ELSEVIER

Antiviral Research 38 (1998) 31–42

Antiviral Research

CL387626 exhibits marked and unusual antiviral activity against respiratory syncytial virus in tissue culture and in cotton rats

Philip R. Wyde [a,*], Donna K. Moore-Poveda [a], Bryan O'Hara [b], Wei-Dong Ding [b], Boris Mitsner [b], Brian E. Gilbert [a]

[a] *Department of Microbiology and Immunology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA*
[b] *Wyeth-Ayerst Research Laboratories, 401 N. Middletown Road, Pearl River, NY 10965, USA*

Received 11 April 1997; accepted 22 December 1997

Abstract

CL387626 (4,4′-Bis[4,6-di[3-aminophenyl-*N*,*N*-bis(2-carbamoylethyl)-sulfonilimino]-1,3,5-triazine-2-ylamino-biphenyl-2,2′-disulfonic acid, disodium salt), a compound synthesized by Wyeth-Ayerst Research Laboratories, was tested for its cytotoxicity and antiviral activity against respiratory syncytial virus (RSV) in tissue culture and in cotton rats. The median cell inhibitory (IC_{50}) and median efficacious (EC_{50}) concentrations of CL387626 against RSV in proliferating HEp2 or Vero tissue culture cells were determined to be 375 and 0.25 μg/ml, respectively, giving the compound an apparent selective index (S.I.) of 1500. This compound also exhibited uncommon antiviral activity against RSV in cotton rats. In multiple experiments, a single 30 mg/kg dose of CL387626 administered intranasally 4 or 5 days prior to virus challenge, significantly inhibited pulmonary replication of RSV compared to that seen in control animals inoculated similarly with placebo (i.e. water). In contrast to these results, most lots of CL387626 failed to significantly inhibit pulmonary RSV replication when administered utilizing therapeutic administration schedules. Although some cytotoxicity was noted in tissue culture assays, no overt toxic effects were noted in any test animal, including those inoculated with >300 mg CL387626/kg, a dose approximately 150 times the apparent minimal efficacious dose (i.e. 1.9 mg/kg).

Keywords: RSV; Cotton rats; Antiviral; Pneumonia; Prophylaxis; Bronchiolitis

1. Introduction

Respiratory syncytial virus (RSV) has been recognized as a leading cause of serious lower res-

* Corresponding author.

0166-3542/98/$19.00
PII S0166-3542(98)00002-3

piratory tract infections in infants and children under 2 years of age (Parrott et al., 1973; Glezen et al., 1982; Chanock and McIntosh, 1990). More recently it has been identified as an important cause of lower respiratory tract infections in adults (Dowell, et al., 1996). Indeed, this virus has been reported to be responsible for 40–50% of hospitalizations for bronchiolitis in the United States, 25% of pediatric hospitalizations for pneumonia and to be an important factor in the development of hyper-reactive airway disease in later life (La Via, et al., 1992). No vaccines are currently available for prevention of RSV infections, and only one antiviral, ribavirin, is approved to treat RSV disease. Unfortunately, ribavirin is licensed for use only when given by continuous small particle aerosol (Committee on Infectious Diseases, 1993), and is a potential teratogen (Bradley et al., 1990). Thus, utilization of this nucleoside analog is both limited and somewhat controversial. The continued medical impact of RSV infections and the problems associated with ribavirin have spurred interest in identifying new, safer and less controversial compounds to prevent or ameliorate these infections.

This report summarizes results of primary in vitro and in vivo studies evaluating the toxicity and antiviral activity against RSV of CL387626, a compound synthesized by Wyeth-Ayerst Research Laboratories (Pearl River, NY) and submitted to the Virology Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, for antiviral testing. In these tests, CL387626 exhibited good selective antiviral activity against RSV in tissue culture (S.I. = 1500 in monolayers of proliferating HEp2 or Vero tissue culture cells) and unusual prophylactic antiviral activity against this virus in cotton rats. In numerous studies, a single 30 mg/kg dose of CL387626 administered intranasally (I.N.) to cotton rats up to 5 days prior to virus challenge, significantly inhibited replication of RSV in the lungs of these animals compared to the replication of this virus in comparably treated control animals given placebo. In contrast, most of these lots of CL387626 did not exhibit significant antiviral activity against RSV when administered to cotton rats using therapeutic administration schedules. No overt toxic effects were noted in any of the test animals, including those administered single doses of CL387626 > 300 mg/kg. These studies are described below in detail.

2. Methods

2.1. Animals

Cotton rats, 50–100 g and 4–12 weeks old (*Sigmoden hispidis*) of either sex were used in these studies. All of the animals were descendants of two pair of cotton rats obtained in 1984 from the Small Animal Section of the Veterinary Research Branch, Division of Research Services, National Institutes of Health. The animals were housed in the Baylor College of Medicine (BCM) vivarium in cages covered with barrier filters, and all were given food and water ad libitum.

