Preclinical Characterization of PC786, an Inhaled Small-Molecule Respiratory Syncytial Virus L Protein Polymerase Inhibitor

Matthew Coates,a Daniel Brookes,a Young-In Kim,b,c Heather Allen,a Euan A. F. Fordyce,d Elizabeth A. Meals,b,c Thomas Colley,a Claire-Lise Ciana,d Guillaume F. Parra,a Vladimir Sherbukhin,d Jennifer A. Stockwell,d Jennifer C. Thomas,a S. Fraser Hunt,d Lindsey Cass,a Peter J. Murray,b Kazuhiro Ito,a Pete Strong,a John P. DeVincenzo,b,c,e Garth Rapeporta

Pulmocide Ltd., London, United Kingdoma; Department of Pediatrics, University of Tennessee Health Science Center, Memphiusb; Children’s Foundation Research Institute at Le Bonheur Children’s Hospital, Memphis, Tennessee, USAc; Sygnature Discovery Ltd., Nottingham, United Kingdomd; Department of Microbiology, Immunology and Biochemistry, University of Tennessee Health Science Center, Memphis, Tennessee, USAe

ABSTRACT Although respiratory syncytial virus (RSV) is the most common cause of lower respiratory tract infection in infants and young children, attempts to develop an effective therapy have so far proved unsuccessful. Here we report the preclinical profiles of PC786, a potent nonnucleoside RSV L protein polymerase inhibitor, designed for inhalation treatment of RSV infection. PC786 demonstrated a potent and selective antiviral activity against laboratory-adapted or clinical isolates of RSV-A (50% inhibitory concentration [IC50], 0.09 to 0.71 nM) and RSV-B (IC50, 1.3 to 50.6 nM), which were determined by inhibition of cytopathic effects in HEp-2 cells without causing detectable cytotoxicity. The underlying inhibition of virus replication was confirmed by PCR analysis. The effects of PC786 were largely unaffected by the multiplicity of infection (MOI) and were retained in the face of established RSV replication in a time-of-addition study. Persistent anti-RSV effects of PC786 were also demonstrated in human bronchial epithelial cells. In vivo intranasal once daily dosing with PC786 was able to reduce the virus load to undetectable levels in lung homogenates from RSV-infected mice and cotton rats. Treatment with escalating concentrations identified a dominant mutation in the L protein (Y1631H) in vitro. In addition, PC786 potently inhibited RSV RNA-dependent RNA polymerase (RdRp) activity in a cell-free enzyme assay and minigenome assay in HEp-2 cells (IC50, 2.1 and 0.5 nM, respectively). Thus, PC786 was shown to be a potent anti-RSV agent via inhibition of RdRp activity, making topical treatment with this compound a novel potential therapy for the treatment of human RSV infections.

KEYWORDS respiratory syncytial virus, polymerase, L protein, PC786, clinical isolate, RNA polymerases, bronchial epithelial cell, inhalation

Received 7 April 2017 Returned for modification 1 May 2017 Accepted 15 June 2017 Accepted manuscript posted online 26 June 2017


Copyright © 2017 Coates et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.

Address correspondence to Kazuhiro Ito, kaz@pulmocide.com.

M.C. and D.B. contributed equally to this article.
(COPD) (3), asthma (4), and cystic fibrosis (5). In immunocompromised adults, approximately 50% of upper respiratory tract infections with RSV progress to pneumonia (6). RSV is the single leading cause of hospitalization of infants (7) and causes nearly 10 times more respiratory deaths than does influenza in this age group (8). RSV exists as two antigenic subgroups: A and B. RSV A viruses were formerly regarded as the subgroup of pathogens responsible for the majority of clinical disease and were reported to produce a slightly more symptomatic pathology (9, 10). However, recent studies have demonstrated that virus strains from the B subgroup also often predominate in medically important afflicted populations (11, 12). In addition, both subgroups exist in differing percentages within a single winter, and available point-of-care rapid diagnostic tests may or may not distinguish between the subgroups, thus necessitating an effective antiviral to have broad activity covering both viral subgroups.

Despite significant efforts to develop a safe and effective treatment against RSV, Food and Drug Administration (FDA)-approved drugs for this indication are limited to (aerosolized) ribavirin and the humanized monoclonal antibody palivizumab (Synagis). The latter agent targets the RSV fusion (F) protein and is restricted to prophylactic use in high-risk, pediatric patients. Furthermore, clinical variants resistant to neutralization by palivizumab have been identified (13), and no effective vaccine is currently available. The use of ribavirin is limited by its low potency against the virus, its questionable clinical benefit, and concerns over its toxicity. In recent years, new compounds intended for the treatment of RSV have been reported. These include the oral F protein inhibitors GS-5806 (phase II) (14), AK0529 (15), BTA-CSR5 (16), JNJ-53718678 (17, 18), and inhaled nanobody ALX-0171 (19) (phase II). In addition, ALS-8176, an orally bioavailable prodrug of the novel nucleoside RSV polymerase inhibitor ALS-8112 (phase II) (20), has entered clinical development.

The initial portal of entry by RSV is through the nose or eye rather than the mouth (21). Once established in the upper respiratory tract, the infection is able to progress readily into the lungs. The pathophysiology of RSV infection was investigated in a study of lung tissues obtained from deceased children who died within their first week of infection (22) or later (23). Samples from these individuals revealed the presence of RSV, by immunostaining, only in epithelial cells, without basal cells being affected. The specific localization of the pathogenic organism provides a particular challenge to treatment, since it requires a supereffective drug concentration to be maintained at the discrete cellular site of virus replication to be effective. Topical therapy is, therefore, an ideal approach for combating RSV infection.

RNA virus polymerase is increasingly recognized as an attractive target for development of antiviral drugs against hepatitis C virus (HCV), and RSV also expresses RNA-dependent RNA polymerase (RdRp), which transcribes and replicates its negative-sense RNA genome. The RSV large polymerase subunit (L) exhibits multiple enzymatic activities, including the capability to synthesize RNA as well as to add and methylate a cap on each of the viral mRNAs. The potential benefits of polymerase inhibition will be expected efficacy even after virus infection has already been established within cells, as opposed to treatments that only prevent infections of new cells, such as virus entry inhibitors, e.g., RSV F protein inhibitors. Recently, several compounds that inhibit RSV RdRp activity by targeting L protein have been described, such as YM-53403 (24), AZ-27 (25, 26), and Boehringer Ingelheim (Bi) compound D (27). However, these agents are generally weak, have poor activity against both A and B strains, and/or have poor retention within the relevant lung tissues.

