Effect of Hemagglutinin-Neuraminidase Inhibitors BCX 2798 and BCX 2855 on Growth and Pathogenicity of Sendai/Human Parainfluenza Type 3 Chimera Virus in Mice

Makiko Watanabe,1 Vasily P. Mishin,‡ Scott A. Brown,2 Charles J. Russell,1 Kelli Boyd,‡ Y. Sudhakara Babu,5 Garry Taylor,6 Xiaoping Xiong,3 Xiaowei Yan,3 Allen Portner,1 and Irina V. Alymova 1*

Departments of Infectious Diseases,1 Immunology,2 and Biostatistics2 and Animal Resource Center,4 St. Jude Children’s Research Hospital, 262 Danny Thomas Place, Memphis, Tennessee 38105-3678; BioCryst Pharmaceuticals, Inc., 2190 Parkway Lake Drive, Birmingham, Alabama 35244; and Centre for Biomolecular Sciences, University of St. Andrews, North Haugh, St. Andrews, Fife KY16 9ST, Scotland

Received 17 February 2009/Returned for modification 2 April 2009/Accepted 17 June 2009

Human parainfluenza virus type 3 (hPIV-3) is a major respiratory tract pathogen that affects young children, but no vaccines or antiviral drugs against it have yet been developed. We developed a mouse model to evaluate the efficacy of the novel parainfluenza virus hemagglutinin-neuraminidase (HN) inhibitors BCX 2798 and BCX 2855 against a recombinant Sendai virus (rSeV) in which the fusion (F) and HN surface glycoproteins (FHN) were replaced by those of hPIV-3 [rSeV(hPIV-3FHN)]. In the prophylaxis model, 129X1/SvJ mice were infected with a 90% or 20% lethal dose of the virus and were treated intranasally for 5 days with 10 mg/kg of body weight/day of either compound starting 4 h before infection. Prophylactic treatment of the mice with either compound did not prevent their death in a 90% lethality model of rSeV(hPIV-3FHN) infection. However, it significantly reduced the lung virus titers, the amount of weight lost, and the rate of mortality in mice infected with a 20% lethal virus dose. In the therapy model, mice were infected with a nonlethal dose of the virus (100 PFU/mouse) and were treated intranasally with 1 or 10 mg/kg/day of either compound for 5 days starting at 24 or 48 h postinfection. Treatment of the mice with either compound significantly reduced the virus titer in the lungs, subsequently causing a reduction in the number of immune cells and the levels of cytokines in the bronchoalveolar lavage fluid and histopathologic changes in the airways. Our results indicate that BCX 2798 and BCX 2855 are effective inhibitors of hPIV-3 HN in our mouse model and may be promising candidates for the prophylaxis and treatment of hPIV-3 infection in humans.
HN complexed with the lead compound 2-deoxy-2,3-dideoxy-N-acetylenuramic acid (Neu5Ac2en), represent this novel class of inhibitors (3). In BCX 2798 and BCX 2855, the hydroxyl group at position 4-0 of Neu5Ac2en is replaced with an azido group and a dichloromethanesulfonylaminogroup, respectively. In both compounds, the methyl group of the acetamido moiety at C-5 is also replaced by an isopropyl group. The conservation of the amino acid residues that form the HN catalytic binding site among all parainfluenza viruses suggested that a single HN inhibitor of all four hPIV serotypes can be used.

We have previously shown that BCX 2798 and BCX 2855 effectively block the hemagglutinin and NA activities and the growth of hPIV serotypes 1, 2, and 3 in vitro (3). Our subsequent studies with mice and a recombinant Sendai virus (rSeV) whose HN gene was replaced with that of hPIV-1 [rSeV(hPIV-1HN)] confirmed the high levels of inhibitory activity of BCX 2798 and BCX 2855 (1–4). The mouse model of chimeric virus infection was developed to test the activities of HN inhibitors because hPIVs do not effectively infect small laboratory animals and produce no clinical symptoms (8, 35, 41). As the background virus (SeV) is a natural mouse pathogen (7, 38) and is also genetically close to hPIVs, the infection of mice with rSeV(hPIV-1HN) causes severe illness and eventually causes death because of the rapid replication of the chimeric virus in the lungs.

Because of the clinical impact of hPIV-3 infections, we evaluated the efficacies of BCX 2798 and BCX 2855 against hPIV-3 HN in in vivo mouse models of prophylaxis and treatment. Using approaches similar to those used for the drug studies with hPIV-1, we established a mouse model of infection with rSeV in which fragments of the HN and F genes were replaced by those of hPIV-3 [rSeV(hPIV-3HN)]. The HN gene in rSeV(hPIV-3HN) contained the ecto- and transmembrane domains and half of the cytoplasmic tail from hPIV-3. The other half of the cytoplasmic tail from SeV was retained because it is required for the incorporation of the HN protein into the virion via an interaction with the SeV matrix protein (44, 47, 48). The F ectodomain of rSeV(hPIV-3HN) was replaced by that of hPIV-3 to maintain a type-specific interaction with HN (from hPIV-3), which is required for efficient membrane fusion (14, 28, 50). rSeV(hPIV-3HN) was biologically functional in vitro in vitro experiments and induced robust infections in mouse lungs, causing pneumonia and leading to weight loss and death in infected animals. We demonstrate that treatment with BCX 2798 and BCX 2855 reduces the rSeV(hPIV-3HN) titer, which then leads to the reduction of the inflammatory immune response in the mouse lungs (thus reducing pathological changes in the respiratory tract), the amount of weight lost, and the rate of mortality in infected mice. Our results strongly support the possibility that HN inhibitors can be effective for the prophylaxis and treatment of hPIV infections.

