RESEARCH PAPER

Late therapeutic intervention with a respiratory syncytial virus L-protein polymerase inhibitor, PC786, on respiratory syncytial virus infection in human airway epithelium

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BACKGROUND AND PURPOSE
Effective anti-respiratory syncytial virus (RSV) agents are still not available for clinical use. Current major targets are virus surface proteins, such as a fusion protein involved in viral entry, but agents effective after RSV infection is established are required. Here we have investigated the effects of late therapeutic intervention with a novel inhaled RSV polymerase inhibitor, PC786, on RSV infection in human airway epithelium.

EXPERIMENTAL APPROACH
Air liquid interface-cultured bronchial or small airway epithelium was infected with RSVA2. PC786 was applied apically or basolaterally once daily following peak virus load on Day 3 post inoculation. Apical wash was collected daily for determination of viral burden by PCR and plaque assay (primary endpoints) and biomarker analyses. The effects were compared with those of ALS-8112, an anti-RSV nucleoside analogue, and GS-5806, a fusion-protein inhibitor, which were treated basolaterally.

KEY RESULTS
Late intervention with GS-5806 did not show significant anti-viral effects, but PC786 produced potent, concentration-dependent inhibition of viral replication with viral load falling below detectable limits 3 days after treatment commenced in airway epithelium. These effects were superior to those of ALS-8112. PC786 showed inhibitory activities against RSV-induced increases of CCL5, IL-6, double-strand DNA and mucin. The effects of PC786 were also confirmed in small airway epithelium.

CONCLUSION AND IMPLICATIONS
Late therapeutic intervention with the RSV polymerase inhibitor, PC786, reduced the viral burden quickly in human airway epithelium. Thus, PC786 demonstrates the potential to be an effective therapeutic agent to treat active RSV infection.

Abbreviations
ALI, air liquid interface; RdRp, RNA-dependent RNA polymerase; RSV, respiratory syncytial virus

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Introduction

Although respiratory syncytial virus (RSV) is the most common cause of acute infection of the lower respiratory tract in children (Nair *et al.,* 2010), attempts to develop an effective therapy have so far proved unsuccessful. Novel anti-RSV agents are normally screened using cancer cell lines and rodent models semi-permissive for replication of human RSV, using prophylactic or simultaneous treatment strategies (Derscheid and Ackermann, 2012; Taylor, 2017). In addition, primary human bronchial cells in monolayer cultures are much less susceptible to RSV infection than the commonly used laryngeal carcinoma cell line, HEp-2 (now known as HeLa cell contaminant) (Wright *et al.,* 2005). Thus, these systems do not provide suitable translational assays.

The primary site of RSV replication in the human respiratory tract is the cytoplasm of epithelial cells comprising the pseudostratified lining of the bronchial airway. Several studies have implied a preferential tropism of RSV for ciliated epithelial cells in particular (Zhang *et al.,* 2002; Villenave *et al.,* 2013). Furthermore, pathophysiological investigations of RSV infection in lower respiratory tract or small bronchiole epithelia, obtained from deceased children who died within their first week of infection (Welliver *et al.,* 2007) or later (Johnson *et al.,* 2007), revealed the presence of RSV, by immunostaining, only in small airway epithelial cells, without basal cell involvement.

The air liquid interface (ALI) culture cellular layer consists of ciliated cells and some goblet cells, and this system shows apical shedding of progeny virions that are subsequently spread by the coordinated motion of the beating cilia, so mimicking human RSV infection. In fact, ALI epithelium was reported to demonstrate robust RSV replication (Villenave *et al.,* 2012; Pickles, 2013), and the level of RSV load was similar to that observed in nasal washes from RSV infected infants (DeVincenzo *et al.,* 2005) or after nasal challenge with RSV Memphis 37 strain to healthy subjects (Kelly *et al.,* 2015). Thus, ALI epithelium is now being used as a model to study the behaviour of respiratory pathogens in human tissue (Essaidi-Laziosi *et al.,* 2017).

Recently, new compounds intended for the treatment of RSV have been reported. Most target surface proteins such as the fusion (F) protein, including the oral inhibitors GS-5806 (Phase II) (DeVincenzo *et al.,* 2014), AK0529 (ClinicalTrial.gov, 2016), BTA-C585 (ClinicalTrials.gov, 2016a) and JNJ-53718678 (ClinicalTrials.gov, 2016b) and the inhaled nanobody ®ALX-0171 (Phase II) (Detalle *et al.,* 2016). RNA virus polymerase is increasingly recognized as an attractive target for antiviral drug development, for instance, against hepatitis C virus. RSV also expresses RNA-dependent RNA polymerase (RdRp) activity within the RSV-large (L) protein, which transcribes and replicates its negative-sense RNA genome. Recently, several compounds that inhibit RSV RdRp activity have been described, including YM-53403 (Sudo *et al.,* 2005), AZ-27 (Xiong *et al.,* 2013; Noton *et al.,* 2015) and Boehringer Ingelheim Compound D (Liuzzi *et al.,* 2005). However, these agents are generally weak, have undesirable selectivity for A versus B strains and/or have poor retention in the lung. ALS-8176, an orally bioavailable prodrug of the novel anti-RSV nucleoside ALS-8112 (Phase II) (DeVincenzo *et al.,* 2015), is the most advanced in clinical development and treatment inhibited RSV replication in healthy subjects experimentally infected with RSV. Effects on active RSV disease have yet to be reported.

PC786 (*N*-(2-fluoro-6-methylphenyl)-6-(4-(5-methyl-2-(7-oxa-2-azaspiro[3.5]nonan-2-yl)nicotinamido)benzoyl)-5,6-dihydro-4*H*-benzo[b]thieno[2,3-d]azepine-2-carboxamide) is a novel non-nucleoside low MW RSV polymerase inhibitor, which demonstrated profound inhibition of both RSV A and B strain replication coupled with a long duration of action, which has been optimized for topical inhalation treatment (Coates *et al.,* 2017). It is currently in early clinical development (clinicaltrials.gov identifier: NCT03236233, NCT03382431) (ClinicalTrial.gov, 2017a; ClinicalTrial.gov, 2017b). The aim of the study described here was to evaluate the effects of a late therapeutic intervention with the RSV polymerase inhibitor, PC786, in a translational model of human infection and compare its effects with those of an F-protein inhibitor, GS-5806, and an anti-RSV nucleoside analogue, ALS-8112, which are in clinical development. Furthermore, as apical treatment of PC786 is used here in the ALI epithelium culture system, the concept of inhaled therapy using PC786 could be validated.

Methods

Cells and virus

Human larynx epithelial (HEp-2) cells (HeLa cell contaminant) (ATCC® CCL-23™) were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA) and maintained in 10% FBS-supplemented DMEM with phenol red (# 4190-094: Life Technologies Ltd, Paisley, UK) at 37°C/5% CO_2. MucilAir™ bronchial epithelium or SmallAir™ small airway epithelium was provided fully differentiated as 24-well plate-sized inserts by Epithelix Sàrl (Geneva, Switzerland). Primary human airway cells were obtained from patients with lung cancer undergoing surgical lobectomy. Those patients were not smokers (except for one donor of SmallAir™) and also not diagnosed as having COPD, asthma or other respiratory diseases. Cells were isolated from the lung tissue without any characteristics of cancer cells based on pathological analysis (Supporting Information Table S1). All experimental procedures were explained in full, and all subjects provided informed consent. The study was conducted according to the declaration of Helsinki on biomedical research (WMA, 1989) and received approval from the local ethical commission. Twice weekly, MucilAir™/SmallAir™ inserts were transferred to a new 24-well plate containing 780 μL of MucilAir™ culture medium (EP04MM), and the apical surface was washed weekly with 400 μL PBS (once). MucilAir™ cultures were incubated at 37°C, 5% CO_2. RSV A2 strain was obtained from the National Collection of Pathogenic Viruses (Public Health England, Salisbury, UK) and passaged in HEp-2 cells containing DMEM supplemented with 2% (v/v) FBS to generate a virus stock solution. Fifty per cent (w/v) sucrose in PBS was added to clarified culture supernatants to a final volume of 12.5% (v/v) sucrose solution.

