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Budesonide and formoterol inhibit inflammatory mediator production by bronchial epithelial cells infected with rhinovirus

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Summary

Background Rhinoviruses (RVs) are responsible for the majority of acute asthma and chronic obstructive pulmonary disease (COPD) exacerbations. RVs infect the lower airways and induce the production of pro-inflammatory and remodelling-associated mediators. Budesonide (BUD) and formoterol (FORM) synergize in controlling asthma and COPD exacerbations; however, their effects on virus-induced inflammation and remodelling are less known.

Objective We investigated whether BUD and FORM synergize in suppressing RV-induced inflammation and remodelling in the airways.

Methods In vitro models of RV infection of BEAS-2B and primary normal human bronchial epithelial (NHBE) cells were used. We assessed the effects of individual and combined drugs administered post-infection, at a clinically relevant concentration range (10^{-6}–10^{-10} M), on the production of CCL5, CXCL10, CXCL8, IL-6 and the remodelling-associated VEGF and bFGF, using ELISA and RT-PCR.

Results BUD effectively suppressed RV-mediated induction of all mediators studied, in a concentration-dependent manner. FORM alone suppressed the production of CXCL8 and bFGF. The combination of BUD and FORM had concentration-dependent, additive or synergistic effects in the suppression of RV-induced CCL5, CXCL8 and CXCL10 in both cell types as well as VEGF in NHBE only. Combination treatment also resulted in an enhanced suppression of RV-induced IL-6, and CCL5 at the mRNA level as compared with BUD or FORM alone.

Conclusion BUD and FORM suppress RV-induced chemokines and growth factors in bronchial epithelial cells in a concentration-dependent, synergistic or additive manner. These data further support the combined use of BUD and FORM in asthma and COPD and intensification of this therapy during exacerbations.

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Introduction

Rhinoviruses (RV), the most frequent causative agents of the common cold, account for 60–65% of exacerbations of both asthma and chronic obstructive pulmonary disease (COPD) [1–3]. RV infect the lower airway epithelium [4], generating a local inflammatory response, inducing the expression of mediators such as CCL5 (RANTES) [5], CXCL8 (IL-8) [6], IL-6 [7], CXCL10 (IP-10) [8] and ICAM-1 [9] as well as remodelling-associated factors such as VEGF [10] and bFGF [11].

Corticosteroids are the major pharmacological agents in the treatment of asthma. Corticosteroids synergize with long-acting β_2-agonists (LABA) in reducing exacerbation frequency in asthma and COPD [12, 13] as well as in exacerbation management [14]. Nevertheless, only little is known about the efficacy of these drugs in the context of virus-induced airway inflammation and remodelling.

Edwards et al. [15] have shown a synergistic suppression of virus-induced chemokines in airway epithelial cells using a combination of fluticasone propionate and salmeterol. We were also able to demonstrate synergistic effects of the above combination on inhibiting RV-induced epithelial production of the remodelling-associated growth factors VEGF and bFGF [16].

In view of these data, we hypothesized that budesonide (BUD), a corticosteroid, and formoterol (FORM), a long-acting bronchodilator, may synergize in suppressing RV-induced inflammation and remodelling. To investigate this hypothesis, we used well-characterized *in vitro* models of RV infection: BEAS-2B cells, a continuous bronchial

epithelial cell line and primary normal human bronchial epithelial cells (NHBE). We assessed the individual and combined effects of the drugs on the production of the T-lymphocyte-attracting chemokines CCL5 and CXCL10, the neutrophil-attracting chemokine CXCL8, IL-6 and the remodelling-associated growth factors VEGF and bFGF.

Materials and methods

Cell cultures and reagents

Human bronchial epithelial cells (BEAS-2B) and Ohio-HeLa cells (ECACC, Salisbury, UK) were grown in a humidified, 5% CO_2 incubator at 37 °C, in Eagle's minimal essential medium (EMEM) buffered with 1% sodium bicarbonate and 0.075% HEPES and supplemented with 10% (v/v) fetal calf serum. All cell culture reagents for these cell lines were purchased from Invitrogen (Paisley, UK). NHBE cells (Clonetics, Wokingham, UK) were obtained from normal non-smoking adult donors and used at passages 3–5. NHBE cells were grown in bronchial epithelial cell basal medium and supplemented with growth supplements, as recommended by the manufacturer. All cell culture reagents for NHBE cells were purchased by Clonetics, while all biochemicals were from Sigma-Aldrich, Steinheim, Germany, unless otherwise specified. BUD and FORM fumarate dihydrate were kindly supplied by AstraZeneca, Lund, Sweden. BUD was dissolved in chloroform at 60 mM and FORM fumarate dihydrate was dissolved in ethanol at 1 mM at the beginning of each experiment, while subsequent dilutions were made in infection media at the required concentrations. Chloroform was present at a final concentration of 20.4 nM–0.2 mM in solutions of BUD at 0.1 nM–1 μM, respectively. Ethanol was present at a final concentration of 1.7 μM–17.1 mM in solutions of FORM at 1 μM–0.1 nM, respectively. Positive controls (RV-infected cells) contained both 0.2 mM chloroform and 17.1 mM ethanol.

Virus cultures and titration

Major and minor RV (RV16 and RV1b, respectively) were propagated in Ohio-HeLa cells and stocks with a 10^6 (RV1b) or 10^7 (RV16) titre were prepared as described previously [4]. Heat-inactivated RV was generated by exposure of RV for 1 h at 58 °C, ultraviolet radiation (UV)-inactivated RV with placing RV at a 4 cm distance from a 100 W UV light source for 8 min, while filtered RV was produced with centrifugation through a 30 kDa membrane (Millipore, Gloucestershire, UK) at 10 000 g for 5 min. Inactivation of RV was effective as assessed by titration assays.

Epithelial cell infection

BEAS-2B and NHBE cells were plated on 48-well plates (Corning, Arlington, UK) and exposed to RVs at a multiplicity of infection of 1 as described previously [4]. Parallel control cultures were exposed to non-infected HeLa cell lysates, heat-inactivated RV, UV-inactivated RV or filtered RV. One hour after infection, cells were washed twice with PBS and fresh medium was added. Cells were then treated with BUD, FORM or both at different concentrations (10^{-6}–10^{-10} M) and plates were incubated at 33 °C. Control cells were treated with drug vehicle instead of drugs. Supernatants (conditioned media, CM) were collected 48 h after addition of drugs, clarified by centrifugation (10 min/3000 g/4 °C) and stored at −80 °C until used in immunoassays. For mRNA analysis, cells were harvested in TRIzol (Invitrogen) at 8 h after addition of drugs and stored at −80 °C for analysis.

