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

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ABSTRACT

Human parainfluenza virus type 3 (hPIV-3) is a major respiratory tract pathogen affecting young children, but no vaccines or antiviral drugs have yet been developed against it. 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 in which the fusion and HN surface glycoproteins were substituted with those of hPIV-3 (rSeV[hPIV-3FHN]). In the prophylactic model, 129x1/SvJ mice were infected with a 90% or 20% lethal dose of the virus and treated intranasally for 5 days with 10 mg/kg/day of either compound, starting 4 h before infection. Prophylactic treatment of mice with either compound did not prevent their death in a 90% lethal model of rSeV(hPIV-3FHN) infection. However, it significantly reduced virus lung titers, weight loss, and mortality in mice infected with a 20% lethal virus dose. In the therapeutic model, mice were infected with a nonlethal dose of the virus (100 PFU/mouse) and treated intranasally with 1 or 10 mg/kg/day of either compound for 5 days, starting 24 or 48 h postinfection. Treatment of mice with either compound significantly reduced virus titer in the lungs, subsequently causing a reduction in the number of immune cells and level of cytokines in the bronchoalveolar lavage, 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 prophylaxis and treatment of hPIV-3 infection in humans.
INTRODUCTION

Human parainfluenza viruses (hPIVs) are members of the family Paramyxoviridae, which consist of single-stranded negative-sense RNA viruses targeting the respiratory tract of humans and animals (27). hPIVs are second only to respiratory syncytial virus as the viral cause of severe acute respiratory tract infections in young children (17, 19, 20). Of the 4 serotypes of hPIV, serotype 3 is the most frequently diagnosed in children (10, 16, 26, 43) and is responsible for approximately 11.5% of all pediatric respiratory tract disease hospitalizations in the US (24). Most hPIV-3 infections occur in the first 6 months of life and are typically associated with lower respiratory tract infections such as bronchiolitis and pneumonia (17, 19, 23). hPIV-3 infection is also a significant cause of morbidity and mortality in immunocompromised patients, patients with chronic diseases, and the elderly (5, 18, 31, 32, 36). At present, no vaccines or drugs have been approved by the Food and Drug Administration to prevent or treat any hPIV infection, thus necessitating the development of novel effective inhibitors against these widespread respiratory viruses.

The envelope of parainfluenza viruses has 2 glycoproteins – fusion (F) and hemagglutinin-neuraminidase (HN). The HN protein has 2 opposing functions, binding to receptors and acting as a neuraminidase, both controlled by the same catalytic-binding site (12). In the early stage of infection, the HN protein binds to sialic acid–containing receptors on the cell surface (46). The HN protein then activates the F glycoprotein to undergo a series of conformational changes that cause membrane fusion (28, 39). In the late stage of infection, HN acts as a neuraminidase to remove sialic acid residues from the surface of virus particles to prevent virus self-aggregation and thus promote efficient
Because of its key role in the parainfluenza virus replication cycle, the HN protein has been considered an attractive target for drug development. The three-dimensional (3D) structure of the Newcastle disease virus (NDV) HN showing that amino acid residues forming the catalytic-binding site are conserved among all paramyxoviruses (12, 49) allowed the structure-based design of selective HN inhibitors. BCX 2798 and BCX 2855, which were designed on the basis of the structure of NDV HN complexed with the lead compound Neu5Ac2en (2-deoxy-2, 3-didehydro-N-acetylneuraminic acid), represent this novel class of inhibitors (3). In BCX 2798 and BCX 2855, the hydroxyl group at position O4 of Neu5Ac2en is replaced with an azido group or a dichloromethanesulfonylaminogroup, respectively. In both compounds, the methyl group of the acetamido moiety at C-5 is also replaced by an isopropyl group. Conservation of amino acid residues that form the HN catalytic-binding site among all parainfluenza viruses suggested that a single HN inhibitor can be used against all 4 hPIV serotypes.

