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Toxicity and antiviral activity of LY253963 against respiratory syncytial and parainfluenza type 3 viruses in tissue culture and in cotton rats

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Summary

LY253963, the sodium salt of 1,3,4-thiadiazol-2-ylcyanamide, was evaluated in tissue culture and in cotton rats for toxicity and antiviral efficacy against respiratory syncytial (RSV) and parainfluenza type 3 (PIV3) viruses. The selective index (ratio of the median toxic dose: median efficacious dose) of LY253963 in HEp2 tissue culture cells was >100 against both RSV and PIV3. When given intraperitoneally to cotton rats, the minimum protective dose of LY253963 against both of these viruses was between 1 and 3 mg/kg/day. In contrast, doses of LY253963 as high as 30 mg/kg/day, administered orally after experimental inoculation of virus, did not significantly reduce pulmonary virus titers in treated animals compared to control animals given placebo. No toxic effects were noted in cotton rats, even in those given 20 mg/kg/day for eight consecutive days.

LY253963; Respiratory syncytial virus; Parainfluenza virus; Cotton rat

Introduction

There is a pressing need to develop new antiviral compounds active against respiratory syncytial (RSV) and parainfluenza type 3 (PIV3) viruses. These viruses remain the leading causes of acute respiratory disease in children under two years of age (Glezen et al., 1982). No vaccines are currently licensed for use in preventing infections by either of these viruses. No antiviral is approved for the treatment of PIV3 infections, and only one, ribavirin, is approved for use against RSV. Unfortunately, ribavirin is licensed for use only when it is delivered by small particle aerosol. Such delivery usually requires long application periods, specialized

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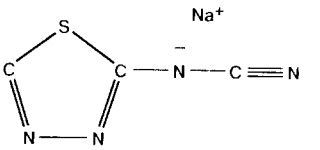

LY253963

Fig. 1. LY253963, the sodium salt of 1,3,4-thiadiazole-2-ylcyanamide (LY217896).

equipment and supervision by trained medical personnel. Thus, treatment of RSV disease with ribavirin is both expensive and practical only for the treatment of the most severe forms of the disease.

LY253963, the sodium salt of 1,3,4-thiadiazole-2-ylcyanamide (structure snown in Fig. 1), would appear to be a compound that could be developed to treat paramyxovirus-induced diseases. LY253963 has been reported to be active in tissue culture and in mice against several related influenza A viruses, with an average median efficacious dose (ED_{50}) of <1 μg/ml in tissue culture and significant reductions in lethal infection of mice with doses of LY253963 ranging from 2 to 256 mg/kg/day (Delong, et al., 1987, Studies on antiviral compound LY253963: II. Evaluations on antiviral activity, 27th Interscience Conference on Antimicrobial Agents and Chemotherapy, October 4–7, N.Y., abstract 988). The compound has also been reported to be active in tissue culture against influenza B (Wu et al., 1987, Studies on antiviral compound LY253963: I. The detection of active compounds, 27th Interscience Conference on Antimicrobial Agents and Chemotherapy, October 4–7, N.Y., abstract 987) and several paramyxoviruses [Nelson, et al., 1988, In vitro antiviral spectrum of LY253963. Antiviral Res. 9:100 (abstract)]. The compound is of additional interest, as its antiviral activity against influenza viruses in vivo was reported to occur whether the compound was given parenterally or orally (see the DeLong, et al., 1987 abstract cited above). To our knowledge, LY253963 has not been tested in vivo against any paramyxovirus.

Based on the promise of these data we were prompted to initiate evaluation of LY253963 in cotton rats for its toxicity and antiviral efficacy against RSV and PIV3. In the present study, the toxicity and antiviral activity of LY253963 were assessed both in tissue culture and in cotton rats. The compound was active against both RSV and PIV3 in HEp2 tissue culture cells, and in cotton rats when given intraperitoneally (i.p.). However, no consistent antiviral activity was seen in experimentally infected cotton rats given LY253963 orally.

Materials and Methods

Animals

All cotton rats (*Sigmoden hispidus*) used in these studies were bred from 2 pairs obtained from the Small Animal Section, Veterinary Research Branch, Division of

Research Services, National Institutes of Health. Test animals were between 40 and 100 g at the start of each experiment and of either sex. All animals were maintained in cages with barrier filters and fed water and food ad libitum.