Enzyme-linked immunosorbent assays

Levels of CCL5, CXCL8, CXCL10, IL-6, bFGF and VEGF were measured in cell supernatants using commercially available sandwich ELISA assays (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. The lower detectable levels were 7 pg/mL for CCL5 and CXCL8, 5 pg/mL for CXCL10, 1 pg/mL for IL-6, 3 pg/mL for bFGF and 9 pg/mL for VEGF.

Semi-quantitative reverse transcriptase-polymerase chain reaction analysis

Total RNA was isolated from BEAS-2B cells using TRIzol, according to the manufacturer's recommendations. The concentration of extracted RNA was determined spectrophotometrically. Two hundred nanograms of RNA were reverse-transcribed using Superscript III and random hexamers (Invitrogen), according to the manufacturer's instructions. Five microlitres (8%) of cDNA was amplified in 50 μL PCR reactions using 1.5 U Platinum Taq, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (all from Invitrogen) and 0.2 μM of each of forward and reverse primers. The primer sequences and thermocycling conditions used in RT-PCR are shown in Table 1. Amplification in each PCR reaction concluded with a final extension step at 72 °C for 5 min. Thermocycling conditions were optimized in order to ensure that all reactions were in the exponential phase of amplification. Following electrophoresis of ethidium bromide-stained gels, the intensity of the RT-PCR signals was measured by densitometry. Cytokine gene expression was determined semi-quantitatively following normalization to the 'housekeeping' gene 18S rRNA [17, 18] using Band Leader v.3.0 software.

Statistical analysis

Distribution normality was assessed using the Kolmogorov–Smirnov test. Data are expressed as means±standard errors of the mean (SEM) and analyzed using a *t*-test for differences between two groups or using one-way ANOVA

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Table 1. Primer sequences and thermocycling conditions used in RT-PCR

Gene	Sequence (5′-3′)	Thermocycling conditions	No. of cycles	Amplicon (bp)
18S rRNA	Fwd: GAAACGGCTACCACATCCAAG	30 s 94 °C	20	250
	Rev: GCTCCCAAGATCCAACTACG	30 s 52 °C		
		30 s 72 °C		
IL-6/IFN-β_2	Fwd: GCAAAGAGGCACTGGCAGAAAAC	30 s 94 °C	36	284
	Rev: CAGGCTGGCATTTGTGGTTGG	30 s 52 °C		
		40 s 72 °C		
CXCL8/IL-8	Fwd: TTGGCAGCCTTCCTGATTTC	30 s 94 °C	35	170
	Rev: ATTTCTGTGTTGGCGCAGTG	30 s 50 °C		
		30 s 72 °C		
CCL5/RANTES	Fwd: CGCTGTCATCCTCATTGCTA	30 s 94 °C	36	196
	Rev: ACACACTTGGCGGTTCTTTC	30 s 52 °C		
		30 s 72 °C		
bFGF/FGF-2	Fwd: AGAAGAGCGACCCTCACATC	30 s 94 °C	32	227
	Rev: TCGTTTCAGTGCCACATACC	30 s 50 °C		
		30 s 72 °C		

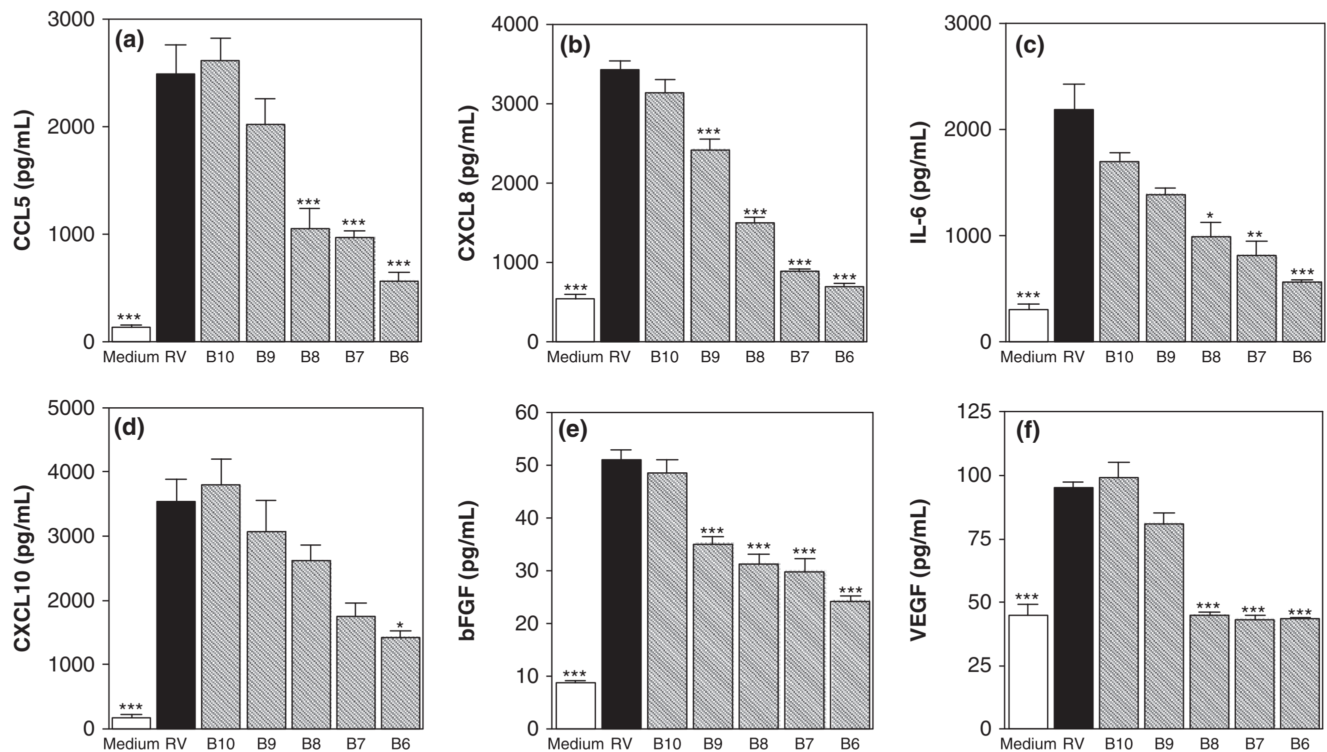

Fig. 1. Concentration responses of budesonide (B) on RV1b-induced CCL5 (a), CXCL8 (b), IL-6 (c), CXCL10 (d), bFGF (e) and VEGF (f) in BEAS-2B cells. Cells were infected with RV1b (RV) or HeLa lysate (Medium; negative control) for 1 h, then the medium was replaced and B was added immediately thereafter and was left on for 48 h. Chemokines were measured by ELISA. Data are presented as mean±SEM B10–B6: 10^{-10}–10^{-6} M; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with RV-infected cultures with addition of drug vehicles (positive control); $n = 3$–7. Linear regression analysis of released cytokine vs. B concentration: (a) $R^2 = 0.5361$, $P<0.0001$; (b) $R^2 = 0.5361$, $P<0.0001$; (c) $R^2 = 0.8289$, $P<0.0001$; (d) $R^2 = 0.4644$, $P<0.0001$; (e) $R^2 = 0.6628$, $P<0.0001$; (f) $R^2 = 0.7695$, $P<0.0001$.

