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# In Vitro Activity of WIN 51711, a New Broad-Spectrum Antipicornavirus Drug

M. J. OTTO,* M. P. FOX, M. J. FANCHER, M. F. KUHRT, G. D. DIANA, AND M. A. McKINLAY

*Department of Microbiology, Sterling-Winthrop Research Institute, Rensselaer, New York 12144*

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**WIN 51711 (5-[7-[4-(4,5-dihydro-2-oxazolyl)phenoxy]heptyl]-3-methylisoxazole), a new antipicornavirus drug, is a potent inhibitor of human entero- and rhinoviruses at concentrations not inhibitory to HeLa cell growth. In plaque reduction assays, WIN 51711 reduced plaque formation by 9 enteroviruses and 33 rhinoviruses, with MICs of 0.004 to 0.17 and 0.004 to 6.2 μg/ml, respectively. Addition of WIN 51711 to infected cells at concentrations of 0.02 to 5.0 μg/ml reduced the yield of picornaviruses by 90%. Other RNA viruses (nonpicornaviruses) and DNA viruses were unaffected by the compound.**

Currently, no drugs are available for the treatment of human diseases caused by the rhino- and enterovirus members of the picornavirus family (7). Based on the finding that arildone is a potent inhibitor of a limited number of enteroviruses in vitro (1, 2) and in vivo (4), a series of analogs was synthesized in an effort to discover a systemically active compound with broad-spectrum activity against the causative agents of viral meningitis, hepatitis A, acute hemorrhagic conjunctivitis, and the common cold (6, 9). This report describes the potent in vitro activity of WIN 51711 (Fig. 1) against representative serotypes of both enteroviruses and rhinoviruses.

## MATERIALS AND METHODS

**Media and solutions.** The following media and solutions were used: minimal essential medium (MEM), medium 199 (M-199), and 2× M-199 (Flow Laboratories, Inc., McLean, Va.) with 10% Bobby calf serum from GIBCO Laboratories (Grand Island, N.Y.), 1% SeaKem agarose in water (FMC Corp., Marine Colloids Div., Rockland, Maine), 3 mg of DEAE-dextran per ml, 3 M $MgCl_2$, 3% formaldehyde with 2% sodium acetate in water for fixing, and 0.25% crystal violet in fixing solution for staining. A set of 200× stock solutions of WIN 51711 was prepared in $Me_2SO$ (dimethyl sulfoxide) and diluted in M-199 to achieve final concentrations of 0.001 to 6.2 μg/ml.

**Viruses and cells.** Poliovirus type 2, MEF strain, was originally obtained from the University of Pittsburgh Medical School. Enterovirus type 70 ($KW_{43}^{1981}HELF_6$) was obtained from L. Anderson at the Centers for Disease Control, Atlanta, Ga., and cultured in Flow 2000 cells. Porcine transmissible gastroenteritis virus was obtained from C. L. Kanitz, Purdue Diagnostic Laboratory, and propagated in porcine turbinate cells. All other viruses were obtained from the American Type Culture Collection, Rockville, Md. Human rhinoviruses, adenovirus 5, and polioviruses were cultured in HeLa (Ohio) cells; herpes simplex virus type 2 (HSV-2) (Curtis strain), echoviruses, and equine rhinovirus in Vero cells; coxsackievirus A-9 in LLC-$MK_2$ cells; vesicular stomatitis virus (VSV) (Indiana) and mengovirus in L-929 cells; respiratory syncytial virus in HEp-2 cells; coronavirus (229E) in L-132 cells; human cytomegalovirus in Flow 2000 cells; and bovine diarrhea virus in BS-C-1 cells.

