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Antiviral efficacy of VP14637 against respiratory syncytial virus in vitro and in cotton rats following delivery by small droplet aerosol

Philip R. Wyde [a,*], Sylvie Laquerre [b], Srikrishna N. Chetty [a], Brian E. Gilbert [a], Theodore J. Nitz [b,c], Daniel C. Pevear [b,d]

[a] *Department of Molecular Virology and Microbiology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA*
[b] *ViroPharma Incorporated, 397 Eagleview Boulevard, Exton, PA 19341, USA*
[c] *Vitex Technologies, 209 Perry Parkway, Gaithersburg, MD 20877, USA*
[d] *Progenics Pharmaceuticals Inc., 777 Old Saw Mill River Road, Tarrytown, NY 10591, USA*

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Abstract

VP14637, the lead compound in a series of substituted bis-tetrazole-benzhydrylphenols developed by ViroPharma Incorporated, was evaluated for antiviral efficacy against respiratory syncytial virus (RSV) in vitro in cell culture and in vivo in cotton rats. A selective index of >3000 (≥2000 times greater than that observed for ribavirin) was determined in the in vitro studies for this compound against both RSV A and B subtypes. In cotton rats, animals given as little as 126 μg drug/kg by small droplet aerosol in divided doses starting 1 day after experimental virus infection with either a RSV A or B subtype consistently had significantly lower mean pulmonary RSV titers and reduced histopathological findings than mock-treated animals or cotton rats given placebo (vehicle-treated animals). No cotton rat treated with aerosols of VP14637 during these studies manifested any evident untoward responses. Thus, VP14637 exhibited good selective antiviral efficacy both in vitro and in vivo.

Keywords: RSV; Antiviral; Cotton rats; Respiratory syncytial virus; VP14637; Aerosol delivery

1. Introduction

Respiratory syncytial virus (RSV) is a widely occurring human pathogen, but is especially perilous to infants and children under 2 years of age (Hall and McCarthy, 2000), the elderly (Agius et al., 1990; Fleming and Cross, 1993; Falsey et al., 1995), certain immunosuppressed populations (Couch et al., 1997), individuals with cystic fibrosis (Abman et al., 1988; Armstrong et al., 1998) and those with chronic pulmonary or cardiac illnesses (Han et al., 1999). No vaccines are currently licensed for preventing RSV infections. However, RSV-neutralizing polyclonal immunoglobulin (IG) preparations (e.g., RSV-IVIG [RespiGam™, MedImmune Inc.]), and humanized monoclonal antibody, palivizumab [Synagis™, MedImmune Inc.]), are licensed in the U.S. for prophylactic treatment to prevent RSV infections in high-risk infant populations. Although shown to be efficacious, the cost/benefit ratios of both of these biologics limit their use (Thakur et al., 1997; Barton et al., 2001; Hashmi et al., 2000; Lofland et al., 2000). Ribavirin (Virazole®, Valeant Pharmaceuticals), the only small molecule drug licensed to treat RSV infections, is expensive and has mutagenic potential (Marquardt, 1995; Crotty et al., 2000, 2002). Thus, its use is limited to high risk or severely ill infants (Committee on Infectious Diseases, 1993). The problems associated with these materials and the continuing medical impact of RSV have spurred efforts to identify safer, more efficacious and less expensive agents to prevent or treat infections caused by this virus.

This report summarizes results of studies performed in tissue culture cells and in cotton rats evaluating VP14637, a small molecule entity that has been reported to prevent RSV replication through inhibition of the viral fusion (F) protein

* Corresponding author. Tel.: +1 713 798 5255; fax: +1 713 798 6802.
E-mail address: pwyde@bcm.tmc.edu (P.R. Wyde).

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function (Pevear et al., 2000b; Douglas et al., 2003). VP14637 was found to significantly inhibit different RSV strains and subtypes both in vitro and in vivo. In cotton rats, delivery in divided doses (b.i.d.) of as little as 126 μg VP14637/kg administrated by small droplet aerosol (SDA) for relatively short periods of time (e.g., two 60-min exposures), consistently and significantly reduced pulmonary virus titers and virus-induced histopathology compared to infected but untreated animals.

2. Materials and methods

2.1. Animals

Fifty to 100 g cotton rats (Sigmoden hispidis) of either sex were used in all in vivo studies. Each of these animals was descended from six 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 (NIH). They were housed in the Baylor College of Medicine (BCM) vivarium in cages covered with barrier filters and given food and water ad libitum. All blood samples obtained from representative animals during the course of these experiments were seronegative for adventitious viruses and other rodent pathogens. The experiments performed for these studies were carried out utilizing NIH and United States Department of Agriculture guidelines and experimental protocols approved by the BCM Investigational Animal Care and Use Committee (IACUC).

