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Anti-influenza virus activity of the compound LY253963

Frederick G. Hayden, Barbara S. Rollins and Alan J. Hay

Departments of Internal Medicine and Pathology, University of Virginia School of Medicine, Charlottesville, Virginia, U.S.A. and Virology Division, National Institute for Medical Research, London, U.K.

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Summary

The compound LY253963 (1,3,4-thiadiazol-2-ylcyanamide) inhibited the in vitro replication of representative influenza A and B viruses in Madin-Darby canine kidney (MDCK) cells at concentrations of 1–3.2 μg/ml. The yield of an influenza A (H3N2) virus in primary rhesus monkey kidney (RMK) cells was inhibited at 0.1–0.3 μg/ml. However, similar concentrations were inhibitory for the growth of uninfected MCDK or RMK cells. Combination drug studies generally found indifferent interactions between LY253963 and ribavirin or rimantadine. In timing of additional studies, hemagglutinin expression was inhibited to the greatest extent when LY253963 exposure was begun at least 8 h before viral infection, which suggested either slow uptake or intracellular metabolism of LY253963 to an active form. Virus-specific protein synthesis was inhibited to a greater extent by ribavirin 10 μg/ml or rimantadine 1 μg/ml than by LY253963 10 μg/ml. No drug-resistant mutants were detected during serial passage of an influenza A (H3N2) virus in the presence of LY253963 1–16 μg/ml. In summary, we found that LY253963 inhibited influenza A and B virus replication in several cell types, but that it was associated with cytostatic effects at low concentrations. These studies failed to identify a selective anti-influenza action.

Influenza virus inhibition; LY253963; Ribavirin; Rimantadine; Cytotoxicity

Correspondence to: F.G. Hayden, Box 473, Department of Internal Medicine, University of Virginia School of Medicine, Charlottesville, VA 22908, U.S.A.

Introduction

Currently the only anti-influenza drugs which are effective orally are amantadine hydrochloride (Symmetrel) and rimantadine hydrochloride. These antivirals are specific for influenza A viruses and provide no protection against influenza B virus infections, which cause substantial morbidity and sometimes mortality in humans. In addition, no treatment of proven clinical value exists for severe influenza A or B virus illness. Recent studies have also found that rimantadine-resistant influenza A viruses can be frequently recovered from treated children (Belshe et al., 1988) and that these viruses can cause typical influenzal illness in household contacts receiving rimantadine prophylaxis (Hayden et al., 1989). Such rimantadine-resistant viruses show cross-resistance to amantadine and related compounds. Thus, a continuing need exists for alternative anti-influenza agents.

The compound LY253963 (sodium salt of 1,3,4-thiadiazol-2-ylcyanamide) is a novel agent possessing broad-spectrum in vitro activity against various DNA (herpes simplex, vaccinia) and RNA (influenza A and B, parainfluenza, respiratory syncytial, measles) viruses (Nelson et al., 1988). Dose-related antiviral effects and reduced mortality have been observed in experimental murine influenza A and B virus infections following parenteral or oral administration (Delong et al., 1987). Single intraperitoneal doses shortly after infection reduce febrile responses in ferrets inoculated with influenza B virus (Tang et al., 1988). Antiviral effects have also been found in animal models of respiratory syncytial virus and parainfluenza virus infection. This agent is currently undergoing preclinical toxicologic evaluation.

The objectives of the current studies were to investigate the anti-influenza action of LY253963, with particular attention to its spectrum of activity, selectivity and specificity of inhibition, and the possible generation of drug-resistant mutants.

Materials and Methods

Compounds

LY253963 (lot no. 882.0T0.132) was provided as a crystalline white powder by W.A. Spitzer (Lilly Research Laboratories, Indianapolis, IN). Ribavirin (lot no. 11102, Viratek Inc., Costa Mesa, CA) and rimantadine hydrochloride (lot no. 001115, Hoffman-LaRoche Inc., Nutley, NJ) were kindly provided by their manufacturers. Each was dissolved in distilled water at a concentration of 10 mg/ml, and aliquots were held frozen at −20°C until further diluted in cell culture media.

Cells and media

Madin-Darby canine kidney (MDCK) cells were passaged weekly in the laboratory with F XV medium containing 10% newborn calf serum (Gibco) and antimicrobics. Primary chick embryo fibroblast (CEF) cell cultures were prepared from day old embryos by previously described techniques (Porterfield, 1960). Tris-

buffered Gey's medium supplemented by 5% calf serum and antimicrobics served as growth medium. Primary rhesus monkey kidney (PRMK) cell monolayers were purchased (Whittaker M.A. Bioproducts, Walkersville, MD) with Eagle's minimal essential medium (EMEM) containing Earle's salts, 2% serum, and antimicrobics. The corresponding media without serum supplementation were used in conducting experiments.