**MATERIALS AND METHODS**

**HN inhibitors.** BCX 2798 (4-azido-5-isobutyrylamino-2,3-dideoxy-2,3,4,5-tetraacetyl-N-glycero-D-galacto-2-nonalopyranoside) and BCX 2855 (4-dichloromethanesulfonylamino-5-isobutyryl-amino-2,3-dIDEOXY-2,3,4,5-tETRAACETOLy-2-glycero-D-galacto-2-nonalopyranoside) were synthesized by BioCryst Pharmaceuticals, Inc. (Birmingham, AL) (3). The compounds, provided as lyophilized powders, were stored at 4°C and solubilized in phosphate-buffered saline (PBS) before each experiment. The toxicities of BCX 2798 and BCX 2855 have been determined previously (3), and it has been demonstrated that 100 μM of either compound does not cause any cell toxicity in vitro cell culture. In mice, 5 days of the intranasal (i.n.) administration of either compound at dosages of up to 50 mg/kg of body weight/day showed no toxicity in terms of weight change and survival.

**Cells and viruses.** LLC-MK2 cells (monkey kidney epithelial cells) and BHK21 cells (baby hamster kidney cells), which were used to rescue rSeV(hPIV-3HN) in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂. hPIV-3 (strain C243) was obtained from the American Type Culture Collection (Manassas, VA). rSeV(hPIV-3HN) was rescued by using a reverse genetics system, as described below. Both viruses were grown in LLC-MK2 cells in DMEM containing 10% FBS. SeV (Enders strain) was propagated in 10-day-old embryonated chicken eggs.

The infectivities of the virus stocks were determined by plaque assays. LLC-MK2 cells, in six-well plates were inoculated with serial 10-fold dilutions of virus in PBS. After incubation for 1 h at room temperature (RT), the inoculum was removed, and 1 × minimum essential medium containing 5% FBS and 0.9% agar was added to the plates. After 5 days of incubation at 33°C, the second overlay, which consisted of minimal essential medium containing 5% FBS, 0.008% neutral red, and 0.9% agar, was added to the plates to help visualize the plaques. The viruses hPIV-3 and rSeV(hPIV-3HN) were concentrated and purified by ultracentrifugation at 96,000 × g for 45 min through 20% sucrose for hemagglutination (HA) inhibition (HI) and NA inhibition (NI) assays.

**Rescue of rSeV(hPIV-3HN).** rSeV(hPIV-3HN) was rescued in BHK21 cells by using a reverse genetics technique. Plasmid pSeV(hPIV-3HN) was generated on the basis of a pSeV(+)AN plasmid (25), which contains the full-length cDNA of SeV. The F gene of pSeV(hPIV-3HN) encodes the first 531 terminal amino acids (ectodomain) of the hPIV-3 F protein and the 66 C-terminal amino acids (amino acids 500 to 565; transmembrane domain and cytoplasmic tail) of the SeV F protein. The HN gene of pSeV(hPIV-3HN) encodes the first 17 N-terminal amino acids (half of the end of the cytoplasmic tail) of the SeV HN protein and the 557 C-terminal amino acids (half of the cytoplasmic tail, transmembrane domain, and ectodomain) of the hPIV-3 HN protein (amino acids 15 to 572).

To rescue rSeV(hPIV-3HN) in BHK21 cells, transfections were performed with 2 μg of pSeV(hPIV-3HN) together with 0.6 μg of each pCAGGS-based plasmid expressing the NP, P, and L genes of SeV and 1 μg of pCAGGS-T-Tpol (the source of bacteriophage T7 RNA polymerase) by using LipoFectamine 2000 (Invitrogen, Carlsbad, CA). After 16 h of incubation at 37°C, the medium was replaced with DMEM supplemented with 0.3% bovine serum albumin and 1 μg/ml acetylated trypsin. Three days after transfection, the supernatant was collected and the rescued rSeV(hPIV-3HN) plaque purified and amplified on LLC-MK2 cells. Subsequent sequencing of the F and HN genes of rSeV(hPIV-3HN) did not reveal any mutations.

**HI assay.** HA assays were performed by using 0.5% turkey red blood cells (RBCs), as described previously (3). For the HI assays, BCX 2798 or BCX 2855 (final concentrations, 40, 10, 2.5, 0.63, 0.16, and 0.04 μM) were preincubated with 4 HA units of virus for 45 min at RT. Turkey RBCs were then added to the mixture. The 50% HA endpoint was recorded after incubation for 40 min at 4°C. The concentration of the compound that showed 50% agglutination was considered the 50% inhibitory concentration (IC₅₀). The data provided represent the mean values ± standard deviations (SDs) from at least three independent experiments.

**NI assay.** Before the NI assay was conducted, the activity of each viral NA was measured by a standard fluorometric assay with 2-(4-methylumbelliferyl)-o-N-acetylenuramic acid (Sigma-Aldrich, Inc., St. Louis, MO) as the substrate (42), as described previously (3). For the NI assays, 10 μl of BCX 2798 or BCX 2855 (final concentrations, 80, 20, 5, 1.25, 0.31, 0.08, and 0.02 μM) was mixed with an equal amount of a standard virus dose (200 relative fluorescence units) for 30 min at RT. The reaction was started by adding substrate and was stopped after 1 h of incubation at 37°C. The extent of NI was defined as the concentration of the compound required to reduce the NA activity of the treated virus to 50% of that of the control virus. The IC₅₀ was calculated by plotting the percentage of fluorescence inhibition (relative to that for the control) versus the log concentrations of the compounds. The data provided represent the mean values ± SDs from at least three independent experiments.