2.2. Tissue culture

All of the tissue culture cell lines used in these studies were started from seed vials obtained from the American Type Culture Collection (ATCC), Rockville, MD. HEp-2 (human epithelial carcinoma; ATCC CCL23) cells were used to grow and titrate RSV, parainfluenza virus type 3 (PIV3) and Coxsackie B3 virus. Vero (epithelial African green monkey kidney, ATCC CCl-81) were used to perform specificity testing for measles, mumps and Herpes simplex viruses, while MA104 (epithelial African green monkey kidney, ATCC CRL-2378.1), and MRC5 (human fibroblast, CRL-171) were used for rotavirus and cytomegalovirus (CMV) testing, respectively. Proliferating HEp2, A549 (human lung carcinoma; ATCC CCL185), Madin Darby canine kidney (MDCK; ATCC CCL34), BSC-40 (epithelial African green monkey, ATCC CRL-2761) and KB (human oral epidermoid carcinoma; ATCC CCL17), were used to determine drug cytotoxicity. Eagle's minimal essential medium (MEM; Sigma Chemical Co., St. Louis; Catalogue no. M4465) supplemented with 2 or 10% fetal bovine serum (FBS; Summit Biotechnology, Fort Collins, CO; Catalogue no. FP-200-05), 100 U/ml penicillin (Sigma; Catalogue no. P-4458), 100 μg/ml gentamicin sulfate (Sigma; Catalogue no. G-1264), 2 mM L-glutamine (Whittaker Bioproducts Inc.; Catalogue no. 17–605A) and 0.2% sodium bicarbonate (Sigma; Catalogue no. S8761) was used to grow and maintain these cells.

2.3. Viruses

Seed vials of RSV strains Long (ATCC VR-26) and B Washington/18537/62 (ATCC VR-1401; [CH 18537]) were purchased from the ATCC. RSV Bernett, RSV 37593 and parainfluenza virus type 3 (PIV3) were clinical isolates that were acquired from the Respiratory Pathogens Research Unit, Department of Molecular Virology and Microbiology, BCM. Herpes simplex virus type 1 (HSV-1; strain KOS, VR-1493), CMV (strain AD-169; VR-538) and rotavirus (strain WA, VR-2018) were also purchased from ATCC. Subtyping of the different RSV strains was performed by Dr. Larry Anderson (Centers for Disease Control and Prevention, Atlanta, GA), or in-house, using the reverse transcriptase-polymerase chain reaction (RT-PCR) assay and synthetic oligonucleotides described by Gottschalk et al. (1996). All of the RSV strains used in these studies were identified as being subtype A except for the Washington/18537/62 virus. Working stocks of each virus were prepared by infecting flasks of HEp-2 cells as described previously (Wyde et al., 1995).

2.4. Virus quantification

Virus titers of cell-culture grown stocks and lung lavage fluids (LF) were determined by serially diluting each sample three-fold in 96-well tissue culture plates (Falcon 3072) as described previously (Wyde et al., 1995). The monolayers in the wells of these plates were observed daily and scored for virus-induced cytopathic effects (CPE; predominantly syncytium formation). On day 7 of incubation, the last well in each replicate row exhibiting virus-induced CPE was determined. This information and the interpolation method of Karber (Rhodes and Van Rooyen, 1953) were used to estimate the amount of virus present in each sample. Titers for virus stocks were expressed as median tissue culture infectious doses ($TCID_{50}$ log_{10})/ml of media and those from LF as $TCID_{50}$ log_{10}/g of lung. In these assays, the minimum detectable virus concentration for virus stocks was 1.8 $TCID_{50}$ log_{10}/ml and for LF it was 2.3 $TCID_{50}$ log_{10}/g lung.

2.5. Compounds

For in vitro testing, VP14637 was suspended in 0.5% dimethyl sulfoxide (DMSO; Sigma Chemical Co.; Catalogue no. D 5879). For in vivo experiments, ViroPharma supplied VP14637 as a 0.2 mg/mL solution in solubilization vehicle (85% ethanol, 10% propylene glycol, 5% water). Solubilized drug and matching vehicle control were stored at room temperature and utilized within 2 days of receipt. The chemical structure and M.W. (i.e., 510.52) of VP14637 (empirical formula $C_{25}H_{22}N_{10}O_3$) are displayed in Fig. 1.

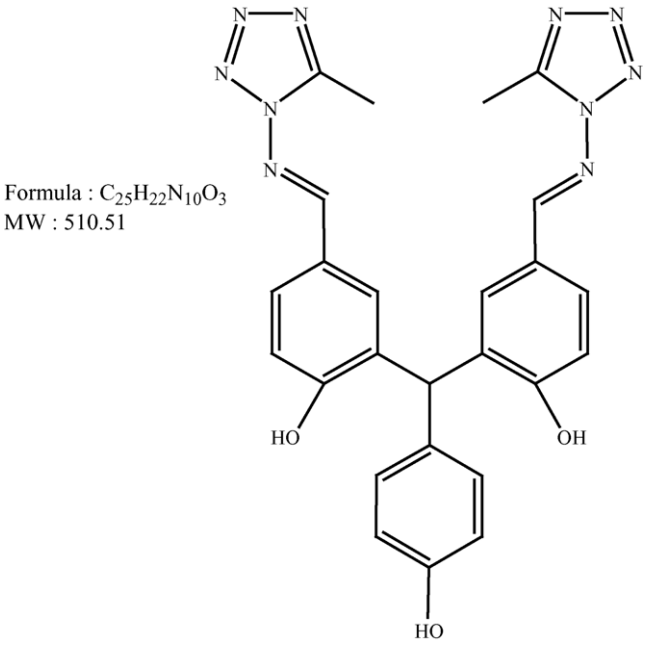

Fig. 1. Structure and molecular weight of VP14637.

Ribavirin (ICN Biochemicals Inc., Costa Mesa, CA; Catalogue no. 196966) was used as a positive control in many experiments. On the morning of an experiment when this compound was included, the amount of ribavirin needed was suspended in sterile distilled water and then filter sterilized using a 0.2 μm DynaGard filter (Spectrum Laboratories, Rancho Dominguez, CA; Catalogue no. DG2M-30-50S).