Viruses

The RSV used in these studies was obtained originally from a patient hospitalized with a severe respiratory infection. Its identity as a RSV virus was made by us using specific neutralizing antisera (Whittaker Bioproducts, Walkersville, MD.; cat. no. 30895). A portion of the working stock was sent to Dr. Larry Anderson of the Communicable Disease Center, Atlanta, GA, who determined this virus to be a type A RSV using virus-specific monoclonal antibodies.

The PIV3 used in these studies was obtained from the American Type Culture Collection (ATCC; Rockville, MD). Stocks of it, and of the RSV, were prepared by infecting flasks of HEp2 tissue culture cells. When the monolayers in these flasks exhibited approximately 90% syncytia formation, the medium from the flasks was collected, pooled and clarified by centrifugation (450 × *g*). The clarified supernatant fluid was passed through a 0.45 μM filter (Acrodisc, Cat. No. 4184, Gelman, Ann Arbor, MI), portioned and stored at −70°C. The titer of the resulting RSV pool was 3×10^6 plaque forming units (pfu)/ml; the titer of the PIV3 pool 1×10^7 pfu/ml.

Tissue culture

Starting cultures of HEp2 (ATCC CCL23), L929 (ATCC CCL1), WI-38 (ATCC CCL75), Madin Darby canine kidney (MDCK; ATCC NBL-2) and A549 (ATCC CCL185) tissue culture cells were obtained from the ATCC. Whenever any flasks containing these cell lines became confluent, they were serially passaged using Eagle's minimum essential medium (MEM) supplemented with fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 μg/ml), sodium bicarbonate (0.2%), and L-glutamine (2 mM/ml).

Compounds

LY253963 was obtained from the Eli Lilly Co. via the Antiviral Research Branch of the National Institutes of Allergy and Infectious Diseases. Initial concentrations of drug were made in sterile distilled water. Additional dilutions were made in 2% FCS-MEM if the compound was to be tested in tissue culture, and in water if it was to be tested in cotton rats.

Ribavirin and carbocyclic 3-deazadenosine (Cc3ado) were used as positive controls in some experiments. Ribavirin was obtained from ViraTek, Covina, CA; Cc3ado was obtained from Drs. John Montgomery and John A. Secrist III of ViraChem Corp. and Southern Research Institute, respectively, Birmingham, AL. Each of these compounds was prepared in 2% FCS-MEM or water as described for LY253963.

Virus quantification

Assays to detect and quantify virus in different preparations were generally performed in 96-well tissue culture plates (Falcon 3072, Lincoln Park, NJ.). In these assays, serial half $\log_{10}$ dilutions of each sample were made in quadruplicate in 2% FCS-MEM. Approximately 3×10^3 HEp2 cells were then added to each well. The plates were placed in a 35°C, 5% CO_2 incubator and observed daily for syncytia formation. At the end of seven days all wells containing syncytia were recorded. Mean virus titers ($\log_{10}$/g of lung or /ml of fluid) were determined by calculating the means of the last dilutions in replicate rows that contained virus.