**Growth inhibition assay.** LLC-MK2 cells in 24-well plates were infected with the virus dose that would provide the standard 16 to 32 HA units at day 4 postinfection (p.i.). BCX 2798 or BCX 2855 (final concentrations, 25, 20, 15, 10, and 5 μM and 10, 8, 6, 4, and 2 μM, respectively) was added to the cells 1 h before infection. The presence of the virus in the cell culture was determined by an HA test performed 4 days p.i. The concentrations required to inhibit virus
replication to 50% of the level of that for the control (without the compound) (EC_{50}) were determined. The data provided represent the mean values ± SDs from at least three independent experiments.

**Animal studies with rSeV(hPIV-3FHN).** Animal studies were performed in a biosafety level 2+ facility at St. Jude Children's Research Hospital and were approved by the institution's Animal Care and Use Committee. In the prophylactic treatment model, 8-week-old female 129X1/SvJ mice (average weight, 17 to 19 g; Jackson Laboratory, Bar Harbor, ME) were anesthetized by isoflurane inhalation (2.5%: Baxter Healthcare Corporation, Deerfield, IL) and were inoculated i.n. with 10^{5} PFU/mouse (20% lethal dose) or 10^{6} PFU/mouse (90% lethal dose) of rSeV(hPIV-3FHN) in 50 μl of sterile PBS. Twice-daily (i.d.) i.n. treatments of the mice with 10 mg/kg/day BCX 2798 or BCX 2855 in 50 μl of PBS were initiated 4 h before infection and were continued for 5 days. Control animals were infected but were treated with PBS only. The efficacies of the compounds in the prophylaxis model were evaluated by measuring the amount of weight lost, the mean number of days to death, the survival rate at day 21 p.i., and the virus titer in the lungs at day 7 p.i. When the weight loss exceeded 30% of the original weight, the mice were euthanized and that point was considered the day of death.

In the treatment model, mice were infected i.n. with 100 PFU/mouse of rSeV(hPIV-3FHN) and were treated i.n. i.d. for 5 consecutive days with 1 or 10 mg/kg/day BCX 2798 or BCX 2855 starting at either 24 or 48 h p.i. The virus and compound volumes were the same as those used in the prophylaxis model. The efficacies of the compounds in the treatment model were evaluated by measuring the reduction of the virus titer in the mouse lungs at days 3, 5, and 7 p.i.; the number of immune cells and cytokine concentrations in bronchoalveolar lavage (BAL) fluid; and the pathological changes in the lungs at day 9 p.i.

**Histopathologic studies.** Mouse lungs. Lungs from three mice per group were harvested after infection with rSeV(hPIV-3FHN) or SeV, and virus growth was determined in at least two independent experiments. The procedure used for preparation of the lung suspension has been described in our previous report (3). Virus titers (PFU/ml) were determined in LLC-MK₂ cells by using plaque assays, as described previously (2). The data provided represent the mean titers ± the standard error of the means (SEMs).

**Immunologic studies.** To measure the cytokines and immune cells in BAL fluid, at least three mice per group were euthanized at day 9 p.i., as described previously (6), with minor modifications. Briefly, the lungs were removed and processed for histopathologic analysis, as described previously (3). The lungs were evaluated blindly by a veterinary pathologist (K.B.) and scored by a semiquantitative method. The scores ranged from 0 to 4 on the basis of severity of the pathology, as follows: 0 indicates a normal lung with no inflammation; 1 indicates mild inflammation around the vessels and airways and rare inflammatory cells in the alveoli; 2 indicates moderate inflammation around the vessels and airways, airway epithelial hyperplasia, and moderate inflammation in the alveolar spaces; 3 indicates increased inflammation around the airways and vessels, airway epithelial hyperplasia, focal airway epithelial necrosis, airway inflammation, and type II pneumocyte hyperplasty; and 4 indicates diffuse bronchointerstitial pneumonia. The data provided represent the mean scores ± SEMs for each group.

**Immunologic studies.** To measure the cytokines and immune cells in BAL fluid, at least three mice per group were euthanized at day 9 p.i., as described previously (6), with minor modifications. Briefly, the lungs were washed twice with 1 ml of PBS (2 ml total); and after removal of the cell materials by centrifugation, the supernatants were tested for eight cytokines (interleukin-2 [IL-2], IL-4, IL-5, IL-10, IL-13, IL-17, gamma interferon [IFN-γ], and tumor necrosis factor alpha [TNF-α]) by using Bioplex assay kits (Bio-Rad Laboratories, Hercules, CA). Samples with known cytokine concentrations were used to prepare standard curves. Individual samples were tested in duplicate. The data provided represent the mean concentrations ± SEMs.

To remove the macrophages, cells from the BAL fluid were incubated in 35-mm culture dishes for 1 h at 37°C in a humidified atmosphere of 5% CO₂. The number of lymphocytes in the supernatant was determined by the use of viable cell counts with trypan blue staining and a hemocytometer. The rest of the nonadherent cells were stained with phycoerythrin-conjugated anti-mouse CD8 and fluorescein isothiocyanate-conjugated anti-mouse CD4 antibodies (BD Biosciences, San Jose, CA) for 20 min in ice. The cells were washed and resuspended in 100 μl of fluorescent-activated cell sorter buffer (2% PBS in PBS). The data were collected with a FACSCalibur instrument (BD Biosciences) and analyzed with CellQuest software (BD Biosciences). The data provided represent the mean numbers of cells ± SEMs.