2.6. In vitro cytotoxicity assay

The cytotoxicity of VP14637 and ribavirin in cell culture was determined in an assay utilizing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction as an endpoint, as described previously (Wyde et al., 1993). After the optical density (570 nM wavelength) in each well was determined, the 50% cytotoxic drug concentration (CC_{50}) of the test compound was calculated by estimating the lowest concentration of compound that resulted in 50% cell growth inhibition compared to that of a matching, no-drug control (Wyde et al., 1993).

2.7. Virus cytopathic effect assay for EC_{50} determination

The susceptibility of RSV to VP14637 and ribavirin were determined in a cell culture assay that measured the prevention of virus-induced CPE by the test drugs. This assay has been described in detail previously (Wyde et al., 1993). In these assays, virus concentrations that were shown in separate experiments to result in 90% destruction of the cell monolayer (CPE assay) after 5 days were added to the appropriate wells and the plates were incubated for 5 days at 36 °C in a humidified, 5% CO_2 atmosphere. The cell monolayers in each well were observed daily and any well exhibiting drug-induced cytotoxicity or viral CPE were recorded. (Because of the conspicuous syncytia induced by RSV, differentiation of the two was readily possible.) On day 5, the monolayers in these plates were fixed with 5% glutaraldehyde and stained with 0.1% crystal violet. After rinsing and drying, the O.D. of the medium in each well was determined using a 570 nm wavelength and a Bio-Tek 300 plate reader. The 50% effective concentration (EC_{50}) was calculated after determining the concentration of compound that protected 50% of the cell monolayer from virus-induced CPE (Wyde et al., 1993).

2.8. Specificity assays

The viral specificity of the anti-viral activity of VP14637 was tested in vitro using multiple RNA and DNA viruses and different tissue culture cell lines (see cell lines and viruses above). In this testing, VP14637 was tested at a concentration range of 50–0.05 uM. All assays measured protection from virus-induced CPE with the exception of the cytomegalovirus-specificity assay, which utilized enzyme linked immunosorbant assay (ELISA) readouts.

2.9. Antiviral activity in cotton rats

In in vivo experiments, cotton rats were lightly anesthetized with Isoflurane (Abbot Laboratories, North Chicago, IL), weighed and then inoculated intranasally (i.n.) with approximately 100 median cotton rat infectious doses ($CRID_{50}$; approximately 10^4 $TCID_{50}$) of RSV in 0.1 ml. Drug administration schedules varied. In some instances the initial aerosol was started within 1 h of virus inoculation and in other experiments it was started 1 or 2 days post virus inoculation (p.i.). When the animals were exposed to more than one treatment per day, the aerosols were given at least 4 h apart. The duration of the drug treatment ranged from 5 to 60 min long. All aerosols were delivered as described previously (Wilson et al., 1980; Knight and Gilbert, 1988), except that an Aero-Mist nebulizer (CIS-US Inc., Bedford, MA; Catalogue no. CA-209) was used to generate them. Virus-infected animals treated with vehicle only were generally used as negative controls in these experiments. However, because lung titers in animals treated with vehicle alone were indistinguishable from those in mock-treated, RSV-infected cotton rats (data not shown), in some experiments virus-infected untreated animals were used as negative controls. Vehicle was always added to the nebulizer delivery reservoir (DR) undiluted and VP14637 at a maximum concentration of 0.2 mg drug/ml of undiluted vehicle.

In all in vivo experiments, the test animals were sacrificed on day 4 p.i., at a time when maximum RSV pulmonary titers are usually observed in untreated or mock-infected animals given this inoculum. The lungs of the sacrificed animals were removed, rinsed in sterile water and weighed. Each set of lungs was then transpleurally lavaged using 3 ml of 2% FBS-MEM as described in detail previously (Numa, 2000).

2.10. Histologic studies for evidence of antiviral efficacy

In experiments designed to include histologic examinations, the lungs were collected to determine virus levels as described above. However, the lower right lobe from each set of lungs was removed for histologic processing. These were inflated with Histochoice fixative (Amresco, Solon, OH), pooled according to their treatment and then submerged in this fixative in 50 ml centrifuge tubes (Corning; Catalogue no. 430921) for at least 24 h. At the end of this time period, the tissues were dehydrated, embedded in paraffin and sectioned at 5 μm thickness. These sections were then rehydrated, stained with hematoxylin and eosin (H&E) and viewed in a blinded fashion using the 10× and 40× objectives on a Bauch and Lomb light microscope. Using the latter lens, 10 consecutive microscopic fields on each section were observed using an up, over, and down pattern. Perivascular and peribronchial infiltrates, septal thickening and the presence of inflammatory cells in the aveoli were the primary variables assessed. For each of the four histopathologic characteristics looked for, 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. Each of the values was recorded and these were then used to obtain a total score for each lung section and an average total score for the lung sections prepared from animals in the same treatment group.

2.11. Toxicity studies in cotton rats

Toxicity studies in cotton rats were limited to observing treated animals daily for unusual behavior (i.e., morbidity, mortality, diarrhea, irritability); comparing weight changes in the animals in the different groups; and observing H&E-stained lung sections from uninfected and virus-infected animals that were exposed to SDA of VP14637 or vehicle for histopathological changes. For these studies, the lungs were removed, rinsed, placed in Histochoice tissue fixative overnight and then processed for histologic study as described above.

