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ORIGINAL ARTICLE

Anti-inflammatory effects of the novel inhaled phosphodiesterase type 4 inhibitor CHF6001 on virus-inducible cytokines

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Abstract

Respiratory virus infections precipitate asthma and chronic obstructive pulmonary disease (COPD) exacerbations, with most exacerbations due to rhinovirus infection. Both asthma and COPD exacerbations are not well controlled by steroid therapies, and there is a much research interest in finding improved therapies or combinations of therapies for controlling exacerbations. CHF6001 is a new, inhaled highly potent and selective phosphodiesterase type 4 (PDE4) inhibitor. Using in vitro human bronchial epithelial cells (BEAS-2B), we investigated the potential anti-inflammatory effects of CHF6001 on rhinovirus (RV1B)-induced cytokines. Cytokine mRNA was measured by real-time PCR, while protein release was measured by ELISA. CHF6001 was used in a 7-point dose–response curve (1000–0.001 nmol/L) as a 1.5-h pretreatment prior to infection in comparison with roflumilast. Both roflumilast and CHF6001 reduced RV1B-induced IL-8, IL-29, IP-10, and RANTES mRNA and protein in a concentration-dependent manner. Generally, CHF6001 was 13- to 16-fold more potent (sub-nanomolar EC_{50} values) than roflumilast at reducing IL-8, IL-29, IP-10, and RANTES mRNA and protein release, but had similar efficacies. In combination with the steroid fluticasone propionate (1 nmol/L), CHF6001 had additive effects, significantly reducing RV-induced cytokines when compared with steroid or CHF6001 alone. Combined low-dose steroid and low-dose CHF6001 had a similar efficacy as high-dose steroid or CHF6001 alone, indicating the combination had steroid and PDE4 inhibitor sparing effects. Overall results indicate that PDE4 inhibitors have anti-inflammatory activity against virus-induced inflammatory mediators and that CHF6001 is more potent than roflumilast.

Abbreviations

COPD, chronic obstructive pulmonary disease; FP, fluticasone propionate; GR, glucocorticoid receptor; IFN, interferon; MOI, multiplicity of infection; PDE, phosphodiesterase; PRRs, pattern recognition receptors; RLHs, RIG-like helicases; RSV, respiratory syncytial virus; RV, human rhinovirus; TLR, toll-like receptor.

Introduction

The majority of exacerbations of asthma and chronic obstructive pulmonary disease (COPD) are triggered by acute respiratory virus infections (Edwards et al. 2012a; Johnston et al. 2005, 1995; Papadopoulos et al. 2010). Respiratory syncytial viruses (RSV), influenza viruses, metapneumoviruses, bocaviruses, and parainfluenza viruses can all precipitate asthma and COPD exacerbations, but the most common virus associated with exacerbations of

either disease is human rhinoviruses (RVs). RVs account for up to 80% of asthma exacerbations in children (Bizzintino et al. 2010; Johnston et al. 1995) and approximately 60% of asthma exacerbations in adults (Nicholson et al. 1993) and up to 50% of total COPD exacerbations (Seemungal et al. 2001).

Asthma and COPD exacerbations represent a major unmet medical need, with current treatments for stable asthma and COPD (inhaled corticosteroids and β_2 agonists) only showing small improvements in both exacerbation rate (Bisgaard et al. 2006; Calverley et al. 2003, 2007a; Dal Negro et al. 2003; Kuna et al. 2007; Nannini et al. 2003; O'Byrne et al. 2005; Vogelmeier et al. 2005) and severity (Calverley et al. 2003; Dal Negro et al. 2003; Kuna et al. 2007) in large clinical trials. The application of additional or new small molecule therapies, in the form of phosphodiesterase (PDE) inhibitors with and without steroid/ β_2 agonist combinations, is of much interest to the pharmaceutical industry and academia to identify improved future therapies that will prevent asthma and COPD exacerbations.

PDEs are enzymes that catalyze the cleavage of a phosphodiester bond, often in the context of a cyclic nucleotide. PDEs comprise a group of enzymes that degrade the phosphodiester bond in the second messenger molecules cAMP and cGMP, thus regulating a range of cellular processes including intracellular signal transduction pathways. There are 11 families in mammals (PDE1-11), and Type 4 (PDE4) is cAMP-specific PDEs encoded by four genes (PDE4A, PDE4B, PDE4C, and PDE4D) sharing a highly conserved catalytic domain. PDE4 is the major cAMP metabolizing enzyme found in immune cells and is also present in lung structural cells, including airway smooth muscle, epithelial cells, endothelial cells, and fibroblasts (Billington et al. 2008; Fuhrmann et al. 1999; Seybold et al. 1998). PDE4 inhibitors have proven potential as anti-inflammatory drugs in chronic airway diseases. By increasing intracellular cAMP levels, PDE4 inhibitors elicit a broad spectrum of anti-inflammatory effects, including the suppression of the release of mucins (Mata et al. 2005), inflammatory cytokines, and other mediators (Buenestado et al. 2011; Burgess et al. 2006; Ouagued et al. 2005), and also inhibit the actions of reactive oxygen species which are proinflammatory in nature (Brown et al. 2007; de Visser et al. 2011; Ortiz et al. 2012).

PDE4 inhibitors have been extensively trialed in asthma and COPD (Bousquet et al. 2006; Compton et al. 2001; Gamble et al. 2003; Louw et al. 2007; van Schalkwyk et al. 2005). In stable disease, roflumilast and cilomilast have shown significant improvements in lung function and symptom score and may reduce rescue medication use (Bousquet et al. 2006; Compton et al. 2001; Louw et al. 2007). PDE4 inhibitors have also reduced COPD exacerbation rates (Calverley et al. 2007b; Rennard et al. 2011) although few studies have specifically investigated the effects of PDE4 inhibitors on virus-induced inflammation (Ikemura et al. 2000; Van Ly et al. 2013). Rolipram was previously shown to reduce RSV-induced eosinophilia and airway hyperresponsiveness in a mouse model (Ikemura et al. 2000) and piclamilast failed to suppress RV-induced inflammatory mediators from airway smooth muscle cells and primary human bronchial epithelial cells, but suppressed inflammatory responses following stimulation with the toll-like receptor (TLR)-3 agonist polyIC (Van Ly et al. 2013). Clearly, little is known about the potential of PDE4 inhibitors to reduce virus-induced inflammation, by what mechanism(s) they act upon, and therefore, how this relates to the observed clinical benefits on exacerbation rates seen in clinical trials.