2.2. Tissue culture

HEp2 (human epithelial carcinoma; ATCC CCL 23) and Vero (ATCC CCL 81) tissue culture cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD). The HEp2 cells were used to prepare stocks of RSV, parainfluenza virus type 3 (PIV3), and adenovirus type 5 (AV5), and to perform assays involving these viruses. The Vero cells were used to prepare stock suspensions of measles virus (MV), and to perform assays including this virus. Both tissue culture cell lines were grown in monolayers using Eagle's minimal essential medium (MEM; BioWhittaker), supplemented with 10% fetal calf serum (FCS; Sigma), 100 U/ml penicillin (GIBCO Laboratories), 100 g/ml streptomycin sulfate (GIBCO), 2 mM L-glutamine (GIBCO), and 0.2% sodium bicarbonate (Sigma). The monolayers were removed from their plastic surfaces and serially passaged whenever they became confluent. MEM supplemented with 2% FCS was used to maintain the cell cultures and as a diluent in all assays.

2.3. Viruses

RSV strains A2 (ATCC VR1302) and Long (ATCC VR26) were purchased from the ATCC. RSV strain 18537, all of the clinical RSV strains and PIV3 were obtained from Dr Tony Piedra, Department of Microbiology and Immunology, BCM. AV5 was acquired from Julius Kasel, Ph.D., also in this department. MV was obtained from Gail Demmler, M.D., Department of Pediatrics, BCM. Working stocks of each of these viruses were prepared as described in detail previously (Wyde et al., 1995). Subtyping of the clinical RSV strains was performed by Dr Larry Anderson of the Centers for Disease Control, Atlanta, GA.

2.4. Virus quantification

RSV levels in virus pools and lung lavage fluids (L.F.) were determined using sterile 96-well, flat bottom tissue culture plates (Falcon 3072), serial 3-fold dilutions and 2% FCS-MEM as described in detail previously (Wyde et al., 1995). The wells in these assay plates were observed for virus-induced cytopathic effects (CPE) including formation of syncytia. After the dilutions in the last wells of replicate rows exhibiting virus-induced CPE were determined, mean virus titers were calculated using the method of Karber (Rhodes and Van Rhodes and Van Rooyen, 1953). The amount of virus in virus pools was expressed as median tissue culture infectious doses ($TCID_{50}$)/ml, ($\log_{10}$). Titers of virus in L.F. were expressed as $TCID_{50}$/g lung tissue ($\log_{10}$). The minimum detectable virus concentration in these assays was 1.3 $\log_{10}$ $TCID_{50}$/ml (virus pools) or 1.6 $\log_{10}$ $TCID_{50}$/g lung.

2.5. Compounds

Six lots of CL387626 (4,4′-Bis[4,6-di[3-aminophenyl-*N*,*N*-bis(2-carbamoylethyl)-sulfonil-imino]-1,3,5-triazine-2-ylamino-biphenyl-2,2′-disulfonic acid, disodium salt) were submitted by Wyeth-Ayerst for antiviral testing. Each lot was stored at 4°C until the morning of an assay. At that time a portion of the material to be tested was weighed and suspended in sufficient sterile distilled water (Baxter Healthcare Corporation, Deerfield, IL; cat. no. 2F7114) to make appropriate working stocks (4 mg/ml for in vitro studies and 30–90 mg/ml for in vivo studies). Each suspension was passed through a 0.2 μm DynaGard filter (Microgon, Laguna, CA; cat. no. DG2M-330-50S). The structure of CL387626 (submitted by Wyeth-Ayerst Research) is shown in Fig. 1.

Ribavirin was used as a positive control in both in vitro and in vivo tests. This compound was obtained from Viratek, Covina, CA. It was stored at 4°C and diluted in sterile distilled water shortly before each assay or experiment. The resulting suspension was filter sterilized using a 0.2 μm DynaGard filter.

2.6. In vitro cytotoxicity assays

The cytotoxicity of CL387626 was evaluated in sterile 96-well plates (Falcon 3072) as described in detail previously (Wyde et al., 1993), with one exception. XTT (2,3-bis[2-methoxy-4-nitro-5-sufophenyl]-2H-tetrazolium-5-carboxanilide; Sigma, cat. no. X4251) was used instead of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma, cat. no. M-2128) to assess mitochondrial respiration and indirectly cell viability. Briefly, CL387626, ribavirin or placebo (distilled water) was serially diluted up the test plate in quadruplicate or duplicate using serial 2-fold dilutions and 2% FCS-MEM as the diluent. After adding approximately 1×10^4 HEp2 or

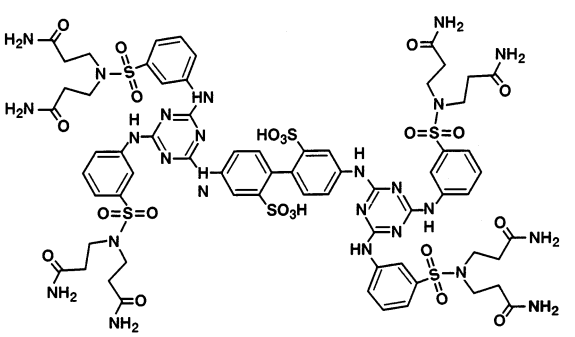

Fig. 1. Structure of CL387626.