Antiviral compounds

PC786, ALS-8112 and GS-5806 (Mackman *et al.*, 2015) were synthesized by Sygnature Discovery Ltd. (Nottingham, UK; with final purities >98%) and reconstituted in DMSO to stock concentrations of 2 mg·mL^{-1} for PC786 and GS-5806 or 20 mg·mL^{-1} for ALS-8112. Single-use aliquots of PC786 (topical medicine) and ALS-8112 or GS-5806 (oral medicines), intended for application to the apical surface or basolateral compartment of MucilAir™/SmallAir™ cultures, respectively, were produced following serial dilution in DMSO and frozen to provide 200-fold the desired final working concentration of compound. When required, aliquots were thawed at room temperature and diluted in PBS (for apical treatments) or MucilAir™ culture medium (for basolateral compartment treatment) to provide the desired concentration of compound and ensure final DMSO concentration of 0.5% for each condition. DMSO was diluted similarly and used as vehicle control for apical or basolateral treatment.

Infection and treatment of MucilAir™ or SmallAir™ culture

Prior to infection, MucilAir™ or SmallAir™ cultures were washed once with PBS and transferred to a new 24-well plate containing MucilAir™ culture medium. Virus was inoculated by adding 2000 plaque-forming unit (PFU; an approximate multiplicity of infection of 0.01) of RSV stock solution to the apical surface of each well for 1 h. Virus inoculum was then removed and the apical surface washed twice with PBS. A third apical wash using 300 μL of PBS was collected and added to 100 μL PBS containing 50% (w/v) sucrose to generate a baseline (Day 0) for viral load and cytokine assessment. On other days (Days 1–10), 300 μL of PBS was applied to apical surface, and the first wash was collected daily for viral load and cytokine assessment. Following sample collection on Days 3–7 (or Day 1 only for single dose experiment), MucilAir™ or SmallAir™ inserts were treated apically with PC786 for 1 h (then removed) or supplemented basolaterally with PC786, ALS-8112 or GS-5806. An apically or basolaterally treated vehicle control infection was performed, against which the appropriate dosing method could be compared. On Day 5 for MucilAir™ or Day 8 for SmallAir™, the basolateral medium was removed from all wells and replenished with fresh MucilAir™ culture medium or SmallAir™ culture medium as a necessary maintenance step for MucilAir™ or SmallAir™ inserts.

Determination of viral load by plaque assay

HEp-2 cells were seeded into 24-well plates (Corning, Corning, NY, USA) at a density of 5–10 × 10^4 cells per well and grown for 48 h prior to infection in 10% FBS DMEM until they attained 100% confluency. Collected samples were thawed at room temperature and 10-fold serial dilutions were prepared in serum-free DMEM. The growth medium from HEp-2 cells was aspirated and replaced with 300 μL of serially diluted virus collections and left to infect at 37°C and 5% CO$_2$ for 4 h. The infectious media were aspirated and replaced with 1 mL of Plaque Assay Overlay [0.3% Avicel RC-591 (FMC Biopolymer UK, Girvan, Scotland) in MEM, supplemented to a final concentration of 2% FBS] and left for 7 days at 37°C/5% CO$_2$. Cells were fixed with ice-cold methanol for

10 min before methanol was removed, and cells were washed twice with sterile PBS. Cells were then stained with 200 μL 0.1% Crystal violet solution (in distilled water) for 1 h. Crystal violet solution was removed, and cells were rinsed with water before plaques were counted and viral load enumerated.

Viral RNA extraction and quantitative RT-PCR (qRT-PCR) for RSV A nucleoprotein

Viral RNA was extracted from collected samples and the RSV A2 inoculation stock solution using a MagMAX™-96 Viral RNA isolation kit (Ambion by Life technologies), according to the manufacturer's instructions before being subjected to quantitative PCR analysis using the One-Step qRT-PCR system (Primer Design Limited, Southampton, UK). Briefly, 5 μL of extracted viral RNA was mixed with 10 μL One-Step qRT-PCR master mix, 4 μL RNase/DNase-free water and 1 μL of the RSV A primer/probe mix (Cat # Path-RSV-A-standard, Primer Design, Southampton, UK) per reaction, with reactions being performed in duplicate. PCR plates were sealed with MicroAmp™ optical adhesive film (Cat #4311971, Life Technologies, Paisley, UK) and briefly centrifuged at 1200 RPM. The One-Step PCR reaction and subsequent amplification analysis was carried out using an Applied Biosystems StepOnePlus™ Real-Time PCR System (Cat #4376598, Life Technologies) using the following condition: 55°C for 10 min and 95°C for 8 min, followed by 50 cycles of qPCR at 10 s at 95°C and 60 s at 60°C. Reactions containing 10-fold serial dilutions of RNA extracted from the stock RSV A2 virus solution were used to generate a standard curve against which the RSV RNA content measured from test samples was quantified.

Cytokine analysis

Collected apical washes were subjected to cytokine analysis using standard Ultra-Sensitive Meso Scale Diagnostics (MSD) assays for **CCL5**, V-Plex IL-8 MSD assays for **CXCL8**, Human IP-10 Tissue Culture Kit for **CXCL10**, and Human proinflammatory 9-plex TC assay or Human **IL-6** Tissue Culture Kit for IL-6 (all: MSD, Rockville, MA, USA). Measurement of CCL5, CXCL10 and CXCL8/IL-6 required dilution of samples 1:2, 1:5 and 1:10, respectively, in Reagent Diluent. The electrochemiluminescence signal of serially diluted standard samples, provided with each assay kit, was measured using an MESO QuickPlex plate reader and used to generate a standard curve using Discovery Workbench 4.0 software for each analyte. Each apical wash sample was quantified using these standard curves.

Mucin quantification

Mucin concentrations were quantified using an enzyme-linked lectin assay based on the protocol previously described (McCoy Jr. *et al.*, 1984). Briefly, samples were sonicated for 10 min and added to high-bind ELISA plates coated in lectin from *Triculum vulgaris*. A standard curve was prepared using serially diluted mucin of known concentration from bovine submaxillary gland (Cat #M3895, Sigma-Aldlich, Dorset, UK). Following incubation at 37°C for 30 min, the plates were washed three times with a wash buffer before the addition of detection reagent (containing HRP-conjugated Glycin max soybean lectin) for a further 30 min at 37°C. Following a

further wash cycle, a substrate solution containing H_2O_2 and tetramethylbenzidine (R&D Systems, Minneapolis, MN, USA) was added and allowed to develop for 5 min. The reaction was terminated using 2 N H_2SO_4 and absorbance read immediately at 450 nM with 570 nM as reference. Standard curves were created using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA), and these were used to calculate the concentration of mucin in all samples.

Double-stranded DNA quantification

The concentration of double-stranded DNA (dsDNA) in apical washes was quantified using a Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies) according to the manufacturer's instructions. Here, a standard curve was prepared by serially dilution of a 2 $\mu g \cdot mL^{-1}$ stock of dsDNA (Phage λ DNA) and then incubated in the presence of the Quant-iT™ PicoGreen® dsDNA reagent for 3 min. Samples were added to the plates at a 1:2 dilution in TE buffer and incubated for 3 min in the presence of Quant-iT™ PicoGreen® dsDNA reagent, and the fluorescence of each well [545 nm (excitation) / 590 nm (emission)] was determined using a monochromator microplate reader (CLARIOstar®: BMG Labtech, Buckinghamshire, UK). A standard curve was created using MARS data analysis software (BMG Labtech, Buckinghamshire, UK) and the equation used to calculate concentration levels including the dilution factor used.

Histology

MucilAir inserts were collected after the final apical wash with PBS on Day 10 post virus inoculation, which were treated with DMSO or PC786 (700 nM) on Days 3–7 post RSV inoculation. The inserts were fixed with 4% paraformaldehyde at 4°C for 24 h and then stored in PBS at 4°C until use. Fixed epithelium with membrane was removed from inserts, processed overnight and embedded in paraffin wax, which was sectioned (5μm) with a microtome. The prepared paraffin sections were stained with haematoxylin and eosin (Gills III haematoxylin, Leica Biosystems, Milton Keyne, UK, and 0.5% eosin, Pioneer Research Chemical, Colchester, UK) or 1% Alcian blue 8GX in 3% acetic acid (pH 2.5) (Alcian blue, Leica Biosystems and acetic acid, Sigma Aldrich, UK) and observed using optical microscope (Olympus, BX40 using DP Controller 1.1.1.65 software).

Determination of PC786 content in cell membrane

RSV A2 virus was inoculated to epithelium in ALI inserts, and a single treatment of PC786 was applied once, 24 h after virus inoculation. Cells from bronchial epithelium sheet were collected at each time point at 24, 48, 60, 72, 84, 96, 108 and 120 h after single compound application, and the content of PC786 in the cells was determined. Assays to determine the concentration of PC786 were conducted by LGC Limited (Fordham, Middlesex, UK). Briefly, frozen cells with membrane were sonicated in 300 μL of methanol for 5 min and centrifuged. The supernatant (250 μL) was removed and blown dry. The sample was reconstituted in 150 μL of MeCN:water (50:50, v/v) and the level of PC786 measured by LC–MS/MS (LLQ was 10 $pg \cdot mL^{-1}$, ULQ was 20 000 $pg \cdot mL^{-1}$).

