Novel Ranking System for Identifying Efficacious Anti-Influenza Virus PB2 Inhibitors

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Through antigenic drift and shifts, influenza virus infections continue to be an annual cause of morbidity in healthy populations and of death among elderly and at-risk patients. The emergence of highly pathogenic avian influenza viruses such as H5N1 and H7N9 and the rapid spread of the swine-origin H1N1 influenza virus in 2009 demonstrate the continued need for effective therapeutic agents for influenza. While several neuraminidase inhibitors have been developed for the treatment of influenza virus infections, these have shown a limited window for treatment initiation, and resistant variants have been noted in the population. In addition, an older class of antiviral drugs for influenza, the adamantanes, are no longer recommended for treatment due to widespread resistance. There remains a need for new influenza therapeutic agents with improved efficacy as well as an expanded window for the initiation of treatment. Azaindole compounds targeting the influenza A virus PB2 protein and demonstrating excellent *in vitro* and *in vivo* properties have been identified. To evaluate the *in vivo* efficacy of these PB2 inhibitors, we utilized a mouse influenza A virus infection model. In addition to traditional endpoints, i.e., death, morbidity, and body weight loss, we measured lung function using whole-body plethysmography, and we used these data to develop a composite efficacy score that takes compound exposure into account. This model allowed the rapid identification and ranking of molecules relative to each other and to oseltamivir. The ability to identify compounds with enhanced preclinical properties provides an opportunity to develop more-effective treatments for influenza in patients.

Seasonal and pandemic influenza virus outbreaks remain a significant challenge to worldwide public health. Due to antigenic drift and shifts, the limitations of annual influenza virus vaccines, and the unpredictable nature of pandemics, there exists a clear unmet need for influenza antiviral agents that are broadly effective prophylactically as well as therapeutically.

Multiple influenza therapeutic agents, including the adamantanes amantadine and rimantadine and the neuraminidase inhibitors (NIs) oseltamivir, zanamivir, peramivir, and laninamivir, have been or are being developed to address in part this unmet medical need. NIs are recommended to be administered within 48 h after infection to be effective (reviewed in references 1 and 2). Therefore, there is an opportunity for therapeutic agents that provide efficacy beyond the 48-hour window for the initiation of treatment and with different mechanisms of action that are not affected by currently circulating resistant variants.

All clinically available influenza therapeutic agents target the neuraminidase or the M2 protein; however, more recent approaches targeting the viral replicase complex through the polymerase (favipiravir [2–7]) or the PB2 cap-snatching components (8–10) and the endonuclease (11–13) demonstrate alternative pathways for the development of anti-influenza agents. While polymerase inhibitors such as favipiravir have been shown to be active against influenza strains A, B, and C, the PB2 inhibitors have demonstrated activity against influenza A strains to date (8, 9) and the spectrum of endonuclease inhibitor activities is largely unknown, although a recent report suggests that anti-influenza activity against A and B strains is possible (14).

Laboratory mice can be experimentally infected with multiple strains of influenza virus and are commonly used for the preclinical evaluation of small-molecule therapeutic agents and antibod-

ies for influenza (reviewed in reference 15). Although oseltamivir has a limited window of opportunity in mice and humans, preclinical data in mice suggest that therapeutic agents that provide an extended window for the initiation of treatment can be developed; favipiravir, monoclonal antibodies, and recently VX-787 have shown survival benefits in the mouse model when administered 48 h postinfection (6, 8, 9, 16).

Infection of mice with the influenza A/Puerto Rico/8/34 strain is associated with inflammation in the alveolar septa by day 2, followed by interstitial pneumonia and alveolar collapse by day 6 and diffuse alveolar damage by day 9. The animals typically succumb to disease by day 10 postinfection (17). Compromised lung function associated with influenza virus infection in mice is due to the loss of type I alveolar pneumocytes. Loss of 10% of type I alveolar pneumocytes is a threshold for the initiation of loss of lung function, as measured by whole-body plethysmography

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(WBP); with a 40% reduction in type I alveolar pneumocytes, there are dramatic decreases in tidal and minute volumes that correlate with reduced oxygen consumption (VO_2) and arterial blood oxygenation. This has led to the overall conclusion that morbidity and death in the mouse model correlate with loss of type I alveolar pneumocytes (18).

Several parameters have been used to monitor influenza virus infections in mice. The most consistently reported parameters for preclinical studies have included mean survival rates, changes in body weight (BW), and lung viral titers. Inclusion of endpoints directly related to the target organ, i.e., lung pathology scores, arterial oxygen saturation, lung weights, and/or histopathological changes in the lungs, have also been reported (reviewed in reference 15). Inclusion of such target-organ-relevant endpoints (15) as measurements of lung damage/function may enhance the ability to differentiate new molecules with respect to their abilities to suppress influenza virus infection-related disease. However, none of the aforementioned parameters provides a convenient or non-destructive means to measure serially, in the same animal, the loss of lung function associated with influenza infection. Noninvasive techniques such as WBP provide an opportunity to follow the development of lung dysfunction during the course of respiratory diseases. This technique has proven useful in understanding lung dysfunction and host responses in the mouse and cotton rat models of respiratory syncytial virus (RSV) and influenza virus (4, 17–23). While multiple parameters of lung function can be measured by WBP, the enhanced pause (Penh), a unitless calculated value that reflects pulmonary resistance, has been used in the majority of respiratory virus infection models (18, 20, 24, 25). Penh is derived from changes in the holding container pressure, which fluctuates as a consequence of changes in the animal's breathing pattern. The changes in pressure are tracked during expiration (i.e., peak expiration pressure [PEP]) and inspiration (i.e., peak inspiration pressure [PIP]). Penh values are calculated according to the formula $\text{Penh} = \text{pause} \times \text{PEP}/\text{PIP}$, where pause reflects the timing of expiration. Therefore, Penh is itself a composite score for lung function.