The clinical dosage and efficacy of the only oral PDE4 inhibitor currently approved in COPD (roflumilast) is limited by target-related side effects, such as nausea, diarrhea, and headaches. Inhibitors specifically designed for inhaled treatment may combine potent anti-inflammatory activity with improved tolerability. One example is CHF6001, a novel highly potent and selective PDE4 inhibitor with robust anti-inflammatory activity and suitable for topical pulmonary administration (Armani et al. 2014). In this study, we compared anti-inflammatory effects of CHF6001 with that of roflumilast, using in vitro models of virus-induced cytokine mRNA induction and protein release. In certain experiments, CHF6001 was used in combination with the inhaled steroid fluticasone propionate (FP). We found that CHF6001 had suppressive effects on RV-induced interferon (IFN)-β, IFN-λ1 (IL-29), IFN-λ2/3 (IL-28A/B), IL-8, IP-10, and RANTES when used as a pretreatment, while roflumilast had no suppressive effects when used as a pretreatment only. In comparisons between CHF6001 as a pretreatment and roflumilast as a pretreatment and posttreatment, CHF6001 had a higher potency but equal efficacy. In combination with low-dose FP, low-dose CHF6001 had additive effects with potential for both steroid and PDE4 inhibitor sparing activity.

Materials and Methods

Reagents and compounds

CHF6001, roflumilast, and FP were obtained from Chiesi Farmaceutici as solid chemicals and were made up in Dimethyl sulfoxide (DMSO) at 0.1 mol/L solutions, aliquoted, and stored at −20°C. A fresh aliquot was used for each experiment.

Cells and viruses

BEAS-2B cells (European Collection of Cell Cultures) were cultured in Roswell Park Memorial Institute medium (RPMI) with 10% FCS (Invitrogen, Paisley, UK). Minor group RV1B (genotype RV-A) was grown in HeLa cells. The RV1B stock was thoroughly assessed prior to use, was *mycoplasma* free, and induced cytokines in a dose responsive and replication-dependent manner. The RV1B stock was diluted ¼ in RPMI medium, an approximate multiplicity of infection (MOI) of 1 for all experiments.

Pretreatment and posttreatment of cells and infection of BEAS-2B cells

BEAS-2B cells seeded into 12-well plates (Nunc) and left overnight. The next day, cells were placed into RPMI medium containing 2% FCS overnight. BEAS-2B cells were then treated with CHF6001, roflumilast, or vehicle with and without FP. Typically, both CHF6001 and roflumilast were used in 7-point dose–response curves (1000–0.001 nmol/L) diluted in RPMI medium as a 1.5-h pretreatment prior to infection or as a pretreatment and posttreatment. The amount of vehicle (DMSO) was kept constant (1/100,000 dilution) for each dose of compound or control used. In certain experiments, FP was also used and diluted in RPMI medium to 1 or 10 nmol/L. Following pretreatment, BEAS-2B cells were then infected with RV1B or treated with medium for 1 h with shaking at room temperature. Virus was washed off cells by 3X washes in RPMI+2% FCS, and fresh medium was replaced and lysates or supernatants harvested at 24 h post infection.

RNA extraction, cDNA synthesis, and quantitative PCR

Total RNA was extracted from BEAS-2B cells (RNeasy kit, Qiagen, Manchester, UK), and approximately 2 μg was used for cDNA synthesis (Omniscript RT kit, Qiagen). Quantitative PCR was carried out using specific primers and probes for each gene (Bartlett et al. 2012; Gielen et al. 2010). Reactions were analyzed using an ABI 7500 TaqMan, (ABI Foster City, CA). Each gene was normalized to 18S rRNA and expressed as copies per μL cDNA reaction using a standard curve based on amplification with plasmid DNA.

ELISA analysis of cytokine production

Enzyme-linked immunosorbent assays (ELISA) for IL-8/CXCL8, IP-10/CXCL10, RANTES/RANTES, and IFN-λ1 (IL-29) were from R&D Systems (Abingdon, UK). The ELISA kit (R&D Systems) for IFN-λ1 measures both IL-29 and IL-28 together and cannot discriminate between the two. All ELISAs were used according to the manufacturer's recommended protocol.

Statistical analysis

All in vitro experiments were performed 4–6 times; all data expressed as mean ± SEM. EC_{50}s rather than IC_{50}s were calculated for all dose–response curves, and this curve considers a plateau for efficacy, as full suppression or suppression beyond 50% of the induced cytokines was not achieved. The equation used describes a sigmoidal dose–response calculating Y = Bottom + (Top−Bottom)/(1 + 10^((LogEC$_{50}$−X))), where X is the logarithm of concentration, Y is the response, Y starts at bottom and goes to top with a sigmoid shape. For multiple comparisons, data were analyzed by one-way analysis of variance and Bonferroni's multiple comparison test. For differences between two groups, a Student's *t*-test was employed. All statistics were performed using Graph Pad Prism 4 software (San Diego, CA USA), with $P < 0.05$ taken as significant.

Results

Human RV1B infection induced IL-8/CXCL8, IL-29, IP-10/CXCL10, and RANTES/RANTES mRNA and protein in bronchial epithelial cell lines

The raw data of cytokine mRNA and protein induction are shown in Table 1. At 24 h, BEAS-2B cells demonstrated a robust, significant induction of each cytokine mRNA and protein after infection with RV1B compared with medium-treated cultures. These data show similar robust levels of induction to previous published data with this cell type following RV infection in vitro (Edwards et al. 2006; Wang et al. 2009), and that the data in this article presented as percentage of RV1B-infected controls are based on statistically significant inductions of each cytokine measured.

CHF6001 reduced RV1B induced IL-8, IL-29, IP-10, and RANTES mRNA and protein in a concentration-dependent manner

We first assessed CHF6001 as a pretreatment and found dose-dependent suppressive effects on RV1B-induced cytokine mRNA and protein. Figure 1 demonstrates 7-point dose–response curves for CHF6001 and shows that CHF6001 as a pretreatment did exhibit suppressive activity against each cytokine mRNA measured.

CHF6001 robustly suppressed IFN-β, IL-29, IL-28, RANTES, and IP-10 mRNA expression and showed less

Table 1. RV1B infection of BEAS-2B cells significantly induced the mRNA and protein of proinflammatory cytokines and IFNs.

Gene/protein	Medium (mean ± SEM)	RV1B (mean ± SEM)	*P*-value
mRNA quantification (copy number per μL cDNA)			
IFN-β	$2.9 \pm 1.0 \times 10^3$	$4.7 \pm 1.1 \times 10^5$	<0.001
IL-29	$0.01 \pm 0.0 \times 10^3$	$1.0 \pm 0.51 \times 10^5$	<0.001
IL-28	$0 \pm 0 \times 10^3$	$3.2 \pm 1.2 \times 10^5$	<0.001
IL-8	$4.9 \pm 3.0 \times 10^5$	$6.5 \pm 0.89 \times 10^6$	<0.001
IP-10	$1.3 \pm 0.81 \times 10^3$	$1.1 \pm 0.31 \times 10^7$	<0.001
RANTES	$1.3 \pm 0.39 \times 10^3$	$1.0 \pm 0.4.1 \times 10^7$	<0.001
Protein Quantification (pg/mL)			
IL-29	$0.97 \pm 0.71 \times 10^1$	$0.35 \pm 0.01 \times 10^3$	<0.001
IL-8	$6.2 \pm 0.62 \times 10^1$	$0.62 \pm 0.12 \times 10^3$	<0.01
IP-10	$1.5 \pm 0.88 \times 10^1$	$1.2 \pm 0.26 \times 10^3$	<0.01
RANTES	$0.18 \pm 0.07 \times 10^1$	$0.60 \pm 0.2 \times 10^3$	<0.05

suppressive effects on IL-8 mRNA (Fig. 1). These trends were also reflected in the respective EC_{50} calculations (Table 2), which were between 0.31 and 0.17 nmol/L. The effects on protein release are shown in Figure 2. We could not detect any IFN-β protein in these supernatants and so could not assess the effects on protein release of this cytokine (data not shown). CHF6001 had similar effects on IL-29, IL-8, IP-10, and RANTES protein when compared with the mRNA data, with similar subnanomolar EC_{50s} (Table 3). The effects of CHF6001 and roflumilast (at doses giving maximal suppression) on raw mRNA and protein levels are additionally shown in Tables S1–S2.