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015). Each treatment was tested using three inserts from three or five different donors, and each marker was measured in duplicate per sample. According to the power calculation ($P < 0.05$, 0.8 power) shown in Supporting Information Table S2, $n = 3$ paired samples are sufficient for the primary and secondary endpoints (viral load AUC, virus reduction slope, viral load reduction in 72 h), although the calculation revealed that the cytokine assays (exploratory endpoints) are inadequately powered. In the work described here, we used five donors for the main studies for all analysis (effects of PC786 on ALI bronchial epithelium), but we have opted to maintain $n = 3$ sample size for ALI small airway analysis and also for comparators. Results were represented as mean ± SEM. AUC (Days 3–10), with or without subtraction of background (the value at Day 0 or lower limit of detection), was calculated using trapezoid area method in Excel template in Dotmatics system (Dotmatics Ltd., Hertfordshire, UK). The percentage inhibition of AUC values was calculated by comparing the values of wells treated with compound with wells treated with vehicle alone (0.5% DMSO). Values were generated from the mean of three individual wells per treatment condition. The IC_{50} values were also calculated using GraphPad Prism. Multiple comparison was performed by Friedman test followed by Dunn's multiple comparison test performed using the PRISM 6® software program. If a parametric test is applicable based on post hoc ANOVA test, Turkey's multiple comparison test was also conducted. The comparison between two groups was performed by unpaired t-test with Welch's correction. Statistical significance was defined as $P < 0.05$.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/2018(Alexander et al., 2017a,b).

Results

PC786 and ALS-8112 inhibit RSV replication determined by plaque assay in bronchial epithelial MucilAir™ cells after late intervention

Inoculation of MucilAir™ ALI bronchial epithelium with RSV A2 resulted in a robust infection that produced detectable virus replication up to Day 10 based on viral load determined by plaque assay (Figure 1A), and each individual demonstrated similar kinetics, although Donor 2 induced weaker RSV replication (Supporting Information Figure S1A).

Apical treatment with PC786 demonstrated a concentration-dependent inhibition of RSV A2 replication following initiation of treatment on Day 3 post virus inoculation, with viral load falling below detectable limits by Day 6 (3 days after treatment commenced) at the maximum concentration of
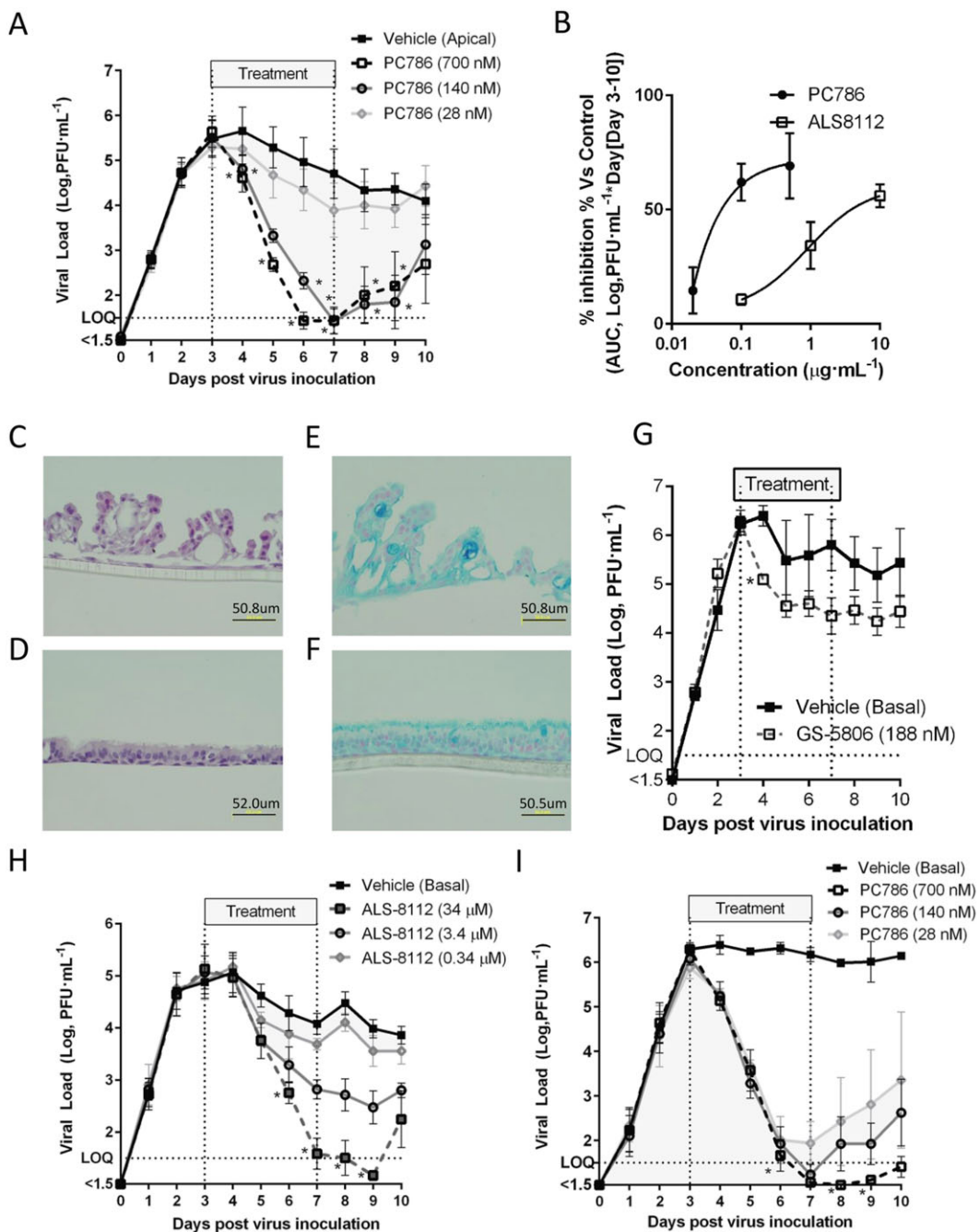
Figure 1

Anti-viral effects of PC786 and comparators on RSV-infected bronchial ALI epithelium. (A) Replication kinetics of RSV A2 in MucilAir™ inserts following virus inoculation on Day 0 and successive daily apical treatment with PC786 from Day 3 to Day 7 post virus inoculation. Replicating virus was quantified *via* plaque assay analysis, and the level of viral load was shown as plaque forming units (PFU). The horizontal dashed line shows the lower limit of quantification (LOQ) for each assay (33 PFU·mL⁻¹). Each point represents the geometric mean value of five independent donors (±SEM). Concentration–response curve of PC786 and ALS8112 on inhibition of viral load AUC (Days 3–10) (B). Representative images (×400) of histology of ALI inserts collected on Day 10. Haematoxylin and eosin staining of RSV-infected control (C) and PC786, 700 nM (D). Alcian blue staining of control (E) and PC786, 700 nM (F). The effects of basolateral treatment of GS-8112 (G), ALS-8112 (H), PC786 (I) or vehicle (0.5% DMSO) from Day 3 to Day 7. *$P < 0.05$, significantly different from infection control.

700 nM (Figure 1A). PC786 inhibited viral load $AUC_{3-10 \text{ days}}$ (Log PFU·mL·days⁻¹) in a concentration-dependent manner (Table 1, Figure 1B), and the IC_{50} value was 154 nM (Table 1). PC786 also showed concentration-dependent responses on the slope of virus reduction a day in the first 48 h, log reduction of viral load during the first 72 h after treatment and the viral load at Day 8 (5 days after the beginning of treatment and 1 day after the final dose) (Table 1).

