The Phosphodiesterase Inhibitor Ensifentrine Reduces Production of Proinflammatory Mediators in Well Differentiated Bronchial Epithelial Cells by Inhibiting PDE4

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ABSTRACT

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel that impair airway salt and fluid secretion. Excessive release of proinflammatory cytokines and chemokines by CF bronchial epithelium during airway infection leads to chronic inflammation and a slow decline in lung function; thus, there is much interest in finding safe and effective treatments that reduce inflammation in CF. We showed previously that the cyclic nucleotide phosphodiesterase (PDE) inhibitor ensifentrine (RPL554; Verona Pharma) stimulates the channel function of CFTR mutants with abnormal gating and also those with defective trafficking that are partially rescued using a clinically approved corrector drug. PDE inhibitors also have known anti-inflammatory effects; therefore, we examined whether ensifentrine alters the production of proinflammatory cytokines in CF bronchial epithelial cells. Ensifentrine reduced the production of monocyte chemoattractant protein-1 and granulocyte monocyte colony-stimulating factor (GM-CSF) during challenge with interleukin-1 β . Comparing the effect of ensifentrine with milrinone and roflumilast, selective PDE3 and PDE4 inhibitors, respectively, demonstrated that the

anti-inflammatory effect of ensifentrine was mainly due to inhibition of PDE4. Beneficial modulation of GM-CSF was further enhanced when ensifentrine was combined with low concentrations of the β_2 -adrenergic agonist isoproterenol or the corticosteroid dexamethasone. The results indicate that ensifentrine may have beneficial anti-inflammatory effects in CF airways particularly when used in combination with β_2 -adrenergic agonists or corticosteroids.

SIGNIFICANCE STATEMENT

Airway inflammation that is disproportionate to the burden of chronic airway infection causes much of the pathology in the cystic fibrosis (CF) lung. We show here that ensifentrine beneficially modulates the release of proinflammatory factors in well differentiated CF bronchial epithelial cells that is further enhanced when combined with β_2 -adrenergic agonists or low-concentration corticosteroids. The results encourage further clinical testing of ensifentrine, alone and in combination with β_2 -adrenergic agonists or low-concentration corticosteroids, as a novel anti-inflammatory therapy for CF.

Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP/protein kinase A-activated channel that plays an important role in the secretion of airway surface liquid, a microscopic layer of fluid that is essential for the innate defenses of the lungs (Elborn, 2016; Saint-Criq and Gray, 2017). Loss-of-function mutations in CFTR cause the autosomal recessive disease cystic fibrosis (CF), which is characterized by reduced airway salt and fluid secretion, increased susceptibility to bacterial infection, and chronic inflammation (Cantin et al., 2015; Roesch et al., 2018). The complex innate immune response to infection begins with

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ABBREVIATIONS: ALI, air-liquid interface; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CI, confidence interval; COPD, chronic obstructive pulmonary disease; CRE, cAMP response element; GM-CSF, granulocyte macrophage colony-stimulating factor; GR, glucocorticoid receptor; IL, interleukin; I_{sc} , short-circuit current; M-BEGM, modified bronchial epithelial cell growth medium; MCP-1, monocyte chemoattractant protein-1; PDE, cyclic nucleotide phosphodiesterase; pHBE, primary human bronchial epithelial; qPCR, quantitative polymerase chain reaction; WT, wild type.

epithelial secretion of proinflammatory cytokines and chemokines. These include interleukin (IL)-8 and tumor necrosis factor alpha (TNF- α), which mediate the chemotaxis and neutrophil activation (Mukaida, 2003), granulocyte macrophage colony-stimulating factor (GM-CSF), which stimulates proliferation and maturation of granulocytes and macrophages (Becher et al., 2016), and monocyte chemoattractant protein-1 (MCP-1), which recruits monocytes (Deshmane et al., 2009). The resulting influx of immune cells to the site of infection helps combat infection; however, in CF it produces a neutrophil-dominated, unopposed inflammation that leads to irreversible lung damage.

A major goal of CF pharmacotherapy is to correct the basic defect in the airways, i.e., restore CFTR-dependent ion and fluid transport. However, drugs that reduce excessive inflammation and promote its resolution after infection could also provide benefit. Several anti-inflammatory drugs, including high-dose ibuprofen, azithromycin, and inhaled corticosteroids, have been clinically tested in patients with CF but were found to be of limited effectiveness or unsafe (Ross et al., 2009; Ratjen et al., 2012; Principi et al., 2015; Lands and Stanojevic, 2019). Clinical trials for several other anti-inflammatory drugs for CF are ongoing, and there is clearly a need for new and better treatments that modulate airway inflammation without compromising the ability to fight infection.

Cyclic nucleotide phosphodiesterases (PDEs) are a family of enzymes that degrade cAMP, cGMP, and other cyclic nucleotides. Eleven members of the cyclic nucleotide PDE family are expressed in humans (Maurice et al., 2014). Inhibition of PDE3 and PDE4 in human bronchial epithelial cells elevates intracellular cAMP levels and increases CFTR activity (Al-Nakkash and Hwang, 1999; Cobb et al., 2003; Barnes et al., 2005; Liu et al., 2005; Blanchard et al., 2014; Tyrrell et al., 2015). Recently, we demonstrated that the PDE inhibitor ensifentrine (RPL554; Verona Pharma, London, UK) stimulates both wild-type CFTR and the disease causing mutant R117H-CFTR when endogenously expressed in well differentiated primary human bronchial epithelial cells (Turner et al., 2016). We further observed that ensifentrine activates the CFTR mutants R334W-, T338I-, S549R-, and G551D-CFTR when expressed in the Fisher rat thyroid cell model (Turner et al., 2020). These data indicate that activation of cAMP/protein kinase A signaling by ensifentrine has therapeutic potential for CFTR mutants with residual trafficking of CFTR to the plasma membrane.

PDE3 inhibitors, including ensifentrine, induce bronchodilation in chronic obstructive pulmonary disease (COPD) and

asthma (Myou et al., 1999; Singh et al., 2018), whereas PDE3 and PDE4 inhibitors have previously been used as anti-inflammatory and immunomodulatory drugs for several respiratory diseases (Banner and Page, 1995; Abbott-Banner and Page, 2014). However, there is surprisingly little information on the anti-inflammatory effects of PDE inhibitors in CF. Therefore, we assessed whether ensifentrine could elicit anti-inflammatory effects in CF airways by measuring proinflammatory cytokine production in CF and non-CF airway epithelia. Our results indicate that ensifentrine causes small but significant decreases in GM-CSF and MCP-1 production and has prominent anti-inflammatory effects when combined with a low concentration of dexamethasone. These results suggest that, in addition to increasing CFTR channel activity, PDE inhibitors have beneficial anti-inflammatory effects in CF when used in combination with corticosteroids.

Methods

Cell Culture. The CFBE41o⁻ cell lines overexpressing wild-type (WT) CFTR (CFBE41o⁻ WT) or F508del CFTR (CFBE41o⁻ F508del), kindly provided by Drs. Jeong Hong and Eric Sorscher (Emory University), were passaged in Eagle's Minimum Essential Media supplemented with FBS (10%), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μ g/ml). For experiments, the cells were seeded on fibronectin-coated, 0.33 cm² permeable Transwell supports (Corning) (80,000 cells/well). After 24 hours, the apical medium was removed, and the basolateral medium was replaced with air-liquid interface (ALI) medium (Lonza) to induce differentiation. The medium was changed every 48 hours, and cells were grown at the air-liquid interface for 7 days prior to experiments. Primary human bronchial epithelial cells from three non-CF donors (one male, two female) and three F508del/F508del patients (two male, one female) were isolated by the Primary Airway Cell Biobank in the Cystic Fibrosis Translational Research Centre at McGill University using lung tissue provided by the biobank of respiratory tissues at the Centre Hospitalier de l'Université de Montréal after informed, written consent from donors. All procedures were approved by the Institutional Review Board of McGill University (A08-M70-14B). Cells were isolated using methods similar to those described by Fulcher et al. (2005). They were seeded on type IV collagen-coated, 0.33 cm² permeable Transwell supports (Corning). The apical media were removed after 3 days, and cells were differentiated at the air-liquid interface for 4 weeks prior to experiments.