**rSeV(hPIV-3FHN)-specific antibodies in mouse blood were detected by an enzyme-linked immunosorbent assay. Blood samples were collected at day 9 p.i., and the sera were clarified by centrifugation. Before analysis, the sera were inactivated by heating at 56°C for 30 min. The enzyme-linked immunosorbent assay plate (Becton Dickinson, Franklin Lakes, NJ) was coated with 0.5 μg/well of concentrated purified rSeV(hPIV3-FHN) and incubated at 4°C overnight.** After disruption of the virus with 0.5% Nonidet P-40 in PBS, blocking buffer (5% skim milk in 0.05% Tween 20 in PBS) was added and the plate was incubated for 3 h at RT. Twofold serial dilutions of serum in blocking buffer (starting from a 1:100 dilution) were added to the plate (100 μl/well) in duplicate, and the plate was incubated for 1 h at RT. After the plate was washed in 0.05% Tween 20 in PBS, 50 μl of horseradish peroxidase-conjugated anti-mouse immunoglobulin G (1:2,000 dilution in blocking buffer; Bio-Rad Laboratories) was added and the plate was incubated for 45 min at RT. After the plate was washed, 100 μl of 3,3',5'-tetramethylbenzidine solution (Sigma-Aldrich, Inc.) was added and the plate was incubated for 30 min at RT. The color development was terminated by adding 0.5 M H₂SO₄ (0.5 μl/well), and the absorbance was read at 450 nm (Emax precision microplate reader; Molecular Devices Corp., Sunnyvale, CA). The threshold was determined by multiplying the mean of the control value by 3. The dilution was considered positive when the mean absorbance was above the threshold. The antibody titer was determined as the end-point dilution for the positive control, and serum was considered positive when the titer was ≥100.

**Statistical analysis.** The Kaplan-Meier method (11) was used to estimate the probability of the survival of the mice at any time point after the infection. The log-rank test (11) was used to compare the survival curves for groups that received different treatments (BCX 2798, BCX 2855, or PBS) during the 21 days after infection. The repeated-measurement model was used to estimate and compare the longitudinal trends of weight loss for the different treatment groups (30), and the fitted longitudinal model was used to compare the weight loss at each time point. An analysis of variance model was used to compare the virus titers in the lungs, the numbers of immune cells, and the cytokine concentrations in the treated and untreated groups. The Kruskal-Wallis test was used to compare the histopathologic scores. A P value of <0.05 was considered significant. The analyses were carried out with the statistical software package SAS for Windows (version 9.1; SAS Institute, Inc., Cary, NC).

**RESULTS**

**Mouse model of rSeV(hPIV-3FHN) infection.** To determine the pathogenicity of rSeV(hPIV-3FHN) in mice, we infected 129X1/SvJ mice (the mouse strain that is highly susceptible to the Sendai virus [15]) i.n. with different doses (100 to 10⁷ PFU/mouse) of the virus and observed them for 21 days for body weight changes and death. The growth of the recombinant virus in the lungs of mice infected with different doses was also examined (on days 1, 3, 5, 7, and 9 p.i.). Our data showed that weight loss, the survival rate, and the level of virus replication in the lungs were dose dependent. Thus, mice infected with the highest virus dose (10⁷ PFU/mouse) lost about 30% of their body weight and 90% of them died by day 12 p.i. (Table 1). Mice infected with 10⁶ PFU had a maximum weight loss of 20% and an 80% survival rate, and those infected with 10⁵ PFU had a maximum weight loss of 10% but did not die. Mice infected with lower doses (<10⁵ PFU) of the recombinant virus did not show clinical symptoms of respiratory distress but showed a moderate level of virus replication in the lungs.

**Table 1**. Pathogenicity of rSeV(hPIV-3FHN) in mice

<table>
<thead>
<tr>
<th>Virus dose (no. of PFU/mouse)</th>
<th>No. of surviving mice/total no. of mice</th>
<th>Survival rate (%)</th>
<th>Mean no. of days to death ± SD</th>
<th>Mean maximum wt loss (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁷</td>
<td>10/10</td>
<td>100</td>
<td>NA</td>
<td>9.7 ± 4.6</td>
</tr>
<tr>
<td>10⁶</td>
<td>20/25</td>
<td>80</td>
<td>19.0 ± 4.1</td>
<td>16.9 ± 8.3</td>
</tr>
<tr>
<td>10⁵</td>
<td>2/20</td>
<td>10</td>
<td>10.8 ± 3.8</td>
<td>27.7 ± 2.8</td>
</tr>
</tbody>
</table>

* Mice were infected with different virus doses and were monitored for 21 days for survival and the number of days to death. The mean number of days to death was the mean of the number of days of survival after the infection. NA, not applicable.