We describe here the in vitro and in vivo profiles of the novel anti-RSV therapeutic agent PC786, N-(2-fluoro-6-methylphenyl)-6-(4-(5-methyl-2-(7-oxa-2-azaspiro[3.5]nonan-2-yl)nicotinamido)benzoyl)-5,6-dihydronaphthalene-4H-benzo[b]thieno[2,3-d]azepine-2-carboxamide (Fig. 1) (28), which targets the RSV L protein and is designed specifically for topical treatment by inhalation.
RESULTS

Antiviral activity of PC786. The antiviral activity of PC786 (Fig. 1A) was assessed in in vitro cytopathic effect (CPE) assays with HEp-2 cells using laboratory-adapted RSV strains (RSV A2 and RSV B Washington [WST]), some known clinical isolates as well as low-passage-number clinical isolates with genotypic diversity, and five isolates each of RSV A and RSV B (University of Tennessee). PC786 potently inhibited CPE induced by RSV A2 and RSV B WST (Table 1). These inhibitory values were on the order of 309 to over 33,000 times more potent than those displayed by ribavirin, much more potent than those of known RSV L protein inhibitors AZ-27 and BI compound D, and equal to those of the F protein inhibitors TMC353121 and GS-5806 on RSV A2. PC786 did not

FIG 1 Antiviral effects of PC786. (A) Chemical structure of PC786. (B) Western blotting image demonstrating the inhibitory effects of PC786 on RSV protein expression in HEp-2 cells infected with RSV A2 for 3 days. *, nonspecific band; F1, disulfide-linked F1 subunit cleaved from RSV F0 protein precursor. NT, no PC786 treatment (0.5% DMSO only). (C) Inhibitory effects of RSV A Memphis 37- and RSV B strain 1262-induced CPE in HEp-2 cells. (D and E) PCR viral loads in the supernatants from HEp-2 cells infected with Memphis 37 (D) and strain 1262 (E) in the presence of PC786. The assay was conducted in duplicate, and individual dots show data from individual wells.

TABLE 1 Comparison of in vitro anti-RSV activity of PC786 with those of known antiviral agents

<table>
<thead>
<tr>
<th>Compound</th>
<th>RSV A2</th>
<th>RSV B WST</th>
<th>Cell viability,</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀ (nM)</td>
<td>IC₉₀ (nM)</td>
<td>IC₅₀ (nM)</td>
</tr>
<tr>
<td>PC786</td>
<td>0.50 ± 0.0014</td>
<td>0.63 ± 0.035</td>
<td>27.3 ± 0.77</td>
</tr>
<tr>
<td>AZ-27</td>
<td>26.4 ± 1.16</td>
<td>60.0 ± 6.75</td>
<td>&gt;1,580</td>
</tr>
<tr>
<td>Bl compound D</td>
<td>57.6 ± 1.53</td>
<td>&gt;333</td>
<td>47.8 ± 2.96</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>&gt;16,400</td>
<td>&gt;16,400</td>
<td>10,300</td>
</tr>
<tr>
<td>ALS-8112</td>
<td>455 ± 16.0</td>
<td>1,130 ± 77.9</td>
<td>647 ± 44.2</td>
</tr>
<tr>
<td>GS-5806</td>
<td>0.45 ± 0.018</td>
<td>0.93 ± 0.098</td>
<td>0.17 ± 0.043</td>
</tr>
<tr>
<td>TMC35315</td>
<td>0.78 ± 0.0093</td>
<td>1.29 ± 0.015</td>
<td>0.85 ± 0.017</td>
</tr>
</tbody>
</table>

⁺CC₅₀ Concentration required for 50% cytotoxicity; IC₅₀ and IC₉₀ concentrations required for 50 and 90% inhibition, respectively; WST, Washington.

ALS-8112 was added 24 h prior to virus inoculation (although all others were treated simultaneously with virus infection).

⁺n = 2 (343 and 857 nM).
affect cell viability at the concentrations up to 14 μM and consequently showed a large safety margin with respect to mammalian cell toxicity (Table 1). PC786 was found to inhibit RSV A2 protein expression in HEp-2 cells 3 days postinfection when used just after virus inoculation, and at concentrations greater than 14 nM, no or little G, N, P, M, M2-1, and F1 protein bands were visible in Western blotting (Fig. 1B). PC786 also exhibited potent inhibition of CPE induced by known RSV A clinical isolates (IC50, 0.14 to 3.2 nM) (Table 2).

PC786 also exhibited potent inhibition of CPE induced by various low-passage-number clinical isolates of RSV A (IC50, 0.42 nM [median]) and RSV B (IC50, 17.5 nM [median]) (Table 3 and Fig. 1C). The antiviral effects of PC786 against RSV A- and RSV B-induced CPE are represented in Fig. S1A and S1B. The supernatant from RSV-infected cells was collected after the end of the CPE analysis and analyzed by reverse transcription-PCR for the RSV N gene. PC786 inhibited, in a concentration-dependent manner, N gene PCR products of the 10 clinical isolates of RSV A and RSV B, and the inhibitory activities, analyzed as IC50 and IC90 values, were consistent with those obtained from the CPE experiments (Table 3 and Fig. 1D and E; see also Table S2).

PC786 exhibited no activity against parainfluenza virus 3 (PIV3), measles virus, influenza A virus (H1N1), rhinovirus 16, human immunodeficiency virus type 1 (HIV-1), and herpesvirus at 14 μM, suggesting a selectivity index of >33,000 in comparison to its efficacy against RSV A Long (Table 4). Although PC786 inhibited hepatitis C virus replication in the replicon assay (rather than in the CPE assay), the effective concentration (IC50 of 0.32 μM) was >760-fold higher than that required to inhibit RSV, and the maximum inhibition achieved was only 74% at 14 μM.

**Kinetics of anti-RSV activity.** PC786 was added to HEp-2 cells at 0 (just after inoculation), 24, 48, and 72 h after inoculation with RSV A2 at multiplicities of infection (MOIs) of 0.02, 0.1, and 1, and the resulting CPE was determined at 144 h (6 days) postinoculation. The influence of PC786 on RSV A2-induced CPE was not affected by the level of infectious virus particles applied to cells (Table 5).

### TABLE 2 Anti-RSV effects of PC786 against RSV clinical isolates in HEp-2 cells, evaluated by CPE assay

<table>
<thead>
<tr>
<th>RSV A strain</th>
<th>Method</th>
<th>IC50 (nM)</th>
<th>ALS-8112</th>
</tr>
</thead>
<tbody>
<tr>
<td>1997/12-35</td>
<td>CPE 1</td>
<td>0.35</td>
<td>693</td>
</tr>
<tr>
<td>2001/2-20</td>
<td>CPE 1</td>
<td>0.53</td>
<td>768</td>
</tr>
<tr>
<td>2000/3-4</td>
<td>CPE 1</td>
<td>0.14</td>
<td>515</td>
</tr>
<tr>
<td>Memphis 37c</td>
<td>CPE 1</td>
<td>0.50</td>
<td>398</td>
</tr>
<tr>
<td>2001/3-12</td>
<td>CPE 2</td>
<td>3.2</td>
<td>3,200</td>
</tr>
<tr>
<td>1998/3-2</td>
<td>CPE 2</td>
<td>2.5</td>
<td>1,500</td>
</tr>
<tr>
<td>USUv1225</td>
<td>CPE 2</td>
<td>1.3</td>
<td>880</td>
</tr>
<tr>
<td>ALS-8112</td>
<td>CPE 1</td>
<td>0.35</td>
<td>693</td>
</tr>
<tr>
<td>ALS-8112</td>
<td>CPE 2</td>
<td>3.2</td>
<td>3,200</td>
</tr>
<tr>
<td>ALS-8112</td>
<td>CPE 2</td>
<td>2.5</td>
<td>1,500</td>
</tr>
<tr>
<td>ALS-8112</td>
<td>CPE 2</td>
<td>1.3</td>
<td>880</td>
</tr>
</tbody>
</table>

*CPE 1, CPE resazurin detection in 384-well format; PC786 was used just after virus infection, and ALS-8112 was added 24 h prior to virus inoculation. CPE 2, CPE neutral red detection in 96-well format; PC786 was used just after virus infection, and ALS-8112 was added 24 h prior to virus inoculation.