Cytotoxicity in vitro

Drug cytotoxicity was determined using the quantitative colorimetric MTT assay of Mosmann (1983). In these assays, LY253963, ribavirin or diluent (sterile water) were added in quadruplicate (LY253963) to the initial wells of 96-well flat-bottomed, tissue culture plates (Falcon 3072) and diluted long ways up the plate in 2% FCS-MEM, using serial 2-fold dilutions. (The final concentration of LY253963 and ribavirin in the first wells was 1 mg/ml). The contents of all wells were then transferred to 96-well plates containing approximately 3×10^3 (approximately 30% confluent) of the appropriate tissue culture cells. Replicate control wells containing linear concentrations of cells (standard curve) and medium, but no antiviral, were included in each assay. These cell control wells were observed daily. When cells in the control wells containing the highest concentration of cells (i.e., 3×10^3 cells) reached confluency, all wells were observed for cytopathic effects (CPE) and for percent monolayer confluency. Results were recorded. At that time 0.05 ml of MTT (3-(4,5-dimethylthiazaol-2-yl)-2,5-diphenytetrazolium bromide; 5 mg/ml) was added to each well. After a 3 h incubation at 37°C during which time a blue color appeared in most wells, acidified isopropanol was added to each well. The OD in each well of each plate was then determined using a 96-well plate reader (Molecular Devices UVMax spectrophotometer). To assure that OD readings were proportional to cell number, in each assay the OD readings obtained for the different cell concentrations in the cell control wells were plotted against the initial cell concentrations in each set of control wells. Only assays in which the resulting correlation coefficients were ≥ 0.90 were used. Toxicity was considered to have occurred if >20% of the cells in a monolayer exhibited CPE or if the monolayer remained <50% confluent. The median inhibitory dose (ID_{50}) was determined by calculating the mean concentration (μg/ml) of test compound in the last wells of the replicate rows exhibiting >20% CPE or <50% confluency.

Antiviral activity in vitro

Assays to assess the antiviral activity of LY253963 in tissue culture were performed in 96-well flat bottom tissue culture plates (Falcon 3072), using conditions similar to those used in the cytotoxicity assays described above. In these assays,

LY253963 was tested in quadruplicate by serially diluting the compound in 2% FCS-MEM using serial two-fold dilutions (0.05 ml/well). A 0.05 ml volume of the appropriate virus containing approximately 100 median tissue culture infectious doses ($TCID_{50}$) was then added to all wells but those set aside as antiviral and tissue control wells. Next, approximately 3×10^3 HEp2 cells (0.1 ml) were added to each well. Control wells containing antiviral and no virus (antiviral control), containing virus but no antiviral (virus control), or containing medium without virus or antiviral (tissue control), were included in each test. The challenge virus was then back titrated. All assay plates were incubated at 35°C for 5 to 7 days in a 5% CO_2 incubator. When virus control wells exhibited 70 to 100% CPE including syncytia, all wells were observed. The median efficacious dose (ED_{50}) was calculated after determining the final concentration of antiviral in the last wells in each set of quadruplicate rows exhibiting <50% CPE compared to the CPE in virus control wells.

Collection of lungs

Lungs were removed, stripped of attached lymph nodes, rinsed in sterile saline, and except for those requiring histologic processing, each was transpleurally lavaged using 3 ml of 2% FCS-MEM as described previously (Wilson et al., 1980).

Histological methods and evaluation

Lungs and kidneys were removed for histologic examination and placed in buffered formalin for a minimum of 24 h. They were then embedded in low-melting point paraffin, sectioned at 5 μM thickness, and stained with hematoxylin and eosin. The stained sections were coded by number and observed in a blinded fashion for histopathologic evidence.

Antiviral activity in vivo

The following protocol was generally used to assess the antiviral activity of LY253963 in cotton rats: on day 0 all animals were anesthetized lightly with ether, weighed and inoculated intranasally (i.n.) with approximately 100 median cotton rat infectious doses ($CRID_{50}$) of RSV or PIV3 in 0.1 ml. On days +1, +2, and +3 after virus inoculation, all animals, except those receiving placebo, were given a single dose LY253963 in 0.1 ml, either i.p. or orally by gavage. (Compound was made fresh each day.) Placebo control animals were given 0.1 ml of sterile water i.p. or by gavage. All animals were killed on day +4, the day of maximum RSV or PIV3 pulmonary infection in untreated cotton rats. The lungs of each animal were then removed, weighed and assayed for virus levels as described above. The minimum efficacious dose of test compound for cotton rats, the $CRED_{50}$, was calculated by determining the minimum dose of compound that caused significant reduction of mean pulmonary virus titer in treated cotton rats compared to the mean pulmonary virus titer in control animals given placebo.

Toxicity studies in cotton rats

Cotton rats were weighed and bled from the retroorbital sinus plexus at the start of each toxicity experiment. For the next eight days each animal was given single i.p. inoculations or oral administrations (gavage) of placebo (water) or LY253963 (20 mg/kg/day). Each animal was observed daily for overt signs of toxicity (i.e., morbidity, death, diarrhea). On day eight, 1 h after the last administration of drug, each animal was reweighed, bled and killed. The lungs from each animal were removed for histologic studies. The sera obtained from these animals were sent to Animal Reference Laboratories, Inc., Houston, Texas for determination of blood urea nitrogen (BUN), aspartate transaminase (AST) and alanine transaminase (ALT) levels.

