The In Vitro Pharmacology of GS-5759, A Novel Bifunctional Phosphodiesterase 4 Inhibitor and Long Acting β₂-Adrenoceptor Agonist


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Running Title: *In Vitro* Pharmacology of GS-5759

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Text pages: 37

Tables: 2

Figures: 5

References: 34

Words in Abstract: 240

Words in Introduction: 594

Words in Discussion: 1466

Abbreviations:

alpha smooth muscle actin (αSMA)

chemokine (C-C motif) ligand 3 (CCL3)

chemokine (C-C motif) ligand 5 (CCL5)

chemokine (C-X-C motif) ligand 10 (CXCL10)

chronic obstructive pulmonary disease (COPD)

(8S,9R,10S,11S,13S,14S,16R,17R)-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-3H-cyclopenta[a]phenanthren-3-one (dexamethasone)
endothelin-1 (ET-1)

fibronectin (FN)

forced expiratory volume in 1 second (FEV₁)

formyl-methionyl-leucyl-phenylalanine (fMLP)

granulocyte macrophage colony-stimulating factor (GM-CSF)

(R)-6-((3-((4-((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)

((2R*,3R*)-1-[(2,3-Dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol) (ICI 118551)

(R)-5-(2-((5,6-diethyl-2,3-dihydro-1H-inden-2-yl)amino)-1-hydroxyethyl)-8-hydroxyquinolin-2(1H)-one (indacaterol)

interleukin 6 (IL-6)

lipopolysaccharide (LPS)

long-acting β₂-adrenoceptor agonist (LABA)

normal human lung fibroblasts (NHLF)

peripheral blood mononuclear cells (PBMC)

phosphorylated cAMP response element-binding protein (pCREB)

3-(cyclopropylmethoxy)-N-(3,5-dichloropyridin-4-yl)-4-(difluoromethoxy)benzamide (roflumilast)

transforming growth factor-β1 (TGF−β1)

tumor necrosis factor-α (TNFα)

Recommended Section: Inflammation, Immunopharmacology, and Asthma
ABSTRACT

Inhaled long acting β₂-adrenoceptor agonists (LABA) which 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 β₂ agonist and PDE4 inhibitory activity. GS-5759 demonstrated potent and full agonist activity at β₂-adrenoceptors (EC₅₀ = 8 ± 4 nM) and is a potent inhibitor of the PDE4 enzyme (IC₅₀ = 5 ± 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 IC₅₀ = 0.3 nM (C.I. 0.1-0.6) and in human neutrophils fMLP-induced super oxide anion production with an IC₅₀ = 3 nM (C.I. 0.8-8). Addition of the β₂ antagonist ICI 118551 shifted the IC₅₀ in these cell assays to 4 and 38 nM respectively, demonstrating the contribution of both β₂ agonist and PDE4 inhibitory activity to GS-5759. GS-5759 was also a potent inhibitor of profibrotic and pro-inflammatory mediator release from human lung fibroblasts. GS-5759 relaxed guinea pig airway smooth muscle strips pre-contracted with carbachol in a concentration-dependent manner with an EC₅₀ = 0.5 µM (C.I. 0.2-2) and had slow dissociation kinetics with an Off T₁/₂ >720 min at an EC₈₀ concentration of 3 µM. GS-5759 is a novel bifunctional molecule with both potent β₂ agonist and PDE4 inhibitor activity which 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 due to inflammation and thickening of the smooth muscle, deposition of extracellular matrix from activated myofibroblasts, and recruitment of inflammatory cells which secrete inflammatory mediators, reactive oxygen species and proteases. All 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 β₂-adrenoceptor agonists (LABA) have been used as the standard of care for COPD to provide bronchodilation and symptom relief (Donohue, 2004; Tashkin and Fabbri, 2010). Indacaterol is a newly approved once daily LABA that shows superior bronchodilatory activity as compared to 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™) has been approved in the US and EU 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), while no direct effect on bronchodilation has been observed. The therapeutic index for roflumilast is narrow due to 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 for COPD has been extensively discussed and is reviewed in Phillips and Salmon (Phillips and Salmon, 2012). The ability of β2-adrenoceptor agonists to also have direct anti-inflammatory activity has been previously described, including inhibition of histamine, arachidonic acid metabolites and TNF-α 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 lab 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 pro-inflammatory mediator release from transforming growth factor-β1 (TGF-β1)-treated normal human lung fibroblasts provides 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 Figure 1) is a bifunctional molecule that has dual pharmacology. It contains a long acting β2-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 as topical delivery may improve the therapeutic window. Additionally, the combination of a $\beta_2$-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 pre-contracted guinea pig smooth muscle strips was evaluated compared to the LABA indacaterol.
METHODS