**Plaque reduction assay.** The methods used in this assay were an adaptation of those described for herpesvirus by Wentworth and French (8). Medium was aspirated from 1-day-old confluent monolayers of cells and infected with 1.0 ml of the appropriate virus (approximately 80 PFU per monolayer) in M-199 containing various concentrations of WIN 51711. The cultures were incubated for 1 h at 37°C for enteroviruses or 33°C for rhinoviruses. The virus inoculum was removed, and the cells were overlaid with M-199 containing 5% Bobby calf serum, 0.5% agarose, and WIN 51711 at various concentrations. The overlay for human rhinovirus-infected cells also contained 30 mM $MgCl_2$ and 15 μg of DEAE-dextran per ml. Enteroviruses were allowed to replicate (forming plaques) for 2 to 3 days at 37°C and rhinoviruses for 3 to 4 days at 33°C in a 2% $CO_2$ atmosphere. Cells were fixed with 3% formaldehyde in 2% sodium acetate and stained with 0.25% crystal violet in the fixing solution. The concentrations of WIN 51711 which inhibited plaque formation by 50% were determined for each virus and recorded as the MICs. The MICs determined in this manner had a variability of less than ±30%.

**Virus yield reduction assay.** Approximately 90% confluent, 1-day-old monolayers of HeLa (Ohio) cells in 25-$cm^2$ flasks (Corning Glass Works, Corning, N.Y.) were infected with virus at a multiplicity of 5 PFU per cell. WIN 51711 (solubilized in $Me_2SO$ as in the plaque reduction assay) was included in the inoculum at the indicated concentrations. After 1 h at 37°C for poliovirus or 33°C for rhinoviruses, the inoculum was removed and the monolayers were washed once with medium containing the appropriate concentration of WIN 51711. Fresh medium containing WIN 51711 was added, and the flasks were incubated for an additional 7 h at 37°C for poliovirus or 13 h at 33°C for rhinovirus and then frozen at −70°C. The virus yield in each flask was quantitated by plaque assay on monolayers of HeLa (Ohio) cells.

**Evaluation of cytotoxicity by measurement of cell growth.** HeLa (Ohio) cells were seeded in 25-$cm^2$ plastic flasks with

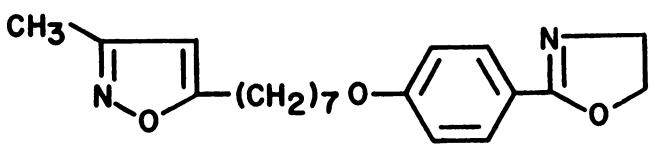


FIG. 1. Chemical structure of WIN 51711 (5-[7-[4-(4,5-dihydro-2-oxazolyl]phenoxy]heptyl]-3-methylisoxazole).

* Corresponding author.

TABLE 1. Effect of WIN 51711 on plaque formation by human enteroviruses

| Enterovirus | MIC (μg/ml)[a] |
|---|---|
| Poliovirus 1 | 0.030 ± 0.009 |
| Poliovirus 2 | 0.004 ± 0.001 |
| Poliovirus 3 | 0.006 ± 0.001 |
| Coxsackie virus A-9 | 0.17 ± 0.04 |
| Echovirus 9 (K-16) | 0.01 ± 0.003 |
| Echovirus 9 (Barty) | 0.02 ± 0.004 |
| Echovirus 11 | 0.06 ± 0.01 |
| Echovirus 12 | 0.03 ± 0.01 |
| Enterovirus type 70 | 0.044 ± 0.001 |

[a] Mean concentration required to reduce plaque number by 50% ± standard deviation.

minimal essential medium plus 5% Bobby calf serum and grown at 37°C to a density of approximately $10^5$ cells per flask at the time of addition of WIN 51711. The drug was dissolved in $Me_2SO$ as 1,000× stock solutions and diluted into medium. A 5-ml sample of each concentration of WIN 51711 or 0.1% $Me_2SO$ (control) in minimal essential medium plus 5% Bobby calf serum was applied to each of four sets of duplicate flasks which were then incubated at 37°C. For sampling at 1, 2, 3, and 4 days after addition of WIN 51711, growth fluid was removed, and the cell layer was rinsed with 0.5 ml of 4 mM disodium EDTA and incubated with 2 ml of a 1:1 mixture of 4 mM disodium EDTA and 0.25% trypsin. After 20 min at 33°C, cells were detached and separated completely by brief, gentle agitation and diluted 10-fold in phosphate-buffered saline. Diluted cell suspensions were counted in duplicate with a model F Coulter Counter equipped with a 100-μm aperture and a 0.5-ml siphon loop.