Statistics

Means, standard errors of the means, standard deviations and Student's *t*-tests were calculated using True Epistat, a statistical program designed by T.L. Gustafson of Epistat Services, Richardson, Texas, for IBM compatible computers.

Results

Cytotoxicity in vitro

A comparison of the cytotoxicity of LY253963 and ribavirin for different tissue culture cell lines is shown in Table 1. With one exception, no significant cytotoxicity (CPE or inhibition of cell growth) was observed for either compound in any of these cell lines, even at the highest concentration of compound tested (1000 μg/ml). The one exception was the marked cytotoxicity of LY253963 for MDCK cells (mean ID_{50} from 3 experiments = 7.8 $\pm$ 4.2 μg/ml).

TABLE 1

Comparison of the cytotoxicity of ribavirin and LY253963 for different tissue culture cell lines[a]

Cell line	Median inhibitory dose (ID_{50}; μg/ml)	
	LY253963	Ribavirin
HEp2	>1000	>1000
A549	>1000	>1000
WI-38	>1000	>1000
L929	>1000	>1000
MDCK	7.8 (4.2)[b]	>1000

[a]All wells were observed for cytopathic effects and/or the inhibition of cellular replication using a quantitative colorimetric MTT assay as described in Materials and Methods. MTT was added and ODs determined when the cell control wells were confluent.

[b]Mean value $\pm$ standard deviation for $\geq$3 experiments; standard deviations for all other values = 0.

TABLE 2

Comparison of the toxicity and antiviral activity of LY253963 in HEp2 tissue culture cells against respiratory syncytial (RSV) and parainfluenza type 3 (PIV3) viruses[a]

Test compound	Virus	ID_{50} (μg/ml)[b]	ED_{50} (μg/ml)	Selective index ID_{50}/ED_{50}
Ribavirin	RSV	>1000	10.7 (9.2)	> 94
LY253963	RSV	>1000	7.8 (5.3)	>128
Ribavirin	PIV3	>1000	12.5 (11.5)	> 87
LY253963	PIV3	>1000	9.6 (6.3)	> 104

[a]Mean values from ≥3 experiments; standard deviations when greater than zero are shown in parentheses.
[b]Abbreviations: ID_{50}, median inhibitory (toxic) dose; ED_{50}, median efficacious dose.

Antiviral activity in vitro

A comparison of the antiviral activity of LY253963 and ribavirin in tissue culture is shown in Table 2. Both compounds had equivalent and significant antiviral activity in HEp2 tissue culture cells. In three experiments, the mean ED_{50} values for ribavirin and LY253963 against RSV were 10.7 and 7.8 μg/ml respectively. The ED_{50} values for these compounds were only slightly higher when they were tested against PIV3 (12.5 and 9.6 μg/ml respectively). The selective indices (ratio of the ID_{50}/ED_{50}) for LY253963 and ribavirin reflect this equivalent activity and ranged only from >87 to >128.

Toxicity in vivo

No toxic manifestations (weight loss, morbidity, mortality, diarrhea, unusual pulmonary histopathology or significantly altered levels of key blood enzymes) were noted in any animal given LY253963 in any experiment performed in these studies, even in those given 20 mg/kg/day LY253963 for eight consecutive days.

Antiviral activity in vivo

Representative data from experiments testing the antiviral efficacy of LY253963 against RSV and PIV3 in cotton rats are shown in Tables 3 and 4. In all five experiments testing LY253963 given i.p., animals given single doses of LY253963 ≥3 mg/kg on days +1, +2 and +3 after experimental infection with either RSV or PIV3 had significantly lower pulmonary titers of virus than comparably inoculated animals given placebo. In these experiments (see experiment 1, Table 3, for an example), animals given 2 mg/kg/day of LY253963 i.p. in divided 1 mg/kg doses had significantly lower pulmonary virus titers than comparably challenged control animals given placebo. Thus, the median efficacious dose for LY253963 in cotton rats against both RSV and PIV3, when given i.p., was between 1 and 3 mg/kg/day. This $CRED_{50}$ value was considerably lower than the one obtained for ribavirin. In similar studies, the $CRED_{50}$ for ribavirin given i.p. against RSV was between 30 and 90 mg/kg/day (see Table 3 for an example).