Viruses

Clinical isolates of influenza A and B viruses were made initially in PRMK or MDCK cells and passaged twice in MDCK cells prior to susceptibility testing. These viruses were influenza A/Virginia/87 (H1N1) (A/Taiwan/1/86-like), A/Virginia/88(H3N2) (A/Sichuan/2/87-like), and three influenza B viruses isolated in Charlottesville during 1984, 1986, and 1988. Influenza A/Singapore/1/57(H2N2) and two avian influenza viruses, A/chicken/Germany/27 ('Weybridge' strain) (H7N7) and A/chicken/Germany/34 ('Rostock' strain)(H7N1) were grown in 12 day old embryonated eggs.

Drug susceptibility testing

A previously described enzyme-linked immunoassay (ELISA) (Belshe et al., 1988, Hayden et al., 1989) which detects expression of viral hemagglutinin (HA) on the surface of infected cell was used to determine the inhibitory effects of the drugs for a range of viruses. LY253963 and ribavirin were diluted (0.5 $\log_{10}$ dilutions) in serum-free F XV medium containing TPCK-treated trypsin 2.5 μg/ml (Cooper Biomedical). Quadruplicate MDCK monolayers in microtiter plates were overlaid with 2× drug-containing medium (50 μl/well) 30 minutes prior to inoculation with an equal volume containing serial $\log_{10}$ dilutions of virus. The final drug concentrations ranged from 0.1 to 32 μg/ml. Monolayers were fixed and assayed for HA after 16–20 h incubation at 37°C. The inhibitory concentration was that causing at least 50% reduction in optical density for monolayers inoculated with approximately 10–100 50% tissue culture infectious doses of virus ($TCID_{50}$).

Inhibition of infectious virus yields were determined for influenza A/Virginia/88(H3N2) in MDCK and PRMK monolayers. Triplicate monolayers were inoculated with 0.1 ml containing approximately 100 $TCID_{50}$. After a 60 min adsorption period, the monolayers were washed and overlaid with plain or drug-containing media. After 24 h incubation at 37°C, the cells and supernatants were pooled after one freeze(−70°C) – thaw cycle and titered on MDCK monolayers by plaque assay. A 90% or greater reduction of virus yield (1.0 $\log_{10}$ PFU/0.1 ml) was considered to indicate a definite antiviral effect.

The plaque inhibitory effects of LY253963 and ribavirin for avian influenza viruses were determined in CEF monolayers (5 cm dishes) overlaid with drug-containing agarose. Monolayers were stained with neutral red after 48 h incubation at 37°C, and the concentrations causing at least 50% reduction in plaque number determined.

Cellular toxicity

In addition to observing uninfected cell monolayers for overt signs of cytotoxicity, quantitative assessments of possible cytostatic effects were made by growing uninfected cells in media containing 0.5 $\log_{10}$ dilutions of LY253963 or ribavirin (0.1–32 μg/ml) or in media containing increasing rimantadine concentrations (10–50 μg/ml). In certain experiments with MDCK and PRMK cells, approximately 5×10^4 cells were seeded into quadruplicate wells of 24-well plates, and viable cell counts determined by hemocytometer before and after five days incubation. Drug concentrations preventing at least 2-fold increases in initial cell counts were considered to be inhibitory.

In other experiments with MDCK and CEF cells, cell growth was determined in 96-well microtiter plates by a tetrazolium-based colorimetric method (Pauwels et al., 1988). This assay detects the in situ reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by viable cells. Approximately 5×10^3 cells were seeded per well and incubated with drug-containing growth medium for 3–4 days until confluent monolayers had formed. After discarding the supernatants, MTT 2 mg/ml in PBS was added and the monolayers incubated 2 h at 37°C. The resulting formazan precipitate was dissolved, and the optical density of the solution determined spectrophotometrically at 540 nm. Concentrations reducing the optical density by 50% or more relative to nondrug-exposed monolayers were considered to be inhibitory.

Drug combination studies

Possible interactions between LY253963 and other anti-influenza agents were determined in the A-inhibition MDCK microtiter assay described above. Quadruplicate monolayers were incubated with 50 μl volumes of 4-fold concentrated drug in trypsin-containing F XV medium for 30 min prior to infection with approximately 100 $TCID_{50}$ of influenza A/Virginia/88(H3N2). The final drug concentrations ranged from 0.1 to 10 μg/ml in 0.5 $\log_{10}$ dilutions for LY253963 and ribavirin, but only three rimantadine concentrations (0.001, 0.01, and 0.1 μg/ml) were tested. Monolayers were assayed for HA expression after 18 h incubation. Interactions were determined by the fractional product method as described previously (Hayden et al., 1984).

