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The In Vitro Pharmacology of GS-5759, A Novel Bifunctional Phosphodiesterase 4 Inhibitor and Long Acting β_2-Adrenoceptor Agonist

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ABSTRACT

Inhaled long-acting β_2-adrenoceptor agonists (LABA) that act as bronchodilators and the oral anti-inflammatory phosphodiesterase 4 (PDE4) inhibitor roflumilast are both approved therapies for chronic obstructive pulmonary disease (COPD). Here we describe the activity of a novel, inhaled, bifunctional, small molecule (*R*)-6-[(3-{[4-(5-{[2-hydroxy-2-(8-hydroxy-2-oxo-1,2-dihydroquinolin-5-yl) ethyl]amino}pent-1-yn-1-yl)phenyl]carbamoyl}phenyl)sulfonyl]-4-[(3-methoxyphenyl)amino]-8-methylquinoline-3-carboxamide (GS-5759), which has specific β_2 agonist and PDE4 inhibitory activity. GS-5759 demonstrated potent and full agonist activity at β_2 adrenoceptors ($EC_{50} = 8 \pm 4$ nM) and is a potent inhibitor of the PDE4 enzyme ($IC_{50} = 5 \pm 3$ nM). In cell assays, GS-5759 inhibited lipopolysaccharide (LPS)-induced tumor necrosis factor α (TNFα) production in human peripheral mononuclear cells (PBMC) with an $IC_{50} = 0.3$ nM [confidence interval (CI) 0.1–0.6] and in human neutrophils formyl-methionyl-leucyl-phenylalanine (fMLP)-induced super oxide anion production with an $IC_{50} = 3$ nM (CI 0.8–8). The addition of the β_2 antagonist ICI 118551 shifted the IC_{50} in these cell assays to 4 and 38 nM, respectively, demonstrating the contribution of both β_2 agonist and PDE4 inhibitory activity to GS-5759. GS-5759 was also a potent inhibitor of profibrotic and proinflammatory mediator release from human lung fibroblasts. GS-5759 relaxed guinea pig airway smooth muscle strips precontracted with carbachol in a concentration-dependent manner with an $EC_{50} = 0.5$ μM (CI 0.2–2) and had slow dissociation kinetics with an Off $T_{1/2} > 720$ minutes at an EC_{80} concentration of 3 μM. GS-5759 is a novel bifunctional molecule with both potent β_2 agonist and PDE4 inhibitor activity that could provide inhaled bronchodilator and anti-inflammatory therapy for COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic respiratory disease in industrial nations characterized by fixed airway obstruction caused by inflammation and thickening of the smooth muscle, deposition of extracellular matrix from activated myofibroblasts, and recruitment of inflammatory cells that secrete inflammatory mediators, reactive oxygen species, and proteases. All of these components of this complex pathophysiology are thought to contribute to the reduced expiratory capacity seen in COPD patients (http://www.goldcopd.org/guidelines/guidelines-gold-summary-2011.html.).

Long-acting β_2-adrenoceptor agonists (LABA) have been used as the standard of care for COPD to provide bronchodilation and symptom relief (Donohue, 2004; Tashkin and Fabbri,

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ABBREVIATIONS: CCL3, chemokine (C-C motif) ligand 3; CCL5, chemokine (C-C motif) ligand 5; CI, confidence interval; COPD, chronic obstructive pulmonary disease; CGP 12177, 4-[3-[(1,1-dimethylethyl)amino]2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazol-2-one hydrochloride; CXCL10, chemokine (C-X-C motif) ligand 10; dexamethasone, (8*S*,9*R*,10*S*,11*S*,13*S*,14*S*,16*R*,17*R*)-9-fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3*H*-cyclopenta[a]phenanthren-3-one; DMSO, dimethylsulfoxide; ET-1, endothelin-1; fMLP, formyl-methionyl-leucyl-phenylalanine; GM-CSF, granulocyte macrophage colony–stimulating factor; GS-5759, (*R*)-6-[(3-{[4-(5-{[2-hydroxy-2-(8-hydroxy-2-oxo-1,2-dihydroquinolin-5-yl)ethyl]amino}pent-1-yn-1-yl)phenyl]carbamoyl}phenyl)sulfonyl]-4-[(3-methoxyphenyl)amino]-8-methylquinoline-3-carboxamide; GSK256066, 6-((3-(dimethylcarbamoyl)phenyl)sulfonyl)-4-((3-methoxyphenyl)amino)-8-methylquinoline-3-carboxamide; ICI 118551, {(2*R**,3*R**)-1-[(2,3-dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol}; IL-6, interleukin 6; LABA, long-acting β_2-adrenoceptor agonist; LPS, lipopolysaccharide; NHLF, normal human lung fibroblasts; PBMC, peripheral blood mononuclear cells; pCREB, phosphorylated cAMP-responsive element–binding protein; PDE4, phosphodiesterase 4; α-SMA, α-smooth muscle actin; TGF-β1, transforming growth factor-β1; TNFα, tumor necrosis factor α.

2010). Indacaterol is a newly approved once daily LABA that shows superior bronchodilatory activity compared with salmeterol or formoterol but equivalency to the muscarinic antagonist tiotropium for forced expiratory volume in 1 second (FEV1) (Jones et al., 2011). Recently, the novel anti-inflammatory phosphodiesterase 4 (PDE4) inhibitor roflumilast (Daxas; Daliresp; Takeda Pharmaceuticals Korea Co., Ltd., Seoul, Korea) has been approved in the United States and the European Union for COPD GOLD stage 3 and 4 patients with bronchitis, where it is used as an add-on therapy to LABA treatment (Cazzola, 2010). Six large studies ranging from 24 weeks to 1 year have demonstrated clinical efficacy for roflumilast on lung function (FEV1) and acute exacerbations in COPD (Gross et al., 2010), although no direct effect on bronchodilation has been observed. The therapeutic index for roflumilast is narrow because of dose-limiting nausea and emesis (Spina, 2008). Attempts to develop an inhaled PDE4 inhibitor have resulted in molecules with an improved side effect profile, while still being selective and potent (Chapman et al., 2010; Nials et al., 2011; Tralau-Stewart et al., 2011).

The combination of a bronchodilator with an anti-inflammatory agent as a treatment of COPD has been extensively discussed and is reviewed in Phillips and Salmon (2012). The ability of β_2-adrenoceptor agonists to also have direct anti-inflammatory activity has been previously described, including inhibition of histamine, arachidonic acid metabolites, and TNFα release from mast cells (Undem et al., 1988; Bissonnette and Befus, 1997; Chong et al., 1998) and on cytokine release from monocytes (Seldon et al., 2005; Donnelly et al., 2010). Previous studies from this laboratory and others have shown the effects of roflumilast combined with salmeterol, formoterol, or dexamethasone on lipopolysaccharide (LPS)-induced peripheral blood mononuclear cells (PBMC) cytokine production, showing additive effects for PDE4 inhibition with either a LABA or glucocorticosteroid (Seldon et al., 2005; Tannheimer et al., 2012a), suggesting an additional anti-inflammatory effect in combination or possible triple combination. Additionally, the additive effects of roflumilast in combination with indacaterol on profibrotic and proinflammatory mediator release from transforming growth factor-β1 (TGF-β1)-treated normal human lung fibroblasts provide supporting evidence for combination therapy (Tannheimer et al., 2012b).

