlay medium added.

[b] Average number of plaques in three replicates.

[c] Corrected for residual compound effect demonstrated in a comparison of conditions E and F. Thus 1 and 0.3 PFU represent only 33.3% and 21.4% respectively of the number of plaques expected in the absence of a residual compound effect.

TABLE 2

Effect of 2 μg MDL 20,610 per ml on RV 1A penetration

Test protocol	Virus titer with designated test condition[1]							
	A		B		C		D	
Step 1 (25 min, 4°C)	Virus		Virus		Virus		Virus	
Step 2 (37°C)	HBSS		Antibody (1:100)		MDL 20,610 (2 μg/ml)		Antibody plus MDL 20,610	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
0 time	57[2]	41	22	12	70	50	23	19
30 min	57	55	41	36	66	67	50	50
60 min	64	55	59	42	60	57	58	51
90 min	72	67	72	58	77	81	78	60

[1] Simultaneous virus titrations give a mean of 54 and 52 PFU per well for tests 1 and 2 respectively. Parallel controls with 2 μg MDL 20,610 present during virus adsorption and replication (Compound in overlay) gave a mean PFU of 6 and 0 for tests 1 and 2 respectively confirming the antiviral effect of MDL 20,610.

[2] Mean PFU per well, 3 wells per experiment.

TABLE 3

MDL 20,610 stabilization of RV 2 against inactivation at pH 5.0 and at 56°C

Experimental condition	Time (Min)	Virus Titer[a]							
		MDL 20,610-Sensitive RV 2				MDL 20,610-Resistant RV 2			
		No Compound		MDL 20,610[b]		No Compound		MDL 20,610	
		Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
pH 5.0[c]	0	6.5	6.5	6.8	7.0	2.6	3.6	6.6	6.5
	15	2.0	3.0	6.5	7.2	2.0	≤2.0	4.5	4.4
	30	0[d]	2.4	≥5.0	6.9	0	0	3.4	3.8
	45	0	2.6	≥5.0	6.7	0	0	3.4	3.3
	60	0	0	≥5.0	6.6	0	0	2.5	3.6
56°C[e]	0	7.0	6.8	6.5	7.3	6.8	6.5	7.0	6.5
	5	2.0	2.6	≥5.0	6.0	0	≤2.0	0	2.5
	10	0	0	≥5.0	5.0	0	0	0	0
	15	0	0	0	5.0	0	0	0	0
	20	0	0	0	0	0	0	0	0

[a] $TCID_{50}$/1.0 ml by the method of Reed and Muench (1938). [b] 25 μg/ml. [c] 37°C. [d] No virus detected. [e] pH 7.0.

Effect of MDL 20,610 on RV 1A penetration

Results showing the effect of 2 μg MDL 20,610/ml on RV 1A penetration are summarized in Table 2. Evaluation of the 0 time data shows that 92.4% of the 53 PFU input virus is cell-associated after the 4°C incubation step (Condition A) and that the compound has no appreciable effect on the virus at this stage. In addition, 35% of the cell-associated virus has undergone penetration and is not accessible to neutralization by antibody (Condition A vs. B). Increasing amounts of virus penetrate as incubation is continued at 37°C before antibody is added (Condition A vs. B, 30–90 min). Finally, MDL 20,610 has little effect on penetration of cell-associated virus (condition A vs. C and B vs. D, 0–90 min).

Effect of preincubation of RV 1A with MDL 20,610 on subsequent virus infectivity

Exposure of RV 1A to 25 μg MDL 20,610/ml for 4 h at 35–37°C has no effect on virus titer. In replicate experiments preincubation titers of 1.0 and 1.4×10^7 PFU/ml and postincubation titers of 1.0 and 1.7×10^7 PFU/ml were obtained.

Effect of MDL 20,610 on pH and heat stability of RV 2

Table 3 shows that MDL 20,610 stabilizes compound sensitive RV 2 against inactivation at pH 5.0 and rapid inactivation at 56°C. In contrast, MDL 20,610-resistant RV 2 mutants are not so stabilized.

Frequency of MDL 20,610-resistant RV 2 mutants

Propagation of an MDL 20,610-sensitive (CPE inhibition IC_{50} of 0.002 μg/ml) RV 2 population in the presence of various concentrations of compound shows the

TABLE 4

Frequency of RV 2-resistant mutants from HeLa cells treated with MDL 20,610

Compound concentration (μg/ml)	Virus titer[a]	Incubation time[b]	Frequency of resista mutants
0	4.0×10^7	48	–
0.25	4.5×10^1	72	1 in 10^6
1.0	1.2×10^1	72	3 in 10^7
4.0	4.7×10^0	96	1 in 10^7

[a] PFU per culture, 5–6 replicates per group. [b] Plaques were counted and titers determined when plaques in a culture reached the optimum size for enumeration.

frequency of resistant mutants to be 1 in 10^6, 3 in 10^7, and 1 in 10^7 at 0.25, 1.0 a 4.0 μg MDL 20,610/ml respectively (Table 4). In untreated cultures RV 2 plaq develop within 48 h whereas the plaques of resistant mutants require 72 to 9 for development and are smaller.