2.12. Pharmacokinetics and drug carry over experiments

Because of the very high potency exhibited by VP14637 in in vitro studies, it was deemed necessary to ensure that any decreased pulmonary virus titers measured in LF in the virus CPE-based assays were not due to residual drug present in the lungs of treated animals at the time that they were sacrificed. Such "carry over" of drug into the assay plates being used to measure pulmonary virus titers in drug-treated animals could inhibit growth of any virus present in the LF collected at this time in the assay plates and make the treatments appear more efficacious than they really were. To determine if this was occurring, several experiments were performed in which 15 cotton rats were exposed to a 60-min aerosol of VP14637 (nebulizer reservoir concentration of 0.2 mg/ml). Three of these animals were sacrificed 5 min after ceasing the aerosolization and again at 2, 4, 24 and 48 h post aerosolization (Table 2). As a control, three cotton rats that were not exposed to any aerosol also were sacrificed at the 5 min interval. The lungs of each of these animals were removed and lavaged. Serial dilutions of the resulting LF were tested for antiviral activity against RSV in 96-well tissue culture plates as described above. Using this experimental design, it was possible to detect drug carry over, measure biologically active drug deposited in the lungs of animals during treatment and to assess the biological half life of active drug in the lungs following aerosol delivery.

2.13. Statistics

The Mann-Whitney nonparametric test was utilized when statistically comparing just two mean geometric virus titers for significance. The Kruskall–Wallis nonparametric analysis of variance (ANOVA) was used to make statistical comparisons of mean geometric virus titers when more than two groups of animals were in a study. Body weights were compared using a parametric ANOVA. Regardless of the test utilized, all comparisons were made to the mean value obtained for either the untreated or placebo control group in that experiment. In addition, all testing was two-tailed and LF with undetectable virus titers were assigned a value of 1.8 $TCID_{50}$ log_{10}/g lung. Instat, a statistical program designed for IBM compatible computers (version 3, Graphpad Software Inc., San Diego, CA) was used to carry out these analyses as well as to obtain all descriptive statistics (e.g., means, geometric mean virus titers and standard deviations).

3. Results

3.1. In vitro antiviral activity of VP14637 and ribavirin

An EC_{50} value of 0.001 ± 0.004 μM was obtained for VP14637 against RSV (Long strain) in the virus CPE-based, EC_{50} assay (Fig. 2; Table 1). By comparison, the EC_{50} obtained for ribavirin against RSV Long in this assay was about 20,000-fold greater, i.e., 20 ± 10 μM (data not shown in table). The antiviral activity of VP14637 was not cell type- or virus subtype-specific since similar inhibition was observed against all of the RSV strains tested in HEp2 (Table 1) and in BSC-40 cells (these data are not shown), including RSV 18573, a subtype B RSV. However, VP14637 was a specific inhibitor of pneumovirus replication. As shown in Table 1, in these assays, the compound did not effectively inhibit the replication of influenza or non-pneumovirus paramyxoviruses (i.e., PIV3, measles virus or mumps virus), even at drug concentrations up to 3 μM. It also did not inhibit Coxsackie B3, HSV-1, rota or CMV (data not shown).

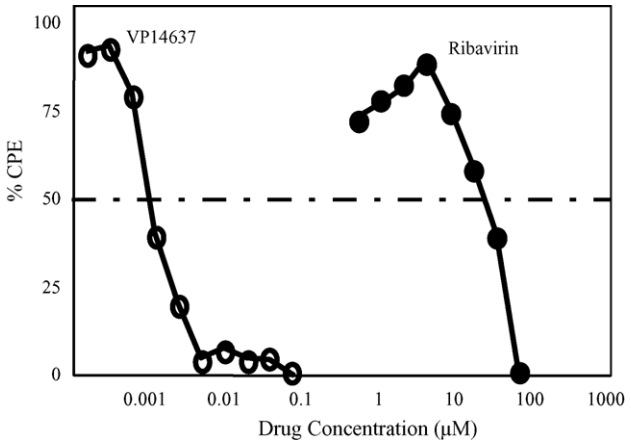

Fig. 2. In vitro anti-viral activity of VP14637 and ribavirin. Duplicate dilutions of VP14637 from 0.1 to 0.000032 μM and of ribavirin from 50 to 0.016 μM were added to HEp-2 cells previously plated in a 96-well plate. Cells were infected at a multiplicity of infection to produce 90% CPE after 5 days. Plates were incubated at 36 °C for 5 days before being fixed and stained with crystal violet. Percent inhibition by drug was compared to uninfected cells. The dotted line marks the median efficacious concentration (EC_{50}) for each compound.

3.2. In vitro cytotoxicity assay

Based on the daily microscopic observation of the cellular monolayers in the EC_{50} plates and the results obtained in the cytotoxicity assays using reduction of MTT as an indicator (Table 1), VP14637 was not cytotoxic to proliferating Hep2, KB, MDCK, BSC-40, Vero, MRC5 or A549 tissue culture cells at compound concentrations up to the limit of solubility of the compound in culture medium (i.e., 3 μM). In contrast, the CC_{50} determined for ribavirin in these cells was about 26 μM (data not shown). Thus, the selective index (SI: CC_{50}/EC_{50}) for VP14637 was >3000 while that of ribavirin was 1.3 (*a* >2000-fold difference).