(*R*)-6-[(3-{[4-(5-{[2-Hydroxy-2-(8-hydroxy-2-oxo-1,2-dihydroquinolin-5-yl)ethyl]amino}pent-1-yn-1-yl)phenyl]carbamoyl}phenyl) sulfonyl]-4-[(3-methoxyphenyl)amino]-8-methylquinoline-3-carboxamide (GS-5759; see Fig. 1) is a bifunctional molecule that has dual pharmacology. It contains a long-acting β_2-adrenoceptor agonist pharmacophore, covalently linked to a PDE4 inhibitor pharmacophore, that has been optimized for inhaled delivery. GS-5759 has the potential for superior anti-inflammatory activity over oral PDE4 inhibitors, because topical delivery may improve the therapeutic window. Additionally, the combination of a β_2 agonist with a PDE4 inhibitor provides the potential to elevate and maintain cellular cAMP levels, which may offer a molecular mechanism for additive or synergistic anti-inflammatory effects through elevation of a common second messenger. The anti-inflammatory and anti-fibrotic properties of GS-5759 were investigated on PBMC, neutrophils, and lung fibroblasts. Evaluation of the compound as a potential component of a triple therapy approach was also evaluated by combination with the glucocorticosteroid (dexamethasone) on LPS-induced PBMC cytokine production. Additionally, relaxation of carbachol precontracted guinea pig smooth muscle strips was evaluated compared with the LABA indacaterol.

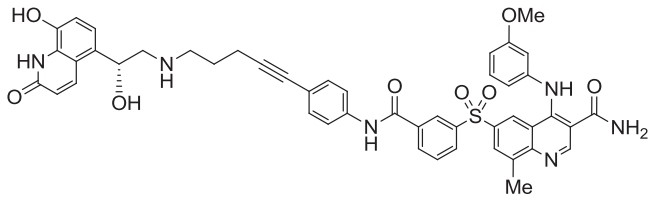

Fig. 1. Chemical structure of GS-5759.

Materials and Methods

Cell culture reagents purchased were RPMI 1640 medium, fetal bovine serum, bovine serum albumin, and penicillin/streptomycin (Life Technologies, Carlsbad, CA). Dimethylsulfoxide (DMSO), LPS, ICI 118551 [{(2*R**,3*R**)-1-[(2,3-dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol}], and dexamethasone were purchased from Sigma-Aldrich (St. Louis, MO); and TGF-β1 and TNFα were from R&D Systems (Minneapolis, MN). ICI 118551 is a potent and selective β_2-adrenoceptor antagonist with pA_2 of 9.3 at guinea pig uterine β_2 adrenoceptor with greater than 100-fold selectivity over β_1 adrenoceptor and does not have partial agonist activity (Bilski et al., 1983). Roflumilast was purchased from Kemprotec Limited (Middlesbrough, UK). Indacaterol and GS-5759 were synthesized in-house.

Compound Synthesis. The synthesis of (*R*)-6-[(3-{[4-(5-{[2-hydroxy-2-(8-hydroxy-2-oxo-1,2-dihydroquinolin-5-yl)ethyl]amino}pent-1-yn-1-yl)phenyl]carbamoyl}phenyl)sulfonyl]-4-[(3-methoxyphenyl) amino]-8-methylquinoline-3-carboxamide (GS-5759) was described by Baker et al. (2011).

PDE Enzyme Assays. The PDE4 isozyme assays were performed based on an assay characterization described previously (Saldou et al., 1998). The assays were optimized for each PDE4 catalytic domain isozyme at an enzyme dilution for which the concentration of product was linearly related to the time of incubation for which the velocity (nmol/min/mg total protein) was proportional to the added enzyme. The PDE4 inhibitors rolipram and 3-isobutyl-1-methylxanthine have been characterized and confirmed to be active against all four of the isozymes tested in these assays. In-house, recombinant human PDE4B2 enzyme (0.12 nM) was combined with compound or DMSO vehicle for 5 minutes at 25°C in PDE Glo Reaction Buffer (Promega, Madison, WI), and cAMP (50 nM) was added to the enzyme mix and incubated for 60 minutes. The enzyme reaction, was terminated and PDE Glo detection buffer containing protein kinase A enzyme was added. Kinase Glo solution (Promega) then was added, and luminescence was measured (EnVision; PerkinElmer Life and Analytical Sciences, Waltham, MA). Data are presented as the mean ± S.E.M. of the vehicle control enzyme activity as measured by increase in luminescence for GS-5759 (n = 3) and roflumilast (n = 19). For assessment of GS-5759 activity on other PDE4 isozymes, screening against human recombinant PDE4A1, -B1, and -D2 isozymes, as well as PDE family members at high concentration only (1 μM) was done by measuring residual cAMP (40 nM, 25°C, 30 minutes) by homogeneous time-resolved fluorescence (Cerep, Poitiers, France) (Saldou et al., 1998; Bender and Beavo, 2006). Additionally, GS-5759 (10 μM) was screened against a large panel of receptors in a competitive radioligand binding assay for off-target activity, with data expressed as specific binding (% of control) (Cerep).

Potency Against β_1 and β_2 Adrenoceptors. Binding of compounds to recombinant human β_2 adrenoceptor expressed on Chinese hamster oocyte cells or recombinant human β_1 adrenoceptor expressed on human embryonic kidney 293 cells was assessed using a competitive receptor binding assay with the radiolabeled ligand [^{3}H](−)CGP 12177 (Cerep). In the same cell systems, elevation of

intracellular levels of the second messenger cAMP was assessed after compound treatment of either cell as a functional readout (Cerep).

PBMC Cytokine Evaluation. All donors gave informed consent and were healthy nonsmoking males. Blood was drawn into K_2EDTA vacutainers, and PBMC were isolated by Ficoll gradient centrifugation (Ficoll Paque Plus; GE Healthcare, Chalfont, St. Giles, UK). PBMC were cultured in RPMI 1640 medium + 10% fetal bovine serum, penicillin/streptomycin in 96-well plates at 1×10^5 cells/well overnight (5% CO_2, 37°C) before LPS stimulation for cytokine evaluation. PBMC were preincubated with ICI 118551 (10 μM) or vehicle for 60 minutes, followed by GS-5759 treatment alone or in combination with dexamethasone for 60 minutes prior to LPS (0.4 ng/ml) stimulation. Supernatants were collected after 6 hours and analyzed for TNFα, interleukin 6 (IL-6), and chemokine (C-C motif) ligand 3 (CCL3) and run in a Luminex bead-based assay, according to manufacturer's instructions (EMD Millipore, Billerica, MA). Results were calculated in picograms per milliliter based on a standard curve, and results were normalized to ICI 118551 or vehicle control. The pretreatment of PBMC with ICI 118551 had no effect on LPS-stimulated cytokine production compared with DMSO vehicle control.

