Novel Strategies in the Treatment of COPD
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2016

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Han, B. (2016). Novel Strategies in the Treatment of COPD: Focus on oxidative stress and a-kinase anchoring proteins. [Groningen]: University of Groningen.

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Exploring the Pharmacological Properties of 4 Sul Compounds as Potential Novel Treatments for COPD

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Abstract

Although chronic obstructive pulmonary disease (COPD) patients are treated with (combinations of) bronchodilators and anti-inflammatory glucocorticosteroids, many patients respond poorly to these drugs. Therefore there is a great need for novel therapy in this disease. Drugs that possess both anti-inflammatory and bronchodilatory properties would yield an optimal treatment of COPD. In the present study, we explored the anti-inflammatory and airway smooth muscle (ASM)-relaxing properties of 4 compounds that have shown anti-oxidative effects (Sul-90, Sul-121, Sul-127 and Sul-136), using in vitro models.

We found that Sul-90 and Sul-121 (but not Sul-127 and Sul-136) dose-dependently prevented cigarette smoke extract (CSE)-induced release of interleukin-8 (IL-8) from human ASM cells, without significantly affecting basal IL-8 release. Moreover, Sul-90 and Sul-121 (but not Sul-127 and Sul-136) both induced relaxation of pre-contracted bovine tracheal smooth muscle (BTSM) strips, which was unaffected by the nonselective β-adrenoceptor antagonist propranolol. In addition, Sul-121 relaxed BTSM strips at a concentration that did not affect cell viability, whereas Sul-90 only induced BTSM relaxation at a concentration that could decreases cell viability.

In conclusion, Sul-121 represents a promising candidate for further screening in cell and animal models of COPD as it possesses the attractive combination of anti-inflammatory and (non-β2-adrenoceptor-mediated) ASM-relaxing properties without a detrimental effect on cell viability. Sul-121 may therefore present a novel compound for the treatment of COPD.
Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by persistent and progressive airflow limitation and a chronic airway inflammation (GOLD 2015). COPD greatly reduces the quality of life of the patients and through its high morbidity and mortality COPD results in a significant clinical, economic and social burden (Mathers and Loncar 2006; Soriano and Rodríguez-Roisin 2011). In the western world, the main risk factor for COPD is exposure to cigarette smoke (GOLD 2015).

Airway inflammation is considered to play a central role in the development of COPD (Barnes et al. 2003) and it is associated with frequent exacerbations (GOLD 2015). Neutrophils are key inflammatory cells in COPD. Both neutrophils and its major chemotactic factor, interleukin 8 (IL-8), are increased in the airways of patients with COPD (Barnes et al. 2003). As a key inflammatory mediator, the role of IL-8 in cigarette smoke-induced lung inflammation has been well studied in several *in vivo* and *in vitro* models (D’hulst et al. 2005; Gosens et al. 2009; Oenema et al. 2010; Ko et al. 2015). Cigarette smoke induces the release of IL-8 from several types of cells, including airway smooth muscle (ASM) cells (Gosens et al. 2009; Oenema et al. 2010; Oldenburger et al. 2012; Pera et al. 2012; Chen et al. 2014; Poppinga et al. 2015). The neutrophils that are recruited by IL-8 may further secrete IL-8 as well as other inflammatory mediators to trigger downstream events involved in the pathophysiology of COPD (Keatings et al. 1996; Tanino et al. 2002; Hoenderdos and Condliffe 2013).

COPD is also featured by a persistent and progressive airflow limitation induced by the chronic inflammation (Barnes et al. 2003). Although often underestimated in clinical assessment, airway hyperresponsiveness (AHR) to ASM contractile stimuli is an important characteristic of COPD (van den Berge et al. 2012) and associates with accelerated lung function decline (Postma et al. 1986; Tashkin et al. 1996). In COPD patients, the ASM responsiveness of ASM is strongly associated with lung neutrophils (van den Berge et al. 2012) and a number of pro-inflammatory cytokines, including IL-8 (Postma et al. 1988; Barnes et al. 2003).

In order to reduce airflow limitation and airway inflammation, (combinations of) bronchodilators, such as β₂-adrenoceptor agonists (β₂-agonists) and anticholinergics and anti-inflammatory glucocorticosteroids are widely prescribed in the first-line pharmacological treatment of COPD (Osthoff et al. 2013; GOLD 2015). However, bronchodilators are not always effective and the majority of patients show resistance to these medications, particularly glucocorticosteroids (Barnes et al. 2003; Broadley 2006; Barnes 2008; Matera et al. 2014). Therefore, studies for novel medications for COPD are highly necessary.

