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Activity of 2-(3,4-Dichlorophenoxy)-5-Nitrobenzonitrile (MDL-860) Against Picornaviruses in Vitro

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The newly synthesized compound 2-(3,4-dichlorophenoxy)-5-nitrobenzonitrile (MDL-860) has been found to inhibit picornavirus replication. In multiple growth cycle experiments, 1 μg of MDL-860 per ml caused a reduction in virus yield of at least 1.0 $\log_{10}$ 50% tissue culture infectious doses per 0.2 ml for 8 of 10 enteroviruses and 72 of 90 rhinovirus serotypes. This antiviral activity was dependent on both compound concentration and virus inoculum size. At concentrations that had no toxic effects on cell cultures, MDL-860 did not inhibit cytopathic effect or hemadsorption activity due to coronavirus 229-E, vesicular stomatitis virus, herpes simplex virus type 1, adenovirus, influenza virus A, or parainfluenza virus 1. Compound concentrations up to 25 μg/ml did not cause cytopathic effect in short-term cultures of rhesus monkey, WI-38, or HeLa cells; 10 μg/ml did not inhibit the replication of HeLa cells. The mechanism of action of MDL-860 has not been defined, although it was not directly virucidal and appeared to inhibit picornaviruses specifically at an early step in the virus-host cell interaction.

The compound 2-(3,4-dichlorophenoxy)-5-nitrobenzonitrile (MDL-860; Merrill-Dow Pharmaceuticals, Indianapolis, Ind.) is the most active of more than 800 newly synthesized nitrobenzene derivatives screened for antiviral activity in vitro (4). The current study reports on its antiviral spectrum and on preliminary studies of its mechanism of action.

MATERIALS AND METHODS

Compound. MDL-860, provided as a crystalline powder, was initially dissolved in dimethyl sulfoxide (10 mg/ml), and further dilutions were then made with warm (37°C) culture medium appropriate to the cell type utilized.

Viruses. Enteroviruses (echoviruses 9 and 16; polioviruses 1 and 3; coxsackieviruses A7, A9, A16, B4, and B5; enterovirus 70) were obtained from the American Type Culture Collection, Rockville, Md. Rhinovirus serotypes 1–81, 83, 84, and 88, coronavirus 229-E, influenza virus A (H1N1 subtype), herpes simplex virus type 1, and adenovirus came from clinical isolates typed and stored at the University of Virginia Common Cold Research Unit. Stocks of vesicular stomatitis virus, rhinovirus serotypes 82, 85, 86, 87, and 88, and parainfluenza virus 1 were obtained from the National Institute of Allergy and Infectious Diseases, Bethesda, Md.

Cell cultures. WI-38 human embryonic lung fibroblasts (HEM Research, Rockville, Md.) were maintained in 49% Eagle minimal essential medium, 49% medium 199, and 2% fetal bovine serum; primary rhesus monkey kidney cells (PRMK; MA Bioproducts, Walkersville, Md.) were maintained in minimal medium. HeLa cells (initially obtained from Vincent Hamparian, Ohio State University) were grown in minimal medium with 10% fetal bovine serum and maintained in minimal medium with 5% fetal bovine serum.

Yield reduction experiments. (i) Rhinovirus. From 10 to 100 50% tissue culture infectious doses ($TCID_{50}$) of virus were inoculated into screw-capped tubes containing WI-38 cell monolayers. After a 1-h adsorption period, five tubes each were overlaid with plain maintenance medium or medium containing MDL-860 at a concentration of 1 μg/ml. Each set of tubes was incubated at 34°C and harvested when cytopathic effect (CPE) was evident in the control tubes. The majority (59 of 85) of the preparations were harvested at 48 h, but several were incubated for up to 144 h. For harvesting the virus, the supernatant liquid was withdrawn from the tubes and pooled. Cell monolayers were then scraped from the tubes, pooled with the supernatants, separated into equal portions, and stored at −70°C. Infectivity titrations were performed with unclarified portions in quadruplicate monolayers of WI-38 cells. After a 1-h adsorption period, the cells were washed three times with phosphate-buffered saline to remove residual drug and incubated at 34°C. Monolayers were observed daily for CPE, and the infectivity titer was calculated by the Kärber formula (2).

(ii) Enterovirus. Experiments were conducted in primary PRMK cell monolayers except for enterovirus 70, which was grown in WI-38 cells. Quadruplicate monolayers were overlaid with tissue culture medium containing 0, 0.2, 1.0, or 5.0 μg of MDL-860 per ml.

Each monolayer was then inoculated with 6 to 126 $TCID_{50}$ of virus and incubated at 36°C. When CPE was evident in control tubes (48 to 96 h), all monolayers were subjected to one freeze-thaw cycle, and virus yields were determined by infectivity titrations in PRMK cells (coxsackieviruses A9 and A16; echoviruses 9 and 16) or WI-38 cells (enterovirus 70) or by plaque formation in HeLa cell monolayers overlaid with medium containing agarose (50 mg/ml), $MgCl_2$ (12 mg/ml), and DEAE-dextran (60 μg/ml) (coxsackieviruses A7, B4, and B5: polioviruses 1 and 3).