Cell Treatment and Sample Collection. Differentiated CFBE41o⁻ and well differentiated primary human bronchial epithelial (pHBE) cells were washed 3 $\times$ with PBS and incubated for 6 hours in modified bronchial epithelial cell growth medium (M-BEGM; Lonza) lacking retinoic acid, hydrocortisone, and antibiotics. These factors were omitted as previous studies had shown that they influence cytokine

Fig. 1. Protocols showing cell differentiation, treatment, proinflammatory challenge, and sample collection.

production and expression (Mathew and Sharma, 2000; Zhao and Ding, 2018). Transwell cultures were treated basolaterally with pharmacological agents for 18 hours, then challenged basolaterally with the proinflammatory agonists. Cells were exposed to IL-1 β (10 ng/ml) or flagellin from *Pseudomonas aeruginosa* (5 ng/ml) for 8 hours for RNA extraction, or 24 hours when collecting supernatant. These concentrations were used based on reported concentrations of IL-1 β and flagellin found in sputum collected from patients with CF (Osika et al., 1999; Eickmeier et al., 2010; Balloy et al., 2014; Eckrich et al., 2017) and are consistent with our previous studies (Dauletbaev et al., 2010). A basolateral challenge was used to 1) mimic IL-1 β release from bloodborne immune cells, notably macrophages, which are elevated in the bronchial mucosae of CF children (Tang et al., 2012) and 2) enable flagellin to activate its cognate receptor toll-like receptor 5. Toll-like receptor 5 is mainly expressed on the basolateral membrane of bronchial epithelia (Shikhagaie et al., 2014) and induces a larger effect on cytokine gene expression when added basolaterally compared with when added apically (Hybiske et al., 2004). A schematic of the experimental design is shown in Fig. 1.

Measurements of cAMP, Cytokines, and Chemokines. Intracellular cAMP ([cAMP]_i) was measured using the Direct cAMP ELISA kit (Enzo Life Sciences) according to the manufacturer's instructions. MCP-1, GM-CSF, IL-10, IL-23, and IL-33 were measured in the basolateral medium bathing air-liquid interface cultures of CFBE41o⁻ cells and well differentiated pHBE cells using the Milliplex Map kit (EMD Millipore) and the manufacturer's instructions. Briefly, samples were added to fluorescent-coded magnetic bead sets coated with specific capture antibodies (MagPlex-C Microspheres) that allowed multiple cytokines to be quantified in one sample. After adding the detection antibody and streptavidin-phycoerythrin reporter molecule, samples were read using the Luminex analyzer MAGPIX and analyzed using the Milliplex Analyst 5.1 Software. Some GM-CSF measurements were also made using the OptEIA Human GM-CSF ELISA set (BD Biosciences) as per the manufacturer's instructions. IL-8 was measured in the basolateral medium of well differentiated CFBE41o⁻ cells and pHBE cells using the OptEIA Human IL-8 ELISA set (BD Biosciences) following methods from the manufacturer.

RNA Purification and Reverse Transcription. Cells were lysed with RNA Lysis Buffer from the Zymogen Quick RNA kit and RNA was purified by following the manufacturer's instructions. One hundred nanogram of RNA were reverse transcribed by adding 4 μ l of 5 $\times$ All-In-One MasterMix (ABM) in a reaction volume of 20 μ l. The reaction was carried out using the following protocol: 25°C for 10 minutes, 42°C for 15 minutes, and 85°C for 5 minutes.

Quantitative Polymerase Chain Reaction. Quantitative polymerase chain reactions (qPCRs) were performed by combining 1.5 μ l cDNA with 10 μ M forward and reverse primer targeting the gene of

interest (Intergrated DNA Technologies) and 10 μ l of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) made up to a 20 μ l reaction volume using molecular grade H₂O. Polymerase chain reactions were performed using a BioRad CFX96 Real-Time PCR Detection System with the following protocol: 95°C for 2 minutes followed by 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds before performing a melt curve analysis. To quantify gene expression, $\Delta\Delta$ CT analysis was performed by normalizing the expression of genes of interest to that of the reference gene Hypoxanthine-guanine phosphoribosyltransferase (HPRT1). The following primer sequences were used: MCP-1, forward, 5'-GCCACCTTCATTCC-3', reverse, 5'-AGCAGCCTCTGCACTGAGATCTTC-3'; GM-CSF, forward, 5'-TGACAA GCAGAAAGTCCTTCAG-3', reverse, 5'-CAGCCTCACCAAGCTCAA G-3'; IL-8, forward, 5'-CGCCAACACAGAAATTTATTGTAAAG-3', reverse, 5'-AACTTCTCCACAACCCTCTG-3'; HPRT1, forward, 5'-GCG ATGTCAATAGGACTCCAG-3', reverse, 5'-TTGTTGTAGGATATG CCCTTGA-3'.

Short-Circuit Current Measurements. For measurements of short circuit current, cells were cultured at the air-liquid interface in ALI medium for 7 days before being washed 3 $\times$ with PBS and incubated for 48 hours in M-BEGM. Short-circuit current (I_{sc}) measurements were then performed as previously described (Turner et al., 2020).

Solutions and Reagents. All reagents were purchased from Sigma Aldrich unless otherwise stated. Ensifentrine was obtained from Verona Pharma. CFTR_{inh}-172 was obtained courtesy of Dr. Robert Bridges (Rosalind Franklin University) and Cystic Fibrosis Foundation Therapeutics Inc. For I_{sc} measurements, the basolateral solution contained (millimolar) 115 NaCl, 25 NaHCO₃, 1.2 MgCl₂, 1.2 CaCl₂, 1.2 K₂HPO₄, 2.4 KH₂PO₄, and 10 D-glucose; pH 7.4. The apical solution contained (millimolar) 1.2 NaCl, NaGluconate, 25 NaHCO₃, 1.2 MgCl₂, 4 CaCl₂, 1.2 K₂HPO₄, 2.4 KH₂PO₄, and 10 D-glucose; pH 7.4.

Statistical Analysis. Data were analyzed using GraphPad Prism 6.0 (GraphPad Software). N indicates the number of independent experiments performed, whereas n signifies the number of technical replicates in one experiment. The sample sizes and number of experiments were set prior to the data being obtained. All data sets were used for statistical analyses. The Students t test was used to assess the statistical significance of differences between data of interest. A P value of <0.05 was considered significant.

Results

Ensifentrine Elevates Intracellular cAMP Levels and Stimulates Wild-Type CFTR in Well Differentiated CFBE41o⁻ Cells. We have previously demonstrated that ensifentrine concentration-dependently stimulates wild-type CFTR overexpressed in CFBE41o⁻ cells or endogenously

Fig. 2. Effect of ensifentrine on [cAMP]_i and CFTR-dependent ion transport in CFBE41o⁻ WT cells. (A) cAMP levels measured after 42-hour exposure to DMSO (vehicle, 0.05%), ensifentrine (Ens; RPL554; concentrations indicated), forskolin (Fsk; 2 μ M), and zardaverine (Zard; 10 μ M) in M-BEGM. *** P < 0.001 vs. DMSO in unpaired t test; * P = 0.0132 in unpaired t test. Individual data points and means $\pm$ S.D. are shown; n = 3–14; N = 3. (B) Dose-response curve of ensifentrine to elevations in [cAMP]_i. Data represent means $\pm$ S.D.; n = 7–14; N = 3. (C) A representative I_{sc} experiment demonstrating the effect of ensifentrine (Ens; 10 μ M) and forskolin (Fsk; 10 μ M) on CFTR channel activity. Inhibition by 10 μ M CFTR_{inh}-172 (172) indicates the currents are CFTR-dependent. n = 6; N = 2.