Roflumilast reduced RV1B-induced cytokine and IFN mRNA and protein in a concentration-dependent manner

We initially used roflumilast as a pretreatment only but found no effect on any RV1B-induced cytokine measured (data not shown). We, therefore, opted to use roflumilast as a pretreatment and posttreatment. Figure 3 demonstrates 7-point dose–response curves for roflumilast, and they indicate that roflumilast as a pretreatment and posttreatment did exhibit suppressive activity against each cytokine mRNA measured. Roflumilast robustly

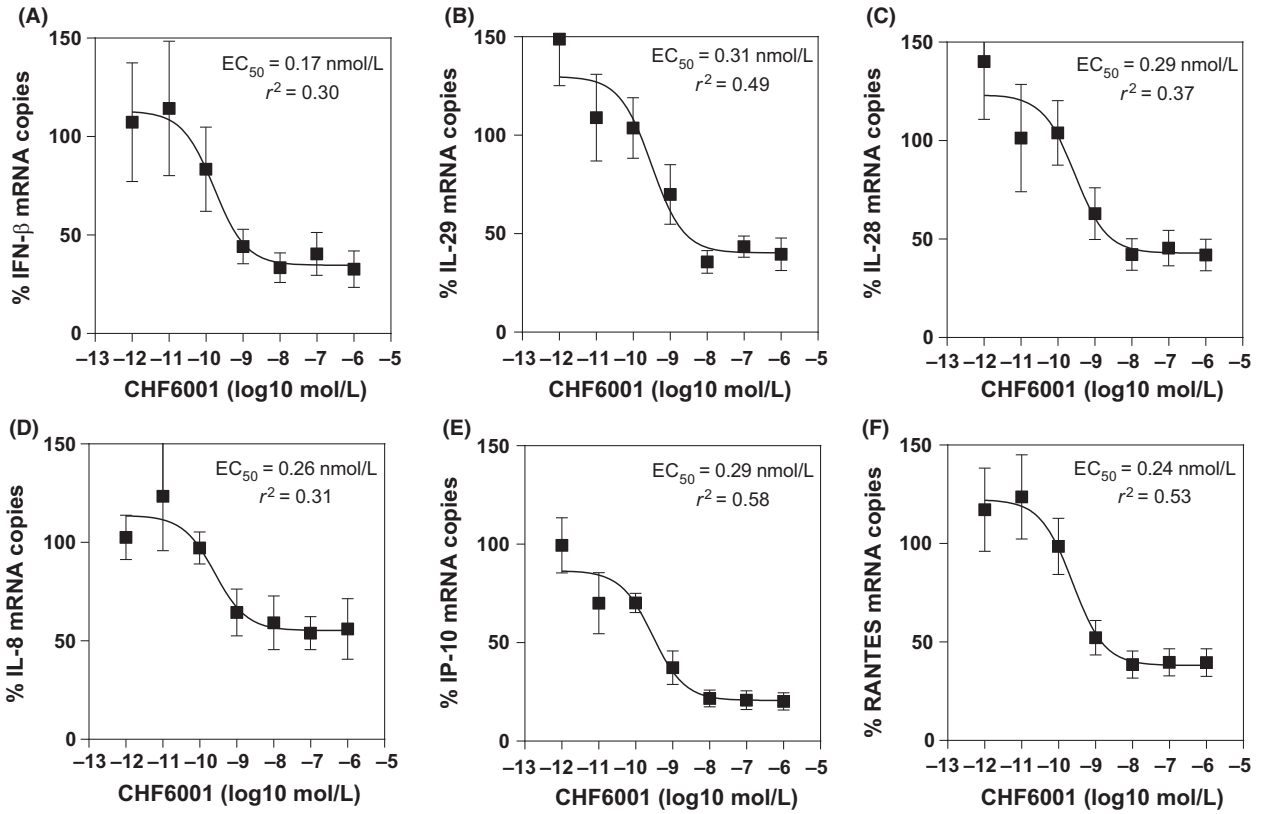

Figure 1. Pretreatment with CHF6001 suppressed RV-induced proinflammatory cytokines and IFN mRNA in a dose-dependent manner. BEAS-2B cells were pretreated with CHF6001 at various doses for 1.5 h and then infected with RV1B (MOI = 1). At 24 h post infection, total RNA was harvested, cDNA synthesized, and cytokine mRNA abundance determined by quantitative PCR. CHF6001 showed a dose-dependent suppression of IFN-β mRNA (A), IL-29 mRNA (B), IL-28 mRNA (C), IL-8 mRNA (D), IP-10 mRNA (E), and RANTES mRNA (F). EC_{50} and r^2 values are shown for each graph $n = 7$ experiments.

Table 2. Comparison of CHF6001 and roflumilast in suppression of RV1B-induced cytokine mRNA.

Gene	CHF6001		Roflumilast		CHF6001 versus Roflumilast	
	EC_{50} (nmol/L)	Maximum suppression (%)[1]	EC_{50} (nmol/L)	Maximum suppression (%)[1]	EC_{50} fold difference	*P*-value[2]
IFN-β	0.17	67 ± 9.2	8.7	59 ± 11	51	0.55
IL-28	0.29	58 ± 8.0	1.1	69 ± 8.5	3.9	0.37
IL-29	0.31	64 ± 5.8	1.3	75 ± 9.0	4.3	0.35
IL-8	0.26	46 ± 8.3	No fit	60 ± 8.6	–	0.25
IP-10	0.29	80 ± 4.4	4.6	78 ± 4.5	16	0.82
RANTES	0.24	61 ± 6.9	0.7	65 ± 8.0	3.0	0.75

[1]At any dose of compound tested.
[2]Comparison of maximum suppression at any dose CHF6001 versus roflumilast by unpaired *t*-test.