Several novel compounds (Sul-90, Sul-121, Sul-127 and Sul-136), which exhibited promising anti-oxidative cell protective effects (Van der Graaf et al. 2014), have recently been developed. Based on these effects the Sul compounds were considered as potential candidate drugs for COPD, which is characterized by a high oxidative stress. In the present study, we investigated the pharmacological potential of the Sul compounds to treat COPD...
symptoms by studying their anti-inflammatory and relaxing properties in ASM. Out of these 4 compounds, we report that Sul-121 prevented cigarette smoke extract (CSE) induced IL-8 release from ASM cells and reduced methacholine-induced ASM contraction, and may therefore represent a potential novel treatment of COPD deserving further studies.

Materials and Methods

Sul compounds

Sul-90, Sul-121, Sul-127 and Sul-136 were developed and produced by Sulfateq (Van der Graaf et al. 2014). We screened these 4 compounds on their effects on cell viability, CSE-induced IL-8 release and methacholine-induced ASM tone.

CSE preparation

CSE was freshly prepared using 3R4F research cigarettes (Reference Cigarette Program, University of Kentucky) as described previously (Oldenburger et al. 2012; Oldenburger et al. 2014; Poppinga et al. 2015). Briefly, the smoke from two combusted cigarettes was pumped (Watson Marlow 323 E/D) through 25 ml of serum-free Dulbecco’s modified Eagle’s medium (DMEM, 11965-092, Life technologies), which was designated as 100% CSE and was further diluted to a 15% working concentration.

Cell culture

Human telomerase reverse transcriptase immortalized ASM cells were used in the study (Oldenburger et al. 2012; Poppinga et al. 2015). For all experiments, ASM cells below passage 30 were used. The cells were maintained in DMEM containing 10% heat-inactivated fetal calf serum supplemented with HEPES (25 mM), L-glutamine (2 mM), amphotericin B (1.5 µg/mL) and penicillin (100 U/ml)/streptomycin (100 µg/ml) in a humidified atmosphere at 37 °C in air/CO₂ (95:5 % vol/vol).

CSE-induced IL-8 release

IL-8 release from ASM cells was used as marker of CSE-induced inflammatory responses. ASM cells were plated on 24-well plates at 20,000 cells/well. After grown to confluence, cells were treated with indicated concentrations of the Sul compounds or 1 µM fenoterol (positive control) for 30 min prior to the addition of 15 % CSE for 24 hours. IL-8 concentration in cell culture medium was measured by using a commercial ELISA kit (PeliKine Compact ELISA kit, Sanquin, The Netherlands).
Cell viability
The effect of the Sul compounds on cell viability was tested using trypan blue and Alamar Blue. Trypan blue is a dye that is not absorbed by viable cells, but traverses the membrane of dead cells thereby staining these cells (Strober 2001). Trypan blue cell counting was performed as previously described (Oldenburger et al. 2012). Briefly, ASM cells were plated on 24-well plates. After grown to confluence, cells were treated with the indicated concentrations of the Sul compounds for 24 hours. At the end of treatment, cells were trypsinized and mixed with a trypan blue solution (0.4 % (w/v) in saline) at a ratio of 1:1 (v/v). Living and dead cells were then determined and counted using a hemocytometer. The effect of the Sul compounds on cell viability was confirmed by an AlamarBlue® assay as described before (Roscioni et al. 2009). Briefly, after the treatment mentioned above, cells were washed twice with PBS and incubated with 5% vol/vol AlamarBlue® (DAL1100, Thermo Fisher) in Hank’s Balanced Salt Solution for about 45 min. AlamarBlue® is converted into its fluorescent form by mitochondrial cytochromes in living cells. Therefore, the amount of fluorescence, measured using a Wallac 1420 Victor 2TM (excitation: 570 nm, emission: 590 nm), is proportional to the number of living cells.