Stability of MDL 20,610 resistance

To study the stability of compound resistance a stock resistant RV 2 isolatec the presence of 0.1 μg MDL 20,610/ml was passed 4 times in the absence of cc pound or in the presence of 4.0 μg MDL 20,610/ml. The resultant titers of the l passage were 4×10^6 PFU/ml and 2×10^7 PFU/ml respectively for virus pro gated in the absence or in the presence of compound when assayed in the prese of compound.

Cross resistance with other antiviral compounds

The data summarized in Table 5 shows that RV 2 MDL 20,610 cross resista occurs with the 4′,6-dichloroflavan BW683C and the 3,4-dichlorophenoxypyrid MDL 055, but not with the *p*-benzoylphenoxypyridine MDL 26,019.

TABLE 5

Comparison of RV 2 CPE reduction with MDL 20,610 and other antiviral compounds

Compound	IC_{50} in μg/ml[a]	
	Parent Virus	Resistant Virus
MDL 20,610	0.002	>1.0
BW683C	0.13	>4.0
MDL 055	0.31	>4.0
MDL 26,019	2.5	5.0

[a] CPE inhibition; 3 replicates per compound concentration tested.

Discussion

Compound MDL 20,610 is one of the more active compounds in a series of 3,4-dihydro-2-phenyl[2,3-*b*]pyridines synthesized as antiviral compounds (Bargar et al., 1986; Kenny et al., 1986a). In addition, MDL 20,610 displays little effect on HeLa cell macromolecular synthesis when evaluated by isotope incorporation or by cloning efficiency (Kenny et al., 1986a). In the present studies we examined the antirhinovirus mechanism of action of MDL 20,610. Analysis of the growth of RV 2 in compound-treated or untreated HeLa cells suggested that MDL 20,610 acts directly on infecting virions or at some early stage in the replicative cycle. Subsequently, it was found that the compound blocks viral (actinomycin D-resistant) RNA synthesis and the uncoating of neutral red photosensitized virions. In addition, it was demonstrated that MDL 20,610 is not directly virucidal, but stabilizes the virion against inactivation at 56°C and at pH 5.0. By comparison, the antirhinovirus compound 44 081 R.P. (Alarcon et al., 1986) and the antipicornavirus flavone Ro 09-0179 (Ishitsuka et al., 1982b) have also been shown to act on some early phase in the viral replicative cycle while the antirhinovirus 4′,6-dichloroflavan BW683C (Tisdale and Selway, 1984) and the antipicornavirus compounds WIN 51711 (Fox et al., 1986; McKinlay, 1985; Otto et al., 1985), Ro 09-0410 (Ishitsuka et al., 1982a; Ninomiya et al., 1984), and arildone (McSharry et al., 1979) bind directly to infecting virions preventing the subsequent pH-mediated uncoating of viral RNA.

Recent elucidation of the three-dimensional structure of the RV 14 capsid (Rossmann et al., 1985) has allowed the study of WIN 51711 binding at the molecular level (Smith et al., 1986). Those studies indicate that virus uncoating may be inhibited by compound binding to the virion VP1 structural protein thus preventing collapse of the VP1 hydrophobic pocket or by blocking the flow of ions into the virus interior during uncoating. Such data are lacking for MDL 20,610 but compound-induced heat and low pH stabilization indicate direct virion binding and protein stabilization. Earlier studies by Dimmock (1967) have shown that RV inactivation above 39°C is due to the degradation of capsid protein. Compound BW683C has also been shown to stabilize RV against heat and low pH inactivation (Tisdale and Selway, 1984). The cross resistance of MDL 20,610, MDL 055 and BW683C, the inability of MDL 20,610 to stabilize compound-resistance mutants as shown in our study, and the cross resistance demonstrated between arildone and BW683C (Tisdale and Selway, 1984) imply a common virion binding site for all four compounds. No such cross resistance was noted with the *p*-benzoylphenoxypyridine MDL 26,019, a compound previously shown to inhibit uncoating of neutral red-sensitized coxsackie A21 (Kenny et al., 1986b).

Compound resistant rhinovirus mutants have been isolated for Ro 09-0410 (Ahmad et al., 1987; Ninomiya et al., 1984), BW683C (Tisdale and Selway, 1984), WIN 51711 (Fox et al., 1986) and for MDL 20,610. The lack of a suitable RV animal infection model makes it difficult to compare the pathogenesis of compound-susceptible parent and resistant mutant RV populations. Reduced virulence of thymidine kinase-negative acyclovir herpes simplex mutants has been noted in mice

(Field and Darby, 1980; Field and Wildy, 1978; Klein et al., 1981). In the present study we observed that MDL 20,610 resistant RV 2 mutants grow poorly in vitro as evidenced by a delay in appearance and reduction in plaque size when compared to the compound-sensitive parent population. Similar changes in growth characteristics have recently been described for Ro 09-0410 resistant RV 9 (Ahmad et al., 1987).

In summary, the data presented here indicate that MDL 20,610 probably acts by stabilization of the rhinovirus capsid against subsequent degradation during the uncoating process.

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