TABLE 1

List of cytokines analyzed in well differentiated human airway epithelia supernatant using MultiPlex ELISAs

Cytokine	Function	References
MCP-1	Expressed in airway epithelia, MCP-1 regulates infiltration of immune cells (i.e., monocytes and macrophages) to site of injury. Found to be significantly higher in sputum and serum of patients with CF vs. healthy persons	Rao et al., 2000; Lundien et al., 2002; Brennan et al., 2009; Velikova et al., 2018
GM-CSF	Produced by airway epithelia, GM-CSF stimulates the production of immune cells, including neutrophils and monocytes. Found to be elevated in patients with CF specifically with chronic <i>P. aeruginosa</i> lung infection	Cox et al., 1991; Jensen et al., 2006
IL-8	Proinflammatory cytokine produced by airway epithelial cells and is elevated in bronchiolar lavage fluid and sputum of patients with CF vs. healthy persons	Dean et al., 1993; Bonfield et al., 1995b; Sagel et al., 2001
IL-10	Anti-inflammatory cytokine that acts to reduce gene expression of proinflammatory cytokines. Shown to be downregulated in CF airway epithelia	Bonfield et al., 1995a
IL-23	Proinflammatory cytokine expressed in airway epithelia	Lee et al., 2017
IL-33	Cytokine expressed in airway epithelia that induces the production of proinflammatory cytokines from mast cells, eosinophils, and helper T cells	Moussion et al., 2008; Préfontaine et al., 2010; Drake and Kita, 2017; Lee et al., 2017

expressed in pHBE cells isolated from non-CF donors, suggesting that ensifentrine-induced elevation of $[cAMP]_i$ activates CFTR (Turner et al., 2016). For the present study, to be compatible with cytokine assays, CFBE41o⁻ cells had to be cultured in different media than in previous studies (i.e., ALI medium for differentiation, followed by M-BEGM for experiments). To confirm that ensifentrine elevates intracellular cAMP and stimulates CFTR in CFBE41o⁻ cells under these conditions, cAMP was measured in cells that were grown for 7 days in ALI medium and then treated for 6 hours with M-BEGM prior to addition of cAMP-elevating agonists for a further 42 hours. Figure 2 shows that ensifentrine concentration-dependently increased $[cAMP]_i$ and, at 10 μ M,

induced a 5.3 ± 0.9 fold increase in $[cAMP]_i$ compared with vehicle control [95% confidence interval [CI]: 4.4–6.1; $P = 0.0002$; $n = 14$; Fig. 2A] confirming that under these conditions inhibiting PDE3/4 with ensifentrine was sufficient to elevate $[cAMP]_i$. Although the dual PDE3/4 inhibitor zardarverine significantly increased $[cAMP]_i$ 3.4 ± 0.7 fold compared with control conditions (95% CI: 2.3–4.4; $P = 0.0132$; $n = 3$), this was significantly lower than the effect of ensifentrine at the same concentration, indicating that ensifentrine is more potent than zardarverine (Fig. 2A). When cells were cultured for 7 days in ALI medium followed by 2 days in M-BEGM, then mounted in Ussing chambers, acute addition of ensifentrine increased the mean I_{sc} by $35.6 \pm 4.5 \mu A cm^{-2}$ (95% CI:

Fig. 3. Effects of ensifentrine on basal and stimulated MCP-1 production in CFBE41o⁻ WT and F508del cells. (A) Schematic of protocol for treating CFBE41o⁻ cells cultured at the air-liquid interface for 7 days. Incubation in M-BEGM was followed by treatment with vehicle (DMSO for ensifentrine and forskolin; 0.05%, ethanol for dexamethasone; 0.05%), ensifentrine (Ens; 10 μ M), dexamethasone (Dex; 5 or 100 nM) or forskolin (Fsk; 2 μ M) for 18 hours. Cells were then challenged with the proinflammatory stimuli IL-1 β (10 ng/ml) or flagellin (Flag; 5 ng/ml) for 24 hours. This was followed by supernatant collection and MCP-1 level quantification by MultiPlex ELISA. (B) Summary of MCP-1 levels in CFBE41o⁻ WT cells. $^{\dagger}P = 0.0001$ in unpaired t test vs. vehicle in basal conditions; $^{\ddagger}P = 0.0067$; $^{\#}P = 0.0009$ in unpaired t tests vs. vehicle after IL-1 β challenge; $^{\S}P = 0.0020$ in unpaired t test vs. vehicle after flagellin challenge. (C) Summary of MCP-1 levels in CFBE41o⁻ F508del cells. $^{\dagger}P = 0.0178$ in unpaired t test vs. vehicle in basal conditions; $^{\ast}P = 0.0324$ in unpaired t test; $^{\ddagger}P = 0.0479$ in unpaired t test and $^{\S}P = 0.0247$ in unpaired t test vs. vehicle after IL-1 β challenge. $^{\#}P = 0.0339$ in unpaired t test vs. vehicle after flagellin challenge. Individual data points and means $\pm$ S.D. are shown; $N = 3$ to 4.

30.8–40.4 $\mu\text{A cm}^{-2}$; $n = 6$; Fig. 2B), a response that was only slightly increased by maximal forskolin stimulation. Together, these observations demonstrate that ensifentrine causes robust elevation of cAMP levels and triggers cAMP-dependent downstream signaling, including activation of CFTR, in CFBE41o[−] cells under these culture conditions.

Production of MCP-1, GM-CSF, and IL-8 in Well Differentiated CFBE41o[−] Cells Is Upregulated by Proinflammatory Challenge. We next wanted to establish that well differentiated cells in our experimental model upregulate production of inflammatory cytokines when exposed to proinflammatory challenges. For this, the cells were cultured in ALI medium for 7 days prior to treatment with M-BEGM for 6 hours, then exposed to vehicle for 18 hours before proinflammatory challenge with either IL-1 β or flagellin for 8 or 24 hours. The outcomes were expression of respective mRNAs (8 hours of stimulation) or production of protein in the supernatant (24 hours of stimulation). The cytokines of interest were studied using SinglePlex or MultiPlex ELISAs (Table 1). Of the six cytokines examined, only MCP-1, GM-CSF, and IL-8 were upregulated by inflammatory challenge. In contrast, levels of IL-10, IL-23, or IL-33 were all either below the detection limit for the assay or were detectable but unaltered by the proinflammatory stimuli (data not shown). We therefore focused the subsequent experiments on measuring MCP-1, GM-CSF, and IL-8 responses to ensifentrine treatment.