TABLE 3

Pulmonary respiratory syncytial (RSV) and parainfluenza type 3 (PIV3) virus titers in cotton rats given LY253963 or ribavirin intraperitoneally following experimental infection[a]

Test compound	Challenge virus	Dose and schedule	Mean pulmonary titer (log_{10}/g lung)
LY253963	RSV	Placebo 2×/day	4.1 (0.7)
		1 mg/kg 1×/day	3.2 (1.3)
		0.3 mg/kg 2×/day	3.6 (1.1)
		1 mg/kg 2×/day	2.8 (0.6)[b]
		3 mg/kg 1×/day	2.9 (0.7)
Ribavirin	RSV	Placebo (daily)	4.5 (0.2)
		10 mg/kg 1×/day	4.5 (0.2)
		30 mg/kg 1×/day	4.0 (0.3)
		90 mg/kg 1×/day	2.8 (0.3)
LY253963	PIV3	None (placebo)	3.8 (0.2)
		0.3 mg/kg 1×/day	3.2 (0.5)
		1 mg/kg 1×/day	3.4 (0.3)
		3 mg/kg 1×/day	2.7 (0.3)

[a]Cotton rats were inoculated with virus intranasally on day 0 and given drug as indicated on days +1, +2 and +3. All animals were killed on day +4, at which time lungs were removed and assessed for virus.
[b]Underlined means are significantly different ($P<0.05$) than placebo control means using Student's t-test; number of animals/group ≥4.

TABLE 4

Pulmonary respiratory syncytial virus (RSV) titers in cotton rat given LY253963 or carbocyclic 3-deazadenosine (Cc3Ado) orally by gavage[a]

Test compound	Challenge virus	Dose and schedule	Mean pulmonary titer (log_{10}/g lung)
LY253963	RSV	Placebo (daily)	4.6 (0.4)
		1 mg/kg 1×/day	4.7 (0.4)
		3 mg/kg 1×/day	5.0 (0.1)
		10 mg/kg 1×/day	4.6 (0.1)
Cc3Ado	RSV	Placebo (daily)	4.2 (0.5)
		1 mg/kg 1×/day	4.0 (0.4)
		3 mg/kg 1×/day	4.3 (0.3)
		10 mg/kg 1×day	2.6 (0.7)[b]

[a]Animals were inoculated with virus intranasally on day 0 and given drug as indicated on days +1, +2 and +3. Animals were killed on day +4, at which time lungs were removed and assessed for virus.
[b]Underlined means are significantly different ($P<0.05$) than placebo control means using Student's t-test; number of animals/group ≥4.

Five experiments were also performed to determine whether LY253963 was efficacious against RSV when given orally. Test cotton rats were given different concentrations of LY253963 or Cc3Ado by gavage. Doses ranged from 0.1 to 30 mg/kg/day. Although significant reductions in pulmonary virus titers were consistently seen in animals given ≥10 mg/kg/day Cc3Ado orally, no consistent reductions in pulmonary virus titers, compared to pulmonary virus titers in placebo controls, were observed in animals given LY253963 similarly. Data from a representative experiment are shown in Table 4.

Discussion

As indicated in the Introduction of this paper, there is a compelling need for the elucidation and development of new antivirals which can be used against pulmonary infections caused by RSV and PIV3. Data presented at recent meetings indicate that LY253963, the sodium salt of 1,3,4-thiadiazole-2-ylcyanamide (LY217896) has a broad spectrum of antiviral activity in vitro, including activity against various paramyxoviruses (abstracts by Delong et al., 1987; Wu et al., 1987; Nelson et al., 1988, cited in the Introduction). The compound has also been reported to have antiviral activity when given orally (Delong et al., 1987, abstract). LY253963 thus appears to be a particularly good candidate compound to evaluate further as a new antiparamyxovirus agent to supercede, or use as an adjunct antiviral with ribavirin.