Table 1
Comparison of the cytotoxicity and antiviral efficacy of VP14637 in different tissues and against different viruses[a]

Virus	CC_{50} (μM)[b]	EC_{50} (μM)	S.I.
RSV Long[c]	>3	0.001 ± 0.004	≥3000
RSV Bernett	>3	0.001 ± 0.006	≥3000
RSV 18537	>3	0.001 ± 0.004	≥3000
RSV 37593	>3	0.001 ± 0.006	≥3000
Parainfluenza type 3 virus	>3	>3	1
Measles virus	>3	>3	1
Mumps virus	>3	>3	1
Influenza A virus	>3	>3	1
Coxsackie B3 virus	>3	>3	1
HSV-1 (KOS)	>3	>3	1
Rotavirus (WA)	>3	>3	1
Cytomegalovirus (Ad169)	>3	>3	1

[a] CC_{50} and EC_{50} values were determined as described in Section 2. Shown are the mean values obtained in two replicate experiments (all standard deviations not shown are 0). Each selective index (S.I.) was calculated by dividing the mean CC_{50} by its respective mean EC_{50}. For comparison, the CC_{50} determined for ribavirin in HEp2 cells in this testing was 26 μM, while its mean EC_{50} value against respiratory syncytial virus (RSV) was 20 ± 10 μM (S.I. = 1.3). Ribavirin was similarly toxic in all of the cell lines, except MDCK.

[b] The antiviral efficacy of VP14637 against the respiratory syncytial virus (RSV) strains, parainfluenza type 3 virus and Coxsackie B3 virus was tested using HEp2 cells, while antiviral testing against measles, mumps and Herpes simplex type 1 (HSV-1) viruses was performed in Vero cells. MA104 and MRC5 cells were used for evaluating the antiviral activity of VP14637 against rotavirus and cytomegalovirus, respectively.

[c] RSV Long, Bernett and 37593 are subtype A RSV strains; RSV 18537 is a subtype B RSV strains.

3.3. Results of "carry over" experiments

The results of two "carry over" experiments are shown in Table 2. As the data indicate, no apparent reduction in RSV-induced CPE, and thus virus replication, was present in any well containing LF obtained from lungs taken from the untreated animals, compared to the degree of CPE seen in control wells containing medium and virus. Similarly, in both experiments, no reduction in virus-induced CPE was evident in any wells containing LF obtained from treated cotton rats 24 or 48 h after ceasing drug aerosolization. In contrast, conspicuous inhibition of CPE (i.e., >20% reduction compared to that seen in the virus-control wells) was evident in these experiments in wells containing dilutions of LF as great as 1:256 if the lungs were collected 5 min after aerosol treatment, in wells containing ≤1:64 dilution of LF if the lungs were taken 2 h post treatment, or in wells with less than or equal to a 1:16 dilution of LF recovered from lungs 4 h after stopping treatment. A similar experiment was performed in which LF were obtained from animals exposed to two 60-min exposures of VP14637 4 h apart. The results of this experiment also resembled those displayed in Table 2 (data not shown).

3.4. Antiviral activity of VP14637 against RSV A subtypes in cotton rats

As indicated by the representative data presented in Fig. 3, VP14637 was administered to RSV-infected cotton rats (four to six animals/group) by SDA using varying administration schedules. In these experiments, if two aerosols of drug were administered on day 0, they were done at one and 4 h p.i. In addition, animals treated more than once a day were exposed to drug aerosol administered once in the morning and once in the afternoon with a minimum of 4 h between treatments. In each experiment, reductions in virus titer in lungs of infected animals following drug treatment were compared with that of vehicle-treated animals. In these studies, the maximum virus replication observed in the lung of animals at 4 days p.i. was about 4.6 $TCID_{50}$ log_{10}/g lung. Since the lower limit of virus detection in lung of infected animals was 2.3 $TCID_{50}$ log_{10}/g lung, the theoretical maximum reduction in virus titer in these tests was 2.3 $TCID_{50}$ log_{10}/g lung.

As the data in Fig. 3 indicate, VP14637 demonstrated marked antiviral effects in the lungs of RSV-infected cotton rats and this effect appeared to follow a dose response pattern. For example, cotton rats that received only one 60-min treat-

Table 2
Biologic assessment of drug levels in lung lavage fluids collected from cotton rats at different time intervals after animals were exposed to a 60-min small droplet aerosol of VP14637[a]

Treatment	Time lung lavage fluids collected post exposure	Highest dilution of lung lavage fluid that inhibited virus replication in	
		Experiment 1[b]	Experiment 2[b]
None	5 min	<1:2/<1:2[c]	<1:2/<1:2[c]
VP14637	5 min	1:128/1:128	1:256/1:128
VP14637	2 h	1:64/1/64	1:64/1:64
VP14637	4 h	1:32/1:32	1:32/1:16
VP14637	24 h	<1:2/<1:2[c]	<1:2/<1:2[c]
VP14637	48 h	<1:2/<1:2[c]	<1:2/<1:2[c]

[a] In each experiment, 15 uninfected cotton rats (CR) were exposed for 60 min to a small droplet aerosol of VP14637 generated from a nebulizer reservoir containing 0.2 mg of this compound/ml. Three of these CR and three unexposed control animals were sacrificed 5 min after ceasing the aerosolization. Three more animals from the group exposed to the 60 min aerosol of VP14637 were sacrificed 2, 4, 24 and 48 h later. At each interval, the lungs of each animal were collected and lavaged. The resulting lung lavage fluids (LF) were serially diluted in duplicate using two-fold dilutions and then transferred to wells of a 96-well tissue culture plate containing HEp2 tissue culture cells. Approximately, 10 $TCID_{50}$ of respiratory syncytial virus (RSV) was then added to each well containing LF and to six virus control wells containing HEp2 cells and medium. The test plate was incubated at 36 °C for 7 days. At that time, the percent of each cell monolayer exhibiting virus-induced cytopathic effects (predominantly syncytia formation) was estimated.