We have previously shown that BCX 2798 and BCX 2855 effectively block the hemagglutinin and neuraminidase activities and growth of hPIV serotypes 1, 2, and 3 in vitro (3). Our subsequent studies on mice with a recombinant Sendai virus (SeV) whose HN gene was replaced with that of hPIV-1 (rSeV[hPIV-1HN]) confirmed the high inhibitory activity of BCX 2798 and BCX 2855 (1-4). The mouse model of chimeric virus infection was developed to test HN inhibitors because hPIVs do not infect small laboratory animals effectively and produce no clinical symptoms (8, 35, 41). As the background virus (SeV) is a natural mouse pathogen (7, 38) (also genetically close to
hPIVs), infection of mice with rSeV(hPIV-1HN) causes severe illness and eventually death because of rapid replication of the chimeric virus in the lungs. Because of the clinical impact of hPIV-3 infections, we evaluated the efficacy of BCX 2798 and BCX 2855 against hPIV-3 HN in in vivo prophylactic and treatment models in mice. Using approaches similar to those for the drug studies on hPIV-1, we established a mouse model of infection wherein a recombinant SeV in which fragments of HN and F genes were substituted with those of hPIV-3 (rSeV[hPIV-3FHN]). The HN gene in rSeV(hPIV-3FHN) contained the ecto- and transmembrane domains and half of the cytoplasmic tail from hPIV-3. The other half of the cytoplasmic tail was retained from SeV because it was required for the HN protein to incorporate into the virion via an interaction with the SeV matrix protein (44, 47, 48). The F ectodomain of rSeV(hPIV-3FHN) was substituted with 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-3FHN) was biologically functional in in vitro experiments and induced robust infection 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-3FHN) titer which then leads to the reduction of the inflammatory immune response in mouse lungs (thus reducing pathologic changes in the respiratory tract), weight loss, and mortality in infected mice. Our results strongly support that HN inhibitors can be effective in the prophylaxis and treatment of hPIV infections.

MATERIALS AND METHODS
**HN inhibitors.** BCX 2798 (4-azido-5-isobutyrylamino-2,3-didehydro-2,3,4,5-tetra(deoxy-D-glycero-D-galacto-2-nonulopyranosic acid) and BCX 2855 (4-dichloromethanesulfonylamino-5-isobutyryl-amino-2,3-didehydro-2,3,4,5-tetra(deoxy-D-glycero-D-galacto-2-nonulopyranosic acid) were synthesized by BioCryst Pharmaceuticals, Inc. (Birmingham, AL) (3). The compounds, provided as lyophilized powder, 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), demonstrating that 100μM of either compound did not cause any cell toxicity in in vitro cell culture. In mice, 5 days of intranasal (IN) administration of either compound showed no toxicity in terms of weight change and survival at dosages up to 50 mg/kg/day.

**Cells and Viruses.** LLC-MK₂ (monkey kidney epithelial) and BHK21 (baby hamster kidney; used to rescue rSeV[hPIV-3FHN]) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂. The hPIV-3 (strain C243) was obtained from the American Type Culture Collection (Manassas, VA). rSeV(hPIV-3FHN) was rescued by using a reverse genetics system as described below. Both viruses were grown in LLC-MK₂ cells in DMEM containing 10% FBS. The SeV (Enders strain) was propagated in 10-day-old embryonated chicken eggs.
Infectivity of virus stocks was determined by plaque assays. LLC-MK2 cells in 6-well plates were inoculated with serial 10-fold virus dilutions 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 plaques.

The viruses hPIV-3 and rSeV(hPIV-3FHN) were concentrated and purified by ultracentrifugation at 96,000 g for 45 min through 20% sucrose for hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays.

Rescue of recombinant Sendai virus carrying the F and HN genes of hPIV-3.

rSeV(hPIV-3FHN) was rescued in BHK21 cells by using a reverse genetics technique. pSeV(hPIV-3FHN) plasmid 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-3FHN) encodes the first 531 N-terminal amino acids (ectodomain) of hPIV-3 F and 66 C-terminal amino acids (500-565 amino acids; transmembrane domain and cytoplasmic tail) of SeV F. The HN gene of pSeV(hPIV-3FHN) encodes the first 17 N-terminal amino acids (half the end of the cytoplasmic tail) of SeV HN and 557 C-terminal amino acids (half of the cytoplasmic tail, transmembrane domain and ectodomain) of hPIV-3 HN (amino acids 15-572).

To rescue rSeV(hPIV-3FHN), BHK21 cells were transfected with 2 µg of pSeV(hPIV-3FHN) together with 0.6 µg of each pCAGGS-based plasmid expressing the
NP, P, and L genes of SeV and 1 µg of pCAGGS-T7pol (source of bacteriophage T7 RNA polymerase) 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 rescued rSeV(hPIV-3FHN) virus was plaque purified and amplified on LLC-MK2 cells. Subsequent sequencing of the F and HN genes of rSeV(hPIV-3FHN) did not reveal any mutations.

**HI assay.** Hemagglutination (HA) assays were performed by using 0.5% turkey red blood cells (RBCs) as described previously (3). For 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 (HAU) of virus for 45 min at RT. Turkey RBCs were then added to the mixture. The 50% HA end point was recorded after incubation for 40 min at 4°C. The concentration of the compound that showed 50% agglutination was considered the 50% inhibition concentration (IC50). Data represent mean values (± the standard deviation [SD]) from at least 3 independent experiments.

