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Antiviral Activity and Mechanism of Action of 2-(3,4-Dichlorophenoxy)-5-Nitrobenzonitrile (MDL-860)

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A nitrobenzene derivative, MDL-860, was found to inhibit plaque formation, cytopathic effect, or both in 11 of 12 picornaviruses at concentrations which did not affect cell growth. The compound did not directly inactivate the virus. MDL-860 inhibited actinomycin D-resistant [^{3}H]uridine uptake in cells infected with coxsackievirus A_{21} or rhinovirus 1-A, whereas incorporation into uninfected cells was not inhibited. With three picornaviruses (echovirus type 12, poliovirus type 2, and rhinovirus type 1-A) made photosensitive with neutral red, MDL-860 did not appear to cause a significant reduction in their loss of photosensitivity (uncoating) during the first 3 h of infection. MDL-860 appears to inhibit some early event in virus replication, after uncoating, which is required for synthesis of a majority of viral RNA.

A search in The Dow Chemical Company laboratories for agents active against the picornaviruses led to the identification of the broadly active 2-(3,4-dichlorophenoxy)-5-nitrobenzonitrile (MDL-860). This agent exhibited high in vitro activity against the picornaviruses but not against viruses of other classes. This report is concerned with the spectrum of activity of MDL-860 and the results of some preliminary observations on its mechanism of action. The accompanying report (5) presents a more extensive evaluation of the antiviral spectrum of the compound.

MATERIALS AND METHODS

Compound. MDL-860 was synthesized by L. Markley of The Dow Chemical Company, Midland, Mich. For easier dissolution in aqueous cell culture medium, the compound is first dissolved in 0.05 ml of dimethyl sulfoxide per 3 mg of MDL-860 and then diluted more than 100-fold in aqueous cell culture medium to produce a suspension of small particles.

Viruses. Rhinovirus (RV) types 5, 8, 64, and Hank and RV types 1-A and 2, obtained from J. Gwaltney (University of Virginia School of Medicine), and B. D. Korant (E. I. Dupont de Nemours and Co.), respectively, were grown in HeLa cells. Coxsackievirus (Cox) A_{21}, obtained from D. C. Delong (Eli Lilly & Co.), was grown in weanling mice and adapted to HeLa cells. Cox B_3 virus, obtained from C. Gauntt (University of Texas Health Science Center), and Cox B_4 virus, obtained from G. Burch (Tulane University Medical School), were grown in Vero cells and adapted to HeLa cells. Echovirus (Echo) type 12 was obtained from G. Schiff (Christ Hospital, Institute of Medical Research, Cincinnati) and grown in Vero cells. Attenuated poliovirus (Polio) type 2, supplied by P. Oriel (The Dow Chemical Company), was initially grown in African green monkey kidney cells and then adapted to growth in HeLa cells. Influenza virus A/PR/8/34 (ATCC VR 95) was grown in embryonated eggs and then adapted to growth in Vero cells. Influenza virus A/NWS, obtained from P. W. Choppin (The Rockefeller University) was grown in Maden-Darby bovine kidney cells and then adapted to growth in Vero cells. Feline calicivirus FPV-255, obtained from E. Hoover (Ohio State University), was initially grown in feline tongue cells FC_3Tg (also obtained from E. Hoover) and then adapted to growth in Vero cells. Newcastle disease virus and mouse mengovirus were obtained from N. A. Miner (Arbrook, Inc., Arlington, Tex.). The Newcastle disease virus strain is an embryonated egg stock. Both Newcastle disease virus and the mengovirus were tested in HeLa cells.

Cell culture. The Vero African green monkey cell line, passage level 140–180, and the HeLa cell line, passage level 10–70 (GIBCO Laboratories, Grand Island, N.Y.), were grown with Eagle minimal essential medium (MEM) with Earle salts (EMEM; K.C. Biologicals, Lenexa, Kans.). The medium was supplemented with 7 to 10% heat-inactivated (HI) fetal calf serum (FCS; GIBCO) for cell growth. The serum concentration was reduced to 1 to 2% for cell maintenance. Both cell lines were grown at 36°C and maintained at 33°C in a 5% CO_2 atmosphere incubator. Cells were maintained in Corning 75-cm^2 tissue culture flasks (Scientific Products, Inc., McGaw Park, Ill.). For testing purposes, the cells were grown either in Costar 24-well plates with 16-mm-diameter wells (MA Bioproducts, Walkersville, Md.) or in Linbro 6-well plates with 35-mm-diameter wells (Flow Laboratories, McLean, Va.). HeLa cells were seeded at 1.0×10^5 to 1.3×10^5 cells per ml, and Vero cells were seeded at 0.8×10^5 to 1.2×10^5 cells per ml when used in 6- or 24-well tissue culture plates.