Screening for activity against non-picornaviruses. Cell culture monolayers were adsorbed for 1 h at 36°C with ~100 $TCID_{50}$ of one of the following viruses (culture cells): coronavirus 229-E (WI-38), vesicular stomatitis virus (HeLa), influenza virus A (PRMK), adenovirus (human embryo kidney), parainfluenza virus 1 (PRMK), or herpes simplex virus type 1 (Vero). The monolayers were then overlaid with maintenance medium containing various concentrations of MDL-860. Control tubes were infected with the same virus inoculum and overlaid with plain maintenance medium. All tubes were incubated on a roller drum at 36°C until CPE was evident in the control monolayers, at which time the extent of CPE or hemadsorbing activity (0.4% guinea pig erythrocytes) in the MDL-860-treated monolayers was compared with that in the control monolayers in a semiquantitative fashion (1).

Direct virus inactivation. To determine whether MDL-860 could directly inactivate picornaviruses, we added 4.5 $\log_{10}$ $TCID_{50}$ of a sensitive enterovirus (coxsackievirus B4) to tubes containing 5 μg of MDL-860 per ml in Hanks balanced salt solution (HBSS). Control tubes containing plain HBSS or 1% dimethyl sulfoxide in HBSS were inoculated with the same amount of virus, and all tubes were incubated at 36°C for 2 h. At the end of this time period portions were serially diluted, and titrations were performed in PRMK monolayers as described above.

Time-of-addition studies. To determine the point in the viral replication cycle at which MDL-860 exerted its maximum effect, we added the compound to cell monolayers at various time intervals after infection (3). Coxsackievirus B4 (multiplicity of infection, ~1) was adsorbed for 1 h at 36°C to HeLa cell monolayers in 16-mm tissue culture wells (Costar, Cambridge, Mass.), which were then washed with HBSS and refed. Maintenance medium was removed from groups of four wells at 1-h intervals and replaced by medium containing 5 μg of MDL-860 per ml. A separate group of four wells was overlaid with medium containing 5 μg of MDL-860 per ml during the 1-h virus adsorption period, then washed five times with phosphate-buffered saline and refed with plain maintenance medium.

TABLE 1. Inhibitory effect of MDL-860 (1 μg/ml) on picornavirus replication in vitro

Virus (no. of strains)	No. (%) of strains with mean decrease in yield ($\log_{10}$ $TCID_{50}$/0.2 ml) of:		
	≥1.0	0.5–0.9	<0.5
Rhinovirus (90)	72 (80)	12 (13)	6 (7)
Enterovirus (10)	8 (80)	1 (10)	1 (10)

Infected control monolayers were subjected to the same cycles of washing and refeeding with maintenance medium. After incubation for 18 h, monolayers were subjected to one freeze-thaw cycle, and the virus yields were determined by titration in HeLa cells.

Effect on HeLa cell growth. Approximately 2×10^4 HeLa cells were added to quadruplicate tissue culture wells containing growth medium and MDL-860 at concentrations of 0, 1, 5, or 10 μg/ml. After a 72-h incubation, cell monolayers were dispersed by trypsinization and counted in a hemacytometer. Viability was determined by trypan blue exclusion.

RESULTS

Inhibition of picornavirus replication. MDL-860 at a concentration of 1.0 μg/ml decreased the yield of the majority of the picornaviruses tested (Table 1). Of 90 rhinovirus serotypes tested, 72 (80%) had their yield reduced by at least 1.0 $\log_{10}$, 12 (13%) were intermediate in their level of inhibition, and only 6 (7%) were not inhibited by this concentration of MDL-860. The yield of 33 (36%) rhinovirus serotypes was reduced by at least 2 $\log_{10}$ at this concentration, but the yields of rhinovirus serotypes 8, 38, 40, 56, 63, and 82 were inhibited by less than 0.5 $\log_{10}$. The yields of 8 of 10 enteroviruses were inhibited by at least 1.0 $\log_{10}$.

The activity of MDL-860 against enteroviruses was found to be related to both drug concentration and virus inoculum. A concentration-dependent reduction in virus yield was observed for all 10 enterovirus strains. The patterns observed with poliovirus 3 and echovirus 16 were typical of most of the strains tested (Fig. 1). In contrast, the yields of enterovirus 70 and coxsackievirus B5 were not significantly altered by MDL-860 concentrations of 0.2 or 1.0 μg/ml, but at 5 μg/ml a greater than 3 $\log_{10}$ reduction of

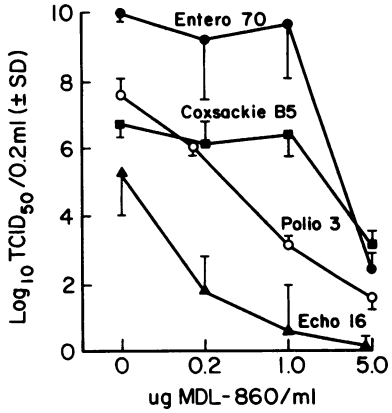

FIG. 1. Effect of various MDL-860 concentrations on enterovirus replication. Symbols: ●, enterovirus 70; ■, coxsackievirus B5; ○, poliovirus 3; ▲, echovirus 16.