**NI assay.** Before the NI assay was conducted, the activity of each viral neuraminidase (NA) was measured by a standard fluorometric assay with 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA; Sigma-Aldrich, Inc., St. Louis, MO) as the substrate (42), as described previously (3). For 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) and incubated for 40 min at 37°C. The reaction was stopped by adding 50 µl of 10% (wt/vol) trichloroacetic acid. The released fluorophore was measured at 450 nm (excitation, 350 nm). Data represent mean values (± the standard deviation [SD]) from at least 3 independent experiments.
units) for 30 min at RT. The reaction was started by adding substrate and 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 the control) versus the log concentrations of the compounds. Data represent mean values (± SD) from at least 3 independent experiments.

**Growth inhibition assay.** LLC-MK₂ cells in 24-well plates were infected with the virus dose that would provide standard 16-32 HAU at day 4 postinfection (p.i.). BCX 2798 or BCX 2855 (final concentrations 25, 20, 15, 10, and 5 µM or 10, 8, 6, 4, and 2 µM, respectively) were added to cells 1 h before infection. The presence of virus in cell culture was determined by an HA test 4 days p.i. The concentrations required to inhibit virus replication to 50% of the level of the control (without the compound) (EC₅₀) were determined. Data represent mean values (± SD) from at least 3 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 approved by the institution’s Animal Care and Use Committee. In the prophylactic treatment model, 8–12 week-old female 129x1/SvJ mice (average weight 17-19 g, Jackson Laboratories, Bar Harbor, ME) were anesthetized by isoflurane inhalation (2.5%; Baxter Healthcare Corporation, Deerfield, IL) and inoculated IN with 10⁶ plaque forming units (PFU)/mouse (20% lethal dose) or 10⁷ PFU/mouse (90% lethal dose) of rSeV(hPIV-3FHN) in 50 µl of sterile PBS. Multiple, twice daily (bid) for 5 days, IN treatments of
mice with 10 mg/kg/day BCX 2798 or BCX 2855 in 50 µl of PBS were initiated 4 h before infection. Control animals were infected but treated with PBS only. The efficacy of the compounds in the prophylactic model was evaluated by measuring weight loss, mean days to death, survival rate determined at day 21 p.i., and the virus titer in lungs at day 7 p.i. When weight loss exceeded 30% of the original weight, mice were euthanized and that point was considered the day of death.

In the treatment model, mice were infected IN with 100 PFU/mouse of rSeV(hPIV-3FHN) and treated IN bid 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. Virus and compound volumes were the same as those used in the prophylactic model. Efficacies of the compounds in the treatment model were evaluated by measuring reduction of the virus titer in the mouse lungs at day 3, 5, and 7 p.i.; number of immune cells and cytokine concentrations in bronchoalveolar lavage (BAL); and pathological changes in the lungs at day 9 p.i.

**Titration of virus from mouse lungs.** Lungs from 3 mice per group were harvested after infection with rSeV(hPIV-3FHN) or SeV and virus growth was determined in at least 2 independent experiments. The procedure for lung suspension preparation 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). Data represent mean titers (± the standard error of the mean [SEM]).

**Histopathologic studies.** Four mice from each rSeV(hPIV-3FHN) infected group were sacrificed at day 9 p.i. The lungs were removed and processed for histopathologic
analysis as described previously (3). Lungs were evaluated blindly by a veterinary pathologist (KLB) and scored by a semiquantitative method. Scores ranged from 0 to 4 on the basis of severity of pathology: 0 = normal lung with no inflammation; 1 = mild inflammation around vessels and airways, rare inflammatory cells in alveoli; 2 = moderate inflammation around vessels and airways, airway epithelial hyperplasia, moderate inflammation in alveolar spaces; 3 = increased inflammation around airways and vessels, airway epithelial hyperplasia, focal airway epithelial necrosis, alveolar inflammation and type II pneumocyte hypertrophy; and 4 = diffuse bronchointerstitial pneumonia. Data represent mean score (± SEM) for each group.

**Immunologic studies.** To measure cytokines and immune cells in BAL, at least 3 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 removing cell materials by centrifugation, supernatants were tested for 8 cytokines (interleukin-2 [IL-2], IL-4, IL-5, IL-10, IL-13, IL-17, interferon [IFN]-γ and tumor necrosis factor [TNF]-α), using Bioplex assay kits (Bio-Rad Laboratories, Hercules, CA). Cytokine samples of known concentrations were used to prepare standard curves. Individual samples were tested in duplicate. Data represent mean concentrations (± SEM).