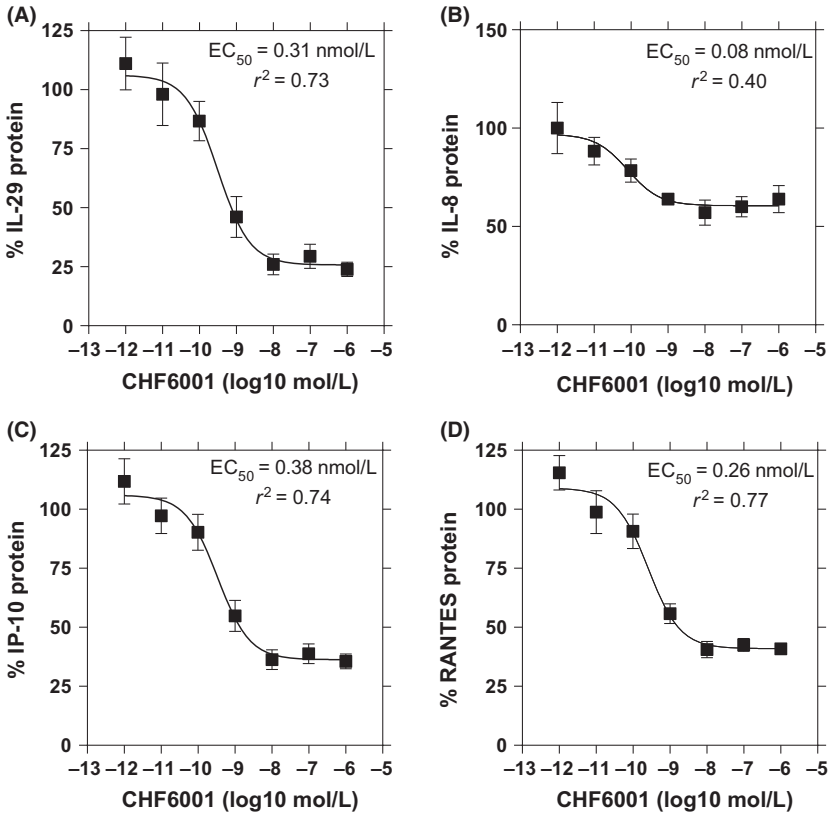

Figure 2. Pretreatment with CHF6001 suppressed RV-induced proinflammatory cytokines and IFN protein in a dose-dependent manner. BEAS-2B cells were pretreated with CHF6001 at various doses for 1.5 h and then infected with RV1B (MOI = 1). At 24 h post infection, cell supernatants were harvested, and cytokine release determined by ELISA. CHF6001 showed a dose-dependent suppression of IL-29 (A), IL-8 (B), IP-10 (C), and RANTES (D). EC_{50} and r^2 values are shown for each graph $n = 7$ experiments.

suppressed IL-29, IL-28, RANTES, and IP-10 mRNA expression and showed less suppressive effects on IFN-β and CXCL8 mRNA (Fig. 3). These trends were also reflected in the respective EC_{50} calculations (Table 2).

We also assessed the effects of roflumilast pretreatment and posttreatment on cytokine release (Fig. 4). In a similar manner to the mRNAs, roflumilast showed dose-dependent suppressive effects on each cytokine protein measured. The EC_{50} values are presented in Table 3.

Comparison between CHF6001 and roflumilast

We next compared relative potencies and efficacies of CHF6001 pretreatment with roflumilast used as a pretreatment and posttreatment. These comparisons are summarized in Tables 2 and 3 for mRNA and protein release, respectively. Table 2 compares both the EC_{50s} and the maximal suppression for mRNA of each

Table 3. Comparison of CHF6001 and roflumilast in suppression of RV1B-induced cytokine release.

Protein	CHF6001		Roflumilast		CHF6001 versus Roflumilast	
	EC_{50} (nmol/L)	Maximum suppression (%)[1]	EC_{50} (nmol/L)	Maximum suppression (%)[1]	EC_{50} fold difference	*P*-value[2]
IL-29	0.41	76 ± 3.0	1.5	85 ± 5.2	3.6	0.15
IL-8	0.08	43 ± 6.4	3.8	40 ± 7.5	48	0.45
IP-10	0.38	64 ± 3.2	2.6	73 ± 4.0	6.8	0.37
RANTES	0.26	59 ± 3.5	1.5	65 ± 6.0	5.8	0.16

[1]At any dose of compound tested.
[2]Comparison of maximum suppression at any dose CHF6001 versus roflumilast by unpaired *t*-test.

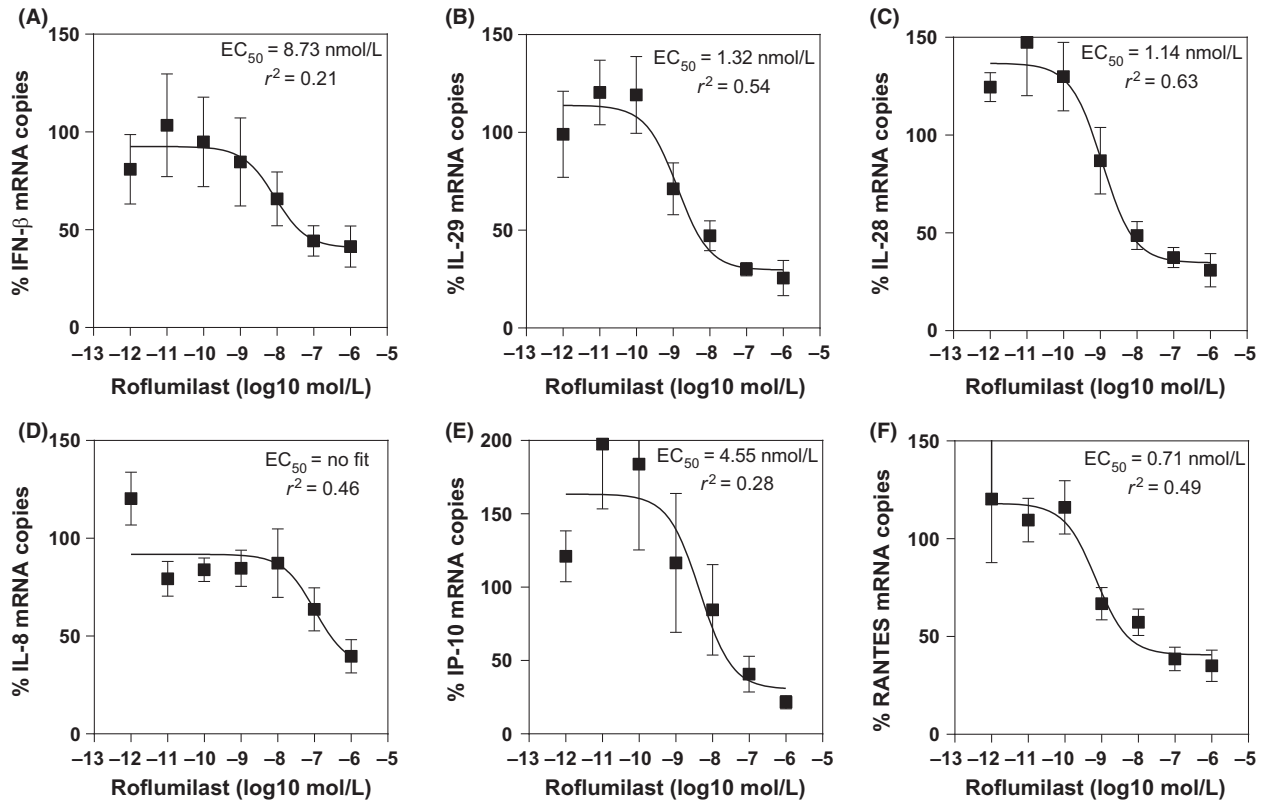

Figure 3. Pretreatment and posttreatment with roflumilast suppressed proinflammatory cytokines and IFN mRNA in a dose-dependent manner. BEAS-2B cells were pretreated with roflumilast at various doses for 1.5 h and then infected with RV1B (MOI = 1). Roflumilast was added back into the culture medium and cells incubated. At 24 h post infection, total RNA was harvested, cDNA synthesized, and cytokine mRNA abundance determined by quantitative PCR. Roflumilast showed a dose-dependent suppression of IFN-β mRNA (A), IL-29 mRNA (B), IL-28 mRNA (C), IL-8 mRNA (D), IP-10 mRNA (E), and RANTES mRNA (F). EC_{50} and r^2 values are shown for each graph $n = 6$ experiments.