[b] Shown is the final dilution of LF in each replicate well that had at least 20% less CPE than was evident in the control wells containing medium and virus; results in replicate wells are separated by a slash bar.

[c] At a 1:2 final dilution, no difference in virus-induced CPE was seen in these wells compared to the CPE seen in the control wells containing medium and virus.

ment/day (Fig. 3, experiments #1 and #2), had consistently higher levels of RSV in their lungs on day 4 than animals exposed twice daily to aerosols of VP14637 for the same length of time (experiments #3 and #7, respectively). Similarly, an additional treatment of 30 min on day 0 (experiment #6) to that of two treatments of 30 min on day 1 (experiment #5) lead to less RSV being measured in the lungs of animals on day 4 p.i. Of these treatment groups, only those given VP14637 twice for 60 min on day 1 had a statistically different mean pulmonary virus titer than the mean titer determined for their respective placebo control group ($p \leq 0.01$ for this group following comparison using a nonparametric ANOVA).

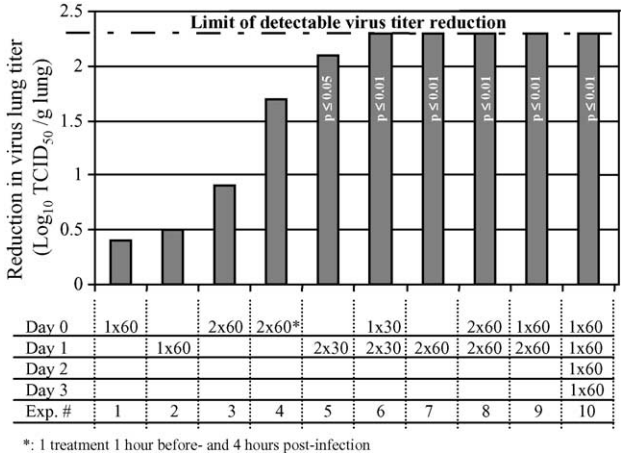

Fig. 3. Representative results obtained testing VP14637 for efficacy against respiratory syncytial virus in cotton rats using different administration schedules. Cotton rats were lightly anesthetized and inoculated intranasally with approximately 100 median cotton rat infectious doses ($CRID_{50}$) of RSV Long in 0.1 ml on day 0. Vehicle or VP14637 (0.2 mg/ml in vehicle) were placed in the nebulizer reservoir and delivered by small droplet aerosol for 30 or 60 min on the day or days indicated. For all experiments (except experiment #4, which was performed as indicated), the single or first exposure to drug on day 0 was started 1 h post virus inoculation (p.i.), while the second drug exposure, if given, was initiated 4 h p.i. For days 1, 2 or 3 p.i., the first exposure was performed in the morning and the second, if given, was started a minimum of 4 h after stopping the first treatment. All of the animals were sacrificed and their lungs tested for virus on day 4 after virus inoculation as described in Section 2. All *p*-values shown were obtained by comparing the pulmonary virus titers obtained for the animal in the test group displayed with those determined for the cotton rat in the placebo control group used in that experiment. A nonparametric analysis of variance was used to do this.

The time that treatment was administered to the animals relative to infection also appeared to have an impact on antiviral activity. For example, 2× 60-min administrations of VP14637 on day 0 at 1 and 4 h p.i. (experiment #3) did not result in a significant reduction in RSV lung titer. However, if the identical treatment regimen was delayed for 24 h (experiment #7), no virus was detectable on day 4 ($p \leq 0.01$). In addition, 2× 30-min administrations on day 1 resulted in more than a 2 $\log_{10}$ reduction in virus titer (experiment #5; $p \leq 0.05$), which was a greater virus titer reduction than a longer treatment (2× 60-min) on day 0 (experiment #3; $p > 0.05$). The results indicated that VP14637's antiviral activity in a therapeutic dosing regimen is most pronounced when treatment is delayed for about 24 h after virus infection.

The minimal drug exposure by SDA to reach maximal reduction in titer was determined in a series of experiments, e.g., experiments #7, #8, #9 and #10 in Fig. 3. As the representative data in this figure indicates, while two 30 min treatments administered only on day +1 consistently significantly reduced mean pulmonary virus titers (in experiment #5, the mean reduction = 2.1 $\log_{10}$/g lung and $p \leq 0.05$ when compared to the mean pulmonary virus titer seen in the animals given vehicle only using the Kruskall–Wallis nonparametric analysis of variance), greater reductions in mean virus titers were generally seen in animals given two treatments of 60 min on day 1 p.i. (e.g., experiment #7 where the