Neutrophil Superoxide Anion. Human neutrophils were purified from whole blood (male nonsmokers) and were preincubated with ICI 118551 (10 μM) or vehicle for 60 minutes followed by GS-5759 treatment of 60 minutes prior to stimulation with formyl-methionyl-leucyl-phenylalanine (fMLP) at the donor-specific EC_{80} concentration (range: 200–700 nM). Superoxide release was measured with a LumiMax Superoxide Anion Detection Kit (Agilent Technologies, Inc., Santa Clara, CA). The pretreatment of neutrophils with ICI 118551 had no effect on fMLP-stimulated superoxide anion production compared with DMSO vehicle control.

Normal Human Lung Fibroblasts Cytokine Production. Normal human lung fibroblasts (NHLF, sex unknown; Lonza Walkersville Inc., Walkersville, MD) were routinely cultured in fibroblast growth media-2 complete media (Lonza Walkersville Inc.) (5% CO_2, 37°C) and used at passages 2–5 for experimentation. NHLF were cultured to subconfluence and then starved in RPMI 1640 medium + 0.1% bovine serum albumin overnight. Cells were pretreated with compounds (0.1 pM–1.0 μM) for 1 hour and then stimulated with TNFα (10 ng/ml). Supernatants were collected at 24-hour poststimulation, and cytokines were measured in a Luminex bead-based assay according to manufacturer's instructions (Millipore). Results were calculated in picograms per milliliter based on a standard curve.

NHLF ET-1 Quantitation and α-SMA Expression. For experimentation, NHLF were cultured to subconfluence, starved in RPMI 1640 medium + 0.1% BSA overnight, pretreated with compound for 1 hour, and stimulated with 10 ng/ml TGF-β1 for 24 hours [endothelin-1 (ET-1) production] or 48 hours [α smooth muscle actin (α-SMA) expression]. ET-1 production was quantitated by use of QuantiGlo ELISA kit (R&D Systems, Inc.) according to manufacturer's instructions. Results were calculated in picograms per milliliter based on a standard curve, and percentage inhibition was calculated relative to TGF-β1-stimulated DMSO control. For α-SMA characterization, cells were trypsinized, fixed (fix buffer 1, 10 minutes, 37°C; BD Biosciences, San Jose, CA), permeabilized (perm buffer III, 30 minutes, 4°C; BD Biosciences), stained with an anti-α-SMA-fluorescein isothiocyanate antibody (Abcam, Cambridge, MA), and visualized on a LSR II flow cytometer (BD Biosciences). Mean fluorescent intensity was determined, and percentage inhibition was calculated from TGF-β1-stimulated DMSO-treated cells. All experimental groups were performed in triplicate.

pCREB Quantitation. NHLF were serum starved and were treated with compound or DMSO (0.1%) (0–120 minutes at 37°C), washed with cold PBS, and then lysed on ice for 20 minutes (Millipore MAP Cell Signaling kit; Millipore). Lysates were analyzed for phosphorylated cAMP-binding protein (pCREB) with Luminex bead-based assay [Milliplex MAP Phospho CREB (Ser133) MAPmate; Millipore], as measured by mean fluorescent intensity. Results were calculated as percentage of DMSO control.

Animals and Airway Smooth Muscle Strip Preparation. Male Dunkin-Hartley guinea pigs (500–700 g), free of guinea pig-specific pathogens, were obtained from Jackson Laboratories (Wilmington, MA). All animal experiments in this study were covered by the protocol approved by the local Institutional Animal Care and Use Committee. Animals were killed by inhalation of CO_2. The trachea was quickly and carefully dissected and immediately immersed in prechilled oxygenated Krebs-Henseleit solution containing indomethacin (10 μM). The connective tissue was trimmed away, and four tracheal rings (3–4 mm) were cut off from the middle portion of one trachea; the cartilage then was cut to make airway smooth muscle strips. Airway smooth muscle strips were then placed in the chambers of the DMT Myograph tissue bath system (ADInstruments, Colorado Springs, CO) and immersed in oxygenated Krebs- Henseleit solution containing indomethacin (10 μM). Baseline tension was artificially set to 14 mN and stabilized for at least 30 minutes before the experiment. The potency at β_2 adrenoceptors was measured as the percent inhibition of carbachol (0.3 μM)-induced contraction in guinea pig tracheal smooth muscle strips preincubated with GS-5759 or indacaterol (1 nM–10 μM). The functional association rate of GS-5759 with β_2 adrenoceptors was measured as the time course of relaxation of carbachol-induced contraction in tracheal smooth muscle strips. The functional disassociation rate of GS-5759 and indacaterol (100 nM, 300 nM, and 3 μM) from β_2 adrenoceptors was measured as the recovery of carbachol-induced contraction in tracheal smooth muscle strips after washout of the compound. To evaluate the contribution of the β_2-adrenoceptor agonist component of GS-5759, guinea pig tracheal smooth muscle strips were incubated with the specific β_2-adrenoceptor antagonist ICI 118551 (10 μM for 1.5 hours) followed by treatment with GS-5759 (1 μM for 3 hours), and then contraction by carbachol (0.3 μM) was measured for 30 minutes. The effect of GS-5759 on the carbachol-induced contraction was compared in the absence and presence of ICI 118551 incubation.

Statistical Analysis. Cytokines and superoxide anion are calculated as the arithmetic mean ± S.E.M. for each experimental group with nonstimulated set as 0% and stimulated DMSO vehicle set as 100%. IC_{50} values are presented as geometric means with 95% confidence intervals. Statistical analysis used a paired two-tailed Student's t test or repeated one-way analysis of variance with Tukey's post hoc test. Differences in mean IC_{50} between experimental groups were also performed using a Student's t test, including Welch correction, as variances were different between groups. Results where the P value was <0.05 were considered significant (GraphPad Software, San Diego, CA).