**TABLE 1**. Pathogenicity of rSeV(hPIV-3FHN) in mice

- Virus dose (no. of PFU/mouse): The virus dose used for infection, expressed as the number of PFUs per mouse.
- No. of surviving mice/total no. of mice: The number of mice that survived the infection divided by the total number of mice infected.
- Survival rate (%): The percentage of mice that survived the infection.
- Mean no. of days to death ± SD: The average number of days until death, along with the standard deviation.
- Mean maximum wt loss (%) ± SD: The percentage of maximum weight loss, along with the standard deviation.
High doses (10^6 and 10^7 PFU) reached the maximum levels (10^5.5 and 10^6 PFU/ml, respectively) at day 1 p.i. and remained at these levels through day 7 p.i. (Fig. 1). During the first 24 h of infection with 10^7 PFU, there was robust virus replication in the mouse lungs, with the mean titers of less than 10^2 PFU/ml being observed at 6 h p.i. and a titer of 10^4.5 ± 10^5 PFU/ml being observed at 12 h p.i. All tested mice that received 10^6 PFU cleared the virus from the lungs by day 9 p.i., whereas at the same time point, the mice challenged with 10^7 PFU had a mean lung virus titer of approximately 10^4 PFU/ml. The virus titers for mice infected with a range from 10^3 to 10^5 PFU peaked on day 3 p.i., with the average peaks in the lungs being 2.2 x 10^5 to 7.0 x 10^5 PFU/ml, whereas those for mice infected with 10^2 PFU peaked on day 5 p.i., with the average peak in the lungs being only 8.0 x 10^4 PFU/ml. Hence, rSeV(hPIV-3FHN) was highly infectious in 129X1/SvJ mice. However, it was less pathogenic than wild-type SeV, which caused 100% lethality at 10^6 PFU (data not shown), whereas that dose of rSeV(hPIV-3FHN) caused 20% lethality. In comparison to the replication of the recombinant, the replication of SeV in the mouse lungs at similar doses was more robust, with higher virus titers (up to 10^7 PFU/ml) and longer times before clearance being detected (data not shown).

Inhibitory effects of BCX 2798 and BCX 2855 on rSeV(hPIV-3FHN) in in vitro tests. Before the efficacies of BCX 2798 and BCX 2855 in mice were evaluated, we compared the sensitivities of rSeV(hPIV-3FHN) and hPIV-3 to HN inhibitors in in vitro assays. We used the HI, NI, and growth inhibition tests to determine the abilities of BCX 2798 and BCX 2855 to inhibit HA and NA activities and the growth of rSeV(hPIV-3FHN) and hPIV-3 in LLC-MK2 cells.

Both inhibitors (at low micromolar concentrations) had high degrees of efficacy against rSeV(hPIV-3FHN) and hPIV-3 in all in vitro assays, suggesting that they might also be efficient in an in vivo model (Table 2). We confirmed our previous data (3) that BCX 2855 is more efficient than BCX 2798 at inhibiting hPIV-3. The IC_{50} in the NI assays and the EC_{50} in the growth inhibition assays for BCX 2855 were approximately four times lower than those for BCX 2798. Both compounds had similar inhibitory activities against rSeV(hPIV-3FHN) and hPIV-3, suggesting that rSeV(hPIV-3FHN) might be useful as a model for evaluations of the effectiveness of novel inhibitors in mice.

Prophylactic efficacies of BCX 2798 and BCX 2855 in mice. To determine the prophylactic efficacies of BCX 2798 and BCX 2855, 129X1/SvJ mice were pretreated i.n. with BCX 2798 and BCX 2855 4 h before lethal rSeV(hPIV-3FHN) infection. Treatment (b.i.d.) was continued for five consecutive days. Data from a 90% lethal infection model (10^7 PFU of virus) showed that there were no significant differences be-

### TABLE 2. Sensitivity of rSeV(hPIV-3FHN) to HN inhibitors in in vitro assays

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mean IC_{50} (μM) ± SD</th>
<th>Mean EC_{50} (μM) ± SD for growth in cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HA activity^b</td>
<td>NA activity^c</td>
</tr>
<tr>
<td></td>
<td>BCX 2798</td>
<td>BCX 2855</td>
</tr>
<tr>
<td></td>
<td>BCX 2855</td>
<td>BCX 2798</td>
</tr>
<tr>
<td>rSeV(hPIV-3FHN)</td>
<td>1.9 ± 0.5</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>0.8 ± 0.5</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>6.4 ± 1.6</td>
<td>5.5 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>1.7 ± 0.1</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>13.1 ± 3.1</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>hPIV-3</td>
<td>2.3 ± 0.6</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>0.8 ± 0.5</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>6.4 ± 1.6</td>
<td>5.5 ± 1.3</td>
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<tr>
<td></td>
<td>1.7 ± 0.1</td>
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<tr>
<td></td>
<td>13.1 ± 3.1</td>
<td>3.1 ± 0.2</td>
</tr>
</tbody>
</table>

^a Each mean concentration was calculated from values obtained from at least three independent experiments.
^b Determined in HI assays with 0.5% turkey RBCs. IC_{50} are the mean concentrations of the compound that caused 50% agglutination.
^c Determined in NI assays. IC_{50} are the mean concentrations of the compound that caused 50% inactivation.
^d Determined by an end-point dilution assay (50% tissue culture infective dose). EC_{50} are the mean concentrations of the compound required to inhibit virus replication in treated LLC-MK2 cells to 50% of that for the untreated control.
th tween mice treated with 10, 25, and 50 mg/kg/day of either compound and untreated mice in terms of the amount of weight lost, the mean number of days to death, the survival rate, and the lung virus titers (data not shown).

Because neither compound demonstrated efficacy against 10^7 PFU of rSeV(hPIV-3FHN), mice were infected with a lower inoculum of 10^6 PFU in the next prophylaxis model experiment. This dose caused robust replication of the recombinant virus in the mouse lungs (Fig. 1), leading to 20% lethality in infected animals. In this model, prophylactic treatment of mice with 10 mg/kg/day BCX 2798 or BCX 2855 extended the survival (by 100%) and significantly reduced the amount of weight lost (starting from day 7 p.i. through the rest of the observation period). Mean lung virus titers in mice treated with either compound and in untreated mice were not significantly different at days 1, 3, and 5 p.i. A significant reduction (by at least 10-fold) of the virus titers in both treated groups was observed only at day 7 p.i. (P < 0.05) (Table 3). There were no statistically significant differences between the inhibitory activities of BCX 2798 and those of BCX 2855 for rSeV(hPIV-3FHN).