Vero cells to each well, the plates were incubated at 36°C in a 5% CO_2 incubator. When the monolayers in the cell control wells (those containing only HEp2 or Vero cells and 2% FCS-MEM) became confluent, usually 48 h later, 50 μg of XTT was added to each well. The optical density (OD) in each test and control well was determined 3 h later using a 96-well plate reader (Molecular Devices UVMax spectrophotometer). The concentration (μg/ml) of CL387626 or ribavirin in the last well of each row exhibiting a $\geq 50\%$ reduction in OD compared to the mean OD obtained in the cell control wells was then noted. From these, the median cell inhibitory concentration (IC_{50}) of each compound was calculated.

2.7. Antiviral activity in tissue culture

The assay used to measure the antiviral activity of CL387626 in tissue culture has also been described in detail previously (Wyde et al., 1993). Briefly, CL387626, ribavirin or distilled water was added in quadruplicate or duplicate to the first wells of 96-well, flat bottom tissue culture plates (Falcon 3072) and serially diluted up the plate using 2% FCS-MEM and serial 2-fold dilutions (0.05 ml/well). The contents of each well were then transferred to parallel plates containing approximately 1×10^4 HEp2 cells (in 0.05 ml)/well. Of the appropriate virus suspension, 50 μl containing approximately 100 $TCID_{50}$ of virus was then added to each well except those set aside as antiviral (wells containing cells and antiviral agent) or tissue (wells containing cells and medium) control wells. These plates were incubated at 36°C in a 5% CO_2 incubator until the virus control wells exhibited 70–100% CPE including syncytia. At that time, each well was observed and scored for viral CPE. The concentration of CL387626 or ribavirin in the last well of each row that completely inhibited virus-induced CPE was noted. The median efficacious concentration (EC_{50}; μg/ml) for CL387626 and ribavirin were then calculated by determining the mean concentration of these compounds that completely inhibited CPE in one-half of the replicate wells. To obtain a S.I. for CL387626, the mean IC_{50} value obtained for this compound in the in vitro cytotoxicity assay was divided by the mean EC_{50} value obtained for CL387626 in these assays. The S.I. for ribavirin was determined in a similar manner.

2.8. Antiviral activity in cotton rats

For intranasal inoculations, animals were anesthetized with Metofane (methoxyflurane, Pitman-Moore, Mundelein, IL). Animals inoculated intraperitoneally (I.P.) were anesthetized using CO_2. In experiments testing therapeutic regimens, the cotton rats were weighed and then inoculated I.N. on Day 0 with approximately 100 median cotton rat infectious doses ($CRID_{50}$; approximately $10^{3.5}$ $TCID_{50}$) of RSV in 0.1 ml. On each of the next 3 days these animals were inoculated once daily, I.P. or I.N., with graded doses of test compound, ribavirin (180 mg/kg per day) or placebo (sterile distilled water). In experiments looking at the prophylactic activity of CL387626, the test animals were similarly anesthetized and inoculated with virus (0.1 ml containing 100 $CRID_{50}$) on Day 0. However, in these experiments, the cotton rats were weighed and inoculated with test compound or placebo, once, 1 h or 1–8 days prior to virus inoculation. Ribavirin was only administered I.P. and only therapeutically. Regardless of whether a therapeutic or prophylactic schedule was used to administer the test compound, all animals were sacrificed on Day + 4 after virus inoculation using CO_2 to asphyxiate the animals. Day 4 was chosen because with the dose of virus used in these experiments, this is the day that maximum RSV pulmonary titers are usually seen in untreated cotton rats.

2.9. Processing of samples

The lungs of sacrificed animals were removed, rinsed in sterile phosphate-buffered saline (PBS; pH 7.2) and weighed. Each set of lungs was then transpleurally lavaged using 3 ml of 2% FCS-MEM media as described previously (Wilson et al., 1980). The resulting L.F. were kept on ice until assayed for RSV.

Table 1
Comparison of the cytotoxic and antiviral activity of CL387626 and ribavirin in HEp2 or Vero tissue culture cells[a]

Virus	RSV sub-group	IC_{50} (μg/ml)		EC_{50} (μg/ml)		Selective index	
		Ribavirin	CL387626	Ribavirin	CL38726	Ribavirin	CL38726
RSV A2	A	750	375	12	0.25	63	1500
RSV 18537	B	750	375	12	0.25	63	1500
RSV 47063	B	750	375	16	0.25	47	1500
RSV 37335	A	750	375	8	0.25	94	1500
RSV 71050	A	750	375	12	0.25	63	1500
MV		750	375	24	175	31	<3
PIV3		750	375	12	>375	62	<1
AV5		750	375	>750	>375	<1	<1

IC_{50}, median cell inhibitory concentration; EC_{50}, median efficacious concentration; S.I., selective index (IC_{50}/EC_{50}); RSV, respiratory syncytial virus; MV, measles virus; PIV3, parainfluenza virus type 3; AV5, adenovirus type 5.
[a] Assays were performed in 96-well tissue culture plates as described in Section 2 using subconfluent (approximately 30% confluent) monolayers of HEp2 (used for all viruses except measles) or Vero (used for measles virus) tissue culture cells.