Results

β-Adrenoceptor and PDE Enzyme Activity. The bifunctional molecule GS-5759 has both β_2-adrenoceptor agonist activity and PDE4 inhibitory activity. The β_2-adrenoceptor activity was demonstrated in binding studies, where GS-5759 has a concentration-dependent inhibition of radioligand binding to β_2 adrenoceptor with an IC_{50} value of 11 ± 2 nM (Table 1). In a functional assay, GS-5759 elevated intracellular cAMP in a concentration-dependent manner with an EC_{50} of 8 ± 4 nM and appeared to be a full agonist at β_2 adrenoceptor, because it attained 100% of the cAMP elevation at 1 μM compared with isoproterenol standard. In a similar experiment, GS-5759 elevated cAMP in a concentration-dependent manner with an EC_{50} of 33 ± 20 nM at β_1 adrenoceptors. GS-5759 was a competitive, concentration-dependent inhibitor of PDE4B2, with an IC_{50} of 5 ± 3 nM, in comparison with the clinically approved roflumilast that demonstrated a similar concentration-dependent inhibition of PDE4B2 of 3 ± 1 nM. GS-5759 also potently inhibited the PDE4A1, -B1, and -D2

TABLE 1
Relative β adrenoceptor and PDE4 enzyme inhibitor potency for GS-5759 and comparator compounds
Data are mean nanomolar values from n = 1–3 separate experiments performed in triplicate.

Compound	Inhibition: IC_{50}				Binding: IC_{50}	Cell cAMP: EC_{50}		β_1 vs. β Fold Ratio
	PDE4A1A Enzyme	PDE4B1 Enzyme	PDE4B2 Enzyme	PDE4D2 Enzyme	β_2 Receptor	β_2 Receptor	β_1 Receptor	
Roflumilast			3 ± 1		NA	NA	NA	NA
GSK256066	0.06 ± 0.002	0.1	<0.1	0.04 ± 0.007	>300	>300	NA	NA
Indacaterol			NA		6 ± 10	1 ± 0.2	26 ± 12	26
GS-5759	67 ± 19	40 ± 6	5 ± 3	56 ± 9	11 ± 2	8 ± 4	33 ± 20	4

isozymes with IC_{50} of 67 ± 19, 40 ± 6, 56 ± 9 nM, respectively. The selectivity of GS-5759 against other PDE enzymes was also assessed at 1 μM, where GS-5759 inhibited PDE2A, -3B, -5, -8A1, and -10A1 by less than 10% and PDE11A4 by 16% and was without effect on PDE1B, -3A, and -7A (data not shown). The IC_{50} comparisons with control compounds roflumilast, GSK256066, and indacaterol are shown in Table 1.

Molecular off-target screening studies evaluated the displacement of radioligand binding by GS-5759 in a panel of 80 receptors. At a concentration of 10 μM, GS-5759 exhibited no significant specific binding to the majority of the receptors (data not shown). The exceptions included the following: benzodiazepine (58% specific binding compared with control), NK_1 (65%), mu opiate receptor (77%), 5-hydroxytryptophan 1B (74%), and Na^+ channel-site 2 (73%) receptors.

Activity of GS-5759 in Human PBMC and Neutrophils. In human PBMC, GS-5759 was a potent, concentration-dependent inhibitor of LPS-induced TNFα production, with a mean IC_{50} of 0.3 nM (CI 0.1–0.6) and a maximum level of inhibition of 95 ± 1% (Fig. 2A). When the assay was performed in the presence of an excess of the β_2-adrenoceptor antagonist ICI 118551 (10 μM) (Bilski et al., 1983; Lemoine et al., 1985), to assess the PDE4-directed inhibition of TNFα production alone, GS-5759 had an IC_{50} of 4 nM (CI 1–15) and a maximum level of inhibition of 68 ± 6%. Statistical analysis of the IC_{50} values and maximum inhibition run in the absence and presence of ICI 118551 demonstrated a statistically significant difference ($P < 0.01$ and 0.001, respectively), indicating that the β_2-adrenoceptor agonist activity of GS-5759 contributed to the increased cellular potency. In comparison, the PDE4 inhibitors roflumilast and GSK256066 demonstrated IC_{50} values of 2 nM (CI 0.9–4) and 0.04 nM (CI 0.02–0.08), respectively, with no effect of ICI 118551 (data not shown).

In studies to evaluate the inhibition of release of superoxide anions from purified human neutrophils, GS-5759 demonstrated a concentration-dependent inhibition of superoxide production with an IC_{50} of 3 nM (CI 0.8–8) and a maximum level of inhibition of 93 ± 5% (Fig. 2B). When the assay was performed with ICI 118551 (10 μM), GS-5759 had an IC_{50} of 38 nM (CI 18–83) and a maximum level of inhibition of 83 ± 6%. A significant difference in the IC_{50} values of GS-5759 in the absence or presence of ICI 118551 was seen ($P < 0.01$). The PDE4 inhibitor roflumilast had an IC_{50} of 7 nM (CI 4–14), and GSK256066 had an IC_{50} of 1 nM (CI 0.5–2) (data not shown).

In additional studies in PBMC, the effect of the glucocorticosteroid dexamethasone was assessed on LPS-induced cytokine production (TNFα, IL-6, and CCL3) in the absence or presence of a submaximal concentration of GS-5759 (0.4 nM, based on the IC_{50} for TNFα production; Fig. 2A). Cytokine induction after 24 hours for GM-CSF, CXCL10 (IP-10), and CCL5 [RANTES (regulated on activation normal T cell expressed and secreted)] was 228 ± 12, 21,042 ± 513, 6220 ± 241 pg/ml, respectively. Dexamethasone alone caused a concentration-dependent inhibition of all three cytokines. In the presence of GS-5759, the IC_{50} for dexamethasone for TNFα was decreased from 2 nM (CI 0.8–6) to 0.6 nM (CI 0.1–4); the IC_{50} for dexamethasone for IL-6 was decreased from 0.5 nM (CI 0.02–12) to 0.2 nM (CI 0.0006–75); and the IC_{50} for dexamethasone for CCL3 was decreased from 5 nM (CI 0.5–39) to 0.6 nM (CI 0.2–1) (Fig. 3, A–C). Additionally, the maximum level of cytokine inhibition achieved by dexamethasone was increased in the presence of GS-5759 from 71 ± 5