To remove macrophages, cells from the BAL were incubated in 35-mm culture dishes for 1 h at 37°C in a humidified atmosphere of 5% CO₂. Number of lymphocytes in the supernatant was determined by viable cell counts with trypan blue staining using a hemocytometer. The rest of the non-adherent 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. Cells were washed and resuspended in 100 µl of FACS buffer (2% FBS in PBS). Data were collected with a FACSCalibur (BD Biosciences, San Jose, CA) and analyzed using CellQuest software (BD Biosciences). Data represent the mean number of cells (± SEM).

rSeV(hPIV-3FHN)-specific antibodies in mouse blood were detected by enzyme linked immunosorbent assay (ELISA). Blood samples were collected at day 9 p.i. and sera were clarified by centrifugation. Before analysis, sera were heat-inactivated at 56°C for 30 min. The ELISA 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 disrupting the virus with 0.5 % NP-40 in PBS, blocking buffer (3% skim milk in PBST [0.05 % Tween 20 in PBS]) was added and the plate was incubated for 3 h at RT. Two-fold serial dilutions of serum in blocking buffer (starting from a 1:100 dilution) were added to the plate (100 µl/well) in duplicate and incubated for 1 h at RT. After washing in PBST, 50 µl of horseradish peroxidase-conjugated anti-mouse IgG (1:2000 dilution in blocking buffer; Bio-Rad Laboratories) was added and incubated for 45 min at RT. After washing, 100 µl of 3,3',5,5'-tetramethylbenzidine solution (Sigma-Aldrich, Inc) was added and 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. Antibody titer
was determined as the end point dilution of the positive and serum was considered positive when the titer was $\geq 100$.

**Statistical analysis.** The Kaplan-Meier method (11) was used to estimate the probability of survival of mice at any time point after the infection. The log-rank test (11) was used to compare the survival curves in 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 in treatment groups (30) and the fitted longitudinal model was used to compare the weight loss at each time point. An ANOVA model was used to compare the virus titers in lungs, numbers of immune cells, and cytokine concentrations in treated and untreated groups. The Kruskal-Wallis test was used to compare histopathologic scores. A $P$ value of $<0.05$ was considered significant. The analyses were carried out using the statistical software package SAS for Windows v9.1 (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 strain that is highly susceptible to the Sendai virus [15]) IN with different doses (100 to $10^7$ PFU/mouse) of the virus and observed them for 21 days for body weight changes and death. The growth of the recombinant virus in 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, survival rate, and the level of virus replication in lungs were dose dependent. Thus, mice infected with the highest virus dose (10^7 PFU/mouse) lost about 30% of their body weight and 90% of them died by day 12 p.i. (Table 1). Mice infected with 10^6 PFU had a maximum weight loss of 20% and an 80% survival rate, and those infected with 10^5 PFU had a maximum weight loss of 10% but did not die. Mice infected with lower doses (<10^5 PFU) of the recombinant virus did not show clinical symptoms of respiratory distress, but showed a moderate level of virus replication in the lungs.

rSeV(hPIV-3FHN) titers in the lungs of mice infected with high doses (10^6 and 10^7 PFU) reached the maximum levels (10^{5.5} and 10^5 PFU/ml, respectively) at day 1 p.i. and remained at these levels through day 7 p.i. (Fig. 1). During the first 24 hours of infection with 10^7 PFU, there was robust virus replication in mouse lungs, with the mean titers of less than 10^2 PFU/ml observed 6 hours p.i. and 10^{4.5\pm0.5} PFU/ml 12 hours p.i. All tested mice receiving 10^6 PFU cleared the virus from the lungs by day 9 p.i., whereas at the same time point, mice challenged with 10^7 PFU had a mean virus lung titer of approximately 10^5 PFU/ml. Virus titers for mice infected with a range from 10^3 to 10^5 PFU peaked on day 3 p.i., with average peaks of 2.2–7.0 \times 10^5 PFU/ml, whereas those for mice infected with 10^2 PFU peaked on day 5 p.i., with an average peak of only 8.0 \times 10^4 PFU/ml in the lungs. Hence, rSeV(hPIV-3FHN) was highly infectious in 129x1/SvJ mice. However, it was less pathogenic than the wild-type SeV which caused 100% lethality at 10^6 PFU (data not shown) versus the 20% lethality caused at this dose by rSeV(hPIV-3FHN). In comparison to the recombinant, replication of the SeV in mouse
Inhibitory effects of BCX 2798 and BCX 2855 against rSeV(hPIV-3FHN) in
in vitro tests. Before evaluating the efficacy of BCXs in mice, we compared the
sensitivity 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 growth of rSeV(hPIV-3FHN) and hPIV-3 in
LLC-MK2 cells.

Both inhibitors (at low micromolar concentrations) had high 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 IC50 in NI assays and the
EC50 in the growth inhibition assays for BCX 2855 were approximately 4 times lower
than those for BCX 2798. Both compounds had similar inhibitory activities for
rSeV(hPIV-3FHN) and hPIV-3, suggesting that rSeV(hPIV-3FHN) might be a useful
model to evaluate the effectiveness of novel inhibitors in mice.