We previously reported the discovery of a class of inhibitors originating from a phenotypic cell protection screening assay (9). The molecular target was identified as the PB2 protein, one of the three subunits (PB1, PB2, and PA) of the viral polymerase complex, which is essential for replication and transcription of the viral RNA genome. In general, rank ordering of potential clinical candidates during the lead optimization process is complicated, because single efficacy parameters fail to capture the complexity of the disease. To address this, we have developed a screening paradigm in mice that takes into consideration survival rates, BW loss, lung dysfunction, and compound exposure, all within a single value, to rank order compounds and to guide the identification process. Additionally, we present data from the mouse model that demonstrate the pharmacokinetic (PK)/pharmacodynamic (PD) relationships for this new class of influenza virus inhibitors.

MATERIALS AND METHODS

Ethics regulation of use of laboratory animals. All studies were conducted with the approval of the Vertex Pharmaceutical Institutional Animal Care and Use Committee (IACUC) and in accordance with its guidelines.

Viral stocks. Influenza A/Puerto Rico/8/34 (VR-1469) was obtained from the ATCC (Manassas, VA). Stocks were prepared by standard meth-

ods (26). Briefly, virus was passaged at low multiplicity of infection (MOI) (MOI of 0.005) in Madin-Darby canine kidney (MDCK) cells (CCL-34; ATCC), and the supernatant was harvested after approximately 48 h and centrifuged at $650 \times g$ for 10 min. Virus stocks were frozen at -80°C until used. Virus titers (in 50% tissue culture infective dose [TCID_{50}] per milliliter) were calculated by the Spearman-Kärber method after serial dilution of the virus sample, infection of replicate MDCK cultures, and measurement of the cytopathic effect (CPE) based on ATP contents at 96 h (CellTiter-Glo; Promega, Madison, WI) (27). Luminescence was measured using an EnVision multilabel reader (PerkinElmer, Waltham, MA).

Compounds. All experimental compounds were synthesized at Vertex Pharmaceuticals (8, 9) and prepared as suspensions in 0.5% methylcellulose. Oseltamivir oral suspension (Tamiflu) was reconstituted according to the manufacturer's instructions and then diluted in water to the appropriate dosing concentration. For efficacy studies, all compounds were dosed orally twice daily (BID) for 10 days, at a final volume of 10 ml/kg. For PK/PD studies, compounds were dosed once daily (QD), once every 12 h, or once every 6 h.

Intranasal infection. Male BALB/c mice (5 to 7 weeks of age, 17 to 19 g) were obtained from Charles River Laboratories or the Jackson Laboratory. For induction of influenza A infections, the mice were anesthetized with ketamine and xylazine (100 and 10 mg/kg, respectively) and inoculated with tissue culture-adapted influenza virus A/Puerto Rico/8/34 (25 $\mu\text{l}/\text{nare}$; 50 $\mu\text{l}/\text{mouse}$), which replicates well in mice and is commonly used as a mouse challenge virus (28). Inoculum infiltration in the lungs is assumed to contribute to the challenge.

Efficacy studies. For all studies with strain A/Puerto Rico/8/34, mice were housed in groups of four animals per cage. Eight mice were enrolled per study group. Mice were weighed and observed daily for signs of morbidity for 21 days after infection. In pilot studies, mice that experienced less than 35% BW loss routinely recovered to their initial BW within 7 to 10 days. Therefore, a mouse was deemed moribund, euthanized, and scored as a death (in accordance with guidelines established with the Vertex IACUC) if it scored positive for four of the following observations: $>35\%$ BW loss, ruffled fur, hunched posture, respiratory distress, reduced mobility, or hypothermia. Typically, in order for a mouse to achieve $>35\%$ BW loss, it had to score positive for at least 3 of the other criteria, and it was usually BW loss that was the final factor for euthanasia of the mice.

Lung function measurements. Respiratory function was measured using a Buxco unrestrained whole-body plethysmography system (DSI, St. Paul, MN). Mice were acclimated in the plethysmography chamber for 15 min, and then data were collected in 1-min intervals, averaged over 10 min, and expressed as absolute Penh values. Measurements were performed every 1 to 4 days postinfection during the 21-day course of the studies. Since mice cannot transmit influenza infection, the chambers were wiped down with a mild detergent at the end of each session, which could include up to 10 to 12 groups of mice.