Timing of addition studies

To determine the effect of varying the time of LY253963 addition on viral replication, a single cycle experiment was performed in microtiter with the ELISA. Quadruplicate MDCK monolayers were overlaid with media containing LY253963 1.0, 3.2, or 10 μg/ml, ribavirin 10 μg/ml, rimantadine 1.0 μg/ml, or no drug beginning at 24, 8, 4, and 1 h before viral infection or 0.5 (end of the adsorption period), 1.5, and 2.5 h after infection. At the time of infection, maintenance media were removed and the monolayers were inoculated with 50 μl of diluted allantoic

fluid containing A/Singapore/1/57 (4.5 $\log_{10}$ $TCID_{50}$/monolayer), held at room temperature for 30 min, washed twice with phosphate-buffered saline (PBS), and overlaid with plain or drug-containing media. The monolayers were fixed at 5.5 h for later assay of HA expression.

Viral protein and RNA synthesis

Single cycle experiments were performed to determine the effect of LY253963 on viral replication and RNA and protein synthesis. Confluent MDCK monolayers in 5 cm dishes were incubated with maintenance medium or medium containing LY253963 1.0, 3.2, or 10 μg/ml or ribavirin 10 μg/ml for 24 h. The medium was aspirated, and the monolayers inoculated with 250 μl of undiluted allantoic fluid containing 7.0 $\log_{10}$ $TCID_{50}$ of A/Singapore/1/57(H2N2). The highest multiplicity of infection(MOI) tested was approximately 2.5 $TCID_{50}$ per cell, but lower MOIs were used in other experiments. After 30 min incubation at room temperature, the monolayers were washed three times with PBS and replenished with methionine-free F XV medium (protein synthesis studies) or plain F XV medium (RNA synthesis, HA yield, and virus yield studies) containing the appropriate drug concentration.

In the protein synthesis experiments, duplicate monolayers were incubated at 37°C for 5 h prior to the addition of approximately 20 μCi ^{35}S-methionine per dish. After 60 min incubation at 37°C, the monolayers were washed three times with ice-cold PBS and then lysed by addition of 0.5 ml of 8 M urea, 1% SDS and 1% B-mercaptoethanol. After one freeze-thaw, the lysates were combined, sonicated briefly, heated in boiling water for 3 min, and centrifuged at 15 000 rpm for 5 min on a microcentrifuge. Fifteen μl volumes were applied to the lanes of a 16% polyacrylamide protein separating gel. The effect of drug exposure on ^{35}S-methionine incorporation in uninfected MDCK cells was determined in monolayers treated in the same fashion. In parallel, duplicate monolayers were incubated for 10 h in drug-containing medium, after which the supernatants were harvested for titration of HA yields by standard methods and for virus yields by endpoint dilution in MDCK monolayers. In one experiment monolayers were exposed to ^{35}S-methionine beginning at 4 h postinfection, and supernatants were harvested at 10 h for quantitation of labelled virus production by previously described methods (Hay and Zambon, 1984).

Analysis of viral mRNA synthesis was done as previously described (Hay et al., 1977). Briefly, duplicate monolayers were incubated for 2 h prior to addition of ^{3}H-uridine (100 μCi/ml) and for a further 2 1/2 h prior to RNA extraction. RNA was annealed with 15 μg virion RNA of A/Singapore/1/57, digested with S1 nuclease, and the double-stranded RNAs separated by electrophoresis on 4.5% polyacrylamide gels. To measure primary viral mRNA synthesis, cells were incubated in the presence of cycloheximide (100 μg/ml) 30 min prior to infection and throughout the duration of the experiment.

The effect of drug exposure on the incorporation of ^{3}H-uridine into the nucleotide pool and RNA of uninfected cells was determined in monolayers preincubated with

drug as described above and then incubated with ^{3}H-uridine (5 μCi/ml) for 15 min. Monolayers were washed successively with 3 ml cold saline 3 times, 1.5 ml cold 5% trichloracetic acid (TCA) 3 times, and 2 ml ethanol twice. The residue was dissolved in 1.5 ml of 0.5 M sodium hydroxide and 0.5 ml aliquots of the pooled TCA-soluble fractions and TCA-insoluble fractions counted.