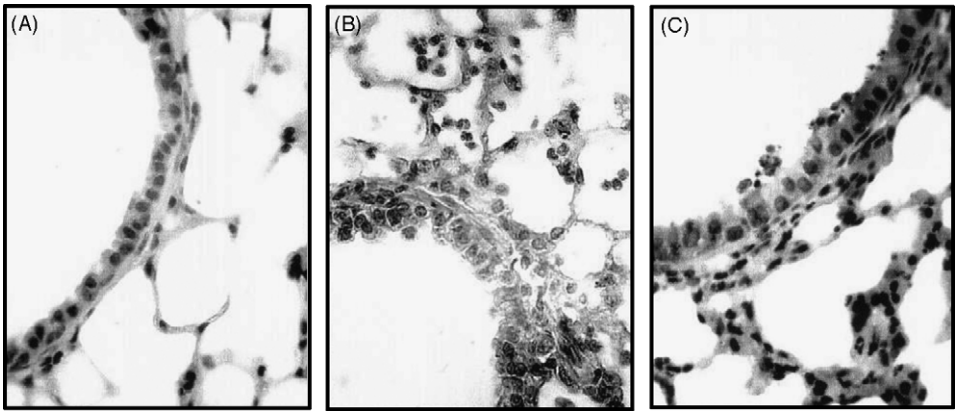

Fig. 4. Hematoxylin and eosin stained lungs from some of the cotton rats used in these studies. Shown are hematoxylin and eosin stained sections of lung from an untreated, uninfected cotton rat (panel A); from an untreated animal 4 days after infection with respiratory syncytial virus (RSV; panel B); and from the lungs of a cotton rat sacrificed 4 days after being experimentally inoculated with RSV and then treated twice for 60 min on day 1 post virus inoculation with VP14637 at 0.2 mg/ml (panel C).

mean reduction in pulmonary virus titer was $\geq$2.3 $\log_{10}$/g and p was $\leq$0.01).

3.5. *In vivo activity of VP14637 versus RSV subtype B*

Two experiments were performed to assess the capability of VP14637 to inhibit RSV subtype B (Washington/18537/'62) replication in lungs of cotton rats. In these experiments, maximal reduction in virus titer (2.3 $TCID_{50}$ $\log_{10}$/g lung) was observed in both experiments in which drug was given twice for 1 h on day 1 ($p \leq 0.05$, data not shown). Thus, in vivo, as in vitro, VP14637 appeared to equivalently inhibit replication of RSV subtypes A and B.

3.6. *Histologic findings*

The effect of dosing with VP14637 under efficacious regimens on RSV-induced cotton rat lung pathology was examined by observing H&E stained tissue sections from these animals (Fig. 4). As shown in Fig. 4 (panel A), stained lung sections from uninfected, untreated animals exhibited little histopathology. Typically the bronchi and bronchioles in these sections were lined with intact tall columnar epithelial cells and few free cells (e.g., lymphocytes or polymorphonuclear neutrophils [PMN]) were seen in or around them or the alveoli, except for an occasional eosinophil, mast cell or pulmonary macrophage. Moreover, no evidence of edema or abnormal pulmonary architecture (e.g., thickening of septae) was observed in any of the lung sections from these animals. Interestingly, no significant histopathological changes were evident in the sections of lung obtained from cotton rats exposed for 60 min twice daily to the alcohol-based vehicle. Indeed, the histopathology scores for lungs taken from these animals ranged from 1 to 3, the same as the scores determined for sections of lung taken from uninfected, untreated animals (data not shown). In contrast, stained sections of lung from untreated, RSV infected cotton rats (Fig. 4, panel B) contained numerous inflammatory cells (e.g., lymphocytes, macrophages and PMN) singly and in foci scattered throughout the lumen of alveoli, both within and adjacent to many bronchi and bronchioles. In many instances, portions of the tall columnar cells lining the lumen of the bronchi or bronchioles were disrupted and scattered patches of edema were evident. Lung sections prepared from animals infected with RSV and treated with VP14637 exhibited fewer foci of inflammatory cells, less edema and thickened septa and fewer bronchioles or bronchi with evident disruption of the tall columnar cells lining them (Fig. 4, panel C). The degree of histopathology (based on histopathology scores) was generally inversely proportional to the virus titers measured in the animals. For example, lungs from virus-inoculated animals treated only with placebo almost always had the highest mean virus titers ranging from 3.5 to 5.0 $\log_{10}$/g lung and mean histopathologic score ranging between 9 and 12; in contrast, virus was never detected in lungs taken from cotton rats exposed twice on day +1 for 60 min and their histopathologic score ranged from 2 to 4; the scores obtained for lung sections from animals with intermediate virus titers (e.g., the animals from experiments #3 and #4 depicted in Fig. 3) generally ranged from 5 to 9 (data not shown).

3.7. *Preliminary cytotoxicity testing of VP14637 in cotton rats*

No diarrhea, weight loss, evident irritability, morbidity, death or unusual behavior was seen in any animal treated with VP14637 by SDA during the course of these experiments. Moreover, there were no significant differences seen in H&E stained sections of lungs processed on day 4 from uninfected animals exposed to four, 60-min treatments of VP14637 (two treatments/day for 2 successive days) and those processed from untreated, uninfected animals (data not shown).