**Materials.** Cell culture reagents purchased were RPMI, fetal bovine serum (FBS), BSA and penicillin/streptomycin (Life Technologies, Carlsbad, CA). Dimethyl sulfoxide (DMSO), LPS, ICI 118551 ((2R*,3R*)-1-[(2,3-Dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol) and dexamethasone were purchased from Sigma-Aldrich (St. Louis, MO), TGF-β1, and TNFα from R&D Systems (Minneapolis, MN). ICI-118551 is a potent and selective β2-adrenoceptor antagonist with pA2 of 9.3 at guinea pig uterine β2-adrenoceptor with greater than 100-fold selectivity over β1-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 in patent WO11143105.

**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 IBMX have been characterized and confirmed to be active against all four of the isozyme tested in these assays. In-house, recombinant human PDE4B2 enzyme (0.12 nM) was combined with compound or DMSO vehicle for 5 min at 25°C in
PDE Glo Reaction Buffer (Promega, Madison, WI), cAMP (50 nM) was added to the enzyme mix and incubated for 60 min. The enzyme reaction was terminated and PDE Glo detection buffer containing protein kinase A (PKA) enzyme was added, followed by Kinase Glo solution (Promega) and luminescence measured (EnVision, Perkin Elmer, Waltham, MA). Data are presented as the mean ± SEM 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 min) by HTRF (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 β1- and β2-adrenoceptors.** Binding of compounds to recombinant human β2-adrenoceptor expressed on Chinese hamster oocyte (CHO) cells or recombinant human β1-adrenoceptor expressed on human embryonic kidney 293 (HEK-293) cells was assessed using a competitive receptor binding assay with the radiolabeled ligand [3H](-)CGP 12177 (Cerep). In the same cell systems, elevation of intracellular levels of the second messenger cAMP was assessed following compound treatment of either cell as a functional readout (Cerep).

**PBMC Cytokine Evaluation.** All donors gave informed consent and were healthy, non-smoking males. Blood was drawn into K2EDTA vacutainers, and PBMC were isolated by Ficoll gradient centrifugation (Ficoll Paque Plus, GE Healthcare, Chalfont St.
Giles, UK). PBMC were cultured in RPMI + 10% FBS, P/S in 96-well plates at 1 x 10^5 cells/well, overnight, 5% CO₂, 37°C, before LPS stimulation for cytokine evaluation. PBMC were pre-incubated with ICI 118551 (10 μM) or vehicle for 60 min, followed by GS-5759 treatment alone or in combination with dexamethasone for 60 min prior to LPS (0.4 ng/mL) stimulation. Supernatants were collected after 6 h 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 pg/mL based on a standard curve, and results normalized to ICI 118551 or vehicle control. The pretreatment of PBMC with ICI 118551 had no effect on LPS-stimulated cytokine production as compared to DMSO vehicle control.

**Neutrophil Superoxide Anion.** Human neutrophils were purified from whole blood (male non-smokers) and were pre-incubated with ICI 118551 (10 μM) or vehicle for 60 min, followed by GS-5759 treatment for 60 min prior to stimulation with formyl-methionyl-leucyl-phenylalanine (fMLP) at the donor-specific EC₈₀ 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 as compared to DMSO vehicle control.