cytokine, found at any dose. We found that the maximal suppression between CHF6001 and roflumilast was not significantly different, indicating an equal efficacy. We did find differences between the EC_{50} values, with CHF6001 having lower EC_{50s}, on average 13-fold lower but with a wide range approximately, 51.3 fold (Table 2). IFN-β was the most differentially affected mRNA by the two drugs (51.4-fold difference). We also compared protein release and found again similar, non-significant maximal levels of suppression but differences in EC_{50}. The average difference in EC_{50} was approximately 16-fold, with a range of 3- to 48-fold. Interestingly, IL-8 was the cytokine most differentially suppressed between CHF6001 and roflumilast (47.5-fold difference), with IL-29 being the least (3.54-fold difference).

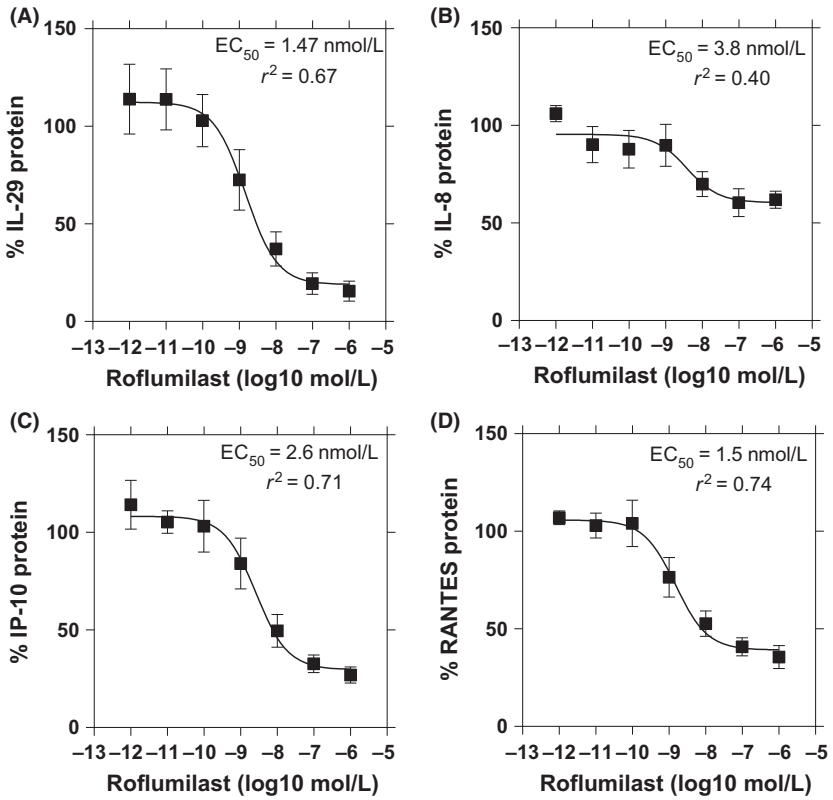

Figure 4. Pretreatment and posttreatment with roflumilast suppressed proinflammatory cytokines and IFN protein in a dose-dependent manner. BEAS-2B cells were pretreated with CHF6001 at various doses for 1.5 h and then infected with RV1B (MOI = 1). Roflumilast was added back into the culture medium and cells incubated. At 24 h post infection, cell supernatants were harvested, and cytokine release determined by ELISA. Roflumilast showed a dose-dependent suppression of IL-29 (A), IL-8 (B), IP-10 (C), and RANTES (D) cytokine release. EC_{50} and r^2 values are shown for each graph $n = 6$ experiments.

CHF6001 had no effect on RV1B replication

As both CHF6001 reduced RV1B-induced IFN-β, IL-29, and IL-28 mRNA and IL-29 protein, we sought to examine the effects on RV replication. At 24 h post infection, we saw no effect on RV replication using both a titration assay measuring virus release and quantitative PCR measuring virus RNA (Table S3).

Additive effects of combined FP and CHF6001 on RV1B-induced IL-8, IL-29, IP-10, and RANTES/RANTES protein

We next assessed if the suppressive effects of CHF6001 could be observed in the presence of steroid and if steroid and CHF6001 had additive or synergistic suppressive effects. We first chose FP at 10^{-9} mol/L (1 nmol/L) as this concentration is approximately the EC_{50} for these cytokines in bronchial epithelial models (Edwards et al. 2007, 2006), and FP has been found at approximately this concentration in lung tissue following inhalation (Esmailpour et al. 1997). For all cytokines measured, FP at 10^{-9} mol/L and CHF6001 at 10^{-6} mol/L used alone significantly reduced cytokine levels compared with RV1B-infected cells without pretreatment (Fig. 5A–D).

Figure 5A also demonstrates that CHF6001 at 10^{-6}–10^{-9} when combined with FP at 10^{-9} mol/L (1 nmol/L) had significantly reduced suppression of IL-29 compared with FP alone but not with CHF6001 alone at 10^{-6} mol/L. CHF6001 at 10^{-6}–10^{-9} with FP showed significantly reduced suppression of IL-8 compared with CHF6001 10^{-6} mol/L but not with FP alone (Fig. 5B). Data for IP-10 and RANTES were quite similar, with CHF6001 at 10^{-6}–10^{-8} mol/L with FP had significantly reduced suppression compared with FP and CHF6001 alone at 10^{-6} mol/L (Fig. 5C); but for RANTES, CHF6001 at 10^{-6}–10^{-8} mol/L with FP had significantly reduced suppression compared with FP alone and reduced suppression compared with CHF6001 10^{-6} mol/L for concentrations 10^{-6}–10^{-9} mol/L (Fig. 5D).