### TABLE 3 Anti-RSV effects of PC786 against low-passage RSV clinical isolates in HEp-2 cells, evaluated in a CPE assay and by reverse transcription-PCR

<table>
<thead>
<tr>
<th>RSV strain</th>
<th>Strain source</th>
<th>CPE assay result</th>
<th>PCR result</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IC50 (nM)</td>
<td>IC90 (nM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td>A2</td>
<td>Laboratory</td>
<td>0.96</td>
<td>NA</td>
</tr>
<tr>
<td>A Long</td>
<td>Laboratory</td>
<td>2.46</td>
<td>NA</td>
</tr>
<tr>
<td>x5 A</td>
<td>Clinical</td>
<td>0.46</td>
<td>&lt;0.09–0.71</td>
</tr>
<tr>
<td>B 9320</td>
<td>Laboratory</td>
<td>29.1</td>
<td>NA</td>
</tr>
<tr>
<td>x5 B</td>
<td>Clinical</td>
<td>20.2</td>
<td>13.4–50.6</td>
</tr>
</tbody>
</table>

*PCR transcripts were present in the supernatant.

**Coates et al. Antimicrobial Agents and Chemotherapy** September 2017 Volume 61 Issue 9 e00737-17 aac.asm.org
PC786 remained even if dosing was initiated as long as 48 h after virus inoculation, and diminished thereafter (Table 5 and Fig. 2A). In contrast, the antiviral activity of the F protein inhibitor GS-5806 decreased when dosing was initiated even as short as 24 h postinoculation, and almost no antiviral activity by the F protein inhibitor was observable when initial dosing was 48 h or more after the virus inoculation (Table 5 and Fig. 2B). Additionally, this sequential diminution of the F protein inhibitor’s activity was more marked at higher viral MOIs (Table 5).

**Persistence of action.** The persistence of action of PC786 was assessed by RSV F protein cell-based enzyme-linked immunosorbent assay (ELISA) on RSV A2-infected bronchial epithelial BEAS-2B cells. The procedure relies upon determining the effect of a 24-h washout period on the potency of PC786 compared with the compound’s activity when used for treatment just after virus inoculation and left in situ, i.e., omitting a washout. Under this protocol, in the sustained presence of drug, the IC50 of PC786 against RSV A2, as measured by F protein expressed on cell surfaces, was 0.129 nM; the IC50 was 0.792 nM following a 24-h washout (Fig. 2C). In contrast, AZ-27’s antiviral effect diminished 35-fold from an initial IC50 of 3.17 nM to 106 nM after the same washout. Under this protocol, in the sustained presence of drug, the IC50 of PC786 compared with the compound’s activity when used for treatment just after virus inoculation and left in situ, i.e., omitting a washout. Under this protocol, in the sustained presence of drug, the IC50 of PC786 compared with the compound’s activity when used for treatment just after virus inoculation and left in situ, i.e., omitting a washout.

**Effects of PC786 on RSV A2 infection in ALI-cultured human airway epithelial cells.** The antiviral effect of PC786 was also evaluated using air-liquid interface (ALI)-cultured fully differentiated human primary airway epithelial cells. These cells undergo extensive mucociliary differentiation, resulting in cultures with morphological characteristics similar to those observed in the normal human respiratory epithelium. Following inoculation with a low level of RSV A2 (MOI, 0.01), the quantity of RSV in apical washes, as determined by plaque assay, in the absence of any antiviral compounds increased from day 1 to a peak at day 3 and then waned gradually and modestly up to day 7 (Fig. 2F). Treatment with PC786 to the apical surface, once daily from day 0 (1 h

A table titled **TABLE 4 In vitro antiviral effects of PC786 on selected RNA and DNA viruses** is presented, listing viruses, family, cell type, and IC50 values for PC786, IC50 or IC90 (µM), CC50 (µM), safety index, and selectivity index vs RSV. The table includes viruses such as RSV A long, Paramyxoviridae, HEp-2, with IC50 values ranging from 0.00042 to 1.4 µM for PC786.

A table titled **TABLE 5 Time-of-addition study for antiviral effects of PC786 and known anti-RSV agents in RSV A infection at different MOIs in HEp-2 cells** is presented, listing compounds (PC786, GS-5806, and more) at indicated treatment time (h) postinoculation, fold increase at indicated time (h) vs time zero, and MOI values. The table includes data for PC786 at MOIs of 0.02, 0.1, and 1.0, with fold increases ranging from 1.1 to 4.3 and 1.4 to 1.6, respectively.
after virus inoculation) to day 6, induced a concentration-dependent inhibition of RSV A2 replication. This effect commenced immediately (observable within 24 h) following drug treatment on day 0 postinoculation and reduced viral quantity to below detectable limits, for the duration of the experiment, at a dose of 140 nM. When PC786 was administered at 28 nM, viral replication was not detected until day 4 and was reduced to below the level of detection once more by day 6. Viral replication was delayed using the lowest dose of PC786 (5.6 nM) and did not peak until day 5 (Fig. 2F).

**In vivo pharmacological effects.** Once-daily treatment with PC786, on days −1 to 3, by either intratracheal (i.t.) or intranasal (i.n.) administration, was found to inhibit viral
TABLE 6: Effects of PC786 administered either intratracheally or intranasally on RSV load (PFU) in the lungs of RSV A2-infected mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PC786 dose, mg/ml (approx mg/kg)</th>
<th>Virus titer, log PFU/ml of lung homogenate</th>
<th>Geometric mean ± SD</th>
<th>Median</th>
<th>Interquartile range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Intratracheal dosing</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle + virus</td>
<td>10</td>
<td></td>
<td>3.1 ± 0.36</td>
<td>3.1</td>
<td>2.7–3.4</td>
</tr>
<tr>
<td>PC786 + virus</td>
<td>9</td>
<td>2.0 (3.2)</td>
<td>&lt;0.33 (LOQ)</td>
<td>&lt;0.33</td>
<td>LOQ</td>
</tr>
<tr>
<td><strong>Intranasal dosing</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle + virus</td>
<td>8</td>
<td></td>
<td>3.0 ± 0.54</td>
<td>3.2</td>
<td>2.8–3.4</td>
</tr>
<tr>
<td>PC786 + virus</td>
<td>8</td>
<td>0.2 (0.19)</td>
<td>2.4 ± 0.59</td>
<td>2.5</td>
<td>1.9–3.0</td>
</tr>
<tr>
<td>PC786 + virus</td>
<td>8</td>
<td>2.0 (1.9)</td>
<td>&lt;0.33 (LOQ)</td>
<td>&lt;0.33</td>
<td>LOQ</td>
</tr>
</tbody>
</table>

**a**Milligrams per kilogram are calculated at a body weight of 25 g.

**b**LOQ, limit of quantification.

loads in the lungs of RSV A2-infected BALB/c mice. The viral load was below the level of detection when the drug was given at 2 mg/ml (40 μg/mouse [approximately 1.6 mg/kg of body weight] for i.t. treatment, or 80 μg/mouse [approximately 3.2 mg/kg] for i.n. treatment) (Table 6).