Lung function is expressed as the enhanced pause (Penh), a unitless calculated value that reflects pulmonary resistance. This value is derived from changes in the holding container pressure, which fluctuates as a consequence of changes in the animal's breathing pattern. Bronchoconstriction of the animal's airways affects the flow of air and, hence, pressure in the holding container. The changes in pressure are tracked during expiration (PEP) and inspiration (PIP). Penh values were calculated according to the formula $\text{Penh} = \text{pause} \times \text{PEP}/\text{PIP}$, where pause reflects the timing of expiration. Data were analyzed using two-way analysis of variance (ANOVA) and the Bonferroni *post hoc* test to compare groups. *P* values of <0.05 were considered significant.

Pharmacokinetics of experimental compounds. The pharmacokinetics of test compounds were investigated with separate groups of male BALB/c mice of the same age as the infected mice. Compounds were administered to mice via oral gavage, at dosage levels of 30 mg/kg or as otherwise indicated ($n = 18$ mice/group). Plasma samples were collected at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 24 h postdose ($n = 3$ mice/time point).

FIG 1 Challenge dose-dependent survival rates and changes in body weight and lung function in influenza-infected mice. The time courses of morbidity/death, body weight loss, and lung function for BALB/c mice challenged with influenza virus are shown. Mice ($n = 8/\text{group}$) were anesthetized and challenged intranasally with influenza strain A/Puerto Rico/8/34 at the concentrations listed below the graphs. Mice were monitored daily for morbidity/death and body weight loss for 21 days, and data were plotted as percentages of survival or body weight change (mean $\pm$ standard error of the mean [SEM]). WBP was performed every 2 or 3 days, and data (mean $\pm$ SEM) were plotted versus study day.

PK sample analysis. The Bioanalytical Sciences Group, Drug Innovation Pharmacokinetics, at Vertex Pharmaceuticals conducted the analysis of mouse plasma samples and dose formulation samples collected from PK studies. Concentrations of PB2 inhibitors in mouse plasma samples and dosing solutions were determined using a high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method. In brief, plasma samples (50 μL) were harvested from whole-blood samples after the addition of anticoagulant (K_2EDTA) and were centrifuged at 3,000 rpm for 10 min. Compounds of interest were extracted from the plasma samples by protein precipitation using acetonitrile/ plasma volume ratio of 4:1). After centrifugation, the supernatant was injected into an Agilent 1100 HPLC system coupled with a tandem mass spectrometer in atmospheric pressure chemical ionization (APCI) positive-ion mode. Chromatography was performed using an Xterra MS C_{18} analytical column (5 μm , 2.1 by 50 mm) and a mobile phase consisting of a mixture of 10 mM ammonium acetate and acetonitrile running in a 3.0-min linear gradient. Compounds of interest were analyzed using an AB Sciex API 4000 mass spectrometer. A standard curve for each compound in the range of 1 to 5,000 ng/ml was established by spiking compound solutions of known concentrations into blank mouse plasma, followed by the protein precipitation procedure and HPLC-MS/MS analysis. The compound concentrations of test samples were determined by interpolation.

PK data analysis. Plasma concentration-time profiles and tissue concentrations of test compounds in mice at scheduled (nominal) sampling times were analyzed by compartmental pharmacokinetic methods using ADAPT II pharmacokinetic/pharmacodynamic systems analysis software, version 4.0 (Biomedical Simulations Resource, University of Southern California, Los Angeles, CA). Briefly, a plasma concentration-time profile was constructed for each compound by naive pooling of plasma concentration data from 18 mice (cohorts of 3 mice per time point). Based on the pharmacokinetic behavior of the compound (exhibiting monoexponential or biexponential decline over time), a one- or two-compartment pharmacokinetic model was selected to fit the plasma concentration-time data. Basic pharmacokinetic parameters such as the area under the plasma concentration-time curve (AUC), the maximum plasma concentration (C_{max}), and the half-life ($t_{1/2}$) for each compound were then determined.

RESULTS

Calibration of influenza mouse model and evaluation of WBP.

To determine the effects of viral challenge doses on survival rates, body weights, and lung function, BALB/c mice were infected with a well-characterized influenza virus strain, A/Puerto Rico/8/34. Mice were anesthetized and administered increasing challenge doses of virus ranging from 5×10^1 to 5×10^4 TCID₅₀/mouse. Morbidity, death, and BW losses were monitored daily, and lung

function, measured by WBP, was assessed every 1 to 3 days (Fig. 1). Challenge doses of 50 and 100 TCID₅₀/mouse did not cause death and resulted in moderate morbidity, with up to 10% loss in body weight on day 8 and loss of lung function that peaked on day 6. At higher challenge doses of 5×10^2 and 1×10^3 TCID₅₀/mouse, there was significant morbidity and death, with increasing body weight loss and a more rapid decline in lung function proportionate with increased viral inoculum size. At challenge doses of 5×10^3 and 5×10^4 TCID₅₀/mouse, the mice rapidly lost up to 35% of their body weight and all animals succumbed to the disease by day 8 to 9. The mice that received the largest viral inoculum declined rapidly and were not analyzed by WBP after day 2. Body weight loss was almost indistinguishable for the challenge doses of 1×10^3 to 5×10^4 TCID₅₀/mouse. With all nonlethal challenge doses, WBP values returned to normal at day 11 to 19, depending on inoculum size, and lung function improvements were correlated with body weight gains for the same animal groups. These data indicated that survival rates, BW, and WBP values are challenge dose dependent and provide us with noninvasive parameters to monitor the impact of antiviral activity.