with Bonferroni's multiple comparison test for subsequent between-group effect evaluation. The effect of a drug combination was defined as a synergistic one when it was greater than the sum of the effects by each drug component (based on average values) and significantly different from the effects of each drug component alone. An additive effect was observed when the overall outcome of a drug combination equalled the sum of the individual components' effect. Linear regression analysis was used to evaluate the effect of drug concentration. Statistical analysis was conducted with

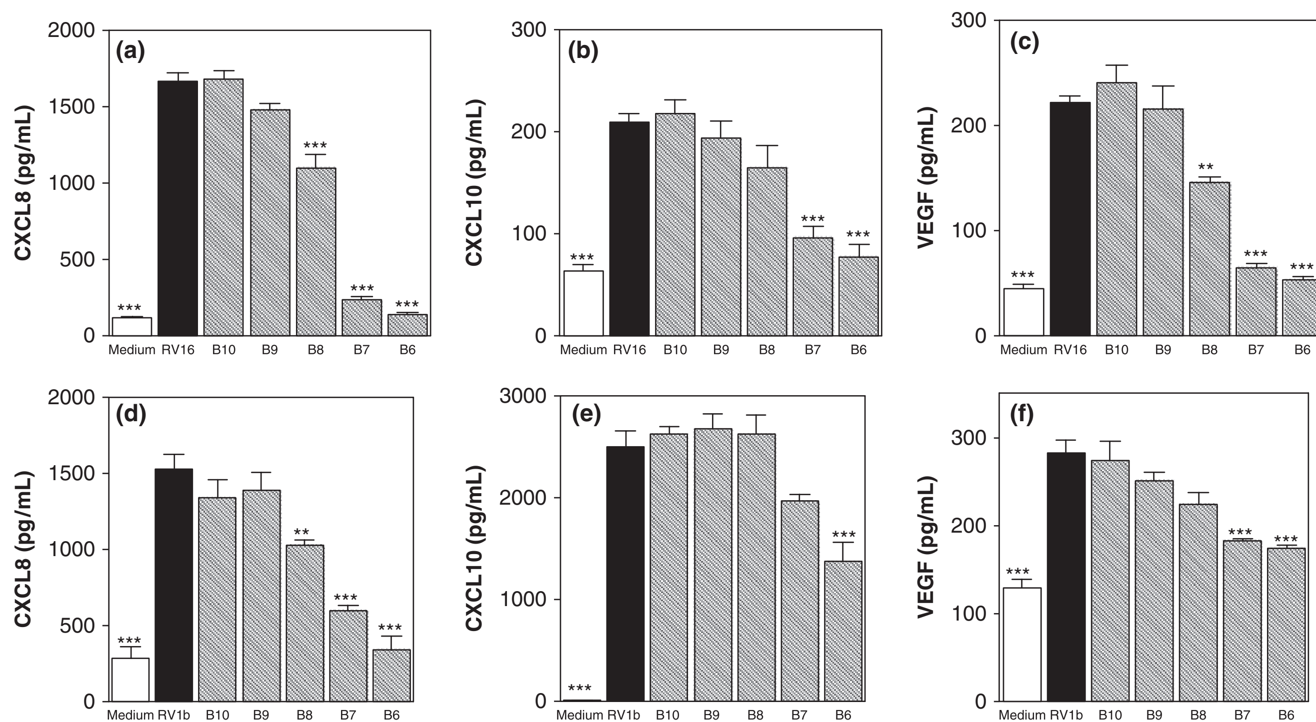

Fig. 2. Concentration-responses of budesonide (B) on RV16-induced CXCL8 (a), CXCL10 (b) and VEGF (c) in BEAS-2B cells and on RV1b-induced CXCL8 (d), CXCL10 (e) and VEGF (f) in NHBE cells. Cells were infected with RV16 or RV1b or HeLa lysate (medium; negative control) for 1 h, then the medium was replaced and B was added immediately thereafter and was left on for 48 h. Chemokines were measured by ELISA. Data are presented as mean±SEM B10–B6: 10^{-10}–10^{-6} M; *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with RV-infected cultures with addition of drug vehicles (positive control); $n=4$. Linear regression analysis of released cytokine vs. B concentration: (a) $R^2=0.9176$, $P<0.0001$; (b) $R^2=0.8119$, $P<0.0001$; (c) $R^2=0.8801$, $P<0.0001$; (d) $R^2=0.8163$, $P<0.0001$; (e) $R^2=0.6810$, $P<0.001$; (f) $R^2=0.7453$, $P<0.0001$.

GraphPad Prism 3.0 software. P-values <0.05 were considered significant.

Results

Rhinoviruses-mediated induction of CCL5 in BEAS-2B is virus specific

Up-regulation of CCL5 production by RV1b in BEAS-2B cells at 48 h after infection was virus specific because it was abolished when UV-irradiated, heat-inactivated or filtered virus was added to the cells (2500±135.7 pg/mL for RV1b-infected cells vs. 96.1±5.7, 127.4±2.2, 100.3±8.7 and 207.6±20.2 pg/mL for cells exposed to UV-irradiated RV, heat-inactivated RV, filtered RV or non-infected HeLa cell lysate, respectively; $n=3$, $P<0.001$).

Budesonide suppresses rhinoviruses-mediated induction of chemokines and growth factors in bronchial epithelial cells in a dose-dependent manner

At 48 h after infection of BEAS-2B cells, RV1b significantly induced the production of all chemokines and growth factors examined. This was effectively suppressed by post-infection treatment with BUD in a concentration-dependent manner at a concentration range of $\leq 10^{-6}$–10^{-8} M (Fig. 1), except for CXCL10, which was significantly suppressed at 10^{-6} M only (Fig. 1d; $P<0.05$). Similar results were obtained with RV1b-infected NHBE cells and when RV16, a major group RV, was used for infection of BEAS-2B cells (Fig. 2).