2.10. Toxicity studies in cotton rats

Minimal toxicity studies were performed. In these studies, the weights of test animals were determined at the beginning and conclusion of each experiment. Each animal was also observed daily for morbidity, mortality, diarrhea or other untoward responses. In two studies, lungs from non-virus-infected and virus-infected cotton rats treated with CL387626 were collected and placed in Omnifix fixative (Zymed Laboratories, San Francisco, CA) for 24 h. These lungs were then embedded in low-melting point paraffin and sectioned at 5 μm thickness. The resulting sections were stained with hematoxylin and eosin, coded and observed in a blinded fashion for perivascular and peribronchial infiltrates, septal thickening and the presence of inflammatory cells in the alveoli. Ten consecutive microscopic fields on each section were observed using the 40× objective and an up, over and down pattern. For each of the four characteristics, a value of 0–3 was assigned, with 0, not evident; 1, evident in 1–3 of the fields observed; 2, evident in 4–6 fields observed, and 3, evident in >6 of the observed fields. Total scores were obtained by adding scores for each of the four characteristics noted above (the maximum score for an individual animal would be 12).

2.11. Statistics

Student's *t*-test was used to compare mean body weights. Geometric mean virus titers, standard deviations and all descriptive statistics were obtained using True Epistat, a statistical program designed by T.L. Gustafson of Epistat Services, Richardson, TX, for IBM compatible computers. In all statistical analyses, lavage fluid samples with undetectable virus titers were assigned a value of 0.8 (the minimal detection limit being 1.6) $\log_{10}$/g lung. Comparison of the mean geometric RSV titers obtained for the different groups in each experiment were made using Epistat's analysis of variance (ANOVA) program. Unless stated otherwise, all comparisons were made to the mean pulmonary virus titer obtained for the group given placebo.

3. Results

3.1. Cytotoxicity and antiviral activity of CL387626 in vitro

As the IC_{50} values shown in Table 1 indicate both ribavirin and CL387626 exhibited some cytotoxicity (IC_{50} values of 750 and 375 μg/ml, respectively) in cultures of proliferating HEp2 and

Table 2
The effect of route of administration and dose on the antiviral efficacy of CL387626 in cotton rats against RSV[a]

Group no.	Treatment	Dose (mg/kg per day)	Route given	Mean titer+S.D. (log_{10}/g lung)	Reduction in titer (log/g lung)
Experiment 1					
1	Placebo	0	I.P. and I.N	4.20.4	—
2	CL387626	30	I.P.	3.7+0.5	0.3
3	**CL387626**	**30**	**I.N.**	**<1.6+0**	**>2.6**[b]
Experiment 2					
1	Placebo	0	I.N.	4.1+0.3	—
2	**CL387626**	**30**	**I.N.**	**0.8+1.3**	**3.3**
3	**CL387626**	**7.5**	**I.N.**	**1.7+1.5**	**2.4**
4	**CL387626**	**1.9**	**I.N.**	**0.9+1.6**	**3.2**
5	CL387626	0.5	I.N.	3.0+0.5	1.1

[a] All animals were administered placebo (sterile water) or CL387626 I.N. and/or I.P. Each cotton rat was inoculated I.N. 60 min later with approximately 100 median cotton rat infectious doses of RSV A2. The animals were sacrificed 4 days later and their lungs were removed, processed and tested for RSV levels. Number of animals per group = 4.
[b] Bold lines indicate test groups in which the mean pulmonary RSV titer was significantly different ($P<0.05$) than the RSV titer seen in animals given placebo (Group 1). Number of animals per group = 4.

Vero cells. However, the CL387626 exhibited ≥ 32 times greater antiviral activity than did ribavirin (EC_{50} CL387626 = 0.25 μg/ml against all of the RSV strains tested versus EC_{50} values ranging from 8 to 16 μg/ml for ribavirin against these viruses). Due to its markedly greater antiviral activity, a much higher S.I. was obtained for CL387626 against RSV than was calculated for ribavirin against these viruses (1500 vs. 47–94). Although CL387626 was more active against RSV than ribavirin, it was notably less effective against MV and PIV3 than the nucleoside analog. Selective indices of <3 and <1 were obtained for CL387626 against MV and PIV3, respectively, compared to selective indices for ribavirin against these viruses of 31 and 64, respectively. Neither CL387626 nor ribavirin exhibited any significant antiviral activity against AV5 (the S.I. for both compounds being <1 versus AV5).

3.2. Effect of route of administration on the antiviral efficacy of CL387626 in cotton rats

Initial experiments in cotton rats compared the antiviral efficacy of CL387626 when administered parenterally (i.e. I.P.) or topically (i.e. I.N.). As the summary data from one of the experiments performed in this series indicates (i.e. Experiment 1, Table 2), only cotton rats given CL387626 topically were protected from pulmonary RSV infection. In the experiment shown, animals administered a single 30 mg/kg dose of CL387626 1 h prior to virus challenge (Group 3) had $>10\,000$-fold less virus on Day + 4 in their lungs than control animals inoculated comparably with placebo (i.e. Group 1; $P<0.01$). In contrast, no significant reduction in mean pulmonary RSV titer was seen in animals given this same dose of CL387626 I.P., 1 h prior to virus challenge ($P>0.05$ compared to the mean virus titer seen in control animals). In some experiments, doses up to 270 mg/kg were administered I.P. Regardless, in none of these experiments did CL387626 exhibit any significant antiviral activity when administered parenterally. However, reductions in pulmonary virus titer similar to those observed in Experiment 1 were observed repeatedly in these experiments when this compound was administered I.N.