A challenge dose of 5×10^3 TCID₅₀/mouse was considered optimal, as it yielded high consistent mortality rates and significant losses of both BW and lung function. We evaluated the correlation of lung function, survival rates, and body weight during prophylactic treatment with oseltamivir. Mice infected with strain A/Puerto Rico/8/34 at 5×10^3 TCID₅₀/mouse were treated prophylactically with oseltamivir twice daily (BID) for 10 days (treatment was initiated 2 h before infection). Death and BW were monitored daily and lung function was monitored every 2 or 3 days for 21 days (Fig. 2). Infected mice that were treated with vehicle BID demonstrated significant morbidity and mortality rates equivalent to those of untreated animals, with all animals succumbing to disease by day 9 (Fig. 2). Vehicle-treated animals demonstrated 35% BW loss by day 8. Those animals showed significant losses of lung function that were well established on day 2, with further decreases by day 4. Prophylactic treatment with oseltamivir showed dose-dependent efficacy in the influenza virus-infected mice. While treatment with oseltamivir at 0.1 mg/kg BID did not provide benefits, doses of 1 mg/kg BID provided partial protection and doses of ≥ 10 mg/kg BID provided complete protection from morbidity and death and dose-dependent reductions in body weight loss and peak lung dysfunction on day 7. Consistent with this, there were dose-dependent delays in loss of lung

FIG 2 Dose-response relationship for oseltamivir against mouse influenza A virus infection. The time courses of morbidity/death, body weight loss, and lung function for BALB/c mice challenged with influenza virus and treated prophylactically with oseltamivir are shown. Mice ($n = 8$ /group) were treated with oseltamivir or vehicle as indicated and 2 h later were anesthetized and challenged intranasally with 5×10^3 TCID₅₀ of influenza strain A/Puerto Rico/8/34; treatment was continued BID for 10 days. Mice were monitored daily for morbidity/death and body weight loss for 21 days, and data were plotted as percentages of survival or body weight change (mean $\pm$ SEM). Mice were also subjected to WBP every 2 or 3 days for 21 days, and data (mean $\pm$ SEM) were plotted versus study day.

function and more rapid recovery, especially at the dose of 120 mg/kg BID. These data demonstrate that WBP values correlate with other measures of disease severity and represent a noninvasive temporal biomarker that can be used to monitor disease in the primary target organ. Additionally, it appears that WBP findings provide additional granularity with respect to differentiation of the dose groups at earlier time points during the course of infection.

Pharmacodynamic studies with zanamivir indicated that the pharmacokinetic/pharmacodynamic driver was AUC (22), suggesting that AUC is the PK driver for neuraminidase inhibitor efficacy. Based on a comparison of AUC values (see Table S2 in the supplemental material) for oseltamivir carboxylate (active component) in humans and mice, 10 mg/kg BID was set as the clinically equivalent dose in mice for subsequent studies in this model.

We next explored the ability of oseltamivir to provide protection when administered after influenza virus infection. Mice were infected with strain A/Puerto Rico/8/34 and treatment was initiated with 10 mg/kg oseltamivir, a clinically relevant dose, 12 to 72 h postchallenge (Fig. 3). When dosing was started 12 to 24 h postchallenge, oseltamivir provided survival benefits but there was over 20% BW loss and extensive lung dysfunction. When oseltamivir was administered at times of >24 h, no significant health benefits were observed.

Efficacy of PB2 inhibitors against strain A/Puerto Rico/8/34 in BALB/c mice. As part of the PB2 inhibitor discovery program, several azaindole molecules were evaluated in the BALB/c mouse

model, at a screening dose of 30 mg/kg BID, for 10 days. To determine the extent of the treatment window, molecules were tested at 48 h postinfection, the time at which oseltamivir no longer provides protective benefits in this model. A screening dose of 30 mg/kg BID was identified as the minimal protective dose for earlier-stage compounds during the lead optimization process, and this dose was used as a starting point for use in the model. Mice treated with PB2 inhibitors were monitored for death and BW loss daily, and WBP was performed every 3 or 4 days. The panel of compounds showed different degrees of efficacy, ranging from complete protection to nonsurvival. Body weight losses at day 8 ranged from 4.9 to 32.7%, and a range of moderate to severe loss of lung function was observed (Fig. 4 and Table 1).

Exposure-based efficacy of PB2 inhibitors for rank ordering compounds. In order to rank order the compounds, we developed a composite score, the efficacy quotient (EQ) ($\text{EQ} = \text{survival rate [\%]} \text{ at day 21} / [\Delta \text{BW at day 8 [\%]} \times \Delta \text{Penh at day 6/7}]$), which incorporates survival rates, BW losses, and changes in Penh values (determined by WBP). The EQ was then normalized to exposure values for the individual molecules, yielding exposure efficiency (EE) ($\text{EE} = \text{EQ}/\text{AUC}$). The study days chosen for measurements of BW and Penh changes were determined because they demonstrated the greatest values observed in treated mice that survived for 21 days.