Ensifentrine Significantly Reduces Production of MCP-1 in Stimulated CFBE41o[−] F508del Cells. We first assessed the effect of ensifentrine on MCP-1 production in CFBE41o[−] cells in both basal and proinflammatory

conditions, in which cells were stimulated basolaterally for 24 hours with either IL-1 β or *P. aeruginosa* flagellin (Fig. 3A). We also compared the effect of ensifentrine with the cAMP-elevating agonist forskolin and two different concentrations of the anti-inflammatory drug dexamethasone (5 or 100 nM). Under basal conditions, both CFBE41o[−] WT and CFBE41o[−] F508del cells produced similar levels of MCP-1 (Fig. 3, B and C). We observed IL-1 β and flagellin to increase MCP-1 levels 4.7 $\pm$ 2.7 fold (95% CI: 2.5–6.9; $P = 0.00009$; $n = 4$) and 3.0 $\pm$ 1.7 fold (95% CI: 1.0–4.9; $P = 0.0242$; $n = 4$) in CFBE41o[−] WT cells, respectively, and 7.2 $\pm$ 3.6 fold (95% CI: 2.9–11.4; $P = 0.0118$; $n = 4$) and 3.0 $\pm$ 1.5 fold (95% CI: 0.5–5.4; $P = 0.0356$; $n = 3$) in CFBE41o[−] F508del cells, respectively (Fig. 3, B and C). Neither vehicle (DMSO or ethanol) altered MCP-1 levels under basal or proinflammatory conditions in the cell lines examined. Although ensifentrine did not affect MCP-1 release under basal conditions, it did reduce MCP-1 levels by 42% $\pm$ 19% in CFBE41o[−] F508del cells when challenged with IL-1 β (95% CI: 20%–64%; $P = 0.0324$; $n = 4$; Fig. 3C). Interestingly, forskolin caused a similar decrease in MCP-1, suggesting that the anti-inflammatory effect of ensifentrine during IL-1 β challenge was mediated by elevation of [cAMP]_i. The anti-inflammatory drug dexamethasone had a concentration-dependent effect such that the higher concentration of 100 nM was significantly anti-inflammatory in both cell lines under basal and proinflammatory conditions (Fig. 3, B and C). Further studies examined modulation of MCP-1 mRNA levels in ensifentrine-treated CFBE41o[−] cells. In this experiment, the cells were pretreated for 18 hours with a vehicle (DMSO) or ensifentrine, challenged with IL-1 β or flagellin for 8 hours, and then collected for mRNA analyses (Fig. 4A). Under control

Fig. 4. Effects of ensifentrine on IL-1 β -stimulated MCP-1 mRNA in CFBE41o[−] WT and F508del cells. (A) Schematic of protocol for treating CFBE41o[−] cells cultured at the air-liquid interface for 7 days. Incubation in M-BEGM was followed by treatment with vehicle (DMSO; 0.05%) or ensifentrine (Ens; 10 μM). Cells were then challenged with the proinflammatory stimuli IL-1 β (10 ng/ml) or flagellin (Flag; 5 ng/ml) for 8 hours. After this, RNA was isolated, and MCP-1 mRNA expression was assessed by qPCR. (B) Summary of MCP-1 gene expression in CFBE41o[−] WT. * $P = 0.0481$ in unpaired t test vs. vehicle after IL-1 β challenge. (C) Summary of MCP-1 gene expression in CFBE41o[−] F508del. * $P = 0.0218$ in unpaired t test vs. vehicle in basal conditions. Individual data points and means $\pm$ S.D. are shown; $N = 3$ to 4.

Fig. 5. Effects of ensifentrine on basal and stimulated GM-CSF production in CFBE41o⁻ WT and F508del cells. (A) Schematic of protocol for treating CFBE41o⁻ cells cultured at the air-liquid interface for 7 days. Incubation in M-BEGM was followed by treatment with vehicle (DMSO for ensifentrine and forskolin; 0.05%, ethanol for dexamethasone; 0.05%), ensifentrine (Ens; 10 μ M), dexamethasone (Dex; 5 or 100 nM), or forskolin (Fsk; 2 μ M) for 18 hours. Some CFBE41o⁻ F508del cells were also treated with VX-809 (1 μ M) and VX-770 (100 nM) in the absence or presence of ensifentrine. Cells were then challenged with the proinflammatory stimuli IL-1 β (10 ng/ml) or flagellin (Flag; 5 ng/ml) for 24 hours. This was followed by supernatant collection and GM-CSF level quantification by MultiPlex ELISA. (B) Summary of GM-CSF levels in CFBE41o⁻ WT cells. $^{\S}P = 0.0001$ in unpaired *t* test vs. vehicle in basal conditions; $^{\dagger}P = 0.0008$; $^{\ast}P = 0.00004$ in unpaired *t* tests vs. vehicle in IL-1 β challenged conditions; $^{\circ}P = 0.0000004$; $^{\#}P = 0.0000002$ in unpaired *t* tests vs. vehicle in flagellin challenged conditions. (C) Summary of GM-CSF levels in CFBE41o⁻ F508del cells. $^{\S}P = 0.0018$ in unpaired *t* test vs. vehicle in basal conditions; $^{\ast}P = 0.0227$; $^{\dagger}P = 0.0073$; $^{\ddagger}P = 0.0058$; $^{\circ}P = 0.000006$ in unpaired *t* test vs. vehicle in IL-1 β challenged conditions; $^{\circ}P = 0.0130$; $^{\#}P = 0.0031$ in unpaired *t* tests vs. vehicle in flagellin challenged conditions. $^{\ast\ast}P = 0.0031$ in unpaired *t* test vs. VX-809 + VX-770 alone in IL-1 β challenged conditions. Individual data points and means $\pm$ S.D. are shown; *N* = 3 to 4.

conditions, IL-1 β and flagellin increased MCP-1 mRNA expression in both cell lines similar to the increases in protein (Fig. 4, B and C vs. Fig. 3, B and C). In CFBE41o⁻ WT cells challenged with IL-1 β , there was a small reduction in MCP-1 mRNA level after ensifentrine treatment compared with cells treated with vehicle ($P = 0.0481$; $n = 4$; Fig. 4B), indicative of transcriptional regulation by ensifentrine. In CFBE41o⁻ F508del cells, ensifentrine only modulated basal MCP-1 mRNA levels (Fig. 4C) and did not affect the upregulation by respective stimuli (Fig. 4C).

Ensifentrine Reduces GM-CSF Production and mRNA Levels in CFBE41o⁻ Cells. The MultiPlex ELISA panel was used to further test ensifentrine modulation of another inflammatory cytokine, namely GM-CSF. A schematic of this protocol shown in Fig. 5A was identical to that for MCP-1. Under basal conditions, both cell lines tested had similar levels of GM-CSF (Fig. 5, B and C). IL-1 β induced a 2.9 ± 1.4 fold increase in GM-CSF production in CFBE41o⁻ WT cells (95% CI: 1.2–4.6; $P = 0.073$; $n = 4$) and a significantly higher 7.0 ± 4.1 fold increase in GM-CSF production in CFBE41o⁻ F508del cells (95% CI: 2.1–11.8; $P = 0.0121$ vs. control; $P = 0.0031$ vs. CFBE41o⁻ WT; $n = 4$; Fig. 5, B and C). Meanwhile, flagellin caused a 2.1 ± 1.0 fold increase in CFBE41o⁻ WT cells (95% CI: 0.9–3.3; $P = 0.0167$; $n = 4$) and

a 3.9 ± 1.9 fold in CFBE41o⁻ F508del cells (95% CI: 1.6–6.1; $P = 0.0069$; Fig. 5, B and C). We were surprised to observe that ethanol enhanced the GM-CSF production in response to proinflammatory stimuli, in CFBE41o⁻ WT cells (Fig. 5B) suggesting that ethanol can modulate inflammatory responses in airway epithelia. Importantly, ensifentrine caused a $37.1 \pm 21.5\%$ decrease in IL-1 β -stimulated GM-CSF production in CFBE41o⁻ F508del cells compared with vehicle control, indicating anti-inflammatory effects (95% CI: 16.7%–57.4%; $P = 0.0227$; $n = 7$; Fig. 5C), whereas forskolin elicited a similar effect, consistent with the anti-inflammatory action of ensifentrine being mediated by elevation of [cAMP]_i (Fig. 5C). Dexamethasone reduced GM-CSF levels under basal and proinflammatory conditions in a concentration-dependent manner in both WT and F508del cells, serving as an anti-inflammatory positive control. We also tested whether there was any effect on GM-CSF production in CFBE41o⁻ F508del cells by treatment with the CFTR-modulating drugs VX-809 and VX-770, which mimic the clinical drug Orkambi (Wainwright et al., 2015). VX-809 + VX-770 did not significantly affect basal or stimulated GM-CSF production in CFBE41o⁻ F508del cells, nor did they enhance the inhibition by ensifentrine (Fig. 5C). Therefore, partial correction of F508del-CFTR does not alter GM-CSF production in CF epithelia.