**Therapeutic efficacies of BCX 2798 and BCX 2855 in mice.** To determine the therapeutic efficacies of BCX 2798 and BCX 2855, we developed a mouse model of nonlethal recombinant virus infection that represents the infectious dose of hPIVs acquired during human-to-human transmission and that mimics the pattern of virus replication in the respiratory tracts of nonimmunocompromised humans (34, 45). In this model, mice were infected with 100 PFU of rSeV(hPIV-3FHN) and were treated i.n. with 1 or 10 mg/kg/day of BCX 2798 and BCX 2855 b.i.d. for five consecutive days starting at 24 h p.i. Because infection of the mice with 100 PFU of rSeV(hPIV-3FHN) did not cause weight loss or death, we evaluated the lung virus titers, the lung histopathologic changes, and the level of immune response to determine the activities of the compounds in the therapy model.

The i.n. administration of either 10 or 1 mg/kg/day BCX 2798 and BCX 2855 to infected mice significantly lowered the virus titers in the lungs through the infection starting at 24 h or 48 h p.i. (P < 0.05) (Fig. 2). With the treatment with 10 mg/kg/day delayed for 24 h, the rSeV(hPIV-3FHN) titers in the mouse lungs were reduced by approximately 10-100-fold from days 3 to 7 p.i. (Fig. 2A). Treatment of the mice with 1 mg/kg/day of either compound starting at 24 h p.i. reduced the mean lung virus titers by approximately 10-fold in the BCX 2855-treated group at days 3 and 5 p.i. and by approximately 100-fold in both treated groups at day 7 p.i. (Fig. 2B). When the treatment with 10 mg/kg/day of either compound was delayed to 48 h p.i., there was an approximately 10-fold reduction in virus titers at day 5 p.i. At day 7 p.i., the lung virus titers in both treated groups were near or under the detection limit (similar to the model in which treatment was delayed by 24 h), whereas the virus titers were 2.0 x 10^6 PFU/ml in the lungs of infected mice treated with PBS only (Fig. 2C).

The histopathologic changes at day 9 p.i. (when the high-level inflammatory response, such as the infiltration of immune cells and cytokine expression, is expected to be observed [6]) in the lungs of mice infected with 100 PFU of rSeV(hPIV-3FHN) and treated with 10 mg/kg/day of either compound or PBS
starting at 24 h p.i. were examined by hematoxylin-eosin staining. The most severe pathology was observed in infected mice that received the PBS treatment. Inflammatory infiltrates consisting of lymphocytes, plasma cells, and macrophages were observed around the vessels and airways of these mice (Fig. 3B). Within the airway, the epithelial cells were hyperplastic and mildly disorganized. Focal areas of epithelial necrosis were also observed. In the interstitium, the alveolar spaces contained macrophages, neutrophils, and lymphocytes. The alveolar walls were thickened due to type II pneumocyte hypertrophy and leukocyte infiltration. In this group, the mean pathology score was 2.5 (score, 2 or 3; Fig. 3E). In contrast, the lungs of mice treated with either BCX 2798 or BCX 2855 had minimal or no inflammatory response (Fig. 3C and D), and the mean scores

FIG. 3. Reduction of histopathologic changes in lungs of mice infected with rSeV(hPIV-3FHN) by treatment with BCX 2798 and BCX 2855. Mice were infected i.n. with 100 PFU of rSeV(hPIV-3FHN) and were treated with either compound at 10 mg/kg/day for 5 days starting at 24 h p.i. Four mice from each group were killed to collect the lungs at day 9 p.i. Sections were stained with hematoxylin-eosin and were examined microscopically. (A) Uninfected mice treated with PBS; (B) infected mice treated with PBS; (C) infected mice treated with BCX 2798; (D) infected mice treated with BCX 2855; (E) histopathologic scoring of infected mouse lungs. Note the infiltration of immune cells, epithelial hyperplasia (arrowheads), and focal necrosis (arrows) (B) and few peribronchiolar lymphocytes (arrows) (C). The degree of histopathologic changes was graded by use of scores of 0 (no change) to 4 (severe pneumonia) (E). Magnifications, ×40. Scale bars, 50 μm.
were 0.8 (BCX 2798) and 0.3 (BCX 2855) (score, 0 or 1 for both groups; Fig. 3E). When leukocyte infiltration was observed in the lungs of the treated mice, it consisted of low numbers of lymphocytes and plasma cells in the perivascular and peribronchiolar spaces and few macrophages in the associated alveoli. There were no signs of lung inflammation in the uninfected control group that received PBS only (Fig. 3A) or the groups treated with either compound (10 mg/kg/day for 5 days i.n.) (data not shown).

In addition to examination of the mice for histopathologic changes, the immune responses in the infected treated and untreated groups of mice were compared by quantifying the immune cells and cytokines in the BAL fluid and the virus-specific antibodies in serum (at day 9 p.i.). In the BAL fluid, the total number of lymphocytes (CD4⁺ and CD8⁺ T cells) was approximately 10-fold lower in the treated mice than in the infected untreated animals (Fig. 4A). BCX 2798 and BCX 2855 treatment also significantly reduced the levels of all detectable cytokines monitored in the BAL fluid (*P* < 0.05) (Fig. 4B). The levels of IL-2, IL-5, and IL-10 were four- to eightfold lower in the treated mice than in the untreated ones. The level of IFN-γ decreased more than 20-fold in mice treated with either compound. The level of TNF-α decreased by approximately fivefold in mice treated with BCX 2798 and was undetectable in mice treated with BCX 2855. IL-4, IL-13, and IL-17 were not detectable in any of the mice examined. There were no countable lymphocytes and no detectable cytokines in the BAL fluid from uninfected mice treated with PBS or either compound.