**NHLF Cytokine Production.** Normal human lung fibroblasts (NHLF, sex unknown, Lonza Walkersville Inc., Walkersville, MD) were routinely cultured in FGM-2 complete media (Lonza), 5% CO₂, 37°C, and used at passage 2-5 for experimentation. NHLF were cultured to subconfluence, and then starved in RPMI + 0.1% BSA overnight. Cells were
pretreated with compounds (0.1 pM – 1.0 µM) for 1 h and then stimulated with TNFα (10 ng/mL). Supernatants were collected at 24 h post-stimulation and cytokines measured in a Luminex bead-based assay, according to manufacturer’s instructions (EMD Millipore). Results were calculated in pg/mL based on a standard curve.

**NHLF ET-1 Quantitation and αSMA Expression.** For experimentation, NHLF were cultured to subconfluence, starved in RPMI + 0.1% BSA overnight, pretreated with compound for 1h, and stimulated with 10 ng/mL TGF-β1 for 24 h (endothelin-1 (ET-1) production) or 48h (α smooth muscle actin (αSMA) 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 pg/mL based on a standard curve, and percent inhibition calculated relative to TGF-β1-stimulated DMSO control. For αSMA characterization, cells were trypsinized, fixed (fix buffer 1, 10 min, 37°C, BD Biosciences, San Jose, CA), permeabilized (perm buffer III, 30 min, 4°C, BD Biosciences), stained with an anti-αSMA-FITC antibody (Abcam, Cambridge, MA) and visualized on a LSR II flow cytometer (BD Biosciences). Mean fluorescent intensity (MFI) was determined and percentage inhibition 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 min at 37°C), washed with cold PBS and then lysed on ice for 20 min (Millipore MAP Cell Signaling kit, EMD Millipore). Lysates were analyzed for phosphorylated cAMP binding protein (pCREB) with Luminex bead-based assay (Milliplex MAP Phospho CREB (Ser133) MAPmate, EMD Millipore), as measured by MFI. 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 sacrificed by inhalation of CO₂. The trachea was quickly and carefully dissected and immediately immersed in pre-chilled oxygenated Krebs-Hunnslet solution containing indomethacin (10 µM). The connective tissue was trimmed away and 4 tracheal rings (3 – 4 mm) were cut off from the middle portion of 1 trachea then the cartilage 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-Hunnslet solution containing indomethacin (10 µM). Baseline tension was artificially set to 14 mN and stabilized for at least 30 min before the experiment. The potency at β₂-adrenoceptors was measured as the percent inhibition of carbachol (0.3 µM)-induced contraction in guinea pig tracheal smooth muscle strips pre-incubated with GS-5759 or indacaterol (1 nM – 10 µM). The functional association rate of GS-5759 with β₂-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 β₂-adrenoceptors was measured as the recovery of carbachol-induced contraction in tracheal smooth muscle strips following washout of the compound. To evaluate the contribution of the β₂-adrenoceptor agonist component of GS-5759, guinea pig tracheal smooth muscle strips were incubated with the specific β₂-adrenoceptor antagonist ICI 118551 (10 µM for 1.5 h) followed by treatment with GS-
5759 (1 µM for 3 h) and then contraction by carbachol (0.3 µM) was measured for 30 min. 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 ± SEM for each experimental group with non-stimulated 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 ANOVA 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 β2-adrenoceptor agonist activity and PDE4 inhibitory activity. The β2-adrenoceptor activity was demonstrated in binding studies, where GS-5759 has a concentration-dependent inhibition of radioligand binding to β2-adrenoceptor with an IC50 value of 11 ± 2 nM (Table 1). In a functional assay, GS-5759 elevated intracellular cAMP in a concentration-dependent manner with an EC50 of 8 ± 4 nM and appeared to be a full agonist at β2-adrenoceptor