Table 1

Effects of PC786 and ALS-8112 on several parameters in RSV viral load (plaques assay)

	PC786 (nM), apical treatment, $n = 5$				ALS-8112 (nM), basolateral treatment, $n = 3$			
	Vehicle	28	140	700	Vehicle	340	3400	34 000
AUC viral load from baseline (Day 3) through Day 10 (Log10 PFU × day·mL^{-1}) and % inhibition	23.6 ± 3.10	20.5 ± 3.29 (15 ± 4.5%) IC$_{50}$: 154 ± 54.9 nM (54–316)	9.36 ± 1.96[a] (62 ± 3.6%)	8.05 ± 2.50[a] (69 ± 6.4%)	20.4 ± 0.906	18.3 ± 0.50 (11 ± 1.0%) IC$_{50}$: 15500 ± 5730 nM (7420–30 200)	11.5 ± 0.953 (34 ± 4.6%)	8.94 ± 0.610[a] (56 ± 2.2%)
Time to be <3 Log PFU·mL^{-1} of RSV load (days post dose)	>7	>6.6 ± 0.87	2.8 ± 0.2[a]	2.2 ± 0.2[a]	>7	>7	4.7 ± 0.93.	3.3 ± 0.26
Viral load slope: baseline to 48 h after first dose (Log10 PFU·mL^{-1}/24 h)	−0.096 ± 0.071	−0.31 ± 0.10	−1.1 ± 0.24	−1.5 ± 0.15[a]	−0.13 ± 0.043	−0.38 ± 0.14	−0.66 ± 0.058	−0.69 ± 0.17
Baseline to 72 h post first dose (Log10 PFU·mL^{-1})	−0.52 ± 0.20	−0.96 ± 0.28	−3.2 ± 0.30	−4.2 ± 0.22[a]	−0.59 ± 0.14	−1.0 ± 0.33	−1.8 ± 0.25	−2.4 ± 0.22[a]
Viral load at 5 days after first dose (24 h after the last treatment) (Log10 PFU·mL^{-1})	4.34 ± 0.471	4.00 ± 0.524 [0.33 Log↓]	1.802 ± 0.404[a] [2.5 Log↓]	2.01 ± 0.629[a] [2.3 Log↓] Concentration for 1Log↓: (37.2 ± 6.88 nM)	4.47 ± 0.171	4.10 ± 0.133 [0.37 Log↓]	2.71 ± 0.240 [1.8 Log↓]	1.50 ± 0.260[a] [3.0 Log↓] Concentration for 1Log↓: (1870 ± 824 nM)

Values are presented as mean ± SEM.
[a]$P < 0.05$, significantly different from infection control.

Although virus load increased again 10 days after virus inoculation (3 days after the final dose) (Figure 1A), a full genome sequence analysis by next-generation sequencing using Illumina confirmed that no mutant virus was present in the population at Day 10 (data not shown). Thus, it seems likely that viral replication re-emerged once apical PC786 treatment was stopped. The ALI inserts from Donors 4 and 5 were collected on Day 10 for histology. As shown in Figure 1C and Supporting Information Figure S2A, most of ciliated cells were disappeared in RSV-infected control ALI inserts in both donors. In contrast, PC786-treated epithelium retained ciliated epithelial cells in both donors (Supporting Information Figure S2A), and especially one of the donors showed no or limited cytopathic effects (Figure 1D), which showed similar morphology to that of ALI insert without any intervention or manipulation (Supporting Information Figure S2B). As PC786 was treated after the peak of viral load, airway epithelium was repaired during PC786 treatment which inhibited virus replication. Alcian blue staining did not show goblet cell hyperplasia in RSV-infected control cultures as the cell damage was too marked (Figure 1E, F).

Although epithelia were also treated basolaterally with GS-5806 (188 nM: 0.1 $\mu g \cdot mL^{-1}$), an F protein inhibitor, using this late intervention regimen (as this is developed as an oral drug), it showed little or no reduction of viral load when treated after the peak of viral load (Figure 1G).

Another comparator, ALS-8112, a nucleoside analogue, was treated in the basal chamber to replicate the systemic delivery conditions of an orally administered drug. The basolaterally treated vehicle control condition showed similar kinetics, with viral load peaking on Day 4, to the apically treated control condition (Figure 1H vs. A, Supporting Information Figure S1A, B). Treatment with ALS-8112 also produced concentration-dependent inhibition of virus replication, but at much higher concentrations, reducing viral load to below detectable limits by Day 9 (6 days after treatment commenced) at the maximum dose of 34 000 nM (Figure 1H). The IC_{50} value of $AUC_{3-10\ days}$ of Log $PFU \cdot mL^{-1}$ was 15 500 nM, 101-fold less potent than PC786, and the dose response curve was shallower for ALS-8112 than PC786 (Table 1, Figure 1B). The further analysis shown in Table 1 demonstrates that treatment with ALS-8112 produced a slower onset and less powerful anti-RSV activity than did PC786.

Thus, apical treatment with PC786 was much more potent than basolateral treatment with ALS-8112. However the different treatment route might affect the outcome. As seen in Figure 1I, basolaterally treated PC786 strongly inhibited virus load, and the difference in antiviral effects between PC786 and ALS-8112 was enhanced. PC786 also strongly inhibited RSV PCR load, CCL5, IL-6, CXCL10 and mucin (Supporting Information Figure S3). Thus, the difference between the effects of PC786 and those of ALS-8112 were not due to the different treatment route.

PC786 and ALS-8112 inhibit the level of RSV genomic material in bronchial epithelial MucilAir™ cells after late intervention

PCR analysis revealed that inoculation of RSV A2 to MucilAir™ ALI bronchial epithelium also resulted in a robust infection that produced detectable virus genome up to Day 10. Both vehicle-treated conditions (apically or basolaterally) displayed similar PCR signal kinetics with viral load peaking on Day 4 (Figure 2A, B). Both PC786 and ALS-8112 showed concentration-dependent inhibition of RSV A2 replication estimated by PCR signal following initiation of treatment on Day 3. By Day 6, PCR analysis showed that PC786 (700 nM) had reduced the measurable genetic material to 2.8 Log, $PFUe \cdot mL^{-1}$, suggesting 2.6 Log, $PFUe \cdot mL^{-1}$ reduction from the level on Day 3 before treatment (Figure 2A). ALS-8112 also showed 1.5 Log, $PFUe \cdot mL^{-1}$ reduction at 34 000 nM on Day 9 (Figure 2B).

PC786 and ALS-8112 inhibited biomarkers associated with RSV replication in human bronchial MucilAir™ cells after late intervention

Inoculation of RSV A2 to ALI cells resulted in an increase in detectable levels of CCL5 which peaked at Day 5 and increased again on Day 10 (Figure 2C). Treatment with PC786 beginning on Day 3 resulted in a concentration-dependent decrease in the levels of CCL5 (Figure 2C). The % inhibition of the $AUC_{3-10\ days}$ calculated using the relative values against the value on Day 3 was 68.9% at 700 nM (Table 2).

Inoculation of RSV A2 also resulted in an increase in detectable levels of IL-6 which peaked at Day 4 (Figure 2D). Treatment with PC786 significantly and concentration-dependently inhibited IL-6 concentrations with 73.6% inhibition at 700 nM (Figure 2D, Table 2). Although inoculation of RSV A2 resulted in an increase in CXCL8 which peaked at Day 4 (Supporting Information Figure S3A), PC786 did not show any effects on CXCL8 production at any concentrations (Table 2, Supporting Information Figure S3A). PC786 also showed a concentration-dependent inhibition of CXCL10, although the sample from Donor 1 was not measured due to insufficient amount of samples (Supporting Information Figure S3B).

As well as cytokines, RSV A2 also increased the levels of dsDNA, a surrogate marker of epithelial cell damage, which gradually increased after inoculation over 10 days (Figure 2 E). PC786 at 700 nM prevented the elevation of dsDNA seen in the vehicle control samples during treatment (Day 3–7) and showed inhibition of AUC that was not statistically significant (Figure 2E, Table 2).

In addition, RSV A2 caused robust mucin production in donors except for Donor 2, who showed the lowest peak viral load and almost no mucus production (Supporting Information Figure S1A and S3C, D). Therefore, the average values of mucus production of four donors (except for Donor 2) were plotted in Figure 2F, although the average of all donors were plotted in Supporting Information Figure S3C. The vehicle-treated condition displayed a sharp peak of mucus production on Day 4 (Figure 2F). PC786 showed a concentration-dependent inhibition of RSV A2-induced mucin release following the commencement of treatment on Day 3 (Figure 2F, Table 2).