Effect of formoterol treatment on rhinoviruses-mediated induction of chemokines and growth factors in bronchial epithelial cells

Post-infection treatment of BEAS-2B cells by FORM resulted in a significant suppression of RV1b-mediated induction of CXCL8 (Fig. 3b; $P<0.001$ at all concentrations) and bFGF at 10^{-6}–10^{-9} M (Fig. 3e; $P<0.01$ to <0.001). On the other hand, FORM treatment tended to enhance IL-6 production concentration dependently; however, the effect was not significant (Fig. 3c). All other mediators were not significantly affected by FORM (Fig. 3). FORM treatment of RV16-infected BEAS-2B cells (Figs 4a–c) yielded similar results, with a strong suppression of CXCL8 at all FORM concentrations (Fig. 4a; $P<0.001$), while VEGF was mildly up-regulated at a high (10^{-6} M) FORM concentration (Fig. 4c; $P<0.05$). Comparable results were obtained with RV1b-infected NHBE cells (Figs 4d–f).

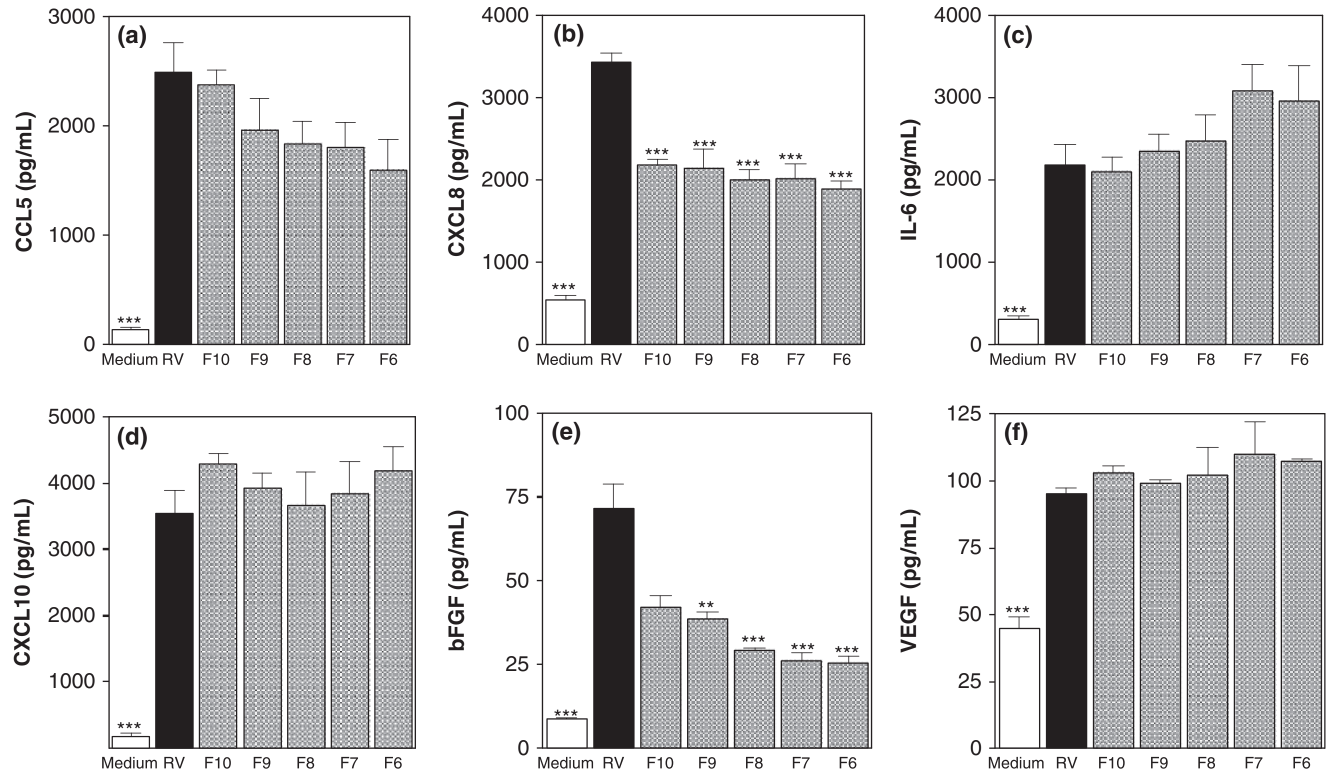

Fig. 3. Concentration responses of formoterol (F) on RV1b-induced CCL5 (a), CXCL8 (b), IL-6 (c), CXCL10 (d), bFGF (e) and VEGF (f) in BEAS-2B cells. Cells were infected with RV1b (RV) or HeLa lysate (medium; negative control) for 1 h, then the medium was replaced and F was added immediately thereafter and was left on for 48 h. Chemokines were measured by ELISA. Data are presented as mean±SEM F10–F6: 10^{-10}–10^{-6} M; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with RV-infected cultures with addition of drug vehicles (positive control); $n = 3$–7. Linear regression analysis of released cytokine vs. F concentration: (a) $R^2 = 0.0524$, $P>0.05$; (b) $R^2 = 0.1006$, $P>0.05$; (c) $R^2 = 0.1260$, $P<0.05$; (d) $R^2 = 0.0039$, $P>0.05$; (e) $R^2 = 0.6589$, $P<0.0001$; (f) $R^2 = 0.0463$, $P>0.05$.

Synergistic effects of the combined budesonide and formoterol treatment on rhinoviruses-mediated induction of chemokines and growth factors in bronchial epithelial cells

Combinations of BUD and FORM (B9F8, B9F7, B8F8 and B8F7; where BxFz: B 10^{-x} M plus F 10^{-z} M) were added to BEAS-2B after RV1b (Fig. 5) or RV16 infection (Figs 6a–c) or to RV1b-infected NHBE cells (Figs 6d–f). In BEAS-2B cells, treatment with B8F8 and B8F7 combinations resulted in a significant down-regulation of RV1b-induced CCL5, CXCL8 and CXCL10 production (vs. control RV-infected cells) that was significantly greater than the effect of either drug component alone (Figs 5a, b, and d). For CCL5, this was also the case for B9F8 and B9F7 and, moreover, the suppression was synergistic (Fig. 5a). Likewise, all drug combinations used, except for the lowest one (B9F8), resulted in a synergistic suppression of RV1b-induced CXCL10 (Fig. 5d). The B8F8 and B8F7 combination treatment resulted in levels of IL-6 similar to 10^{-8}M BUD alone, overcoming the trend for induction of this cytokine by FORM (Fig. 5c). The reduction of bFGF levels by the combination treatment was similar to that of FORM alone and was lower than or similar to that of BUD alone (Fig. 5e). The VEGF levels after combination treatment were similar to or slightly higher than those after BUD alone and similar or lower to those after FORM alone (Fig. 5f).