as it attained 100% of the cAMP elevation at 1 µM compared to isoproterenol standard. In a similar experiment GS-5759 elevated cAMP in a concentration-dependent manner with an EC50 of 33 ± 20 nM at β1-adrenoceptors. GS-5759 was a competitive, concentration-dependent inhibitor of PDE4B2 with an IC50 of 5 ± 3 nM, in comparison to the clinically approved roflumilast which demonstrated a similar concentration-dependent inhibition of PDE4B2 of 3 ± 1 nM. GS-5759 also potently inhibited the PDE4A1, -B1 and -D2 isozymes with IC50 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, -10A1 by less than 10%, PDE11A4 by 16 % and was without effect on PDE1B, -3A and -7A (data not shown). The IC50 comparison to 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, as
compared to control), NK₁ (65%), mu opiate receptor (77%), 5-HT₁B (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 IC₅₀ of 0.3 nM (C.I. 0.1-0.6), and a maximum level of inhibition of 95 ± 1% (Figure 2A). When the assay was performed in the presence of an excess of the β₂-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 IC₅₀ of 4 nM (C.I. 1-15), and a maximum level of inhibition of 68 ± 6%. Statistical analysis of the IC₅₀ values and maximum inhibition run in the absence and presence of ICI 118551 demonstrated a statistically significant difference (p<0.01 and p<0.001, respectively), indicating that the β₂-adrenoceptor agonist activity of GS-5759 contributed to the increased cellular potency. In comparison, the PDE4 inhibitors roflumilast and GSK256066 demonstrated IC₅₀ values of 2 nM (C.I. 0.9-4) and 0.04 nM (C.I. 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 IC₅₀ of 3 nM (C.I. 0.8-8) and a maximum level of inhibition of 93 ± 5% (Figure 2B). When the assay was performed with ICI 118551 (10 μM), GS-5759 had an IC₅₀ of 38 nM (C.I. 18-83) and a maximum level of inhibition of 83 ± 6%. A significant difference in the IC₅₀ values of GS-5759 in the absence or presence of ICI 118551 was seen (p<0.01). The PDE4 inhibitor roflumilast had an IC₅₀ of 7 nM (C.I. 4-14) and GSK256066 an IC₅₀ of 1 nM (C.I. 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 sub-maximal concentration of GS-5759 (0.4 nM, based on the IC₅₀ for TNFα production, Figure 2A). Cytokine induction after 24 h for GM-CSF, CXCL10 (IP-10), and CCL5 (RANTES) was 228 ± 12, 21042 ± 513, 6220 ± 241 pg/mL, respectively. Dexamethasone alone caused a concentration-dependent inhibition of all three cytokines. In the presence of GS-5759 the IC₅₀ for dexamethasone for TNFα was decreased from 2 nM (C.I. 0.8-6) to 0.6 nM (C.I. 0.1-4), for IL-6 from 0.5 nM (C.I. 0.02-12) to 0.2 nM (C.I. 0.0006-75) and for CCL3 from 5 nM (C.I. 0.5-39) to 0.6 nM (C.I. 0.2-1) (Figure 3A-C). Additionally, the maximum level of cytokine inhibition achieved by dexamethasone was increased in the presence of GS-5759 from 71 ± 5% 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 Pro-inflammatory 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 IC₅₀ of 0.1 ± 0.05 nM for CXCL10, 0.04 ± 0.02 nM for CCL5 and 0.2 ± 0.07 nM for GM-CSF (Figure 4A-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 β₂-adrenoceptor agonist indacaterol was, however, a potent inhibitor, with IC₅₀ 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 αSMA 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 non-stimulated versus TGF-β1-stimulated NHLF, respectively. GS-5759 inhibited ET-1 production in a concentration-dependent manner with an IC₅₀ of 6 pM (C.I. 3 - 9 pM) and maximum inhibition of 98 ± 1% (Figure 4D). Evaluation of indacaterol on ET-1 production showed an IC₅₀ of 61 pM (C.I. 0.04 – 86 pM) and maximum inhibition of 85 ± 9%, while a 10 μM concentration of roflumilast produced very little inhibition (12 ± 4%). The IC₅₀ of GS-5759 was significantly different (p<0.05) to indacaterol, while the maximum level of inhibition was similar.