ALS-8112 also caused a concentration-dependent inhibition of CCL5, IL-6 and CXCL8 at much higher concentrations than those of PC786, but it did not show any obvious effects on either dsDNA or mucin (Table 2).
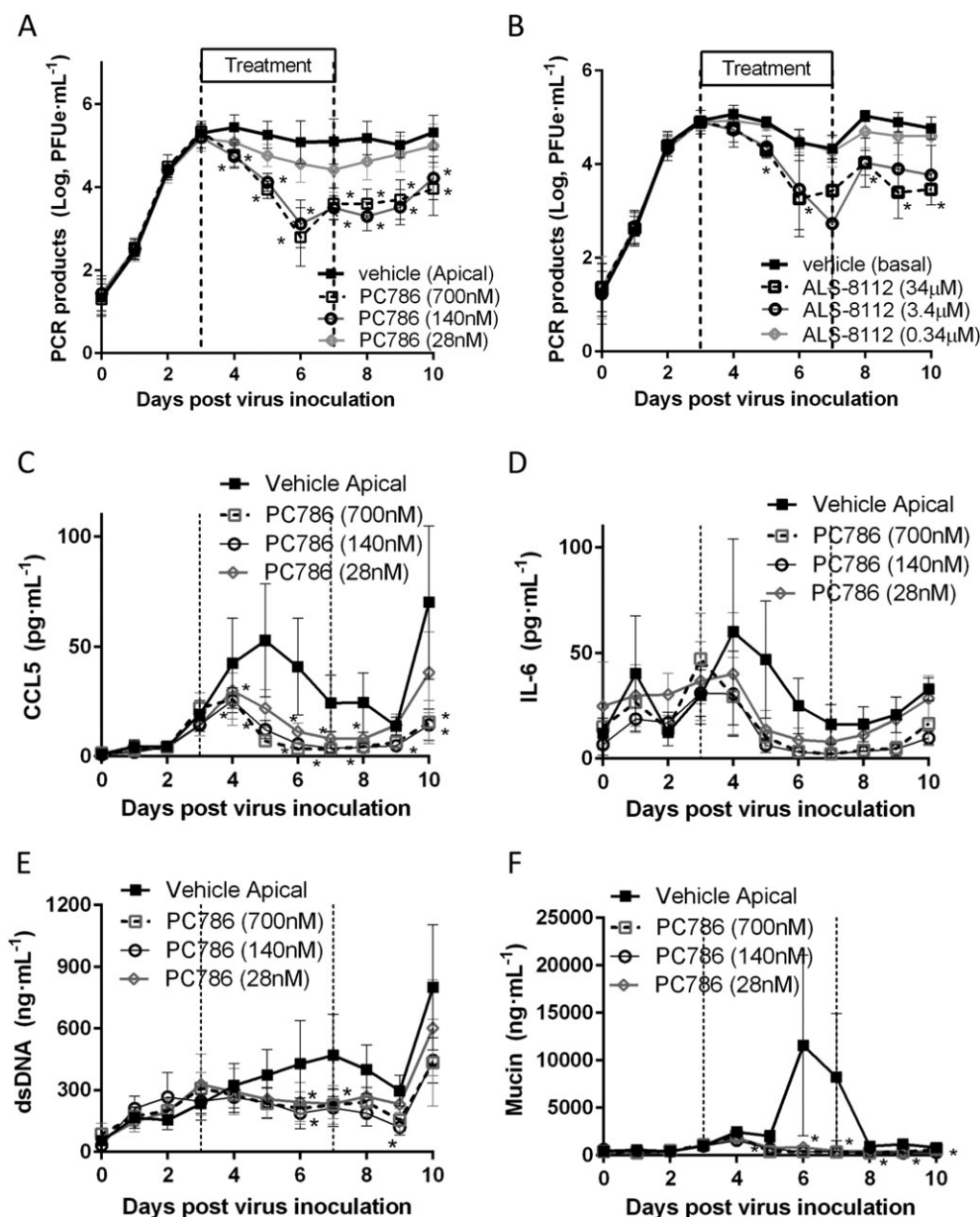
Figure 2

Effects of PC786 on viral genome and biomarkers. Viral genome concentrations in apical wash was quantified by quantitative RT-PCR using primers targeted to the N gene for the experiment with PC786 (A) and ALS-8112 (B). Each experiment was conducted in triplicate, and three to five donors were used for this study. CCL5 (C), IL-6 (D), dsDNA (E) and mucin (F) production in apical washes collected post RSV inoculation (Day 0) with treatment with either vehicle or PC786 (3–7 days post-inoculation). Each point represents the mean ±SEM, from five independent donors, except in (F) from four donors. *$P < 0.05$, significantly different from infection control.

The relationship between pharmacokinetics (PK) and pharmacodynamics (PD) of PC786 in RSV A2 infected MucilAir™ cells after single application

To investigate the PK/PD relationship, a simple pilot study was designed. A single treatment of PC786 was applied once, 24 h after virus inoculation, and the samples were collected *via* daily washes from the apical surface of MucilAir™ inserts. A single application with either 140 or 700 nM of PC786 on

Day 1 post infection resulted in an immediate inhibition of RSV A2 replication to below detectable levels in this system, with the highest dose (700 nM) preventing subsequent re-detection of replicating virus for 5 days (Figure 3A). Once the antiviral effect of the single dose had been lost, RSV A2 demonstrated similar growth characteristics to the vehicle-treated control infection, implying a similarity of this virus to the original inoculum. The PCR products targeting RSV N gene also showed similar kinetics to the plaque assay

Table 2

Effects of PC786 and ALS-8112 on the levels of biomarkers in apical wash [AUC Days 3–10 (treatment phase)]

Compound	Concentration nM	% inhibition of virus-infected control (AUC$_{Days\ 3\ to\ 10}$, relative value vs. Day 3)					
		CCL5	IL-6	CXCL8	CXCL10	dsDNA	Mucin
PC786	28	18.1 ± 21.2	54.9 ± 8.41	4.3 ± 14.7	10.6 ± 26.2[a]	10.0 ± 28.3	44.5 ± 19.9 [47.7 ± 22.7][c]
(n = 5)	140	50.1 ± 15.1	69.3 ± 11.5	11.3 ± 21.3	24.8 ± 37.5[a]	20.5 ± 19.8	54.9 ± 15.1 [57.6 ± 17.2][c]
–	700	68.9 ± 5.73*	73.6 ± 7.67*	2.2 ± 17.2	64.0 ± 11.2[a]	47.6 ± 8.07	42.3 ± 20.4 [60.4 ± 14.0][c]
ALS-8112	340	−33.8 ± 29.8	−19.5 ± 52.3	4.87 ± 46.4	−30.0[b]	−64.1 ± 54.9	−36.0 ± 17.8 [−47.8][d]
(n = 3)	3400	30.5 ± 11.4	44.1 ± 3.04	23.2 ± 29.0	27.4[b]	−12.3 ± 35.7	4.71 ± 19.1 [23][d]
–	34 000	34.1 ± 10.9	55.7 ± 3.54	45.4 ± 23.9	11.0[b]	−34.7 ± 27.4	−11.4 ± 25.6 [13][d]

Values are presented as means ± SEM (n = 2–5).

[a]n = 4 (except for Donor 1);
[b]n = 2 (except for Donor 1);
[c]n = 4 (except for Donor 2),
[d]n = 2 (except for Donor 2),
*P < 0.05, significantly different from infection control.

(Figure 3B). The level of PC786 was 393.3 pg per epithelium-sheet, 24 h after treatment, which was 8% of the amount originally applied, and reduced gradually over 5 days to 53.4 pg per epithelium-sheet. Thus, high levels of PC786 persisted in epithelium for several days after treatment. As shown in Figure 4C, there was a good correlation between PC786 content in cells and reduction of viral load determined by plaque assay and RT-PCR. In addition, this early intervention with a single dose of PC786 was found to markedly inhibit production of CCL5, IL-6, CXCL8, dsDNA and mucin (Figure 3D–H).

PC786 inhibits RSV A2 replication in SmallAir™, human small airway epithelium after late intervention

We also studied ALI-cultured small airway epithelium (SmallAir™), which were characterised with a population of CC-10 positive Club cells rather than goblet cells (Huang *et al.*, 2017). Inoculation of RSV A2 to these cells (*n* = 3 donors) also resulted in a robust infection that produced detectable replicating virus up to Day 10 based on viral load determined by plaque assay. The peak load (Log, PFU·mL^{-1}) of vehicle control (0.5% DMSO in media treated apically) was 5.73 on Day 4, which gradually reduced to 4.56 on Day 10 post inoculation, therefore showing similar kinetics to that in bronchial epithelial cells (MucilAir™) (Figure 4A vs. 1A). PC786 demonstrated a concentration-dependent inhibition of RSV A2 replication following initiation of treatment on Day 3 post virus inoculation, with PC786 reducing viral load to below detectable limits on Day 6 at both 140 and 700 nM and on Day 7 at 700 nM (Figure 4A), as observed in MucilAir™ (Figure 1A). The IC$_{50}$ of AUC (Log, PFU·mL·Day$_{3-10\ days}^{-1}$) was 108 nM, again similar to the value for bronchial epithelial cells (MucilAir™). The inhibitory activity was also confirmed by viral PCR analysis (Figure 4B). The epithelium also produced CCL5 and dsDNA (Figure 4C, D), which were both inhibited by PC786.