PK studies with single oral doses of 30 mg/kg were carried out with separate uninfected cohorts of mice, and AUC and C_{max} values for the compounds were determined (Table 1). Direct com-

FIG 3 Oseltamivir treatment window. The time courses of morbidity/death, body weight loss, and lung function for BALB/c mice challenged with influenza virus and treated with oseltamivir (10 mg/kg) are shown. Mice ($n = 8$ /group) were anesthetized and challenged intranasally with 5×10^3 TCID₅₀ of influenza strain A/Puerto Rico/8/34; treatment with oseltamivir was initiated at the indicated time and continued BID for 10 days. Vehicle treatment (10 mL/kg) was initiated 12 h postchallenge and continued BID for 10 days. Mice were monitored daily for morbidity/death and body weight loss for 21 days, and data were plotted as percentages of survival or body weight change (mean $\pm$ SEM). Mice were also subjected to WBP every 2 or 3 days for 21 days, and data (mean $\pm$ SEM) were plotted versus study day.

FIG 4 PB2 inhibitor efficacy at 48 h. The efficacy of select PB2 inhibitors was studied in the 48-h start-to-treatment BALB/c mouse model with strain A/Puerto Rico/8/34. Mice ($n = 8/\text{group}$) were anesthetized and challenged intranasally with 5×10^3 TCID₅₀ of influenza virus strain A/Puerto Rico/8/34; treatment with PB2 inhibitors (30 mg/kg) was initiated 48 h postchallenge and continued BID for 10 days. Vehicle treatment (10 ml/kg) was initiated 48 h postchallenge and continued BID for 10 days. Mice were monitored daily for morbidity/death and body weight loss for 21 days, and data were plotted as percentages of survival or body weight change (mean $\pm$ SEM). Mice were also subjected to WBP every 3 or 4 days for 21 days, and data (mean $\pm$ SEM) were plotted versus study day.

parisons with data from infected mice indicated that infection did not affect the PK parameters for these PB2 inhibitors (data not shown). The observed PK profiles covered a broad range, which is not uncommon during compound optimization, with AUC values ranging from 3.7 to 500 $\mu\text{g} \cdot \text{h}/\text{ml}$. C_{max} values ranged from 1.3 to 61.1 $\mu\text{g}/\text{ml}$, and $t_{1/2}$ values ranged from 1 to 23 h. EE-based ranking (Table 1) allows us to equate the observed efficacy with exposure and allows more precise comparison of compounds and identification of promising molecules for further evaluation.

Several compounds, covering a range of EE values, were then chosen for dose-down experiments to determine the minimally

efficacious dose and to examine how well the EE values correlated with the results of these more detailed experiments. Compound O, compound J, compound N, and compound E (Fig. 5A to D) provided complete survival when dosed at 30 mg/kg BID starting 48 h postinfection, with modest to significant BW and lung function losses. Compound O at 100 mg/kg BID showed complete survival, with minimal BW loss and lung dysfunction. While these compounds were efficacious at 30 mg/kg BID, dose reductions rapidly resulted in loss of survival, BW, and lung function.

The most efficacious compounds identified were compound B, compound A, and VX-787 (Fig. 5E to G). All three compounds

TABLE 1 Efficacy and pharmacokinetic parameters^a

Compound	Survival rate (%) ^b	Weight loss (%) ^c	Penh change (%) ^d	AUC ($\mu\text{g} \cdot \text{h}/\text{ml}$)	C_{max} ($\mu\text{g}/\text{ml}$)	$t_{1/2}$ (h)	Efficacy quotient ^e	Exposure efficiency [$\text{ml}/(\mu\text{g} \cdot \text{h})$] ^f
VX-787	100	4.9 $\pm$ 3.9	220 $\pm$ 62	29.9	24.2	4.0	9.28	0.310
A	100	8.2 $\pm$ 11	400 $\pm$ 52	10.1	3.67	5.7	3.08	0.305
B	100	14 $\pm$ 6.1	450 $\pm$ 120	7.01	11.8	4.6	1.60	0.228
C	100	27 $\pm$ 7.8	490 $\pm$ 98	4.65	3.33	5.4	0.767	0.165
D	100	25 $\pm$ 2.7	480 $\pm$ 37	6.79	1.31	3.9	0.840	0.124
E	100	14 $\pm$ 1.6	580 $\pm$ 130	14.3	7.37	3.0	1.23	0.086
F	100	21 $\pm$ 1.6	580 $\pm$ 90	17.4	8.29	7.7	0.828	0.048
G	100	28 $\pm$ 2.8	530 $\pm$ 56	19.4	13.4	2.5	0.669	0.034
H	75	31 $\pm$ 3.4	410 $\pm$ 55	25.9	6.41	3.7	0.588	0.023
I	100	23 $\pm$ 11	420 $\pm$ 42	44.5	32.1	2.4	1.03	0.023
J	100	21 $\pm$ 4.9	460 $\pm$ 34	47.2	17.2	2.1	1.05	0.022
K	37.5	33 $\pm$ 1.3	500 $\pm$ 48	21.6	5.16	8.7	0.229	0.011
L	62.5	30 $\pm$ 0.7	440 $\pm$ 38	57.1	25.6	1.0	0.486	0.009
M	100	23 $\pm$ 3.0	450 $\pm$ 32	111	41.2	2.2	0.984	0.009
N	100	13 $\pm$ 5.4	340 $\pm$ 73	336	61.1	2.8	2.29	0.007
O	75	27 $\pm$ 2.3	440 $\pm$ 80	180	18.2	24	0.627	0.003
P	37.5	32 $\pm$ 2.7	520 $\pm$ 110	83.2	33.8	2.6	0.228	0.003
Q	25	28 $\pm$ 3.1	480 $\pm$ 43	113	50.9	1.4	0.184	0.002
R	0	33 $\pm$ 3.1	540 $\pm$ 140	8.97	3.37	1.6	0	0
S	0	33 $\pm$ 1.5	630 $\pm$ 70	3.70	3.90	2.0	0	0
T	0	31 $\pm$ 1.5	680 $\pm$ 170	71.1	35.0	2.3	0	0
U	0	31 $\pm$ 1.9	530 $\pm$ 100	218	58.5	1.6	0	0