A study was also conducted with cotton rats, infected intranasally with RSV A Long, in which the animals were sacrificed 4 days after the infection. Once-daily intranasal treatment on days 0 to 3 with PC786 was found to reduce the viral load in lung homogenates on day 4, and the viral load was lower than the detection limit at doses of more than 3.3 mg/ml (165 μg/cotton rat [approximately 1.7 mg/kg, i.n.]) (Fig. 3A). In addition, PC786 showed a dose-dependent inhibition of RSV NS-1 gene transcripts (Fig. 3B) and of RANTES (Fig. 3C) and IP-10 (Fig. 3D) transcripts in lung homogenates.

**Inhibitory effects of PC786 on RSV polymerase and virus gene transcription by the RSV A-derived L protein-P complex.** PC786 concentration-dependently inhibited RSV RdRp activity in crude RSV A L-P complex, exhibiting an IC₅₀ and IC₉₀ of 2.1 nM and 21.8 nM, respectively. This profile is on the order of 75-fold to 163-fold more potent than that shown by AZ-27 (IC₅₀, 342 nM; IC₉₀, 1632 nM [Fig. 4A]). PC786 at 100 nM, which induces almost complete inhibition of RdRp activity, did not inhibit the signal from the extracts of nontreated cells, cells infected with the vaccinia virus (T7) only, or cells infected with vaccinia virus and transfected with P plasmid only (see Fig. S2).

PC786 was also found to produce potent, concentration-dependent inhibition of the expression of luciferase driven by a minigenome construct comprising the L protein, M2-1, N, and P of RSV A2 in HEp-2 cells (Fig. 4B). The maximum inhibition achieved by PC786 was 100% ± 0.09% at 140 nM, while the IC₅₀ and IC₉₀ were 0.476 nM and 1.54 nM, respectively. PC786 was found to be much more potent than the known RSV L protein inhibitors AZ-27 (IC₅₀, 10.2 nM; IC₉₀, 37.0 nM [Fig. 4B]) and BI compound D (IC₅₀, 55.3 nM; IC₉₀, 233 nM [Fig. 4B]). Furthermore, PC786 was also 435-fold to 540-fold more potent than ALS-8112 (IC₅₀, 207 nM; IC₉₀, 831 nM) in this minigenome assay. Only a little luciferase signal was observed in vaccinia virus only and M2-1/N/P complex only (without L protein), and PC786 at 140 nM did not show any inhibitory activities (see Fig. S3).

**Analysis of PC786 escape mutant.** RSV A2 viruses were repeatedly passaged in HEp-2 cells in the presence of increasing concentrations of PC786 (from 1.4 nM to 4,366 nM [up to 8,700-fold higher than the IC₅₀] [see Table S3]). After the 6th passage, escape mutants were obtained, which exhibited IC₅₀ in the CPE assays that were >650-fold weaker than those of the control virus (PC786: IC₅₀ of 0.027 nM against vehicle control virus and IC₉₀ of 17.9 nM against PC786 mutant virus) (Fig. 4C). Although the inhibitory activity of BI compound D on CPE was slightly reduced for the PC786 escape mutant, the same virus remained susceptible to an F protein inhibitor, GS-5806 (see Table S4). When the complete RSV genome was sequenced, the mutant at the 6th passage showed that two amino acid substitutions had occurred with high frequency; the first
was tyrosine 1631 to histidine or leucine (Y1631H, Y1631L) in the RSV L gene product and the second was the change of valine 153 to alanine or methionine (V153A or V153M) in the RSV M gene product. This mutation was also confirmed by single nucleotide polymorphism (SNP) genotyping analysis using PCR (Fig. 4D and E). Both mutants arose partially in the 3rd passage, but neither was confirmed in the 2nd passage (Fig. S4). Therefore, both mutants arose at the same passage, and it appeared to happen independently rather than consequently after one mutation. Phenotypic validation of the Y1631 mutation was conducted using the RSV minigenome assay, in which the plasmid encoding the Y1631H mutated RSV L gene together with wild type N, P, and M2-1 plasmids was cotransfected into HEp-2 cells. It was found that PC786 showed a 187-fold shift in the resulting IC50 obtained from the minigenome assay when run against the mutated L protein (IC50s, 0.48 nM in wild-type L protein and 89.7 nM in mutant L protein) (Fig. 4F).

**DISCUSSION**

Despite considerable effort, no safe and effective antiviral drugs to successfully treat RSV infections have emerged to date. Currently, surface proteins of RSV, such as the G and F proteins, are the principal molecular targets for the development of vaccines and small-molecule inhibitors. The RSV polymerase, which plays a central role in RSV transcription and genomic replication, is also an attractive, yet somewhat underexplored, target for therapeutic intervention. Here we report the discovery of PC786, a novel nonnucleoside small-molecule agent, and evaluate its effects. PC786 appears to inhibit the activity of the RSV RNA-dependent RNA polymerase within the large
polymerase subunit (L protein). It is an in vitro inhibitor of virus-induced CPE in HEp-2 cells resulting from the infection with laboratory-adapted RSV A2 as well as B Washington (WST) strains. PC786 demonstrates potent anti-RSV activities against several genetically diverse low-passage-number clinical isolates in the CPE assay, although the molecule is somewhat more potent against RSV A than against B strains (Table 3). We show the antiviral activity of PC786 to be highly specific for RSV, with no inhibition observed up to 14 μM against virtually all other viruses screened (Table 4). The exception is hepatitis C virus, against which PC786 shows modest activity in the replicon assay. Furthermore, PC786 demonstrates no measurable direct cytotoxic effects in cellular activity assays conducted at concentrations up to 14 μM in vitro (data not shown). Thus, PC786 inhibits RSV polymerase with high specificity over polymerases expressed by other viruses and human cells.

A highly desirable feature of inhaled medicines is extended longer duration of...

FIG 4 Mode of action of PC786. (A) Inhibition of PC786 on RSV RdRp activity in crude extracts from RSV L protein-P overexpressed in HEp-2 cells. (B) Inhibition of reporter fluorescent signal in RSV RdRp-driven minigenome assay. (C) Inhibitory activity of PC786 on CPE induced by control RSV A2 virus and by a PC786 escape mutant (Y1631H). (D) SNP genotype PCR analysis of viruses with wild-type and Y1631H mutant L protein. (E) SNP genotype PCR analysis of viruses with wild-type and V153A mutant M protein. (F) Minigenome analysis in HEp-2 cells transfected with wild-type and Y1631H mutant L protein plasmid.
action, thereby ensuring that therapeutic activity is maintained throughout the dosing interval. The persistence of action of PC786 was demonstrated in a washout study using BEAS2B cells (Fig. 2C). The observed persistence of effect of PC786 in the absence of RSV may be particularly valuable in the context of extended treatment dosing interval and the potential use of PC786 in prophylaxis. Once-daily treatment retains effective antiviral activity in both a human air-liquid interface (ALI)-cultured fully differentiated bronchial epithelial cells in vitro, as well as in mice and cotton rats in vivo. Of particular note is that PC786 maintains the ability to inhibit virus production irrespective of infectious particles (shown as multiplicity of infection [MOI]) and when added after peak virus production has occurred (Table 5 and Fig. 2A). In contrast, the antiviral activity of the F protein inhibitor GS-5806 was progressively diminished with increasing level of infectious particles (shown as MOI) or when administered after infection, as shown in Table 5 and Fig. 2B and previously reported (29). Because humans who are ill with RSV infection may come to medical attention at various times after the infection has started, this profile indicates that PC786 may be therapeutically advantageous for active RSV disease. Furthermore, its antiviral activity against low-passage-number human clinical isolates (Table 3) of RSV highlights these potential human therapeutic advantages.