We also assessed potential additive/synergistic effects using FP at a lower dose, 10^{-10} mol/L: (0.1 nmol/L). For

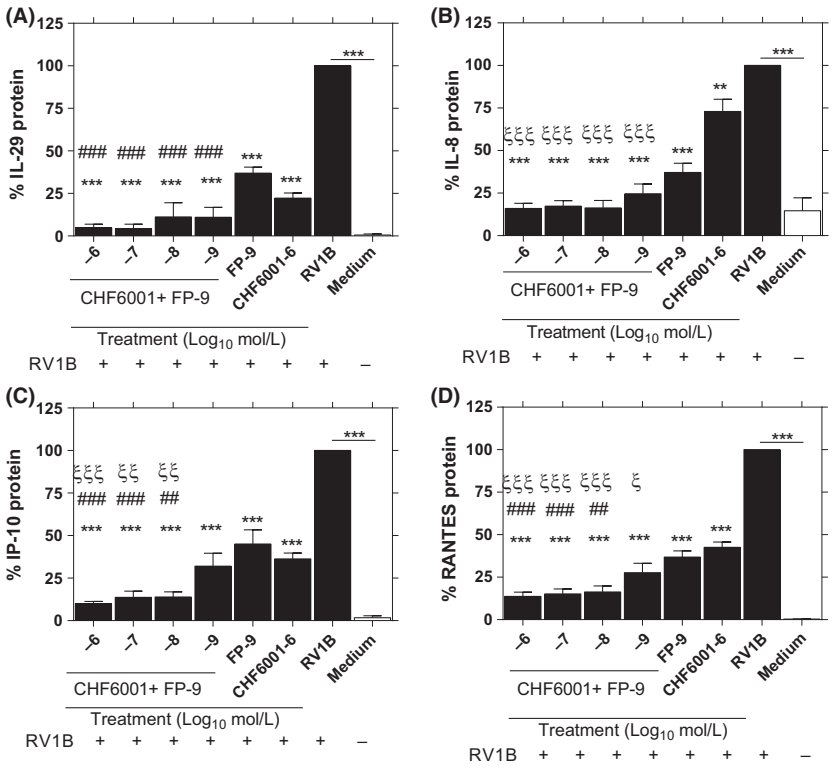

Figure 5. Combined effects of CHF6001 and FP 10^{-9} mol/L on RV induced IFN and inflammatory cytokine protein release. BEAS-2B cells were pretreated with CHF6001 at various doses (CHF6001-6, −7, −8, −9) and/or FP at 10^{-9} mol/L (FP-9), for 1.5 h and then infected with RV1B (MOI = 1). At 24 h post infection, cell supernatants were harvested, and cytokine release determined by ELISA. Combined CHF6001 and FP at all doses showed enhanced suppression of IL-29 that was significantly different from RV1B infection alone, and FP but not CHF6001 at 10^{-6} mol/L (A). Combined CHF6001 and FP at all doses showed enhanced suppression of IL-8 that was significantly different from RV1B infection alone, FP, and also CHF6001 at 10^{-6} M alone (B). Combined CHF6001 and FP at all doses showed enhanced suppression of IP-10 that was significantly different from RV1B infection alone, and FP and for CHF6001 with FP at doses 10^{-6}–10^{-8} M but not at 10^{-9} M. Enhanced suppression versus FP was also seen for CHF6001 with FP at 10^{-6}–10^{-8} M but not for 10^{-9} M. (C) Combined CHF6001 and FP at all doses showed enhanced suppression of RANTES that was significantly different from RV1B infection alone, and FP, and for CHF6001 with FP at doses 10^{-6}–10^{-8} M but not at 10^{-9} M. Enhanced suppression versus FP was also seen for CHF6001 with FP at every dose tested (D). ***$p < 0.001$, **$p < 0.01$ versus RV1B infection alone, ###$p < 0.001$, ##$p < 0.01$ versus RV1B with FP 10^{-9} M; ξξξ$p < 0.001$ ξξ$p < 0.01$ ξ$p < 0.05$ versus RV1B with CHF6001 10^{-6} M $n = 5$.

all cytokines tested, FP showed no significant reduction, while CHF6001 failed to reduce IL-8 but significantly reduced the other three cytokines tested (Fig. 6A–D).

CHF6001 at 10^{-6}–10^{-8} when cultured with FP significantly reduced suppression of IL-29 compared with FP alone but not with CHF6001 alone at 10^{-6} mol/L. (Fig. 6A). CHF6001 at 10^{-6}–10^{-9} with FP showed significantly reduced suppression of IL-8 compared with CHF6001 10^{-6} mol/L but not with FP alone (Fig. 6B). Data for IP-10 and RANTES again were quite similar, with CHF6001 at 10^{-6}–10^{-9} mol/L with FP significantly reducing suppression compared with FP but not CHF6001 alone at 10^{-6} mol/L (Fig. 6C). For RANTES, CHF6001 at 10^{-6}–10^{-8} mol/L with FP at 10^{-10} mol/L had significantly reduced suppression compared with FP alone but not when compared with CHF6001 10^{-6} mol/L for concentrations at any concentration tested (Fig. 6D).

Steroid and PDE4 inhibitor sparing effects of CHF6001 and FP when used in combination

We also compared the effects of combined, low-dose CHF6001 10^{-9} mol/L and FP 10^{-10} mol/L with that of higher dose CHF6001 (10^{-6} mol/L) and FP (10^{-9} mol/L, Table 4). For IL-29, we found that the low-dose combination gave similar levels of suppression when compared with FP at 10^{-9} mol/L and CHF6001 at 10^{-6} mol/L. The levels of suppression for the combination when compared with CHF6001 10^{-6} mol/L were not as impressive but were not statistically different. For all other cytokines tested, the data were similar in that the combination gave similar levels to either higher dose FP at 10^{-9} mol/L or high-dose CHF6001 at 10^{-6} mol/L. Of note, the levels of suppression for the combination for IP-10 were better,