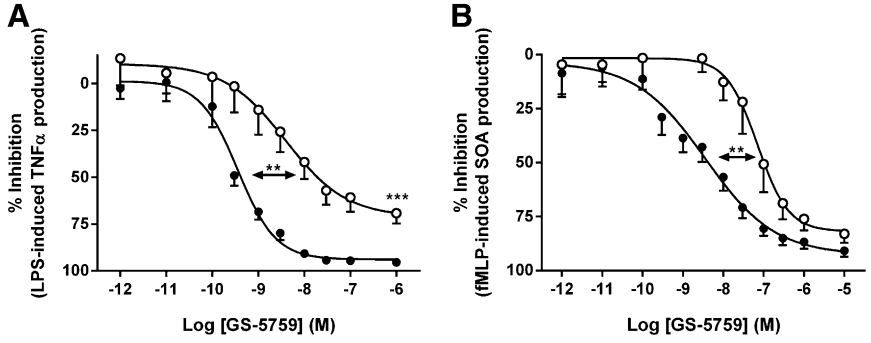

Fig. 2. Concentration-dependent inhibition of LPS-induced TNFα production from PBMC and fMLP-induced superoxide anion production from neutrophils by GS-5759 in the absence or presence of ICI 11855. (A) Data represent the percent maximum inhibition of TNFα release from human PBMC compared with ICI 118551- or vehicle-treated cells after stimulation with LPS (mean ± S.E.M., n = 14–18 donors). (B) Data represent the percentage maximum inhibition of superoxide anion release from human neutrophils compared with ICI 118551- or vehicle-treated cells after stimulation with fMLP (mean ± S.E.M., n = 9 donors). ●, GS-5759 alone; ○, GS-5759 in the presence of ICI 118551. **$P \leq 0.01$ GS-5759 IC_{50} compared with ICI 118551 treated; ***$P \leq 0.001$ maximal inhibition of GS-5759 compared with ICI 118551 treated.

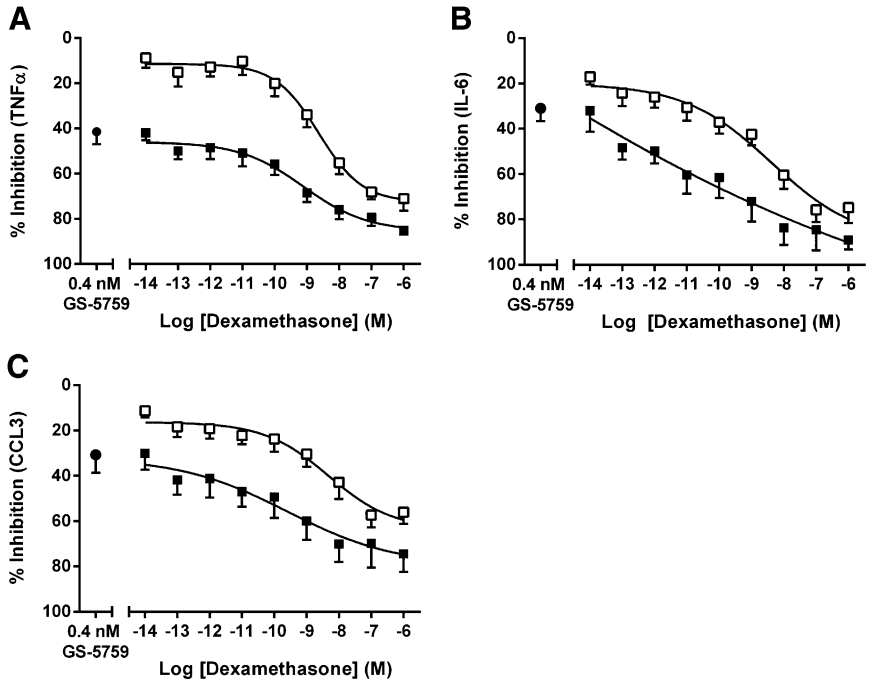

Fig. 3. Concentration-dependent inhibition of LPS-induced proinflammatory cytokine production from PBMC by GS-5759 in combination with dexamethasone. (A–C) Data represent the percentage maximum inhibition of cytokine release from human PBMC compared with vehicle-treated cells after stimulation with LPS (mean ± S.E.M., n = 6). ●, GS-5759 alone (0.4 nM); □, dexamethasone alone or in the presence of GS-5759 (0.4 nM) (■).

to 85 ± 2% for TNFα, from 75 ± 7 to 89 ± 4% for IL-6, and from 56 ± 5 to 74 ± 5% for CCL3.

GS-5759 Inhibition of Proinflammatory Cytokine Production from NHLF. The ability of GS-5759 to inhibit the release of the chemokine (C-C motif) ligand 5 (CCL5), chemokine (C-X-C motif) ligand 10 (CXCL10), and the immune cell survival factor granulocyte macrophage colony–stimulating factor (GM-CSF) after TNFα stimulation of human normal lung fibroblasts was investigated. GS-5759 was a potent, concentration-dependent inhibitor of all three cytokines, with an IC_{50} of 0.1 ± 0.05 nM for CXCL10, 0.04 ± 0.02 nM for CCL5, and 0.2 ± 0.07 nM for GM-CSF (Fig. 4, A–C). In these fibroblast assays, the PDE4 inhibitor roflumilast demonstrated poor activity with levels of inhibition below 30% for all cytokines measured at concentrations up to 1 μM. The β_2-adrenoceptor agonist indacaterol was, however, a potent inhibitor, with IC_{50} values of 0.1 ± 0.04 nM for CXCL10, 0.07 ± 0.03 nM for CCL5, and 0.2 ± 0.06 nM for GM-CSF.

Inhibition of ET-1 Production and α-SMA Expression in NHLF. TGF-β1 stimulation of NHLF resulted in an increased production of the profibrotic molecule ET-1, with a change from 1 ± 0.3 to 18 ± 3 pg/ml for nonstimulated versus TGF-β1-stimulated NHLF, respectively. GS-5759 inhibited ET-1 production in a concentration-dependent manner, with an IC_{50} of 6 pM (CI 3–9 pM) and maximum inhibition of 98 ± 1% (Fig. 4D). Evaluation of indacaterol on ET-1 production showed an IC_{50} of 61 pM (CI 0.04–86 pM) and maximum inhibition of 85 ± 9%, whereas a 10 μM concentration of roflumilast produced very little inhibition (12 ± 4%). The IC_{50} of GS-5759 was significantly different ($P < 0.05$) from indacaterol, whereas the maximum level of inhibition was similar.

After TGF-β1 treatment, fibroblasts acquire a more contractile phenotype, which is characterized by expression of α-SMA. Treatment of NHLF with TGF-β1 caused a 12 ± 2-fold upregulation of α-SMA expression. GS-5759 exhibited a concentration-dependent inhibition of α-SMA expression, with an IC_{50} of 7 pM (CI 0.07–783) and maximum inhibition of 49 ± 3% (Fig. 4E). Indacaterol inhibited α-SMA expression, with an IC_{50} of 60 pM (CI 0.02–230), which was not statistically different than GS-5759, and had a maximum inhibition or 42 ± 8% (Fig. 4E). Roflumilast alone at any concentration tested up to 10 μM failed to inhibit α-SMA expression.