^a All efficacy endpoints were determined following a 10-day treatment with the specified compound at 30 mg/kg BID. Standard deviations for PK parameters could not be determined due to sparse sampling of cohorts of animals at each time point. PK parameters were determined after 30-mg/kg oral doses of the compounds. Compound structures are provided in Table S1 in the supplemental material.

^b Survival rates were measured on day 21 postinfection.

^c BW losses (mean $\pm$ SD, $n = 8$) were measured on day 8.

^d Penh values (mean $\pm$ SD, $n = 8$) were measured on day 6 or 7, as the changes relative to control.

^e EQ = survival rate/(BW loss $\cdot$ Penh change).

^f EE = EQ/AUC.

provided complete survival and significant reductions in BW loss and lung dysfunction when dosed at 10 mg/kg BID. Both compound A and VX-787 were effective at doses as low as 3 and 1 mg/kg BID. When compounds were compared at equal levels of exposure, compound B, compound A, and VX-787 showed similar efficacies (Table 1), with the highest EE scores (i.e., 0.228, 0.305, and 0.310, respectively) underlying the need to interpret efficacy data in the context of exposure. Importantly, these dose-down studies support the data obtained in the studies with treatment with 30 mg/kg BID at 48 h postchallenge, indicating that compound doses that provide lower exposures can still be efficacious.

Extended start-to-treatment window. Expanding the treatment window could provide significant benefits for the treatment of influenza virus infections. To evaluate extended start-to-treatment windows, dose-response studies were conducted with compound O, compound B, compound A, and VX-787, in which dosing was initiated 72 h postinfection (see Fig. S1 in the supplemental material). Compound O at 100 mg/kg BID provided complete survival and moderate BW loss and lung dysfunction, while only 50% survival and significant weight loss and lung dysfunction were observed with 30 mg/kg (see Fig. S1A in the supplemental material). Compounds A and B were able to provide complete survival at doses as low as 10 mg/kg BID (see Fig. S1B and C in the supplemental material). VX-787 provided complete protection with a dose as low as 1 mg/kg BID (see Fig. S1D in the supplemental material). Further extension of the start-to-treatment times demonstrated that compound B and VX-787 could provide complete survival and significant reductions in BW loss and lung dysfunction when dosing was initiated 96 h postinfection (data not shown) (8). Maintained protection with extended start-to-treatment time points was consistent with the rank ordering observed in the screening model with doses of 30 mg/kg BID at 48 h and confirmed the value of the exposure-based rank ordering based on the screening model with treatment at 48 h.

Effects on viral titers. To confirm the ability of the PB2 inhibitors to reduce lung viral titers directly, we used a short-term lung infection model in which mice were infected with the standard viral inoculum and treatment was initiated either 2 h prior to infection or 24 h postinfection, with subsequent harvesting of the lungs 48 h postinoculation and analysis of lung viral titers (Table 2). Consistent with previously published data (6, 15, 29–32), oseltamivir had minimal effects on lung viral titers at doses as high as 120 mg/kg BID, even when dosing was initiated prophylactically (2 h prior to infection) (Table 2).

Compound B, compound A, and VX-787 were identified to be the most efficacious molecules from the studies described above (Tables 1 and 2), and extensive dose-response relationships were explored for these molecules. All three molecules provided significant reductions in lung viral titers.