Passage of virus in presence of LY253963

In order to determine the genetic basis of susceptibility to LY253963, an effort was made to select mutant virus resistant to its action. Serial $\log_{10}$ dilutions of influenza A/Virginia/88(H3N2) were inoculated onto MDCK monolayers (0.1 ml) simultaneously exposed to EMEM containing 2.5 μg/ml trypsin and LY253963 at final concentrations of 0, 1, 2, 4, 8, or 16 μg/ml. After 2–3 days incubation, supernatants from the monolayers inoculated with the highest dilution of virus that resulted in viral cytopathic effect were harvested, and serial dilutions inoculated onto fresh MDCK monolayers in the presence of the same LY253963 concentration. This process was repeated through a total of 13 passages, after which the parent virus and harvests from the final passage were tested for drug susceptibility in the ELISA.

Results

Susceptibility of influenza viruses to LY253963

In assays using low multiplicities of infection, the in vitro inhibitory activity of LY253963 was similar against a range of clinical isolates of influenza A and B viruses in MDCK cells (Table 1). No differential sensitivity among virus strains was apparent. Inhibition of representative influenza A and B viruses was comparable whether determined by yield reduction or ELISA methods. However, the degree of inhibition for particular drug concentrations was highly dependent upon the viral inoculum and the cell type. A 10-fold increase in the amount of input virus was associated with increases of the IC_{50} to 10 μg/ml or higher in the ELISA for both influenza A and B viruses (data not shown). The compound appeared marginally more active in PRMK than MDCK cells in yield reduction experiments but was inactive against several avian influenza A viruses in CEF monolayers (Table 1). In contrast, the A/Rostock(H7N1) and A/Weybridge(H7N7) viruses were inhibited by over 90% at 10 μg/ml in MDCK cells when determined by ELISA.

The anti-influenza spectrum of LY253963 was similar to that of ribavirin (Table 1). When compared to the potency of other anti-influenza agents, the inhibitory effects of LY253963 were comparable to ribavirin in both MDCK and PRMK cells, but were much lower in CEF cells ($P< 0.01$, paired t-test) (Table 1). In PRMK cells, LY253963 was associated with a 1.0 $\log_{10}$ reduction in virus yield at a slightly lower concentration than ribavirin (Table 1), but its inhibitory activity was less at higher drug concentrations. In three experiments conducted in PRMK cells, the reduction (mean $\pm$ SD) in virus titers compared to controls were 1.2 ± 0.3 $\log_{10}$

TABLE 1
Inhibition of influenza virus replication in vitro by LY253963

Cell type	Assay	Virus	Inhibitory concentration (μg/ml)[a]		
			LY253963	Ribavirin	Rimantadine
MDCK	Yield reduction	A/Virginia/88(H3N2)	1.0, 1.0	1.0, 1.0	NT
		B/Virginia/88	1.0	1.0	NT
	ELISA	A/Virginia/88(H3N2)	3.2, 3.2	3.2, 3.2	<0.1, <0.1
		A/Virginia/87(H1N1)	3.2, 3.2	3.2, 3.2	<0.1, <0.1
		A/Singapore/57(H2N2)	3.2	3.2	<0.1
		B/Virginia/88	3.2	3.2	>10
		B/Virginia/86	3.2	3.2	>10
		B/Virginia/84	3.2	<10	>10
		A/Rostock(H7N1)	<10	<10	<1
		A/Gendon(H7N7)	<10	<10	<1
PRMK	Yield reduction	A/Virginia/88(H3N2)	0.1–0.3[b]	1.0[b]	NT
CEF	ELISA	A/Rostock(H7N1)	>32	10	<0.1
	Plaque inhibition	A/Rostock(H7N1)	>32, >32	10, 10	NT
		A/Weybridge(H7N7)	>32	10	NT

NT = not tested, MDCK = Madin-Darby canine kidney, PRMK = primary rhesus monkey kidney, CEF = chick embryo fibroblast.
[a]Defined as reduction of at least 90% in infectious virus yield in the yield reduction assay or at least 50% in OD value or plaque count in the ELISA or plaque assays, respectively. The results of individual experiments are listed.
[b]Range of 3 experiments.

PFU/0.1 ml for LY253963 and 0.7±0.1 $\log_{10}$ PFU/0.1 ml for ribavirin at 0.3 μg/ml concentrations, 2.0±0.4 and 2.2±0.7 at 1.0 μg/ml, 2.7±0.4 and 3.9±1.4 at 3.2 μg/ml, and 2.4±0.5 and 5.3±0.5 at 10.0 μg/ml ($P< 0.01$), respectively. Rimantadine was much more active against influenza A viruses than either LY253963 or ribavirin in MDCK ($P< 0.01$, paired t-test) and CEF cells.