Before being sampled, each flask was inspected microscopically for visible signs of toxicity, such as swelling, shrinkage, granularity, and floating cells.

## RESULTS

**Inhibition of human enterovirus and rhinovirus plaque formation.** The ability of WIN 51711 to inhibit multiple rounds of replication by enteroviruses was ascertained by a plaque reduction assay. The data in Table 1 demonstrate that WIN 51711 is a potent inhibitor of the human enteroviruses tested. The MICs ranged from 0.006 μg/ml for poliovirus 3 to 0.17 μg/ml for coxsackievirus A-9. WIN 51711 inhibited plaque production by 33 human rhinovirus serotypes (Table 2), with MICs ranging from 0.004 μg/ml (rhinovirus 6) to 6.2 μg/ml (rhinovirus 8). Only rhinoviruses 8 and 31 had MICs greater than 3.0 μg/ml.

**Effect of WIN 51711 on the yield of virus from a single round of replication.** The effect of WIN 51711 on the replication of selected picornaviruses in a single round of replication was determined. The viruses selected represent a range of susceptibilities to WIN 51711 (MICs, 0.004 to 1.8 μg/ml) as determined by plaque reduction assay. For all the viruses tested, WIN 51711 was effective in reducing the virus yield by 90% (Fig. 2). The 90% effective doses ranged from approximately 0.02 μg/ml for poliovirus 2 to 4.0 μg/ml for rhinovirus 1A.

**HeLa (Ohio) cell growth in the presence of WIN 51711.** The in vitro cytotoxicity of WIN 51711 was determined by assessing the effects of the drug on cell growth. The composite data from two experiments are presented in Fig. 3. In each case, the cell number for the untreated control (0.1% $Me_2SO$) increased approximately 10-fold above the day 0 level. Inhibition of cell growth, manifested by an 80% decrease in cell number between days 3 and 4, occurred in the presence of 12.5 μg of WIN 51711 per ml, while 6.25 μg/ml was marginally inhibitory, with a 10 to 29% reduction in cell number on day 4. By day 3, most of the cells in the 12.5-μg/ml samples were detached and rounded.

**Specificity of antipicornavirus activity of WIN 51711.** The ability of WIN 51711 to inhibit plaque formation of other RNA and DNA viruses was determined. A number of representatives from different RNA and DNA viruses were tested. It was found that the RNA viruses (respiratory syncytial, vesicular stomatitis, influenza A, bovine diarrhea, and porcine transmissible gastroenteritis viruses and mengovirus, equine rhinovirus, and coronavirus) were insensitive to WIN 51711. Likewise, HSV-2, human cytomegalovirus, and adenovirus 5 were not inhibited by the drug. These results indicate that WIN 51711 was limited in its spectrum of activity to two groups within the picornavirus family, the enteroviruses and the human rhinoviruses.