Prophylactic efficacy of BCX 2798 and BCX 2855 in mice. To determine the
prophylactic efficacy of BCX 2798 and BCX 2855, 129x1/SvJ mice were IN pretreated
with BCX 2798 and BCX 2855 4 h before lethal rSeV(hPIV-3FHN) infection. Treatment
(bid) was continued for 5 consecutive days. Data from a 90% lethal infection model (10^7
PFU of virus) showed that there were no significant differences between mice treated
with 10, 25, and 50 mg/kg/day of either compound and untreated mice in terms of weight loss, mean days to death, and survival rate, and virus lung 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 prophylactic model experiment. This dose caused robust replication of the recombinant virus in 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 survival rate (by 100%) and significantly reduced weight loss (starting from day 7 p.i. through the rest of the observation period). Mean virus lung titers in mice treated with either compound and in untreated mice were not significantly different at postinfection days 1, 3, and 5. A significant reduction (by at least 10-fold) of 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 BCX 2855 for rSeV(hPIV-3FHN).

**Therapeutic efficacy of BCX 2798 and BCX 2855 in mice.** To determine the therapeutic efficacy of BCX 2798 and BCX 2855, we developed a model of nonlethal recombinant virus infection in mice that represents an infectious dose of hPIVs during human-to-human transmission and mimics the pattern of virus replication in the respiratory tract of nonimmunocompromised humans (34, 45). In this model, mice were infected with 100 PFU of rSeV(hPIV-3FHN) and treated IN with 1 or 10 mg/kg/day of BCX 2798 and BCX 2855 bid for 5 consecutive days starting 24 or 48 h p.i. Because infection of mice with 100 PFU of rSeV(hPIV-3FHN) does not cause weight loss or
death, we evaluated the virus lung titers, lung histopathologic changes, and the level of
immune response to determine the activities of the compounds in the therapeutic model.

IN administration of either 10 or 1 mg/kg/day BCX 2798 and BCX 2855 to infected mice significantly lowered virus titers in the lungs through the infection starting
24 h or 48 h p.i. \((P < 0.05)\) (Fig. 2). With the 24 h delayed treatment at 10 mg/kg/day,
rSeV(hPIV-3FHN) titers in the mouse lungs were reduced by approximately 10- to 100-
fold from day 3 to 7 p.i. (Fig. 2A). Treatment of mice with 1 mg/kg/day of either compound starting 24 h p.i. reduced mean virus lung 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 hours p.i., there was approximately a 10-fold reduction in virus titers at day 5 p.i. At day 7 p.i., virus lung titers in both treated groups were near or under the detection limit (similar to the 24 h delayed treatment model) compared to virus titers of \(2.0 \times 10^4\) 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 of inflammatory responses such as infiltration of immune cells or cytokine expression are 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 24 h p.i. were examined by hematoxylin and eosin staining. The most severe pathology was observed in infected mice that received PBS treatment. Inflammatory infiltrates consisting of lymphocytes, plasma cells, and macrophages were observed around vessels and airways of these mice (Fig. 3B). Within the airway, epithelial cells were hyperplastic and mildly disorganized. Focal areas of epithelial necrosis were also observed. In the interstitium, alveolar spaces
contained macrophages, neutrophils, and lymphocytes. 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, lungs from mice treated with
either BCX 2798 or BCX 2855 had minimal or no inflammatory response (Fig. 3, C and
D), and mean scores were 0.8 (BCX 2798) and 0.3 (BCX 2855) (score 0 or 1 in both
groups; Fig. 3E). When leukocyte infiltration was observed in the lungs of treated mice, it
consisted of low numbers of lymphocytes and plasma cells in perivascular and
peribronchiolar spaces and few macrophages in associated alveoli. There were no signs
of lung inflammation in the uninfected control group that received PBS only (Fig. 3A) or
those treated with either compound (10 mg/kg/day for 5 days, IN) (data not shown).

In addition to examining histopathologic changes, immune responses in infected
treated and untreated groups of mice were compared by quantifying immune cells and
cytokines in the BAL and virus-specific antibodies in serum (at day 9 p.i.). In the BAL,
the total number of lymphocytes, CD4⁺ and CD8⁺ T cells, was approximately 10-fold
lower in treated mice than infected untreated animals (Fig. 4A). BCX 2798 and BCX
2855 treatment also significantly reduced the level of all detectable cytokines monitored
from the BAL (P < 0.05) (Fig. 4B). Levels of IL-2, IL-5, and IL-10 were 4- to 8-fold
lower in treated mice than 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 5-fold 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 all examined mice.
There were no countable lymphocytes and no detectable cytokines in the BAL from
uninfected mice treated with PBS or either compound.
To determine the total humoral immune response in mice infected with $10^2$ 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 1600 to 34,100. There were no significant differences in the mean titer of virus-specific antibodies in the serum from infected PBS-treated and 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 treatment but the local immune response decreased.