Similarly as after RV1b-infection, in RV16-infected BEAS-2B cells the B8F8 and B8F7 treatments resulted in an additive suppression of CXCL8, which was significantly greater in comparison with either BUD or FORM alone (Fig. 6a). Combinations also yielded lower levels of CXCL10 compared with either drug alone, although differences were statistically significant only in comparison with FORM (Fig. 6b). Combination B8F8 and B8F7 treatments did not further suppress RV16-induced VEGF by BEAS-2B cells as compared with BUD alone but showed significantly greater suppression than F alone (Fig. 6c).

In RV1b-infected primary epithelial cells, the B8F8 and B8F7 treatment led to suppression of CXCL8, which was significantly greater in comparison with BUD and FORM alone (Fig. 6d). For CXCL10 and VEGF, all drug combinations applied resulted in a strong synergistic reduction of the respective mediator levels, leading to a 40–80% suppression while BUD alone showed only

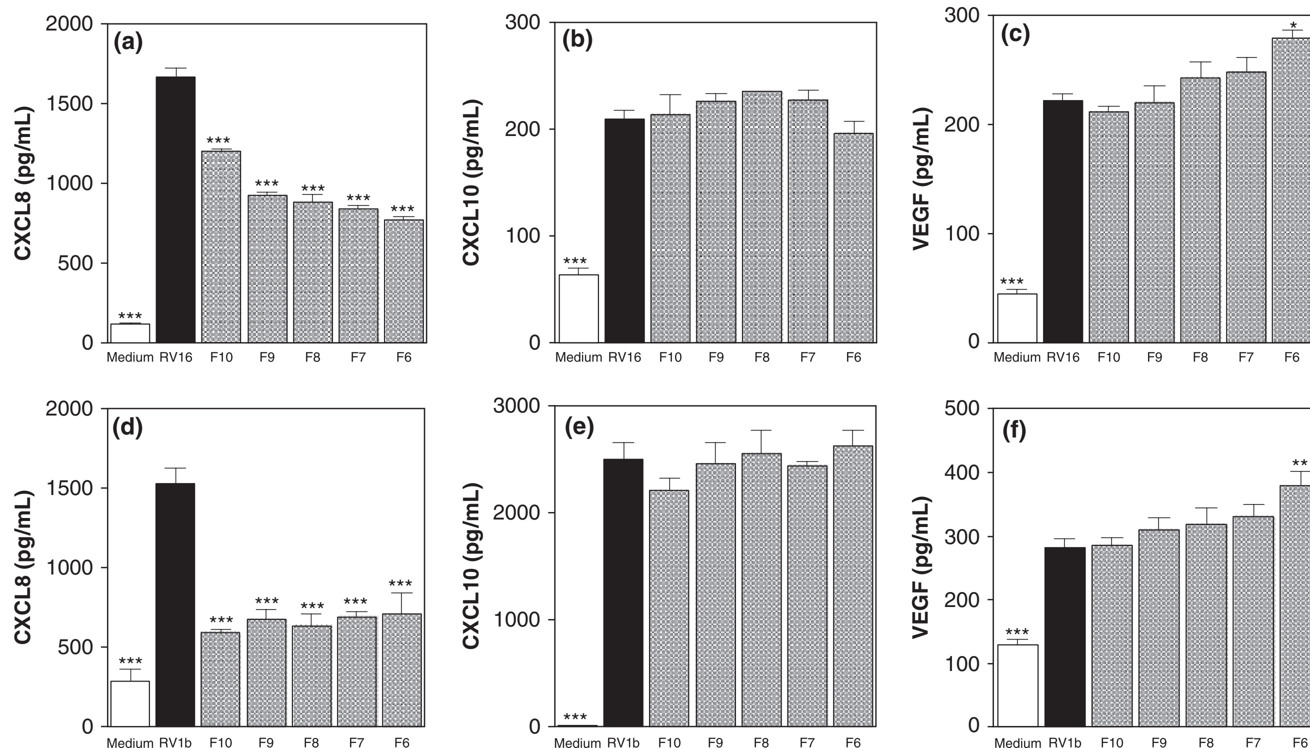

Fig. 4. Concentration responses of formoterol (F) on RV16-induced CXCL8 (a), CXCL10 (b) and VEGF (c) in BEAS-2B cells and on RV1b-induced CXCL8 (d), CXCL10 (e) and VEGF (f) in NHBE cells. Cells were infected with RV16 or RV1b or HeLa lysate (medium; negative control) for 1 h, then the medium was replaced and F was added immediately thereafter and was left on for 48 h. Chemokines were measured by ELISA. Data are presented as mean±SEM (F10–F6: 10^{-10}–10^{-6} M; *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with RV-infected cultures with addition of drug vehicles (positive control); $n=4$. Linear regression analysis of released cytokine vs. F concentration: (a) $R^2=0.0610$, $P>0.05$; (b) $R^2=0.1776$, $P>0.05$; (c) $R^2=0.3877$, $P<0.01$; (d) $R^2=0.7294$, $P<0.0001$; (e) $R^2=0.0786$, $P>0.05$; (f) $R^2=0.5231$, $P<0.001$.

modest effects (if any) and FORM alone had no suppressive effects whatsoever (Figs 6e and f).

Suppression of rhinoviruses1b-mediated induction of IL-6, CXCL8, CCL5 and bFGF mRNA in BEAS-2B cells

We evaluated the ability of B9F8 and B8F8 to suppress RV1b-induced IL-6, CXCL8, CCL5 and bFGF mRNA up-regulation in BEAS-2B cells (Fig. 7). Both combinations examined resulted in a significant down-regulation of RV1b-induced IL-6 in BEAS-2B cells that was significantly greater than the effect of any drug component alone (Fig. 7b). Moreover, the suppression was additive for B8F8 and synergistic for B9F8 and, in both cases, resulted in a more than twofold down-regulation compared with either drug alone. The combinations also demonstrated further significant suppression of RV1b-induced CCL5 compared with individual drug treatments alone (Fig. 7b). A similar trend was observed for CXCL8, where all drug treatments showed significant suppression; however, only the B8F8 combination led to a significant difference ($P<0.05$) over the effect of F8 (but not the effect of B8). The levels of bFGF gene expression, although attenuated further by drug combinations, were not statistically different from those after treatment with individual drug components. All drug treatments, however, resulted in significant suppression of RV-induced bFGF up-regulation, and this was especially evident in the case of the combination treatment ($P<0.001$ for each vs. RV control).