Effects of combination of PC786 and ALS-8112

In this ALI system, the effects of the combination of a low, non-effective, concentration of apical PC786 with a high concentration of basolateral ALS-8112 were investigated (Figure 5A). As expected, 28 nM of PC786 showed only little or marginal effects, and ALS-8112 at 34 μM showed a marked anti-viral effect, but it did not inhibit the viral load to undetectable level. However, combination of PC786 and ALS-8112 strongly inhibited viral load to the lower limit of quantification on Day8 to Day10. These results suggested that the combination had eliminated virus completely. AUC viral load calculations also showed significant anti-viral effects only in combination (Figure 5B).

Discussion

We have previously published a 'basic' or 'standard' biological data set outlining the profile of PC786, using cancerous or immortalised cell lines *in vitro* and mouse or cotton rat *in vivo*, which are semi-permissive to human RSV infection (Coates *et al.*, 2017). However, the viral replication period is too short in all of these systems to evaluate a therapeutic treatment regimen, as opposed to prophylaxis. This is a
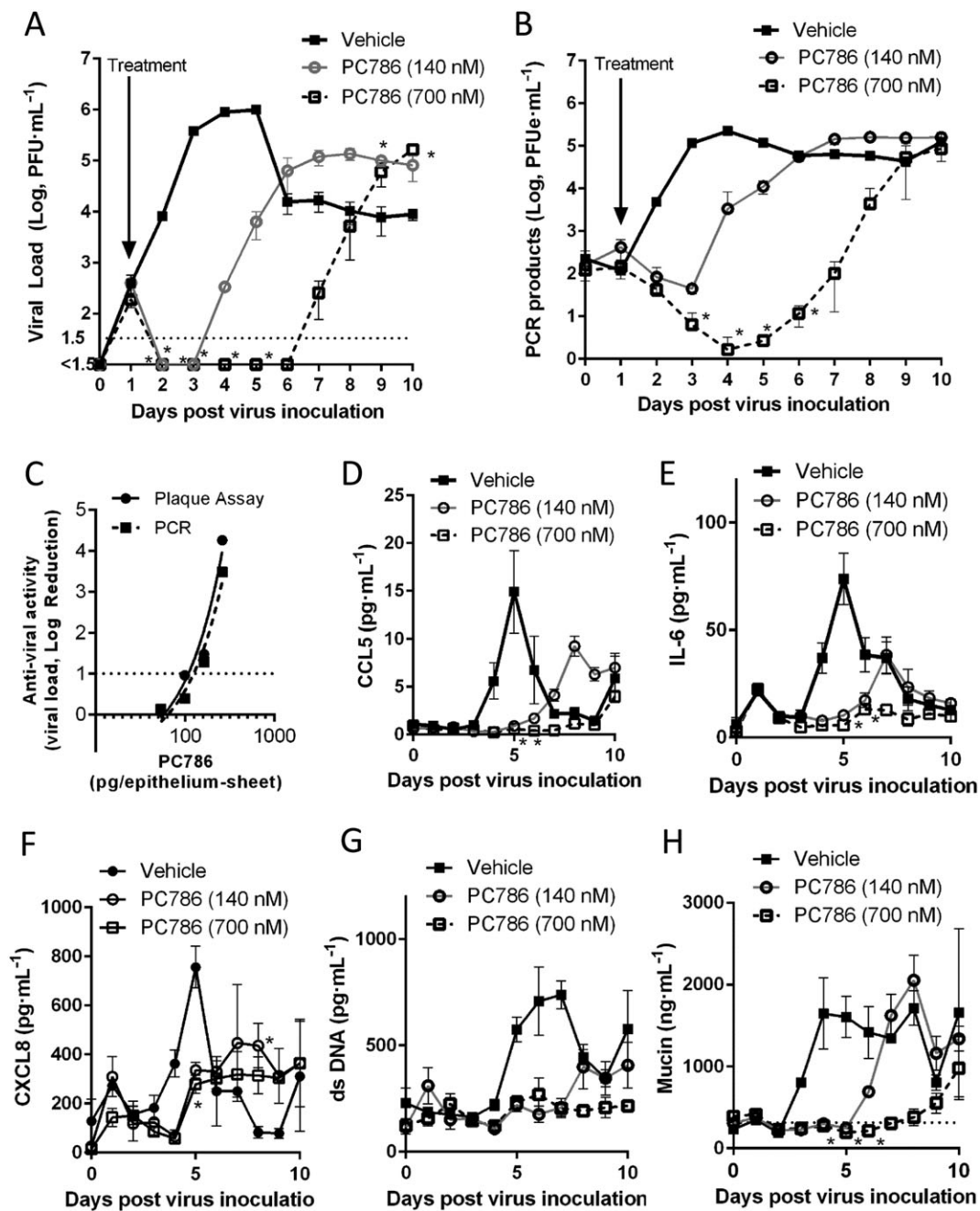
Figure 3

Effects of single dose of PC786 on RSV A2 replication assessed by plaque assay (A) and PCR targeting RSV N genes (B) in MucilAir™. PC786 was administered apically a day after virus inoculation. Apical wash was collected daily and used for analysis. (C) The correlation between PC786 contents in epithelium sheet in MucilAir™ and anti-viral activities of PC786 determined by plaque assay and PCR. Effects of a single dose of PC786 on concentrations of biomarkers, such as CCL5 (D), IL-6 (E), CXCL8 (F), ds DNA (G) and mucin (H) in MucilAir™. PC786 was applied apically only once a day after virus inoculation, and apical wash was collected daily. Each point represents the geometric mean±SEM (for viral load) and mean±SEM (others). *$P < 0.05$, significantly different from infection control.

fundamental problem for current RSV anti-viral research in the absence of a translational model. The situation is exacerbated here, as PC786 is an inhaled compound, and the standard systems do not adequately test the concept. ALI epithelium is a primary cell culture and closely mimics human trachea or small airway architecture and reported to

show apical shedding of progeny virions that are subsequently spread by the coordinated motion of the beating cilia, so mimicking human RSV infection (Villenave et al., 2012; Pickles, 2013). Using these systems, we have been able to treat compounds at the peak of virus load, so mimicking the clinical situation of treating an established infection.
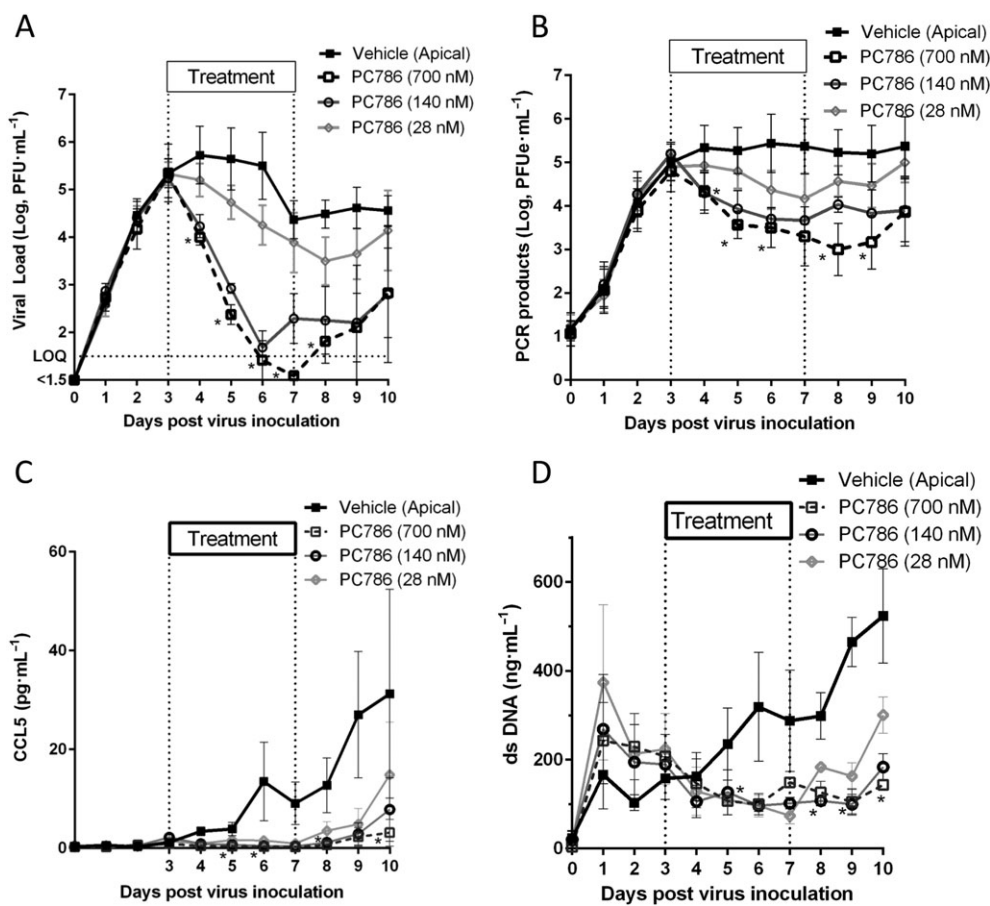
Figure 4