3.3. The effect of dose on the antiviral efficacy of CL387626 in cotton rats

An approximate minimum efficacious dose (MED) for CL387626 was determined by inoculating groups of cotton rats I.N. with graded

Table 3
Comparison of the antiviral efficacy of different lots of CL387626 against RSV when administered to cotton rats just prior to, or following, virus inoculation[a]

Group no.	Lot no.	Dose (mg/kg per day)	Days given	Mean titer (log_{10}/g lung)	S.D.	Reduction in titer (log_{10}/g lung)
1	Placebo	0	0, 1–3	4.0	0.1	—
2	**1**	**30**	**0, 1–3**	**<1.6**	**0**	**>2.4**[b]
3	1	30	1–3	3.8	0.4	0.2
4	**2**	**30**	**0, 1–3**	**<1.6**	**0**	**>2.4**
5	2	30	1–3	3.8	0.4	0.2
6	**3**	**30**	**0, 1–3**	**<1.6**	**0**	**>2.4**
7	3	30	1-3	3.7	0	0.3

[a] Animals in Groups 1, 2, 4 and 6 were inoculated I.N. on Day 0 with placebo (sterile water) or 30 mg/kg of one of the lots of CL387626. All of the cotton rats were inoculated I.N. 60 min later with approximately 100 median cotton rat infectious doses of RSV A2. On days 1–3 all of these animals were inoculated once daily with either placebo or 30 mg/kg of lot 1, 2 or 3 of CL387626. Number of animals per group = 4.
[b] Bold lines indicate test groups in which the mean pulmonary RSV titer was significantly different ($P<0.05$) than the RSV titer seen in animals given placebo (Group 1). The number of animals per group = 4.

doses of this compound, once, 1 h prior to virus challenge, and then sacrificing the animals 4 days later. At that time the lungs of these animals were removed and the levels of RSV in each assessed. In the representative experiment shown in Table 2 (Experiment 2), statistically significant reductions in mean pulmonary RSV titer occurred in the groups of cotton rats administered single doses of CL387626 ranging from 1.9 to 30 mg/kg (i.e. Groups 2–4). The 1.1 log_{10}/g of lung reduction in mean virus titer calculated for the group of animals given 0.5 mg CL387626/kg (Group 5), although substantial, was not statistically different than the mean pulmonary virus titer seen in the placebo control group ($P > 0.05$). In ensuing experiments using this experimental design, 1.9 mg/kg was confirmed as the MED.

It should be noted that in Experiment 2, and in many of the experiments performed with CL387626, the reductions in pulmonary virus seen did not correlate well with the doses of drug given. For example, in Experiment 2, although significant reductions in pulmonary virus titer were seen in the three groups of animals given 1.9, 7.5 and 30 mg of CL387626/kg, the reductions in pulmonary virus titers seen in all three groups were equivalent and not significantly different from each other.

3.4. Effect of inoculation schedule on the antiviral efficacy of CL387626

In the experiment shown in Table 3, the antiviral efficacy of three different lots of CL387626 when given prior to, or subsequent to, virus challenge was compared. As the data in this table indicates, significant reductions in pulmonary virus titers were observed only in groups of animals administered the test compound 1 h prior to virus inoculation, in addition to the daily inoculations on Days + 1 through + 3 (i.e. Groups 2, 4 and 6). The use of different lots of CL387626 did not seem to matter. In contrast, only minimal and non-significant reductions in pulmonary RSV titers (i.e. 0.2–0.3 log_{10}/g lung) were seen in these experiments in animals comparably administered these lots of CL387626 only on Days + 1 through + 3. Similar testing was performed on a total of six lots of CL387626. Significant antiviral activity was seen in only two of these experiments when the test compound was administered only after virus inoculation (data not shown).

Further evidence of the prophylactic activity of CL387626 can be seen in Tables 4 and 5. In the first experiment shown in Table 4, significant reductions in mean pulmonary virus titers were detected in the groups of cotton rats given single inoculations of CL387626 I.N. 3 or 5 days prior

Table 4
Comparison of the antiviral efficacy of CL387626 seen in cotton rats following use of different prophylactic administration schedules[a]

Group no.	Treatment	Dose (mg/kg)	Route administration	Day(s) administration	Mean RSV titer[b] (log_{10}/g lung)	Reduction titer (log_{10}/g lung)
Experiment 1						
1	Placebo	0	I.N.	−7, −5, −3	4.3+0.6	—
2	CL387626	30	I.N.	−7	3.1+1.0	1.2
3	**CL387626**	**30**	**I.N.**	**−5**	**2.8+0.2**	**1.5**
4	**CL387626**	**30**	**I.N.**	**−3**	**3.0+0.5**	**1.3**
Experiment 2						
1	Placebo	0	I.N.	−8, −4, −2, −1	3.6+0.4	—
2	CL387626	30	I.N.	−8	3.5+0.6	0.1
3	**CL387626**	**30**	**I.N.**	**−4**	**1.6+1.3**	**2.0**
4	**CL387626**	**30**	**I.N.**	**−2**	**0.7+0.9**	**2.9**
5	**CL387626**	**30**	**I.N.**	**−1**	**1.7+1.0**	**1.9**