In the therapeutic treatment model, BCX 2798 and BCX 2855 were significantly different in reducing virus titers against rSeV(hPIV-3FHN) in mouse lungs through the infection only when the dose was reduced to 1 mg/kg/day ($P < 0.05$). Virus titers in the lung were 6- to 8-fold lower in BCX 2855-treated mice than BCX 2798-treated mice at day 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 from 129x1/SvJ mice indicate that BCX 2798 and BCX 2855 are effective for both prophylaxis and therapy as hPIV-3 HN inhibitors.

**DISCUSSION**

hPIV-3 causes severe lower respiratory tract infections in children, the elderly, and immunocompromised people (10, 17, 19, 26, 43). Although the importance of
selective high-efficacy antivirals targeting hPIVs has been long recognized, resolution of the 3D structure of the NDV HN protein (12) has allowed for 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 (at low micromolar concentrations) inhibit binding, NA activity, and growth in cell culture of different hPIVs (3).

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

To determine the efficacy of the HN inhibitors BCX 2798 and BCX 2855 against hPIV-3 HN in mice, we used a reverse-genetics approach successfully applied by us previously to determine the efficacy 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 extracellular domains of F and HN genes of SeV were substituted with those of hPIV-3. rSeV(hPIV-3FHN) replicated robustly in mouse lungs and 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 was, therefore, a valuable tool to evaluate the efficacy of BCX 2798 and BCX 2855.

In the 20% lethal model of rSeV(hPIV-3FHN) infection (10^6 PFU/mouse), prophylactic treatment (starting 4 h before infection) of mice with 10 mg/kg/day of either compound significantly reduced virus titers in the lung, which in turn, reduced weight
loss and mortality. However, prophylactic administration of either compound at dosages up to 50 mg/kg/day did not significantly affect these parameters in the 90% lethal model of rSeV(hPIV-3FHN) infection (10⁷ PFU/mouse). This result is in contrast to data published previously for a 90% lethal infection with the rSeV(hPIV-1HN) — a virus in which the HN gene of SeV was substituted with 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 100% and 83% survival, respectively, of mice infected with 10⁶.5 PFU of rSeV(hPIV-1HN). This suggests that both BCX 2798 and BCX 2855 (designed on the basis of NDV HN) may be less efficient at binding the catalytic-binding site of hPIV-3 HN than of hPIV-1 HN, thus higher efficacy against hPIV-3 infection could be achieved by modifying the architecture of these compounds based on the recently discovered crystal structure of hPIV-3 HN (29).

The therapeutic efficacy of the novel compounds was 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 24 or 48 h p.i. with 1 or 10 mg/kg/day of either compound also significantly reduced recombinant virus load in the lungs compared with 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) infection with BCX compounds in mice was also evaluated by measuring the local immune response. The immune system plays an important role in parainfluenza virus infection. Studies on 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 cellular and humoral immune responses can be both beneficial and harmful to lung pathology; that is, they eliminate pathogens from the host but in doing so can damage host tissues. Our study demonstrates that both compounds (10 mg/kg/day, 24 h delayed-treatment) significantly reduced 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 as compared with 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 at day 9 p.i. between treated and untreated groups, probably due to lower levels of antigen and lower CD4^+ T cell response. However, the equivalent antibody titers seen in both groups, as well as a complete clearance 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 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, the only significant difference being an isoleucine at position 175 in NDV HN but a threonine at the equivalent residue, 193, in hPIV-3 HN. Figure 5 shows a superposition of 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 O4 hydroxyl of Neu5Ac2en, whereas hPIV-3 HN can more easily
accommodate the bulkier dicholoromethanesulfonylamino group in place of O4. 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 isoleucine, respectively, at position 175 of NDV HN. In our prophylactic and therapeutic in vivo models, both HN inhibitors showed similar efficacy against rSeV(hPIV-3FHN) at 10 mg/kg/day. However, at 1 mg/kg/day, BCX 2855 showed significantly higher efficacy than BCX 2798 in the therapeutic model ($P < 0.05$), even though the BCX 2798 eventually lowered virus titer to the same level as BCX 2855 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 is involved (in addition to the antiviral activity of the compound) in lowering virus lung titers at this time point. The difference in the in vivo activity of BCXs correlated with their in vitro activity 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 activity of HN inhibitors on rSeV(hPIV-1HN) (3), BCX 2798 showed significantly higher activity than BCX 2855 for hPIV-1 HN. Since it is possible that the diversity in the specificity of compounds in vivo was due to the subtle variation of structure in the catalytic-binding site of different hPIVs, our earlier idea about a single HN inhibitor being effective against all 4 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 difference in the efficacies of compounds against each serotype is significant for treating hPIVs in humans.
Our studies on the efficacy of structure-based HN inhibitors were initiated in 2001, after the 3D structure of NDV HN was resolved. Since then, we have shown the efficacy of these novel compounds \textit{in vitro}, and by using reverse genetics, have developed novel approaches to test compounds in a mouse model, wherein BCXs have been shown to have high efficacy against rSeV(hPIV-1HN) (3) and rSeV(hPIV-3FHN). We have also demonstrated that BCX 2798 can prevent lethal parainfluenza - \textit{S. 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 the drug resistant variant, N173S, which possesses an unmasked second receptor-binding site on hPIV-1 HN, retains the sensitivity to BCX 2798 \textit{in vivo} (2).