GS-5759 Induces pCREB in NHLF. GS-5759 has the potential to both elevate and maintain intracellular cAMP through its activity on β_2 adrenoceptors and PDE4, respectively. GS-5759 was evaluated for its ability to phosphorylate CREB in NHLF downstream of cAMP. GS-5759 (1 μM) was added to serum-starved NHLF over a time course of 120 minutes, causing an induction of pCREB that was maximal at 30–45 minutes after compound addition (Fig. 4G). A 30-minute time point was chosen to assess GS-5759 concentration-dependent induction of pCREB, with a 3-fold increase in pCREB seen at the top concentration of 0.1 μM (3 ± 1-fold, Fig. 4F). Indacaterol also achieved a similar maximal level of pCREB modulation at 0.1 μM (3 ± 0.3-fold), but there was a pronounced difference in the potency of GS-5759 versus indacaterol (EC_{50} = 22 vs. 368 nM, respectively).

Effect of GS-5759 on Carbachol-Induced Contraction of Guinea Pig Smooth Muscle Strips. A guinea pig model of bronchoconstriction was used to evaluate the bronchodilatory potential of GS-5759 in vitro. Guinea pig airway smooth muscle strips were previously determined to have an EC_{80} for carbachol-induced constriction of 0.3 μM (data not shown). Treatment with GS-5759 or indacaterol demonstrated a concentration-dependent inhibition of carbachol-induced contraction, with IC_{50} values of 0.5 μM (CI 0.2–2) and 0.5 μM (CI 0.1–20), respectively (P = not significant) (Fig. 5A). To evaluate the contribution of the β_2-adrenoceptor agonist component, airway smooth muscle strips were treated with the specific β_2-adrenoceptor antagonist ICI 118551 (10 μM) followed by treatment with GS-5759 (1 μM) or vehicle control. The inhibition by GS-5759 on the carbachol-induced contraction was totally abolished by preincubation with ICI 118551

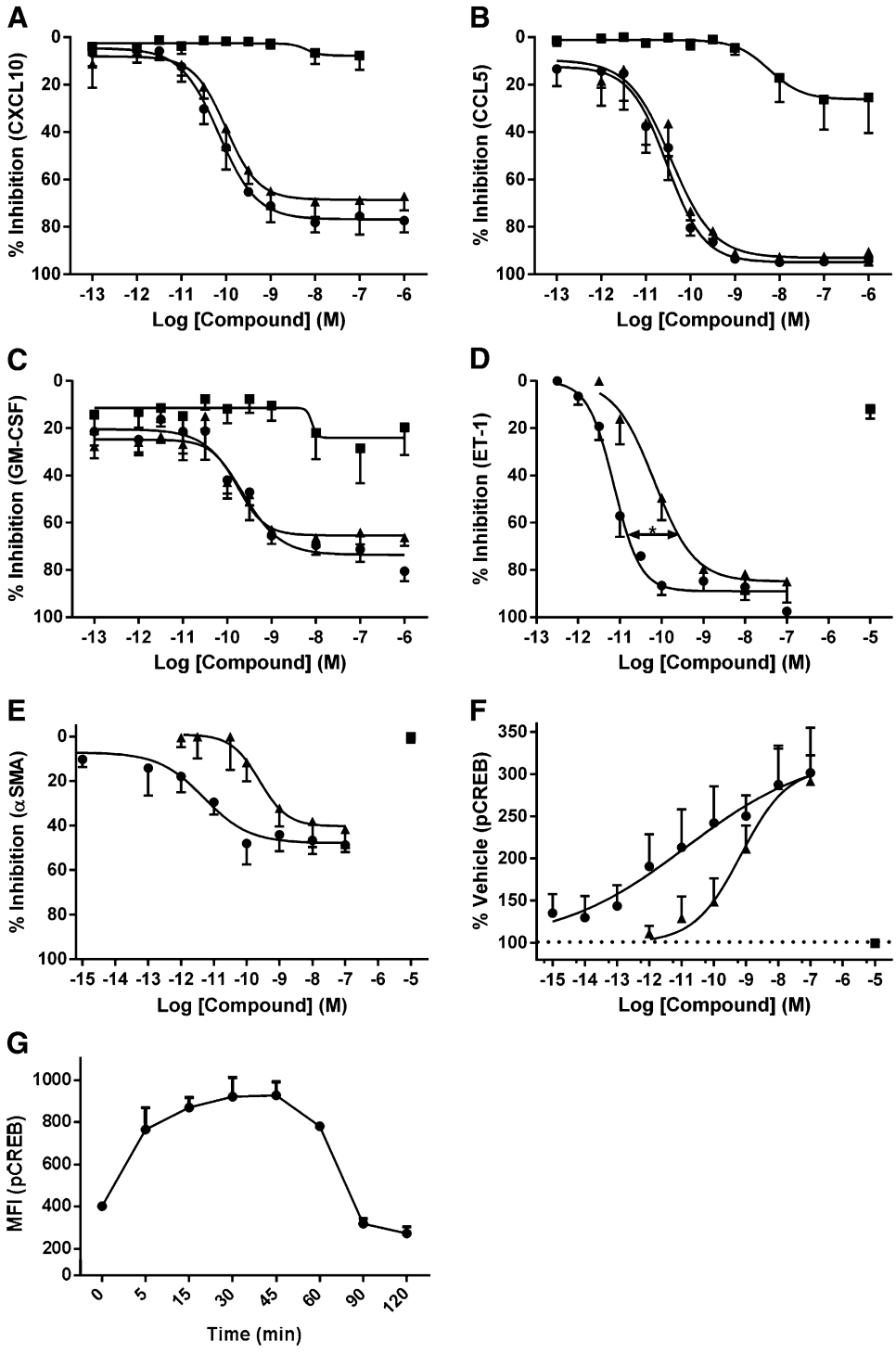

Fig. 4. Concentration-dependent inhibition of TNFα-induced proinflammatory cytokine production and profibrotic mediator production by GS-5759 in NHLF. (A–C) Cytokine production is calculated as the percent inhibition relative to TNFα-stimulated DMSO control (mean ± S.E.M., n = 6). (D) ET-1 production is calculated as the percentage inhibition relative to TGF-β1-stimulated DMSO control (mean ± S.E.M., n = 6-10). (E) α-SMA expression is calculated as the percent inhibition relative to TGF-β1-stimulated DMSO treated cells (mean ± S.E.M., n = 5). (F–G) Concentration-dependence of GS-5759 and indacaterol was assessed after 30 minutes of treatment. Time course of pCREB induction was done with GS-5759 (1 μM). pCREB induction is calculated as percentage of DMSO control (mean ± S.E.M., n = 5–12). Compound treatments were GS-5759 (●), indacaterol (▲), or roflumilast (■). *$P < 0.05$ for GS-5759 IC_{50} as compared with indacaterol.

($P < 0.05$), indicating that the effect of GS-5759 on the airway smooth muscle was specifically via β_2 agonism (Fig. 5B).