[a] In both Experiment 1 and 2, cotton rats were inoculated I.N. with placebo (sterile water) or CL387626 on the days indicated. On Day 0 each animal was inoculated I.N. with approximately 100 median cotton rat infectious doses of RSV A2.
[b] Mean+S.D. Bold lines indicate test groups in which the mean pulmonary RSV titer was significantly different ($P<0.05$) than the RSV titer seen in animals given placebo (Group 1). The number of animals per group = 4.

Table 5
The antiviral efficacy of CL387626 against a B subtype RSV when given prophylactically[a]

Group no.	Treatment	Dose (mg/kg)	Route administration	Day administration	Mean RSV titer[b] (log_{10}/g lung)	Reduction titer (log_{10}/g lung)
1	Placebo	0	I.N.	0	4.3+0.4	—
2	**CL387626**	**30**	**I.N.**	**−5**	**2.6+0.7**	**1.7**
3	**CL387626**	**30**	**I.N.**	**−3**	**<1.6+0**	**>2.7**
4	**CL387626**	**30**	**I.N.**	**0**	**0.5+1.1**	**3.6**

[a] Animals were inoculated I.N. with approximately 100 median cotton rat infectious doses of RSV 18537, a subtype B RSV, on Day 0. These animals were also inoculated I.N. 1 h or 3 or 5 days before virus challenge with placebo (sterile water) or 30 mg/kg of CL387626.
[b] Mean+S.D. Bold lines indicate test groups in which the mean pulmonary RSV titer was significantly different ($P<0.05$) than the RSV titer seen in animals given placebo (Group 1). The number of animals per group = 4.

to inoculation with RSV A2. In the second experiment shown in this table, animals given this compound 1–4 days before virus had significantly reduced mean pulmonary virus titers compared to the mean RSV titers seen in control animals. Comparable antiviral activity was seen against subtype B RSV. In the experiment shown in Table 5, a 1.7 log_{10}/g lung reduction in RSV titer was observed in the lungs of animals given a single dose of CL387626 5 days prior to challenging these animals with RSV 18537, a RSV B subtype. Even greater reductions in mean pulmonary virus titers were seen in this experiment in cotton rats given CL387626 3 or 1 day prior to virus challenge.

3.5. Results of preliminary cytotoxicity studies in cotton rats

In no experiment was the mean change in weight of any test group significantly different from that observed in the placebo control group (comparisons made using Student's *t*-test; data not shown). Moreover, no morbidity, mortality, diarrhea or apparent untoward behavior was observed in any of the test animals.

Inflammatory cells (i.e. polymorphonuclear neutrophils (PMN), lymphocytes or macrophages) were occasionally seen in stained sections of lungs from untreated, uninfected cotton rats (histologic score = 2). These cells were more abundant in lung sections from non-virus-infected cotton rats inoculated I.N. once with placebo or 300–330 mg of CL387626/kg (these sections were prepared from animals sacrificed 2–4 days after the administration of the test compound). However, there were no significant differences in the histology scores of these animals and those of the uninfected, untreated control animals (histologic score = 4). As in previous studies (Piedra et al., 1993), significantly increased numbers of inflammatory cells and histolopathology were seen 4 days after virus inoculation in untreated virus-infected animals, compared to the histopathology seen in the normal control animals (histologic scores for the untreated, virus-infected animals = 12). The inflammatory cells seen in the stained lung sections from these animals were most evident in and adjacent to the bronchioles and bronchi, but they were also seen scattered throughout the alveolar spaces. Histologic scores on Day + 4 for cotton rats given differing doses of CL387626 prior to virus inoculation correlated well with the mean lung virus titers obtained for these animals (i.e. the higher the mean pulmonary virus titer, the greater the histopathology scores obtained).

4. Discussion

This report summarizes results of studies evaluating CL387626 for selective antiviral activity against paramyxoviruses. Although the com-

pound was found to have some cytotoxic activity in proliferating HEp2 and Vero tissue culture cells (Table 1), this activity occurred at concentrations ≥ 1500 times higher than that found to completely inhibit replication of RSV in these cells (i.e. 0.25 μg/ml). The antiviral activity seen in these assays was virus-, but not strain- or subtype-specific. Thus laboratory and clinical RSV strains belonging to both RSV subtypes (i.e. A and B) were equally inhibited by CL387626, while no significant selective antiviral activity was seen in these tests against MV, PIV3 or AV5 (S.I. < 3 vs. MV and < 1 vs. PIV3 and AV5).