Human RSV is able to infect and replicate in a number of animal species used for preclinical evaluation (30). Although primate species can be studied, most work of this nature is conducted with mice or cotton rats. Both standard, inbred mouse strains and cotton rats are characterized as semipermissive for the replication of human RSV, although significantly greater viral replication is seen in cotton rats than in mice. The anti-RSV activity of PC786 in vivo was therefore investigated in these species. Topical administration of PC786 to the lungs of mice or cotton rats subsequently challenged with RSV produced profound inhibition of virus production and exhibited a dose-dependent steep inhibition curve. In the cotton rat, PC786 was found to inhibit the RSV load, as determined by culture and PCR, in addition to gene expression of the proinflammatory molecules RANTES and IP-10 in lung homogenates. RANTES has been shown to correlate with RSV disease severity (31) and with RSV load in humans (32). Once-daily dosing indicated that treatment with PC786 resulted in sustained antiviral activity, a profile consistent with its persistence of action in vitro (Fig. 2C). Given that these rodent species require high inocula to establish an infection and have limited replication of RSV after infection, these models are more suitable for assessing treatments that target virus entry, rather than those reducing active intracellular replication. In fact, if administered early, several small-molecule F protein inhibitors and RSV monoclonal antibodies work well in these models (33). Therefore, we also tested the effects of PC786 on air-liquid interface-cultured fully differentiated human bronchial epithelial cells (ALI system), in which a high level of RSV replication can be sustained (Fig. 2E). Addition of RSV A2 at a low level of infectious particles (MOI, 0.01) resulted in a robust, persistent infection which generated amplified viral concentrations over 7 days. The cellular layer consisted of ciliated cells and some goblet cells, and the system shows apical shedding of progeny virions that are subsequently spread by the coordinated motion of the beating cilia, so mimicking human RSV infection (34, 35). The ALI model has been used for the evaluation of anti-RSV activities of several agents, such as RSV604 (36), ALS-8112 (37), and GS-5806 (38). In the ALI system, PC786 displayed a concentration-dependent inhibition of RSV A2 replication determined by plaque assay following daily apical exposure (mimicking a topical/aerosol delivery to the respiratory tract) commencing on day 0 and ending on day 7 postinoculation. Viral concentrations were reduced to below detectable limits for the duration of the experiment at the maximum dose investigated, 140 nM. Thus, PC786 showed a rapid onset of action (as it worked after short exposure) and produced sustained inhibition of RSV in this paradigm.

To determine the mode of action of PC786, an escape mutant was generated by passaging virus with escalating concentrations of PC786 (see Table S3). After the 6th passages with 4.4 μM PC786 (8,700× higher than the IC₅₀), we were able to isolate a
PC786-resistant virus that was >660-fold less sensitive to PC786 than was wild-type RSV A2 in a CPE assay (Fig. 4C). Sequencing analysis revealed that the resistant virus possessed a mutation at position 1631 in the L protein, from tyrosine to a histidine (Y1631H). This is the exact location described for mutants obtained using the other nonnucleoside polymerase inhibitors YM53403 and AZ-27 (24, 29). Phenotypic evaluation in a minigenome assay using a mutated L protein plasmid revealed that PC786 was 190-fold less active against this protein than against wild-type L protein, indicating that RSV L protein is a molecular target for PC786.

The active form of RSV polymerase is reported to be a heterodimer composed of the L and P proteins; the L protein is the catalytic subunit responsible for the RdRp function. The RSV L protein also has domains necessary for mRNA cap addition and methylation. AZ-27 was demonstrated to inhibit an early stage in mRNA transcription as well as genome replication by inhibiting initiation of RNA synthesis from the promoter. The compound did not inhibit back priming, mRNA capping, and it also did not affect association between the L and P proteins (25). AZ-27 has been also shown to inhibit synthesis of transcripts from the 3' end of the genome to a greater extent than those from the 5' end, indicating that it inhibits transcription initiation. AZ-27 was found to inhibit equally both mRNA transcription and genome replication in cell-based minigenome assays, indicating that it steps a step common to both of these RNA synthesis processes and that AZ-27 is an allosteric inhibitor of the RdRp function of RSV polymerase acting by a noncompetitive mechanism without affecting mRNA capping (39). Although a direct interaction of PC786 with a target RSV protein has not yet been demonstrated, PC786 has been shown to inhibit RSV RdRp activity in crude extracts from HEp-2 cells overexpressed with RSV L protein and P and to be highly active in a cell-based minigenome analysis (Fig. 3A and B). PC786 has therefore been shown to be an inhibitor of virus genomic RNA replication and mRNA transcription by its action on the RSV L protein. This is particularly important, as reduction in cellular expression of viral markers may be a key in rapid reduction of host immunopathology accomplishable by PC786 as opposed to nonreplication inhibitor antivirals. As PC786 induced an escape mutation at the same site as that elicited by AZ-27, the mode of action of PC786 may be similar to that of AZ-27. This allosteric inhibition by conformational change of a target protein is a common mechanism of action for nonnucleoside inhibitors of viral polymerases, such as HCV NS5B (40) and HIV reverse transcriptase (41). The L protein inhibitor BI compound D (27) was demonstrated to inhibit mRNA capping and may therefore be a different class of nonnucleoside inhibitor.

During the in vitro studies exposing RSV to PC786 (forced-mutation studies), a second genetic difference was observed in the M protein (change from valine 153 to alanine [V153A]), which was not reported for any anti-RSV agents, including AZ-27, BI compound D, and ALS-8112. Passage-dependent analysis revealed that the M protein mutation arose at the same time at which the L protein mutant arose, suggesting that it did not arise as a consequence of the L protein mutation (see Fig. S4). Matrix protein is known to be a key factor in virion morphogenesis (42). At an early stage of RSV replication, it is shuttled to the host cell nucleus, where it can cause modest inhibition of cellular transcription. Later, the M protein is found associated with cytoplasmic viral inclusion bodies (the site of viral RNA synthesis), in addition to the plasma membrane (the site of virion formation) (42). The M protein appears to silence viral RNA synthesis by nucleocapsids, presumably in preparation for their packaging into virions. Thus, the M protein has multiple functions which change at different stages of infection. We also conducted a minigenome assay in the presence or absence of M protein plasmid, but it did not affect the signal, suggesting that M protein did not affect promoter activation directly (Fig. S5). In addition, Do et al. have reported that the M protein is the most conserved protein between RSV A and RSV B and suggested it to be a promising target for novel anti-RSV agents (43), although genetic variations within M even occurring within a single patient with chronic RSV infection have been reported (44). It is, therefore, inferred that the principal target of PC786 inhibition arose from its action on the RSV L protein, and thereby the polymerase activity, and is probably attributable to...
disruption of the RSV M protein, which is involved in transcription repression, virus maturity, and budding. Presently, the significance of the M protein mutation found in association with exposure to PC786 is not fully understood, and further studies will be required.

In summary, we have identified a novel, selective, and highly potent small-molecule inhibitor that targets the replication complex of RSV and constitutes a promising candidate for the treatment of established RSV infection and disease in humans. Development to allow the study of PC786 in the clinic is under way.