(A) Replication kinetics of RSV A2 in SmallAir™ inserts following virus inoculation on Day 0 and successive daily apical treatment with PC786 from Day 3 to Day 7 post virus inoculation. Replicating virus was quantified *via* plaque assay analysis, and the level of viral load was shown as PFU. The horizontal dashed line shows the lower limit of quantification (LOQ) for each assay (33 PFU·mL^{-1}). Each point represents the mean (±SEM) from three independent inserts. (B) RSV-N gene was detected by RT-PCR in same apical wash. CCL5 (C) and dsDNA (D) were determined, and the relative value to the value on Day 3 when the treatment has been commenced was shown. Each point represents the geometric mean (±SEM) for viral load and mean (±SEM) for others, from three independent donors. *$P < 0.05$, significantly different from infection control.

Thus, these results provide an important advance in the characterization of PC786 over our already published study. We believe that the widespread use of the ALI-cultured epithelium systems has the potential to reduce animal work substantially and can have a significant effect on future drug development, as well as helping to predict the likely clinical performance of PC786, specifically.

As indicated above, ALI airway epithelium is a promising translational model in preclinical studies of RSV infection. We found a robust and persistent infection after addition of RSV A2 at a low level of infectious particles (0.01 MOI) (Figure 1A). This system also offers several advantages for experimental design that can be exploited while assessing the antiviral activity of novel agents. First, the characteristic apical facing surface of ALI cultures allows for direct application of topical agents, such as PC786, to this surface of infected cells, so serving as a model for the planned route of delivery. Second, RSV infection of ALI epithelium does not result in extensive cytopathic destruction of cellular tissue, allowing for repeated manipulation of ALI inserts for an

extended duration of time compared with immortalised (cancer) cell lines. Consistent with the anticipated use of PC786 as a therapeutic intervention, our experiments were designed to mimic the circumstance of viral infection being well established before treatment could commence by allowing viral replication to proceed for 72 h before the first application of PC786. This model has been used to evaluate the anti-RSV activities of several agents, such as RSV604 (Chapman *et al.,* 2007), ALS-8112 (Deval *et al.,* 2015), PC786 (Coates *et al.,* 2017) and GS-5806 (Perron *et al.,* 2015) albeit not using therapeutic treatment.

Using our therapeutic regimen, significant antiviral effects of GS-5806, an F-protein inhibitor, were not observed (Figure 1G). *In vitro* time-of-addition studies revealed that the anti-viral activity of F-protein inhibitors was dependent on the time of treatment, relative to time of virus inoculation (Tiong-Yip *et al.,* 2014; Coates *et al.,* 2017). Thus, as this compound inhibits virus entry, therapeutic treatment of ongoing virus replication was ineffective. In contrast, late therapeutic intervention with PC786 displayed a concentration-dependent,
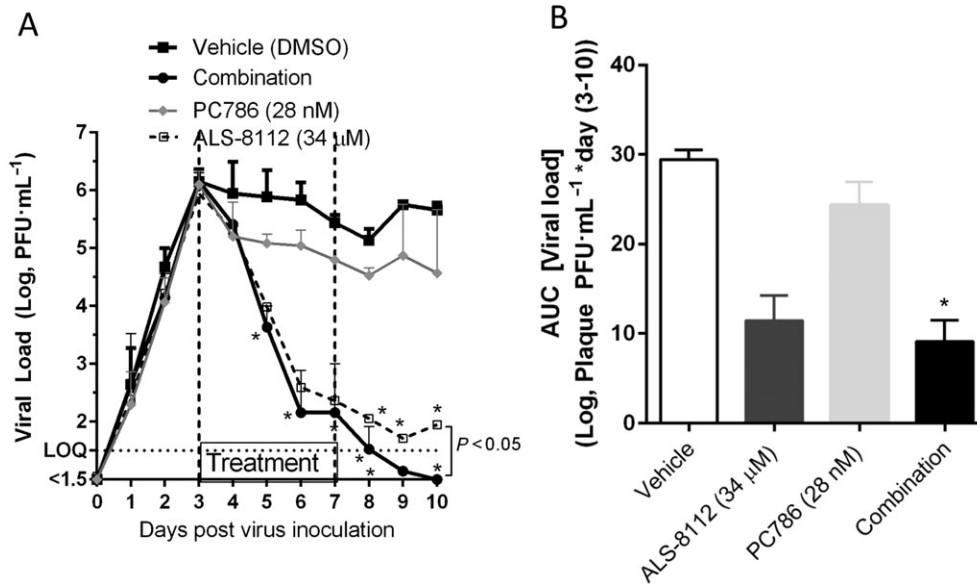
Figure 5

Effects of combination of PC786 (28 nM) and ALS-8112 (34 μM) on replication kinetics of RSV A2 in MucilAir™ inserts following virus inoculation on Day 0. For control, vehicle (0.5% DMSO in PBS) was applied both apically and basally from Day 3 to Day 7 post virus inoculation daily in three donors. PC786 (28 nM) was applied apically, and vehicle was applied basolaterally. ALS-8112 (34 μM) was applied basally, and vehicle was applied apically. For combination group, PC786 (28 nM) was applied apically, and ALS-8112 (34 μM) was applied basally. Replicating virus in daily apical wash was quantified *via* plaque assay analysis in daily apical (A), and AUC of viral load (Days 3–10) was also calculated (B). The horizontal dashed line shows the limited of quantification (LOQ) for each assay (33 PFU·mL^{-1} = 1.5 Log, PFU·mL^{-1}). Each point represents the geometric mean (±SEM) for viral load and mean (±SEM) for others, from three independent donors. *$P < 0.05$, significantly different from infection control.

rapid inhibition of RSV A2 replication with the AUC-IC$_{50}$ value of 154 nM. As the AUC was calculated using log values of PFU·mL^{-1}, this IC$_{50}$ value is equivalent to inhibition of half of the peak log viral load, suggesting more than 2.5 log reduction over 7 days (>99.5% inhibition), a much greater effect than a simple 50% reduction of total viral load. Despite the fact that PC786 was transiently exposed to epithelium daily (1 h only) to avoid cell disruption resulting from consequent hypoxic conditions, PC786 produced sustained anti-viral activity, a profile that is fully consistent with the persistence of action *in vitro* shown previously (Coates *et al.,* 2017). In addition, the anti-viral activities were confirmed in small airway epithelium. Notably, the specific localisation of the pathogenic organism provides a particular challenge to treatment, as it requires a super-effective drug concentration to be maintained at the discrete cellular site of virus replication. Topical therapy is, therefore, an ideal approach for combating RSV infection.

The antiviral activity of PC786 was readily apparent in the histology of the cells. RSV-infected ALI epithelium is reported to show remarkably similar histological changes to those observed in lung tissues from fatal cases of RSV-induced bronchiolitis (Villenave *et al.,* 2012). These include substantial damage to the respiratory epithelium including cell sloughing and apoptotic epithelial cell death (Welliver *et al.,* 2007), albeit without inflammatory cell accumulation, because this model uses only epithelium. The current study also showed a complete loss of ciliated epithelial cells 10 days post virus inoculation and abnormal structure with regenerated basal cells. These changes presumably reflect the loss of

elastic fibres of basement membrane to support the epithelium structure because of severe cytopathic effects following extensive apical shedding of progeny virions. In contrast to control, when PC786 was administered from the virus peak (Day 3) to Day 7 for 5 days apically (1 h exposure a day), the ALI structure was protected. Ciliated epithelial cells were retained and showed no or limited cytopathic effects, showing similar morphology to that of ALI inserts without any manipulation (Supporting Information Figure S2B). Late intervention with PC786 therefore helped to retain a normal morphology of epithelium as a consequence of inhibition of virus replication. However, we did not observe syncytia and goblet cell hyperplasia/metaplasia in our study on Day 10 post infection. To investigate the similarity of experimental virus infection in ALI to the clinical setting and assess the effects of PC786, a time course study is required in the future.