Discussion

Viruses are the most frequent precipitants of acute asthma exacerbations and in this context RVs are the most common isolates [1, 2]. RVs also seem to be important in exacerbating COPD [3]. We examined the effect of BUD, a corticosteroid and FORM, a rapid-acting long-lasting bronchodilator, alone and in combination, in two *in vitro* models of RV infection of bronchial epithelial cells. To our knowledge, this is the first study on the effects of these drugs on mediators associated with virus-induced inflammation and remodelling.

RV-induced CCL5 release was sensitive to UV irradiation and filtration through a 30 kDa membrane, confirming that the presence of a live, replicating virus is needed for chemokine production by bronchial epithelial cells

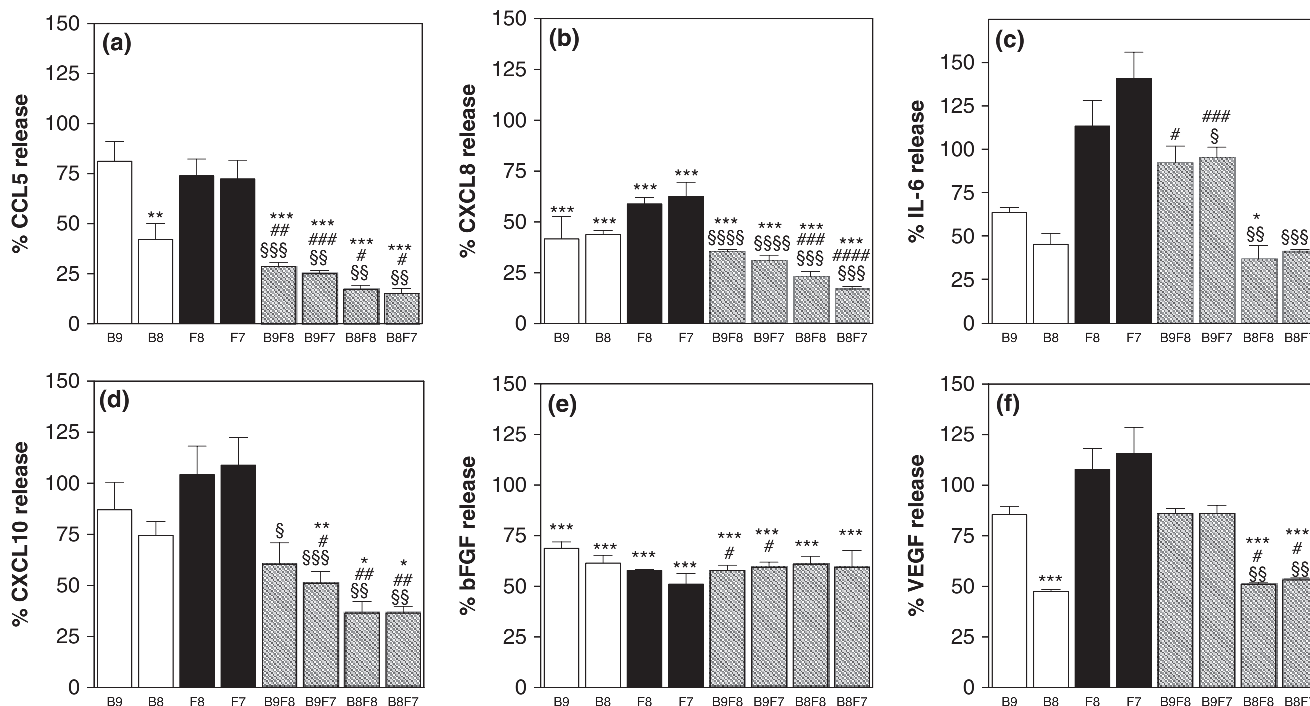

Fig. 5. Suppression of RV1b-induced CCL5 (a), CXCL8 (b), IL-6 (c), CXCL10 (d), bFGF (e) and VEGF (f) release from BEAS-2B cells by treatment with budesonide (B), formoterol (F) or their combinations (BF). Cells were infected with RV1b (RV) for 1 h, then the medium was replaced and B, F or BF were added immediately thereafter and left on for 48 h. Chemokines were measured by ELISA. Data are presented as mean±SEM. Chemokine release is expressed as % of RV-induced release with drug vehicles; B or F9–7: 10^{-9}–10^{-7} M; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with RV-infected cultures with addition of drug vehicle (positive control); $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P\leqslant0.001$, $^{\#\#\#\#}P<.0001$ compared with the effect of the B drug component; $^{§}P<0.05$, $^{§§}P<0.01$, $^{§§§}P<0.001$, $^{§§§§}P<0.0001$ compared with the effect of the F drug component; $n=3$–7.

[19]. Furthermore, we used BUD and FORM at concentrations in the 0.1–1000 nM range because this is representative for airway levels after inhalation treatment [20, 21]. In this study, BUD treatment of either cell system (BEAS-2B or NHBEs) after infection with either major or minor RV subtype resulted in a concentration-dependent suppression of chemokines (CCL5, CXCL8, IL-6 and CXCL10) and growth factors (bFGF and VEGF). We have previously shown that RV-induced VEGF levels were effectively suppressed, while bFGF was not altered, after post-infection treatment with fluticasone [16].

In the case of FORM, treatment of RV-infected bronchial epithelial cells did not significantly suppress CCL5, while it strongly and significantly down-regulated CXCL8 production. Pre- or post-infection treatment with salmeterol as well as pre-infection treatment with salbutamol have been previously reported to lack any anti-inflammatory effects on virus-induced release of CCL5 and CXCL8 by BEAS-2B cells [15]. In the present study, besides CXCL8, FORM also suppressed bFGF in a concentration-dependent manner up to a 10^{-9}M concentration. Our previous work has shown that salmeterol treatment had no effect on bFGF production by BEAS-2B cells [16]. We now also demonstrate that FORM does not significantly influence IL-6 or CXCL10 levels in RV1b-infected BEAS-2B cells although a tendency towards an increase of IL-6 was observed. Similarly, IL-6 production has been recently shown to be up-regulated in RV-infected BEAS-2B and primary bronchial epithelial cells following pre-infection treatment with salmeterol or salbutamol [22]. In contrast, Edwards et al. [15] have previously demonstrated an effective inhibition of CXCL10 with pre-infection salmeterol treatment.