Although it is difficult to extrapolate the trend obtained in animals to the human population (especially without the availability of drug pharmacokinetics data), the results of our previous studies and this study strongly support that HN inhibitors can provide a breakthrough in the prophylaxis and treatment of hPIV infections.

\section*{ACKNOWLEDGEMENTS}

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Figure Legends

Fig. 1. Growth of rSeV(hPIV-3FHN) in mouse lungs. Mice were intranasally inoculated with different doses of rSeV(hPIV-3FHN). The titers of inocula are indicated on the right of each graph. Lungs were collected at days 1, 3, 5, 7 and 9 p.i. Values are mean titers of virus from at least 3 animals, plotted with error bars indicating the SEM.

Fig. 2. Reduction of rSeV(hPIV-3FHN) virus titers in the lung by treatment with BCX 2798 and BCX 2855. Mice infected with 100 PFU of virus were treated with 10 (panel A) or 1 (panel B) mg/kg/day of either compound starting at 24 h p.i. or with 10 mg/kg/day of either compound starting 48 h p.i. (panel C). Animals were treated bid for 5 consecutive days. At least 3 mice from each group per experiment (at least 2) were sacrificed at each time point, and virus titers in the mouse lungs were determined by plaque assays. The mean values for each group are plotted with error bars indicating the SEM. Viral titers in lungs between groups were compared by the two-way ANOVA model. ( -Δ -) – mice treated with BCX 2798; ( -□-) – mice treated with BCX 2855; ( -●-) – mice treated with PBS (control). An asterisk indicates a significant difference in virus titers in the lung between the drug-treated and control groups at \( P < 0.05 \).

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 intranasally infected with 100 PFU of rSeV(hPIV-3FHN) and treated with either compound at 10 mg/kg/day for 5 days starting 24 h p.i. Four mice from each group were sacrificed to collect the
lungs at day 9 p.i. Sections were stained with hematoxylin and eosin and 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; and (E) histopathologic scoring of infected mouse lungs. Note the infiltration of immune cells, epithelial hyperplasia (arrowheads) and focal necrosis (small arrows) (B); few peribronchiolar lymphocytes (arrows) (C). Magnification: 40×. The degree of histopathologic changes was graded by a score of 0 (no change) to 4 (severe pneumonia) (E).

Fig. 4. Effect of treatment with BCX 2798 and BCX 2855 on the number of immune cells and cytokines in the BALs of rSeV(hPIV-3FHN) infected mice. Mice were infected with 100 PFU of the chimera virus and intranasally treated with either compound (10 mg/kg/day for 5 days bid) starting 24 h p.i. BALs from at least 3 mice from each group were collected at day 9 p.i. (A) Total number of immune cells in BAL from untreated and treated groups. Cells were stained with anti-CD4 and anti-CD8 antibodies and populations of CD4+/CD8- and CD8+/CD4- cells were calculated by flow cytometry analysis. (B) Cytokine concentrations in BALs from untreated and treated groups. An asterisk indicates a significant difference in virus lung titers between the drug-treated and control groups at \( P < 0.05 \).

Fig. 5. Superposition of the catalytic-binding sites of NDV HN (green) and hPIV-3 HN (blue). (A) The 2 structures were determined in the presence of the lead compound Neu5Ac2en that sits in an identical position in both active sites. A solvent-accessible
surface is shown for hPIV-3 HN, and the only significant sequence difference is
highlighted for Ile175 of NDV HN, showing how it reduces the size of the cavity around
O4 of Neu5Ac2en. The analysis was performed by PyMOL (12). (B) Chemical structures
of BCX 2798 and BCX 2855.
TABLE 1. Pathogenicity of rSeV(hPIV-3FHN) in mice