Cytotoxicity studies

In subconfluent MDCK cell monolayers LY253963 was associated with alterations in cellular morphology at concentrations of 3.2 μg/ml and higher. After overnight incubation, microscopic inspection revealed increased numbers of round, refractile cells, and decreased cell sheet density was apparent after several days of incubation.

Quantitative studies of the effects of LY253963 on the growth of uninfected cells found that it was inhibitory for MDCK and RMK cells at low concentrations, comparable to those associated with antiviral effects, but not inhibitory for CEF cells (Table 2). Ribavirin was associated with cytostatic effects at 3–10-fold higher concentrations. As shown in Fig. 1, no clear distinction existed between the antiviral and cytostatic effects of LY253963 in MDCK cells across a range of drug concentrations. Parallel testing of ribavirin found that concentrations exerting antiviral effects were approximately 3-fold lower than those associated with inhi-

TABLE 2
Inhibition of uninfected cell growth by LY253963

Cell type	Days of exposure	Assay	Inhibitory concentration (μg/ml)[a]	
			LY253963	Ribavirin
MDCK	5	Cell count	0.3–1.0[b]	3.2–10[b]
MDCK	3	MTT	3.2, 3.2	10, 10
RMK (secondary)	5	Cell count	1.0, 1.0	10
CEF	4	MTT	>32	>32

See text for description of MTT assay.
[a]Defined as the concentration preventing at least 2-fold increases in viable cell counts from time of seeding or for the MTT assay as the concentration reducing the OD value by at least 50% compared to control. The results of individual assays are listed.
[b]Range of results from 3 separate experiments. In MDCK cells the combined results of the cell counting and MTT assays indicated that the inhibitory concentrations of LY253963 were significantly lower than those of ribavirin ($P < 0.02$, paired t-test).

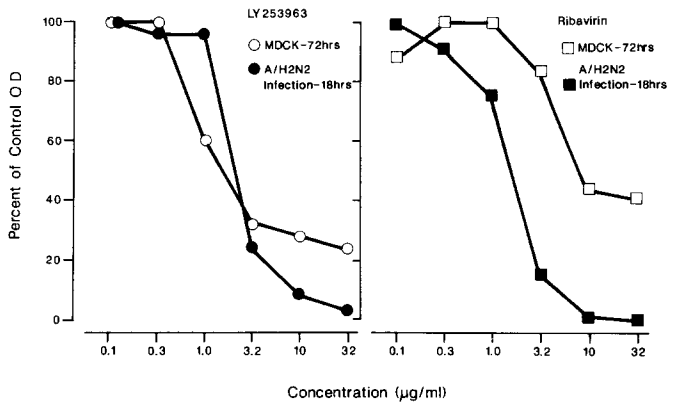

Fig. 1. Comparison of the antiviral and cytostatic effects of LY253963 and ribavirin in MDCK cells. The solid circles (LY253963) and squares (ribavirin) represent the optical densities, expressed as percent of nondrug-exposed virus-infected monolayers (control), found in the ELISA for HA expression after infection of MDCK monolayers with A/Singapore/57(H2N2). The open circles and squares represent the optical densities, also expressed as percent of nondrug-exposed control monolayers, found in the MTT assay for measuring growth of uninfected cells (Pauwels et al., 1988). Each point represents the average value of two experiments performed in quadruplicate wells.

bition of cell growth. In the same type of assays, rimantadine showed cytotoxic effects at 30–40 μg/ml concentrations and anti-influenza A virus effects at 0.01–0.1 μg/ml concentrations, so that its therapeutic index was approximately 1000 (data not shown).

Drug combination studies

Various combinations of LY253963 with ribavirin or rimantadine were tested in the ELISA to assess whether selective antiviral effects could be achieved at non-

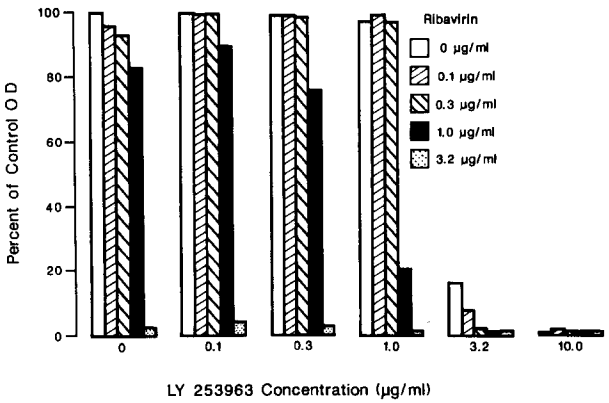

Fig. 2. Effect of LY253963 and ribavirin alone and in combination on HA expression in MDCK cell monolayers infected with A/Virginia/88(H3N2) virus. The mean optical densities, expressed as a percent of control values (non-drug exposed, virus infected monolayers), are shown for one experiment involving eight replicate wells per combination.

cytostatic concentrations of LY253963. As shown in Fig. 2, concentration-related reductions in HA expression were observed for LY253963 or ribavirin alone. The IC_{50} values were between 1.0 and 3.2 µg/ml for each single agent.