Such differences in the control of different mediators may reflect the impact of drug treatment timing. Indeed, gene expression of different cytokines requires activation of different transcription factors [23, 24]. We have applied a post-infection treatment scheme, because this is more relevant in the clinical setting. Differences between the effects of drugs within the same class, such as FORM, salmeterol and salbutamol, may be explained by differences in their chemical structure, which may result in variable interactions with β_2-receptors. The electrochemical shape of their head groups may influence the strength of interaction with the receptor, which in turn also affects the degree of signal transduction [25]. Of note, FORM is nearly a full β_2-adrenoceptor agonist whereas salmeterol and salbutamol are partial agonists.

Combination treatment with BUD and FORM proved to have additive or synergistic and concentration-dependent

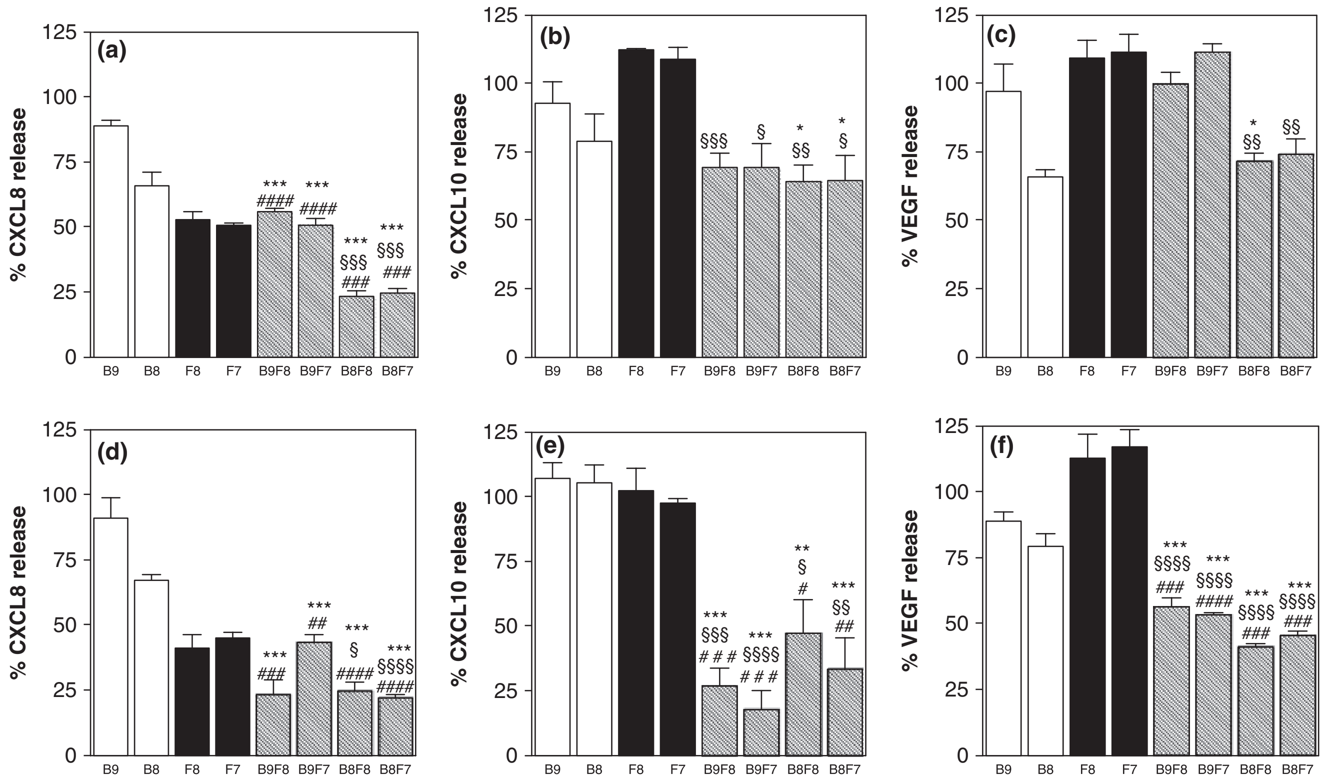

Fig. 6. Suppression of RV16-induced CXCL8 (a), CXCL10 (b) and VEGF (c) from BEAS-2B and suppression of RV1b-induced CXCL8 (d), CXCL10 (e) and VEGF (f) release from NHBE cells by treatment with budesonide (B), formoterol (F) or their combinations (BF). Cells were infected with RV1b or RV16 for 1 h, then the medium was replaced and B, F or BF was added immediately thereafter and left on for 48 h. Chemokines were measured by ELISA. Data are presented as mean±SEM. Chemokine release is expressed as % of RV-induced release with drug vehicles; B or F9–7: 10^{-9}–10^{-7} M; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with RV-infected cultures with addition of drug vehicle (positive control); $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P\leqslant0.001$, $^{\#\#\#\#}P<.0001$ compared with the effect of the B drug component; $^{\S}P<0.05$, $^{\S\S}P<0.01$, $^{\S\S\S}P<0.001$, $^{\S\S\S\S}P<0.0001$ compared with the effect of the F drug component; $n=4$. Medium, RV, B9, B8, F8 and F7 groups are the same as those included in Figs 4 and 5.

effects for suppressing CCL5, CXCL8 and CXCL10 in RV1b-infected BEAS-2B cells. For IL-6, bFGF and VEGF, the drug combination resulted generally in levels similar to the levels obtained by any of the two drugs alone. A similar outcome as in RV-1b-infected BEAS-2B cells was also obtained in RV16-infected BEAS-2B and RV1b-infected NHBE cells, with the exception of VEGF production synergistically suppressed in NHBE cells by the drug combination (despite the fact that FORM alone has no effect). Variability between different RV serotypes has also been reported in several other *in vitro* and *in vivo* studies [16, 26]. Furthermore, BEAS-2B cells represent an SV (simian virus)-40 transformed NHBE cell line, a fact that may account for the observed variation in their responses to RV infection.

At the mRNA level, combination treatment also resulted in significant suppression compared with the effect of the individual drug component on the expression of IL-6, CCL5, and in part CXCL8, with a synergistic effect observed for IL-6. Cytokine suppression at the mRNA level after combined drug treatment with salmeterol/fluticasone of BEAS-2B has also been reported earlier [15].