MATERIALS AND METHODS

Materials. PC786, GS-5806 (45), AL5-8112 (46), BI compound D (27), AZ-27 (compound 11f) (26), and AZ compound 11j (26) were prepared by Sgnature Discovery Ltd. (Nottingham, UK; final purities, >98%). RSV A2 large polymerase (L) helper plasmid (NR-36461), RSV A2 matrix 2-1 (M2-1) helper plasmid (NR-36464), RSV A2 nucleoprotein (N) helper plasmid (NR-36462), and RSV A2 phosphoprotein (P) helper plasmid (NR-36463) were obtained through BEI Resources, NIAID, NIH (Manassas, VA). Plasmids with an RSV trailer/promoter-conjugated luciferase gene (47), mutant L protein expression vector, and wild-type M protein expression vector were obtained from Creative Biogene (Shirley, NY).

Cells and virus. Human larynx epithelial (HEp-2) cells (ATCC CCL-23) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in 10% fetal bovine serum (FBS)-supplemented Dulbecco modified Eagle medium (DMEM) with phenol red (4190-094: Life Technologies Ltd, Paisley, UK) at 37°C and 5% CO2. The simian virus 40 (SV40)-immortalized human bronchial epithelial cell line BEAS-2B (ATCC) was maintained in LHC-8 medium (Invitrogen, Paisley, UK) at 37°C and 5% CO2. MucliAir cells were provided as 24-well plate-sized inserts by Epithelix Sàrl (Geneva, Switzerland). Twice weekly, MucliAir inserts were transferred to a new 24-well plate containing 780 µl of MucliAir culture medium (EP04MM), and once weekly the apical surface was washed once with 400 µl of phosphate-buffered saline (PBS). MucliAir cultures were incubated at 37°C and 5% CO2. RSV A2 (National Collection of Pathogenic Viruses [NCPV], Public Health England, Wiltshire, UK) and RSV B Washington/1837/1962 (WST; VR1580; ATCC) were propagated in HEp-2 cells. Clinical RSV A isolates 1997/12-35, 2001/2-20, and 200/3-4 were obtained from BEI Resources, NIAID, NIH (Manassas, VA), and Memphis 37c was isolated and maintained at the University of Tennessee. RSV A 2001/3-12, 1998/3-2, and USU:v1225 were sourced and used at Utah State University. Low-passage clinical isolates (RSV A Memphis 37, 1211, 689, 492, and VAN1160 and RSV B 1276, 1262, 1000, HAN1135, and PEP121) were isolated and maintained in the University of Tennessee. Vaccinia virus (ATCC; VR-2153, vTF7-3[Wr] strain) expressing bacteriophage T7 RNA polymerase was also purchased from the ATCC and used as a donor of T7 polymerase.

CPE assay and cell viability in HEp-2 cells. For the assay of RSV A2 and RSV B Washington (WST) strains as well as low-passage-number clinical isolates, a 96-well-format resazurin-based cytopathic effect (CPE) assay was conducted. Approximately 24 h after the cells were seeded into 96-well black plates (200 µl of 5% FBS phenol red-free DMEM/well) at a density of 3 × 104/ml, the cells were infected with RSV A2, RSV A Long, or RSV A clinical strains (multiplicity of infection [MOI], 0.2), RSV A Memphis 37 (MOI, 0.6), RSV B WST (MOI, 0.2), or 9320 (VR955) or other RSV clinical strains (MOI, 1). Plain medium was also added to nontreatment wells as noninfection controls. Immediately after the infection, the compounds (except for ALS-8112) and neat dimethyl sulfoxide (DMSO; 0.5 µl/well) were added as appropriate to obtain a final concentration of 0.5% DMSO across all wells. ALS-8112 was applied 24 h before virus inoculation. The plates were incubated for 5 days for RSV A and clinical RSV B strains and 6 days for RSV B WST (37°C and 5% CO2). After removal of supernatant or collection of supernatant for subsequent PCR analysis, 200 µl of resazurin solution (0.0015% in PBS) was added to each well and the plates were incubated for a further 1 h at 37°C and 5% CO2. The fluorescence of each well (545 nm [excitation] and 590 nm [emission]) was then determined. The percent inhibition for each well was calculated against infection control and the IC50 and IC90 were calculated from the concentration-response curve generated for each test compound. For assessment of cell viability, test compounds or neat DMSO as a vehicle (1% final concentration) were added to each well of confluent HEp-2 cell culture in 96-well plates (200 µl/well in phenol red-free DMEM/well) and incubated for 5 or 6 days (37°C and 5% CO2). Cells were then incubated with resazurin solution and the fluorescence level of resorufin (metabolized materials) was determined as described above. Where appropriate, a 50% cytotoxicity concentration (CC50) was calculated from the concentration-response curve generated for each test compound.

For RSV A 2001/3-12, 1998/3-2, and USU:v1225, CPE was induced in HEp-2 cells for 7 days and the plates were then stained with neutral red dye for approximately 2 h. The intracellular incorporated dye was extracted in 50:50 Sorensen citrate buffer-ethanol and the absorbance read on a spectrophotometer. The optical density (OD) of test wells was converted to percentage of cell and virus controls. The concentration of test compound required to inhibit CPE by 50% (EC50) was calculated by regression analysis. The virus inocula were approximately 63 50% cell culture infectious doses (CCID50) per well for 2001/3-12, 2000 CIID50/well for 1998/3-2, and 32 CCID50/well for USU:v1225. This assay was conducted in Utah State University (Logan, UT).

For known clinical strains (RSV A 1997/12-35, 2000/2-20, 200/3-4, and Memphis 37), a 384-well-format resazurin-based CPE assay was conducted. Approximately 24 h after the cells were seeded in 384-well black plates (353962; BD Falcon, Oxford, UK) at a density of 2 × 104/ml (50 µl/well in phenol red-free DMEM [31053-028; Life Technologies Ltd.] with 5% FBS, 2 mM l-glutamine [Life Technologies Ltd.], and...
1 mM sodium pyruvate (Life Technologies Ltd.), the cells were infected with RSV at an MOI of 1 (50 μl/well in phenol-free DMEM, 2 mM L-glutamine, and 1 mM sodium pyruvate). The plain medium was also added to nontreatment wells as noninfection controls. The compounds and neat DMSO (as a vehicle) were added to appropriate wells (0.5 μl/well) using the Integra ViaFlo (Integra Biosciences, Hudson, NH) to obtain a final concentration of 0.5% DMSO across all wells. The plates were incubated for 5 days (37°C and 5% CO₂). Resazurin solution (5 μl of 0.03% solution in PBS; R7017; Sigma-Aldrich, Dorset, UK) was then added to each well using a Thermo Multidrop Combi (Thermo Fisher Scientific), and the plates were incubated for a further 6 h at 37°C and 5% CO₂. The fluorescence of each well (545 nm [excitation] and 590 nm [emission]) was determined using a multiscanner (Clariostar; BMG, Buckinghamshire, UK).