Fig. 6. Effects of ensifentrine on the IL-1 β -stimulated GM-CSF mRNA in CFBE41o⁻ WT and F508del cells. (A) Schematic of protocol for treating CFBE41o⁻ cells cultured at the air-liquid interface for 7 days. Incubation in M-BEGM was followed by treatment with vehicle (DMSO; 0.05%) or ensifentrine (Ens; 10 μ M) for 18 hours. Cells were then challenged with the proinflammatory stimuli IL-1 β (10 ng/ml) or flagellin (Flag; 5 ng/ml) for 8 hours. After this, RNA was isolated, and GM-CSF mRNA expression assessed by qPCR. (B) Summary of GM-CSF gene expression in CFBE41o⁻ WT cells. (C) Summary of GM-CSF gene expression in CFBE41o⁻ F508del cells. * $P = 0.0025$ in unpaired t test vs. vehicle after IL-1 β challenge; ‡ $P = 0.0177$ in unpaired t test, vs. vehicle after flagellin challenge. Individual data points and means $\pm$ S.D. are shown; $N = 3$ to 4.

We next assessed the expression of GM-CSF mRNA in CFBE41o⁻ cells with or without ensifentrine, and in the presence or absence of proinflammatory stimuli (Fig. 6A). Increases in GM-CSF mRNA induced by IL-1 β and flagellin closely mirrored increases in protein production (Fig. 6, B and C vs. Fig. 5, B and C). Furthermore, in both tested cell lines, ensifentrine reduced the IL-1 β -stimulated elevation of GM-CSF mRNA levels, with this inhibition reaching statistical significance in CFBE41o⁻ F508del cells (Fig. 6C). Therefore, ensifentrine mediates its effects on GM-CSF production at the transcriptional level.

Ensifentrine has No Effect on IL-8 Protein and mRNA Expression in CFBE41o⁻ Cells. As the last inflammatory cytokine in our MultiPlex ELISA panel, we quantified the production of IL-8 in CFBE41o⁻ cell lines under basal and stimulated conditions. Cells were pretreated with vehicle, ensifentrine, different concentrations of dexamethasone or forskolin before a 24-hour proinflammatory challenge and IL-8 quantification (Fig. 7A). Interestingly, basal IL-8 production was higher under control conditions in CFBE41o⁻ WT cells than in CFBE41o⁻ F508del cells. However, IL-1 β induced similar elevations in IL-8 production in CFBE41o⁻ WT and CFBE41o⁻ F508del cells with respective fold increases of 20.6 ± 6.9 (95% CI: 12.5–28.8; $P = 0.0008$; $n = 4$) and 23.4 ± 8.9 (95% CI: 12.9–34.0; $P = 0.0000004$; $n = 4$; Fig. 7, B and C). Likewise, flagellin also induced similar respective fold increases in IL-8 production of 15.7 ± 6.9 (95% CI: 8.8–22.7; $P = 0.00002$; $n = 4$) and 12.8 ± 4.2 (95% CI: 7.9–17.7; $P = 0.0021$; $n = 4$; Fig. 7, B and C). Note that the robust effect of

proinflammatory stimuli on IL-8 production contrasts with the relatively small upregulation of MCP-1 and GM-CSF (Fig. 7, B and C vs. Figs. 3, B and C and 5, B and C). As was the case with GM-CSF, CFBE41o⁻ WT cells under proinflammatory challenge had enhanced IL-8 production in the presence of ethanol consistent with ethanol modulating cytokine production in these cells. In both cell lines, neither IL-1 β nor flagellin-stimulated upregulation of IL-8 was altered by ensifentrine treatment. Dexamethasone induced only a small downregulation of IL-8 at 5 nM with a greater effect at 100 nM (Fig. 7, B and C). These concentration-dependent effects resemble those observed for MCP-1 and GM-CSF. Similar to GM-CSF, VX-809 + VX-770 did not significantly affect basal or stimulated IL-8 production in CFBE41o⁻ F508del cells, nor did they modulate the response to ensifentrine (Fig. 7C). Therefore, partial correction of F508del does not modulate IL-8 production in CF epithelia.

We also examined ensifentrine modulation of IL-8 mRNA transcript levels during stimulation (Fig. 8A). As expected, challenge with both IL-1 β or flagellin increased IL-8 mRNA expression but this upregulation was not significantly altered by treatment with ensifentrine (Fig. 8, B and C).

Effects of Ensifentrine on MCP-1, GM-CSF, and IL-8 Production in pHBE Cells. We next examined responses in primary bronchial epithelial cells isolated from CF and non-CF donors. The overall experimental design is presented in Fig. 9A. When comparing cytokine production in control conditions in non-CF pHBE cells and CF pHBE cells, we observed IL-1 β -stimulated MCP-1 and GM-CSF levels to be

Fig. 7. Effects of ensifentrine on basal and stimulated production of IL-8 in CFBE41o⁻ WT and F508del cells. (A) Schematic of protocol for treating CFBE41o⁻ cells cultured at the air-liquid interface for 7 days. Incubation in M-BEGM was followed by treatment with vehicle (DMSO for ensifentrine and forskolin; 0.05%, ethanol for dexamethasone; 0.05%), ensifentrine (Ens; 10 μ M), dexamethasone (Dex; 5 or 100 nM), or forskolin (Fsk; 2 μ M) for 18 hours. Some CFBE41o⁻ F508del cells were also treated with VX-809 (1 μ M) and VX-770 (100 nM) in the absence or presence of ensifentrine. Cells were then challenged with the proinflammatory stimuli IL-1 β (10 ng/ml) or flagellin (5 ng/ml) for 24 hours. This was followed by supernatant collection and IL-8 level quantification by MultiPlex ELISA. (B) Summary of IL-8 levels in CFBE41o⁻ WT cells. [§] P = 0.0002 in unpaired t test vs. vehicle in basal conditions; [†] P = 0.0195; [‡] P = 0.0021 in unpaired t tests vs. vehicle in IL-1 β challenged conditions; [#] P = 0.0004 in unpaired t test vs. vehicle in flagellin challenged conditions. (C) Summary of IL-8 levels in CFBE41o⁻ F508del cells. [§] P = 0.005 in unpaired t test vs. vehicle in basal conditions; [#] P = 0.0441 in unpaired t test vs. vehicle in flagellin challenged conditions. Individual data points and means $\pm$ S.D. are shown; N = 3 to 4.

~2-fold higher in the CF pHBE cells, suggesting that CF airway epithelia mount a greater inflammatory response when challenged with proinflammatory stimuli (compare Fig. 9, B and C and Fig. 9, D and E). Production of MCP-1, GM-CSF, and IL-8 was largely unaffected by ensifentrine treatment, whether it was used alone or in combination with VX-809 + VX-770, although there was a tendency for ensifentrine to reduce IL-1 β -stimulated GM-CSF production in CF pHBE cells both when used alone and with VX-809 + VX-770 (Fig. 9E) indicating a small positive effect of ensifentrine. The fact that VX-809 + VX-770 alone did not cause the same trend suggests that merely correcting F508del CFTR is not sufficient to dampen proinflammatory cytokine production. A low concentration of dexamethasone (5 nM) also did not have significant anti-inflammatory effects but tended to reduce IL-1 β -stimulated GM-CSF (Fig. 9, D–G). The lack of a robust effect of 5 nM dexamethasone in pHBE cells is consistent with results in CFBE41o⁻ cells.