The effective suppression of growth factors described in the present study (VEGF by BUD alone, bFGF by BUD and FORM alone and suppression of both growth factors by the drug combination) may actually represent plausible mechanisms through which these drugs affect remodelling processes. Importantly, in human primary bronchial epithelial cells, we have observed a strong synergy in the suppression of RV1b-induced VEGF by the BUD and FORM combination. Synergistic or additive inhibition was also seen for CXCL8, which was recently described as having angiogenic properties in various studies [27, 28]. Addition of LABAs to corticosteroid treatment has also been earlier reported to be beneficial for the vascular component of airway remodelling [29, 30].

Additive and/or synergistic interactions between corticosteroid and LABAs may be mediated by a number of molecular mechanisms reported previously [31, 32]. Importantly, local pharmacokinetics of these drugs in the airways may also be involved, as recently shown by Horvath et al. [33]. Such mechanistic approaches were beyond the scope of this article but are being investigated by our group currently.

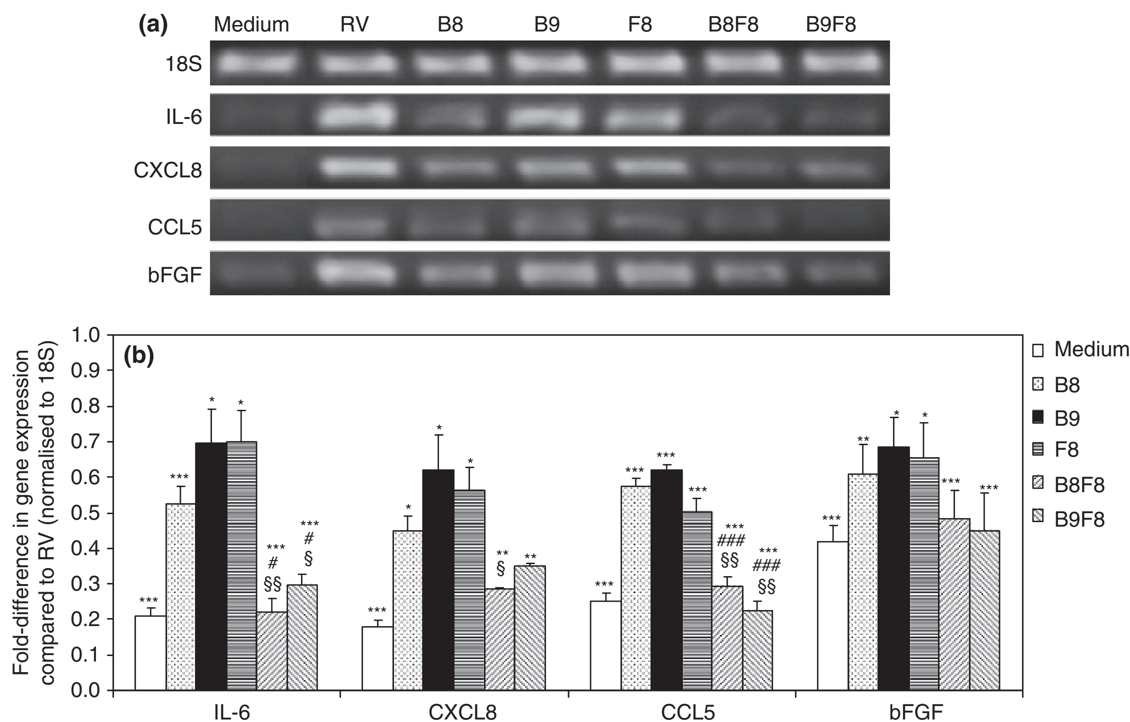

Fig. 7. Effects of budesonide (B), formoterol (F) and their combinations (BF) on chemokine/growth factor gene expression of BEAS-2B cells. Cells were infected with RV1b or HeLa lysate (medium; negative control) for 1 h, then the medium was replaced and B, F or BF was added immediately thereafter and left on for 8 h. (a) Representative RT-PCR bands following gel electrophoresis (b) Gene expression levels in drug-treated cells are expressed as fold-differences compared with control RV-infected cells treated with drug vehicles (set as 1), after normalization to the housekeeping gene 18S rRNA. Differences in gene expression were measured by semi-quantitative RT-PCR. Data are presented as mean±SEM B8–B9: 10^{-8}–10^{-9} M; F8: 10^{-8} M; *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with RV-infected cultures with addition of drug vehicle (positive control); #$P<0.05$, ###$P<0.001$, compared with the effect of the B drug component; §$P<0.05$, §§$P<0.01$, compared with the effect of the F drug component; $n=3$.

Clinical studies support the use of BUDFORM combination treatment at increased dose and dosage frequency when asthma exacerbation symptoms start to appear [14, 34]. Besides the well-known anti-inflammatory effects of BUD, our findings on FORM-mediated suppression of CXCL8 and of bFGF, and concentration-dependent, additive or synergistic effects of BUDFORM combination on other cytokines and growth factors, may represent an additional explanation for the clinically observed, enhanced control of asthma exacerbations by Symbicort® maintenance and reliever therapy [14].

The effect of corticosteroid/LABA treatment on viral clearance from the airways and the probability of acquiring a secondary infection is largely unknown. One may argue that administration of these agents may suppress a beneficial inflammatory host reaction by interfering with interferon production and the generation of a secondary immune response. On the other hand, virus-induced chemokines have been associated with impaired lung function in asthma patients [35], which underlines their contribution to asthma exacerbations. Moreover, numerous clinical studies [13, 36] performed during the last 12 years have shown that administration of the BUD/FORM drug combination results in a reduction of exacerbations' frequency as compared with monotherapy with even double doses of BUD. Because the majority of asthma and COPD exacerbations are caused by respiratory viruses (most commonly RVs), it is unlikely that corticosteroid/LABA therapy may have an unfavourable clinical outcome with regard to long-term impact of RV infection.

In conclusion, we have demonstrated for the first time that BUD effectively suppresses RV-mediated induction of pro-inflammatory and remodelling-associated mediators in bronchial epithelial cells in a concentration-dependent manner. FORM treatment alone may exert anti-inflammatory effects by suppressing the production of CXCL8 by the bronchial epithelium as well as may have anti-remodelling effects by suppressing bFGF. Most importantly, the combination of BUD and FORM has shown concentration-dependent, additive or synergistic effects in the suppression of RV-induced CCL5, CXCL8, CXCL10 and VEGF. These data may have important implications in the treatment of respiratory virus-induced exacerbations of asthma and COPD and the prevention of structural changes in the airway walls, and further support the combined use of BUD and FORM *in vivo* and intensification of this therapy early in the exacerbation course.

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