TABLE 2. Effect of WIN 51711 on plaque formation by human rhinovirus serotypes

| Rhinovirus | MIC (μg/ml)[a] |
|---|---|
| 6 | 0.004 ± 0.001 |
| 86 | 0.015 ± 0.004 |
| 25 | 0.03 ± 0.009 |
| 5 | 0.05 ± 0.01 |
| 14 | 0.06 ± 0.01 |
| 3 | 0.09 ± 0.02 |
| 16 | 0.10 ± 0.02 |
| 2 | 0.12 ± 0.03 |
| 89 | 0.16 ± 0.04 |
| 29 | 0.20 ± 0.06 |
| 15 | 0.24 ± 0.06 |
| 33 | 0.28 ± 0.08 |
| 1B | 0.30 ± 0.07 |
| 72 | 0.33 ± 0.06 |
| 30 | 0.40 ± 0.10 |
| 22 | 0.40 ± 0.08 |
| 36 | 0.55 ± 0.16 |
| 50 | 0.65 ± 0.17 |
| 18 | 0.69 ± 0.2 |
| 21 | 0.70 ± 0.16 |
| 49 | 0.72 ± 0.14 |
| 67 | 0.80 ± 0.20 |
| 39 | 0.95 ± 0.24 |
| 13 | 0.96 ± 0.20 |
| 20 | 1.30 ± 0.30 |
| 61 | 1.40 ± 0.28 |
| 1A | 1.76 ± 0.31 |
| 10 | 1.90 ± 0.20 |
| 75 | 2.70 ± 0.10 |
| 17 | 2.80 ± 0.2 |
| 41 | 3.00 ± 0.1 |
| 31 | 6.0 ± 0.2 |
| 8 | 6.2 ± 0.2 |

[a] Mean concentration required to reduce plaque number by 50% ± standard deviation.

## DISCUSSION

WIN 51711 represents a new class of antiviral drugs with broad-spectrum in vitro efficacy against clinically significant members of the picornavirus family. The data presented here show that WIN 51711 is effective in inhibiting the replication of the enteroviruses (poliovirus 1, 2, and 3; echoviruses 9, 11, and 12; and EV-70), with MICs as low as 0.004 μg/ml in plaque reduction assays. Studies carried out by C. Wilfert (Duke University Medical Center) showed that the replication of other coxsackie and echovirus serotypes are also inhibited by WIN 51711 (personal communication). The majority of the rhinoviruses tested, while generally less susceptible than the enteroviruses, are inhibited by drug concentrations of less than 1 μg/ml in plaque reduction assays. Similar results were obtained in virus yield reduction assays, indicating the efficacy of the compound in single-round, high-multiplicity infections as well as in multiround, low-multiplicity infections. In addition, studies with poliovirus 2 and echovirus 9 have shown that WIN 51711 is systemically active in preventing disease caused by these viruses in mice (B. A. Steinberg, A. A. Visosky, and M. A. McKinlay, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 432, 1984; B. A. Steinberg, A. A. Visosky, J. A. Frank, Jr., and M. A. McKinlay, 24th ICAAC, abstr. no. 433, 1984).

The effects of WIN 51711 on cell growth over a 4-day period were examined under conditions used to quantitate the inhibitory effects of the drug on virus yield. Inhibition of cell growth was apparent on days 1 to 4 in the presence of 12.5 μg of WIN 51711 per ml. Concentrations of 6.25 μg/ml had an inhibitory effect on the growth of HeLa cells, which was observed only on day 4. Similar results were obtained with [$^{35}$S]methionine incorporation as a measure of protein synthesis (data not shown). Incorporation of [$^{35}$S]methionine into cellular proteins was inhibited at 6.2 and 12.5 μg/ml, but not at 3.1 μg/ml. DNA and RNA synthesis, however, was not inhibited at 12.5 μg/ml. Since the inhibitory effects of WIN 51711 on virus replication are observed at concentrations substantially below 6.25 μg/ml, and since nonsusceptible picornaviruses as well as other RNA and DNA viruses were able to replicate normally in the presence of 6.2 μg/ml, the antiviral activity cannot be attributed to cytotoxic effects of WIN 51711 on the host cell.