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**FIG. 4.** Effects of treatment with BCX 2798 and BCX 2855 on the number of immune cells and cytokines in the BAL fluid of rSeV(hPIV-3FHN)-infected mice. Mice were infected with 100 PFU of the chimera virus and treated i.n. with either compound (10 mg/kg/day for 5 days b.i.d.) starting at 24 h p.i. The BAL fluid from at least three mice from each group was collected at day 9 p.i. (A) Total number of immune cells in BAL fluid from the untreated and the treated groups. Cells were stained with anti-CD4 and anti-CD8 antibodies, and the populations of CD4⁺/CD8⁺ and CD8⁺/CD4⁺ cells were calculated by flow cytometry analysis. (B) Cytokine concentrations in BAL fluid from the untreated and the treated groups. *, significant difference in lung virus titers between the drug-treated and control groups at *P* < 0.05.
To determine the total humoral immune response in mice infected with 10² PFU of rSeV(hPIV-3FHN) and treated with PBS or 10 mg/kg/day of BCX 2798 or BCX 2855, the level of virus-specific antibodies in serum was measured at day 9 p.i. All mice were seropositive, with antibody titers ranging from 1,600 to 34,100. There was no significant difference in the mean titer of virus-specific antibodies in the serum from infected PBS-treated mice and that from infected BCX 2798- or BCX 2855-treated mice (data not shown). Thus, the total humoral immune response in infected mice was not affected by BCX 2798 or BCX 2855 treatment, but the local immune response decreased.

In the therapeutic treatment model, BCX 2798 and BCX 2855 were significantly different in reducing the titers against rSeV(hPIV-3FHN) in the mouse lungs through the infection only when the dose was reduced to 1 mg/kg/day (P < 0.05). The virus titers in the lungs were eight- and sixfold lower in the BCX 2855-treated mice than in the BCX 2798-treated mice at days 3 and 5 p.i., respectively. This result correlated with our in vitro data showing that BCX 2855 was more effective than BCX 2798 against hPIV-3 and rSeV(hPIV-3FHN) (Table 2).

Taken together, our data for 129X1/SvJ mice indicate that BCX 2798 and BCX 2855 are effective as hPIV-3 HN inhibitors for both prophylaxis and therapy.

**DISCUSSION**

hPIV-3 causes severe lower respiratory tract infections in children, elderly individuals, and immunocompromised people (10, 17, 19, 26, 43). Although the importance of selective high-efficacy antivirals targeting hPIVs has long been recognized, resolution of the 3D structure of the NDV HN protein (12) has allowed the design of structure-based inhibitors such as BCX 2798 and BCX 2855 (3). Both HN inhibitors block the catalytic binding site of the HN molecule to effectively inhibit (at low micromolar concentrations) the binding, NA activity, and growth of different hPIVs in cell culture (3).

The lack of a convenient in vivo model has been a major hindrance in the development of drugs that are active against hPIVs. Several rodent species such as cotton rats, hamsters, and guinea pigs have been used as in vivo models of hPIV infections (8, 9, 37, 41); but they develop no clinical signs. A mouse model is also best suited for initial studies of novel inhibitors that require extensive animal resources.

To determine the efficacies of the HN inhibitors BCX 2798 and BCX 2855 against hPIV-3 HN in mice, we used a reverse genetics approach that we successfully applied previously to determine the efficacies of BCX 2798 and BCX 2855 against hPIV-1 (3). For this study, we developed a mouse model using rSeV(hPIV-3FHN), a virus in which the extracellular domains of the F and HN genes of SeV were replaced by those of hPIV-3. rSeV(hPIV-3FHN) replicated robustly in mouse lungs, caused weight loss and death (at high doses) in mice, and was also as sensitive as hPIV-3 to BCX 2798 and BCX 2855 in all in vitro tests. The mouse model of rSeV(hPIV-3FHN) infection is therefore a valuable tool for evaluation of the efficacies of BCX 2798 and BCX 2855.

In the 20% lethal infection model of rSeV(hPIV-3FHN) infection (achieved with 10⁶ PFU/mouse), prophylactic treatment (starting 4 h before infection) of the mice with 10 mg/kg/day of either compound significantly reduced the virus titers in the lungs, which in turn reduced the amount of weight lost and the rate of mortality. However, prophylactic administration of either compound at dosages up to 50 mg/kg/day did not significantly affect those parameters in the 90% lethal infection model of rSeV(hPIV-3FHN) infection (achieved with a dose of 10⁷ PFU/mouse). This result is in contrast to data published previously for a 90% lethal infection with rSeV(hPIV-1HN), a virus in which the HN gene of SeV was replaced by that of hPIV-1 (3). In that study, treatment with 10 mg/kg/day of BCX 2798 and 50 mg/kg/day of BCX 2855 resulted in rates of survival of 100% and 83%, respectively, for mice infected with 10⁶.⁵ PFU of rSeV(hPIV-1HN). This suggests that both BCX 2798 and BCX 2855 (designed on the basis of the NDV HN) may be less efficient at binding to the catalytic binding site of hPIV-3 HN than at binding to that of hPIV-1 HN; thus, a higher degree of efficacy against hPIV-3 infection could be achieved by modifying the architecture of these compounds on the basis of the recently discovered crystal structure of hPIV-3 HN (29).