TABLE 2. Effect of inoculum size on inhibition of coxsackievirus B5 by MDL-860

Inoculum ($TCID_{50}$)	Culture duration (h)	Mean (SD) virus titer (log_{10} PFU/0.2 ml) at MDL-860 concn (μg/ml):		
		0	1.0	5.0
3.2	72	7.2 (0.9)	0.3 (0.1)	0.2 (0.2)
126	48	10.3 (0.2)	9.6 (1.5)	2.4 (0.8)

virus yield was apparent for these as well as for the other strains.

Table 2 lists the effects of varying the virus inoculum on the yield of coxsackievirus B5. At a low inoculum (3.2 $TCID_{50}$ per monolayer), the coxsackievirus B5 yield at 72 h after infection was reduced by over 6 log_{10} at either concentration of MDL-860. In contrast, at an approximately 40-fold-higher virus input, the virus yield was not substantially reduced at the lower compound concentration despite a shorter duration of culture. A fivefold-higher compound concentration reduced viral replication by more than 7 log_{10}.

Inhibition of non-picornaviruses. MDL-860 at the concentrations indicated in parentheses did not decrease the CPE or hemadsorbing activity in vitro of herpes simplex virus type 1 (50 μg/ml), adenovirus (50 μg/ml), influenza virus A (50 μg/ml), parainfluenza virus type 1 (50 μg/ml), coronavirus 229-E (6 μg/ml), or vesicular stomatitis virus (12.5 μg/ml).

Mechanism of action. (i) Direct inactivation. After 2 h of exposure to 5 μg of MDL-860 per ml, no direct inactivation of coxsackievirus B4 was observed. Analysis of three separate experiments revealed that the mean titer (± standard deviation) of MDL-860-exposed virus (3.9 ± 0.1) log_{10} $TCID_{50}$) was similar to that of virus incubated in HBSS containing 1% dimethyl sulfoxide (3.9 ± 0.1 log_{10} $TCID_{50}$) or in plain HBSS (4.0 ± 0.1 log_{10} $TCID_{50}$).

(ii) Time of addition. The effects of adding MDL-860 to HeLa cell monolayers at various times after infection are shown in Table 3. The presence of the compound during the adsorption period was associated with a 1.0 log_{10} reduction in yield for coxsackievirus B4, but the greatest inhibitory effect was noted when MDL-860 was present during the first 2 h after adsorption. Addition of MDL-860 after this time interval had a less apparent effect on viral replication.

Cell culture toxicity. MDL-860 concentrations of 25 μg/ml did not cause CPE in short-term cultures (3 to 5 days) of rhesus monkey kidney, WI-38, or HeLa cells. In quantitative tests of HeLa cell proliferation, the mean number of viable cells per monolayer after a 72-h incubation period were 13 × 10^4, 12 × 10^4, 12 × 10^4, and 11 × 10^4 for monolayers exposed to MDL-860 supernatant concentrations of 0, 1.0, 5.0 and 10 μg/ml, respectively.

TABLE 3. Effect on replication of coxsackievirus B4 by the addition of MDL-860 (5 μg/ml) at various time intervals after HeLa cell monolayer infection

MDL-860 added (h after monolayer infection)	Reduction in virus titer (log_{10} $TCID_{50}$/0.2 ml) relative to control[b]
0[a]	1.0
1	2.5
2	2.5
3	1.2
4	0.8
6	0.5

[a] MDL-860 was added at the time of infection and removed 1 h later.

[b] Mean of quadruplicate monolayers harvested 18 h after infection.

DISCUSSION

In the present study the nitrobenzene compound MDL-860 proved to be a potent inhibitor of picornavirus replication in vitro. MDL-860 demonstrated broad-spectrum activity that was dependent on both virus inoculum and compound concentration. At 1 μg/ml, it inhibited 80% of the rhinovirus and enterovirus strains tested. In addition, the inhibitory concentrations were 10- to 50-fold lower than those having no effect on HeLa cell proliferation, which indicates that MDL-860 has a high therapeutic index in vitro.

Although its exact mechanism of antiviral action remains to be elucidated, the inhibitory effect of MDL-860 appears to be specific for members of the picornavirus family. Although the compound does not directly inactivate coxsackievirus B4, time-of-addition studies indicated that it inhibits an early step(s) in the replication process. The observation that MDL-860 has its antiviral effect within the first few hours of cell infection is consistent with earlier work showing inhibition by MDL-860 of virus-directed RNA synthesis (4). The suitability of this compound for clinical use remains to be determined, but its specificity and high therapeutic index in vitro make it a promising new antiviral agent.

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