In the present studies, LY253963 was relatively nontoxic (ID_{50} >1000 μg/ml) in tissue culture, with one, possibly important exception. LY253963 was markedly toxic (ID_{50} = 7.8 ± 4.2 μg/ml) to MDCK cells. The reason for this unusual cytotoxicity is not known. It is known that MDCK cells differ from most cultured cells in that they maintain some of their specialized in vivo (kidney) functions in vitro (Cereijido et al., 1978; Leighton et al., 1970; Lever, 1979; Misfeld et al., 1976). We do not know if this in vitro toxicity to kidney cells is predictive of potential kidney toxicity in vivo. No evidence of toxicity (i.e., weight loss, diarrhea, death, histopathology, or significant changes in certain serum enzyme levels) was seen in cotton rats, even in those given 20 mg/kg/day LY253963 for eight consecutive days. No kidney function tests were performed, however, as we lack this capability. The significance of the cytotoxicity to MDCK tissue cells thus remains to be determined.

Regardless of the cytotoxicity observed in MDCK tissue culture cells, LY253963 exhibited significant antiviral activity against both RSV and PIV3 in HEp2 cells and in cotton rats. The mean ED_{50} values obtained for LY253963 in HEp2 tissue culture cells against RSV and PIV3 were 7.8 and 9.6 μg/ml respectively (Table 2), and the selective indices in HEp2 cells against both viruses were >100. These values were comparable to those obtained for ribavirin in these same tests (ED_{50} values of >10.5 and >12.7 μg/ml versus RSV and PIV3, respectively, and selective indices of >94 and >87).

More importantly, RSV- or PIV3-inoculated cotton rats given single doses of LY253963 ≥3 mg/kg/day, or two 1 mg/kg/day daily, consistently had significantly lower pulmonary virus titers than comparably inoculated cotton rats given placebo. The resulting $CRED_{50}$ value (between 1 and 3 mg/kg/day) compares quite favorably with the $CRED_{50}$ obtained by us for ribavirin against RSV in cotton rats (30–90 mg/kg/day) in the present study (see data in Table 3), and in previous studies (Wyde et al., 1987; Gruber et al., 1987).

Unfortunately, no similar antiviral activity was observed in virus-infected animals given up to 30 mg/kg/day LY253963 orally by gavage. These data are disappointing, as a compound which can be administered orally and be active against paramyxovirus-induced diseases would be highly desirable, and have many advantages over ribavirin, the only compound currently licensed for use in treatment of

RSV-induced disease.

The cause(s) for the differences seen in antiviral activity following oral administration of LY253963 observed by us and the Lily investigators who report good antiviral activity against influenza viruses following oral administration of drug to mice (DeLong et al., 1987 abstract cited in introduction) is not known. It is not due to our gavage technique. We are able to show consistent reduction of pulmonary RSV titers in cotton rats given ≥10 mg/kg/day Cc3Ado by gavage compared to pulmonary virus titers in control cotton rats given placebo (see Table 4). It is possible that the differences noted are related to species differences, particularly to the marked differences in the gastrointestinal flora of cotton rats and mice. It has been reported by Itoh et al., (1989) that cotton rats have very different gastrointestinal flora than most other rodents. Such differences could result in markedly different degradation and/or transport of drug in or through the gastrointestinal tracts of mice and cotton rats.

These studies confirm that LY253963 has significant anti-paramyxovirus activity in tissue culture. The studies extend previous findings by showing that the compound is also active against this group of viruses in cotton rats. Because the minimal efficacious dose for LY253963 against paramyxovirus virus induced infection in cotton rats is significantly less than that seen with ribavirin, the only compound currently available for use against RSV disease, further testing of LY253963 seems reasonable. One major area of interest is the mechanism of action by which LY253963 inhibits virus replication. This is currently not known (Nelson, et al., 1988 abstract), and given its unusual structure, is of great interest. Knowledge of the mechanism of how LY253963 inhibits virus might also help in understanding its cytotoxic effects on MDCK tissue culture cells.

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