<table>
<thead>
<tr>
<th>Virus dose (PFU/mouse)</th>
<th>Number of surviving mice/total mice</th>
<th>Survival rate (%)</th>
<th>Mean days to death ± SD</th>
<th>Mean maximum weight loss (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>10³</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>10⁷</td>
<td>2/20</td>
<td>10</td>
<td>10.8 ± 3.8</td>
<td>27.7 ± 2.8</td>
</tr>
</tbody>
</table>

a Mice were infected with different virus doses and monitored for 21 days for survival and days to death.

b The mean days to death was the mean of the number of days of survival after the infection. NA, not applicable.
<table>
<thead>
<tr>
<th>Virus</th>
<th>HA activity (IC\textsubscript{50})\textsuperscript{a} (µM)</th>
<th>NA activity (IC\textsubscript{50})\textsuperscript{b} (µM)</th>
<th>Growth in cells (EC\textsubscript{50})\textsuperscript{c} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BCX 2798 BCX 2855 BCX 2798 BCX 2855 BCX 2798 BCX 2855</td>
<td>BCX 2798 BCX 2855 BCX 2798 BCX 2855 BCX 2798 BCX 2855</td>
<td>BCX 2798 BCX 2855 BCX 2798 BCX 2855 BCX 2798 BCX 2855</td>
</tr>
<tr>
<td>rSeV(hPIV-3FHN)</td>
<td>1.9 ± 0.5 0.8 ± 0.5 6.4 ± 1.6 1.7 ± 0.1 14.2 ± 3.8 3.5 ± 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hPIV-3</td>
<td>2.3 ± 0.6 1.9 ± 0.5 5.5 ± 1.3 1.4 ± 0.3 13.1 ± 3.1 3.1 ± 0.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Each mean concentration was calculated from values obtained from at least 3 independent experiments.

\textsuperscript{b} Determined in HI assays with 0.5% turkey RBCs. IC\textsubscript{50} values are the mean concentrations of the compound that caused 50% agglutination.

\textsuperscript{c} Determined in NI assays. IC\textsubscript{50} values are the mean concentrations of the compound required to reduce NA activity to 50% of that of untreated control.

\textsuperscript{d} Determined by endpoint dilution assay (TCID\textsubscript{50}). EC\textsubscript{50} values are the mean concentrations of the compound required to inhibit virus replication in treated LLC-MK\textsubscript{2} cells to 50% of that in untreated control.
TABLE 3. Effect of prophylactic treatment with BCX 2798 or BCX 2855 on rSeV(hPIV-3FHN) infection in mice\textsuperscript{a}

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. of surviving mice/total no. of mice</th>
<th>Mean weight loss (% ± SEM) on postinfection day\textsuperscript{b}</th>
<th>Mean virus titer at day 7 p.i\textsuperscript{c} (Log\textsubscript{10}, PFU/ml ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>20/25</td>
<td>17.6 ± 1.2</td>
<td>17.2 ± 2.2</td>
</tr>
<tr>
<td>BCX 2798</td>
<td>20/20</td>
<td>9.6 ± 1.3\textsuperscript{d}</td>
<td>3.0 ± 1.3\textsuperscript{d}</td>
</tr>
<tr>
<td>BCX 2855</td>
<td>20/20</td>
<td>7.9 ± 1.2\textsuperscript{d}</td>
<td>3.6 ± 1.4\textsuperscript{d}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} BCX 2798 or BCX 2855 at a dosage of 10 mg/kg/day were administered intranasally to 129x1/SvJ mice for 5 days beginning 4 h before infection with 10\textsuperscript{6} PFU of virus. Control mice were infected but were treated only with sterile PBS on the same schedule.

\textsuperscript{b} The fitted longitudinal model was used to estimate and compare the weight changes of control and treated groups.

\textsuperscript{c} The virus titers were determined using six mice per each group in two independent experiments.

\textsuperscript{d} \( P < 0.05 \) compared to the control group.
Mean Virus Lung Titer ($\log_{10}$ PFU/ml ± SEM)

Days postinfection

$10^2$ PFU
$10^3$ PFU
$10^4$ PFU
$10^5$ PFU
$10^6$ PFU
$10^7$ PFU
Days postinfection

Mean Virus Lung Titer
(Log10 PFU/ml ± SEM)

A

C

B

Mean Virus Lung Titer
(Log10 PFU/ml ± SEM)

<2
3
4
5
6
3 5 7

**
Mean histopathology score ± SEM

- PBS
- BCX 2798
- BCX 2855

* * *

A
B
C
D
E
Lymphocyte CD4$^+$ CD8$^+$ Cell Type:
Mean number of cells in BAL (Log10 ± SEM)

A

IL-2

IL-5

IFN-γ

TNF-α

IL-10

Concentration in BAL (pg/ml ± SEM)