Ensifentrine Mediates Its Effects through Inhibition of PDE4. Although the effect was small, ensifentrine was able to significantly reduce IL-1 β -stimulated MCP-1 and GM-CSF production in well differentiated CFBE41o⁻ F508del cells. Ensifentrine is marketed as a dual PDE3/4 inhibitor but is 3440 times more potent toward PDE3 than PDE4 (Boswell-Smith et al., 2006). We compared IL-1 β -stimulated GM-CSF levels in CFBE41o⁻ cells treated with ensifentrine, the PDE4

selective inhibitor roflumilast, the PDE3 selective inhibitor milrinone and the dual PDE3/4 inhibitor zardaverine to gain further insights into the mechanism of action (Fig. 10A). In both CFBE41o⁻ WT and CFBE41o⁻ F508del cells, we observed significant reductions in IL-1 β -stimulated GM-CSF production by ensifentrine and roflumilast but there was no effect of milrinone (Fig. 10, B and C). Zardaverine elicited significant effects in CFBE41o⁻ WT cells but, surprisingly, not in CFBE41o⁻ F508del cells. Together, these data demonstrate that PDE4 inhibition underlies the anti-inflammatory effects on ensifentrine in CFBE41o⁻ cells.

Combining Ensifentrine with Isoproterenol or Low-Concentration Dexamethasone Causes Enhanced Downregulation of GM-CSF in CFBE41o⁻ Cells. We next explored whether the anti-inflammatory effects of ensifentrine could be enhanced by combination therapies. Several studies have demonstrated that PDE inhibition can enhance the ability of corticosteroids and/or β_2 -adrenergic agonists to modulate the expression of genes under the regulation of the glucocorticoid response element and cAMP response element (CRE) (Moodley et al., 2013; BinMahfouz et al., 2015; Reddy et al., 2020). We therefore tested the effects of combining ensifentrine with low concentrations of dexamethasone and/or isoproterenol on IL-1 β -stimulated GM-CSF production in CFBE41o⁻ F508del cells. A schematic of the experimental protocol is shown on Fig. 11A. As previously shown, addition of

Fig. 8. Effects of ensifentrine on the IL-1 β -stimulated IL-8 mRNA in CFBE41o⁻ WT and F508del cells. (A) Schematic of protocol for treating CFBE41o⁻ cells cultured at the air-liquid interface for 7 days. Incubation in M-BEGM was followed by treatment with vehicle (DMSO; 0.05%) or ensifentrine (Ens; 10 μ M) for 18 hours. Cells were then challenged with the proinflammatory stimulus IL-1 β (10 ng/ml) or flagellin (Flag; 5 ng/ml) for 8 hours. After this, RNA was isolated, and IL-8 mRNA expression assessed by qPCR. (B) Summary of IL-8 gene expression in CFBE41o⁻ WT cells. (C) Summary of IL-8 gene expression in CFBE41o⁻ F508del. Individual data points and means $\pm$ S.D. are shown; $N = 3$ to 4.

ensifentrine induced a small but significant $22.5\% \pm 16.5\%$ reduction on IL-1 β -stimulated GM-CSF levels compared with vehicle control (95% CI: 6.8%–38.1%; $P = 0.0170$; $n = 7$; Fig. 11B). However, in the presence of isoproterenol, this was further enhanced to a $47.6\% \pm 5.1\%$ reduction (95% CI: 40.0%–55.0%; $P = 0.0336$ vs. ensifentrine alone; $n = 3$; Fig. 11B), demonstrating that isoproterenol could potentiate the actions of ensifentrine. An even greater inhibition of GM-CSF production of $91.4\% \pm 0.8\%$ (95% CI: 90.1%–92.5%; $P = 0.0000004$ vs. vehicle; $n = 3$; Fig. 11B) was observed when ensifentrine was combined with a low concentration of dexamethasone which suggests additive mechanisms between the two drugs. Combining all three agonists did not further reduce IL-1 β -stimulated GM-CSF levels, presumably because ensifentrine and dexamethasone had already induced maximal downregulation (Fig. 11B). We also assessed GM-CSF mRNA expression after 8 hours challenge with IL-1 β . Under these conditions, ensifentrine reduced GM-CSF gene expression by $46.4\% \pm 15\%$ compared with cells treated with vehicle (95% CI: 24.6%–68.0%; $P = 0.0002$; $n = 4$; Fig. 11C), as we have previously shown in Fig. 6C. However, combining dexamethasone with ensifentrine and isoproterenol caused gene expression to be reduced to levels observed in nonchallenged cells. These findings are consistent with the protein levels and indicate that ensifentrine and dexamethasone act at the transcriptional level to downregulate GM-CSF production in response to inflammatory challenge.

Effect of Ensifentrine and Low-Concentration Dexamethasone Combination on GM-CSF Production by pHBE Cells. Encouraged by powerful anti-inflammatory

effects of ensifentrine and dexamethasone in combination in CFBE41o⁻ cells, we tested the combination in well differentiated pHBE cells. The protocol is shown in Fig. 12A. For these experiments, generally lower absolute levels of GM-CSF were measured compared with the experimental data presented in Fig. 9. This possibly reflects batch to batch variations in components of cell culture media; bovine serum albumin, for example, causes variable biologic effects between batches (Kane, 1983). Nevertheless, the fold changes in response to IL-1 β remain comparable, indicating that the cultures responded to proinflammatory challenge in the same way with respect to GM-CSF production. Consistent with preceding experiments on CFBE41o⁻ cells, the effect of each drug alone was not significant. However, in combination, ensifentrine and dexamethasone significantly reduced GM-CSF levels compared with vehicle control in non-CF pHBE cells ($P = 0.0219$; $n = 3$; Fig. 12). Furthermore, the extent of the reduction in IL-1 β -stimulated GM-CSF production by ensifentrine + dexamethasone was significantly greater than by ensifentrine alone, demonstrating combining ensifentrine and low dose dexamethasone was an effective drug combination therapy to reduce proinflammatory mediators in airway epithelia.

Given that ensifentrine elevates intracellular cAMP and that cAMP can upregulate expression of the glucocorticoid receptor (GR) (Dong et al., 1989; Peñuelas et al., 1998), we tested if the effects observed when combining ensifentrine and dexamethasone might involve upregulation of GR expression. After 24 treatment with IL-1 β , GR mRNA expression was not altered by the presence of ensifentrine (Fig. 11, F and G). We

Fig. 9. Effects of ensifentrine on IL-1 β -stimulated production of MCP-1, GM-CSF, and IL-8 in well differentiated pHBE cells. (A) Experimental protocol for well differentiated pHBE cells cultured at the air-liquid interface for 28 days prior to experiments. Incubation in M-BEGM is followed by treatment with vehicle (DMSO; 0.05%), ensifentrine (Ens; 10 μ M) or dexamethasone (Dex; 5 nM) for 18 hours. Some CF pHBE cultures were also treated with VX-809 (1 μ M) + VX-770 (100 nM), or VX-809 + VX-770 + ensifentrine. Cells were then challenged with the proinflammatory stimulus IL-1 β (10 ng/ml) for 24 hours. This was followed by supernatant collection, and cytokine quantification by MultiPlex ELISA. (B, F, and J) Summaries of absolute MCP-1, GM-CSF, and IL-8 levels in pHBE non-CF cells, respectively; these data are displayed as percent changes when normalized to IL-1 β -stimulated control cells in (C), (G), and (K). (D, H, and L) Summaries of absolute MCP-1, GM-CSF, and IL-8 levels in pHBE CF cells, respectively; these data are displayed as percent changes when normalized to IL-1 β -stimulated control cells in (E), (I), and (M). Individual data points and means $\pm$ S.D. are shown; $N = 3$.

therefore conclude that anti-inflammatory effects of ensifentrine in the presence of the low-concentration dexamethasone occur through some other mechanism.