**Time-of-addition assay and MOI dependency.** Twenty-four hours after seeding of HEp-2 cells in a 96-well plate, culture medium was aspirated from cells and replaced with 100 μl of phenol red-free DMEM containing 5% FBS. The appropriate virus concentration was diluted in 100 μl of phenol red- and serum-free DMEM to obtain final MOIs of 0.02, 0.1, and 1.0 (1,200, 6,000, and 60,000 PFU, respectively). This diluted medium was also added to noninfected wells to yield a final PBS concentration of 2.5% via an addition of 200 μl of medium to all wells. Then, 1-μl quantities of compounds and the vehicle (DMSO) were also added to obtain appropriate doses, such that the final DMSO concentration was constant across the plate at 0.5%. Compound addition was repeated on separate plates at 24, 48, or 72 h postinoculation. All plates were incubated for 6 days at 37°C and 5% CO₂. Following the aspiration of all supernatants, 50 μl of methylene blue solution (2% formaldehyde, 10% methanol, and 8% methylene blue in 100 μl of PBS) was added to each well for 1 h. Methylene blue solution was then aspirated and cells were washed twice with 200 μl of PBS before being solubilized using 100 μl of 1% SDS solution for 1 h. The absorbance of each well was then read using a multiscanner at a wavelength of 663 nm (Clariostar; BMG, Buckinghamshire, UK). The percent inhibition for each well was calculated against vehicle-treated infected control wells.

**qPCR.** Viral RNA was extracted using EZ1 virus minikit v2.0 (Qiagen, Hilden, Germany) and EZ1 Advanced XL robot (Qiagen) per the manufacturer’s protocol. Viral RNA was eluted with 60 μl of AVE elution buffer and stored at −80°C. The reverse transcription and quantitative PCR (qPCR) employed an ABI 7500 FAST real-time PCR system (Applied Biosystems International, Waltham, MA). The reverse transcription and qPCR were performed in one step by amplifying a portion of the N gene using RSV A- and B-specific primers and probes (48). The PCR mixture contained the following: 12.5 μl of 2 × reaction mix (Invitrogen), 0.5 μl of SuperScript III RT/Platinum Taq mix (Invitrogen), 0.75 μl of ROX reference dye (Invitrogen), 0.25 μl of RNase inhibitor (Applied Biosystems; 20 U/μl), 0.045 μl (for A strains) and 0.015 μl (for B strains) of forward primers (Invitrogen; 500 nM), 0.05 μl of each primer pair (A strains: Biosearch Technologies, Petaluma, CA; 50 μM), 10 μl of template RNA, and 0.76 μl (for A strains) and 0.79 μl (for B strains) of RNase-free water (Invitrogen). Each PCR plate contained a negative control in which RNase-free water was substituted for RNA. Each sample was run in duplicate in 96-well plates with internal standards comprising duplicate pairs of six 10-fold dilutions of RSV RNA extracted from parallel aliquots containing a known quantity of RSV A Long (ATCC VR-26) and RSV 9320 (ATCC VR-955), as defined by and as used in the plaque assay. Results are presented as means of duplicates in log (base 10) PFU equivalents per milliliter (log PFUe/ml). The PCR employed the following thermal cycler settings: 15 min at 50°C and 2 min at 95°C, followed by 45 cycles of 15 s at 95°C, 33 s (AT) and 30 s (AT) for A strains, and 15 s at 95°C, 30 s (AT) and 30 s (AT) for B strains. The resultant signal was determined colorimetrically (OD at 450 nm [OD₄₅₀] with a reference wavelength of 655 nm) in a microplate reader (Multiskan FC; Thermo Fisher Scientific). Cells were then washed, and 0.5% crystal violet solution (50 μl/ml) was applied for 30 min. After two washings with PBS (200 μl), 1% SDS (100 μl) was added to each
well, and the plates were shaken lightly for 1 h prior to reading the absorbance at 595 nm as an indicator of cell number in each well. The measured F protein signals (OD_{450} – OD_{655} readings) were corrected for cell number (OD_{595} readings) by dividing the OD_{450} readings by the OD_{595} readings.

Virus infection on ALI-cultured bronchial epithelial cells and plaque assay. On the day of infection (day 0), the apical surface of each insert was washed once with 300 µl of sterile PBS and the inserts were then transferred to new 24-well plates containing 780 µl of fresh MucilAir culture medium (Epithelix Sârl). RSV A2 was diluted in MucilAir culture medium to obtain a final inoculation concentration of 2,000 PFU in 50 µl (an approximate MOI of 0.01) and incubated onto cells for 1 h at 37°C and 5% CO₂. The virus inoculum was removed with a pipette and inserts were washed twice with 300 µl of sterile PBS. A day 0 sample was harvested by adding 300 µl of sterile PBS to the apical surface of each well for 5 min. The 300-µl sample was then removed and transferred to 0.5-ml tubes containing 100 µl of 50% sucrose dissolved in PBS, and the tubes were stored at −80°C. This harvesting procedure was repeated daily until day 7. PC786 was dosed apically onto MucilAir inserts. PC786 solution diluted with PBS at 1 in 200 (50 µl; 0.5% DMSO in final solution) was applied to the apical surface and incubated at 37°C and 5% CO₂ for 1 h before being removed. The dosing regimen was performed daily on day 0 to day 6 following each sample collection. On day 5, the basal medium was removed from every well and replenished with fresh MucilAir culture medium as a necessary maintenance step for ALI culture cells. Virus titer in the apical sample collection. On day 5, the basal medium was removed from every well and replenished with fresh MucilAir culture medium as a necessary maintenance step for ALI culture cells. Virus titer in the apical sample collection.

Coates et al. Antimicrobial Agents and Chemotherapy
September 2017 Volume 61 Issue 9 e00737-17 aac.asm.org

Downloaded from http://aac.asm.org/ on August 24, 2017 by Imperial College London
CTP (catalog number NEL581001EA; PerkinElmer), 200 nM RNA (5'-UUUGUUCGCGCU; Sigma-Aldrich), and 4 µM 5' biotin-conjugated RNA primer (5'-biotin-ACGC; Sigma-Aldrich) and then mixed and incubated in the dark for 5 min at RT. The primer extension reaction was started by adding crude RSV polymerase L protein-P complex (20 µg of protein in 3 µl) to the reaction buffer prepared as described above, the buffer was incubated at 30°C for 120 min, and the reaction was stopped by the addition of 10 µl of 50 mM EDTA. The solution was transferred to a streptavidin-coated 96-well black plate (Pierce 15119; Thermo Fisher Scientific), incubated for 45 min in the dark, and then washed twice with 20 mM Tris-HCl containing 0.1% Tween 20 (pH 7.5). Diethyl pyrocarbonate (DEPC)-treated H2O (60 µl) was added to the plate to aid detection, and the fluorescence level of Cy5 was measured using the Clariostar reader (BMG Labtech, Buckinghamshire, UK), with the following wavelengths (bandwidth): excitation, 610 (30 nm); emission, 675 (50 nm); and dichroic, 637.5 nm.