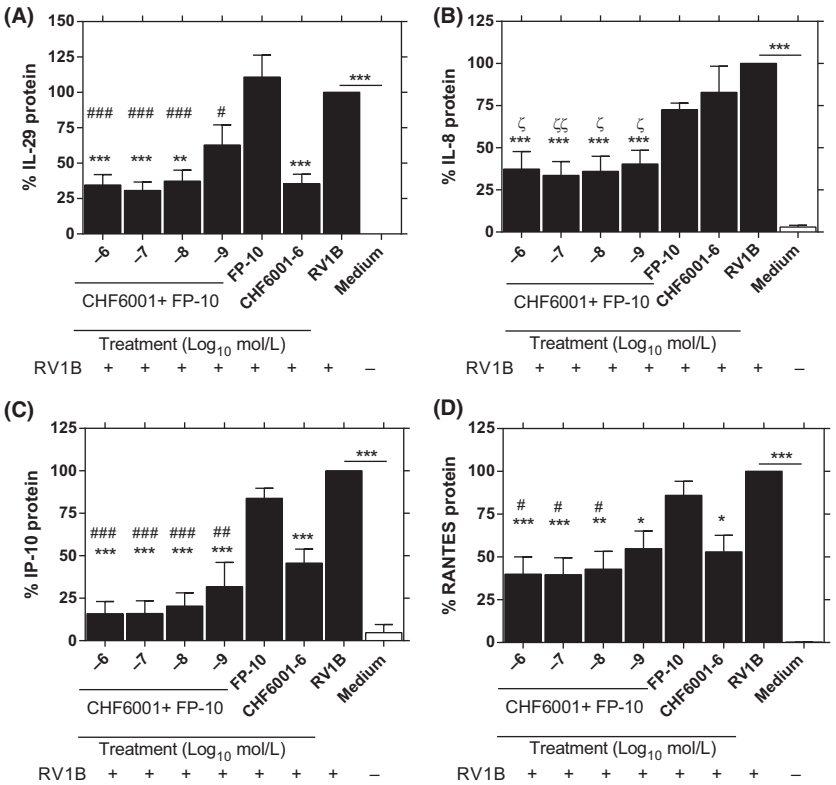

Figure 6. Combined effects of CHF6001 and FP 10^{-10} M on RV-induced IFN and inflammatory cytokine protein release. BEAS-2B cells were pretreated with CHF6001 at various doses (CHF6001 −6, −7, −8, −9) and or FP at 10^{-10} M (FP-10), for 1.5 h and then infected with RV1B (MOI=1). At 24 h post infection, cell supernatants were harvested, and cytokine release determined by ELISA. Combined CHF6001 and FP at 10^{-6}-10^{-8} M showed enhanced suppression of IL-29 that was significantly different from RV1B infection alone, and FP but not CHF6001 at 10^{-6} M (A). Combined CHF6001 and FP at all doses showed enhanced suppression of IL-8 that was significantly different from RV1B infection alone, also CHF6001 at 10^{-6} M alone, but not FP alone (B). Combined CHF6001 and FP at all doses showed enhanced suppression of IP-10 that was significantly different from RV1B infection alone, and FP, but not for CHF6001 at 10^{-6} M (C). Combined CHF6001 and FP at all doses showed enhanced suppression of RANTES that was significantly different from RV1B infection alone, combinations of CHF6001 10^{-6}-10^{-8} M showed significantly enhanced suppression when compared with FP, but not for CHF6001 at 10^{-6} M (D). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ versus RV1B infection alone, $^{###}p < 0.001$, $^{##}p < 0.01$, $^{#}p < 0.05$ versus RV1B with FP 10^{-10} M; $^{\zeta\zeta\zeta}p < 0.01$ $^{\zeta}p < 0.05$ versus RV1B with CHF6001 10^{-6} M $n = 5$.

but not statistically different to either high-dose treatment used alone.

Discussion and Conclusions

In this study, we compared two PDE4 inhibitors, roflumilast and CHF6001, for suppressive activity of virus-induced cytokines and IFNs. While showing potential as therapies in stable asthma and COPD and also exacerbations of COPD (Calverley et al. 2007b; Rennard et al. 2011), few studies have investigated the effects of PDE4 inhibitors in the control of virus-induced inflammation (Ikemura et al. 2000; Van Ly et al. 2013). CHF6001 is a new inhaled highly specific PDE4 inhibitor that may have improved activity and potency (Moretto et al., 2015; Villetti et al., 2015).

In our present study, we compared the relative efficacy and potency of CHF6001 and roflumilast on virus-induced cytokines. Unfortunately, we did not plan experiments to observe the effects of either roflumilast or CHF6001 on basal cytokine expression in the absence of infection. Considering the doses used, we would not expect either of these agents to spontaneously induce cytokine expression. Consistently for all cytokines tested, CHF6001 as a pretreatment had a similar level of efficacy to roflumilast, when used as a pretreatment and posttreatment. The fact that, unlike for CHF6001, pretreatment only with roflumilast gave no suppression for any cytokine measured suggests that CHF6001 is more tightly engaged to the target than roflumilast, thus resistant to the washing step that followed virus infection. This phenomena may be explained by the previously described very slow dissociation kinetic ($t_{1/2} > 20$ h) of CHF6001 (compound 32a) (Armani et al. 2014). When we compared a pretreatment of CHF6001 with pretreatment and posttreatment of

Table 4. Steroid and PDE4 inhibitor sparing effects of combined low-dose FP + CHF6001 in suppression of RV1B-induced cytokine release.

Protein	FP at 10^{-9} mol/L Suppression (%)	CHF6001 at 10^{-6} mol/L Suppression (%)	FP 10^{-10} + 10^{-9} mol/L CHF6001 Suppression (%)	FP + CHF6001 *P*-value	
				Versus FP[1]	Versus CHF6001[2]
IL-29	63 ± 3.5	78 ± 3.0	65 ± 6.7	0.84	0.07
IL-8	63 ± 5.4	27 ± 7.2	50 ± 12	0.28	0.14
IP-10	55 ± 8.3	64 ± 3.5	79 ± 12	0.14	0.12
RANTES	63 ± 3.6	58 ± 3.2	50 ± 13	0.25	0.40

[1]Comparison of suppression of high-dose FP 10^{-9} mol/L versus combined low-dose FP 10^{-10} mol/L and CHF6001 10^{-9} mol/L by unpaired *t*-test.
[2]Comparison of suppression of high-dose CHF6001 10^{-9} mol/L versus combined low-dose FP 10^{-10} mol/L and CHF6001 10^{-9} mol/L by unpaired *t*-test.

roflumilast, levels of suppression where not significantly different however, and no treatment reduced cytokine mRNA and protein levels to baseline. In the best case scenario, the maximum suppression was approximately 80–85% for both compounds; however, not every cytokine exhibited this level of suppression. We did not investigate the effects of CHF6001 as a pretreatment and posttreatment, and it is possible that this level of suppression could be improved further if this strategy was undertaken. CHF6001 produced lower EC_{50} values than roflumilast, on average approximately 13- to 16-fold lower; definitive evidence that CHF6001 is more potent than roflumilast. CHF6001 (compound 32a) was selected from a screen partly on its ability to make extended interactions with all three regions of the PDE4B catalytic binding pocket, resulting in potentially improved inhibitory potency (Armani et al. 2014). Furthermore, CHF6001 potently (subnanomolar IC_{50} values) inhibited TNF-α release from human peripheral blood mononuclear cells and the release of IFN-γ from CD4(+) T cells (Moretto et al. 2015). In all these functional assays, CHF6001 was more potent than previously described PDE4 inhibitors, including roflumilast (Moretto et al. 2015). In summary, the improved potency and implied better efficacy versus roflumilast seen in our studies is in line with other studies.

We also assessed CHF6001 in combination with the steroid, FP. Previous studies have shown that other cAMP elevating agents such as β_2 agonists can synergize with steroids, further reducing virus-induced inflammation (Edwards et al. 2006; Skevaki et al. 2009). We found evidence that low-dose CHF6001 (10^{-8} mol/L), when used together with FP (10^{-9} mol/L), gave a greater suppressive effect than FP 10^{-9} mol/L or high-dose CHF6001 10^{-6} mol/L. Additionally, high-dose CHF6001 (10^{-6} mol/L), when used together with low dose of FP (10^{-10} mol/L), gave a comparable suppressive effect to that of FP 10^{-9} mol/L. This possibly suggests a steroid sparing effect, and that as a combination, it might be possible that doses of both agents could be reduced (up to 10-fold) for the same therapeutic effect. The effect of both drugs was additive rather than synergistic. Complete comparisons between how FP affects the CHF6001 dose–response curve or how CHF6001 might alter the FP dose–response curve were not made, however, and would be needed to ascertain a definitive understanding of the pharmacology of both drugs. However, the data do point to a potential beneficial suppressive effect of CHF6001 when used in conjunction with steroids. How both agents work together are worthy of further study. Other studies have reported additive effects of cAMP elevating agents on both expression and activation of the glucocorticoid receptor (GR), including both β_2 agonists and PDE4 inhibitors (Ortiz et al. 2012; Roth et al. 2002). Investigating either expression or activation of the GR was, however, beyond the scope of our experiments. It is possible though that CHF6001 could be acting via the GR, such as increasing its activation, or by acting on the same pathways thus having an additive suppressive effect. In support of this, Moodley et al. (2013) have shown that combined roflumilast and FP treatment enhanced GRE-dependent reporter activity and several GRE responsive anti-inflammatory genes. Further studies are required to investigate the potential mechanism responsible for steroid sparing effects of CHF6001.