Virus plaque reduction. Plaquing methods were adaptations of those described by Wentworth and French (7). The medium was aspirated from 6-well tissue culture plates containing monolayers of Vero or HeLa cells, and 0.1 ml of the appropriate virus dilution in maintenance medium was added to each cell monolayer. All plates were incubated for 90 min at 36°C in a humidified incubator containing a 5% CO_2 atmosphere to allow virus adsorption. Each monolayer then was overlaid with MDL-860 in 2 ml of a medium composed of 87% (vol/vol) McCoy 5a medium supplemented with 2% HIFCS, penicillin and streptomycin (50 μg/ml each), neomycin (100 μg/ml), 10% of a 3.5% ion agar solution, 2% of a 1.5 M solution of $MgCl_2$, and 1% of a 0.2% dextran solution. After incubation at 36°C in a 5% CO_2 atmosphere incubator for 48 to 72 h (Cox B_4 and Echo 12) or 96 h at 33°C (RV Hank), the overlay medium was removed by inverting the plate. At this time, each cell layer was stained with 1 to 2 ml of 0.1% crystal violet (Allied Chemical, New York, N.Y.) in ethanol-water (20:80). After 1 min, the dye was removed by gently rinsing the plates with water, the plates were allowed to dry, and the plaques were counted.

Virus CPE inhibition. The growth medium was removed from the 24-well plates when the cells had reached 60 to 75% of a monolayer and was replaced with EMEM containing 1 to 2% HIFCS and MDL-860 at various concentrations. Cell culture plates were returned to the 36°C incubator for approximately 1 h, and then 0.05 to 0.1 ml of challenge virus (10 to 1,000 tissue culture 50% infective doses [$TICD_{50}$]) was added to all but the control wells. The plates were incubated at 36 or 33°C for the duration of the test (2 to 4 days). Cells were observed microscopically at 24-h intervals for viral cytopathic effect (CPE). Compounds are considered active at concentrations which reduce viral CPE in infected cultures by 50% or more compared with untreated virus-infected cultures.

Cell growth test. HeLa cells were seeded at 25 to 100 cells per well in 24-well tissue culture plates in growth medium (EMEM with 5% HIFCS). After 24 h at 36°C, the growth medium was removed, and the cells were refed with 1 ml of growth medium supplemented with 0 to 25 μg of MDL-860 per ml. Cell culture plates were incubated at 36°C for 8 days; the medium was then removed, and the cells were fixed and stained with 1% crystal violet in ethanol-water (20:80). HeLa cell colonies were counted macroscopically, and comparisons of colony number and size between treated and control wells were made.

Direct inactivation of virus. RV 1-A was incubated in medium containing 20 μg of MDL-860 per ml and in medium without MDL-860 for 4 h at 35°C. The two virus preparations were then titrated for infectivity in HeLa cells, with seven wells used for each dilution. Virus titers were calculated by the method of Reed and Meunch (6).

RNA synthesis. RNA synthesis was measured indirectly by determining the uptake of [5-^{3}H]uridine into a trichloroacetic acid (TCA)-insoluble fraction of cells (2). HeLa cells were grown to confluent monolayers in 6-well culture plates. The growth medium was aspirated, the cells were inoculated at a multiplicity of infection of 1 to 10 with Cox A_{21} or RV 1-A, and the virus was allowed to adsorb for 1 h at 36°C. After the adsorption, each of three wells then received 2 ml of MEM containing 2% FCS and the indicated concentrations of MDL-860. As controls, and for determining the effect of MDL-860 on cellular RNA synthesis, another set of plates was prepared without virus inoculation. In the appropriate medium, 5 μg of actinomycin D (ICN Pharmaceuticals, Inc.) per ml was added to block DNA-directed cellular RNA synthesis to allow determination of the non-blocked viral RNA-

TABLE 1. Effect of MDL-860 on virus plaque formation

MDL-860[a] (μg/ml)	No. of PFU[b]		
	RV Hank	Echo 12	Cox B_4
0	38	37	44
0.06	37	33	45
0.125	16	21	40
0.25	9	13	30
0.5	6	2	17
1.0	0	0	3
2.0	0	0	3

[a] MDL-860 was present only in the overlay medium.
[b] Average number of PFU in three replicate cultures.