Based on the apparent selective antiviral activity seen in the tissue culture assays, testing of CL387626 against RSV in cotton rats was started. It was determined that this compound was efficacious when administered I.N., particularly if started prior to virus challenge (Tables 3–5). It was not active when given to cotton rats (at doses up to 270 mg/kg) I.P. (Table 2). Nor was significant protection seen with most (i.e. 66%) lots of CL387626 if administration of the compound was started more than 16 h after virus challenge (Table 3). The reason(s) for the general failure of CL387626 to significantly inhibit virus replication when given after virus is not known. This compound is thought to work by inhibiting the cell membrane fusing activity of the virus' fusion (F) protein.

The prolonged prophylactic activity observed in cotton rats in these studies (i.e. protection lasting between 5 and 8 days following a single administration of compound) is unusual for a chemotherapeutic agent in our experience. (Such activity has been reported in vivo for interferons (Wyde et al., 1984), immunoglobulins (Prince and Porter, 1996) and monoclonal antibodies (Wyde et al., 1995; Johnson et al., 1997).) Despite its apparent extended efficacy, it should be noted that the compound appeared to be more effective the closer to virus challenge that it was administered. This can be seen in Fig. 2, which compares decreases in mean pulmonary virus titers seen in several experiments with the time that CL387626 was given before virus challenge. (In all cases the animals were administered a single 30 mg/kg dose of the test compound.) As the slope of the line and positive correlation coefficient (0.7) indicates, the greatest reductions in mean pulmonary RSV titers occurred in animals given CL387626 shortly before (i.e. 1 h) to 2 days before virus and generally decreased as the interval between drug administration and virus challenge increased. Regardless, in multiple experiments, single 30 mg/kg doses of CL387626 administered once I.N. 4 or 5 days prior to virus challenge, repeatedly resulted in significant reductions in mean pulmonary RSV titer compared to mean virus titers seen in control animals comparably inoculated with placebo.

Minimal cytotoxicity was observed in tissue culture assays, and no apparent toxic effects due to CL387626 were evident in cotton rats. In no instance was mortality, morbidity, decreased weight gain, weight loss, diarrhea or unusual activity observed in any test animal. This was true of animals used in the antiviral studies, or those utilized in a simple toxicity study in which ani-

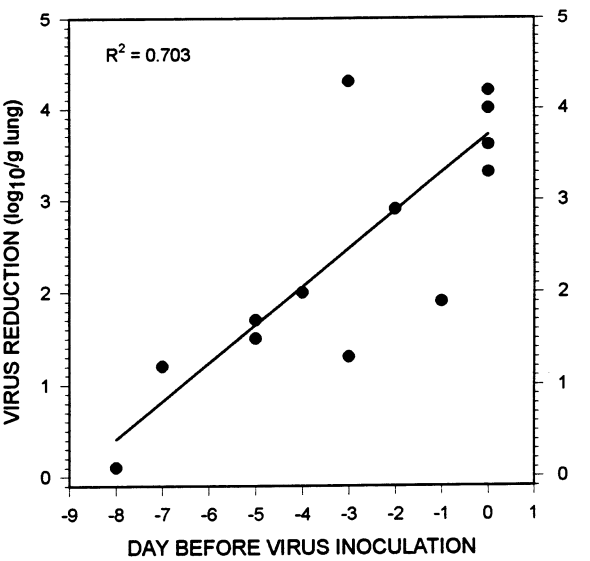

Fig. 2. Comparison of the reduction in mean pulmonary respiratory syncytial virus titers (compared to the mean virus titers seen in control animals given placebo) observed in cotton rats in several experiments testing CL387626 for prophylactic antiviral activity. In each experiment the animals were administered a single 30 mg/kg dose of the test compound intranasally. Only the interval between the time that the drug and virus was administered varied. (R^2 = correlation coefficient.) On Day 0, the cotton rats were inoculated with the test compound 1 h prior to virus challenge.

mals were given single doses ranging from 310 to 350 mg/kg (i.e. doses more than 10 times that used in most of the antiviral studies). In addition, no significant histopathology was observed in stained sections of lungs obtained from any of the animals given CL387626 alone. Nor was increased pulmonary pathology seen in sections of lung obtained from animals given CL387626 and RSV compared to the histopathology seen in stained lung sections from animals given virus alone. It is emphasized that these data are only preliminary. The consequences of multiple injections of CL387626 or prolonged treatment with this compound (such as the weeks and months of treatment required by immunosuppressed patients infected with RSV) were not determined. Nor were the effects of CL387626 on rapidly proliferating tissues (e.g. bone marrow or T- and B-lymphocytes) evaluated. Such studies may be needed if development of this or related compounds is pursued, particularly since the prolonged antiviral state induced by CL387626 seen in these studies suggest that this compound is not readily degraded or cleared from the lungs.

The apparent stability of CL387626 when delivered topically may have contributed to the general lack of correlation seen in many experiments between the amount of compound given and the degree of virus inhibition that occurred (Table 2). One hypothesis is that above a certain level, excess compound is removed from the lungs resulting in similar amounts of test compound becoming residual in the lungs of animals receiving different doses of drug. Then there is a slow decline in active compound over the next 5–8 days (due to removal or inactivation) until protection is lost. Interestingly, the failure to see any significant antiviral activity when CL387626 was administered I.P. (Table 2) suggests that following parenteral inoculation this compound is either bound or altered in a manner that prevents its transport to the lungs, or that it is rapidly degraded or cleared.