Previous studies have explored how RV induces IFNs and proinflammatory cytokines (Bartlett et al. 2012; Slater et al. 2010; Wang et al. 2011, 2009; Zhu et al. 1997, 1996). Pattern recognition receptors (PRRs), such as TLR-3 in the endosome, and the RIG-like helicases (RLHs) retinoic acid-inducible gene-I and melanoma-differentiated gene-5 in the cytoplasm sense viral RNA and elicit a signaling pathway involving activation of the transcription factors IRF3 and NF-κB p65/p50 that lead to IFN and proinflammatory cytokine transcription, respectively (Bartlett et al. 2012; Slater et al. 2010; Wang et al. 2011, 2009). While there is some controversy over whether or not NF-κB p65 is required for RV-induced IFN transcription (Bartlett et al. 2012), it is possible that CHF6001 and roflumilast are suppressing both IRF3- and NF-κB-mediated gene transcription, as we observed

impressive suppression of proinflammatory cytokines and IFNs using either treatment. Studies in other systems have shown that PDE4 inhibitors can inhibit NF-κB p65 activation and NF-κB-driven inflammatory mediator release (Herve et al. 2008; Kwak et al. 2005). Other studies suggest that PDE4 inhibition may affect signaling intermediates upstream of NF-κB, such as the mitogen-activated protein kinases (Kwak et al. 2005), and interfere with reactive oxygen generation (Cheng et al. 2005). The effects of PDE4 inhibitors on IRF3 signaling have not yet been performed, although it is possible that activation of this transcription factor is also targeted by PDE4 inhibitors. More studies are required to understand how CHF6001 and roflumilast suppress virus-induced cytokines and IFNs; in our experiments, it would also be interesting to investigate if both TLR3 and RLH signaling are affected, as previous studies suggest that TLR3 signaling may be suppressed specifically (Van Ly et al. 2013). Our studies were limited to NF-κB induced cytokines and IFNs. It would be of interest to extend these studies to other epithelial-derived cytokines, notably the pro-Th2 factors IL-33 and IL-25.

We found that both CHF6001 and roflumilast had potent suppressive effects on RV-induced IFNs. Type I IFN-β and type III IFN-λs are potent antiviral cytokines involved in upregulating the intracellular antiviral response as well as inducing other cytokines and chemokines that activate various aspects of the innate and adaptive immune system (Schneider et al. 2014). It could be argued that suppressing beneficial antiviral immunity in the context of asthma and COPD exacerbations would be an unwanted outcome of a new therapy. In fact, impaired antiviral immunity has been reported in both asthmatic (Contoli et al. 2006; Edwards et al. 2012b; Uller et al. 2010) and COPD tissues (Mallia et al. 2010), and IFN-β has been recently trialed in a Phase I/II study for efficacy on naturally occurring asthma exacerbations (Djukanovic et al. 2014). Nevertheless, suppression of the IFN pathways seen in our experiments could simply be a necessary by-product for a potent anti-inflammatory response to the viral infection elicited by PDE4 inhibition. The clinical ramifications of suppressing IFN are currently unknown and require further study. Indeed, we also found that FP suppressed RV-induced IFNs, alone and in combination with CHF6001, yet steroids have been the traditional mainstay of asthma and COPD treatment, and in some studies have a beneficial effect in controlling exacerbation rate or severity (Calverley et al. 2007a; Pauwels et al. 2003). In addition, the use of roflumilast was associated with reduced COPD exacerbations, which might be triggered by viral infections, with the greatest benefits observed in patients with severe COPD who had chronic bronchitis and a history of frequent exacerbations (Lipari et al. 2013). In addition, no increase in the incidence of either influenza-like symptoms or upper respiratory tract infections in COPD patients treated with roflumilast was observed (Chong et al. 2013).

We also found that CHF6001 did not significantly alter RV replication in our experiments, measured either as a titration assays or qPCR for virus RNA. Considering the suppressive effects of both treatments on IFNs, this result is perhaps surprising. A possible explanation for these results is the fact that we only measured virus release or RNA levels at 24 h. Changes in virus induced IFN at 24 h are unlikely to alter virus replication at 24 h, although we cannot exclude an effect on virus replication at later time points (Edwards et al. 2012b). Roflumilast was previously shown to suppress RSV infection at day 10 post infection using airway epithelial cells in air–liquid interface cultures (Mata et al. 2013). While the exact mechanism was not defined in this study, the authors believed that inhibition of RSV attachment and entry may at least, in part, explain these phenomena. Our own results are clearly different, which may reflect virus- or culture-specific effects as well a study design that was not set up to examine the effects of PDE4 inhibitors on RV replication.

In summary, have shown that a new PDE4 inhibitor CHF6001 has anti-inflammatory activity using in vitro models of virus infection, providing evidence that this class of inhibitor may be effective in clinical studies of asthma and COPD exacerbations. Comparisons with the PDE4 inhibitor roflumilast, which is used in severe COPD patients with frequent exacerbations, showed that CHF6001 had greater potency and likely improved efficacy as an anti-inflammatory. Importantly, future experiments should be performed in preclinical animal models to investigate how CHF6001 (topical) compares to roflumilast which is given systemically. In conjunction with steroids, CHF6001 gave enhanced anti-inflammatory activity compared with equivalent doses of steroid or CHF6001 when used alone, in an additive manner, and was strongly suggestive of both a steroid and PDE4 inhibitor sparing effect. Although the exact mechanisms of how PDE4 inhibitors suppress virus-induced inflammation remain to be elucidated, the data support the use of PDE4 inhibitors in exacerbations of chronic airways diseases.

Author Contributions

MRE performed the experimental work. MRE, SLJ, FF, MC, and GV designed the experimental plan. FF, MC, and VG contributed reagents. MRE analyzed the data. MRE, SLJ, FF, MC, and GV wrote the article.

Disclosures

MRE and SLJ each received a research grant and personal consultancy fees from Chiesi Farmaceutici S.p.A. for performing these studies. FF, MC, and GV are employees of Chiesi Farmaceutici S.p.A.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Effects of CHF6001 and roflumilast on cytokine mRNA expressed as raw data.
Table S2. Effects of CHF6001 and roflumilast on cytokine protein release expressed as raw data.
Table S3. Effects of CHF6001 on RV RNA levels and virus release at 24 h.