**Minigenome analysis.** The RSV trailer/promoter-conjugated luciferase plasmid reported previously (47) was manufactured by Creative Biogene (NY). Briefly, four overlapping oligonucleotides were annealed to form a 238-bp DNA fragment containing a terminal BamHI site, the upstream 32-nucleotide (nt) nonstructural protein 1 (NS1) nontranslated region, the 10-nt RSV NS1 gene start signal, a 44-nt RSV leader sequence, a 94-bp hammerhead ribozyme, a 47-nt T7 terminator, and a NotI-compatible end. A 191-bp DNA fragment was synthesized in vitro, containing a terminal HindIII site, a 155-nt RSV trailer sequence, the 12-nt RSV L gene end sequence, a 12-nt nontranslated region of RSV L, and an Xhol site (Integrated DNA Technologies, Skokie, IL). These two fragments were ligated along with a BamHI/Xhol fragment of firefly luciferase cDNA (pGEM-luc; Promega Corp., Fitchburg, WI) into the NotI and HindIII sites of pcDNA3.1 such that an antisense copy of luciferase flanked by RSV leader and trailer regulatory elements was produced by T7 polymerase transcription. On the day of transfection, growth medium was removed from HEp-2 cells and replaced with 50 µl of serum-free DMEM containing vaccinia virus (expressing T7 polymerase) at an MOI of 0.1, with one row of cells receiving serum-free medium only as uninfected controls. Cells were incubated at 37°C and 5% CO2 for 1 h, during which time plasmids were prepared for transfection using Lipofectamine 2000 as per the manufacturer’s instructions. A master mix was prepared allowing for 0.00625 µg of M2-1, N-, P-, and Luc-encoding plasmids and 0.003215 µg of L protein-encoding plasmid per 60 µl of Opti-MEM to be administered per well, with a final ratio of Lipofectamine 2000 to DNA of 3 µl to 1 µg. Medium containing vaccinia virus was removed and the transfection reagents were added to the cells and left to 2 h at 37°C and 5% CO2 before a further 140 µl of DMEM containing 2% FBS was added to the cells. Compounds were administered in 1-µl volumes at this time point and cells were further incubated for 48 h at 37°C and 5% CO2. Luminescence was then detected using the luciferase assay system (Promega Corp.) as follows. Cells were lysed in 100 µl of 1% Triton X-100 (Promega Corp.), and 20 µl of each well was transferred to a black flat-bottomed 96-well plate (Thermo Fisher Scientific, Waltham, MA). Each well was injected with 100 µl of luciferase assay reagent, and luminescence was detected for 5 s by a multiscaner (ClarioStar). A single nucleotide change, T13388C (full genome numbering), was engineered into the L protein sequence to generate the Y163H mutation in the L protein (Creative Biogene), and a pcDNA3.1(+) vector harboring mutated L protein was also used for a minigenome analysis.

**Mutation induction.** HEp-2 cells, maintained in DMEM with phenol red supplemented with 10% FBS at 37°C and 5% CO2, were subcultured twice a week. Approximately 72 h prior to the beginning of the experiment, cells were seeded in T-75 flasks in 10% FBS DMEM to be fully confluent on the day of infection. RSV A2 (MOI of 2.0) was added to T-75 flask of HEp-2 cells and incubated for 2 h at 37°C and 5% CO2, after which the virus was removed and replaced with 12 ml of the medium containing the appropriate concentration of drug. Following drug treatment, the virus growth milieu was replaced with 0.003215 g of M2-1-, N-, P-, and Luc-encoding plasmids and 0.003215 g of L protein-encoding plasmid per 60 µl of Opti-MEM to be administered per well, with a final ratio of Lipofectamine 2000 to DNA of 3 µl to 1 µg. Medium containing vaccinia virus was removed and the transfection reagents were added to the cells and left to 2 h at 37°C and 5% CO2 before a further 140 µl of DMEM containing 2% FBS was added to the cells. Compounds were administered in 1-µl volumes at this time point and cells were further incubated for 48 h at 37°C and 5% CO2. Luminescence was then detected using the luciferase assay system (Promega Corp.) as follows. Cells were lysed in 100 µl of 1% Triton X-100 (Promega Corp.), and 20 µl of each well was transferred to a black flat-bottomed 96-well plate (Thermo Fisher Scientific, Waltham, MA). Each well was injected with 100 µl of luciferase assay reagent, and luminescence was detected for 5 s by a multiscaner (ClarioStar). A single nucleotide change, T13388C (full genome numbering), was engineered into the L protein sequence to generate the Y163H mutation in the L protein (Creative Biogene), and a pcDNA3.1(+) vector harboring mutated L protein was also used for a minigenome analysis.

**Virus full-genome sequence.** Viral RNA was extracted from serially passaged virus stocks using a QiAamp viral RNA minikit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Approximately 55 µl of extracted RNA was then dispatched to GENEWIZ Inc. (South Plainfield, NJ) for full-genome sequencing using the MiSeq technique, and single nucleotide polymorphisms (SNPs) were identified against a template RSV A2 sequence (GenBank accession number M745661). SNPs that appeared with a frequency of more than 20% in a routinely used laboratory strain of RSV were identified as drift mutations that emerged in-house during routine passage at Pulmocide Ltd. These nucleotide changes were used to amend the submitted RSV A2 sequence template so that a direct comparison could be made against in-house RSV A2 isolates as opposed to the published sequence mentioned above. SNPs which appeared with a frequency of over 10%, or that matched previously reported
mutation sites, were analyzed to see if they elicited a codon change. Silent mutations were excluded from the report.

PCR genotype assay. To confirm the genotype of the compound-treated viruses, a TaqMan SNP genotype assay to detect mutant (Y163H) and wild-type viornas was designed. Viral RNA was extracted from the appropriate virus samples by MagMAX Express, and these were used to produce cDNA by reverse transcription using Invitrogen SuperScript VILO MasterMix (SuperScript; Life Technologies) as per the manufacturer’s instructions. The TaqMan SNP genotyping PCR assay was run using TaqMan genotyping master mix (catalog number 4371355; Life Technologies) in the StepOne Plus PCR instrument, with enzyme activation at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and then 60 s at 60°C, with fluorescence measured. The data were analyzed using StepOne software V2.3 to determine which allele of the target gene was present in each solution. The primers used for this SNP assay were as follows: forward (GAAGACACTCAACCTTACACATGAT), reverse (ACACTGATGGATCTTGGAGTCTT), reporter VIC conjugated (TGGTGGATGATAAATTGTTGAA), reverse (TCCCATTTCAACAGATCTATATAATAATATTGCTTTC), reporter VIC conjugated (TGTGGATGATAACT ATG), and reporter FAM conjugated (TTGAGTGATCATATG) for L protein.

In vivo RSV infection. BALB/c mice (male, 20 to 30 g) were inoculated intranasally on day 0 with RSV A2 (0.055 × 10^5 PFU/mouse) in Pneumolabs UK. Animals were sacrificed 4 days after the inoculation, and the lungs were collected for preparation of lung homogenate. PC786 was prepared as suspension in 10% DMSO–90% isotonic saline and delivered intranasally by installation of 20 μl/mouse. The comparison between two groups was performed by unpaired ANOVA, nonparametric Kruskal-Wallis analysis followed by Dunn’s multiple-comparison test was conducted. Multiple comparison was performed by analysis of variance (ANOVA) followed by Dunnett’s multiple-comparison test performed using the PRISM 6 software program. If no significance was achieved using ANOVA, nonparametric Kruskal-Wallis analysis followed by Dunn’s multiple-comparison test was conducted. The comparison between two groups was performed by unpaired t test with Welch’s correction or the Mann-Whitney test. Statistical significance was defined as a P value of <0.05.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00737-17.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

ACKNOWLEDGMENT
The following reagents were obtained through BEI Resources, NIAID, NIH: RSV A2 large polymerase helper plasmid (NR-36461), RSV A2 matrix 2-1 helper plasmid (NR-36464), RSV A2 nucleoprotein helper plasmid (NR-36462), and RSV A2 phosphoprotein helper plasmid (NR-36463).

REFERENCES
8. Thompson WW, Shay DK, Weintraub E, Brammer L, Cox N, Anderson...
RSV L Protein Polymerase Inhibition by PC786

Antimicrobial Agents and Chemotherapy

September 2017 Volume 61 Issue 9 e00737-17 aac.asm.org