Discussion

Novel therapeutic strategies are needed for the safe modulation of airway inflammation in CF. Suppression of the production of proinflammatory cytokines needs to not be to the extent where the immune response of the lung becomes compromised. The PDE inhibitor ensifentrine has previously been shown to reduce LPS-induced neutrophil and macrophage levels in the sputum of healthy patients, suggesting potential anti-inflammatory properties (Franciosi et al., 2013). In the present study, ensifentrine increased [cAMP]_i in CFBE41o⁻ WT cells and stimulated robust, CFTR-dependent secretion, demonstrating its ability to induce cAMP signaling in well differentiated human airway epithelia. These cells produced low levels of IL-10, IL-23 and IL-33 that were not modulated by 24-hour proinflammatory challenge. In mice lungs, flagellin-induced increases in IL-33 are rapid and transient that, if similar in cultured human airway epithelia, would have been missed at the time points in our study (Wilson et al., 2012). The high levels of MCP-1, GM-CSF and IL-8 measured after 24 hours suggest these cytokines may be upregulated later or remain elevated for a longer time period than IL-33. This would be consistent with results from *P. aeruginosa*-infected mouse airways (Morris et al., 2009) and suggests that maintaining high levels of these cytokines may be required for late immune responses.

Basal levels of MCP-1, GM-CSF or IL-8 were not different between CFBE41o⁻ WT and CFBE41o⁻ F508del cells, nor between non-CF and CF pHBE cells implying that CF epithelia are not hyperinflammatory under basal conditions. This is consistent with a previous study demonstrating IL-6 and IL-8 are not elevated in CF pHBE cells (Becker et al., 2004). However, IL-1 β -induced elevations in MCP-1 and GM-CSF levels were more pronounced in in CFBE41o⁻ F508del cells compared with CFBE41o⁻ WT cells and GM-CSF levels were significantly higher in IL-1 β -challenged CF pHBE cells compared with non-CF pHBE cells. IL-8 levels did not show these differences, indicating that CF epithelia are hyperinflammatory when challenged with proinflammatory stimuli but only with respect to specific cytokines. In well differentiated CFBE41o⁻ F508del cells, ensifentrine reduced IL-1 β -induced MCP-1 and GM-CSF production to that of those measured in control, IL-1 β -challenged CFBE41o⁻ WT cells, suggesting that ensifentrine can dampen the heightened response to proinflammatory stimuli in CF airway epithelia. The effects of ensifentrine were mediated exclusively through PDE4 inhibition, even though ensifentrine exhibits greater potency for PDE3 versus PDE4 (Boswell-Smith et al., 2006). These findings are consistent with our previous studies that detected little PDE3A expression in well differentiated airway epithelia and showed that ensifentrine-stimulated CFTR activity is identical to stimulation by selective PDE4 inhibitors (Turner et al., 2016). Furthermore, the anti-inflammatory effects of ensifentrine are comparable to those of alternative PDE4 inhibitors reported in other models of lung inflammation (Profita et al., 2003; Sasaki and Manabe, 2004; Buenestado

Fig. 9. Continued.

et al., 2012, 2013). Surprisingly, cytokine production in CFBE41o⁻ WT cells was modulated by ethanol which may be a consequence of previously described ethanol-induced upregulation of immune receptors (Bailey et al., 2009).

In well differentiated pHBE cells from non-CF and CF donors, we observed ensifentrine to not significantly alter MCP-1, GM-CSF or IL-8 under basal or inflammatory conditions. However, there was a trend for ensifentrine to reduce

IL-1 β -stimulated GM-CSF production in CF pHBE cells to levels seen in control, IL-1 β -stimulated non-CF pHBE cells. These observations support data from CFBE41o⁻ cells and suggests that ensifentrine can diminish the hyperinflammation in response to proinflammatory stimuli observed in CF airway epithelia. Levels of proinflammatory mediators were not affected by partial correction of F508del CFTR using VX-809 + VX-770. These results were unexpected as VX-809 + VX-770

Fig. 10. Effects of other PDE3 and PDE4 inhibitors on stimulated GM-CSF production in CFBE41o⁻ cells. (A) Schematic of protocol for treating CFBE41o⁻ cells cultured at the air-liquid interface for 7 days. Incubation in M-BEGM was followed by treatment with vehicle (DMSO; 0.05%), ensifentrine (Ens; 10 μ M), roflumilast (Rofl; 1 μ M), milrinone (Mil; 10 μ M), and zardaverine (Zard; 10 μ M) for 18 hours. Cells were then challenged with IL-1 β (10 ng/ml) before the supernatants were collected, and GM-CSF levels were quantified by SinglePlex ELISA. (B) Summary of GM-CSF levels in CFBE41o⁻ WT cells. * P = 0.0025; § P = 0.0032; $^{\Psi}$ P = 0.0012 in unpaired t tests vs. vehicle control. (C) Summary of GM-CSF levels in CFBE41o⁻ F508del cells. * P = 0.0016; § P = 0.0017 in unpaired t tests vs. vehicle control. Individual data points and means $\pm$ S.D. are shown; N = 3.

Fig. 11. Effects of combining ensifentrine with isoproterenol and low-concentration dexamethasone on stimulated GM-CSF production in CFBE41o⁻ F508del cells. (A) Schematic of protocol for treating CFBE41o⁻ cells cultured at the air-liquid interface for 7 days. Incubation in M-BEGM was followed by treatment with vehicle (DMSO; 0.05% + ethanol; 0.05%), ensifentrine (Ens; 10 μ M), isoproterenol (Iso; 10 nM), dexamethasone (Dex; 5 nM), ensifentrine + isoproterenol, ensifentrine + dexamethasone, and ensifentrine + isoproterenol + dexamethasone for 18 hours. Cells were then challenged with IL-1 β (10 ng/ml) for either 8 hours or 24 hours before the RNA lysate or supernatants were collected, GM-CSF mRNA levels were quantified by qPCR, and protein levels were quantified by SinglePlex ELISA. Note that RNA was only collected for selected outcomes. (B) Summary of GM-CSF levels in CFBE41o⁻ F508del cells. * P = 0.017 in unpaired t test vs. vehicle control; § P = 0.0427 in unpaired t test vs. ensifentrine alone; ‡ P = 0.00000044 in unpaired t test vs. vehicle control; $^{\#}$ P = 0.00000061 in unpaired t test vs. vehicle control. (C) Summary of GM-CSF gene expression levels in CFBE41o⁻ F508del cells. ** P = 0.002 in unpaired t test vs. vehicle control; ‡ P = 0.000002 in unpaired t test vs. vehicle control. Individual data points and means $\pm$ S.D. are shown; N = 3 to 4.

have been shown to reduce IL-8 mRNA levels in well differentiated CF pHBE cells exposed to *P. aeruginosa* exoproducts (Ruffin et al., 2018). The different results in the two studies could be due to the use of different proinflammatory stimuli and/or exposure conditions.