Most interactions between these compounds were indifferent, that is, no greater inhibition than that produced by the more effective single agent. Certain combinations (ribavirin 1.0 µg/ml + LY 1.0 µg/ml ribavirin 0.3 + LY 3.2, or ribavirin 1.0 + LY 3.2) were associated with synergistic effects according to the fractional product method of analysis. The calculated/observed fractional products were 0.81/0.28, 0.14/0.01, and 0.13/0 for these three combinations, respectively. In no instance was an antagonistic interaction identified. In the same experiment, rimantadine alone at concentrations of 0.001, 0.01, and 0.1 µg/ml was associated with 29, 45, and 93% reductions, respectively, in HA expression. The addition of LY253963 at 0.1, 0.3, or 1.0 µg/ml did not result in any increase or decrease in the effect of rimantadine.

Timing of addition studies

In single cycle studies, the antiviral effect of LY253963 depended on both the drug concentration and the length of monolayer pretreatment. As shown in Fig. 3, inhibition of HA expression on the surface of MDCK cells at 5 1/2 h post-infection was highest when the monolayers had been exposed to LY253963 for at least 8 h before viral infection. Concentrations of 10 µg/ml were associated with greater than 50% reductions, when drug treatment was begun 1 h before viral infection but not at the end of the adsorption period. The time-related patterns observed with ribavirin or rimantadine exposure were different (Fig. 3). The inhibitory effects of ribavirin did not appear to relate to duration of preinfection exposure and gradually declined in relation to increasing time after infection. In accord with its proposed mechanism of action against virus uncoating (Hay, 1989), the inhibitory effects of

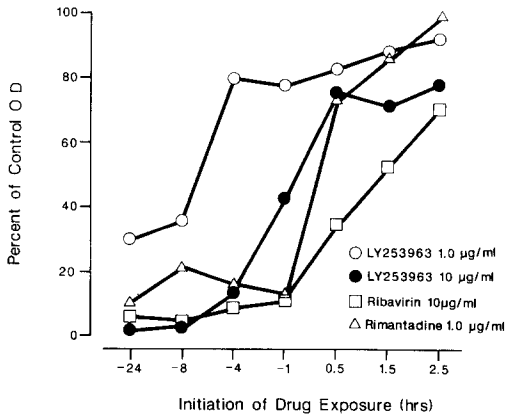

Fig. 3. Effects of increasing duration of LY253963 exposure on inhibition of influenza A/Singapore/57(H2N2) hemagglutinin expression in single cycle infection (5.5 h) of MDCK cell monolayers. The time of initiating drug exposure is indicated on the horizontal axis. The end of the 30 min adsorption period is 0.5 h. The multiplicity of infection was approximately 0.2 $TCID_{50}$/cell. Each point represents the mean of four replicate wells in one experiment. The mean optical densities are expressed as a percent of non-drug exposed, virus-infected monolayers.

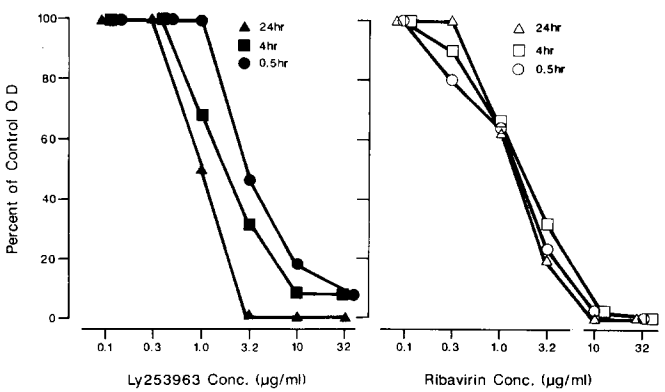

Fig. 4. Effects of increasing the duration of drug exposure on the inhibition of influenza A/Singapore/57(H2N2) hemagglutinin expression in the multiple cycle ELISA (18 h infection). Each point represents the mean of four replicate wells in one experiment and is expressed as a percent of the optical density in non-drug exposed, virus-infected monolayers.

rimantadine were similar over a range of preinfection times (−24 to −1 h) but were largely lost when exposure was begun at the end of the absorption period. Because of these observations, subsequent studies of the effects of LY253963 on protein and RNA synthesis (see Methods section) incorporated preinfection treatment of cells for 24 h.