More comprehensive cytotoxicity and pharmacokinetic studies on CL387626 are not currently planned. This is because the cytotoxicity and antiviral efficacy of derivatives of CL387626 are presently being evaluated and compared to that of the lead compound, and several seem more promising. More extensive cytotoxicity, pharmacokinetic and mechanism of action studies will be performed on the most promising compound(s) elucidated in these tests.

In summary, the data presented in this report indicate that CL387626 has significant selective antiviral activity against RSV in vitro and in vivo. This activity is most pronounced and reproducible when administered prior to virus challenge. The compound's prolonged protective activity following topical delivery makes further studies with this compound, or related compounds, of great interest and a priority. Efforts are now being directed to compounds related to CL387626 to determine if they have equivalent or better prophylactic activity than the parent compound, and optimistically, have more consistent antiviral activity than CL387626 when administered therapeutically.

Acknowledgements

This work was supported by the Virology Branch, National Institute of Allergy and Infectious Disease and National Institutes of Health contract NO1-AI-65292.

References

Bradley, J.S., Conner, J.D., Campogiannis, L.M., 1990. Exposure of health care workers to ribavirin during therapy for respiratory syncytial virus infection. Antimicrob.Agents Chemother. 34, 68–70.

Chanock, R.M., McIntosh, K., 1990. Parainfluenza viruses. In: Fields, B.N. (Ed.), Virology. Raven Press, New York, pp. 963–988.

Committee on Infectious Diseases, 1993. Use of ribavirin in the treatment of respiratory syncytial virus in infection. Pediatrics 92, 501–504.

Dowell, S.F., Anderson, L.J., Gary, H.E. Jr., Erdman, D.D., Plouffe, J.F., File, T.M. Jr., Marston, B.J., Breiman, R.F., 1996. Respiratory syncytial virus is an important cause of community-acquired lower respiratory infection among hospitalized adults. J. Infect. Dis. 174, 456–462.

Glezen, W.P., Loda, F.A., Denny, F.W., 1982. The parainfluenza viruses. In: Evans, A.S. (Ed.), Viral Infections of Humans: Epidemiology and Control. Plenum Press, New York, pp. 337–349.

Johnson, S., Oliver, C., Prince, G.A., Hemming, V.G., Pfarr, D.S., Wang, S.C., Dormitzer, M., O'Grady, J., Koenig, S., Tamura, J.K., Woods, R., Bansal, G., Couchenour, D., Tsao, E., Hall, W.C., Young, J.F., 1997. Development of a humanized monoclonal antibody (MEDI-493) with potent in vitro and in vivo activity against respiratory syncytial virus. J. Infect. Dis. 176, 1215–1224.

La Via, W.V., Marks, M.I., Stutman, H.R., 1992. Respiratory syncytial virus puzzle: clinical features, pathophysiology, treatment and prevention. J. Pediatr. 121, 503–510.

Parrott, R.H., Kim, H.W., Arrobio, J.O., Hodes, D.S., Murphy, B.R., Brandt, C.D., Camargo, E., Chanock, R.M., 1973. Epidemiology of respiratory syncytial virus infection in Washington, DC. Am. J. Epidemiol. 98, 289–300.

Piedra, P.A., Wyde, P.R., Castleman, W.L., Ambrose, M.W., Jewell, A.M., Speilman, D.J., Hildreth, S.W., 1993. Enhanced pulmonary pathology associated with the use of formalin-inactivated respiratory syncytial virus vaccine in cotton rats is not a unique viral phenomenon. Vaccine 11, 1415–1423.

Prince, G.A., Porter, D.D., 1996. Treatment of parainfluenza virus type 3 bronchiolitis and pneumonia in a cotton rat model using topical antibody and glucocorticosteroid. J. Infect. Dis. 173, 598–608.

Rhodes, A.J., Van Rooyen, C.E., 1953. Textbook of Virology, 2nd ed. Williams and Wilkins, Baltimore, MD, pp. 66–69.

Wilson, S.Z., Knight, V., Wyde, P.R., Drake, S., Couch, R.B., 1980. Amantadine and ribavirin aerosol treatment of influenza A and B infection in mice. Antimicrob. Agents Chemother. 17, 642–648.

Wyde, P.R., Wilson, S.Z., Kramer, M.J., Sun, C.-S., Knight, V., 1984. Duration of effect of interferon aerosol prophylaxis of vesicular stomatitis virus infection in mice. Antimicrob. Agents Chemother. 27, 60–64.

Wyde, P.R., Meyerson, L.R., Gilbert, B.E., 1993. An in vitro evaluation of the antiviral activity of SP-303, an euphorbiaceae shrub extract, against a panel of respiratory virus. Drug Dev. Res. 28, 467–472.

Wyde, P.R., Moore, D.K., Hepburn, T., Silverman, C.L., Porter, T.G., Gross, M., Taylor, G., Demuth, S.G., Dillon, S.B., 1995. Evaluation of the protective efficacy of reshaped human monoclonal antibody RSHZ19 against respiratory syncytial virus in cotton rats. Pediatr. Res. 38, 543–550.