Association and Disassociation Kinetics of GS-5759 on Guinea Pig Smooth Muscle Strips. The association and disassociation rates of compounds with β_2 adrenoceptors was evaluated on guinea pig airway smooth muscle strips contracted to carbachol. GS-5759 demonstrated a time- and concentration-dependent relaxation with times to 50% association (On $T_{1/2}$) of 64 ± 4 and 10 ± 2 minutes at 300 nM and 3 μM, respectively (Table 2). In comparison, indacaterol demonstrated a faster association rate at 300 nM with an On $T_{1/2}$ = 6 ± 0.3 minutes ($P < 0.05$), but at 3 μM, it had a similar On $T_{1/2}$ of 6 ± 0.3 minutes. The disassociation rate of GS-5759 from endogenous β_2 adrenoceptors expressed on guinea pig airway smooth muscle strips was measured by monitoring the recovery of functional carbachol-induced contraction after washout of the compound from tissue baths. GS-5759 demonstrated a time- and concentration-dependent recovery of functional carbachol-induced contractions, with Off $T_{1/2}$ times of 603 ± 61, 649 ± 54, and >720 minutes at 100 nM, 300 nM,

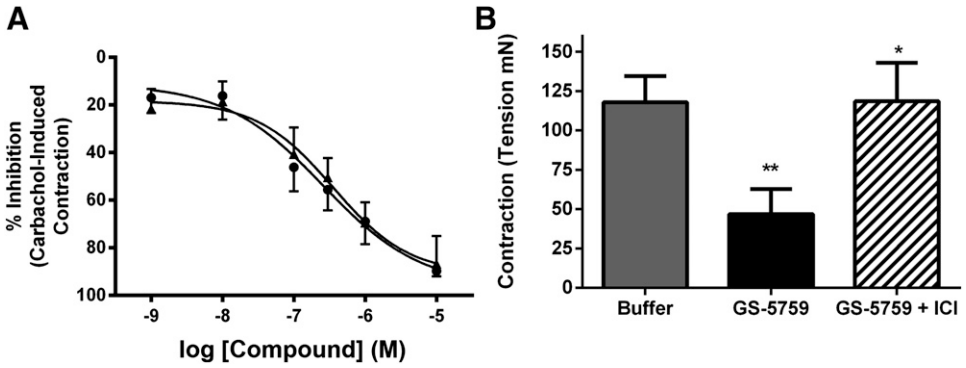

Fig. 5. Dose-related inhibition of carbachol-induced smooth muscle constriction by GS-5759 in the absence or presence of ICI 118551 in guinea pig tissue baths. (A) Percentage inhibition of carbachol-induced contraction was calculated compared with vehicle control. GS-5759 (●), indacaterol (▲), n = 5, mean ± S.E.M. (B) Airway smooth tissue strips were contracted with carbachol (0.3 μM) and contractive response (tension, mN) was recorded at 20 minutes. *$P \leq 0.05$ compared with GS-5759; **$P \leq 0.01$ compared with buffer control.

and 3 μM, respectively (approximate EC_{30}, EC_{50}, and EC_{80} concentrations). The Off $T_{1/2}$ times for indacaterol were 318 ± 129, 646 ± 47, and >720 minutes at 100 nM, 300 nM, and 3 μM, respectively (Table 2). Over the concentration range used in these studies, GS-5759 appeared to have a slow functional dissociation rate from guinea pig tracheal smooth muscle, similar to indacaterol and consistent with a long duration of effect at β_2 adrenoceptors.

Discussion

In the present study, we evaluated the in vitro pharmacology of GS-5759, a novel bifunctional molecule designed for oral inhalation delivery. GS-5759 was designed by covalently linking a PDE4 inhibitor pharmacophore with a β_2-agonist pharmacophore, with retained functional activity at both targets. GS-5759 is a potent and full agonist of β_2 adrenoceptors and is a low nanomolar inhibitor of the PDE4 enzyme. GS-5759 demonstrated potent anti-inflammatory activity in PBMC and neutrophils with apparent contributions from both the PDE4 inhibitor and β_2-agonist components. In functional studies, GS-5759 relaxed precontracted guinea pig airway smooth muscle strips in a concentration-dependent manner and appeared to have slow dissociation kinetics in washout studies.

Previous studies in our laboratory have evaluated the combined effects of the PDE4 inhibitor roflumilast and a β_2 agonist in a number of primary cell types (Tannheimer et al., 2012a,b). In the present studies, we evaluated the combined effect of modulating both targets using the bifunctional GS-5759, with or without the β_2 antagonist ICI 118551, to differentiate the effects of both pharmacophores. In PBMC and neutrophils, we observed a rightward shift in the IC_{50} in the presence of ICI 118551, suggesting that the β_2-agonist component could contribute to the anti-inflammatory activity in these cell types. Although we did not look at diseased cells in the present studies, the anti-inflammatory activity of GS-5759 on cytokine release from LPS-stimulated macrophages from COPD patients has been reported (Kaur et al., 2012).

In similar experiments, we evaluated another relevant cell type, NHLF, which may have a different β_2-expression profile than immune cells, and here we showed that GS-5759 inhibition of TNFα-driven proinflammatory cytokine production was equivalent to indacaterol when levels of GM-CSF, CXCL10, and CCL5 were evaluated. Roflumilast had no significant activity in this cellular assay, suggesting that under these experimental conditions, only the β_2-agonist pharmacophore was driving the inhibition seen with the GS-5759. When a different stimulation, TGF-β1, was used to induce the profibrotic molecule ET-1 and expression of α-SMA, whereas indacaterol showed good inhibition on both mediators, the activity of GS-5759 showed an increase in potency over indacaterol. This is a clear example of the additional effects that both components of GS-5759 can have mechanistically in relation to different stimuli, on the same cell type. This additive or cooperative effect was demonstrated previously using separate chemical entities in the same experimental cellular assays (Tannheimer et al., 2012b), whereas the effects of roflumilast as a single agent have shown little effect in our system, an observation reported by others (Togo et al., 2009; Sabatini et al., 2010). The antifibrotic and anti-inflammatory properties of GS-5759 on multiple cell types that may be contributing factors to the small airway disease seen in COPD patients suggest that this molecule could improve bronchodilation beyond just a direct effect on smooth muscle relaxation.

Tissue bath experiments in guinea pig airway smooth muscle suggested that GS-5759 retained a very similar potency to the parent β_2-agonist comparator indacaterol. On- and off-rate studies were performed to compare GS-5759 with indacaterol, and our data indicated that at the lower concentration, indacaterol had a significantly faster On $T_{1/2}$, more rapid Off $T_{1/2}$, whereas at the higher concentrations the kinetics were similar for both. The observation of the slower on- and off-rates for GS-5759 at the lower concentration tested may reflect the differences in tissue rather than receptor kinetics, although further experiments would be warranted to support this. In the present studies, we also evaluated the effect of blockade of the β_2 adrenoceptors using ICI 118551 on the ability of GS-5759 to relax airway smooth muscle and observed a complete abolition of this response that confirmed that this effect was completely β_2-adrenoceptor mediated.