Preparation of bovine tracheal smooth muscle (BTSM) strips
Bovine tracheal smooth muscle (BTSM) was prepared as described previously (Gosens et al. 2002). Briefly, bovine tracheal were purchased from local slaughter house and immediately transported to the laboratory in Krebs-Henseleit buffer (composition in mM: 117.5 NaCl, 5.60 KCl, 1.18 MgSO4, 2.50 CaCl2, 1.28 NaH2PO4, 25.00 NaHCO3, and 5.50 glucose), pre-gassed with 5 % CO2 and 95 % O2, pH 7.4. After dissection of the smooth muscle layer and careful removal of connective tissues, BTSM strips of approximately 1 cm length and 2 mm width were prepared. Tissue strips were maintained in DMEM supplemented with nonessential amino acid mixture (1 mM for all: glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, L-serine), sodium pyruvate (1 mM), gentamicin (45 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (1.5 µg/ml) apo-transferrin (5 µg/ml) and ascorbic acid (100 µM), in an Innova 4000 incubator shaker 37 oC, 55 rpm). The BTSM strips that maintained in above medium for no more than 3 days were used in present study.

Isometric contraction and relaxation measurements
Strips were mounted for isometric recording (Grass force-displacement transducer FT03) in 20 ml water-jacked organ baths containing KH buffer at 37°C, continuously gassed with 5 % CO2 and 95 % O2, pH 7.4. Before isometric tension measurement, BTSM strips were calibrated as described previously (Dekkers et al. 2007). Briefly, each strip was gradually adjusted to a resting tension of 3 gram. Then, the strips were equilibrated for 60 minutes
with washouts every 20 min. Subsequently, muscle strips were pre-contracted with 0.1, 1 and 10 μM methacholine. Following two washouts, maximal relaxation was established by the addition of 0.1 μM (-)-isoproterenol. Subsequently, tension was readjusted to 3 gram, followed by two changes of fresh KH buffer. After another equilibration period of 30 min, muscle strips preparation were ready for following test. In the end of each following protocols, through washout were performed and isoprenaline was added to set the basal line.

To analyze acute effects of the Sul compounds on the airway smooth muscle isometric tension, the strips were first pre-contracted by 0.3 μM methacholine, followed by accumulative doses of the Sul compounds (1 - 600 μM).
To analyze the potential role of the β2-AR in the effects induced by the Sul compounds, after pre-contracted with 0.3 μM methacholine, the strips were incubated with 1 μM nonselective β-AR antagonist propranolol for 30 minutes prior addition of accumulative doses of the Sul compounds. Doses responsive curve of classic β2-agonist isoprenaline (1 x 10^{-10} – 3 x 10^{-4} M) was served as the positive control.

**Statistics**

Data are expressed as means ± SEM from n individual experiments. Statistical significance of differences was evaluated by a one-way or two way ANOVA with Bonferroni post-hoc tests as appropriate, using Prism 5 software. Differences were considered to be statistically significant when P<0.05.

**Results**

**Effect of the Sul Compounds on IL-8 release**

Treatment with 15% CSE increased the release of IL-8 from ASM cells on average by 6.6-fold (P<0.001, Figure 1A-D). The Sul compounds exerted differential effects on the cellular release of IL-8 induced by CSE. Treatment of cells with Sul-121 or Sul-90 dose-dependently reduced CSE-induced IL-8 release up to 90%, which were comparable to that of fenoterol (1 μM) as a positive control (Figure 1A, B). Sul-90 started to significantly reduce CSE-induced IL-8 at 100 μM, and almost fully abrogating IL-8 release at 300 μM (Figure 1A). Sul-121 significantly reduced CSE-induced IL-8 release at 300 μM (Figure 1B). On the contrary, while 300 μM Sul-127 caused a small, non-significant reduction of the CSE-induced IL-8 release (Figure 1C). Sul-136 even increased the CSE-induced IL-8 release from the cells by 1.4-fold (Figure 1D).
Figure 1. Sul-90 and Sul-121, but not Sul-127 and Sul-136, inhibit CSE-induced IL-8 release from ASM cells. Human ASM cells were incubated for 24 hours with indicated concentrations of Sul-90 (A), Sul-121 (B), Sul-127 (C) or Sul-136 (D) in the absence of presence of 15 % CSE. The β2-agonist fenoterol (1 µM) served as positive control. Data are expressed as means ± SEM of n=7-8 experiments. *P<0.05 and ***P<0.001, compared to CSE vehicle, two way ANOVA with Bonferroni post-tests. ##p<0.01 and ###p<0.001, compared to corresponding basal IL-8 release, two way ANOVA with Bonferroni post-tests.
Effect of the Sul Compounds on cell viability

Although the cell viability was not affected at the lower concentrations, 300 µM Sul-90 significantly decreased viability of ASM cells by 65% (P<0.05, Figure 2A). The viability of ASM cells was not affected by any of the tested concentrations (10, 30, 100 and 300 µM) of Sul-121, Sul-127 or Sul-136 (Figure 2B-D). Similar effects were obtained using the AlamarBlue® assay (data not shown).