The therapeutic efficacies of the novel compounds were evaluated by using the nonlethal rSeV(hPIV-3FHN) infection model. We have previously applied this model to show that therapeutic treatment with BCX 2798 significantly reduces the growth of rSeV(hPIV-1HN) in mice infected with a nonlethal dose (4). Treatment of mice infected with a nonlethal dose (100 PFU) of rSeV(hPIV-3FHN) starting at 24 or 48 h p.i. with 1 or 10 mg/kg/day of either compound also significantly reduced the recombinant virus load in the lungs compared with that in the lungs of infected mice treated with PBS only (P < 0.05), suggesting that these compounds could be used to treat hPIV-3 infection.

The effect of treating rSeV(hPIV-3FHN) infections in mice with BCX compounds was also evaluated by measuring the local immune response. The immune system plays an important role in parainfluenza virus infection. Studies of SeV have shown that the immune response is critical to the pathogenesis of virus infection, as determined by the magnitude of virus replication (7, 21, 33, 38). These in vivo experiments show that the cellular and humoral immune responses can be both beneficial and harmful to the lung pathology; that is, they eliminate pathogens from the host but in so doing can damage host tissues. Our study demonstrates that both compounds (10 mg/kg/day, treatment delayed by 24 h) significantly reduced the virus titers, leading to a decrease in the integrated cellular immune response (CD4⁺ and CD8⁺ T cells; both Th1 and Th2 cytokines) at the lesion site in treated mice compared with the response in untreated mice; therefore, there were only minor or no histopathologic changes in the lungs of treated mice.

We were unable to detect a difference in antibody titers in serum between the treated and the untreated groups at day 9 p.i., probably due to the lower levels of antigen and the lower CD4⁺ T-cell response. However, the equivalent antibody titers seen in both groups, as well as the complete clearance of virus in mice that survived the challenge by day 9, suggest that the compounds did not directly abrogate the immune response. Thus, our study clearly shows that the treatment of rSeV (hPIV-3FHN) infection with novel HN inhibitors is a complex process that influences different aspects of virus pathogenesis.

The catalytic binding sites of hPIV-3 HN (29) and NDV HN...
(12) are very similar, with the only significant difference being an isoleucine at position 175 in NDV HN but a threonine at the equivalent residue, residue 193, in hPIV-3 HN. Figure 5 shows a superposition of the monomers of NDV HN (PDB code 1e8v) and hPIV-3 HN (PDB code 1v3d), both complexed with the lead compound Neu5Ac2en. The presence of the bulkier isoleucine at position 175 in NDV HN probably favors the less bulky azide group of BCX 2798 in place of the O-4 hydroxyl of Neu5Ac2en, whereas the hPIV-3 HN can more easily accommodate the bulkier dicholoromethanesulfonyl amino group in place of O-4 hydroxyl. The crystal structures of hPIV-1 HN and hPIV-2 HN are not yet available, but sequence alignments suggest that they have a leucine and an isoleucine, respectively, at position 175 of the NDV HN. In our models of in vivo prophylaxis and therapy, both HN inhibitors showed similar efficacies against rSeV(hPIV-3FH) at 10 mg/kg/day. However, at 1 mg/kg/day, BCX 2855 showed a significantly higher degree of efficacy than BCX 2798 in the therapy model ($P < 0.05$), even though BCX 2798 eventually lowered the virus titer to the same level that BCX 2855 did at day 7 p.i. We do not know the exact reason for this result at day 7 p.i., but this observation could be because the immune response (in addition to the antiviral activity of the compound) is involved in lowering the lung virus titers at this time point. The difference in the in vivo activities of the BCX compounds correlated with their in vitro activities in this study as well as our previous study (3). Therefore, our in vivo data indicate that BCX 2855 is slightly more effective than BCX 2798 at ameliorating hPIV-3 infection. In our previously published results of in vivo studies of the activities of HN inhibitors against rSeV(hPIV-1HN) (3), BCX 2798 showed a significantly higher level of activity than BCX 2855 for hPIV-1 HN. Since it is possible that the diversity in the specificities of the compounds in vivo was due to the subtle variations in the structures of the catalytic binding sites of different hPIVs, our earlier idea that a single HN inhibitor is effective against all four types of hPIV (3) may not be feasible, and the best way to develop more effective inhibitors would be to base them on the crystal structure of each hPIV HN. However, more studies are required to determine whether the differences in the efficacies of compounds against each serotype is significant for the treatment of hPIV infections in humans.

Our studies of the efficacies of structure-based HN inhibitors were initiated in 2001, after the 3D structure of NDV HN was resolved. Since then, we have shown the efficacies of these novel compounds in vitro, and by using reverse genetics, we have developed novel approaches to the testing of the compounds in a mouse model, wherein the BCX compounds have been shown to have high degrees of efficacy against rSeV (hPIV-1HN) (3) and rSeV(hPIV-3FH). We have also demonstrated that BCX 2798 can prevent lethal parainfluenza virus-Streptococcus pneumoniae synergism in 80% of dually infected mice (1). Using both cell culture and a mouse model, we have determined that resistance to these inhibitors does not develop easily and that the drug-resistant variant, N173S, which possesses an unmasked second receptor-binding site on hPIV-1 HN, retains the sensitivity to BCX 2798 in vivo (2). Although it is difficult to extrapolate the trend obtained in studies with animals to the human population (especially without the availability of drug pharmacokinetic data), the results of our previous studies and this study strongly support the possibility that HN inhibitors can provide a breakthrough in the prophylaxis and treatment of hPIV infections.

**ACKNOWLEDGMENTS**

This work was supported by research grant 160244010 from BioCryst Pharmaceuticals, Inc., Cancer Center support grant CA 21765 from the U.S. National Cancer Institute, and the American Lebanese Syrian Associated Charities. We thank Jennifer Rogers and Pamela Freiden for technical assistance and Vani Shanker for editing the manuscript.

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