After TGF-β1 treatment, fibroblasts acquire a more contractile phenotype, which is characterized by expression of αSMA. Treatment of NHLF with TGF-β1 caused a 12 ± 2 fold upregulation of αSMA expression. GS-5759 exhibited a concentration-dependent inhibition of αSMA expression with an IC₅₀ of 7 pM (C.I. 0.07 – 783) and maximum inhibition of 49 ± 3% (Figure 4E). Indacaterol inhibited αSMA expression with an IC₅₀ of 60 pM (C.I. 0.02 – 230) which was not statistically different to GS-5759 and had a maximum inhibition or 42 ± 8%, (Figure 4E). Roflumilast alone at any concentration tested up to 10 μM failed to inhibit αSMA expression.

GS-5759 Induces pCREB in NHLF. GS-5759 has the potential to both elevate and maintain intracellular cAMP through its activity on β₂-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 min, causing an induction of pCREB that was maximal at 30-45 min after
compound addition (Figure 4G). A 30 min 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, Figure 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 (EC50=22 versus 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 EC80 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 an IC50 value of 0.5 µM (C.I. 0.2-2) and 0.5 µM (C.I. 0.1-20), respectively (p=N.S.) (Figure 5A). To evaluate the contribution of the β2-adrenoceptor agonist component, airway smooth muscle strips were treated with the specific β2-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 pre-incubation with ICI 118551 (p<0.05), indicating that the effect of GS-5759 on the airway smooth muscle was specifically via β2 agonism (Figure 5B).

**Association and Disassociation Kinetics of GS-5759 on Guinea Pig Smooth Muscle Strips.** The association and disassociation rates of compounds with β2 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 T1/2) of 64 ± 4 and 10 ± 2 min at 300 nM and 3 µM, respectively (Table 2). In comparison, indacaterol demonstrated a faster association rate at 300 nM with an On T1/2 = 6 ± 0.3 min (p<0.05), but at 3 µM it had a similar On T1/2 of 6 ± 0.3 min. The disassociation rate of GS-5759 from endogenous β2-adrenoceptors expressed on guinea pig airway smooth muscle strips was measured by monitoring the recovery of functional carbachol-induced contraction following wash-out of the compound from tissue baths. GS-5759 demonstrated a time- and concentration-dependent recovery of functional carbachol-induced contractions, with Off T1/2 times of 603 ± 61, 649 ± 54 and >720 min at 100 nM, 300 nM and 3 µM, respectively (approximate EC30, EC50 and EC80 concentrations). The Off T1/2 times for indacaterol were 318 ± 129, 646 ± 47 and >720 min 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 β2-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 $\beta_2$ agonist pharmacophore, with retained functional activity at both targets. GS-5759 is a potent and full agonist of $\beta_2$-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 $\beta_2$ agonist components. In functional studies, GS-5759 relaxed pre-contracted guinea pig airway smooth muscle strips in a concentration-dependent manner and appeared to have slow dissociation kinetics in wash-out studies.