Figure 2. Effect of Sul-90, Sul-121, Sul-127 and Sul-136 on cell viability. Human ASM cells were incubated for 24 hours with the indicated concentrations of Sul-90 (A), Sul-121 (B), Sul-127 (C) or Sul-136 (D). Cells were trypsinized for trypan blue staining and cell counting to determine cell viability. Data are expressed as means ± SEM of n=4-5 experiments. *P<0.05 compared to untreated (0 µM), one way ANOVA with Bonferroni post-test.

Effects of Sul compounds on BTSM contractility

Sul-90 and Sul-121 significantly decreased methacholine-induced contraction in BTSM strips (Figure 3A and 3B). This effect was statistically significant at the higher concentrations: 600 µM for Sul-90, and 300 and 600 µM for Sul-121. The maximal relaxation (E_{max}) induced by Sul-121 as - compared to the effect of DMSO as a vehicle control was significantly
larger than for Sul-90 (~67% and ~25%, respectively, P<0.05). Sul-127 and Sul-136 did not significantly affect methacholine-induced contraction at any concentration (Figure 3C and 3D). The vehicle control DMSO by itself caused a small, but non-significant increase in methacholine-induced contraction (Figure 3A-D).

Figure 3. Sul-90 and Sul-121, but not Sul-127 and Sul-136, induced relaxation of methacholine-pre-contracted BTSM strips. BTSM strips were pre-contracted with 0.3 µM methacholine and cumulative concentration-response curves were constructed for Sul-90 (A), Sul-121 (B), Sul-127 (C) and Sul-136 (D). Cumulative addition of DMSO (up to 0.5%) served as vehicle control. Data are expressed as means ± SEM of n=5-6 experiments. **P<0.01 and ***P<0.001 compared to DMSO, two way ANOVA with Bonferroni post-tests.

The β2-agonist isoprenaline induced a potent and full relaxation of methacholine-induced BTSM contraction (Figure 4A), which was shifted to the right by the 1 µM β-adrenoceptor antagonist propranolol (pD$_2$-values 7.7±0.2 vs 5.4±0.1, P<0.001). Treatment with propranolol did not affect the relaxation induced by Sul-90 or Sul-121 (Figure 4B).
Figure 4. The relaxation of BTSM strips induced by Sul-90 and Sul-121 is not mediated by the β2-adrenoceptor. BTSM strips were pre-contracted with 0.3 µM methacholine and cumulative concentration-response curves were constructed for isoprenaline (A) and Sul-90 and Sul-121 (B) in the absence and presence of 1 µM propranolol. Cumulative addition of DMSO (up to 0.5 %) in the absence and presence of propranolol served as a vehicle control. Data are expressed as mean ±SEM of n=3 experiments. *P<0.05 and *** P<0.001, compared to the isoprenaline control group, two way ANOVA with Bonferroni post-test.

Discussion

The role of ASM in the pathophysiology of COPD is well recognized as it contributes to aspects of airway constriction, airway remodeling, and, through the production of inflammatory chemokines, cytokines, proteases, and growth factors, airway inflammation (Chung 2005). Cigarette smoke is the most important risk factor for COPD and treatment of ASM cells with CSE leads to the production of various inflammatory mediators, including IL-8 (Oldenburger et al. 2012; Pera et al. 2012; Chen et al. 2014; Poppinga et al. 2015). Neutrophilic infiltration within the ASM was found to be higher in smokers with COPD compared to non-smokers without COPD (Baraldo et al. 2004). In the present study, we found that 15% CSE induced a 6.6-fold increase in IL-8 release from ASM cells, which is in line with previous studies from our group (Oldenburger et al. 2012; Pera et al. 2012; Poppinga et al. 2015). Treatment with 100 µM Sul-90 or 100 µM Sul-121 (a concentration that did not alter cell viability) almost completely prevented this CSE-induced IL-8 release, demonstrating that Sul-90 and Sul-121 possess anti-inflammatory properties. Sul-127 did not significantly alter CSE-induced IL-8 release, suggesting that these compounds specifically inhibit CSE-induced pro-inflammatory pathways. Sul-127 did not significantly alter CSE-induced IL-8 release, indicating that Sul-127 does not have anti-inflammatory properties at the tested concentration, whereas 300 µM Sul-136, on the other hand, even further increased CSE-induced IL-8 release. Based on these
results, Sul-90 and Sul-121, but not Sul-127 and Sul-137, may have therapeutic potential for the treatment of inflammation in COPD.