4. Discussion

VP14637 is a lead drug candidate that emerged from high-throughput screening of a random small molecule chemical library against whole RSV replication in Hep2 cells. The high in vitro selective indices (SI = 3000) obtained for VP14637 in these studies and others (McKimm-Breschkin, 2000; Pevear et al., 2000a) was reflected in the in vivo (cotton rat) studies reported herein where significant anti-RSV activity and low toxicity were observed. Reductions in mean pulmonary virus titer of greater than 2.3 $TCID_{50}$ log_{10}/g lung compared to titers in untreated or placebo control groups frequently occurred in groups of cotton rats exposed to two or more 60 min treatments, in particular if the test animals were treated on day 1 (see Fig. 3, experiment #7). Interestingly, similar treatment on day 0 (Fig. 3, experiment #3) did not reduce significantly (only 0.8 $TCID_{50}$ log_{10}/g lung) virus titer. The reason(s) why treatment administered only on day 0 did not provide significant protection is not clear, especially since there was likely to be much less virus present in the lungs of inoculated animals on this day than on day 1 by which time the initial inoculum of 10^4 $TCID_{50}$ had time to replicate and increase in titer. One possibility is that any virus in the inoculum that successfully infected host cells after the first aerosol was likely to be in a replicative phase in which the fusion (F) protein of RSV does not play an important role (e.g., eclipse, transcription or translation) and thus not susceptible to inhibition by the VP14637 administered during the second aerosolization. Then if no more drug, or insufficient drug, was administered, this sequestered virus had 3 days in which to replicate to higher titers. The fact that virus levels were so well reduced following treatment with two 60-min aerosols on day 1 suggests that VP14637 can interfere with virus spread via cell-to-cell fusion (syncytia formation) as well as during normal virus entry from without.

It is important to note that because VP14637 is so poorly soluble in aqueous solvents, this compound was tested using an ethanol-based vehicle. Regardless, use of this vehicle, combined with the aerosolized delivery, permitted good distribution (data not shown) and good efficacy of the compound in cotton rats. Moreover, the formulation used had no apparent cytotoxicity to a number of different tissue culture cell lines (Table 2) or to cotton rats (no significant abnormal histopathology was seen in virus-infected, treated animals (e.g., Fig. 4, panel C). The latter may have been due to the fact that this material was only administered a maximum of two times for relatively short periods of time (i.e., $\leq 2\times$ 60 min). Regardless, using this vehicle, the test compound appeared to have selective antiviral activity that was at least 2000-fold more selective in vitro against RSV than ribavirin and it was much more active than this guanosine analog in cotton rats (see Wyde et al. (1987) for a comparison). However, in contrast to the broad spectrum antiviral activity of ribavirin, the virus-inhibitory activity of VP14637 was specific for RSV and was not detectable against the other paramyxoviruses tested (i.e., measles, mumps and PIV3) or the other RNA and DNA viruses included in the testing (i.e., Coxsackie B3, influenza, rota, Herpes simplex or CMV).

Although simple in design, the carry over experiments provided much useful information. First, because virus replication was not overtly reduced in these experiments by any LF collected at greater than 24 h after ceasing aerosolization, it is unlikely that the inhibition of RSV seen throughout our studies in the assays performed by us to quantify pulmonary virus levels was an artifact due to carry over of drug still present in the lungs at the time that animals were sacrificed. This is especially true of animals in which no drug was administered later than 2 days following virus inoculation, since more than 48 h would have elapsed between the last treatment and the time that the animals were harvested and LF obtained. Second, these experiments provided clear evidence that VP14637 was being successfully delivered to the lungs by SDA. Finally, the data obtained provides some idea of the half life of this compound in the lungs. Based on the data obtained in several replicate experiments (the one shown in Table 2 being representative), it would appear that VP14637 is inactivated, bound or cleared to below levels of biologic detection within 4–24 h of dosing. Supporting this hypothesis is the fact that no significant inhibition of virus was ever seen in cotton rat experiments in which drug was administered prophylactically 24 h or more prior to virus challenge (data not shown). These data, however, do not exclude the possibility that a higher concentration of drug delivered to the site of infection, or delivery of a more active drug (or one with longer residency time in the lung) would not demonstrate prophylactic efficacy.

As discussed above, the protection from virus seen in these studies generally correlated with the dose of compound administered. Thus, throughout these studies, cotton rats exposed to two 60 min aerosols of VP14637 on day 1 consistently had significantly reduced mean pulmonary virus titers compared to these titers in the placebo control group, while animals similarly treated with just one 60 min, or two 30 min aerosols of this compound on this day did not. These data suggest that the lower efficacious dose of VP14637 to obtain maximal efficacy was two, 60-min exposures by SDA to 0.2 mg/ml of VP14637 given on day one following infection. Because the estimated dosage of VP14637 per hour of aerosol given to the cotton rats utilized in these experiments is 63 μg/kg/h (the formula used to obtain this calculation has been described in detail previously (Wyde et al., 1987)), the minimal protective dose for VP14637 would appear to be approximately 126 μg/kg/day when administered in divided doses, a value between 64 and 120 times lower than the minimal protective dose of ribavirin (i.e., 8–15 mg/kg) when administered by SDA (Gilbert et al., 1992).

Only preliminary toxicity testing was performed in cotton rats. Regardless, no animal involved in these studies exhibited any overt toxic effects (i.e., morbidity, mortality, irritability, significant change in body weight or any other observable untoward response). This included animals exposed once daily for 4 successive days to one 60-min SDA of VP14637,

the maximum exposure that any animals received in these tests (data not shown). In addition, H&E stained sections of lungs obtained from these animals appeared to be no different than comparably stained lung sections prepared from untreated animals (data not shown). Despite these findings, it is clear that the vehicle used in these studies to suspend the VP14637 is not likely to be approved for clinical studies, and that a more acceptable (i.e., non-alcohol-based) carrier needs to be found. This is particularly true if one wished to give more prolonged treatment with VP14637 such as might be required for an immunosuppressed patient infected with RSV. However, based on the extraordinary selective antiviral activity seen in tissue culture, the marked inhibition of RSV infection in cotton rats and the absence of overt toxicity in these animals, it seems that the search for a superior vehicle, or for more water-soluble derivatives of this material, are warranted.

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