Furthermore, an experiment was performed to determine whether a longer duration of LY253963 pretreatment would reduce the IC_{50} value found in the multiple cycle ELISA used for susceptibility testing. As shown in Fig. 4, modest shifts in

TABLE 3
Inhibition of A/Singapore/57(H2N2) haemagglutinin in virus production during single cycle infection (10 hours) of MDCK cells

Drug	Concentration (μg/ml)	Change compared to virus control		
		HA titer[a] ($\log_2$)	Virus yield[a] ($\log_{10}TCID_{50}$/0.2ml)	^{35}S-methionine-labelled virus[b]
LY253963	1.0	0	+0.10	86
	3.2	−0.5	−0.07	102
	10	−1.5	−0.75	71
Ribavirin	10	−2.5	−1.67	25

[a]Results represent means of two separate experiments. In control monolayers, the HA titers were 8.0 and 6.0 $\log_2$, respectively, and the virus yields were 6.17 and 6.00 $\log_{10}$ $TCID_{50}$/0.2 ml, respectively, in the first and second experiments.
[b]Results of one experiment performed in duplicate.

the inhibition curves were observed for LY253963 when the duration of drug exposure was increased from 30 min (standard conditions) to 24 h. This represented a reduction of approximately one 0.5 $\log_{10}$ dilution in the IC_{50} value from 3.2 μg/ml to 1 μg/ml with 24 h pretreatment in this experiment. No shift was observed for ribavirin tested in parallel.

Inhibition of viral protein synthesis

Studies were conducted to determine the effect of LY253963 on the production of infectious virus and on the expression of virus-specific proteins in MDCK cells following a single cycle of virus replication. Under conditions of high MOI, only modest decreases in virus yields, assessed by HA, ^{35}S-labelled virus, or infectivity, were observed at concentrations of 10 μg/ml (Table 3). Lower concentrations were not inhibitory. Ribavirin 10 μg/ml was associated with greater inhibitory effects under the same experimental conditions (Table 3).

Similarly, ribavirin 10 μg/ml or rimantadine 1.0 μg/ml inhibited the expression of virus-specific proteins to a greater extent than observed with LY253963 (Fig. 5). The degree of inhibition for all three agents depended on the viral input; inhibition by LY253963 was only apparent when the MOI was reduced by a factor of 1.0 $\log_{10}$ (Fig. 5). No effects on ^{35}S-methionine incorporation into the proteins of uninfected, confluent MDCK monolayers were observed for any of the agents (data not shown).

Inhibition of viral RNA synthesis

Similarly, only at cytostatic concentrations of 3.2 μg/ml or greater did LY253963 influence primary or secondary viral messenger RNA synthesis following high multiplicity of infection, as assessed by incorporation of ^{3}H-uridine (data not shown). However, as shown in Table 4, the reduced incorporation of ^{3}H-uridine into the nucleotide pool (trichloracetic acid-soluble) of the cell may account largely

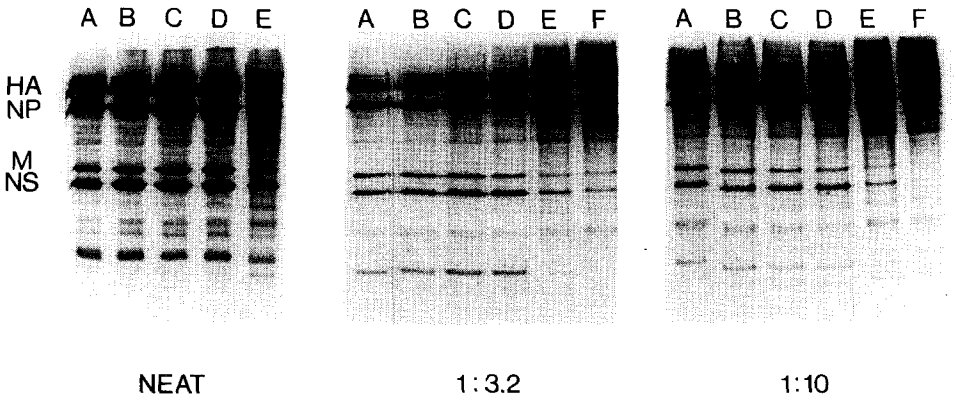

Fig. 5. Synthesis of ^{35}S-methionine labelled influenza virus proteins in MDCK cells infected by A/Singapore/1/57. Drug was continuously present beginning 24 h prior to infection until harvest of monolayers 5 1/2 h after infection. The inoculum was allantoic fluid used undiluted (neat) or diluted 0.5 or 1.0 $\log_{10}$ in three separate experiments. The major protein bands are indicated on the left: HA, hemagglutinin; NP, nucleoprotein; M, matrix; NS, nonstructural proteins. The lane markers indicate harvests from untreated, virus-infected monolayers (A); infected monolayers treated with LY253963 1.0 μg/ml (B) 3.2 μg/ml (C), or 10 μg/ml (D); and infected monolayers treated with ribavirin 10 μg/ml (E) or rimantadine 1.0 μg/ml (F).