For compound B and VX-787, we evaluated the changes in viral titers, lung function, and body weight over time (Fig. 6). An

TABLE 2 Reductions in lung viral titers

Compound and dose (mg/kg BID) ^a	Start-to-treatment time (h) ^b	Log titer (mean ± SD)		Log ₁₀ reduction versus vehicle
		Vehicle control	Compound treated	
Oseltamivir	–2	7.70 ± 0.31		
10			7.51 ± 0.34	–0.19
30			7.20 ± 0.61	–0.50
120			7.05 ± 0.22	–0.65
Compound O	–2	7.70 ± 0.31		
30			7.10 ± 0.22	–0.60
100			3.95 ± 0.43	–3.75
Compound J	–2	6.78 ± 0.52	3.74 ± 1.27	–3.04
30			2.32 ± 0.31	–4.46
60			2.37 ± 0.30	–4.42
120				
30	+24	6.78 ± 0.52	6.12 ± 0.77	–0.67
60			4.37 ± 1.58	–2.42
120			3.28 ± 0.20	–3.50
Compound N	–2	6.78 ± 0.52	2.37 ± 0.41	–4.42
30			2.20 ± 0.00	–4.58
60			2.28 ± 0.13	–4.50
120				
30	+24	6.78 ± 0.52	3.62 ± 1.44	–3.17
60			3.07 ± 1.05	–3.71
120			2.45 ± 0.39	–4.33
Compound B	+24	6.55 ± 0.38		
1			5.65 ± 0.45	–0.90
3			5.25 ± 0.65	–1.30
10			5.15 ± 0.37	–1.40
30			4.40 ± 0.27	–2.15
60			3.80 ± 0.52	–2.75
Compound A	+24	7.70 ± 0.34		
0.1			7.25 ± 0.74	–0.45
0.3			7.15 ± 0.37	–0.55
1			7.05 ± 0.34	–0.65
3			6.30 ± 0.14	–1.40
10			5.45 ± 1.16	–2.25
VX-787	+24	7.70 ± 0.34		
0.1			7.00 ± 0.33	–0.70
0.3			7.10 ± 0.14	–0.60
1			6.55 ± 1.32	–1.15
3			6.40 ± 0.21	–1.30
10			3.60 ± 0.72	–4.10

^a *n* = 6 mice/group.

^b –2, 2 h prior to infection; +24, 24 h postinfection.

untreated group was used to establish lung viral burdens at the initiation of treatment. Cohorts were treated with compound or vehicle as indicated for up to 8 days, with groups taken for lung viral titer measurements on days 2, 4, 6, and 8. In addition, BW

FIG 5 Dose-response relationships for select PB2 inhibitors administered to influenza A/Puerto Rico/8/34-infected mice. The time courses of morbidity/death, body weight loss, and lung function for BALB/c mice challenged with influenza virus are shown. (A) Mice (*n* = 8/group) were treated with compounds 2 h prior to infection with strain A/Puerto Rico/8/34 and dosing was continued BID for 10 days, as indicated. (B to G) Mice (*n* = 8/group) were infected with strain A/Puerto Rico/8/34, and treatment was initiated 48 h postinfection as indicated. For all compounds, treatment was continued BID for 10 days (shaded boxes). All mice were monitored daily for morbidity/death and body weight loss for 21 days, and data were plotted as percentages of survival or body weight change (mean ± SEM). WBP was performed every 2 or 3 days, and data (mean ± SEM) were plotted versus study day.

FIG 6 Lung viral titer time courses. The time courses of viral titers, body weight loss, and lung function for BALB/c mice challenged with influenza virus are shown. Mice ($n = 8$ /group) were treated with compound B (A, C, and E) or VX-787 (B, D, and F) at the indicated doses starting 2 h prior to infection with strain A/Puerto Rico/8/34, and dosing was continued BID for 8 days. Lung viral titers were determined on days 1, 2, 4, 6, and 8 (A and B), and WBP data (C and D) and body weights (E and F) were determined in parallel. All data points are presented as mean $\pm$ SEM.

and lung function were measured each day for up to 8 days. Compound B and VX-787 were dosed to achieve equivalent exposures, with 30 and 60 mg/kg BID, respectively, as the top doses. Both compounds demonstrated significant dose-dependent reductions in viral titers versus vehicle controls on study day 2, which continued through day 6 (Fig. 6A and B).

Vehicle-treated animals demonstrated continuous severe loss in lung function, as demonstrated by a $>700\%$ increase in Penh scores at study day 2, which further increased by study day 4. Lung function was not measured at later time points due to the poor health of these groups (Fig. 6C and D). In contrast, animals that received the lowest doses of compound B or VX-787 showed a slow onset of lung dysfunction that peaked at 400% increases in Penh, whereas mice treated with higher doses of the same compounds showed minimal increases in Penh, compared with uninfected controls, through the whole course of the experiment. Body weights showed patterns similar to those of WBP data (Fig. 6E and F). These data suggest that PB2 inhibitors have direct antiviral effects that lead to improved outcomes for influenza A virus-infected mice.

PK/PD analysis of PB2 inhibitors. In an effort to elucidate and to identify the best predictor of *in vivo* antiviral efficacy for PB2 inhibitors, we studied the correlations between the viral burdens in the lungs and several pharmacokinetic measures (i.e., minimum plasma concentration [$C_{\min}$], $C_{\max}$, and AUC) in the mouse model for VX-787 and compound A (Fig. 7), with doses administered QD, once every 12 h, or once every 6 h. The reductions in the terminal lung viral burden correlated best with $C_{\min}$, and a weak correlation with the overall exposure (AUC) was identified; $C_{\max}$ was not correlated with reductions in viral titers. The $C_{\min}$ level required to attain a 1-log-unit reduction in lung viral titers coincided with the *in vitro* branched DNA (bDNA) 50% effective concentration (EC_{50}), after correction for the lung/plasma ratio and protein binding, indicating good *in vitro-in vivo* correlation for these PB2 inhibitors.