Previous studies in our laboratory have evaluated the combined effects of the PDE4 inhibitor roflumilast and a $\beta_2$ agonist in a number of primary cell types (Tannheimer et al., 2012a; Tannheimer et al., 2012b). In the present studies, we evaluated the combined effect of modulating both targets using the bifunctional GS-5759, with or without the $\beta_2$ 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 the $\beta_2$ agonist component could contribute to the anti-inflammatory activity in these cell types. While 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 $\beta_2$ expression profile than immune cells, and here we showed that GS-
5759 inhibition of TNFα-driven pro-inflammatory 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 β2 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 αSMA, while 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 has been demonstrated previously using separate chemical entities in the same experimental cellular assays (Tannheimer et al., 2012b), while the effects of roflumilast as a single agent has shown little effect in our system, an observation reported by others (Togo et al., 2009; Sabatini et al., 2010). The anti-fibrotic 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 suggests 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 β2 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 T1/2, more rapid Off T1/2, while 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 $\beta_2$-adrenoceptors using ICI 118551 on the ability of GS-5759 to relax airway smooth muscle and observed a complete abolition of this response which confirmed that this effect was completely $\beta_2$-adrenoceptors-mediated. PDE4 inhibitors have been reported to have activity in reducing bronchoconstriction induced by pro-inflammatory 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 biological 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 to a mixed fixed-dose combination (Matera et al., 2011). For the combination of a $\beta_2$ 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; Tannheimer et al., 2012b). 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 compartmentalised cAMP signaling, any cooperative interactions between a β2-adrenoceptor agonist and a PDE4 inhibitor could be cell type- and compartment-specific and that they may also act independently in many settings (Houslay, 2009).

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 asthmatics. However, when dosed in combination with β2 agonists, they do improve lung function and health status in moderate to severe COPD patients (Calverley et al., 2007). Now that Daxas® has been approved for treatment of specific COPD patient subtypes, it is of interest to understand if addition of a PDE4 inhibitor to a β2 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 IC50 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 (GRE) 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 (GR) activation, or up-regulation of anti-inflammatory genes independent of GR activation, could lead to enhanced anti-inflammatory gene expression to a level that can’t 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.

β2 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 (CRE) in their promoter regions (Johannessen et al., 2004). Previously, evaluation of the mechanism of action for a β2 agonist in combination with a PDE4 inhibitor showed an increase in CREB phosphorylation in both PBMC and NHLF (Tannheimer et al., 2012a; Tannheimer et al., 2012b). In the present studies GS-5759 also increased phosphorylated CREB by three-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 β2 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 as compared to 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 have demonstrated that GS-5759 is a potent relaxer of airway smooth muscle strips and has both anti-inflammatory and anti-fibrotic activity in a variety of cell types. While 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 (Salmon et al., 2013). GS-5759 is a bifunctional molecule which has the potential as a novel therapy for COPD providing both bronchodilator and anti-inflammatory activity.
Acknowledgements

We would like to thank Professors Mark A. Giembycz and Robert Newton from the University of Calgary who provided critical discussions around the interpretation of the data described.
Authorship Contribution

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

Conducted experiments: Sorensen, Cui, and Tannheimer

Contributed new reagents: Kim and Patel

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

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


Footnotes

This study was funded by Gilead Sciences, Inc.

Portions of this work were presented at the ATS 2012 and ACS 2012 Annual meetings.

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FIGURE LEGENDS

Figure 1
Chemical structure of GS-5759

Figure 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 to ICI 118551- or vehicle-treated cells following stimulation with LPS (mean ± SEM, n=14-18 donors). (B) Data represent the percent maximum inhibition of superoxide anion release from human neutrophils compared to ICI 118551- or vehicle-treated cells following stimulation with fMLP (mean ± SEM, n=9 donors). Closed circles represent GS-5759 alone, open circles represent GS-5759 in the presence of ICI 118551. * p≤0.05, ** p≤0.01 GS-5759 IC_{50} as compared to ICI 118551-treated, *** p≤0.001 maximal inhibition of GS-5759 as compared to ICI 118551-treated.