TABLE 2

Associate and disassociate rate of GS-5759 with β_2 adrenoceptors

GS-5759 demonstrated a concentration- and time-dependent relaxation of carbachol precontracted tracheal smooth muscle (On $T_{1/2}$). In the presence of carbachol, GS-5759 demonstrated a concentration- and slow time-dependent loss of relaxation of airway smooth muscle after washout (Off $T_{1/2}$). Data represent the mean ± S.E.M., n = 3–7.

Compound	On $T_{1/2}$		Off $T_{1/2}$		
	300 nM	3 μM	100 nM	300 nM	3 μM
			min		
GS-5759	64 ± 4*	10 ± 2	603 ± 61	649 ± 54	>720
Indacaterol	6 ± 0.3	6 ± 0.3	318 ± 129	646 ± 47	>720

*$P < 0.05$ versus the same concentration of indacaterol (unpaired t test).

PDE4 inhibitors have been reported to have activity in reducing bronchoconstriction induced by proinflammatory stimuli, but our experiments confirmed the findings of others that PDE4 inhibition does not have a direct effect on contractile agonist-induced contraction in airway smooth muscle strips (Underwood et al., 1998; Hatzelmann et al., 2010).

Development of an inhaled bifunctional molecule composed of two pharmacophores that are covalently linked and can retain the ability to engage two biologic targets has the potential for some distinct advantages over single molecule combination approaches. These include enhanced lung retention times due to the increased molecular size and slower release into the systemic circulation, which could improve the therapeutic window (Robinson et al., 2011). A single bifunctional molecule will also have matched pharmacokinetics, simplified formulation, and a more straightforward regulatory path compared with a mixed fixed-dose combination (Matera et al., 2011). For the combination of a β_2 agonist and a PDE4 inhibitor, where both act through modulation of cAMP, there is an opportunity to provide additive or synergistic interactions, as has been previously reported (Seldon et al., 2005; Tannheimer et al., 2012a,b). This could be further enhanced, as a bifunctional molecule with a balanced optimal pharmacology could be delivered throughout the lung microenvironment, maintaining the ratio of interaction at the targets to provide maximal opportunity for their molecular interactions (Phillips and Salmon, 2012). It should be acknowledged, however, that from our current understanding of compartmentalized cAMP signaling, any cooperative interactions between a β_2-adrenoceptor agonist and a PDE4 inhibitor could be cell type- and compartment-specific and that they may also act independently in many settings (Houslay, 2010).

Many COPD patients are treated with an inhaled combination of a LABA for symptom relief and a glucocorticosteroid, which provides the anti-inflammatory activity. The magnitude of the anti-inflammatory benefit of glucocorticosteroids in COPD patients remains a matter of debate, in contrast to their efficacy in the majority of patients with asthma. However, when dosed in combination with β_2 agonists, they do improve lung function and health status in patients with moderate to severe COPD (Calverley et al., 2007). Given that Daxas (Takeda Pharmaceuticals Korea Co., Ltd.) now has been approved for treatment of specific COPD patient subtypes, it is of interest to understand whether addition of a PDE4 inhibitor to a β_2 agonist and a glucocorticosteroid might provide additional efficacy as a triple combination. This was addressed in studies using PBMC stimulated with LPS, where GS-5759 was added in combination with the glucocorticosteroid dexamethasone. The addition of GS-5759 caused an increased inhibition of cytokine production without shifting the IC_{50} for dexamethasone, showing that there is the potential for gain of anti-inflammatory activity. The use of the bifunctional molecule with a glucocorticosteroid may exert their anti-inflammatory effects through different mechanisms of action. It has been shown in bronchial epithelial cells transfected with a glucocorticoid response element reporter that GS-5759, in combination with dexamethasone, can increase activation by a 4-fold higher level over dexamethasone alone (Joshi et al., 2012). A further recent study evaluating the individual components of a triple combination in the same reporter system suggests that direct effects on glucocorticosteroid receptor activation or upregulation of anti-inflammatory genes independent of glucocorticosteroid receptor activation could lead to enhanced anti-inflammatory gene expression to a level that cannot be achieved by any of the drugs alone (Moodley et al., 2013). Such interactions could be cell- and stimulus-specific and need to be studied further in functional cellular assays, but they underscore the potential for increased anti-inflammatory activity using such a triple combination.

β_2 Agonists and PDE4 inhibitors cause a downstream elevation and maintenance, respectively, of the second messenger cAMP, which leads to phosphorylation of the transcription factor CREB and activation of genes containing a cAMP-responsive element in their promoter regions (Johannessen et al., 2004). Previously, evaluation of the mechanism of action for a β_2 agonist in combination with a PDE4 inhibitor showed an increase in CREB phosphorylation in both PBMC and NHLF (Tannheimer et al., 2012a,b). In the present studies, GS-5759 also increased phosphorylated CREB by 3-fold in NHLF and was more potent than indacaterol. These data suggest that the addition of a PDE4 inhibitor to help maintain the cAMP levels induced by a β_2 agonist could promote significant increases in CREB-mediated signaling, and such an effect may explain the superior inhibitory effect on fibrotic mediator release seen with GS-5759 compared with indacaterol in this cell system.

GS-5759 is a novel, bifunctional molecule designed for inhalation, containing a LABA pharmacophore covalently linked to a PDE4 inhibitor pharmacophore. In these studies, we demonstrated that GS-5759 is a potent relaxer of airway smooth muscle strips and has both anti-inflammatory and antifibrotic activity in a variety of cell types. Although the in vitro assays described here demonstrate strong evidence for the bifunctional nature of this molecule, it will be important to see if this in vitro profile translates to the in vivo setting, and this will be the focus of a further publication (M. Salmon, S.L. Tannheimer, T.T. Gentzler, Z.-H. Cui, E.A. Sorensen, K.C. Hartsough, M. Kim, L.J. Purvis, J.A. Kaplan, E.G. Barrett, J.D. McDonald, K. Rudolph, M. Doyle-Eisele, P.J. Kuehl, C.M. Royer, W.R. Baker, G.B. Phillips, and C.D. Wright, manuscript submitted for publication). GS-5759 is a bifunctional molecule that has the potential as a novel therapy for COPD, providing both bronchodilator and anti-inflammatory activity.

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Authorship Contributions

Research design: Tannheimer, Baker, Phillips, Wright, Salmon.

Conducted experiments: Tannheimer, Sorensen, Cui.

Contributed new reagents: Kim, Patel.

Performed data analysis: Tannheimer, Sorensen, Cui, Phillips, Salmon.

Wrote or contributed to writing of manuscript: Tannheimer, Sorensen, Cui, Phillips, Salmon.

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