TABLE 2. Effect of MDL-860 treatment on virus CPE in vitro

Cell line[a]	Virus[b]	MIC_{50}[c] of MDL-860 (μg/ml)
HeLa	RV 1-A	0.1
HeLa	RV 2	0.1
HeLa	Cox A_{21}	0.2
HeLa	RV 8	0.2
HeLa	RV 64	0.2
HeLa	Polio 2	≤0.6
Vero	Echo 12	≤0.6
HeLa	RV 5	0.6
HeLa	RV Hank	1.5
HeLa	Cox B_3	1.5
HeLa	Cox B_4	1.5
HeLa	Mengovirus	NA[d]
HeLa	Newcastle disease virus	NA
Vero	Influenza virus A/NWS	NA
Vero	Influenza virus A/PR/8/34	NA
Vero	Calicivirus FPV-255	NA

[a] Cell cultures of 60 to 75% confluency were treated with 1 ml of EMEM with 2% HIFCS supplemented with MDL-860 at concentrations of 0 to 12.5 μg/ml.
[b] At 1 to 2 h after treatment, cells were challenged with 0.05 to 0.1 ml of virus preparation (10 to 1,000 $TCID_{50}$).
[c] MIC_{50}, Minimum concentration of MDL-860 required to inhibit viral CPE on cells by 50% or more as compared with infected controls.
[d] NA, Not active against this virus.

directed RNA synthesis (actinomycin D-resistant) (2). [^{3}H]uridine ([5-^{3}H]uridine, TRK 178; Amersham Corp., Arlington Heights, Ill.) was diluted to 0.11 mCi/ml and 4.03 meq/ml in phosphate-buffered saline containing 200 μg of uridine per ml to avoid depletion of the intracellular uridine pool. A 0.05-ml portion of this preparation was added to each well 3 h after virus had been added to the cells. At 8.5 h after the cells had been inoculated with virus, the period for uridine uptake was terminated. An equal volume of 10% TCA was added to the contents of each well. The 5% TCA-insoluble materials were collected on 0.45-μm filters (25 mm, GN6; Gelman Instrument Co., Ann Arbor, Mich.) with the model 3025 Millipore sampling manifold (Millipore Corp., Bedford, Mass.), and the precipitate and filters were then washed and dried. To measure the [^{3}H]uridine incorporation, each filter was placed into a 10-ml volume of Permafluor (Packard Instrument Co., Inc.) diluted 25-fold in toluene (T313; Fisher Scientific Co., Pittsburgh, Pa.), and the disintegrations per minute were determined with the model 6847 Searle liquid scintillation system.

Inhibition of viral uncoating. The production, testing, and use of photosensitive viruses to determine the ability of a compound to inhibit the uncoating of a virus were performed as described by Mandel (3) and McSharry et al. (4). RV 1-A, Echo 12, and Polio 2 were grown for three serial passages in cell cultures containing 10 μg of neutral red (Nutritional Biochemicals Corp., Cleveland, Ohio) per ml. The viruses were tested after the third passage, and all were found to be photosensitive (data not shown).

The viruses, at dilutions suitable for plaque determinations, were added to cell cultures with and without MDL-860 (10 μg/ml). One set of plates was exposed to light within 5 to 10 min after addition of the virus. A duplicate set of plates was exposed to light 3 h after addition of the virus to destroy any virus retaining photosensitivity (coated). The cells then were washed three times to remove the compound, which could inhibit plaque formation by any virus not destroyed by light. Overlay medium was then added, and the plaques were allowed to develop. Details of the incubation and subsequent staining of the cells were described above.

RESULTS

Inhibition of viral plaque formation. The incorporation of MDL-860 into the agar overlay medium produced a significant reduction in Echo 12, RV Hank, and Cox B_4 plaque formation at compound concentrations from 0.125 to 0.5 μg/ml (Table 1).

Inhibition of viral CPE. MDL-860 incorporated in the cell culture medium inhibited viral CPE for 11 of 12 picornaviruses at concentrations of 0.1 to 1.5 μg/ml. The compound did not inhibit the CPE of four non-picornaviruses tested (Table 2).

HeLa cell growth. Concentrations of MDL-860 up to and including 25 μg/ml of medium did not reduce the number or size of colonies which developed over an 8-day period of growth. There was no apparent adverse effect on the cells as determined by microscopic examination.