FIG 7 PK/PD relationships. Relationships between three PK measures ($C_{\max}$, $C_{\min}$, and AUC) and the PD endpoint (lung viral titer) determined 24 h after the initiation of treatment for mice challenged with the influenza A/Puerto Rico/8/34 strain are shown. Observed data are presented for the PB2 inhibitors compound A (A) and VX-787 (B), stratified by dosing regimens (i.e., every 6 h [q6h], every 12 h [q12h], or every 24 h [q24h]).

DISCUSSION

The continuing threat of a new influenza pandemic, the increase in viral resistance to NIs and adamantanes, and a continuing need for alternatives to vaccines for special populations (e.g., immunocompromised, very old, and very young patients) present an unmet need for effective small-molecule therapeutic agents with novel mechanisms of action. To enhance our ability to identify potent PB2 inhibitors *in vivo* (6, 8, 9, 16), we developed an influenza A mouse model that utilizes WBP to assess lung function in infected mice. WBP has been used to assess lung function during RSV infection in mice (25, 33, 34) and, to a lesser extent, during influenza infection in mice (4). Others have used WBP to characterize respiratory viruses; to our knowledge, WBP has not been utilized extensively during an antiviral discovery process for influenza or other respiratory infections.

To extend the use of WBP into the treatment setting, we initially validated the model with oseltamivir. The key benefit of incorporating such a biomarker is the ability to monitor noninvasively the time course of disease progression and the reversal of disease severity due to treatments. Consistent with previously published data (4), prophylactically administered oseltamivir provided dose-dependent reductions in the severity of lung dysfunction on day 7. In addition, very high doses of oseltamivir (120 mg/kg BID) delayed the loss of lung function and accelerated recovery, compared to lower doses. These data demonstrate that WBP can be used to assess the outcomes of therapeutic interventions in the mouse influenza model.

Here we demonstrate that loss of lung function during the progression of influenza in mice is challenge dose dependent, i.e., at higher challenge doses (5×10^4 TCID₅₀), mice rapidly develop high Penh scores, a measure of lung dysfunction (Fig. 1). At lower challenge doses (5×10^2 to 5×10^3 TCID₅₀), the progression of disease is less rapid and peaks with lower Penh levels. Nonlethal challenge doses were characterized by peak Penh scores around days 6 to 8, which then resolved by day 21 (4). With these data in hand, we developed a screening model to identify active molecules with increased efficacy versus oseltamivir. To that end, we developed a protocol in which PB2 inhibitors of interest were used to treat strain A/Puerto Rico/8/34-infected mice 48 h postinfection. We then developed a multiparameter measure of efficacy corrected for exposure, i.e., the EE score, which allowed us to rank order multiple PB2 inhibitors (described in reference 9).

During the lead optimization process, the ability to select rapidly the most promising candidates for drug development from a group of closely related and highly efficacious molecules is extremely important. The EE scoring system allowed us to utilize all available efficacy endpoints and PK exposure data to rank order compounds. With the use of only survival rates, body weight losses, Penh scores, or survival rates and body weight losses (survival/BW), after correction for exposure, VX-787 would have ranked eighth, fifth, seventh, or fourth, respectively. Ranking compounds based on survival/AUC data alone did not provide sufficient resolution for late-stage compounds, which were all efficacious at the standard screening dose of 30 mg/kg BID. However, such measures should be incorporated in the composite score to eliminate compounds with poor survival benefits, such as compound S, which would have ranked as one of the top contenders if survival rates had not been considered. While body weight losses and Penh scores seemed to correlate well, the Penh score

was a direct measurement of the lung function in response to treatment and was therefore a useful efficacy endpoint. Incorporating all available efficacy endpoints combined with PK exposure data allowed us to identify VX-787 as the clinical candidate.

Furthermore, we confirmed the antiviral activity of the PB2 inhibitors by directly measuring the viral titers in the lungs of infected mice. All of the PB2 inhibitors, albeit at higher doses for some compounds, reduced viral titers measured 48 h postinfection, compared to the vehicle control. Examining the time courses of lung viral titers in response to PB2 inhibitors revealed rapid sustained reductions in viral titers throughout the treatment course (day 2 to day 8). The lung viral titer reduction on day 6 postinfection in mice treated with VX-787 reported in this study is in agreement with a previous report (8). The rapid reductions in lung viral titers and the agreement with the overall *in vivo* efficacy presented an opportunity for us to devise a short-term 2-day PK/PD study to correlate PK measures (C_{min} , C_{max} , and AUC) with PD data (lung viral titers) following a dose-fractionation study design. We determined that C_{min} is the driver for the antiviral activity of PB2 inhibitors.

To our knowledge, this is the first report of an exposure-based scoring function for the systematic rank ordering of potential anti-influenza preclinical candidates. VX-787 was identified as the most potent PB2 inhibitor via this ranking method and is currently being developed for clinical treatment of influenza infections. Similar strategies that take advantage of more translational endpoints could be employed for the development of other therapeutic agents for infectious and noninfectious diseases.

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