Figure 3
Concentration-dependent inhibition of LPS-induced pro-inflammatory cytokine production from PBMC by GS-5759 in combination with dexamethasone. (A-C) Data represent the percent maximum inhibition of cytokine release from human PBMC compared to vehicle-treated cells following stimulation with LPS (mean ± SEM, n=6). Closed circles represent GS-5759 alone (0.4 nM), open squares represent dexamethasone alone, or in the presence of GS-5759 (closed squares).
Figure 4

Concentration-dependent inhibition of TNFα-induced pro-inflammatory 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 ± SEM, n=6). (D) ET-1 production is calculated as the percent inhibition relative to TGF-β1-stimulated DMSO control (mean ± SEM, n=6-10). (E) αSMA expression is calculated as the percent inhibition relative to TGF-β1-stimulated DMSO treated cells (mean ± SEM, n=5). (F-G) Concentration-dependence of GS-5759 and indacaterol was assessed after 30 min of treatment. Time course of pCREB induction was done with GS-5759 (1 μM). pCREB induction is calculated as percentage of DMSO control (mean ± SEM, n=5-12). Compound treatments were GS-5759 (close circles), indacaterol (closed triangles), or roflumilast (closed squares). *p<0.05 for GS-5759 IC₅₀ as compared to indacaterol.

Figure 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) Percent inhibition of carbachol-induced contraction was calculated as compared to vehicle control. GS-5759 (closed circle), indacaterol (closed triangle), n=5, mean ± SEM. (B) Airway smooth tissue strips were contracted with carbachol (0.3 μM) and contractive response (tension, mN) was recorded at 20 min. * p≤0.05 as compared to GS-5759, ** p≤0.01 as compared to buffer control.
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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>PDE4A1A Enzyme Inhibition: IC₅₀</th>
<th>PDE4B1 Enzyme Inhibition: IC₅₀</th>
<th>PDE4B2 Enzyme Inhibition: IC₅₀</th>
<th>PDE4D2 Enzyme Inhibition: IC₅₀</th>
<th>β₂ Receptor Binding: IC₅₀</th>
<th>β₂ Receptor Cell cAMP: EC₅₀</th>
<th>β₁ Receptor Cell cAMP: EC₅₀</th>
<th>β₂ vs β₁ fold-ratio</th>
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<tbody>
<tr>
<td>Roflumilast</td>
<td>3 ± 1</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>GSK256066</td>
<td>0.06 ± 0.002</td>
<td>0.1</td>
<td>&lt;0.1</td>
<td>0.04 ± 0.007</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Indacaterol</td>
<td>NA</td>
<td>NA</td>
<td>6 ± 10</td>
<td>1 ± 0.2</td>
<td>26 ± 12</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GS-5759</td>
<td>67 ± 19</td>
<td>40 ± 6</td>
<td>5 ± 3</td>
<td>56 ± 9</td>
<td>11 ± 2</td>
<td>8 ± 4</td>
<td>33 ± 20</td>
<td>4</td>
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</table>
Table 2

Associate and disassociate rate of GS-5759 with β2-adrenoceptors

<table>
<thead>
<tr>
<th>Compound</th>
<th>On T½ (min)</th>
<th>Off T½ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>300 nM 3 μM</td>
<td>100 nM 300 nM 3 μM</td>
</tr>
<tr>
<td>GS-5759</td>
<td>64 ± 4* 10 ± 2</td>
<td>603 ± 61 649 ± 54 &gt;720</td>
</tr>
<tr>
<td>Indacaterol</td>
<td>6 ± 0.3 6 ± 0.3</td>
<td>318 ± 129 646 ± 47 &gt;720</td>
</tr>
</tbody>
</table>

GS-5759 demonstrated a concentration- and time-dependent relaxation of carbachol pre-contracted tracheal smooth muscle (On T½). In the presence of carbachol, GS-5759 demonstrated a concentration- and slow time-dependent loss of relaxation of airway smooth muscle following washout (Off T½). Data represent the mean ± SEM, n=3 – 7.

*p<0.05 vs the same concentration of indacaterol (unpaired t test).
Figure 1