Direct inactivation studies. Exposure of RV 1-A to 20 μg of MDL-860 per ml for 4 h at 35°C resulted in no loss of infectivity. RV 1-A incubated without the compound had a titer of 6.3 $TCID_{50}$/ml versus 6.84 $TCID_{50}$/ml for virus incubated with the compound.

Cell RNA synthesis. Incorporation of [^{3}H]uridine into the TCA-insoluble fraction of cells over a 5.5-h incubation period was not significantly inhibited by concentrations of MDL-860 from 0.048 to 25 μg/ml.

Viral RNA synthesis. Actinomycin D-resistant incorporation of [^{3}H]uridine into the TCA-insoluble fraction of RV 1-A- and Cox A_{21}-infected cells was inhibited by MDL-860. At a concentration of 0.048 μg/ml, MDL-860 inhibited actinomycin D-resistant [^{3}H]uridine uptake in Cox A_{21}-infected cells by more than 50% (Table 3).

TABLE 3. Effect of MDL-860 on viral-directed RNA synthesis

MDL-860 (μg/ml)	Viral RNA synthesis[a] (% control)	
	RV 1-A	Cox A_{21}
0.048	89	32
0.097	80	29
0.19	66	17
0.39	63	12
0.78	56	0.9
1.56	17	0
3.125	25	0
6.25	18	0
12.5	13	0.9
25.0	13	0

[a] Actinomycin D-resistant incorporation of [^{3}H]uridine into the 5% TCA-insoluble fraction of RV 1-A- and Cox A_{21}-infected cells was an indirect determination of viral-directed RNA synthesis.

TABLE 4. Effect of MDL-860 on uncoating of picornaviruses

Infected cells exposed to light (h postinfection)	MDL-860 (10 μg/ml) added[a]	Avg no. of plaques[b]			
		RV 1-A		Polio 2	Echo 12
		Expt 1	Expt 2		
0	Yes	0	0	0	0
0	No	0	0	0	0
3	Yes	25	17	18	25
3	No	38	24	52	57
Not exposed	Yes	41	31	15	40
Not exposed	No	60	50	49	57

[a] MDL-860 was added before virus adsorption and light inactivation. All plates were washed just before the medium was overlaid.

[b] Average number of plaques in three replicate cultures.

Effect of MDL-860 on uncoating of picornaviruses. Incorporation of neutral red by picornaviruses during replication renders the progeny virus sensitive to light until the neutral red is lost after the virus infects cells and becomes uncoated (3, 4). Three photosensitive viruses (RV 1-A, Polio 2, and Echo 12) were tested for their ability to lose photosensitivity (or uncoat) after being inoculated into cells in the presence of 10 μg of MDL-860 per ml (Table 4). Cells exposed to light within 5 to 10 min (time zero) after virus inoculation produced no viral plaques, which indicated that the uncoated viruses were destroyed by light. A comparison of the number of plaques in control cultures with and without MDL-860 but not exposed to light indicated that MDL-860 had some plaque-inhibiting effect which was not completely removed from the cells by washing them as performed in these experiments. Such intracellular persistence of an antiviral compound has been reported for acyclovir (1). Although there was a reduction in the number of plaques in the treated cells due to residual MDL-860, no further reduction was effected by light. This indicated that MDL-860 does not inhibit the uncoating of the virus.

DISCUSSION

MDL-860 at relatively low concentrations was found to effectively inhibit 11 of 12 picornaviruses and not to inhibit other viruses. The accompanying report (5) extends these findings to include its activity against a larger number of picornaviruses. The compound had no discernible adverse effects on RNA or protein synthesis by or growth or colony formation of HeLa cells. Even a subtle effect through many cell divisions might be expected to reduce the size or number of colonies if the compound were affecting cellular metabolism. These results in conjunction with the compound's selective activity against picornaviruses suggest that it does not inhibit virus replication by inhibiting cellular metabolism.

MDL-860 did not directly inactivate virus, suggesting an action in the infected cell. In studies of single-cycle viral replication which used a high multiplicity of infection, MDL-860 was found to inhibit viral RNA synthesis when it was added shortly after virus adsorption. Picornaviruses (Echo 12, Polio 2, and RV 1-A) made photosensitive with neutral red to measure viral uncoating were capable of losing photosensitivity during the first 3 h after infection of HeLa cells in the presence of antiviral concentrations of MDL-860. This was interpreted as evidence that viral uncoating was not affected (3, 4). MDL-860 appeared to inhibit some early event in replication, after uncoating, which is required for synthesis of a majority of viral RNA.

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