ALS-8112, a nucleoside-analogue RSV polymerase chain terminator, also demonstrated a concentration-dependent inhibition of viral load. The effect was 101-fold weaker than PC786 based on viral AUC, and ALS-8112 showed slower onset of action than PC786. ALS-8112 requires conversion into the triphosphate active form by human kinases, such as deoxycytidine kinase, in epithelial cells (Jordan *et al.,* 2017), and this may explain its delayed onset of anti-viral activity. ALS-8176 (ALS-8112 prodrug) was tested in RSV human challenge in healthy subjects and eliminated virus 2–3 days after initiation of treatment (DeVincenzo *et al.,* 2015). However, the compound was given at an early stage of RSV infection, and the contrasting demands of our protocol may have prevented treatment inhibiting total viral load

promptly. Although both PC786 and ALS-8112 inhibited RSV virus replication, they work through different molecular mechanisms (Deval *et al.,* 2015; Coates *et al.,* 2017). Interestingly, the synergistic effects of ALS-8112 and AZ-27, another RSV polymerase inhibitor, have been reported (Deval *et al.,* 2016). We also found beneficial effects of combined treatment with PC786 and ALS-8112 (Figure 5), but further studies are required to clarify a possible clinically relevant regimen.

Although PC786 has been designed for inhalation delivery (poor oral bioavailability and extensive plasma protein binding) and basolateral administration of this compound would not be clinically relevant, it is difficult to appreciate the differences between PC786 and ALS-8112 as the compounds were delivered *via* different routes. Therefore, we evaluated the effects of basolateral treatment of PC786. As seen in Figure 1I, PC786 demonstrated much stronger anti-viral effects with basolateral treatment compared with apical treatment, and consequently, the difference in antiviral effects between PC786 and ALS8112 was enhanced. In fact, for apical treatment, dosing was limited to 1 h before washing, but for basolateral treatment, compounds were exposed to the epithelium for a much longer period (without daily wash). Thus, the stronger effects of PC786 compared with ALS8112, were not due to the different modes of administration.

ALI cultures were also characterised by increases in the concentrations of several biomarkers. The chemokine CCL5 has been shown to correlate with RSV disease severity (DeVincenzo *et al.,* 2010) and with RSV load in humans (Thompson *et al.,* 2015). CXCL8 and IL-6 were also reported to increase in nasal aspirate after infection and show a strong correlation with symptoms (DeVincenzo *et al.,* 2010). Here, PC786 inhibited CCL5 and IL-6 strongly but did not inhibit CXCL8. As PC786 was treated after the peak of virus replication, a considerable amount of virus-derived RNA already existed and probably stimulated CXCL8 production through signalling *via* **toll-like receptors** rather than through mechanisms dependent on virus replication. CXCL10 is one of the important factors for host defence such as recruiting NK cells, and probably any drugs to inhibit interferon signalling such as corticosteroid will cause impaired host defence. Although PC786 showed inhibitory effects on CXCL10, we believe that the inhibition is a consequence of reduction of viral load by PC786 rather than its immunosuppressive effects. We have tested PC786 in CD3/**CD28**-activated human PBMCs and found that PC786 did not show any effects on cytokine production, although corticosteroid substantially inhibited a wide range of cytokines (unpublished data).

Despite serving as a high-fidelity model of the human airway, there were some limitations observed with ALI cultures that hindered data interpretation. First, compared to viral load (our primary endpoint), biomarker concentrations varied between different donors. This was notable for mucus production, although substantial inter-individual variation in mucus levels has been reported clinically (Bagga *et al.,* 2013). Thus, power calculations using our data indicate that a larger sample size is needed to investigate the effects of PC786 appropriately. Second, we observed limited decrease of PCR product, compared with viral load assessed by plaque assay, during the late intervention protocol (Figure 2A). In contrast, strong, rapid inhibitory effects on PCR products

were observed after single treatment of PC786, a day after virus inoculation when viral load is still modest (Figure 3B). On Days 3 to 4 at viral peak, some of the infected cells are probably dying and being shed into the apical medium, so RT-PCR results would include signal from nucleocapsids within or released from the dead cells. In fact, we presented evidence for dead cell release through our double-strand DNA assay (Figure 2E). Third, although treatment with PC786 produced very strong anti-viral effects, virus load increased when treatment ceased. We believe the following considerations may explain this finding. The apical dosing period (1 h daily only) was limited, so the increase in virus load might reflect a technical problem. Current therapeutic late intervention provides an extreme challenge, and longer exposure to treatment will be required. In addition, this profile may well be cell-model specific. Unlike these cultures, in the whole body, adaptive immune cells also participate in virus elimination. Thus, some technical problems are likely to affect the outcome. Finally, the peak virus load was similar to that observed in nasal wash samples collected from healthy subjects challenged with RSV Memphis 37 strain (Kelly *et al.,* 2015) or from RSV-infected infants (DeVincenzo *et al.,* 2005). However, the viral load was sustained for more than 10 days in ALI epithelium whereas the virus infection resolved in 10 days in healthy subjects *in vivo*. This difference is likely to be due to the lack of immune cells in the ALI system, such as cytotoxic T cells which eliminate virus-infected cells (Russell *et al.,* 2017).

In summary, PC786 achieved a superior anti-viral profile (onset of action and potency) in airway epithelium when administered after virus infection was established, which replicates treatment of established clinical disease, compared with those of an RSV nucleoside analogue and an F protein viral entry inhibitor. Therefore, a topically administered, highly potent, low MW inhibitor targeting the replication complex of RSV is 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 underway.

Author contributions

D.W.B., P.S., G.W.R. and K.I. conceived and designed the experiments. D.W.B., M.C., H.A., L.D., I.K. and M.H. performed the experiments. S.C. and S.H. prepared cell models. D.W.B., M.C., L.D., H.A., M.H., J.A., A.D., L.C., J.A. and K.I. analysed and interpreted the data. D.W.B., M.C. and K.I. drafted the manuscript. P.S., G.R. and K.I. supervised the project.

Conflict of interest

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

https://doi.org/10.1111/bph.14221

Figure S1 Replication kinetics of RSV A2 in MucilAir™ inserts following virus inoculation on Day 0 and successive daily apical treatment with vehicle (0.5% DMSO in PBS) (A) or basolateral treatment with vehicle (B) from Day 3 to Day 7 post virus inoculation in individual donor. Replicating virus was enumerated via plaque assay analysis, and the level of viral load shown as plaque forming units. The horizontal dashed line shows the limited of quantification (LOQ) for each assay (33 PFUml^{-1} = 1.5 Log, PFUml^{-1}). The value expressed as the mean of Log value of viral load ± SEM from 3 independent samples (3 inserts) in each donor.

Figure S2 Histology of MucilAir™ Bronchial epithelium (A) Histology image of MucilAir™ Bronchial epithelium on Day 10 after RSV A2 inoculation. 0.5% DMSO (vehicle) or PC786 (700nM) were applied on Day 3 –7 daily for 1hr just after daily apical wash (x100 magbitude). Ciliated bronchial epithelial cells were damaged and disappeared in RSV infected control, but PC786 treated epithelium retained ciliated bronchial epithelial cells. (B) Representative histology image of MucilAir™ Bronchial epithelium without any manipulation (EpithelixSarl).

Figure S3 Effects of PC786 on CXCL8 (A), CXCL10 (B) and mucin (C) production in apical washes collected post RSV inoculation (day 0) with treatment with either vehicle or PC786 (3-7 days post-inoculation). Each point represents the mean value of five independent donors, with the standard error of mean shown. *P <0.05 versus infection control. Individual mucus production kinetics (D).

Figure S4 Effects of basolateral treatment of PC786 on virus genome (A), CCL5 (B), IL-6 (C), CXCL10 (D), mucin (E) and dsDNA (F) in apical washes collected post RSV inoculation (day 0). Either vehicle or PC786 were treated from Day 2 to Day 7 once daily. Each point represents the mean value of three independent donors, with the standard error of mean. *P <0.05 versus infection control.

Table S1 Characteristics of donors of bronchial ALI epithelium and small airway ALI epithelium.

Table S2 Sample size determination by power calculation (P <0.05, 0.8 power).