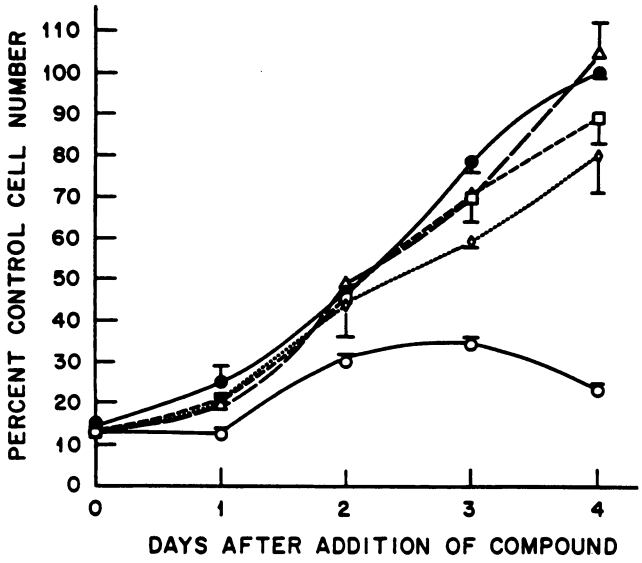


FIG. 3. The effect of WIN 51711 on the growth of HeLa (Ohio) cells. HeLa cells were seeded in 25-cm$^2$ flasks and grown to approximately $10^5$ cells per flask. The cells were exposed to WIN 51711 at the indicated concentrations, and the cell number was determined daily for 4 days. The results are the composite of two experiments and are plotted as the percentage of control cell number from day 4. Symbols: ●, dimethyl sulfoxide control; △, WIN 51711 at 0.8 μg/ml; □, WIN 51711 at 3.1 μg/ml; ◇, WIN 51711 at 6.2 μg/ml; ○, WIN 51711 at 12.5 μg/ml.

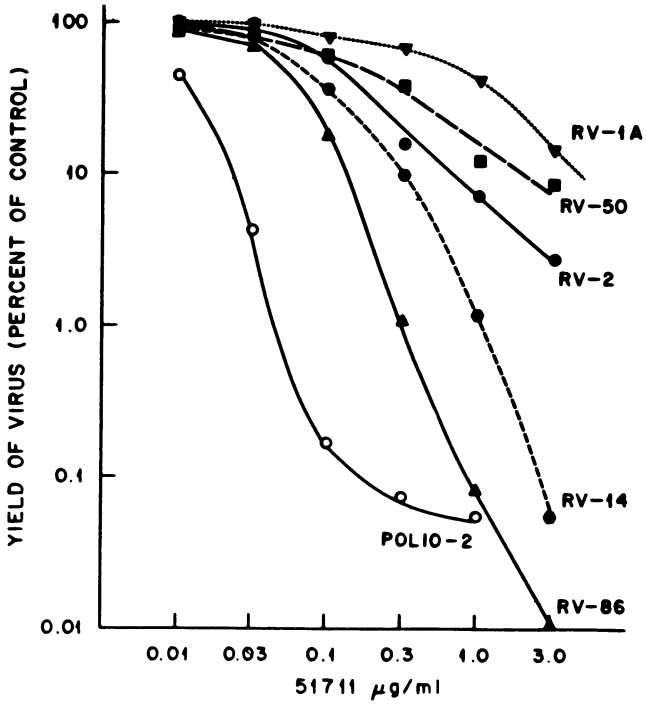


FIG. 2. Effect of WIN 51711 on the yield of selected picornaviruses in vitro. Cells were infected with the indicated virus in the presence of the appropriate concentration of WIN 51711. After incubation of the cells at either 37°C for 8 h (poliovirus) or 33°C for 14 h (rhinoviruses), the cells were frozen and thawed, and the yield of infectious virus was determined by plaque assay. Each point is the average of two determinations.

Mechanism of action studies indicate that WIN 51711, like its predecessor arildone (3, 5), inhibits virus replication by preventing uncoating of the virion and subsequent release of the viral RNA into the cytoplasm (manuscript in preparation). Currently, studies are in progress to further elucidate the mechanism and site of action at the molecular level.

The need exists for a potent, nontoxic drug for the treatment of picornavirus diseases, which range from neonatal sepsis, aseptic meningitis, and hepatitis A to the more common upper respiratory tract disease (colds) (6, 9). WIN 51711, with its broad in vitro spectrum and in vivo efficacy, is a candidate for development to treat these diseases.

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