TABLE 4
Incorporation of ^{3}H-uridine in uninfected MDCK cells

Drug	Concentration (μg/ml)	Incorporation (% of control)	
		TCA-soluble	TCA-insoluble
LY253963	1.0	101	99
	3.2	94	85
	10	62	37
Ribavirin	10	65	64

for the apparent reduction in synthesis of both viral and cellular RNAs.

Passage of virus in presence of LY253963

The end-point harvests from an influenza A(H3N2) virus passaged 13 times in the absence or presence of LY253963 1.0, 2.0, 4.0, 8.0, or 16 μg/ml were tested for drug susceptibility in the ELISA. For all harvests the IC_{50} values were less than 3.2 μg/ml but greater than 1.0 μg/ml. No evidence for the selection of resistant variants was detected in these studies. In comparison, similar methods readily select rimantadine-resistant influenza A(H3N2) virus after one or two passages in the presence of 0.1 or 1.0 μg/ml concentrations (data not shown).

Discussion

These studies found that the in vitro anti-influenza virus activity of LY253963 was similar in spectrum and potency to that of ribavirin. The inhibitory effects of LY253963 were comparable to those of ribavirin at lower concentrations in multiple cycle experiments but less at higher concentrations in either single or multiple cycle studies. Both agents were much less active than rimantadine against influenza A viruses. More importantly, LY253963 exhibited cytostatic effects on proliferating cells at concentrations similar to those associated with antiviral effects. This was also observed in rhesus monkey kidney cells, which indicates that this cytostatic action extends to primate cells. In growing MDCK cells the inhibitory concentrations of LY253963 as determined by cell counting were approximately 10-fold lower than those of ribavirin (Table 2). Of note, the inhibitory concentrations observed for ribavirin in MDCK cells are comparable to those reported previously (Browne, 1981; Larsson et al., 1978). In contrast to its effects on proliferating cells, LY253963 does not cause cytotoxic changes (Nelson et al., 1988) or inhibit cellular protein synthesis (data not shown) in stationary MDCK monolayers at much higher concentrations. Its cytostatic effects at relatively low concentrations suggest that LY253963 may be associated with significant toxicity in preclinical testing.

The lack of selective inhibition of influenza virus replication is also indicated by the failure to select drug-resistant mutants despite long-term passage of virus in the presence of the drug and the lack of differential sensitivity among both type A and B virus strains. Although our limited studies do not exclude the possibility that such mutants could be generated, the non-availability of LY253963-resistant viruses precluded the use of genetic studies in defining its mechanism of action.

Drug interaction studies suggested that selected combinations of LY253963 and ribavirin may have synergistic antiviral effects over a very narrow concentration range. Since these combinations represented drug concentrations just below those having antiviral effects when used alone, it may be that the agents share certain inhibitory actions. However, these studies did not identify a substantially reduced LY253963 concentration that would have antiviral without antiproliferative effects.

Prolonged exposure of cells to LY253963 before infection increased its anti-influenza inhibitory effects. Other studies found that it had cell type-dependent actions, in that it exhibited both antiviral and cytostatic effects in MDCK and RMK cells but not in CEF cells. Together these observations suggest that the compound is taken-up or metabolized differently in different cell types. Recent studies have shown that LY253963 metabolites accumulate in cells in which the compound exerts antiviral and cytostatic actions but not in those in which it lacks these effects (W. Plunkett, unpublished observations). In accord with LY253963's apparent lack of selective anti-influenza activity in MDCK cells, no differences were found in the accumulation of metabolites in uninfected and influenza B virus-infected MDCK cells (W. Plunkett). Such findings indicate that careful studies in human cell systems are warranted.

The lack of selective anti-influenza activity of LY253963 in our in vitro studies contrasts with its protective effects in murine and ferret models of infection (Delong

et al., 1987; Tang et al., 1988). Differences in activity in cells of different species, in vivo metabolism of the compound, and nonselective immunomodulating or anti-inflammatory effects in the animals are possible reasons for the inconsistencies observed. Detailed studies of species-related pharmacokinetics, antiviral effects, and effects on host responses to infection would help to address these possibilities.

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