Although ensifentrine and a low concentration of dexamethasone had only small effects on GM-CSF production when used separately, in combination we observed a significant downregulation of GM-CSF. The effects were mirrored in well differentiated pHBE cells, indicating that using drugs in combination has more potent anti-inflammatory effects than either drug alone. PDE4 inhibition has previously been shown to enhance corticosteroid-induced activation of the glucocorticoid response element in BEAS-2B and pHBE cells and potentiate dexamethasone-induced downregulation of IL-8 and TNF α in pHBE cells from patients with COPD (Moodley et al., 2013; BinMahfouz et al., 2015; Reddy et al., 2020). Furthermore, in human airway epithelia, TNF α -stimulated GM-CSF production is significantly reduced in cells treated with both the glucocorticoid budesonide and the cAMP-elevating formoterol (Korn et al., 2001) with these effects of budesonide and formoterol further enhanced when MRP4-dependent cAMP efflux is also blocked (Huff et al., 2018). These data are in good agreement with our findings and imply combination therapies of glucocorticoids and cAMP-elevating agents are an effective approach to modulate airway inflammation. The mechanism of the interaction between corticosteroids

and cAMP-elevating agonists remains uncertain (Taylor and Hancox, 2000). In neurons, synergism between forskolin and dexamethasone has been ascribed to cAMP upregulation of GR expression (Scarcériaux et al., 1995), whereas roflumilast enhances the expression of GR α in COPD pHBE cells after 6-hour treatment (Reddy et al., 2020). However, we did not observe an effect of ensifentrine on GR mRNA expression, perhaps due to our analysis of GR expression occurring later (24 hours) than in previous studies. Alternatively, ensifentrine may increase GR efficiency, e.g., by altering its intracellular localization. Dexamethasone reduces proinflammatory cytokine production in CF airway epithelia in vitro (Rebeyrol et al., 2012), however the concentrations of dexamethasone used generally exceed the clinical dose. The glucocorticoid prednisone has been studied in clinical trials for CF. Although it slowed the progression of lung disease at high doses, adverse effects were observed (Eigen et al., 1995; Cheng et al., 2015). Our results suggest that it may be possible to reduce inflammation in CF airways and avoid harmful side effects of glucocorticoids by combining a low dose of prednisone with a PDE4 inhibitor (i.e., ensifentrine). Recently, preliminary investigations into the use of dexamethasone as a treatment of Covid-19 has suggested potential benefits (Horby et al., 2020). Therefore, combining dexamethasone with PDE4 inhibitors would be interesting to examine in these patients.

Isoproterenol was also able to enhance the effect of ensifentrine on IL-1 β -stimulated GM-CSF production. PDE4 inhibitors

Fig. 12. Effects of combining ensifentrine and low-concentration dexamethasone on GM-CSF production in well differentiated pHBE cells. (A) Experimental protocol for well differentiated pHBE cells cultured at the air-liquid interface for 28 days prior to experiments. Incubation in M-BEGM is followed by treatment with vehicle (DMSO; 0.05% + ethanol; 0.05%), ensifentrine (Ens; 10 μ M), dexamethasone (Dex; 5 nM), or ensifentrine + dexamethasone for 18 hours. Cells were then challenged with the proinflammatory stimulus IL-1 β (10 ng/ml) for 24 hours. This was followed by supernatant collection for GM-CSF level quantification by MultiPlex ELISA, and RNA collection for assessment of GR mRNA expression. (B and C) Summaries of absolute GM-CSF levels in, respectively, non-CF and CF pHBE cells. * P = 0.0219 in unpaired t test vs. vehicle. (D and E) GM-CSF levels presented as % of IL-1 β -challenged and untreated cells (=control). * P = 0.0294 in unpaired t test vs. vehicle; $^{\dagger}P$ = 0.0362 in unpaired t test vs. ensifentrine alone. Individual data points and means $\pm$ S.D. are shown, N = 3. (F and G) GR mRNA expression in, respectively, non-CF and CF pHBE cells. Individual data points and means $\pm$ S.D. are shown; N = 3.

can enhance the expression of genes under control of the CRE in BEAS-2B cells and enhance the sensitivity of CRE-dependent gene expression to β_2 receptor agonists. Interestingly, this effect is most prominent when both PDE3 and PDE4 inhibition is achieved (BinMahfouz et al., 2015). Although their effectiveness is disputed, long acting, inhaled bronchodilators, including β_2 agonists, are prescribed for patients with CF (Smith and Edwards, 2017). Ensifentrine is also clinically administered by inhalation and thus could be combined with inhalation of β_2 agonists, either simultaneously or sequentially, to enhance the anti-inflammatory effects of ensifentrine.

Elevated $[Cl^-]_i$ in CF bronchial epithelia has been attributed to contributing to hyperinflammation in CF airways in which $[Cl^-]_i$ per se can regulate the expression of proinflammatory cytokines (Valdivieso et al., 2016; Clauzure et al., 2017). As a result, stimulation of Cl^- secretion through CFTR has been proposed to dampen inflammation in CF airway epithelia (Perez et al., 2007; Zhang et al., 2018). However, although ensifentrine stimulated CFTR activity in CFBE41o-WT cells, ensifentrine and forskolin elicited their anti-inflammatory effects in CFBE41o⁻ F508del cells, in which cAMP agonists induce negligible Cl^- secretion (Bebok et al., 2005). Therefore, these data indicate that elevated $[cAMP]_i$ per se mediates the anti-inflammatory effects and not the downstream activation of CFTR. The lack of an effect of VX-809 + VX-770 is also consistent with CFTR activity being independent of proinflammatory cytokine production. Instead, cAMP-dependent regulation of NF κ B signaling and/or regulation of genes regulated by the cAMP-sensitive transcription factor CREB (Rovin et al., 1995; Koga et al., 2016) are more likely to underlie the effects of ensifentrine.

Although in bronchial epithelial cells ensifentrine elicited similar anti-inflammatory effects to selective PDE4 inhibitors, CF pathology and inflammation also involve other cell types that express PDE3. For instance, recent reports have indicated that CFTR dysfunction in platelets contribute to the excessive inflammation in CF airways (Ortiz-Muñoz et al., 2020). PDE3 inhibition can modulate platelet function (Gresele et al., 2011); therefore, PDE3 inhibitors may exert anti-inflammatory effects in CF platelets rather than directly on bronchial epithelium. Furthermore, our model of cultured bronchial epithelial cells lacks the surrounding tissue, notably submucosal glands that secrete the majority of the airway surface liquid and airway smooth muscle. Unpublished observations from our laboratory show that ensifentrine stimulates CFTR-dependent fluid and HCO_3^- secretion from Calu-3 cells, a cell line often used as a model for submucosal gland serous cells (Shen et al., 1994) through inhibition of PDE3 rather than PDE4, whereas PDE3 inhibitors, including ensifentrine, induce significant bronchodilation in COPD (Page, 2014). Thus, dual PDE3/4 inhibitors are attractive anti-CF therapeutics given their ability to inhibit PDEs expressed in different cell types to elicit multiple beneficial effects across a range of tissue involved in CF pathology. Given the broad range of tissues that ensifentrine can modulate, administration by inhalation enables it to be restricted to the airways and avoid potential adverse effects in other nonairway tissues.

In summary, we have demonstrated that ensifentrine both stimulates CFTR and beneficially modulates the release of proinflammatory factors in well differentiated bronchial epithelial cells, particularly when combined with low concentrations

of β_2 -adrenergic or corticosteroids. Although the precise molecular mechanisms of ensifentrine-inhibited cytokine production remain to be elucidated, the present study is the first to examine PDE inhibitor effects on inflammatory responses in well differentiated human bronchial epithelia that express F508del CFTR. Given its ability to stimulate mutant CFTR and reduce airway epithelial inflammatory responses, our findings encourage further clinical testing of ensifentrine as a CF therapeutic.

Authorship Contributions

Participated in research design: Turner, Dauletbaev, Lands, Hanrahan.

Conducted experiments: Turner.

Performed data analysis: Turner, Dauletbaev.

Wrote or contributed to the writing of the manuscript: Turner, Dauletbaev, Lands, Hanrahan.

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