Another feature of COPD is chronic airflow limitation which is treated with inhaled bronchodilators such as β₂-agonists (GOLD 2015). To circumvent the limited access to human ASM strips, we used BTSM strips as an established model to test bronchodilatory responses (Boterman et al. 2005). Importantly, Sul-90 and especially Sul-121 were able to relax BTSM precontracted with methacholine, demonstrating that they have bronchodilating properties in addition to their anti-inflammatory properties. For Sul-90, the concentration needed to significantly relax the precontracted BTSM strips (600 µM) was higher than the dose that had strong anti-inflammatory effects (100 and 300 µM). Sul-121 induced a significantly larger relaxation than Sul-90 and the effect was already present at the same concentration that inhibited CSE-induced IL-8 release (300 µM). The effects of Sul-90 and Sul-121 cannot be explained by a vehicle effect, because cumulative addition of the vehicle (DMSO, maximum concentration: 0.5%) even slightly increased the metacholine-induced contraction. Sul-127 and Sul-136 did not alter BTSM contraction at any tested concentrations.

Inhaled β₂-agonists are potent bronchodilators, mainly due to their effective relaxation of ASM (GOLD 2015). β₂-agonists bind and activate β₂-adrenoceptors on ASM cells, thereby triggering a signaling cascade involving cAMP which induces ASM relaxation (Cazzola et al. 2013). To test whether the effects of Sul-90 and Sul-121 on ASM tone are mediated by activation of the β₂-adrenoceptor, the effect of the nonselective β-antagonist propranolol on Sul-90 and Sul-121 induced relaxation was tested. Whereas propranolol strongly counteracted the relaxation induced by the non-selective β-agonist isoprenaline, it left the relaxation induced by Sul-90 and Sul-121 unaffected, demonstrating that Sul-90 and Sul-121 induce relaxation of ASM independent of the β₂-adrenoceptor. There results suggested that Sul-90 and Sul-121, next to anticholinergics, could possess several advantages over β₂-agonists for the treatment of COPD. β₂-Agonists, although a potent bronchodilators, should be used with caution in patients with diabetes, hypokalemia, hyperthyroidism and certain cardiovascular diseases due to the activation of β₂-adrenoceptors in peripulmonary organs (Broadley 2006; Cazzola et al. 2013). Moreover, repeated and prolonged treatment of β₂-agonists can cause desensitization of the β₂-adrenoceptor, which is believed to be one of the main mechanisms of β₂-agonists tolerance (Broadley 2006). Currently, the mechanisms of actions for Sul-90 and Sul-121 in inducing ASM relaxation is still unknown, and it would be interesting to test it on other novel bronchodilating pathways, including the bitter taste receptor. As COPD is featured with both chronic inflammation and progressive airflow limitation in the lungs (Soriano and Rodríguez-Roisin 2011; GOLD 2015), the most effective pharmacological therapy for COPD would be with a drug that possesses both anti-inflammatory and bronchodilating properties. Here, we reported that both Sul-90 and particularly Sul-121 show promising anti-inflammatory and some ASM-relaxing properties.
However, to screen the potential candidates for novel therapies, it is necessary to take into account not only the beneficial effects but also the adverse effects. We also found Sul-90 decreased cell viability at concentrations needed to induce ASM relaxation, making Sul-90 a less favorable candidate for further studies. Importantly, this adverse effect on cell viability was not observed for Sul-121, which has similar anti-inflammatory actions as Sul-90 and is the stronger bronchodilator of the two.

In conclusion, we identify Sul-121 as the most promising candidate of a series of Sul compounds for further screening for therapeutic potential in COPD using cell models \textit{in vitro} and animal models of COPD \textit{in vivo}, as it possesses an attractive combination of anti-inflammatory and bronchodilating properties.

\textbf{Acknowledgments}

This study was financially supported by the Ubbo Emmius program of the University of Groningen and by Sulfateq (Groningen, The Netherlands). This study was financially supported by grants from Biobrug (BB-50 and BB060).
References


Chapter 5


